Advances in Experimental Medicine and Biology 1062

Rolf Hilgenfeld · Subhash G. Vasudevan
Editors

Dengue and Zika: Control and Antiviral Treatment Strategies

Advances in Experimental Medicine and Biology

Volume 1062

Editorial Board
IRUN R. COHEN, *The Weizmann Institute of Science, Rehovot, Israel*
ABEL LAJTHA, *N.S. Kline Institute for Psychiatric Research, Orangeburg, NY, USA*
JOHN D. LAMBRIS, *University of Pennsylvania, Philadelphia, PA, USA*
RODOLFO PAOLETTI, *University of Milan, Milan, Italy*
NIMA REZAEI, *Tehran University of Medical Sciences Children's Medical Center, Children's Medical Center Hospital, Tehran, Iran*

More information about this series at http://www.springer.com/series/5584

Rolf Hilgenfeld
Subhash G. Vasudevan
Editors

Dengue and Zika: Control and Antiviral Treatment Strategies

Editors
Rolf Hilgenfeld
Institute of Biochemistry
University of Lübeck
Lübeck, Germany

Subhash G. Vasudevan
Emerging Infectious Diseases Program
Duke-NUS Medical School Singapore
Singapore, Singapore

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-981-10-8726-4 ISBN 978-981-10-8727-1 (eBook)
https://doi.org/10.1007/978-981-10-8727-1

Library of Congress Control Number: 2018940143

© Springer Nature Singapore Pte Ltd. 2018
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Printed on acid-free paper

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Many emerging viruses have their origin in Africa. For example, Ebola virus was first recorded after the outbreak in the Democratic Republic of Congo (then known as Zaire) in 1976 and Chikungunya virus emerged and was isolated in Tanzania (formerly known as Tanganyika) in 1952. During that outbreak, a number of cases also occurred in Northern Mozambique. Furthermore, a particularly virulent strain of Dengue virus (serotype 3) first appeared in Mozambique, and antibodies to Zika virus have been detected in the Mozambican population decades ago (see Chap. 2 of this book).

Mozambique, with its tropical climate, seems to be an ideal breeding ground for newly emerging viruses. Yet, even though Africa, perhaps along with South East Asia and Latin America, carries the largest burden of outbreaks of emerging viruses, almost all scientific conferences dealing with these viruses take place in the USA or Europe. To address this gap, one of us (RH) started the Tofo Advanced Study Week (TASW) series on Emerging Viral Diseases in 2015, during the Ebola outbreak in West Africa, and managed to convince about 50 prominent Ebola researchers from around the world to gather in Tofo, Mozambique, for an in-depth discussion of the scientific and public health issues connected with the epidemic. Very importantly, this workshop provided a forum for many African scientists to report their results, when most of them did not have the opportunity to speak at the large Ebola conferences in Paris or Washington, D.C.

Building on this success, the 2nd Tofo Advanced Study Week on Emerging Viruses was held from Aug 28 to Sep 01, 2016, when the Zika virus epidemic was gripping the world. The meeting brought together experts from around the world to discuss Dengue and Zika epidemiology, diagnosis, immunopathology, mosquito control, and antiviral targets ranging from entry mechanism to viral replication enzymes or their protein interactions. The discussions also briefly considered the merits and issues of the Dengvaxia® vaccine that has been approved in more than 10 countries.

Given the very unique presence of experts from around the globe, this time we also endeavored to produce a scientific book that would collate the contributions written by the lecturers. Today, we are proud to present a collection of papers in this book entitled ***Dengue and Zika: Control and Antiviral Treatment Strategies*** arising from the Proceedings of the 2nd Tofo Advanced Study Week on Emerging Viral Diseases, published by Springer as a contributed volume in the Advances in Experimental Medicine and Biology series.

The industry perspective on antiviral development against flaviviral diseases was discussed briefly at the meeting and a comprehensive chapter on this topic from industry-based authors is included in the book. As in the first TASW meeting, the attendance and participation of young scientists from the African continent, as well as international scientists working on epidemiological aspects of arboviruses in Africa, was a key component. The abstracts of the presentations by these scientists are collectively provided in Chap. 25, and we envisage that these should allow international scientists interested in emerging viruses to connect, collaborate, and help extend and build virology-related research capacity in Africa. In total, the book contains 25 chapters written by leading international experts and reflects the current state of knowledge on Dengue and Zika and occasionally also other arboviruses. Moreover, we include transcripts of the discussions following each presentation that were recorded during the conference. In fact, Chap. 8 comprises the transcript of an in-depth discussion on flavivirus entry and on the NS1 protein as potential drug targets, where you can read the opinion of leading experts, which to a large part you will not find published anywhere else. We consider these lively discussions the “salt in the soup” of this book and very much hope that you will equally enjoy them.

We believe that the wide spectrum of topics covered in this book will bring the field up to date to consider the merits of a three-pronged approach to Dengue and Zika control involving vaccines, antivirals, and mosquito control. We would like to thank all the authors who contributed to this volume, as well as people who helped with producing it: Nina Eichler, Charlotte Flory, Antje Lindae, Jan Magonov, and the dedicated staff of Springer. Others have to be mentioned because they provided essential support with the organization of the meeting: Charlotte Flory, Nobina Morimoto, Linda Ngoromani, Eduardo Samo Gudo, and the staff of the conference venue, Hotel Tofo Mar. We also thank the organizations and companies that sponsored the meeting: Casa do Mar Guesthouse, Euroimmun AG, Gilead, Hotel Tofo Mar, Instituto Nacional de Saúde de Mocambique, Terra Agua Ceu Travel Agency, and Top-of-the-Dune Guesthouse. The German Centre for Infection Research (DZIF) supported the conference as a co-organizer.

We sincerely hope that you will be delighted by this lively and comprehensive compendium of flavivirus science and decide to join us at future Tofo Advanced Study Week meetings to discuss emerging viral diseases.

Lübeck, Germany — Rolf Hilgenfeld
Singapore, Singapore — Subhash G. Vasudevan

Photo taken by Subhash G. Vasudevan during a post-conference tour of the Kruger National Park. Dedicated to Dr. Wilfred LF Armarego (The Australian National University).

Participants of the 2nd Tofo Advanced Study Week on Emerging Viral Diseases, Praia do Tofo, Inhamabane, Mozambique, August 28 - September 01, 2016:
First row: Vanessa Monteiro, Imelda Rogério Chelene, Flora Inalda Mula, Onélia Cecília Guiliche, Aruna Sampath, Suzanne Kaptein, Maurice Demanou
Second row: Sádia Ali, Laura Rivino, Argentina Felisbela Muianga, Nicole Zitzmann, Edwige Rances, Francis Maluki Mutuku
Third row: Katja Fink, Katell Bidet, Beate Kümmerer, Anuja Mathew, Claudia Ohst, Ana Rita Chico, Aravinda de Silva, David Jans
Fourth row: Inocêncio Chongo, Paul Young, Subhash G. Vasudevan, Edward Jaszi, Sailas Witimosi, Scott O'Neill, Félix Rey
Fifth row: Vijaykrishna Dhanasekaran, Robert Lowen, John Oludayo Oludele, Jonas Schmidt-Chanasit, Charlotte Flory, Christoph Nitsche, Linda Ngoromani, Yi Shi, Norbert Heinrich
Sixth row: Joanna Miller, Kerstin Falk, Xavier Gundana, Jan Magonov, Rolf Hilgenfeld

Contents

1 Arboviruses: A Family on the Move 1
Paul R. Young

2 Historical Perspective of Arboviruses in Mozambique and Its Implication for Current and Future Epidemics 11
Eduardo Samo Gudo, Kerstin Falk, and Julie Cliff

3 Reliable Serological Testing for the Diagnosis of Emerging Infectious Diseases 19
Claudia Ohst, Sandra Saschenbrecker, Konstanze Stiba, Katja Steinhagen, Christian Probst, Christiane Radzimski, Erik Lattwein, Lars Komorowski, Winfried Stöcker, and Wolfgang Schlumberger

4 Flaviviral RNA Structures and Their Role in Replication and Immunity 45
Katell Bidet and Mariano A. Garcia-Blanco

5 The Molecular Specificity of the Human Antibody Response to Dengue Virus Infections 63
Emily N. Gallichotte, Ralph S. Baric, and Aravinda M. de Silva

6 Structures of Zika Virus E & NS1: Relations with Virus Infection and Host Immune Responses 77
Yi Shi, Lianpan Dai, Hao Song, and George F. Gao

7 Plugging the Leak in Dengue Shock 89
Daniel Watterson, Naphak Modhiran, David A. Muller, Katryn J. Stacey, and Paul R. Young

8 Viral Entry and NS1 as Potential Antiviral Drug Targets 107
Aravinda M. de Silva, Félix A. Rey, Paul R. Young, Rolf Hilgenfeld, and Subhash G. Vasudevan

9 The Dengue Virus Replication Complex: From RNA Replication to Protein-Protein Interactions to Evasion of Innate Immunity 115
Julien Lescar, Sherryl Soh, Le Tian Lee, Subhash G. Vasudevan, Congbao Kang, and Siew Pheng Lim

10 The Structure of the Zika Virus Protease, NS2B/NS3^{pro} 131
Rolf Hilgenfeld, Jian Lei, and Linlin Zhang

11 The Transactions of NS3 and NS5 in Flaviviral RNA Replication 147
Moon Y. F. Tay and Subhash G. Vasudevan

12 Establishment and Application of Flavivirus Replicons 165
Beate M. Kümmerer

13 Strategies Towards Protease Inhibitors for Emerging Flaviviruses 175
Christoph Nitsche

14 Discovery of Potent Non-nucleoside Inhibitors of Dengue Viral RNA-Dependent RNA Polymerase from Fragment Screening and Structure-Guided Design 187
Siew Pheng Lim, Christian G. Noble, Shahul Nilar, Pei-Yong Shi, and Fumiaki Yokokawa

15 Nucleocytoplasmic Trafficking of Dengue Non-structural Protein 5 as a Target for Antivirals 199
David A. Jans and Alexander J. Martin

16 Animal Models for Dengue and Zika Vaccine Development 215
Eduardo Alves dos Santos and Katja Fink

17 Understanding the Human T Cell Response to Dengue Virus 241
Laura Rivino

18 Regulation and Function of NK and T Cells During Dengue Virus Infection and Vaccination 251
Anuja Mathew

19 Structural Insights into the Broad-Spectrum Antiviral Target Endoplasmic Reticulum Alpha-Glucosidase II 265
Alessandro T. Caputo, Dominic S. Alonzi, John L. Kiappes, Weston B. Struwe, Alice Cross, Souradeep Basu, Benoit Darlot, Pietro Roversi, and Nicole Zitzmann

20 Mechanisms of Antiviral Activity of Iminosugars Against Dengue Virus 277
Joanna L. Miller, Beatrice E. Tyrrell, and Nicole Zitzmann

21 Countering Zika Virus: The USAMRIID Response 303
Robert G. Lowen, Thomas M. Bocan, Christopher D. Kane, Lisa H. Cazares, Krishna P. Kota, Jason T. Ladner, Farooq Nasar, Louise Pitt, Darci R. Smith, Veronica Soloveva, Mei G. Sun, Xiankun Zeng, and Sina Bavari

22 Dengue Antiviral Development: A Continuing Journey 319
Jenny G. Low, Rene Gatsinga, Subhash G. Vasudevan, and Aruna Sampath

23 An Industry Perspective on Dengue Drug Discovery and Development 333
Ilane Hernandez-Morales and Marnix Van Loock

24 The Use of *Wolbachia* by the World Mosquito Program to Interrupt Transmission of *Aedes aegypti* Transmitted Viruses 355
Scott L. O'Neill

25 Seroepidemiological Studies of Arboviruses in Africa 361
Eduardo Samo Gudo, S. Ali, V. S. António, I. R. Chelene, I. Chongo, M. Demanou, K. Falk, O. C. Guiliche, N. Heinrich, V. Monteiro, A. F. Muianga, J. Oludele, F. Mula, F. Mutuku, N. Amade, P. Alho, E. Betsem, Z. Chimbuinhe, A. J. Cristovam, G. Galano, A. Gessain, E. Harris, M. Heise, F. Inalda, I. Jala, E. Jaszi, C. King, U. Kitron, B. M. Kümmerer, A. D. LaBeaud, N. Lagerqvist, G. Malai, M. Mazelier, S. Mendes, D. Mukoko, B. Ndenga, R. Njouom, G. Pinto, A. Tivane, D. M. Vu, and J. Vulule

Index 373

1 Arboviruses: A Family on the Move

Paul R. Young

Abstract

Arboviruses are a diverse group of vector-borne viruses, many of whose members are the cause of significant human morbidity and mortality. Over the last 30 years, the emergence and/or resurgence of arboviruses have posed a considerable global health threat. The ongoing geographical expansion of the dengue viruses (DENV), along with the explosive outbreaks of West Nile virus (WNV), Chikungunya virus (CHIKV) and more recently, Zika virus (ZIKV) have all served as reminders that new epidemics may emerge at any time from this diversity. A clearer understanding of what mechanisms drive these dramatic changes in vector-host transmission cycles that result in the human population becoming significantly more exposed, will help to prepare us for the next emerging epidemic/pandemic. This Chapter seeks to provide a brief overview of the arboviruses, their mode of transmission and some of the known factors that drive their expansion.

Keywords

Arthropod-borne viruses · Zoonotic infections · Virosphere · Arbovirus transmission · Climate change impacts

1.1 Introduction

Arboviruses (a term derived from the descriptor, **ar**thropod-**bo**rne **viruses**) are an amazingly diverse group of viruses that are transmitted from infected to susceptible hosts by a range of arthropod vectors that include mosquitoes, ticks, sand flies or biting midges [20, 21]. Following ingestion of a blood meal from an infected host, viruses multiply in the insect mid-gut and then invade underlying tissues to cause a spreading infection (collectively referred to as the extrinsic incubation period) that ultimately results in a high-titred viral load, particularly in the salivary glands. They are then passed on to humans or other vertebrates during insect biting. Most diseases caused by arboviruses are zoonoses, primarily infections of vertebrates that can occasionally cause incidental infection and disease in humans. Notable exceptions to this are the dengue viruses (DENV), as humans are the primary vertebrate host. Indeed, passage through humans is essential in maintaining the virus transmission cycle. The nature of this two-way dependency prompted Duane Gubler to once remark that "humans could

P. R. Young (✉)
Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia
e-mail: p.young@uq.edu.au

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_1

be considered the vector for dengue virus infection in mosquitoes”. While monkeys have been implicated as an alternative vertebrate host to humans for dengue in rural settings, it is unlikely that this sylvatic cycle contributes much to the current global impact of this apex arbovirus.

By definition, arboviruses are arthropod-borne, however some are grouped within the arboviruses despite no apparent association with an arthropod vector, primarily because of their close genetic relationship. The naming of individual arboviruses has had a somewhat eclectic history with no formal taxonomic approach having been established. Some refer to dialect names after the illness they induce (chikungunya, o’nyong-nyong, dengue), others recognise the name of the location where they were first discovered (West Nile, Bwamba, Ross River, Zika) and some reflect a characteristic clinical presentation (Western equine encephalitis, yellow fever) [21].

Over the course of the last two decades, a dramatic expansion in the territorial range of a number of arboviruses has seen a significant increase in global epidemic activity. These include West Nile virus and its emergence in New York in 1999 and subsequent march across the North American continent over the next 4 years and subsequent spread, both north and south over the following decade. Chikungunya virus with its sudden expansion on La Reunion in 2005 and spread across the Indian subcontinent, South East Asia and globally. The ongoing expansion of the dengue viruses across the tropical zone and beyond, and of course, the recent explosive epidemic of Zika virus in South America, on the other side of the world from its first isolation in an African forest some 70 years previously. One thing is certain; we will see more of these outbreaks in the years to come [1, 9]. As a brief introduction to the research efforts detailed in the following Chapters, this review provides an overview of the group of viruses we collectively refer to as arboviruses, and addresses some of the issues that are helping to drive their expansion.

1.2 Who Are They?

More than 500 arboviruses have been recognised worldwide [21], a number that is undergoing rapid and exponential revision as researchers interrogate the virosphere using deep sequencing [19]. Estimates have suggested that the arboviruses we have recognised to date may represent less than 1% of the total. Only some of the currently known arboviruses, some 150, are known to cause human disease [21]. Some infect humans only occasionally or cause only mild illness, whereas others are of significant medical importance, causing large epidemics.

Most arboviruses causing human disease belong to three families; Togaviridae (genus *Alphavirus*), Flaviviridae (genus *Flavivirus*) and Bunyaviridae (*Bunyavirus*, *Orthobunyavirus*, *Nairovirus* and *Phlebovirus* genera), with members of three further families, Rhabdoviridae, Orthomyxoviridae and Reoviridae also contributing (Fig. 1.1). The alphaviruses and flaviviruses are enveloped, linear single-stranded, positive-sense RNA viruses. They are spherical in shape, with an underlying capsid and measure from 40 to 70 nm. The bunyaviruses are enveloped, segmented, circular negative-strand RNA viruses. They are generally spherical and measure 80–120 nm in diameter.

The most important group, at least from a human disease perspective, are the flaviviruses with a number of viruses in this group being of global health concern; dengue virus (DENV), West Nile virus (WNV), Zika virus (ZIKV) and yellow fever virus (YFV) [11]. Others, including Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), Venezuelan equine encephalitis virus (VEEV) and St. Louis encephalitis virus (SLEV) are usually restricted to specific regions. However, the spread of arboviruses across several regions have lead to major international health concerns. WNV with its jump from the Middle-East into the Americas, chikungunya virus (CHIKV) moving into islands in the southwest Indian Ocean, and from there to Southeast

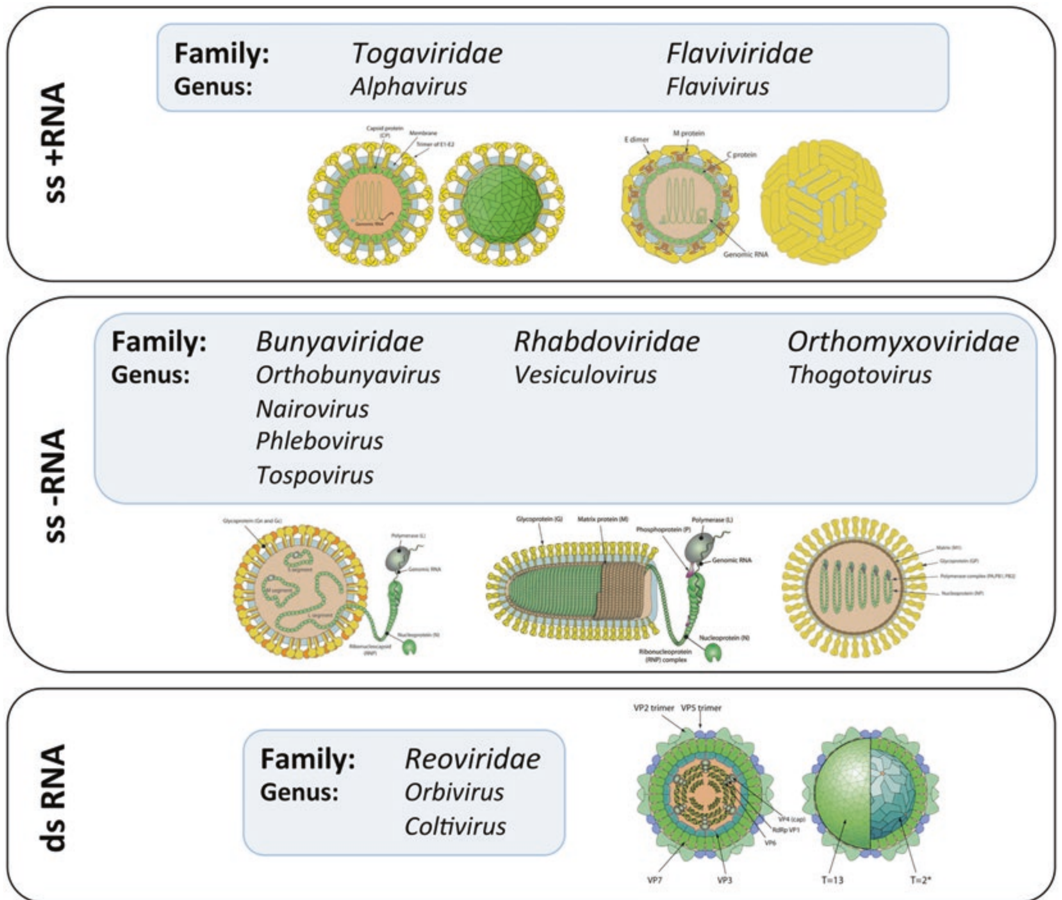


Fig. 1.1 **Arboviruses and virion schematics.** Viruses are grouped according to genome composition: single-stranded positive-sense RNA, ss + RNA; single-stranded negative-sense RNA, ss-RNA; double-stranded RNA, dsRNA. Arboviruses that are associated with human disease are mostly found within the Togaviridae, Flaviviridae and Bunyaviridae families. Virus schematics provided by ViralZone, Swiss Institute of Bioinformatics

Asia and the Americas, and Zika virus which spilled out of Africa to Southeast Asia, the islands of Polynesia and then to Brazil in an explosive epidemic in 2015–2016 (Fig. 1.2).

1.3 How Are They Maintained and Spread?

Three key elements are required for effective maintenance of arbovirus transmission: the vector (mosquito, tick, sandfly, biting midge), the vertebrate host(s) and appropriate environmental conditions. Some transmission cycles are relatively simple (involving one vector and one host, e.g., DENV and ZIKV) while some are highly complex (involving multiple vectors and hosts, e.g., JEV, WNV and Rift Valley Fever virus, RVFV). The epidemiology of human arboviral disease usually involves one of two transmission cycle scenarios (Fig. 1.3). In the first, the virus is stably and naturally maintained via transmission between vectors and wild animals in a sylvatic (jungle) cycle with spillover occurring when an infected arthropod bites either a domestic animal or human that has strayed into that ecological niche. This mode of infection results in small clusters of cases initiated at the same site. The second is the urban cycle where a person or domestic animal, infected via the sylvatic mode

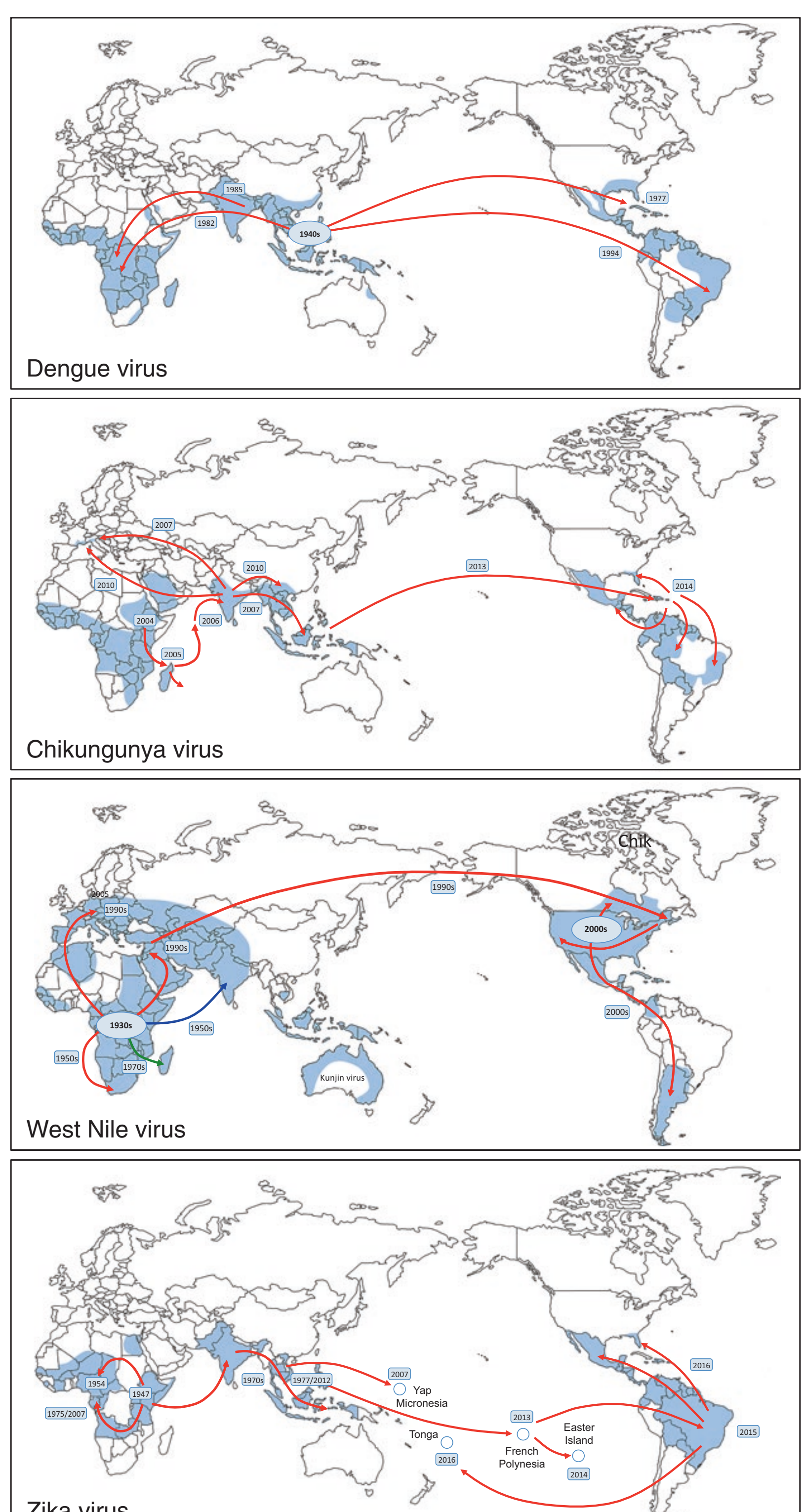


Fig. 1.2 Arbovirus epidemiology. Four examples of arboviruses that have emerged as globally distributed threats to human health. Geographical regions shaded in blue indicate historical, ongoing and recent viral activity with dates highlighting key epidemic translocation events. Dengue virus (DENV); the four serotypes of dengue virus

or moving from another area with urban activity, acts as an amplifier host in the transfer of the virus to other persons or domestic animals in the community. These cases occur as epidemics or epizootics in nature (Fig. 1.3) The vector involved in the urban cycle may be the same or different to that in the sylvatic cycle and indeed, there may be multiple vector species playing a role in transmission in either cycle.

The primary arboviral hosts are mammals and birds with the potential for virus dispersal depending on the type of vertebrate host involved [21]. Migratory birds can facilitate virus movement over large distances, such as occurred with the spread of WNV through the Americas, whereas transmission through most terrestrial hosts result in virus activity that is restricted to a particular region.

Animal hosts that are essential for arbovirus transmission and for the maintenance of virus populations are referred to as reservoir hosts, with the immune status of these hosts impacting on transmission rates. Their long co-evolution with their viral passengers is characterised by high titre viraemia that enables vector mediated virus transmission to occur, often in the absence of overt disease. A wide variety of reservoir host species have been implicated in arbovirus diseases. These include birds, mammals (including primates), rodents, marsupials and bats. Individual arboviruses may have more than one host species involved in transmission cycles. For example, birds (herons in particular) are considered to be the major maintenance hosts for the flavivirus JEV. In Asia however, pigs have also been shown to amplify the virus to high titres. Feeding mosquitoes can therefore be readily infected, with transmission of the virus to humans who live in close proximity. The life cycle of Ross River virus (RRV) in Australia involves complex relationships between multiple vectors and zoonotic (marsupials, horses, possums, bats) reservoirs across multiple environments including urban, inland (freshwater wetlands) and coastal (estuarine wetlands) regions [3].

Host species may move virus from an area of active transmission to another location. Movement by viraemic waterbirds has been suggested as a mechanism of spread for a number of arboviruses including Murray Valley encephalitis virus (MVEV), JEV, WNV and Eastern equine encephalitis virus (EEEV). Arboviruses can also be introduced into new areas by the movement of humans, particularly as air travel now enables movement between two destinations anywhere in the world, all within the time window of a typical viraemic period. Infected arthropod vectors may also disseminate disease if they are carried on air, marine, rail or road transport. This has been pro-

Fig. 1.2 (continued) continue to spread across the globe, with serotype subsets cycling in sequence with developing local herd immunity and virus evolution. The sudden and dramatic expansion of dengue in the early 1940s with the influx of naive adult hosts during the Pacific campaign of WWII seeded much of the subsequent global epidemic activity. After successful vector eradication programs in the first half of the twentieth century, dengue was re-introduced into the Americas, first into Cuba in 1977 with subsequent spread throughout tropical South America as its vector, *A. aegypti* reclaimed its earlier territory. Chikungunya virus (CHIKV); CHIKV exploded out of Africa following a large epidemic on the island of La Reunion in 2005. A single mutation in the virion surface protein facilitated a spillover into a new mosquito host, *A. albopictus* and further, global spread, reaching the Americas in 2014. West Nile virus (WNV); WNV was known to circulate within Africa from the 1930s when it was first isolated, spreading to the Middle East and Europe in the 1990s. What is thought to be a single transportation event resulted in WNV landing in New York from Israel in 1999. The subsequent march of WNV west across the North American continent was driven primarily by migration of its bird hosts, resulting in its wide distribution across the Americas over the subsequent decade. Zika virus (ZIKV); ZIKV was first isolated in 1947 but it wasn't until 1954 that the first human cases were reported in Nigeria. While its spread across Africa and into India and South East Asia were noted, it wasn't until a large epidemic on the island of Yap in 2007 highlighted the potential importance of ZIKV to human health. The subsequent epidemic in French Polynesia in 2013/2014 was thought to be the seed for its emergence in Brazil in 2015. The cause of the explosive nature and severity of the resulting epidemic over 2015–2016 is still the subject of considerable conjecture – the presence of a naïve population primed with a high level of potentially enhancing dengue-specific antibody, viral genome mutation or a combination of both along with additional factors remain possibilities

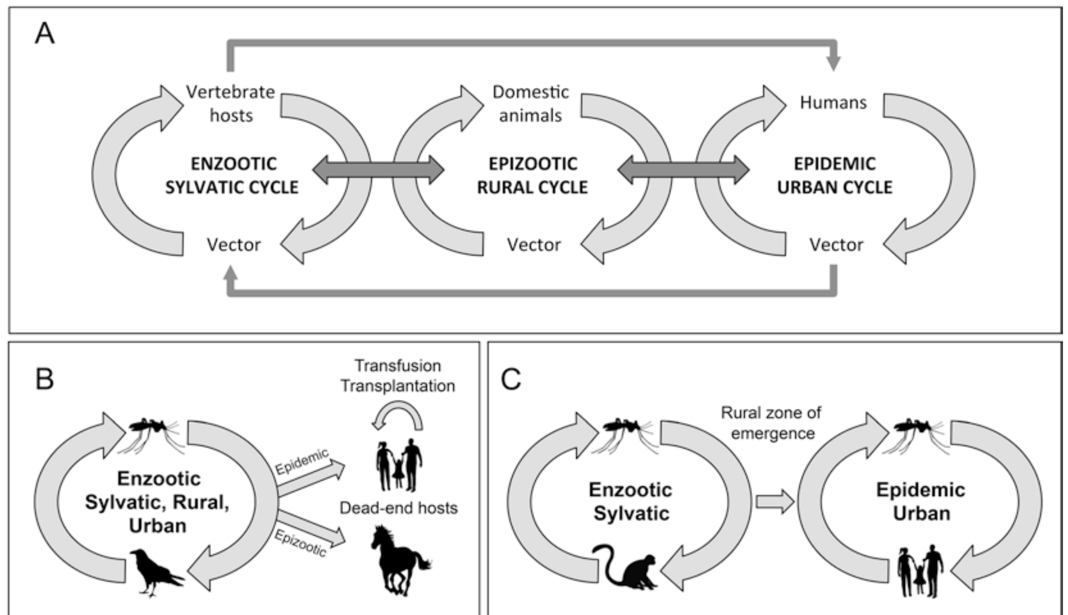


Fig. 1.3 Arbovirus transmission cycles. A. Enzootic (low level endemic virus transmission within native animals), epizootic (higher level epidemic transmission, usually within domestic animals) and epidemic cycles within humans are inextricably linked for many arboviruses, with spillover events driving the dynamics of each cycle. B. For some arboviruses (e.g., WNV) the epidemic and epizootic spillover from the enzootic cycle are unimportant for arbovirus survival, as these are dead-end hosts that do not act as reservoirs for further rounds of transmission. Exceptions are driven by specific human activity; e.g., transfusion and transplantation. C. For some arboviruses (e.g., DENV and ZIKV), the epidemic cycle in humans can be self-sustaining given the high levels of viraemia resulting in efficient transmission between vector and humans without the need for an enzootic amplification host. Nevertheless, occasional spillover events from the enzootic sylvatic cycle have been recorded

posed as the most likely mechanism for introduction of WNV into the USA in 1999.

Some hosts that become infected may not be sufficiently viraemic or may not be infected with sufficient regularity to contribute to the stable maintenance of virus populations and are referred to as incidental hosts. Incidental hosts may or may not show symptoms. For many arbovirus infections, humans are usually an incidental host, often being a dead end in the transmission chain.

Arthropod-borne viruses are distinguished from other animal viruses because of their ability to infect both vertebrate and invertebrate hosts. The virus replicates within the cells of the arthropod vector before being transferred to a susceptible host [16]. Occasionally, arthropods may also transmit viruses by mechanical transmission with the vector simply transferring the virus from an infected to a susceptible host without replication in the vector itself. Direct transfer from an infected to an uninfected vector during co-feeding on a naïve host has also been reported.

Invertebrate hosts include mosquitoes, sandflies, ticks and culicoides (biting midges) although most arboviruses have been recovered from mosquitoes. While transmission of arboviruses most often follows the bite of the infected arthropod, transmission has also been reported in other ways. European TBEV can be acquired by drinking the milk of infected goats, VEEV (in cotton rats) apparently via urine or faeces infecting the nasopharynx, WEEV possibly through aerosol from a patient and WNV and DENV has been transmitted by blood transfusion. DENV, JEV, WNV and CHIKV have all been transmitted from mother to foetus following infection during pregnancy, but this is considered rare. In contrast, an unusually high rate of maternal to foetal transmission has been observed in the recent ZIKV outbreak in Brazil. The finding of Zika virus in a

range of bodily fluids including semen, tears and sweat, as well as the apparently high rate of CNS invasion following foetal infection remains to be fully explained [18].

1.4 What Diseases Do They Cause?

The vast majority of arboviral infections lead to either an asymptomatic or non-specific mild illness. Only a handful of those who are infected develop clinical symptoms for which the individual arbovirus is known. For the flaviviruses, the case to infection ratio varies considerably, from very low (e.g. around 1:300 for encephalitis due to JEV) to quite high (1:4 for fever as a result of DENV infection). It may be higher during epidemic (rather than endemic) disease activity, and will be modified by a range of other factors, including host susceptibility and virus strain. The major burden of disease is at the extremes of life, the very young and the elderly. For alphavirus infections, particularly those causing arthritis, the ratio of symptomatic to asymptomatic infection is typically higher than that of the flaviviruses, from 1:40 to 1:3. If clinical manifestations arise after infection they do so after an intrinsic incubation period lasting from a few days to a week or more. During that time the virus replicates at the site of inoculation, then further amplifies within the reticuloendothelial system before it becomes viraemic and spreads to target organs.

Symptomatic arbovirus infection often presents as a systemic febrile illness. In the early stages, this illness may be non-specific or even suggestive of other viral illnesses, including gastrointestinal and respiratory infections. In a developing world setting featuring an increased burden of disease, this can be particularly problematic, often delaying appropriate clinical management. On-going development of low cost, point-of-care diagnostics to provide early and effective diagnosis, remains an important goal of current research efforts. Headache is common and may be severe and accompanied by meningitis. Muscle and joint aches and pains are common, especially with alphavirus infections where many also develop joint swelling and stiffness. Rash may be present and is usually generalised and maculopapular, although occasionally vesicular. Petechial rashes are less common and may be an early indicator of haemorrhagic fever. In the vast majority of cases, febrile illness is followed by recovery. In the remainder, illness may progress to one of the more severe forms of disease, sometimes following a few days of remission. These can be broadly grouped into those arboviruses causing haemorrhagic fever, encephalitis or polyarthralgic illness (for further discussion see [21]).

1.5 What Is Driving Arbovirus Expansion?

As noted above, humans are often no more than incidental hosts for arbovirus infection. However, their behaviour, along with environmental factors can play a significant role in the activity and spread of these viruses [20] with many human activities known to encourage transmission [4, 7, 17, 21]. The construction of dams and extensive areas of irrigation promotes the breeding of large numbers of mosquitoes that is otherwise unusual for these geographical locations. For instance, the development of rice fields encourages breeding of *Cx. tritaeniorhynchus* in Sarawak that in turn fosters the spread of JEV, and *Mansonia uniformis* and *Anopheles gambiae* in Kenya spreading CHIKV, o'nyong-nyong virus (ONNV) and Sindbis virus (SINV). The seasonal removal of old vegetation in Sarawak leads to heavily polluted pools that support large populations of culicines. Driving cattle into marginal forest areas in India promotes the growth and transport of ticks, and the incursion of people into forest areas exposes them to infection with YFV and the tick-borne diseases. In many countries, the practice of using large containers for water storage has helped to increase *Aedes aegypti* populations and the consequent transmission of DENV, CHIKV and other viruses vectored by this species.

Environmental conditions, particularly rainfall, temperature and humidity, also have an important role to play in arbovirus transmission

cycles with the result that arbovirus activity is often seasonal. For example, the alphaviruses transmitted by mosquitoes in temperate regions cause disease in summer during periods of increased vector activity [2]. In tropical areas, human infections caused by arboviruses usually occur during the wet season, with increased virus activity again coinciding with periods of high vector numbers. As mosquito larvae and pupae are aquatic, the abundance of arthropod vectors is directly affected by the amount of rainfall and flooding in a particular region. Rainfall is also required to maintain permanent water bodies, or in some cases create temporary water bodies that provide a sanctuary and breeding grounds for water birds that act both as mechanisms for introducing the virus into that area and for amplifying the virus. Humidity can also play a role, with increased humidity facilitating increased survival of mosquitoes. Temperature can also affect the length of the extrinsic incubation period with most studies showing that the extrinsic incubation period for mosquitoes is shorter at 30 °C than at lower temperatures thereby ensuring that mosquitoes become 'infectious' in a shorter time after ingestion of an infected blood meal. High external temperatures on the other hand may have adverse effects on vector survival.

Global climate change will significantly impact on arbovirus transmission cycles over time [7]. The amount and extent of rainfall, frequency and heights of high tides, temperature, humidity and consequent movement of vertebrate hosts and human populations will all contribute. The extent and timing of these environmental changes is unknown, but because of the complex interactions between these viruses, their hosts and vectors as well as the environment, it is likely that even minor changes will affect arbovirus activity in different regions. This may result in an increased number of cases and/or a greater geographical spread of these viruses [5, 12–14]. Climate change impacts on arbovirus transmission are already being played out, such as in the dramatic resurgence of West Nile virus in the US in 2012. This emergence was linked to a record-breaking drought across the US in combination with sporadic, end of season rains and local complacency with regards vector control. Mosquito numbers in metropolitan areas surged, with consequent increased transmission of WNV.

As noted above, the last two decades have seen a dramatic increase in the emergence and/or re-emergence of a number of serologically distinct arboviruses [6, 15, 21]. Ecological factors have played a pivotal role in this expansion with a rich array of demographic, cultural and societal changes impacting arbovirus transmission between vectors and hosts. Understanding some of these mechanisms will provide insight into future predictions of arboviral activity, disease risk assessment and control.

Southeast Asia has experienced an exponential increase in the number of arbovirus related epidemics; YFV and RVFV cases are on the rise in Africa; South America has seen the re-emergence of DENV and YFV and the emergence of ZIKV; and the incursion into North America and Europe of some arboviruses previously restricted to the tropical zone (e.g., CHIKV and DENV) all serve to emphasize that no region of the globe is resistant to these threats. Their spread has been linked to a range of complex factors.

It is recognized that biodiversity plays an important role for arbovirus maintenance with African, Southeast Asian and South American tropical regions, particularly their rainforests, considered reservoirs for many of these arboviruses. However, it is the demographic and societal changes in the human population during the past two to four decades that has had the biggest impact on the revival of arbovirus infections. Unprecedented population growth has been the underlying driver of many of the changes that have affected transmission dynamics. These include rapid urbanization, deforestation, new dams, an expansion in irrigation, and a lack of closed water storage containers. The resulting increase in mosquito populations and their closer contact with human communities has contributed to increased virus, and hence disease transmission. The changing demographics that have resulted from modern transportation have also played a significant role in the distribution and transmission dynamics of arboviruses. While the

geographic distribution of some arboviruses and their mosquito vectors has expanded, resulting in recurrent and larger outbreaks (e.g., DENV), others have invaded new geographic regions having taken advantage of susceptible mosquito vectors and hosts to become established (e.g., WNV, CHIKV and ZIKV). Clearly, factors such as the absence of herd immunity and a lack of vector control have been instrumental in the re-emergence of several arboviral infections (e.g., CHIKV, JEV, and more recently, ZIKV).

The changing epidemiological patterns of arboviruses are complex and unique to each virus, however virus evolution can also be an important driver of the emergence of these new disease threats. One clear example of how virus evolution has re-defined the epidemiology of an arbovirus infection is the re-emergence and spread of CHIKV. Sequence analyses have shown that CHIKV originated from Africa and was later introduced in to Asia with the delineation of three phylogenetic distinct clusters: East-, Central- and South-African (ECSA), Asian, and West-African clusters [10]. Analysis of CHIKV strains isolated from the Indian Ocean outbreaks indicated that it was more closely related to the ECSA cluster than the Asian or West African clusters. However, 90% of the CHIKV strains isolated revealed a nucleotide mutation leading to an alanine to valine change at position 226 in the virus E1 glycoprotein. This single amino acid change was of particular interest as it was exclusively found in CHIKV isolated from *Ae. albopictus*. This mutation was subsequently shown to be associated with adaptation to *Ae. albopictus* with an increased fitness in this vector attributable to the loss of cholesterol dependence for virus growth. This adaptation has allowed CHIKV to replicate and disseminate more efficiently in *Ae. albopictus*.

More recently, another arbovirus that has generated significant interest is ZIKV. First isolated from sentinel primates in the Zika forest of Uganda in 1947, it was also isolated in sub-Saharan Africa and South East Asia [8]. Few human cases were previously noted but in 2007, major human outbreaks were reported on Yap Island, Micronesia. Preliminary phylogenetic data showed two distinct ZIKV lineages circulating in Africa and a third lineage formed by the Micronesia and Malaysia strains [8]. The subsequent spread of ZIKV to the Americas in 2015 and the extensive epidemic it caused is now being attributed, in part, to specific mutations found in these circulating South American viruses.

1.6 Conclusion

In a world of rapid travel and transportation, many other arboviruses have the potential to spread geographically and cause serious outbreaks. What is of concern is that most of these new introductions are not detected until an epidemic or some unusual situation signals the alarm, often too late to effect control. The world is finally coming to grips with the notion of epidemic preparedness and the realization that significant and coordinated effort will be required to effectively deal with the inevitable future threats to global health posed by arboviruses on the move.

Acknowledgements This brief overview has been adapted, in part from [21]. The author wishes to express his thanks for ongoing funding support from the Australian National Health and Medical Research Council and to ViralZone, SIB Swiss Institute of Bioinformatics for granting their permission to use the virus schematics in Fig. 1.1.

References

1. Calisher CH, Karabatsos N (1988) Arbovirus serogroups: definition and geographic distribution. In: Monath TP (ed) The Arboviruses: epidemiology and ecology, vol I. CRC Press, Florida, pp 19–58
2. Calisher CH (1995) Alphavirus infections (family Togaviridae). In: Porterfield JS (ed) Kass handbook of infectious diseases: exotic viral infections. Chapman & Hall, London, pp 1–18
3. Claflin SB, Webb CE (2015) Ross river virus: many vectors and unusual hosts make for an unpredictable pathogen. PLoS Pathog 11(9):e1005070
4. Dunn FL (1988) Human factors in arbovirus ecology and control. In: Monath TP (ed) The Arboviruses: epidemiology and ecology, vol I. CRC Press, Florida, pp 281–290

5. Githeko AK, Lindsay SW, Confalonieri UE, Patz JA (2000) Climate change and vector-borne diseases: a regional analysis. Bull World Health Organ 78:1136–1147
6. Gonzalez JP (1997) Arboviruses and related viruses as emerging pathogens in Southeast Asia. In: Saluzzo JF, Dodet B (eds) Factors in the emergence of Arbovirus diseases. Elsevier, Paris, pp 117–122
7. Gould EA, Higgs S (2009) Impact of climate change and other factors on emerging arbovirus diseases. Trans R Soc Trop Med Hyg 103:109–121
8. Haddow AD, Schuh AJ, Yasuda CY, Kasper MR, Heang V, Huy R, Guzman H, Tesh RB, Weaver SC (2012) Genetic characterization of Zika virus strains: geographic expansion of the Asian lineage. PLoS Negl Trop Dis 6:e1477
9. Gould E, Pettersson J, Higgs S, Charrel R, de Lamballerie X (2017) Emerging arboviruses: why today? One Health 4:1–13
10. Her Z, Kam YW, Lin RTP, Ng LFP (2009) Chikungunya: a bending reality. Microbes Infect 11:1165–1176
11. Mayer SV, Tesh RB, Vasilakis N (2017) The emergence of arthropod-borne viral diseases: a global prospective on dengue, chikungunya and zika fevers. Acta Trop 166:155–163
12. McMichael AJ, Woodruff RE, Hales S (2006) Climate change and human health: present and future risks. Lancet 367:859–869
13. Mills JN, Gage KL, Khan AS (2010) Potential influence of climate change on vector-borne and zoonotic diseases: a review and proposed research plan. Environ Health Perspect 118:1507–1514
14. Morin CW, Comrie AC (2013) Regional and seasonal response of a West Nile virus vector to climate change. Proc Natl Acad Sci USA 110:15620–15625
15. Pfeffer M, Dobler G (2010) Emergence of zoonotic arboviruses by animal trade and migration. Parasit Vectors 3:35–50
16. Porterfield JS (1986) Comparative and historical aspects of the Togaviridae and Flaviviridae. In: Schlesinger S, Schlesinger MJ (eds) The Togaviridae and the Flaviviridae. Plenum Press, New York, pp 1–19
17. Simpson DIH (1972) Arbovirus diseases. Br Med Bull 28:10–15
18. Sharma A, Lal SK (2017) Zika virus: transmission, detection, control, and prevention. Front Microbiol 8:110
19. Shi M, Lin X-D, Vasilakis N, Tian J-H, Li C-X, Chen L-J, Eastwood G, Diao X-N, Chen M-H, Chen X, Qin X-C, Widen SG, Wood TG, Tesh RB, Xu J, Holmes EC, Zhang Y-Z (2016) Divergent viruses discovered in arthropods and vertebrates revise the evolutionary history of the Flaviviridae and related viruses. J Virol 90:659–669
20. World Health Organization (1985) Arthropod-borne and rodent-borne viral diseases. Report of a WHO Scientific Group. World Health Organisation Technical Report Series 719: 1–114
21. Young PR, Ng LFP, Hall RA, Smith DW, Johansen CA (2014) Arbovirus infections. In: Farrar J, Hotez PJ, Junghans T, Kang G, Lalloo D, White NJ (eds) Manson's tropical diseases, 23rd edn. Elsevier, Amsterdam, pp 129–161

2 Historical Perspective of Arboviruses in Mozambique and Its Implication for Current and Future Epidemics

Eduardo Samo Gudo, Kerstin Falk, and Julie Cliff

Abstract

Mozambique is a tropical country situated in the Southern part of Africa, a region where data on the burden and epidemiology of arbovirus is presently quite scarce although the frequency of outbreaks caused by arboviruses is rapidly increasing. Outbreaks of dengue fever have been reported in Mozambique, Angola and Tanzania and a recent unprecedented outbreak of Yellow fever has been recorded in Angola. These new outbreaks collectively suggest that arboviruses, and specifically flavivirus infections, are endemic in Mozambique.

Although recent data on arbovirus activity is scarce, the work of *Kokernot* et al. [R.H. Kokernot, K.C. Smithburn, A.F. Gandara, B.M. Mc'Intosh and C.S. Heymann Anais Inst Med Trop (1960), 17:201–230] describes sero-epidemiological and entomological studies carried out in several parts of Mozambique during the 1950s. Complementary seroepidemiological investigations on arboviruses that were conducted in the early 1980s also found serological evidence of several arboviruses which included Dengue, Chikungunya, Zika, Rift Valey Fever, Sinbdis virus, Wesselsbron, Bunyamwera, Pongola and Bawamba Fever and Yellow Fever.

Notably the first description of Chikungunya virus in 1952–1953 in Tanzania also included reported cases in northern Mozambique. Furthermore, DENV serotype 3 was for the first time described in northern Mozambique in 1984 and 1985. Since several arboviral infections result in acute self limiting fever they have remained unsuspected for several decades. However, it is well known that during the 1980's intensive malaria control initiatives which included massive distribution of bed nets, community education and indoor and outdoor spraying campaigns were implemented. It is possible that these measures may have influenced the epidemiology of arboviruses. However, the impact of these interventions in controlling the spread of arboviruses is not known.

In conclusion, the old literature on arboviruses in Mozambique is relevant for assessing the gaps and current risk of occurrence of these pathogens at the region, particularly in a time in which they are spreading worldwide.

E. S. Gudo (✉)
National Institute of Health, Maputo, Mozambique

K. Falk
The Public Health Agency of Sweden and Karolinska Institute, Solna, Sweden

J. Cliff
Faculty of Medicine, Eduardo Mondlane University, Maputo, Mozambique

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_2

Keywords

Arboviruses · *Dengue* · Chikungunya · Zika virus · Malaria seroepidemiology

2.1 General Description of Mozambique in the Context of Spread of Arboviruses

Mozambique is situated on the southeast coast of Africa, with a total land area of around 800,000 sq. km, which is slightly less than the size of the state of California, and has a population of close to 30 million people. The climate is tropical with two distinct seasons: the rainy season from November through April and the dry season during the rest of the year. Due to its geographical location, Mozambique has long been considered a unique and important hub-"tropical corridor" – for the movement of arboviruses in the region. Its ecological characteristics combined with its geographical location, make Mozambique a player in the international epidemiology and spread of arboviruses. Mozambique is a multi-cultural country with strong commercial and cultural trade with different regions in the world, specially with Asia where strong relationships with countries such as India and China have been established. Because of this, Mozambique is at particular risk of importation from and exportation of infectious diseases to Asia and other places in the region. For this reason, tracking the epidemiology of arboviruses in Mozambique is of utmost importance to understanding the regional and global pattern of their spread. Historical data demonstrate an active arboviral acitivity in Mozambique, which may have played an important role in the current epidemiology of arboviruses in the region and other continents with intense past and current trade links with Mozambique. In this context, we review the detailed studies in order to identify the gaps that exist at present to control arbovirus activity in Mozambique.

The seroepidemiological study conducted by [9] was an exhaustive investigation based on detecting, in residents of Mozambique, neutralizing antibodies against arboviruses that had previously been isolated in East Africa and the Union of South Africa. The study was undertaken to obtain information on the etiology of "unexplained fevers", so that useful information can be obtained, and to draw the attention of the medical authorities to the potential role of these viruses as causal agents of human diseases in Mozambique [9].

Arbovirus Activity in Mozambique The study conducted by Kokernot et al. was carried out in the months of July and August of 1957 and covered 29 different localities stretching over 8000 km of the entire country of Mozambique (Fig. 2.1). The map in the figure shows that both coastal and interior terrains were sampled. In this study Kokernot et al. collected blood samples from indigenous Mozambicans and then tested for different arboviruses using serological assays for detection of neutralizing antibodies. A total of 29 localities across the country were surveyed, which gives a good representativeness of the country.

The study enrolled 30 local residents in each of the selected locations who had never traveled outside the residence area in their lifetime. These samples were subjected to neutralization tests with 15 different viruses and a summary of the results is presented in Table 2.1. The viruses with high prevalence rates of neutralizing antibodies included Chikungunya (21.0%), Wesselsbron (15.9%), Bunyamwera (24.1%), Pongola (23.2%) and Bwamba Fever (24.7%) (Table 2.1).

This study, together with a concurrent entomological survey conducted in several localities across the country by [17] between 1957 and 1959, found that *Aedes* mosquitoes with potential to transmit different species of arbovirus were present in several parts of the country. The fact that these two studies were conducted in the same period, reinforced significantly the belief that the country was an important tropical corridor with potentially high arboviral activity. However, since then, research into arbovirus prevalence has been abandoned in Mozambique and there are no

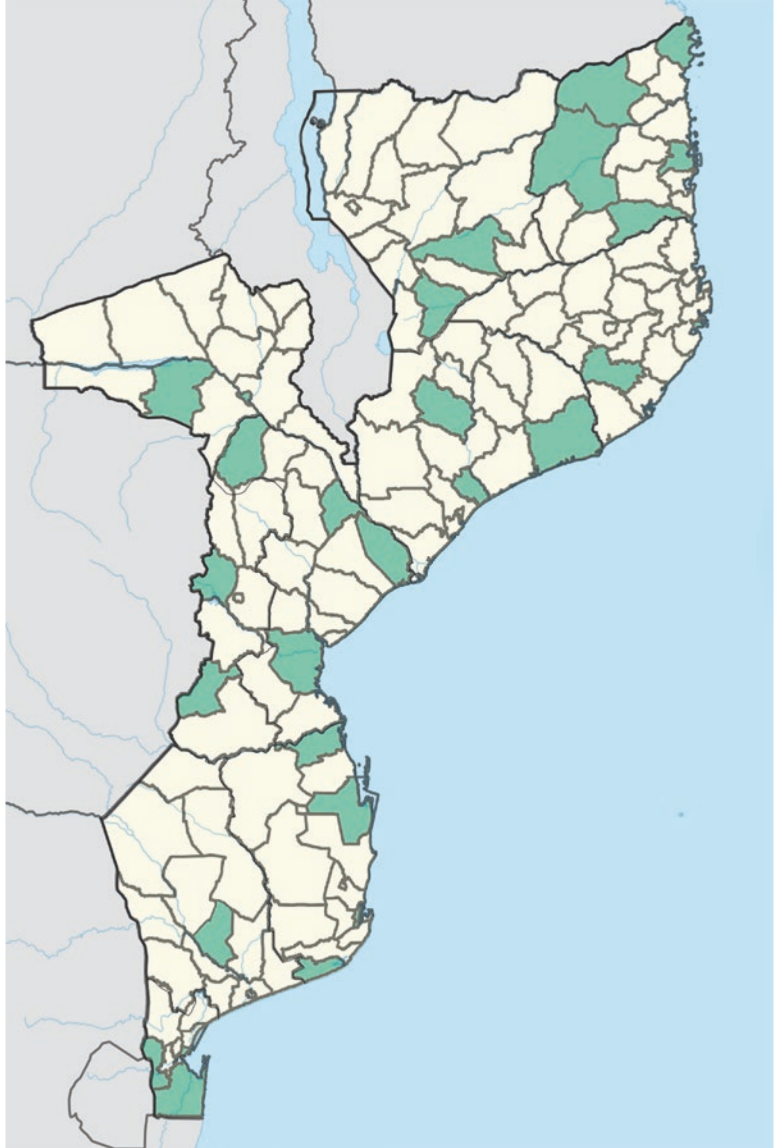


Fig. 2.1 Administrative districts of Mozambique. In 1957, shaded areas have been surveyed for CHIKV antibodies (all with positive results) by [9]. (Taken from [5]. © Gudo, E.S.. Original image credit: Americo Feriano Jose)

records of similar detailed surveys covering the entire country. This long drought in information on arboviral activity was broken very recently when a Dengue outbreak caused by Dengue serotype 2 was recorded in Pemba city in northern Mozambique [11], where a high density of *Aedes* was also found during the outbreak [8]. There is strong reason to believe that several other outbreaks of dengue and other arboviruses occurred in the 1980s and 1990s, but due to lack of diagnostic capacity and lack of awareness as well as other health priorities, they were under-reported.

2.2 Initial Report of Zika in Mozambique

Public health interest in ZIKV has increased dramatically since 2015 when the virus experienced an explosive spread in south and central America and the Caribbean [3, 12], with reports of its association with an increase of cases of microcephaly and other neurologic disorders in Brazil prompting the World Health Organization (WHO) to declare the ZIKV epidemic in South

Table 2.1 Summary of neutralization tests performed with 15 viruses in the presence of sera from indigenous donors living in Mozambique. [9]

Virus per group	Children			Adults			All ages		
	Number of participants	Neut.poisitive		Number of participants	Neut.poisitive		Number of participants	Neut.poisitive	
		No.	%		No.	%		No	%
Group A									
Chikungunya	404	16	4.0	467	175	37.5	871	191	21.9
Semliki	388	13	3.4	449	31	6.9	837	44	5.3
Sindbis	399	21	5.3	456	43	9.4	855	64	7.5
Middelburg	142	1	0.7	160	2	1.3	302	3	1.0
Group B									
Wesselsbron	403	40	9.9	467	98	21.0	870	138	15.9
Spondweni	399	5	1.3	459	30	6.5	858	35	4.1
H 366	404	10	2.5	467	29	6.2	871	39	4.5
Uganda S	151	2	1.3	185	10	5.4	336	12	3.6
Zika	107	2	1.9	142	8	5.6	249	10	4.0
Bunyamwera group									
Bunyamwera	404	66	16.3	466	144	30.9	870	210	24.1
Without group									
Pongola	403	65	16.1	467	137	29.3	870	202	23.2
Bwamba fever	149	20	13.4	142	52	36.6	291	72	24.7
Rift valley fever	397	5	1.3	436	18	4.1	833	23	2.8
Simbu	57	0	0	61	0	0	118	0	0
AR 344	53	0	0	57	0	0	110	0	0

Note: This table is translated and reproduced from Ref. [9]

America a Public Health Emergency of International Concern (PHEIC) [15].

Despite the fact that ZIKV was first described in Africa in 1947 [13] few studies had been conducted on the continent to understand its epidemiology. However, the study published by [9] was one of the few conducted on the continent at that time and demonstrated that neutralizing antibodies against ZIKV were found in Mozambicans in several parts of the country [6]. We recently noticed that the Kokernot et al. study had been ignored by most current descriptions or reviews on historical data of ZIKV on the continent and worldwide [7, 16]. We believe that the study conducted by Kokernot et al. has been ignored, most likely because the manuscript was published in Portuguese and also due to its poor indexing [6]. However this study highlights that Mozambique was considered a hotspot for the occurrence of arboviruses, including ZIKV since the 1950s, and may have played a role in the spread of the virus in the region and to other regions. Although no recent study has been conducted to assess circulation of ZIKV in Mozambique and few studies had been conducted in the region, old data on ZIKV in Mozambique may suggest that the risk of current circulation of the virus in Mozambique is considerable.

Mozambique does not have a surveillance system for birth defects, which makes it difficult to correlate any potential link between microcephaly cases and Zika. On the other hand, there is much discussion in the scientific arena suggesting that the most studied African strains of ZIKV may not be involved in causing microcephaly. To confirm this, studies should be conducted in Mozambique and other countries in sub- Saharan Africa using newly isolated Zika virus strains as

well as historic isolates to investigate the potential for these strains to cause Zika-associated microcephaly.

2.3 First Report of Chikungunya in 1953 Is Tightly Bounded to Mozambique

Chikungunya virus (CHIKV) has re-emerged in 2005 as an important cause of infectious disease, mostly after the occurrence of large epidemics of CHIKV on the Indian Ocean islands and in Asia [2, 14]. Since then, the virus has rapidly expanded to become endemic in South America [14]. We revisited old literature to understand the potential role of Mozambique in the current global epidemiology of CHIKV and found that indeed, during the initial discovery of CHIKV in 1952/3 in Tanzania, a few cases were also reported in villages in northern Mozambique. This is reflected in the serosurvey by [9] that, conducted a few years later, detected neutralizing antibodies for CHIKV in all sampled places with a prevalence rate of 21.9%. Given the geographical situation, together with its commercial trade relationships to other parts of the region and the world, there is the potential that Mozambique may have contributed to the spread of CHIKV. Molecular epidemiological studies would be needed to explore this further.

The recent report of a severe case of CHIKV case with intestinal bleeding in northern Mozambique [1] suggests that several cases of severe CHIKV infection had been under-reported or misdiagnosed as malaria for many years and decades. Severe disease caused by CHIKV had not been considered until 2005 when a massive CHIKV outbreak hit Reunion Island [10]. This recent report of a severe case of CHIKV has increased the concern that CHIKV may be the etiological agent of severe disease in sub-Saharan Africa, where similar reports have not been published, mostly due to lack of a surveillance systems for CHIKV.

2.4 First Report of Dengue-3 in Mozambique

Dengue is the most-widespread arbovirus worldwide and historical data on its epidemiology is important to understand current and future trends of the virus. The epidemiology of Dengue is complex due to serological and genetic diversity. Mozambique has played an important role in the global epidemiology of Dengue, as the first time that Dengue serotype 3 was described in Africa was during a 1984/5 outbreak of febrile illness in Pemba city, the capital of Cabo Delgado Province in northern Mozambique [4]. This further suggests that northern Mozambique was in the past a hotspot of arboviral activity. Since Mozambique has intense trade with countries in the region as well as in Asia and South America, we believe that the country played a relevant role in the spread of this serotype. Of the two deaths reported during this outbreak, one was a Chinese traveler, and the strain circulating in Mozambique was later shown to be similar to that circulating in southeast Asia, suggesting that trade with China and other countries in Asia could have played an important role in the import of dengue-3 into Africa in the early 1980s. An entomological investigation conducted during this outbreak showed a high density of *Aedes aegypti* [4] suggesting that Mozambique had ecological conditions favorable for *Aedes* breeding and arbovirus transmission.

2.5 Summary

The presentation revisits history of arboviruses in Mozambique and highlights that the country may have played an important role in spread of several arboviruses such as Dengue and Zika in sub Saharan Africa and other regions. Despite of this, research and investigation on arbovirus in Mozambique was abandoned for several decades due to a changed focus to the spread of HIV/AIDS and the continuing challenges of parasitic

diseases like malaria. For this reason, there is a current lack of knowledge of arboviral activity in Mozambique. The TASW meeting highlights the past and current importance of arboviruses and the need to implement surveillance similar to the work done by [9] to better understand the current risk of arbovirus in the country and region.

Acknowledgement The authors thank the library of the National Institute of Health (Mozambique) for providing old literature about arbovirus in Mozambique.

Discussion of Chapter 2 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Eduardo Samo Gudo.

Aravinda de Silva: Just a quick comment about those Dengue 3 strains from the mid 1980s and their importance for Mozambique, because we showed subsequently that those strains were first described in Mozambique. I think some of those isolates came from Chinese workers in Mozambique and they spread it to the Indian subcontinent. In fact, the first major epidemic of severe Dengue caused by serotype 3 in the Indian subcontinent was very closely related to those strains from Mozambique. And then subsequently, those same strains were introduced into Panama and Nicaragua. And the severe Dengue epidemics in Latin America were also caused by a very close relative of the Mozambique viruses and so this highlights the importance of the circulating strains here in Mozambique. What kind of work is going on in terms of looking at enzootic cycles? For Dengue 3, we still do not know the sylvatic cycle – the non-primate cycle. Also for Chikungunya virus, you indicated that very early isolates came from this part of the world. Is there any work being done in this area?

Eduardo Samo Gudo: Not yet. This is the first and only description of Dengue 3 in Mozambique as you can see in the map in my presentation. So nothing happened after that and it's unclear and we do not know if we eliminated DENV3 from Mozambique.

Aravinda de Silva: In terms of understanding the enzootic cycles of Dengue and Chickungunya – it may be an important area to focus in terms of non-human primate studies.

Gao George: So where did the strains in Mozambique come from?

Aravinda de Silva: That strain was first described in East Africa in Mozambique. It was subsequently described in Kenya, it went up to Somalia. It moved into the Indian subcontinent, where the very first description of the 1985 isolates came from.

Subhash Vasudevan: So you said it came from China?

Aravinda de Silva: No, I thought the Mozambican isolates came from Chinese workers in Mozambique, who got very sick, who got something like severe Dengue. But they were here working in Mozambique.

Maurice Demanou: It is incredible, because the figures you presented are similar to the picture in Cameroon. But in the 60's there was a lot of research on arboviruses done by medical research institutes. Later on in the 80's, probably because of the outbreak of HIV, the arbovirus surveillance ceased. Even though there were some surveillances on yellow fever, nothing at all was done for other arbovirus research. In the year 2000, with the spread of epidemic arboviruses worldwide, people started to be interested. I think we have the same problem in Cameroon, but I do not know how you explain the 40 years' gap in the attention.

Félix Rey: Is there a political will now to change the situation and focus more on arboviruses?

Eduardo Samo Gudo: Arboviruses are seriously neglected in Mozambique, as malaria, tuberculosis and HIV are the leading cause of morbi-mortality in the country and will for sure be the focus of intervention for many

decades to come. Not just in Mozambique, this is similar in many countries in Africa. When we raise the issue of arboviruses, we are simply asked the question "How many people die per year because of Dengue and other arboviruses and how many from malaria, tuberculosis and HIV?" The fact is that it is much less than malaria, tuberculosis and HIV, so that is the end of he conversation.

Subhash Vasudevan: What about co-infections of malaria with Chickungunya or Dengue?

Eduardo Samo Gudo: There is high coinfection rates between malaria and arboviruses. This actually is something that we are very interested in, but I did not show any data here in the talk. If you are familiar with asymptomatic malaria infection – it's a problem that is mostly being ignored. We did some research in Mozambique, back in 2005, where we went to our primary schools in areas of high transmission for malaria. We took blood samples from the children – otherwise healthy looking children – and we tested for malaria not using rapid tests, but by using blood smear. We found that 55% of the children showed parasitemia in the blood and then there were some with high parasitemia in their blood. So coming back to your question of coinfection: People that have immunity against malaria, because of the continous exposure, can have parasitemia and not be sick. Imagine now if they got fever and they go to the hospital. The hospital will test first for malaria and 55% of the children that go to the hospital will be positive but mainly have other causes of fever. So that means that they will never be picked for other possible infections. It is not bad that the patients are treated for malaria because of their parasitemia, because at some point they can develop malaria or they can transmit. But other diseases would be ignored and arbovirus problems will expand. It is amazing that in several parts of Mozambique where we have carried our vector survellence studies, the population of *Aedes* is high, but the control is based on Anopheles. So arborviral diseases are ignored and have been ignored for very long because of these reasons.

Félix Rey: Is there any interference between the circulating parasites and arboviruses? Nobody knows?

Eduardo Samo Gudo: That's right, nobody knows.

References

1. Aly MM, Ali S, Muianga AF, Monteiro V, Gallego JG, Weyer J et al (2017) Severe Chikungunya infection in northern Mozambique: a case report. BMC Res Notes 10(1):88. https://doi.org/10.1186/s13104-017-2417-z. PubMed PMID: 28179029; PubMed Central PMCID: PMCPMC5299683
2. Burt FJ, Rolph MS, Rulli NE, Mahalingam S, Heise MT (2012) Chikungunya: a re-emerging virus. Lancet 379(9816):662–671. https://doi.org/10.1016/S0140-6736(11)60281-X. PubMed PMID: 22100854
3. Chang C, Ortiz K, Ansari A, Gershwin ME (2016) The Zika outbreak of the 21st century. J Autoimmun 68:1–13. https://doi.org/10.1016/j.jaut.2016.02.006. PubMed PMID: 26925496
4. Gubler DJ, Sather GE, Kuno G, Cabral JR (1986) Dengue 3 virus transmission in Africa. Am J Trop Med Hyg 35(6):1280–1284. PubMed PMID: 3789276
5. Gudo ES, Black JF, Cliff JL (2016) Chikungunya in Mozambique: a forgotten history. PLoS Negl Trop Dis 10(11):e0005001. https://doi.org/10.1371/journal.pntd.0005001. PubMed PMID: 27855168; PubMed Central PMCID: PMCPMC5113865
6. Gudo ES, Falk KI, Ali S, Muianga AF, Monteiro V, Cliff JA (2016) Historic report of Zika in Mozambique: implications for assessing current risk. PLoS Negl Trop Dis 10(12):e0005052. https://doi.org/10.1371/journal.pntd.0005052. PubMed PMID: 27930650; PubMed Central PMCID: PMCPMC5145135
7. Gyawali N, Bradbury RS, Taylor-Robinson AW (2016) The global spread of Zika virus: is public and media concern justified in regions currently unaffected? Infect Dis Poverty 5(1):37. https://doi.org/10.1186/s40249-016-0132-y. PubMed PMID: 27093860; PubMed Central PMCID: PMCPMC4837632
8. Higa Y, Abilio AP, Futami K, Lazaro MA, Minakawa N, Abundant Aedes GES (2015) (Stegomyia) aegypti aegypti mosquitoes in the 2014 dengue outbreak area of Mozambique. Trop Med Health 43(2):107–109. https://doi.org/10.2149/tmh.2014-29. PubMed PMID: 26060423; PubMed Central PMCID: PMCPMC4458510
9. Kokernot RH, Smithburn KC, Gandara AF, McIntosh BM, Heymann CS (1960) Neutralization tests with sera from individuals residing in Mozambique against specific viruses isolated in Africa, transmitted by arthropods. Inst Med Trop (Lisb) 17:201–230. PubMed PMID: 13757663

10. Larrieu S, Balleydier E, Renault P, Baville M, Filleul L (2012) Epidemiological surveillance du chikungunya on Reunion Island from 2005 to 2011. Med Trop (Mars) 72 Spec No:38–42. PubMed PMID: 22693926
11. Massangaie M, Pinto G, Padama F, Chambe G, da Silva M, Mate I et al (2016) Clinical and epidemiological characterization of the first recognized outbreak of dengue virus-type 2 in Mozambique, 2014. Am J Trop Med Hyg 94(2):413–416. https://doi.org/10.4269/ajtmh.15-0543. PubMed PMID: 26643534; PubMed Central PMCID: PMCPMC4751938
12. Musso D, Gubler DJ (2016) Zika virus. Clin Microbiol Rev 29(3):487–524. https://doi.org/10.1128/CMR.00072-15. PubMed PMID: 27029595
13. Simpson DI (1964) Zika virus infection in man. Trans R Soc Trop Med Hyg 58:335–338. PubMed PMID: 14175744
14. Wahid B, Ali A, Rafique S, Idrees M (2017) Global expansion of Chikungunya virus: mapping the 64-year history. Int J Infect Dis 58:69–76. https://doi.org/10.1016/j.ijid.2017.03.006. PubMed PMID: 28288924
15. WHO Director-General summarizes the outcome of the emergency committee regarding clusters of microcephaly and Guillain-Barré syndrome [Internet]. 2016 [cited April 14, 2016]. Available from: http://www.who.int/mediacentre/news/statements/2016/emergency-committee-zika-microcephaly/en/
16. World Health Organization ROfA (2016) Zika virus risk assessment in the WHO African region: a technical report. World Health Organization, Regional office for Africa
17. Worth CB, de Meillon B (1960) Culicine mosquitoes (Diptera: Culicidae) recorded from the province of Mozambique (Portuguese East Africa) and their relationship to arthropod-borne viruses. An Inst Med Trop (Lisb) 17:231–256. PubMed PMID: 13786708

3 Reliable Serological Testing for the Diagnosis of Emerging Infectious Diseases

Claudia Ohst, Sandra Saschenbrecker, Konstanze Stiba, Katja Steinhagen, Christian Probst, Christiane Radzimski, Erik Lattwein, Lars Komorowski, Winfried Stöcker, and Wolfgang Schlumberger

Abstract

Climate change, increased urbanization and international travel have facilitated the spread of mosquito vectors and the viral species they carry. Zika virus (ZIKV) is currently spreading in the Americas, while dengue virus (DENV) and chikungunya virus (CHIKV) have already become firmly established in most tropical and also many non-tropical regions. ZIKV, DENV and CHIKV overlap in their endemic areas and cause similar clinical symptoms, especially in the initial stages of infection. Infections with each of these viruses can lead to severe complications, and co-infections have been reported. Therefore, laboratory analyses play an important role in differential diagnostics. A timely and accurate diagnosis is crucial for patient management, prevention of unnecessary therapies, rapid adoption of vector control measures, and collection of epidemiological data.

There are two pillars to diagnosis: direct pathogen detection and the determination of specific antibodies. Serological tests provide a longer diagnostic window than direct methods, and are suitable for diagnosing acute and past infections, for disease surveillance and for vaccination monitoring. ELISA and indirect immunofluorescence test (IIFT) systems based on optimized antigens enable sensitive and specific detection of antibodies against ZIKV, DENV and CHIKV in patient serum or plasma. In recent years, Euroimmun (Lübeck, Germany) has developed numerous test systems for the serological diagnosis of (re-) emerging diseases, including a very sensitive and specific anti-ZIKV ELISA.

Keywords

Antibody detection · Serological tests · Indirect immunofluorescence test · ELISA · NS1 antigen specific test for Dengue and Zika

C. Ohst · S. Saschenbrecker (✉) · K. Stiba
K. Steinhagen · C. Probst · C. Radzimski
E. Lattwein · L. Komorowski · W. Stöcker
W. Schlumberger
Institute for Experimental Immunology, Euroimmun AG, Lübeck, Germany
e-mail: s.saschenbrecker@euroimmun.de

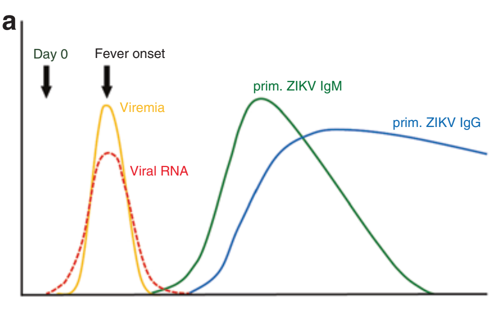
© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_3

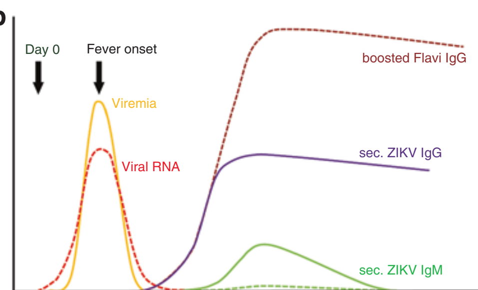
3.1 Laboratory Testing for ZIKV Infections

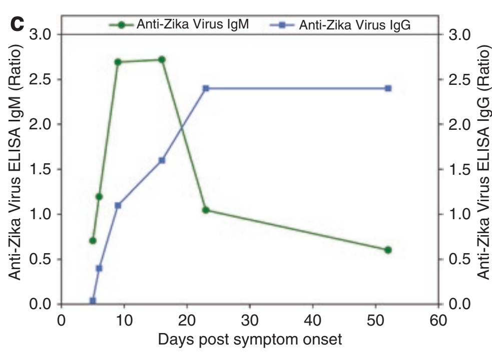
Diagnostic testing for ZIKV infection is accomplished using mainly genome detection and serological methods [48, 63, 71, 91]. Recently, a number of in-house and commercial *in vitro* diagnostic assays for direct and indirect ZIKV detection have been developed and widely applied in routine laboratories [95, 96]. However, no single test is capable of accurately diagnosing ZIKV infections over the whole course of disease. Rather, a combination of direct and indirect detection methods is preferable to either approach alone, with serological methods being effective from soon after clinical onset to beyond convalescence. In the differential diagnosis of ZIKV infections, evaluation for DENV and CHIKV should be included because of their clinical similarity and co-endemicity [60]. Careful result interpretation is crucial as it will guide the clinical management regarding, for example, possible adverse pregnancy outcomes.

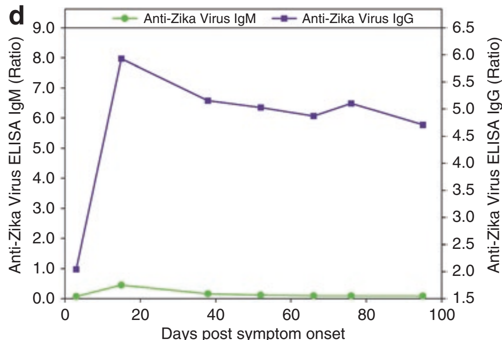
3.1.1 Direct ZIKV Detection

Only within the first week after symptom onset, ZIKV can be isolated from infected individuals via cell culture [2, 28, 33, 56]. Within the same time frame, highly specific reverse transcription-polymerase chain reaction (RT-PCR) assays allow for the detection of ZIKV RNA in serum samples [15, 46] (Fig. 3.1a, b). In saliva, urine, semen or amniotic fluid, the viral genome may be detectable for a prolonged time [4, 16, 37, 72]. In contrast to DENV diagnostics, where the determination of virus antigen is a common approach, ZIKV antigen assays are not yet available [54]. Due to the short viremic phase, the high number of asymptomatic infections and the frequent difficulty in precisely determining the onset date of









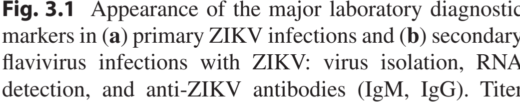


Fig. 3.1 Appearance of the major laboratory diagnostic markers in (**a**) primary ZIKV infections and (**b**) secondary flavivirus infections with ZIKV: virus isolation, RNA detection, and anti-ZIKV antibodies (IgM, IgG). Titer course of anti-ZIKV IgM and IgG in (**c**) a European traveler after primary infection with ZIKV, and (**d**) a Columbian resident who contracted ZIKV as secondary flavivirus infection

symptoms, the utility of direct ZIKV detection is limited. Negative results obtained by direct techniques do not rule out a ZIKV infection and require additional serological testing.

3.1.2 Indirect ZIKV Detection

Serology is the most commonly applied technique for diagnosing both recent and past ZIKV infections [48]. Serum samples obtained after the first week of clinical illness as well as samples that were tested negative or not tested by RT-PCR are most relevant for serological examination. If serology is negative in the first instance, particularly in the early phase of infection, antibody testing should be repeated on a serum sample taken between one and 2 weeks later. The detection of anti-ZIKV IgM, seroconversion, or a significant increase in the IgG titer in paired samples are indicative of an acute infection [48, 64, 67].

In primary ZIKV infections that often occur among travelers without previous flavivirus infection, anti-ZIKV IgM antibodies appear 4–7 days after disease onset, peak within 2 weeks, and remain detectable for approximately 12 weeks. Anti-ZIKV IgG antibodies appear shortly after IgM and are expected to persist lifelong (Fig. 3.1a, c) [4, 46, 81]. In contrast, most residents of ZIKV-endemic areas have preexisting antibodies against DENV or some other flavivirus (e.g., yellow fever virus) and contract ZIKV as a secondary flavivirus infection. According to our findings, these patients typically develop very high anti-ZIKV IgG titers early after the onset of symptoms, paralleled by a boost in IgG against the virus of the primary flavivirus infection, while the anti-ZIKV IgM response is often low or absent (Fig. 3.1b, d) [81]. Similar kinetics have been demonstrated for patients with secondary DENV infections, as described below. Considering the possibility of IgM negative results in the setting of secondary infections, the "IgM only" strategy, as currently recommended for ZIKV serological testing [60, 64], bears a high risk of misdiagnosis of active infections. Rather, additional IgG analysis is regarded mandatory [14, 75].

ZIKV-specific antibodies are most commonly analyzed by ELISA and IIFT, including in-house assays (e.g., CDC Zika MAC ELISA) and an increasing number of commercial tests. Immunochromatographic assays for point-of-care rapid diagnostic testing (RDT) are also available [95, 96] (Table 3.1). Most of these assays, however, are based on whole-virus antigen or virus glycoproteins and consequently suffer from extensive serological cross-reactivity with other flaviviruses (e.g., DENV, yellow fever, West Nile, and Japanese encephalitis virus), which leads to false-positive or uninterpretable data. Thus, cross-reactivity presents a major challenge for the interpretation of results and may preclude the identification of the specific infecting virus, especially in individuals previously infected with or vaccinated against a related flavivirus. Even when using plaque-reduction neutralization tests (PRNT) to confirm the diagnosis of ZIKV, as recommended in current testing algorithms, cross-neutralization cannot be excluded for secondarily-infected patients so that PRNT results may not always distinguish ZIKV from other flavivirus or multi-flavivirus infections [13, 17, 22, 46, 48, 69–71]. Therefore, efforts have been made in the development of widely applicable serological assays that provide high diagnostic sensitivity and specificity for ZIKV. So far, however, data reflecting assay performance is rare and has to be interpreted with caution as it depends on the validation cohort.

3.1.2.1 ZIKV Differential Diagnosis Using IIFT Biochip Mosaics

IIFT biochip mosaics (Euroimmun, Lübeck, Germany) provide a suitable test platform for standardized multiparametric testing, and have been developed and manufactured in cooperation with the Robert Koch Institute (RKI, Berlin, Germany). Every test field contains a combination of biochips each of which is coated with another substrate (virus-infected cells). Thus, in a single analysis, a patient sample can be examined simultaneously for antibodies against all infectious agents relevant for differential diagnosis, i.e. identifying one specific pathogen amongst pathogens occurring in the same geographic

Table 3.1 Available serological tests for the diagnosis of ZIKV infections (11/2016)

Company	Assay*	Ig class	Format	References
ADI	RecombiVirus Zika Virus Envelop antibody ELISA[c]	IgM	ELISA	
	RecombiVirus Zika Virus Envelop antibody ELISA[c]	IgG	ELISA	
	RecombiVirus Zika Virus Envelop domain III ELISA[c]	IgM	ELISA	
	RecombiVirus Zika Virus Envelop domain III ELISA[c]	IgG	ELISA	
	RecombiVirus Zika Virus PrM antibody ELISA[c]	IgM	ELISA	
	RecombiVirus Zika Virus PrM antibody ELISA[c]	IgG	ELISA	
	RecombiVirus Zika Virus NS1 antibody ELISA[c]	IgM	ELISA	
	RecombiVirus Zika Virus NS1 antibody ELISA[c]	IgG	ELISA	
	RecombiVirus Zika Virus Capsid antibody ELISA[c]	IgM	ELISA	
	RecombiVirus Zika Virus Capsid antibody ELISA[c]	IgG	ELISA	
	RecombiVirus Zika Virus Env+NS1+Prm+Capsid antibody Combo ELISA[c]	IgM	ELISA	
	RecombiVirus Zika Virus Env+NS1+Prm+Capsid antibody Combo ELISA[c]	IgG	ELISA	
Artron	One Step Zika Virus Test Kit[a]	IgM/IgG	ICA (RDT)	
Biocan	Zika Virus IgG/IgM Antibody Test[a]	IgM/IgG	ICA (RDT)	
	Zika/Dengue/Chikungunya Combo Tests[a]	IgM/IgG	ICA (RDT)	
Chembio	DPP Zika IgM/IgG Assay[a]	IgM/IgG	ICA (RDT)	
CD	Zika Virus IgM ELISA	IgM	ELISA	
CDC	Zika MAC-ELISA[b]	IgM	ELISA	[23, 25]
CTK Biotech	RecombiLISA Zika IgM Test[d]	IgM	ELISA	
DA/CD	OneStep Zika Virus IgG/IgM RapiCard[c]	IgM/IgG	ICA (RDT)	
DIACHECK	DIACHECK Anti-ZIKA IgM[c]	IgM	ELISA	
	DIACHECK Anti-ZIKA IgG[c]	IgG	ELISA	
	DIACHECK Anti-ZIKA IgA[c]	IgA	ELISA	
Dia.Pro	ZIKA Virus IgM[a]	IgM	ELISA	
	ZIKA Virus IgG[a]	IgG	ELISA	
	Zika Virus IgG Avidity[a]	IgG	ELISA	
DRG	Zika Virus IgM μ-capture ELISA	IgM	ELISA	

(continued)

Table 3.1 (continued)

Company	Assay*	Ig class	Format	References
Euroimmun	Anti-Zika virus IIFT[a]	IgM/IgG	IIFT	
	Arbovirus Fever Mosaic 2 IIFT[a]	IgM/IgG	IIFT	
	Arbovirus Profile 3 IIFT[a]	IgM/IgG	IIFT	
	Anti-Zika virus ELISA (IgM)[a]	IgM	ELISA	[2, 14, 34, 35, 38, 41, 55, 72, 81, 86, 100]
	Anti-Zika virus ELISA (IgG)[a]	IgG	ELISA	[41, 81, 86, 100]
IBL	Zika Virus IgM μ-capture ELISA[a]	IgM	ELISA	
InBios	ZIKV Detect IgM Capture ELISA[b]	IgM	ELISA	
LumiQuick	QuickProfile Zika Virus IgG/IgM Combo Test[a]	IgM/IgG	ICA (RDT)	
Mikrogen	alphaWell Zika Virus IgM μ-capture	IgM	ELISA	
	recomLine Tropical Fever IgM[a]	IgM	LIB	
	recomLine Tropical Fever IgG[a]	IgG	LIB	
MyBioSource	Qualitative Human Zika Virus IgM (ZV-IgM) ELISA[c]	IgM	ELISA	
	Qualitative Human Zika Virus IgG (ZV-IgG) ELISA[c]	IgG	ELISA	
NovaTec	NovaLisa Zika Virus IgM μ-capture ELISA[a]	IgM	ELISA	
R-Biopharm	RIDASCREEN Zika Virus IgM μ-capture	IgM	ELISA	
SD Biosensor	STANDARD E Zika IgM ELISA	IgM	ELISA	
	STANDARD Q Zika IgM/IgG	IgM/IgG	ICA (RDT)	
Viramed	Zika Virus ViraStripe IgM[a]	IgM	LIB	
	Zika Virus ViraStripe IgG[a]	IgG	LIB	
Vircell	ZIKV-DENV-CHIKV IFA IgM[a]	IgM	IIFT	
	ZIKV-DENV-CHIKV IFA IgG[a]	IgG	IIFT	
	ZIKA ELISA IgM[d]	IgM	ELISA	
	ZIKA ELISA IgG[d]	IgG	ELISA	
	ZIKA VIRCLIA IgM MONOTEST[d]	IgM	CLIA	
	ZIKA VIRCLIA IgG MONOTEST[d]	IgG	CLIA	
Viro-Immun	VIR-ELISA Zika Virus[c]	IgM	ELISA	
	VIR-ELISA Zika Virus[c]	IgG	ELISA	
	VIR-ELISA Zika Virus[c]	IgA	ELISA	

CLIA chemiluminescence assay, *ELISA* enzyme-linked immunosorbent assay, *ICA* immunochromatographic assay, *IIFT* indirect immunofluorescence test, *LIB* line immunoblot, *RDT* rapid diagnostic test

*Regulatory status (if available) according to suppliers' official websites and test instructions

[a]CE/IVD certified

[b]Authorized by FDA under an Emergency Use Authorization for use by authorized laboratories only [32]

[c]For research use only

[d]Coming soon

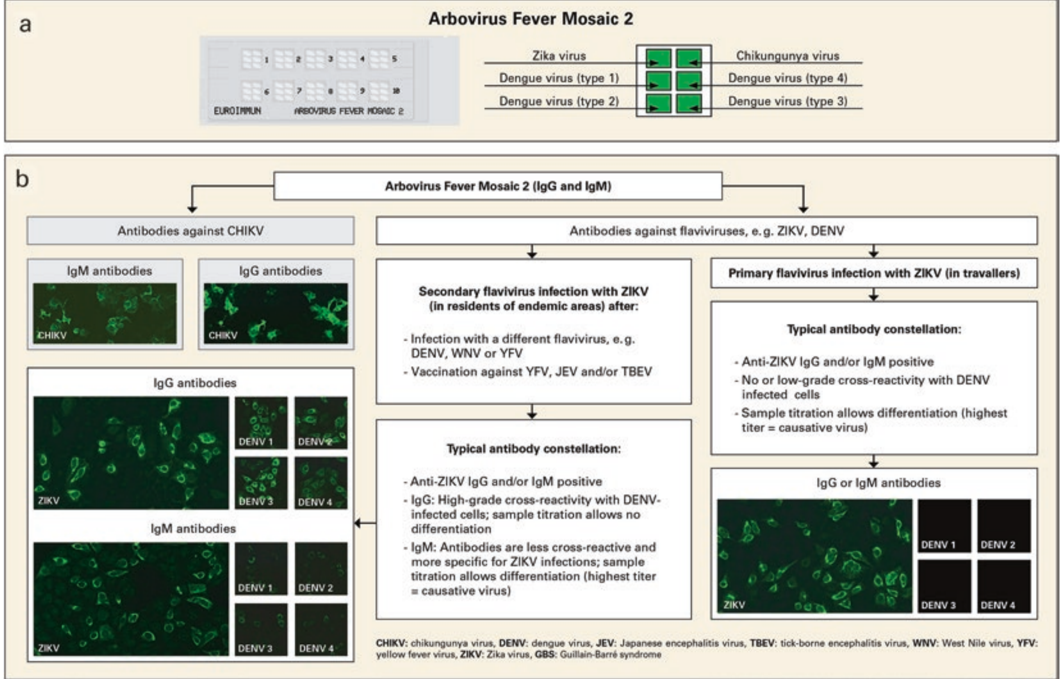


Fig. 3.2 Arbovirus Fever Mosaic 2 (Euroimmun) for the detection of antibodies against ZIKV, DENV and CHIKV by indirect immunofluorescence. (**a**) Microscope slide with ten analysis fields each containing six biochips coated with differently infected cells (ZIKV, DENV 1, DENV 2, DENV 3, DENV 4, CHIKV). (**b**) Flow chart for the differential diagnosis of arbovirus infections, focusing on the characteristic antibody constellations and reactivity patterns in primary and secondary ZIKV infections

region and causing similar clinical symptoms. Further advantages as compared to in-house IIFTs are high-level standardization for increased reproducibility among patients and laboratories, rapid performance, storability, and inclusion of uninfected control cells for the recognition of unspecific reactivity.

The **Euroimmun Arbovirus Fever Mosaic 2 IIFT** consists of a combination of six cell substrates infected with ZIKV, CHIKV or DENV serotypes 1–4 (Fig. 3.2a). The mosaic's diagnostic performance is indicated in Table 3.2, including assay sensitivity in detecting confirmed infections with ZIKV, DENV or CHIKV, as well as assay specificity among healthy blood donors from non-endemic areas. The combination of antigenically related viruses on adjacent biochips also enables the investigation of potentially cross-reactive antibodies: Whilst there is generally no or only low-grade cross-reactivity in a primary flavivirus infection, high grade cross-reactivity is typical for secondary infections (e.g., a ZIKV infection following a DENV infection), usually being stronger for IgG than for IgM antibodies. Titration of the patient sample on the mosaic may enable the determination of a dominant end-point titer for the virus causing the infection (Fig. 3.2b). If IIFT does not allow unambiguous differentiation, an ELISA-based approach is appropriate (Fig. 3.3).

3.1.2.2 Detection of Anti-ZIKV IgM and IgG Using a Highly Specific NS1-Based ELISA

In order to overcome the problem of serological cross-reactivity associated with whole virus-based assays, recombinant proteins have recently been given priority in the development of more efficient antigenic substrates for ZIKV serodiagnosis. Since the non-structural protein 1 (NS1)

Table 3.2 Sensitivity and specificity of the Arbovirus Fever Mosaic 2 IIFT (Euroimmun)

Substrate	Ig class	Sensitivity		Specificity	
		n	%	n	%
Zika virus	IgM	97[a-c]	96.9	211[a,i,k]	98.1
	IgG	104[a-c]	96.8	258[a,i,k]	93.4
Dengue virus (types 1–4)	IgM	65[a,d,e]	98.5	184[e,i]	96.2
	IgG	59[a,d,e]	96.6	251[e,i]	96.4
Chikungunya virus	IgM	174[a,f-h]	97.7	256[g-i]	99.6
	IgG	117[a,f,g,i]	95.7	200[a,i]	98.5

[a]Samples pre-characterization using in-house methods at the WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research (WHOCC, Hamburg, Germany)
[b]Samples from ZIKV-RT-PCR-confirmed patients from the Dominican Republic (BocaBiolistics, USA)
[c]Samples from ZIKV-RT-PCR-confirmed German and Italian patients
[d]Samples pre-characterization using in-house methods at the Robert Koch Institute (RKI, Berlin, Germany)
[e]Samples pre-characterization using Dengue IgM Capture ELISA or Dengue IgG Capture ELISA (Panbio Diagnostics, Brisbane, Australia) at the University Jedda (Saudi Arabia)
[f]Samples pre-characterization using in-house anti-CHIKV ELISA at the Centre National de Référence des Arbovirus (Marseille, France)
[g]Samples pre-characterization using in-house anti-CHIKV ELISA at Cerba Specimen Services (France)
[h]Samples pre-characterization using in-house CHIKV MAC-ELISA at the Centers of Disease Control and Prevention (CDC) Arboviral Diseases Branch (Fort Collins, Colorado, USA) [43]
[i]Samples from healthy blood donors provided by the University Medical Center Schleswig-Holstein (Lübeck, Germany)
[k]Samples from healthy pregnant women provided by Laboratory Schottdorf (Augsburg, Germany)

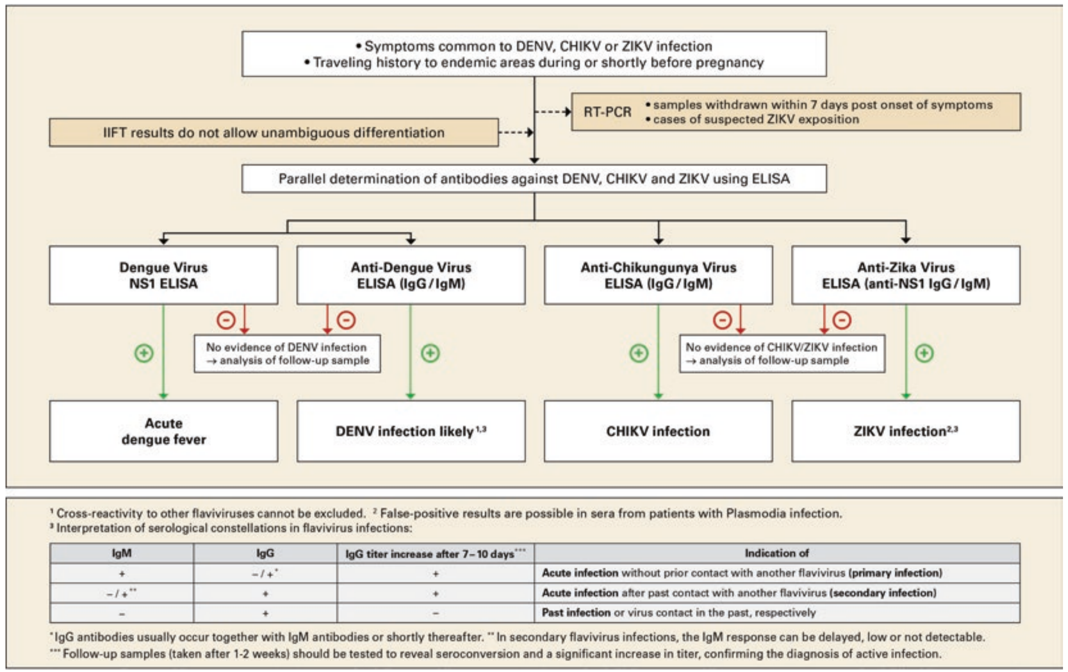


Fig. 3.3 Algorithm for the serological differential diagnosis in suspected cases of DENV, CHIKV or ZIKV infections using Euroimmun ELISA. IgG and IgM antibodies against DENV, CHIKV and ZIKV should be determined in parallel

Table 3.3 Sensitivity of the NS1-based Anti-Zika Virus ELISA (Euroimmun)

Cohort	Origin of ZIKV infection	n	Anti-ZIKV ELISA sensitivity (CI 95%)[c]			References
			IgM	IgG	IgM/IgG	
Travelers returning from ZIKV-endemic areas (primary ZIKV infection)						
RT-PCR-confirmed ZIKV infection[a]	Haiti or N/A	5	100% (51.1–100%)	60.0% (22.9–88.4%)	100% (51.1–100%)	[81]
IIFT-confirmed ZIKV infection[b]	Brazil, Colombia or N/A	26	80.8% (61.7–92.0%)	69.2% (49.9–83.7%)	96.2% (79.6–100%)	[81]
Residents in ZIKV-endemic areas (secondary ZIKV infection)						
RT-PCR-confirmed ZIKV infection[a]	Suriname, Colombia, Dominican Republic	12	41.7% (19.3–68.1%)	100% (71.8–100%)	100% (71.8–100%)	[81]
IIFT-confirmed ZIKV infection[a]	Colombia	38	7.9 % (2.0–21.5%)	89.5% (75.3–96.4%)	89.5% (75.3–96.4%)	[81]
IIFT-confirmed acute ZIKV infection	Brazil	10	100% (67.9–100%)	50% (23.7–76.3%)	100% (67.9–100%)	[41]

CI confidence interval, *ELISA* enzyme-linked immunosorbent assay, *IIFT* indirect immunofluorescence test, *N/A* not available, *RT-PCR* reverse transcription-polymerase chain reaction, *ZIKV* Zika virus

[a]Samples taken from different patients ≥6 days post symptom onset

[b]Samples taken from different patients ≥1 days post symptom onset (no data available for ≥6 days)

[c]Cut-off ratio for positivity: ≥1.1

had been shown to be a suitable candidate [21], the **Euroimmun Anti-Zika Virus ELISA** was developed based on recombinant ZIKV NS1 as solid-phase antigen [81]. Meanwhile, this ELISA has become widely established in prenatal and routine diagnostic settings [2, 14, 34, 35, 55, 72, 100] (Fig. 3.3). Its diagnostic performance was evaluated in cooperation with renowned European institutes for tropical and travel medicine using sera from residents in ZIKV-endemic areas, returning travelers, individuals with potentially cross-reactive antibodies, and blood donors of different age groups and origin [41, 81, 86]. Highest ELISA sensitivity (100% in RT-PCR-confirmed ZIKV infections) was achieved by combined IgM/IgG testing (Table 3.3). In cohorts of healthy individuals, ELISA specificity amounted to ≥99.0% for IgM and 96.7–100% for IgG (Table 3.4). Referring to potentially cross-reactive samples, specificity was 97.1–100% for IgM and 96.0–100% for IgG. Positivity in very few samples from patients infected with another flavivirus (WNV, JEV) may be due to co-infection with ZIKV (true-positive results) or due to cross-reactivity (false-positive results). Importantly, ELISA reactivity was neither observed in DENV-infected patients from ZIKV non-endemic regions nor in individuals with high-titer anti-DENV antibodies (including secondary infections), irrespective of the DENV serotype [38, 81] (Table 3.4). Furthermore, Granger *et al*. reported an excellent overall agreement of the Euroimmun Anti-Zika Virus IgM ELISA with the CDC Zika MAC-ELISA (100% correlation) [38].

Analysis of potential interfering factors (polyclonal B-cell stimulation triggered by infections with Epstein-Barr virus, Mycoplasma pneumoniae, cytomegalovirus, human immunodeficiency virus or Plasmodium spp.; rheumatoid factor; autoimmune antibodies) revealed false-positive anti-ZIKV IgM/IgG results in about 20–30% of patients with acute and <2% in those with past Plasmodium infection. Among all other factors, interferences were observed in ≤6% for IgM and 0% for IgG [41, 81, 86] [and unpublished data].

Table 3.4 Specificity of the NS1-based Anti-Zika Virus ELISA (Euroimmun)

Cohort	Anti-ZIKV ELISA IgM		Anti-ZIKV ELISA IgG		References[e]
	n	Specificity (CI 95%)[c]	n	Specificity (CI 95%)[c]	
Healthy control samples					
German pregnant women	100	100% (95.6–100%)	100	100% (95.6–100%)	[81]
Healthy prepartal women[b]	20	100% (83.2–100%)	20	100% (83.2–100%)	[38]
Zimbabwean blood donors	128	100% (96.5–100%)	128	100% (96.5–100%)	[81]
Argentinian blood donors	99	99.0% (94.0–100%)	99	100% (95.5–100%)	[81]
US-American blood donors	100	100% (95.6–100%)	100	99.0% (94.0–100%)	[81]
German blood donors	500	99.8% (98.8–100%)	500	99.8% (98.8–100%)	[81]
Healthy blood donors[b]	30	100% (86.5–100%)	30	96.7% (81.9–100%)	[38]
German children	88	100% (95.0–100%)	88	100% (95.0–100%)	[81]
Potentially cross-reactive samples					
DENV[a] infection (high median anti-DENV IgM)	47	100% (91.0–100%)	47	100% (91.0–100%)	[81]
DENV[a] infection (high median anti-DENV IgG)	46	100% (90.8–100%)	46	100% (90.8–100%)	[81]
DENV[a] infection	16	100% (77.3–100%)	10	100% (67.9–100%)	[41]
Early convalescent DENV[a] infection in individuals from a ZIKV non-endemic region	7	100% (59.6–100%)	7	100% (59.6–100%)	[38]
Suspected secondary DENV[a] infection in individuals from a ZIKV non-endemic region	13	100% (73.4–100%)	13	100% (73.4–100%)	[38]
DENV (type 1) infection	3	100% (29.2–100%)	3	100% (29.2–100%)	[86]
DENV (type 1) infection	8	100% (62.8–100%)	8	100% (62.8–100%)	UD
DENV (type 2) infection	4	100% (45.4–100%)	4	100% (45.4–100%)	[86]
DENV (type 2) infection	10	100% (67.9–100%)	10	100% (67.9–100%)	UD
DENV (type 3) infection	2	100% (29.0–100%)	2	100% (29.0–100%)	[86]
DENV (type 3) infection	5	100% (51.1–100%)	5	100% (51.1–100%)	UD
DENV (type 4) infection	1	100% (16.8–100%)	1	100% (16.8–100%)	[86]
DENV (type 4) infection	3	100% (38.3–100%)	3	100% (38.3–100%)	UD
YFV vaccination	12	100% (71.8–100%)	12	100% (71.8–100%)	[81]
YFV vaccination	15	100% (76.1–100%)	15	100% (76.1–100%)	[41]
YFV vaccination	10	100% (67.9–100%)	10	100% (67.9–100%)	[86]
WNV infection	34	97.1% (83.8–100%)[d]	34	100% (87.9–100%)	[81]
WNV infection	10	100% (67.9–100%)	13	100% (73.4–100%)	[38]
JEV infection	25	100% (84.2–100%)	25	96.0% (78.9–100%)[d]	[81]
CHIKV infection	19	100% (80.2–100%)	19	100% (80.2–100%)	[81]
CHIKV infection	4	100% (45.4–100%)	5	100% (51.1–100%)	[38]
SLEV infection	2	100% (29.0–100%)	6	100% (54.1–100%)	[38]
TBEV infection	38	100% (89.1–100%)	21	100% (81.8–100%)	[41]
TBEV vaccination		N/A	52	100% (91.8–100%)	[41]
HCV infection		N/A	16	100% (77.3–100%)	[41]

CHIKV, chikungunya virus, *CI* confidence interval, *DENV* dengue virus, *ELISA* enzyme-linked immunosorbent assay, *HCV* hepatitis C virus, *JEV* Japanese encephalitis virus, *N/A* not available, *SLEV* St. Louis encephalitis virus, *TBEV* tick-borne encephalitis virus, *UD* unpublished data, *WNV* West Nile virus, *YFV* yellow fever virus, *ZIKV* Zika virus

[a]DENV serotype not known

[b]Origin of sample donors not available

[c]Cut-off ratio for positivity: ≥1.1

[d]Anti-ZIKV reactivity may be due to ZIKV/WNV or ZIKV/JEV co-infections (true-positive results) or due to cross-reactivity (false-positive results)

[e]Unpublished data (UP) were provided by K. Steinhagen

3.2 Laboratory Testing for DENV Infections

Any of the four DENV serotypes can cause asymptomatic infections, dengue fever (DF) or dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Importantly, a secondary infection by a heterologous DENV serotype is associated with a high risk for DHF/DSS due to immune enhancement [10, 79]. Diagnostics can be performed through isolation of the virus, detection of viral RNA or antigen, detection of specific antibodies, or (optimally) a combination thereof [27, 39, 64, 66, 82, 84]. Evidence of the value of combining DENV antigen- and antibody-based test results has been comprehensively described in previous publications [8, 36, 61].

3.2.1 Direct DENV Detection

Direct methods, including virus isolation and detection of viral RNA by RT-PCR are most efficient during the first 4–5 days after the onset of symptoms [18, 20, 45, 47, 74]. RT-PCR is both sensitive and specific with respect to DENV confirmation and serotyping, therefore representing the method of choice for the examination of patient samples obtained in the early acute stage of illness [15, 63]. However, RT-PCR requires specific reagents and laboratory equipment as well as trained personnel, while virus isolation is time consuming. Due to the short viremic phase, RT-PCR may already be negative by the time a patient consults a doctor.

Alternatively, many standardized commercial assays for the detection of DENV antigens, particularly NS1, have been developed [3, 29, 30, 61, 65]. NS1 is detectable at the onset of symptoms in both primary and secondary DENV infections and remains detectable past the viremic phase (up to 9 days from disease onset), thus offering a larger time frame for diagnosis than other direct techniques. In addition, high NS1 levels correlate with the development of DHF [1, 29, 52, 99]. Importantly, sera from patients with acute ZIKV infection do not appear to cross-react with DENV NS1 antigen tests, indicating high specificity [54]. Rapid diagnostic tests (RDT) for DENV NS1 detection provide opportunities for point-of-care diagnosis, but should be interpreted with caution since their sensitivity is inherently limited and inferior to ELISA testing [5, 42, 65]. Negative results obtained by direct methods do not rule out a DENV infection and require additional serological testing.

3.2.1.1 Detection of DENV NS1 Antigen Using ELISA

The **Euroimmun Dengue Virus NS1 ELISA** is coated with monoclonal anti-DENV NS1 antibodies that specifically bind NS1 of all four DENV serotypes and enable highly sensitive antigen detection. Analysis of 35 clinically and serologically pre-characterized sera provided by the INSTAND quality assessment scheme (2010 to 2016) revealed 100% agreement between the INSTAND target values and the results obtained using the Euroimmun Dengue Virus NS1 ELISA, indicating 100% sensitivity and specificity. Parallel investigation of specific antibodies against DENV, ZIKV and CHIKV is recommended considering the relevance of early diagnosis and the possibility of co-infections (Fig. 3.3).

3.2.2 Indirect DENV Detection

Serology is frequently used for routine diagnosis of DENV infections and preferentially applied after the time slot for direct virus detection (>3–4 days post symptom onset) or subsequent to negative testing by direct methods [39]. In primary DENV infections, specific IgM antibodies are detectable in 50% of patients by the third day after symptom onset, increasing to 80–99% by day five to ten. IgM levels peak after approximately 2 weeks of illness and decline to undetectable levels after 2–3 months. Anti-DENV IgG starts to increase at the end of the first week of illness and remains detectable for months and years [39, 63, 84]. In contrast, during secondary infection, the IgM response is often delayed, low or undetectable, while IgG titers rise rapidly within the first 2 days after symptom onset, the

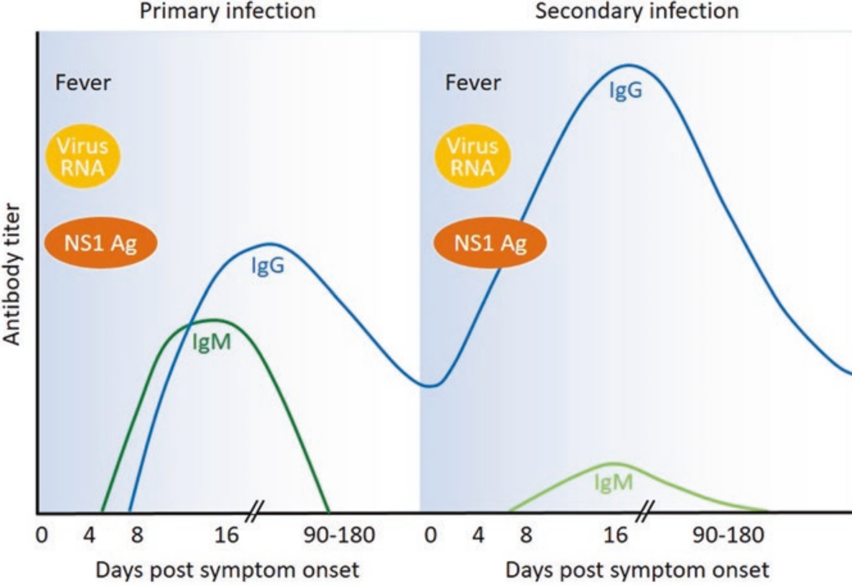


Fig. 3.4 Time course of serological parameters in primary and secondary DENV infection. In secondary DENV infections, the IgM response is variable, and in some cases undetectable

latter showing high cross-reactivity with other flaviviruses (Fig. 3.4) [19, 39, 75, 77, 87].

Acute DENV infections are indicated serologically by the presence of specific IgM, seroconversion or a fourfold or greater increase in IgG titer. Since the delay or absence of detectable IgM levels in some secondary infections may lead to misdiagnosis, the combined testing of IgM and IgG has been proposed as an effective strategy [75, 76]. As DENV-specific antibodies of class IgA are produced in parallel to IgM in about 70% of cases, the additional determination of IgA can assure the diagnosis of a recent primary infections, especially when a follow-up sample is not yet available or if the IgG response is not yet detectable [57]. For the classification of primary and secondary DENV infections, some protocols use IgM/IgG ratios, with capture ELISAs being the most common assays for this purpose [26, 31, 78, 84]. Alternatively, a hemagglutination inhibition (HI) titer exceeding 1:1280 in convalescent serum is considered indicative of a secondary DENV infection [84].

A large number of commercial assays for DENV serological testing with variable degrees of sensitivity and specificity are currently available, with ELISA, IIFT and immunochromatographic RDTs representing preferred methods for rapid, simple and high-throughput testing (Table 3.5) [5, 8, 40, 83, 84]. HI assays and PRNTs are further options, but not routinely used because they are not standardized, time consuming, labor intensive, low throughput and restricted to Biosafety Level 3. Noteworthy, the performance of anti-DENV RDTs appears problematic [5]. This was also reflected by the INSTAND DENV proficiency testing in 2016, demonstrating average pass rates for anti-DENV IgG positive samples of 8.3–70.8% by laboratories using rapid tests compared to 97.7–100% using ELISA and 90.0–100% using IIFT [42]. In a WHO multicenter study, anti-DENV IgM RDTs showed mean sensitivities ranging from 20.5% to 97.7% and specificities between 76.6% and 90.6%, while IgM ELISA kits had higher sensitivities of 61.5–99.0% and specificities of 79.9–97.8% [40].

Table 3.5 Available serological tests for the diagnosis of DENV infections (11/2016)

Company	Assay	Ig class	Format	References [5, 80, 83]
Access Bio	CareStart Dengue Combo (NS1+IgM/IgG)	IgM/IgG	ICA (RDT)	
ADI	Human Anti-Dengue Virus IgM ELISA	IgM	ELISA	
	Human Anti-Dengue Virus IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 1 Envelop protein IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 2 Envelop protein IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 3 Envelop protein IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 4 Envelop protein IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 1 prM protein IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 2 prM protein IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 3 prM protein IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 4 prM protein IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 1+2+3+4 Envelop protein IgG ELISA	IgG	ELISA	
	Human Anti-Dengue virus 1+2+3+4 prM protein IgG ELISA	IgG	ELISA	
Artron	Dengue Virus IgG/IgM Antibody	IgM/IgG	ICA (RDT)	
	Dengue IgG/IgM/NS1 Combo	IgM/IgG	ICA (RDT)	
Bio-Rad	Platelia Dengue IgA Capture	IgA	ELISA	[26]
	Platelia Dengue IgG Capture	IgG	ELISA	
	RDT Dengue IgA/IgG	IgA/IgG	ICA (RDT)	
Biocan	Dengue IgG/IgM Antibody	IgM/IgG	ICA (RDT)	
	Dengue IgA/IgG/IgM Antibody (Triplex)	IgM/IgG/IgA	ICA (RDT)	
	Dengue IgA/IgG/IgM Antibody & NS1 (Fourplex)	IgM/IgG/IgA	ICA (RDT)	
	Zika/Dengue/Chikungunya Combo Tests	IgM/IgG	ICA (RDT)	
Biosynex	Immunoquick Dengue Fever IgG and IgM	IgM/IgG	ICA (RDT)	[7]
Calbiotech	Dengue Virus IgM ELISA	IgM	ELISA	
	Dengue Virus IgG ELISA	IgG	ELISA	
CD	Dengue IgM Capture ELISA	IgM	ELISA	
	Dengue IgG ELISA	IgG	ELISA	
	Dengue Virus IgG Human ELISA	IgG	ELISA	
	Dengue Fever IgG-IgM (S/WB/P) Rapid Test (Cassette)	IgM/IgG	ICA (RDT)	
Core	Core Dengue (IgG+IgM)	IgM/IgG	ICA (RDT)	[6]

(continued)

Table 3.5 (continued)

Company	Assay	Ig class	Format	References [5, 80, 83]
CTK Biotech	RecombiLISA Dengue IgM Test	IgM	ELISA	
	RecombiLISA Dengue IgG Test	IgM	ELISA	
	OnSite Dengue IgG/IgM Combo Rapid Test	IgM, IgG	ICA (RDT)	[8]
	OnSite Duo Dengue Ag-IgG/IgM Rapid Test	IgM, IgG	ICA (RDT)	
	OnSite Duo Dengue IgG/IgM-CHIK IgM Rapid Test	IgM	ICA (RDT)	
DA/CD	AccuDiag Dengue IgM ELISA	IgM	ELISA	
	AccuDiag Dengue IgG ELISA	IgG	ELISA	
	Dengue IgG/IgM	IgM/IgG	ELISA	
	OneStep Dengue NS1 Antigen IgG/ IgM Antibody Duo Panel RapidCard	IgM/IgG	ICA (RDT)	
	OneStep Dengue Fever IgG/IgM RapiCard	IgM/IgG	ICA (RDT)	
Dia.Pro	DENM – IgM	IgM	ELISA	
	DENG – IgG	IgG	ELISA	
DiaSorin	Dengue IgM	IgM	ELISA	
	Dengue IgG	IgG	ELISA	
	Dengue Virus IgM μ-capture	IgM	ELISA	
DRG	Dengue (1–4) IgM capture ELISA	IgM	ELISA	
	DengueVirus IgM ELISA	IgM	ELISA	[42]
	Dengue Virus IgG ELISA	IgG	ELISA	[42]
	Dengue IgG/IgM Cassette Test	IgM/IgG	ICA (RDT)	
	Dengue Rapid Test	IgM/IgG	ICA (RDT)	
Euroimmun	Mosaic: Dengue virus types 1–4 (IgG or IgM)	IgM/IgG	IIFT	[42]
	Flavivirus Profile 2 (IgG or IgM)	IgM/IgG	IIFT	
	Flavivirus Profile 3 (IgG or IgM)	IgM/IgG	IIFT	
	Arbovirus Fever Mosaic 1 (IgG or IgM)	IgM/IgG	IIFT	
	Arbovirus Fever Mosaic 2 (IgG or IgM)	IgM/IgG	IIFT	
	Arbovirus Profile 3 (IgG or IgM)	IgM/IgG	IIFT	
	Anti-Dengue virus ELISA (IgM)	IgM	ELISA	[42]
	Anti-Dengue virus ELISA (IgG)	IgG	ELISA	[42]
Focus	Dengue Virus IgM Capture DxSelect	IgM	ELISA	[40, 42]
	Dengue Virus IgG DxSelect	IgG	ELISA	[42]
	Dengue Dx IgG/IgM Rapid Test	IgM/IgG	ICA (RDT)	[42]
GenWay	Dengue Virus IgM-ELISA	IgM	ELISA	
	Dengue Virus IgG-ELISA	IgG	ELISA	
IBL	Dengue Virus IgM μ-capture ELISA	IgM	ELISA	
	Dengue Virus IgM ELISA	IgM	ELISA	[42]
	Dengue Virus IgG ELISA	IgG	ELISA	[42]
InBios	DENV Detect IgM Capture ELISA	IgM	ELISA	[94]
	DENV Detect IgG ELISA	IgG	ELISA	

(continued)

Table 3.5 (continued)

Company	Assay	Ig class	Format	References [5, 80, 83]
J. Mitra & Co	Dengue IgM Microlisa	IgM	ELISA	
	Dengue IgG Microlisa	IgG	ELISA	
	Dengue Day1 Test	IgM, IgG	ICA (RDT)	
	Diagnos Dengue Card	IgM, IgG	ICA (RDT)	
LumiQuick	QuickProfile Dengue IgG/IgM Combo Test	IgM/IgG	ICA (RDT)	
	QuickProfile Dengue NS1 Antigen & IgG/IgM Duo Panel	IgM/IgG	ICA (RDT)	
MIROGEN	alphaWell Dengue IgM µ-capture	IgM	ELISA	
	alphaWell Dengue IgM	IgM	ELISA	
	alphaWell Dengue IgG	IgG	ELISA	
	recomLine Tropical Fever IgM	IgM	LIB	
	recomLine Tropical Fever IgG	IgG	LIB	
MP Diagnostics	Dengue Virus IgG ELISA	IgG	ELISA	
MyBioSource	Dengue Virus IgM ELISA	IgM	ELISA	
	Dengue Virus IgG ELISA	IgG	ELISA	
NovaTec	NovaLisa Dengue Virus IgM µ-capture ELISA	IgM	ELISA	
	NovaLisa Dengue Virus IgM ELISA	IgM	ELISA	[42]
	NovaLisa Dengue Virus IgG ELISA	IgG	ELISA	
Omega	Pathozyme-Dengue M Capture	IgM	ELISA	[40, 59]
	Pathozyme-Dengue G	IgG	ELISA	
	Visitect Dengue	IgM/IgG	ICA (RDT)	
OrangeLife	OL Dengue IgG/IgM	IgM/IgG	ICA (RDT)	
	OL Combo Dengue NS1/IgG/IgM	IgM/IgG	ICA (RDT)	
Orgenics/ Alere	ImmunoComb II Dengue IgM & IgG BiSpot	IgM/IgG	EIA	[8, 73]
Panbio/Alere	Dengue IgM Capture ELISA	IgM	ELISA	[8, 26, 40, 42, 59, 89]
	Dengue IgG Capture ELISA	IgG	ELISA	[8, 26, 89]
	Dengue IgG Indirect ELISA	IgG	ELISA	[42]
	Dengue Duo IgM and IgG Capture ELISA	IgM/IgG	ELISA	[24, 73, 76, 88]
	Japanese Encephalitis – Dengue IgM Combo ELISA	IgM	ELISA	[51]
	Dengue Duo Cassette	IgM/IgG	ICA (RDT)	[7, 36, 40, 42, 59, 80]
PROGEN	Dengue Virus Type 2 Antibody detection kit	IgM/IgG	IIFT	[42]
R-Biopharm	RIDASCREEN Dengue Virus IgM	IgM	ELISA	
	RIDASCREEN Dengue Virus IgG	IgG	ELISA	
SD Standard/ Alere	SD Dengue IgM Capture ELISA	IgM	ELISA	[8, 40]
	SD Dengue IgG Capture ELISA	IgG	ELISA	[8]
	SD BIOLINE Dengue Duo (Dengue NS1 Ag + IgG/IgM)	IgM/IgG	ICA (RDT)	[7, 8, 42, 61, 85, 90, 93]
	SD BIOLINE Dengue IgG/IgM	IgM/IgG	ICA (RDT)	[6, 40, 42]
	SD BIOLINE Dengue IgG/IgM WB	IgM/IgG	ICA (RDT)	

(continued)

Table 3.5 (continued)

Company	Assay	Ig class	Format	References [5, 80, 83]
SD Biosensor	STANDARD E Dengue IgM ELISA	IgM	ELISA	
	STANDARD E Dengue IgG ELISA	IgG	ELISA	
	STANDARD Q Dengue IgM/IgG	IgM/IgG	ICA (RDT)	
	STANDARD Q Dengue Duo	IgM/IgG	ICA (RDT)	
Virion\Serion	SERION ELISA classic Dengue Virus IgM	IgM	ELISA	
	SERION ELISA classic Dengue Virus IgG	IgG	ELISA	
Vircell	ZIKV-DENV-CHIKV IFA IgM	IgM	IIFT	
	ZIKV-DENV-CHIKV IFA IgG	IgG	IIFT	
	DENGUE ELISA IgM CAPTURE	IgM	ELISA	
	DENGUE ELISA IgG	IgG	ELISA	
	DENGUE VIRCLIA IgM MONOTEST	IgM	CLIA	
	DENGUE VIRCLIA IgG MONOTEST	IgG	CLIA	
Zephyr	Denguecheck Combo	IgM/IgG	ICA (RDT)	[40]

CLIA chemiluminescence assay, *ELISA* enzyme-linked immunosorbent assay, *ICA* immunochromatographic assay, *IIFT* indirect immunofluorescence test, *LIB* line immunoblot, *RDT* rapid diagnostic test

Since cross-reactivity between the DENV serotypes and within the flaviviruses has been reported as a major limitation of DENV serological assays, differential diagnosis with respect to all four DENV serotypes and other co-endemic viruses causing similar clinical manifestations (e.g., ZIKV, CHIKV, yellow fever, Japanese encephalitis virus,) is of particular importance, just like the determination of the pathogenic agent using direct techniques [6, 8, 27] .

3.2.2.1 DENV Serotyping and Differential Diagnosis Using IIFT Biochip Mosaics

In the **Euroimmun Anti-Dengue Virus IIFT**, every test field contains four biochips, each coated with cells infected with one of the four DENV serotypes. This enables the simultaneous examination of a patient sample for reactivity (IgM or IgG) against DENV types 1–4 and in some cases serotyping by endpoint titration. Other Euroimmun IIFT biochip mosaics (e.g., Flavivirus Profiles, Arbovirus Profiles and Arbovirus Fever Mosaics) combine DENV-infected cells with further flavivirus substrates (Fig. 3.2, Tables 3.2 and 3.5). They are helpful for differential diagnosis and in consideration of potential cross-reactions with related viruses, representing a fast and simple alternative to more elaborate methods.

3.2.2.2 Detection of Anti-DENV Antibodies Using ELISA

The **Euroimmun Anti-Dengue Virus ELISA** is based on highly purified virus particles of serotype 2. Because of the structural similarity between DENV 1 to 4, use of a single serotype is sufficient for the reliable detection of antibodies (IgM or IgG) against any of the virus types. In clinically characterized sera the IgM and IgG ELISA demonstrated 100% sensitivity and 99% specificity. The correlation between the Euroimmun Anti-Dengue Virus ELISA and the PANBIO Dengue Capture ELISA amounted to 97% for IgM and 99% for IgG (unpublished data). Due to use of whole-virus antigen, cross-reactions with other flavivirus antibodies, however, cannot be excluded (Fig. 3.3).

3.3 Serological Testing for CHIKV Infections

The confirmation of CHIKV infection by laboratory diagnostic means is analogous to ZIKV and DENV infections, as described above. In brief, during the first 5 days of infection, the identification of CHIKV is most sensitive using RNA detection (RT-PCR) or viral culture. Thereafter, specific antibodies against CHIKV are reliable indicators of disease, and can be detected from about 3–5 days after clinical onset [12, 50, 62, 92]. The determination of specific IgM and IgG can be performed using HI, virus neutralization and also by ELISA or IIFT which are preferable tests in the routine diagnostic settings. In addition, rapid point-of-care assays are available but suffer from poor performance [11, 43]. Table 3.6 presents current commercial assays for CHIKV serology. Differential diagnosis should include other co-circulating infections causing similar symptoms (e.g., dengue fever, Fig. 3.3) and should take into account cross-reactivity within the alphavirus genus. Reliable interpretation of test results and differential diagnosis can be achieved using, among others, neutralization, antibody profiles or IIFT biochip mosaics (e.g., Euroimmun Arbovirus Fever Mosaics and Arbovirus Profiles; Fig. 3.2; Table 3.6).

The **Euroimmun Anti-Chikungunya Virus IIFT** with CHIKV-infected and uninfected cell substrates coated on separate biochips is a valuable tool for the diagnosis of CHIKV infections and antibody seroprevalence studies. Evaluation of this assay for the detection of IgM antibodies revealed a sensitivity and specificity of 96.9% and 98.3%, respectively. The IgG IIFT showed a sensitivity of 95.4% and a specificity of 100% [53]. Another study demonstrated increasing IgM assay sensitivity along with the progression of the disease, improving from 75.6% by day five to 100% by day seven, at a specificity of 100% [98]. Assay accuracy for anti-CHIKV IgM detection amounted to 96–97%, which was similar to the accuracy demonstrated for commercial IgM ELISAs (95–100%) [43]. Other Euroimmun IIFT biochip mosaics are suitable for differential diagnosis analyses, such as Arbovirus Fever Mosaics and Arbovirus Profiles (Fig. 3.2, Tables 3.2, 3.6 and 3.7).

The **Euroimmun Anti-Chikungunya Virus ELISA** is based on a recombinant CHIKV-specific structural protein. When compared to other commercial anti-CHIKV assays, it was shown to provide excellent overall agreement as well as very high sensitivity and specificity of up to 100% depending on the cohort (Fig. 3.3) [43, 68].

3.4 Summary

Biochip mosaics for IIF-based detection of antibodies against ZIKV, DENV and CHIKV enable the determination of the infectious agent by means of titration. Alternatively, the combination of the highly specific and non-cross-reactive NS1-based anti-ZIKV ELISA with anti-DENV- and CHIKV-ELISAs allows for reliable differential diagnosis, also in regions where these infections are co-endemic.

Table 3.6 Available serological tests for the diagnosis of CHIKV infections (11/2016)

Company	Assay	Ig class	Format	References
Abcam	Human Anti-Chikungunya Virus IgM ELISA	IgM	ELISA	[43]
	Human Anti-Chikungunya Virus IgG ELISA	IgG	ELISA	
ADI	Human Anti-Chikungunya virus (CHIKV) IgM capture ELISA	IgM	ELISA	
	Human Anti-Chikungunya virus (CHIKV) IgG capture ELISA	IgG	ELISA	
	Human Anti-Chikungunya virus E1 (CHIKV-E1) IgM capture ELISA	IgM	ELISA	
	Human Anti-Chikungunya virus E1 (CHIKV-E1) IgG capture ELISA	IgG	ELISA	
	Human Anti-Chikungunya virus E2 (CHIKV-E2) IgM capture ELISA	IgM	ELISA	
	Human Anti-Chikungunya virus E2 (CHIKV-E2) IgG capture ELISA	IgG	ELISA	
Artron	Chikungunya IgG/IgM Antibody	IgM/IgG	ICA (RDT)	
Biocan	Chikungunya IgG/IgM Antibody	IgM/IgG	ICA (RDT)	
Biocan	Zika/Dengue/Chikungunya Combo Tests	IgM/IgG	ICA (RDT)	
CD	Chikungunya IgM ELISA	IgM	ELISA	
	Human Chikungunya IgG ELISA	IgG	ELISA	
	Chikungunya Virus IgG capture ELISA	IgG	ELISA	
CTK	RecombiLISA CHIK IgM Test	IgM	ELISA	[43]
	OnSite Chikungunya IgM Combo Rapid Test	IgM	ICA (RDT)	[11, 43, 68]
	OnSite Duo Dengue IgG/IgM-CHIK IgM Rapid Test	IgM	ICA (RDT)	
DA/CD	AccuDiag Chikungunya IgM ELISA	IgM	ELISA	
	AccuDiag Chikungunya IgG ELISA	IgG	ELISA	
Diasorin	Chikungunya Virus IgM μ-capture	IgM	ELISA	
DRG	Chikungunya IgM	IgM	ELISA	
	Chikungunya IgG	IgG	ELISA	
Euroimmun	Anti-Chikungunya virus IIFT (IgG or IgM)	IgM/IgG	IIFT	[43, 53, 58, 97, 98]
	Arbovirus Fever Mosaic 1 (IgG or IgM)	IgM/IgG	IIFT	
	Arbovirus Fever Mosaic 2 (IgG or IgM)	IgM/IgG	IIFT	
	Arbovirus Profile 3 (IgG or IgM)	IgM/IgG	IIFT	
	Anti-Chikungunya Virus ELISA (IgM)	IgM	ELISA	[43, 68]
	Anti-Chikungunya Virus ELISA (IgG)	IgG	ELISA	[68]
GenWay	Chikungunya IgM μ-capture ELISA	IgM	ELISA	[43]
	Chikungunya IgG capture ELISA	IgG	ELISA	
IBL	Chikungunya IgM μ-capture ELISA	IgM	ELISA	[68]
	Chikungunya IgG capture ELISA	IgG	ELISA	[68]
InBios	CHIKjj Detect IgM ELISA	IgM	ELISA	[43]
	CHIKjj Detect IgG ELISA	IgG	ELISA	
J. Mitra & Co	Advantage Chikungunya IgM Card	IgM	ICA (RDT)	
LumiQuick	QuickProfile Chikungunya IgG/IgM Combo Test	IgM/IgG	ICA (RDT)	
Mikrogen	alphaWell Chikungunya IgM μ-capture	IgM	ELISA	
	alphaWell Chikungunya IgG	IgM	ELISA	
	recomLine Tropical Fever IgM	IgM	LIB	
	recomLine Tropical Fever IgG	IgG	LIB	
MyBioSource	Chikungunya Virus IgM μ-capture ELISA	IgM	ELISA	
	Chikungunya Virus IgG capture ELISA	IgG	ELISA	
NovaTec	NovaLisa Chikungunya Virus IgM μ-capture ELISA	IgM	ELISA	[49]
	NovaLisa Chikungunya Virus IgG capture ELISA	IgG	ELISA	[49]

(continued)

Table 3.6 (continued)

Company	Assay	Ig class	Format	References
OrangeLife	OL Chikungunya IgM	IgM	ICA (RDT)	
R-Biopharm	RIDASCREEN Chikungunya Virus IgM μ-capture	IgM	ELISA	
	RIDASCREEN Chikungunya Virus IgG capture	IgG	ELISA	
SD Standard/ Alere	Chikungunya IgM ELISA	IgM	ELISA	[9, 43]
	BIOLINE Chikungunya IgM	IgM	ICA (RDT)	[9, 43, 44, 68]
Vircell	ZIKV-DENV-CHIKV IFA IgM	IgM	IIFT	
	ZIKV-DENV-CHIKV IFA IgG	IgG	IIFT	

ELISA enzyme-linked immunosorbent assay, *ICA* immunochromatographic assay, *IIFT* indirect immunofluorescence test, *LIB* line immunoblot, *RDT* rapid diagnostic test

Table 3.7 Suppliers of serological assays (supplement to Tables 3.1, 3.5 and 3.6)

Notation	Company
Abcam	Abcam plc, Cambridge, UK
Access Bio	Access Bio, Inc., Somerset, NJ, USA
ADI	Alpha Diagnostic International., Inc., San Antonio, TX, USA
Artron	Artron Laboratories, Inc., Burnaby, BC, Canaca
Biocan	Biocan Diagnostics, Inc, Coquitlam, BC, Canada
Bio-Rad	Bio-Rad Laboratories, Marnes La Coquette, France
Biosynex	Biosynex, Strasbourg, France
Calbiotech	Calbiotech, Inc., El Cajon, CA, USA
Chembio	Chembio Diagnostic Systems, Inc., Medford, NY, USA
CD	Creative Diagnostics, Shirley, NY, USA
CDC	CDC, Centers for Disease Control and Prevention, Atlanta, GA, USA
Core	Core Diagnostics, Birmingham, UK
CTK Biotech	CTK Biotech Inc., San Diego, CA, USA
DA/CD	Diagnostic Automation/Cortez Diagnostics, Inc., Woodland Hills, CA, USA
DIACHECK	Dr. Julio Moran, Laboratories, Herrliberg (Zurich), Switzerland
Dia.Pro	Dia.Pro, Diagnostic Bioprobes Srl., Sesto San Giovanni (MI), Italy
DiaSorin	DiaSorin, Saluggia, Italy
DRG	DRG Diagnostics, Marburg, Germany
Euroimmun	Euroimmun, Lübeck, Germany
Focus	Focus Diagnostics, Cypress, CA, USA
GenWay	GenWay Biotech Inc., San Diego, CA, USA
IBL	IBL International, Hamburg, Germany
InBios	InBios International Inc., Seattle, WA, USA
J. Mitra & Co	J. Mitra & Co. Pvt. Ltd., New Delhi, India
LumiQuick	LumiQuick Diagnostics, Inc., Santa Clara, CA, USA
MIKROGEN	MIKROGEN, Neuried, Germany
MP	MP Biomedicals, Santa Ana, CA, USA
MyBioSource	MyBioSource, Inc., San Diego, CA, USA
NovaTec	NovaTec Immundiagnostica, Dietzenbach, Germany
Omega	Omega Diagnostics Ltd., Alva, UK
OrangeLife	OrangeLife, Rio de Janeiro, Brazil
Orgenics/Alere	Orgenics Ltd. (Alere Inc.), Yavne, Israel
Panbio/Alere	Panbio Diagnostics (Alere Inc.), Brisbane, Australia
PROGEN	PROGEN Biotechnik, Heidelberg, Germany
R-Biopharm	R-Biopharm AG, Darmstadt, Germany
SD Standard/ Alere	SD Standard Diagnostics, Inc. (Alere Inc.), Yongin-si, Gyeonggi-do, Republic of Korea
SD Biosensor	SD Biosensor, Inc., Suwon-si, Gyeonggi-do, Republic of Korea
Viramed	Viramed Biotech AG, Planegg, Germany
Vircell	Vircell, Granada, Spain
Viro-Immun	Viro-Immun Diagnostics, Oberursel, Germany
Virion\Serion	Virion\Serion, Würzburg, Germany
Zephyr	Zephyr Biomedicals, Verna, Goa, India

(continued)

Discussion of Chapter 3 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Claudia Ohst.

Aruna Sampath: Most of the tests that you have shown are based on serology. Do you have any tests that can specifically look at infectious virus particles?

Claudia Ohst: The only test that we have is the Dengue NS1 test, that shows the NS1 for all four types when the virus breaks down. We only have this test for Dengue virus and not for Zika virus or Chikungunya virus. We do not do any PCRs either.

Aruna Sampath: Ok, so for the Dengue, what sensitivity do you have? What is the sensitivity for the NS1 antigens?

Claudia Ohst: Our Dengue NS1 test looks into detecting the Dengue NS1 antigen, so it is not an antibody ELISA here, but an antigen test. Compared to the antibody ELISA, the correlation is quite good and even slightly more sensitive. This has been confirmed by serology where both, the antibody and the NS1 antigen test have been performed.

Subhash Vasudevan: And do you think you can find the serotype as well?

Claudia Ohst: No. This one is for all of the four, so you cannot distinguish the serotypes with this, it is just a screening test.

Kerstin Falk: But you have this mosaic slide with Dengue 1, 2, 3, and 4. But then you said you have to titrate it out. And how much work is that and how reliable is that?

Claudia Ohst: You see, in immunofluorescence you always have crossreactions between the different serotypes and all of them will come up positive in your screening dilution. That's why you have to titrate. Obviously you can take bigger steps and just see where you end up. And then the one with the highest titer should be the virus in your serotype that you are dealing with. Maybe PCR is the better method, but at least there is an option to do this via serology.

Kerstin Falk: So what antigen are you using? So what part of the genome? For the Dengue 1, 2, 3 and 4?

Claudia Ohst: So we are using Dengue 2, I think. For the NS1 antigen ELISA we used antibodies from all four Dengue serotypes, for the antibody ELISA we used purified Dengue 2 virus particles (now purified particles from all four Dengue types).

Kerstin Falk: But when you have Dengue 1, 2, 3 and 4?

Claudia Ohst: Yes, but it does detect all of them.

Aravinda de Silva: But you have done this with IFA. IFA is immunofluorescence.

Claudia Ohst: Well, the IFA uses infected cells, there are four Biochips on each field of the Mosaic slide, each chip is coated with cells which were infected with only one of the four Dengue serotypes.

Aravinda de Silva: The Zika virus data looks very encouraging, much better than Dengue.

Claudia Ohst: We put a lot of work into it.

Aravinda de Silva: Have you looked at Zika virus in late convalescence, in other words, in people 6 months, 1 year after exposure and see how the NS1 antibodies change over the long term?

Claudia Ohst: I think, these data might just be on their way. You need to get the specimens for this. And everything is from the first half basically of 2016 and that is when we start to get the specimens. I cannot give an answer now.

Aravinda de Silva: The other question I have had is that you showed that in secondary cases the Zika IgM, you could not really detect, it was not very sensitive. Is it because there is no IgM or is it because the IgG antibodies are binding to your NS1 antigen, because you have very high IgG levels. And are the IgG antibodies binding the NS1 and preventing IgM from binding?

Claudia Ohst: No. There apparently is not very much IgM. What we do in our IgM tests, is to pre-absorb all the IgG, so they should not

really interfere with the assay. We really only catch the IgM antibodies that are present in your specimen.

Aravinda de Silva: So even in the commercial assay, you pre-absorb the IgG?

Claudia Ohst: Yes. So our dilution solution provided in the test kit contains something that pre-absorbs the IgGs.

George Gao: I missed your NS1 antigen preparation. Did you use the mammalian-expressed protein or insect cells expressed proteins for your antigen, NS1 antigen? How do you prepare the antigen?

Claudia Ohst: Are you referring to the NS1 antigen for Dengue or for Zika?

George Gao: For both. Dengue 1–4 and the Zika. How do you prepare the antigen?

Claudia Ohst: For Dengue, it is an antigen capture test, so obviously we coat our ELISA plates with antibodies that catch the antigen. In the case of Zika, where we detect antibodies against the NS1 antigen, Zika NS1, we have NS1 protein coated on our plate. And this protein is prepared by our company, in our own molecular biology department that produces these recombinant antigens.

Paul Young: I am a little confused by the IgM and IgG antibody data against Zika. NS1. In Dengue, in primary infected patients at least, there is very little if any, and In many cases no, early IgG or IgM response to NS1. This comes along much later in convalescence. Very strong anti-E and sometimes prM, but very little NS1. Is it completely different in Zika? The patients actually mount a good antibody response to the NS1 protein?

Claudia Ohst: Well, we are using the NS1, because that is the one where we really get specific results. Otherwise it would not really help.

Paul Young: I understand completely, but it is not there in Dengue, so I am just surprised.

Claudia Ohst: I cannot tell you. You see, in our Dengue ELISAs, we do not even use the Dengue NS1. That might be the reason why.

Paul Young: Yes, that is why no one uses it. And I am just very surprised that Zika infection would actually induce such a high response, given that the viral load is lower in Zika virus. I suspect, no one has really done that analysis yet. And I know there is really quantitative data on the amount of NS1 in patients, but I suspect it is lower than in Dengue virus. So again it is still confusing.

Claudia Ohst: Maybe we were just lucky with our NS1.

Jonas Schmidt-Chanasit: I would support this comment, because I think it was neglected that the sensitivity especially for IgG is not that good. In combination with IgM, this Zika assay is okay, but if you only perform the IgG, you lose like 30–40% sometimes. Then, what is also important to mention is that the plaque titers do not correlate with the reactivity in the Zika virus ELISA. So you have sometimes very high viral titers and the ELISA is completely negative. So it is quite interesting and it might be related to what was just mentioned before. I just wanted to highlight that. High sensitivity is only achieved if you do this in combination, IgG and IgM together. We have a lot of samples and this was also very nicely illustrated by the data from Mozambique, that they were completely negative in IgG ELISA, but positive in the IFA. So this is due to a lack of sensitivity.

References

1. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M (2002) Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 40:376–381
2. Barzon L, Pacenti M, Berto A, Sinigaglia A, Franchin E, Lavezzo E, Brugnaro P, Palu G (2016) Isolation of infectious Zika virus from saliva and prolonged viral RNA shedding in a traveller returning from the dominican republic to Italy, January 2016. Euro Surveill 21(10):pii=30159
3. Bessoff K, Delorey M, Sun W, Hunsperger E (2008) Comparison of two commercially available Dengue virus (DENV) NS1 capture enzyme-linked immu-

nosorbent assays using a single clinical sample for diagnosis of acute DENV infection. Clin Vaccine Immunol 15:1513–1518
4. Bingham AM, Cone M, Mock V, Heberlein-Larson L, Stanek D, Blackmore C, Likos A (2016) Comparison of test results for Zika virus RNA in urine, serum, and saliva specimens from persons with travel-associated Zika virus disease – Florida, 2016. MMWR Morb Mortal Wkly Rep 65:475–478
5. Blacksell SD (2012) Commercial Dengue rapid diagnostic tests for point-of-care application: recent evaluations and future needs? J Biomed Biotechnol 2012:151967
6. Blacksell SD, Newton PN, Bell D, Kelley J, Mammen MP Jr, Vaughn DW, Wuthiekanun V, Sungkakum A, Nisalak A, Day NP (2006) The comparative accuracy of 8 commercial rapid immunochromatographic assays for the diagnosis of acute Dengue virus infection. Clin Infect Dis 42:1127–1134
7. Blacksell SD, Jarman RG, Bailey MS, Tanganuchitcharnchai A, Jenjaroen K, Gibbons RV, Paris DH, Premaratna R, de Silva HJ, Lalloo DG, Day NP (2011) Evaluation of six commercial point-of-care tests for diagnosis of acute Dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy. Clin Vaccine Immunol 18:2095–2101
8. Blacksell SD, Jarman RG, Gibbons RV, Tanganuchitcharnchai A, Mammen MP Jr, Nisalak A, Kalayanarooj S, Bailey MS, Premaratna R, de Silva HJ, Day NP, Lalloo DG (2012) Comparison of seven commercial antigen and antibody enzyme-linked immunosorbent assays for detection of acute Dengue infection. Clin Vaccine Immunol 19:804–810
9. Blacksell SD, Tanganuchitcharnchai A, Jarman RG, Gibbons RV, Paris DH, Bailey MS, Day NP, Premaratna R, Lalloo DG, de Silva HJ (2011) Poor diagnostic accuracy of commercial antibody-based assays for the diagnosis of acute Chikungunya infection. Clin Vaccine Immunol 18:1773–1775
10. Bravo JR, Guzman MG, Kouri GP (1987) Why Dengue haemorrhagic Fever in Cuba? 1. Individual risk factors for Dengue haemorrhagic Fever/Dengue shock syndrome (DHF/DSS). Trans R Soc Trop Med Hyg 81:816–820
11. Burdino E, Calleri G, Caramello P, Ghisetti V (2016) Unmet needs for a rapid diagnosis of Chikungunya virus infection. Emerg Infect Dis 22:1837–1839
12. Caglioti C, Lalle E, Castilletti C, Carletti F, Capobianchi MR, Bordi L (2013) Chikungunya virus infection: an overview. New Microbiol 36:211–227
13. Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, Brandt WE (1989) Antigenic relationships between flaviviruses as determined by cross neutralization tests with polyclonal antisera. J Gen Virol 70:37–43
14. Calleri G, Burdino E, Bonora S, Raso R, Ghisetti V, Caramello P (2016) Zika virus infection in two travelers returning from an epidemic area to Italy, 2016: algorithm for diagnosis and recommendations. Travel Med Infect Dis 14:506–508
15. Calvo EP, Sanchez-Quete F, Duran S, Sandoval I, Castellanos JE (2016) Easy and inexpensive molecular detection of Dengue, Chikungunya and Zika viruses in febrile patients. Acta Trop 163:32–37
16. Campos RDM, Cirne-Santos C, Meira GL, Santos LL, de Meneses MD, Friedrich J, Jansen S, Ribeiro MS, da Cruz IC, Schmidt-Chanasit J, Ferreira DF (2016) Prolonged detection of Zika virus RNA in urine samples during the ongoing Zika virus epidemic in Brazil. J Clin Virol 77:69–70
17. CDC (2016) Guidance for U.S. laboratories testing for Zika virus infection. Available from: http://www.cdc.gov/zika/pdfs/laboratory-guidance-zika.pdf. Accessed 16 Dec 2016
18. Chan SY, Kautner I, Lam SK (1994) Detection and serotyping of Dengue viruses by PCR: a simple, rapid method for the isolation of viral RNA from infected mosquito Larvae. Southeast Asian J Trop Med Public Health 25:258–261
19. Chanama S, Anantapreecha S, Nuegoonpipat A, Sa-gnasang A, Kurane I, Sawanpanyalert P (2004) Analysis of specific IgM responses in secondary Dengue virus infections: levels and positive rates in comparison with primary infections. J Clin Virol 31:185–189
20. Chien LJ, Liao TL, Shu PY, Huang JH, Gubler DJ, Chang GJ (2006) Development of real time reverse transcriptase PCR assays to detect and serotype Dengue viruses. J Clin Microbiol 44:1295–1304
21. Cleton NB, Godeke GJ, Reimerink J, Beersma MF, Doorn HR, Franco L, Goeijenbier M, Jimenez-Clavero MA, Johnson BW, Niedrig M, Papa A, Sambri V, Tami A, Velasco-Salas ZI, Koopmans MP, Reusken CB (2015) Spot the difference-development of a syndrome based protein microarray for specific serological detection of multiple flavivirus infections in travelers. PLoS Negl Trop Dis 9:e0003580
22. Corbett KS, Katzelnick L, Tissera H, Amerasinghe A, de Silva AD, de Silva AM (2015) Preexisting neutralizing antibody responses distinguish clinically inapparent and apparent Dengue virus infections in a Sri Lankan pediatric cohort. J Infect Dis 211:590–599
23. Cordeiro MT, Pena LJ, Brito CA, Gil LH, Marques ET (2016) Positive IgM for Zika virus in the cerebrospinal fluid of 30 neonates with microcephaly in Brazil. Lancet 387:1811–1812
24. Cuzzubbo AJ, Vaughn DW, Nisalak A, Solomon T, Kalayanarooj S, Aaskov J, Dung NM, Devine PL (2000) Comparison of PanBio Dengue Duo IgM and IgG capture ELISA and venture technologies dengue IgM and IgG Dot Blot. J Clin Virol 16:135–144

25. De Araujo TV, Rodrigues LC, de Alencar Ximenes RA, de Barros Miranda-Filho D, Montarroyos UR, de Melo AP, Valongueiro S, de Albuquerque MF, Souza WV, Braga C, Filho SP, Cordeiro MT, Vazquez E, Di Cavalcanti Souza Cruz D, Henriques CM, Bezerra LC, da Silva Castanha PM, Dhalia R, Marques-Junior ET, Martelli CM, Investigators from the Microcephaly Epidemic Research Group, Brazilian Ministry of Health, Pan American Health Organization, Instituto de Medicina Integral Professor Fernando Figueira and State Health Department of Pernambuco (2016) Association between Zika virus infection and microcephaly in Brazil, January–May, 2016: preliminary report of a case-control study. Lancet Infect Dis (in press)
26. De Decker S, Vray M, Sistek V, Labeau B, Enfissi A, Rousset D, Matheus S (2015) Evaluation of the diagnostic accuracy of a new Dengue IgA capture assay (Platelia Dengue IgA Capture, Bio-Rad) for Dengue infection detection. PLoS Negl Trop Dis 9:e0003596
27. De Paula SO, Fonseca BA (2004) Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. Braz J Infect Dis 8:390–398
28. Dupont-Rouzeyrol M, Biron A, O'Connor O, Huguon E, Descloux E (2016) Infectious Zika viral particles in breastmilk. Lancet 387:1051
29. Dussart P, Labeau B, Lagathu G, Louis P, Nunes MR, Rodrigues SG, Storck-Herrmann C, Cesaire R, Morvan J, Flamand M, Baril L (2006) Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin Vaccine Immunol 13(1185):1189
30. Dussart P, Petit L, Labeau B, Bremand L, Leduc A, Moua D, Matheus S, Baril L (2008) Evaluation of two new commercial tests for the diagnosis of acute Dengue virus infection using NS1 antigen detection in human serum. PLoS Negl Trop Dis 2:e280
31. Falconar AK, de Elsa P, Romero-Vivas CM (2006) Altered enzyme-linked immunosorbent assay immunoglobulin M (IgM)/IgG optical density ratios can correctly classify all primary or secondary Dengue virus infections 1 day after the onset of symptoms, when all of the viruses can be isolated. Clin Vaccine Immunol 13:1044–1051
32. FDA (2016) Emergency use authorizations. Available at http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm#zika. Accessed 16 Dec 2016
33. Fonseca K, Meatherall B, Zarra D, Drebot M, Macdonald J, Pabbaraju K, Wong S, Webster P, Lindsay R, Tellier R (2014) First case of Zika virus infection in a returning canadian traveler. Am J Trop Med Hyg 91:1035–1038
34. Fourcade C, Mansuy JM, Dutertre M, Delpech M, Marchou B, Delobel P, Izopet J, Martin BG (2016) Viral load kinetics of Zika virus in plasma, urine and saliva in a couple returning from martinique, french West Indies. J Clin Virol 82:1–4
35. Frank C, Cadar D, Schlaphof A, Neddersen N, Gunther S, Schmidt-Chanasit J, Tappe D (2016) Sexual transmission of Zika virus in Germany, April 2016. Euro Surveill 21(23):pii=30252
36. Fry SR, Meyer M, Semple MG, Simmons CP, Sekaran SD, Huang JX, McElnea C, Huang CY, Valks A, Young PR, Cooper MA (2011) The diagnostic sensitivity of dengue rapid test assays is significantly enhanced by using a combined antigen and antibody testing approach. PLoS Negl Trop Dis 5:e1199
37. Gourinat AC, O'Connor O, Calvez E, Goarant C, Dupont-Rouzeyrol M (2015) Detection of Zika virus in urine. Emerg Infect Dis 21:84–86
38. Granger D, Gómez LJ, Schimek M, Dubbels M, Mosquera JA, Christensen J, Bistodeau S, Strain A, Theel ES (2016) Zika virus antibody detection: evaluation of three different serologic methodologies. Mayo Clinic, MN, USA. Presentation at the 32nd clinical virology symposium, May 19–22, 2016, Daytona Beach, Florida.
39. Guzman MG, Kouri G (2004) Dengue diagnosis, advances and challenges. Int J Infect Dis 8:69–80
40. Hunsperger EA, Yoksan S, Buchy P, Nguyen VC, Sekaran SD, Enria DA, Pelegrino JL, Vazquez S, Artsob H, Drebot M, Gubler DJ, Halstead SB, Guzman MG, Margolis HS, Nathanson CM, Rizzo Lic NR, Bessoff KE, Kliks S, Peeling RW (2009) Evaluation of commercially available Anti-Dengue virus immunoglobulin M tests. Emerg Infect Dis 15:436–440
41. Huzly D, Hanselmann I, Schmidt-Chanasit J, Panning M (2016) High specificity of a novel Zika virus ELISA in European patients after exposure to different flaviviruses. Euro Surveill 21(16):pii=30203
42. INSTAND (2016) Report: external auality assessment scheme, group 350, virus immunology Dengue virus, March 2016. Available at http://www.instand-ev.de/ringversucheonline/ringversuche-service.html. Accessed 16 Dec 2016
43. Johnson BW, Goodman CH, Holloway K, de Salazar PM, Valadere AM, Drebot MA (2016) Evaluation of commercially available Chikungunya virus immunoglobulin M detection assays. Am J Trop Med Hyg 95:182–192
44. Kosasih H, Widjaja S, Surya E, Hadiwijaya SH, Butarbutar DP, Jaya UA, Nurhayati AB, Williams M (2012) Evaluation of two IgM rapid immunochromatographic tests during circulation of Asian lineage Chikungunya virus. Southeast Asian J Trop Med Public Health 43:55–61
45. Kuno G, Gubler DJ, Velez M, Oliver A (1985) Comparative sensitivity of three mosquito cell lines for isolation of Dengue viruses. Bull World Health Organ 63:279–286

46. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, Stanfield SM, Duffy MR (2008) Genetic and serologic properties of Zika virus associated with an Epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis 14:1232–1239
47. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of Dengue viruses from clinical samples by using reverse transcriptase polymerase chain reaction. J Clin Microbiol 30:545–551
48. Landry ML, St.George K (2016) Laboratory diagnosis of Zika virus infection. Arch Pathol Lab Med 141:60–67
49. Latz A, Vögler J (2015) Development and evaluation of a serological IgG and IgM Chikungunya antibody detection assay. Presentation at the 25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) April 25–28, 2015, Copenhagen, Denmark.
50. Laurent P, Le RK, Grivard P, Bertil G, Naze F, Picard M, Staikowsky F, Barau G, Schuffenecker I, Michault A (2007) Development of a sensitive real-time reverse transcriptase PCR assay with an internal control to detect and quantify Chikungunya virus. Clin Chem 53:1408–1414
51. Lewthwaite P, Shankar MV, Tio PH, Daly J, Last A, Ravikumar R, Desai A, Ravi V, Cardosa JM, Solomon T (2010) Evaluation of two commercially available ELISAs for the diagnosis of Japanese encephalitis applied to field samples. Trop Med Int Health 15(811):818
52. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S, Green S, Vaughn DW, Nisalak A, Ennis FA, Rothman AL (2002) High circulating levels of the Dengue virus nonstructural protein NS1 early in Dengue illness correlate with the development of Dengue hemorrhagic fever. J Infect Dis 186:1165–1168
53. Litzba N, Schuffenecker I, Zeller H, Drosten C, Emmerich P, Charrel R, Kreher P, Niedrig M (2008) Evaluation of the dirst commercial Chikungunya virus indirect immunofluorescence test. J Virol Methods 149:175–179
54. Matheus S, Boukhari R, Labeau B, Ernault V, Bremand L, Kazanji M, Rousset D (2016) Specificity of Dengue NS1 antigen in differential diagnosis of Dengue and Zika virus infection. Emerg Infect Dis 22:1691–1693
55. Meltzer E, Lustig Y, Leshem E, Levy R, Gottesman G, Weissmann R, Rabi DH, Hindiyeh M, Koren R, Mendelson E, Schwartz E (2016) Zika virus disease in traveler returning from Vietnam to Israel. Emerg Infect Dis 22:1521–1522
56. Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau VM (2015) Potential sexual transmission of Zika virus. Emerg Infect Dis 21:359–361
57. Nawa M, Takasaki T, Ito M, Inoue S, Morita K, Kurane I (2005) Immunoglobulin A antibody responses in Dengue patients: a useful marker for serodiagnosis of Dengue virus infection. Clin Diagn Lab Immunol 12:1235–1237
58. Niedrig M, Zeller H, Schuffenecker I, Drosten C, Emmerich P, Rumer L, Donoso-Mantke O (2009) International diagnostic accuracy study for the serological detection of Chikungunya virus infection. Clin Microbiol Infect 15:880–884
59. Nunes MR, Nunes Neto JP, Casseb SM, Nunes KN, Martins LC, Rodrigues SG, Matheus S, Dussart P, Casseb LM, Vasconcelos PF (2011) Evaluation of an immunoglobulin M specific capture enzyme-linked immunosorbent assay for rapid diagnosis of Dengue infection. J Virol Methods 171:13–20
60. Oduyebo T, Petersen EE, Rasmussen SA, Mead PS, Meaney-Delman D, Renquist CM, Ellington SR, Fischer M, Staples JE, Powers AM, Villanueva J, Galang RR, Dieke A, Munoz JL, Honein MA, Jamieson DJ (2016) Update: interim guidelines for health care providers caring for pregnant women and women of reproductive age with possible Zika virus exposure – United States, 2016. MMWR Morb Mortal Wkly Rep 65:122–127
61. Osorio L, Ramirez M, Bonelo A, Villar LA, Parra B (2010) Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early Dengue infection. Virol J 7:361
62. Pabbaraju K, Wong S, Gill K, Fonseca K, Tipples GA, Tellier R (2016) Simultaneous detection of Zika, Chikungunya and Dengue viruses by a multiplex real-time RT-PCR assay. J Clin Virol 83:66–71
63. PAHO (2016) Dengue: guidelines for patient care in the region of the Americas. 2nd ed. Pan American Health Organization (PAHO), Washington, DC. Available at http://iris.paho.org/xmlui/bitstream/handle/123456789/31207/9789275118900eng.pdf?sequence=1&isAllowed=y. Accessed 16 Dec 2016
64. PAHO/WHO (2015) Zika virus (ZIKV) surveillance in the Americas: interim guidance for laboratory detection and diagnosis. Available at http://iris.paho.org/xmlui/bitstream/handle/123456789/18602/zikavirusinterim_jan2015.pdf?seuence=1&isAllowed=y. Accessed 16 Dec 2016
65. Pal S, Dauner AL, Mitra I, Forshey BM, Garcia P, Morrison AC, Halsey ES, Kochel TJ, Wu SJ (2014) Evaluation of Dengue NS1 antigen rapid tests and ELISA kits using clinical samples. PLoS One 9:e113411
66. Peeling RW, Artsob H, Pelegrino JL, Buchy P, Cardosa MJ, Devi S, Enria DA, Farrar J, Gubler DJ, Guzman MG, Halstead SB, Hunsperger E, Kliks S, Margolis HS, Nathanson CM, Nguyen VC, Rizzo N, Vazquez S, Yoksan S (2010) Evaluation of diagnostic tests: Dengue. Nat Rev Microbiol 8:S30–S38
67. Petersen LR, Jamieson DJ, Powers AM, Honein MA (2016) Zika Virus. N Engl J Med 374:1552–1563

68. Prat CM, Flusin O, Panella A, Tenebray B, Lanciotti R, Leparc-Goffart I (2014) Evaluation of commercially available serologic diagnostic tests for Chikungunya virus. Emerg Infect Dis 20:2129–2132
69. Priyamvada L, Quicke KM, Hudson WH, Onlamoon N, Sewatanon J, Edupuganti S, Pattanapanyasat K, Chokephaibulkit K, Mulligan MJ, Wilson PC, Ahmed R, Suthar MS, Wrammert J (2016) Human antibody responses after Dengue virus infection are highly cross-reactive to Zika virus. Proc Natl Acad Sci U S A 113:7852–7857
70. Puschnik A, Lau L, Cromwell EA, Balmaseda A, Zompi S, Harris E (2013) Correlation between Dengue-specific neutralizing antibodies and serum avidity in primary and secondary Dengue virus 3 natural infections in humans. PLoS Negl Trop Dis 7:e2274
71. Rabe IB, Staples JE, Villanueva J, Hummel KB, Johnson JA, Rose L, Hills S, Wasley A, Fischer M, Powers AM (2016) Interim guidance for interpretation of Zika virus antibody test results. MMWR Morb Mortal Wkly Rep 65:543–546
72. Reusken C, Pas S, GeurtsvanKessel C, Mogling R, van Kampen J, Langerak T, Koopmans M, van der Eijk A, van Gorp E (2016) Longitudinal follow-up of Zika virus RNA in semen of a traveller returning from Barbados to the Netherlands with Zika virus disease, March 2016. Euro Surveill 21(23):pii=30251
73. Rivetz B, Siman-Tov D, Ambal E, Jaramillo AC, Ben-Zvi A, Tartakovsky B, Fish F (2009) New Dengue antibody assay with unique sifferential detection of IgG and IgM antibodies. Clin Biochem 42:180–184
74. Rosen L (1981) The use of Toxorhynchites mosquitoes to detect and propagate Dengue and other arboviruses. Am J Trop Med Hyg 30:177–183
75. Sa-Ngasang A, Anantapreecha S, Nuegoonpipat A, Chanama S, Wibulwattanakij S, Pattanakul K, Sawanpanyalert P, Kurane I (2006) Specific IgM and IgG responses in primary and secondary Dengue virus infections determined by enzyme-linked immunosorbent assay. Epidemiol Infect 134:820–825
76. Sang CT, Cuzzubbo AJ, Devine PL (1998) Evaluation of a commercial capture enzyme linked immunosorbent assay for detection of immunoglobulin M and G antibodies produced during Dengue infection. Clin Diagn Lab Immunol 5:7–10
77. Schilling S, Ludolfs D, Van AL, Schmitz H (2004) Laboratory diagnosis of primary and secondary Dengue infection. J Clin Virol 31:179–184
78. Shu PY, Chen LK, Chang SF, Yueh YY, Chow L, Chien LJ, Chin C, Lin TH, Huang JH (2003) Comparison of capture immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA) and nonstructural protein NS1 serotype-specific IgG ELISA for differentiation of primary and secondary Dengue virus infections. Clin Diagn Lab Immunol 10:622–630
79. Sierra B, Perez AB, Alvarez M, Garcia G, Vogt K, Aguirre E, Schmolke K, Volk HD, Guzman MG (2012) Variation in inflammatory/regulatory cytokines in secondary, tertiary, and quaternary challenges with Dengue virus. Am J Trop Med Hyg 87:538–547
80. Smith MD, Azizan A (2015) Current global status of Dengue diagnostics. J Adv Biol Biotechnol 2:79–95
81. Steinhagen K, Probst C, Radzimski C, Schmidt-Chanasit J, Emmerich P, Van Esbroeck M, Schinkel J, Grobusch MP, Goorhuis A, Warnecke JM, Lattwein E, Komorowski L, Deerberg A, Saschenbrecker S, Stöcker W, Schlumberger W (2016) Serodiagnosis of Zika virus (ZIKV) infections by a novel NS1-based ELISA devoid of cross-reactivity with Dengue virus antibodies. A multicohort study of assay performance, 2015–2016. Euro Surveill 21(50):pii=30426
82. Tang KF, Ooi EE (2012) Diagnosis of Dengue: an update. Expert Rev Anti Infect Ther 10:895 907
83. TDR/PDVI/WHO (2009) Evaluation of commercially available anti-dengue virus immunoglobulin M tests (Diagnostic evaluation series, 3). World Health Organization (WHO), Geneva, Switzerland. Available at http://www.who.int/tdr/publications/documents/diagnostics evaluation-3.pdf?ua=1. Accessed 16 Dec 2016
84. TDR/WHO (2009) Dengue: gudelines for diagnosis, tratment, prevention and control. new ed. World Health Organization (WHO), Geneva. Available at https://www.ncbi.nlm.nih.gov/books/NBK143157/pdf/Bookshelf_NBK143157.pdf. Accessed 16 Dec 2016
85. Tricou V, Vu HT, Quynh NV, Nguyen CV, Tran HT, Farrar J, Wills B, Simmons CP (2010) Comparison of two Dengue NS1 rapid tests for sensitivity, specificity and relationship to viraemia and antibody responses. BMC Infect Dis 10:142
86. Van Esbroeck M, Meersman K, Michiels J, Arien KK, Van den Bossche D (2016) Letter to the editor: specificity of Zika virus ELISA: interference with Malaria. Euro Surveill 21(21):pii=30237
87. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, Rothman AL, Ennis FA, Nisalak A (1997) Dengue in the early febrile phase: viremia and antibody responses. J Infect Dis 176:322–330
88. Vaughn DW, Nisalak A, Solomon T, Kalayanarooj S, Nguyen MD, Kneen R, Cuzzubbo A, Devine PL (1999) Rapid serologic diagnosis of Dengue virus infection using a commercial capture ELISA that distinguishes primary and secondary infections. Am J Trop Med Hyg 60:693–698
89. Vazquez S, Hafner G, Ruiz D, Calzada N, Guzman MG (2007) Evaluation of immunoglobulin M and G capture enzyme-linked immunosorbent assay Panbio kits for diagnostic Dengue infections. J Clin Virol 39:194–198

90. Vickers IE, Harvey KM, Brown MG, Nelson K, DuCasse MB, Lindo JF (2015) The Performance of the SD BIOLINE Dengue DUO(R) rapid immunochromatographic test kit for the detection of NS1 Antigen, IgM and IgG antibodies during a Dengue type 1 epidemic in Jamaica. J Biomed Sci 22:55
91. Waggoner JJ, Pinsky BA (2016) Zika virus: diagnostics for an emerging pandemic threat. J Clin Microbiol 54:860–867
92. Wang SM, Ali UH, Sekaran SD, Thayan R (2016) Detection and quantification of Chikungunya virus by real-time RT-PCR assay. Methods Mol Biol 1426:105–117
93. Wang SM, Sekaran SD (2010) Early diagnosis of Dengue infection using a commercial Dengue duo rapid test kit for the detection of NS1, IGM, and IGG. Am J Trop Med Hyg 83:690–695
94. Welch RJ, Chang GJ, Litwin CM (2014) Comparison of a commercial Dengue IgM capture ELISA with Dengue antigen focus reduction microneutralization test and the centers for disease control Dengue IgM capture-ELISA. J Virol Methods 195:247–249
95. WHO (2016) Current Zika Product Pipeline (21 June 2016). Available at http://www.who.int/csr/research-and-development/zika_dx_landscape_report.pdf?ua=1. Accessed 16 Dec 2016
96. WHO (2016) Current Zika Product Pipeline (3 March 2016). Available at http://www.who.int/csr/research-and-development/zika-rd-pipeline.pdf?ua=1. Accessed 16 Dec 2016
97. Yagci CD, Uyar Y, Korukluoglu G, Ertek M, Unal S (2012) An Imported Chikungunya fever case from New Delhi, India to Ankara, Turkey: the first imported case of Turkey and review of the literature. Mikrobiyol Bul 46:122–128
98. Yap G, Pok KY, Lai YL, Hapuarachchi HC, Chow A, Leo YS, Tan LK, Ng LC (2010) Evaluation of Chikungunya diagnostic assays: differences in sensitivity of serology assays in two independent outbreaks. PLoS Negl Trop Dis 4:e753
99. Young PR, Hilditch PA, Bletchly C, Halloran W (2000) An antigen capture enzyme-linked immunosorbent assay reveals high levels of the Dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol 38:1053–1057
100. Zavattoni M, Rovida F, Campanini G, Percivalle E, Sarasini A, Cristini G, Tomasoni LR, Castelli F, Baldanti F (2016) Miscarriage following Dengue virus 3 infection in the first six weeks of pregnancy of a Dengue virus-naive traveller returning from Bali to Italy, April 2016. Euro Surveill 21(31):pii=30308

Check for updates

Flaviviral RNA Structures and Their Role in Replication and Immunity

4

Katell Bidet and Mariano A. Garcia-Blanco

Abstract

More than simple vectors of genetic information, flaviviral RNAs have emerged as critical regulators of the virus life cycle. Viral RNAs regulate interactions with viral and cellular proteins in both, mosquito and mammalian hosts to ultimately influence processes as diverse as RNA replication, translation, packaging or pathogenicity. In this chapter, we will review the current knowledge of the role of sequence and structures in the flaviviral RNA in viral propagation and interaction with the host cell. We will also cover the increasing body of evidence linking viral non-coding RNAs with pathogenicity, host immunity and epidemic potential.

Keywords

Flavivirus · RNA · Structures · Replication · Pathogenesis

K. Bidet
Infectious Diseases IRG, Singapore-MIT Alliance for Research and Technology, Singapore, Singapore

M. A. Garcia-Blanco (✉)
Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore, Singapore

Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA

Abbreviations

(+)gRNA	Positive-stranded genome RNA
(−)gRNA	Negative-stranded antigenome RNA
3′DB	3′ untranslated region dumbbell region
3′HP	3′ hairpin
3′SL	3′ terminal stem loop
3′VR	3′ untranslated region variable region
3′xrRNA	3′ exonuclease-resistant RNA structure
5′cHP	5′ capsid hairpin
5′ and 3′CS	5′ and 3′ complementary cyclization sequences
5′ and 3′UAR	5′ and 3′ complementary upstream of A regions
5′SLA/SLB	5′ terminal stem loop A/B
DENV	Dengue virus
DIG	Defective interfering genome
dsRNA	Double-stranded RNA
HCV	Hepatitis C virus
IFN	Interferon type I (IFN-α and IFN-β)
ISG	Interferon-stimulated genes
JEV	Japanese encephalitis virus
KUNV	Kunjin virus
MTase	Methyltransferase
m^6A	N-6 methyladenosine
ncRNA	Non-coding RNA

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_4

NTPase	Nucleoside triphosphatase
ORF	Open reading frame
PRR	Pathogen recognition receptor
RdRp	RNA-dependent RNA-polymerase
RNAi	RNA interference
sfRNA	Subgenomic flaviviral RNA
UTR	Untranslated region
vsRNA	Viral small RNA
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

4.1 Flaviviral RNA Structures Are Critical Regulators of the Viral Life Cycle

The flaviviral genome is a ~11 kb positive single-stranded RNA ((+)gRNA), which is capped like cellular mRNAs, but not polyadenylated. It encodes for a single open reading frame (ORF) flanked by highly conserved and structured 5′ and 3′ untranslated regions (UTRs). Since it serves multiple functions during infection – template for RNA synthesis, translation of viral proteins and encapsidation into new virions – its life cycle must be tightly regulated by viral RNA-RNA and viral RNA-viral proteins interactions [8, 71]. In this section, we describe how flaviviral RNA sequences and structures participate in regulating the generation of a progeny of infectious viruses from the initial infecting particle.

4.1.1 Life Cycle of the Flaviviral Genome

The flaviviral RNA life cycle begins with uncoating of the viral genome and its release in the cytosol of the host cell. There, it undergoes an initial round of translation to produce the viral proteins with enzymatic activities required for viral RNA replication. Viral RNA translation depends solely on cellular factors and is thought to proceed by mechanisms similar to those used for translation of cellular mRNAs: translation initiation factors bind to the cap structure and in turn recruit ribosomes, which decode and elongate the polypeptide chain. It must be noted that both translation initiation and elongation flaviviruses may involve non-canonical mechanisms that require host factors not needed for bulk cellular translation [9, 10]. Flaviviral RNAs are translated as a single polyprotein, which is subsequently cleaved in 10 individual proteins. Two of these, the RNA-dependent RNA-polymerase (RdRp) and methyltransferase (MTase) NS5 and the nucleoside triphosphatase (NTPase) and helicase NS3, are required for viral RNA synthesis [50, 52, 97, 123, 124].

After formation of replication complexes in membrane structures of the endoplasmic reticulum (ER) [41], RNA replication begins with synthesis of a full length negative strand genomic RNA ((−)gRNA), or antigenome, from the positive stranded template. During this process, long double stranded RNA (dsRNA) intermediates are formed. The newly synthesized (−)gRNA is in turn used as a template for synthesis of multiple genomes in an asymmetric replication cycle, producing ~10 times more (+)gRNA than (−)gRNA [71]. Capping of the (+)gRNA – the successive removal of the 5′ phosphate from the first nucleotide of the genome, followed by transfer of a 5′-5′-linked guanine residue, N-7 and 2′-O methylation – is performed by the viral NTPase and MTase [50, 68, 97]. Although little is known about when capping occurs during the viral replication process, recent structural studies of NS5 support a model in which capping is performed concurrently with the initiation of RNA synthesis and that this short capped RNA is then elongated into full-length genome [57].

After many rounds of translation of the (+)gRNA, structural proteins are present in large numbers allowing the viral capsid protein to encapsidate the (+)gRNA, further coated with membrane and envelope proteins and released as new infectious particles capable of starting new infection cycles [15, 56].

It should be noted that a strict temporal division of translation, replication and genome packaging is unlikely during the viral lifecycle, which may resemble more an oscillating system with populations of (+)gRNAs sorting into the three aforementioned activities in a somewhat stochastic fashion [38].

4.1.2 The Role of RNA Structures in Regulating Replication, Translation and Packaging

Decades of extensive characterization have allowed gaining a deeper understanding of the importance RNA elements in flavivirus replication, especially in the UTRs. It is now clear that the 5′, 3′ UTR and the ORF contain highly conserved secondary structures with critical roles in viral RNA replication, translation and encapsidation (Fig. 4.1).

At the 5′ end of the genome, the 5′ stem loops A and B (5′SLA and 5′SLB) contain binding sites to the NS5 RdRp domain, allowing initiation of RNA synthesis.

[27, 33, 40, 66, 77, 126]. Downstream of the start codon is the capsid hairpin (5′cHP), which directs ribosome biding to the correct site of translation initiation [17, 18]. The existence of additional structures further in the capsid coding regions have been more recently reported, such as CCR1, which is required for viral encapsidation [45].

The 3′UTR is divided in three regions: the variable region (3′VR), containing a succession of exonuclease resistant structures (xRNAs), appears more dispensable to viral replication but plays critical functions in virus-host interactions (see Sect. 4.3) [37, 75, 93, 106]. The dumbbell region (3′DB) contains two copies of the DB structure with A-rich stretches required for PABP interaction and therefore translation [2, 95, 119]. Both the 3′VR and 3′DB also contain highly conserved pseudoknots [37, 93, 106, 108, 117]. PKs are tertiary structures formed by complementarity between free nucleotides in loops and sequences downstream the stem and play important roles in stabilizing the RNA and potentially mediate important RNA-protein interactions. Atomic resolution models for two xRNA strutures from Murray Valley Encephalitis virus and ZIKV have been determined revealing the mechanistic basis for their impressive nuclease resistance [1, 13]. The 3′ short hairpin (3′HP) is required for viral replication in mosquito but not mammalian cells [115]. Finally, at the end of the genome the 3′ stem loop (3′SL) is one of the best-characterized structural elements and plays critical roles in both RNA replication and translation [2, 27, 40, 47, 62, 63, 84, 98, 110, 119, 125, 126].

In addition to these linear structures, it is well documented that flaviviral RNAs adopt a circular, or panhandle structure (Fig. 4.2) and that this transition is absolutely required for RNA synthesis [3, 24, 36, 55, 94]. The cyclization of the genome is mediated by long-range RNA-RNA interactions between several sets of complementary sequences in the 5′ and 3′UTRs. The 5′-3′-upstream of A region (5′UAR-3′UAR)

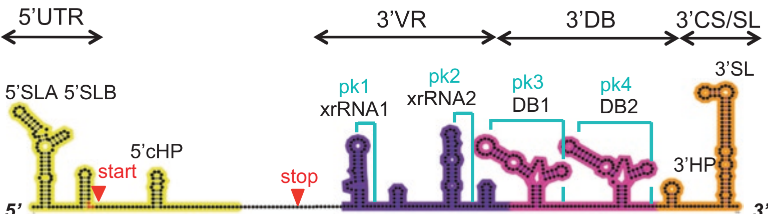


Fig. 4.1 Conserved structures in mosquito-borne flaviviruses UTRs. Model of sequence and structural elements in mosquito-borne flaviviruses UTRs, based on the DENV-2 NGC sequence. The start and stop codons are indicated by red arrows. The 5′terminal region (yellow) encompasses the 5′SLA and 5′SLB in the 5′UTR and the 5′cHP in the capsid-coding region. The 3′UTR is divided into three regions: the 3′VR (purple) with exonuclease-resistant stem-loops xrRNA1 and xrRNA2 (previously named SL-II and SL-IV) the 3′DB region (pink) and the 3′ cyclization and terminal stem-loop region (3′CS/SL, orange). The position of conserved pseudoknot structures is indicated in aqua blue. Complementary sequences in the 5′ and 3′UTRs allowing genome cyclization are underlined (5′ and 3′UAR in light green; 5′ and 3′CS in dark green). This figure is adapted from Bidet and Garcia-Blanco, Biochemical Journal, 2014 to update nomenclature of stem loops in the VR

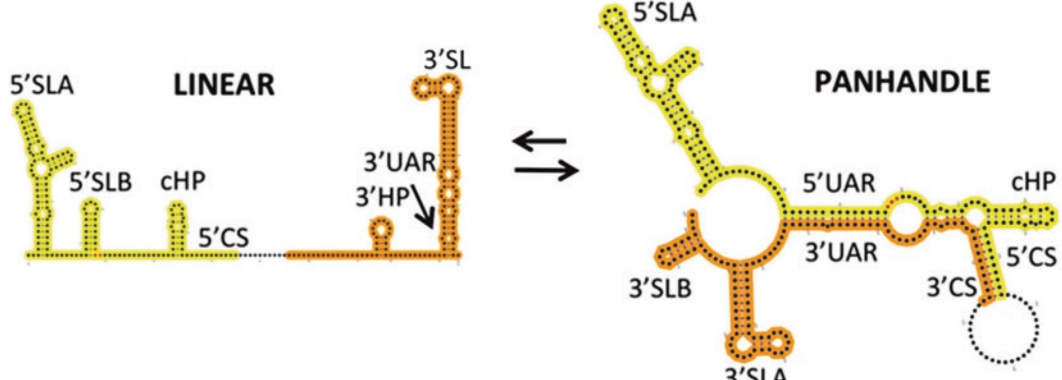


Fig. 4.2 Cyclization of the flavivirus genome. Long range RNA-RNA interactions between the CS and UAR sequences circularize the flaviviral genome into a panhandle structure, inducing conformational changes in the 5′UTR and 3′SL. Notably, the 5′SLB unfolds to allow 5′-3′UAR interactions, the 3′HP unfolds to mediate 5′-3′CS annealing and the 3′SL refolds into 3′SLA and 3′SLB. The model presented is based on the DENV-2 NGC sequence. This figure was originally published in Bidet and Garcia-Blanco, Biochemical Journal, 2014

interaction results in unfolding of the 5′SLB and of the 3′SL into 3′SLA and 3′SLB, while the 5′-3′-cylyzation sequence (5′CS-3′CS) interaction disrupts the 3′hairpin (3′HP) [3, 4, 24, 34, 36, 74, 94]. Other paired regions, such as interactions between the capsid coding region and DB1, might also participate in the conservation of these mutually exclusive structures [76].

Circularization of the flaviviral genome is necessary for RNA synthesis for several reasons. First, NS5 RdRp only binds to the 5′SL; thus circularization is needed to bring it in close proximity to the 3′end where antigenome synthesis starts [27, 33, 66]. In addition, in the linear structure the last nucleotides of the 3′UTR are involved in double stranded interactions in the 3′SL and cannot enter the active site of the RdRp domain to initiate (−)gRNA synthesis [33, 94]. Therefore, the conformation changes in the 3′SL allows to free these nucleotides to serve as template for priming of RNA replication. Importantly, any changes to the complementary sequences that allow formation of the panhandle structure, but also mutations that perturb the switch between the linear and circular conformation result in dead viruses, indicating that a dynamic balance between both structures is critical in the viral life cycle [36, 76].

4.1.3 Conservation and Plasticity of RNA Sequences and Structures Determines Viral Fitness

As most RNA viruses, flaviviruses evolve rapidly due to the error-prone nature of their RdRp. In vitro experiments using DENV-2 NS5 RdRp estimated one misincorporation every 75,000 nucleotides and with hundreds of ~11 kb new genomes synthesized in each infected cell, this implies the generation of several mutant viruses for each round of infection [52]. While this diversity might be one of the main drivers in the success of flaviviruses as human pathogens, it is also restricted by their circulation between two hosts and thus the need to maintain fitness in very different organisms to be successfully transmitted.

Host-specific mechanisms and constraints have been observed in several instances and are particularly apparent at the level of RNA structure conservation. Although many structures described above, such as the 5′SL, 3′SL or 5′-3′ cyclization sequences are required for replication in both hosts, for instance, the DENV-2 capsid coding region element CCR1 appears to affect virion assembly to a greater extend in mosquito

cells [45]. More strikingly, the 3′HP is dispensable for RNA replication in mammalian cells but its absence completely abrogates RNA synthesis in mosquito cells, denoting clear differences in host-specific requirements for RNA elements [115]. This entails added selection pressure on the RNA sequences and structures compared to RNA viruses with only one obligatory host.

Another evidence of this differential selection pressure comes from the study of the 3′VR. Structures in the 3′VR are significantly less conserved than their counterparts in the 5′UTR or 3′SL [37, 93, 116]. While DENV-1, −2, −3 and Zika virus (ZIKV) have two large loops after the stop codon in the 3′VR (xrRNA1 and xrRNA2, as depicted in Fig. 4.1), likely evolving from duplication of an original structure, DENV-4 and yellow fever virus (YFV) only possess one copy whereas Japanese encephalitis virus (JEV), West Nile virus (WNV) and Kunjin virus (KUNV) have an extra large loop inserted in between. Mutagenesis studies on several flaviviruses revealed that depletion of structures in the VR had only minimal effects on intracellular RNA synthesis, translation or encapsidation [2, 37, 75, 93]. As expected in view of this less critical role in viral replication, deep sequencing analysis of DENV-2 sequences showed that the VR rapidly accumulates mutations in mosquito cells. However, most of these variants were deleterious for replication in mammalian cells and were rapidly deselected after host switch [32, 116]. This suggests that the 3′VR plays important roles in virus-host interaction, especially in the human host, and that the sets of duplicated structures it contains might have evolved as a strategy to alleviate evolutionary constraints due to species-specific adaptation mechanisms.

4.2 Flaviviral RNAs Mediate Virus-Host Interaction

The mechanisms described in the previous section imply that flaviviruses possess all enzymatic activities required and thus are self-sufficient for RNA synthesis and encapsidation. However, in infected cells viral RNAs interact with a large variety of host proteins that can dramatically influence – positively or negatively – the outcome of infection. Various studies have attempted to catalogue proteins bound to flaviviral RNAs in artificial cellular systems or during *bona fide* infection and validate their role in viral replication (Table 4.1). In this section, these findings are reviewed with a focus on events occurring in mammalian cells.

4.2.1 Interactions with Host Proteins Modulate Viral Propagation

Flaviviral RNA translation depends entirely on host factors, therefore it is not surprising that a number of RNA-interacting host proteins are involved in modulation of this step of the life cycle. Flaviviral RNAs are capped and must recruit factors such as the cap-binding EIF4E, the translation initiation complex EIF4F and ribosomes for efficient translation. Interaction with the canonical poly A-binding protein PABP is also required for flaviviral RNA translation and it possible through binding to the A-rich DB structures in the 3′UTR, as mentioned previously [95]. It is interesting to note that DENV-2 RNA have been shown to translate efficiently in limiting EIF4E conditions, suggesting that EIF4E independent translation initiation mechanisms can also occur [29]. Indeed, recently some cellular mRNAs have been shown to carry out cap-dependent initiation using EIF3D recognition of the cap [60]. In addition to these basic mechanisms, binding of NFKB2 to the 3′SL of DENV-2 was also shown to enhance translation, while binding of YBX1 binding to the same structure of DENV-2 or FBP1 to the JEV 3′SL inhibited it [16, 61, 90]. Many more host proteins modulating translation of cellular mRNAs have been identified as host factors for DENV-2, YFV or WNV, although their direct interaction and effect on the viral RNAs translation has not been confirmed [58, 59, 79, 100, 105, 117, 127]. This indicates that a wide range of cellular factors, possibly competing with each other for binding to structures in the UTRs, will influence translation of the viral RNAs.

Table 4.1 Host flaviviral RNA-interacting proteins with validated role in viral replication

	Protein	Binding and requirements	Step	References
Proviral	PABP	DENV-2 3′DB	Translation	[95, 92]
	NFKB2	DENV-2 3′SL		[61]
	LSM1	DENV-2 3′UTR		[28, 11]
		Required for DENV-2 and WNV		
	NF90 (ILF3)	DENV-2 3′SL	Replication	[44]
	ERI3	DENV-2, YFV 3′SL		[118]
		Required for DENV-2 and YFV		
	SSB	DENV-2, −4, JEV UTR		[92, 39, 112, 113]
	PTBP1	DENV-2 antigenome		[5]
		Required for DENV-2 not YFV		
	hnRNPA2,C	DENV-2, JEV, not mapped		[25, 54, 92]
	hnRNP G (RBMX)	DENV-2, not mapped		[114]
	EEF1A	DENV-2, YFV, WNV 3′SL		[23]
	TIAR	WNV antigenome		[31, 64]
	CSDE1	DENV-2, not mapped		[92]
	HMCES	DENV-2, not mapped		[114]
	DDX6	DENV-2 3′DB	Post-replication	[117]
	hnRNP M	DENV-2, not mapped		[114]
	NONO	DENV-2, not mapped		[114]
Antiviral	YBX1	DENV-2 3′SL	Translation	[90]
	FBP1	JEV 3′SL		[16]
	G3BP1/2, CAPRIN1 (see Sect. 4.3)	DENV-2 3′VR	Indirect (immune response)	[7]
		Antagonized by DENV-2 not DENV-3, YFV		
	TRIM25 (see 4.3)	DENV-2 3′VR		[78]
	TLR3	dsRNA		[21, 111]
	RIG-I, MDA-5	dsRNA		[87]
	RNase L	WNV, not mapped	RNA degradation	[101]
		Acts on WNV not DENV-2 or JEV		
	MCPIP1 (ZC3H12A)	DENV-2, JEV, not mapped		[70]
		Acts on DENV-2 and JEV		

A large variety of host proteins involved in mRNA metabolism, from heterogenous nuclear ribonucleoproteins (hnRNP) to stress granules and P-body proteins, have been reported to influence flaviviral RNA replication. hnRNPA2, hnRNPC, hnRNPF, RBMX, SSB, HMCES, CSDE1, splicing factor PTBP1, exonuclease ERI3 or RNA degradation-associated LSM1 are required for the replication of some, but not all flaviviral replication [5, 25, 28, 51, 54, 92, 114, 118]. Interestingly, NF90, EEF1A and the stress granule protein TIAR, which are generally associated with translational repression of cellular mRNAs, were also pro-RNA replication factors in the case of flaviviral RNAs [23, 30, 31, 44, 64]. The helicase DDX6, NONO and hnRNPM were dispensable for RNA synthesis but required for the production of infectious particles, suggesting roles in viral encapsidation [92, 114, 117]. For all these proteins though, the precise mechanisms on how they influence viral replication remains to be fully characterized.

Additional studies also revealed that a large number of RNA-binding proteins were recruited to sites of replication and could influence the outcome of infection, although their interaction with the viral RNA and/or direct effect on viral replication was not confirmed. These include stress granule proteins G3BP1, G3BP2, CAPRIN1 (with an indirect effect on replication as dis-

cussed in Sect. 4.3), USP10, FMR1 and NUFIP2 and P-body proteins EDC2, LSM10, LSM12 for DENV-2 infection [117]; P-body proteins GW182, LSM1, DICER, DDX3 for WNV infections [11, 30] and Golgi component ERI3 [118]. Clearly, viral RNA-binding proteins in the host cell will still require more studies to understand the landscape, function and conservation of requirements during flaviviral infections.

4.2.2 Compartmentalization and Switches in the Viral Life Cycle

One of the particularities of the flaviviral life cycle is that it occurs in specialized replication complexes (RCs), a relatively isolated microenvironment of ER-derived membrane vesicles containing high concentrations of both viral and host proteins [41, 120, 121]. Electron microscopy studies showed that the ER compartments contain mostly genome-antigenome dsRNAs intermediates; genomic RNAs associated with ribosomes are located outside in association with the ER membrane and finally encapsidated genomes traffic back to the ER lumen [81, 83, 120]. This implies that genomes could interact with different sets of host factors at different stages of their life cycle, and that these interactions could act to tightly coordinate these steps and ensure optimal viral propagation.

One of the mechanisms described in Sect. 4.1 that could ensure switching between antigenome synthesis and genome synthesis or translation is the dynamic interplay between genome circularization and linearization. Phosphorylation status of viral proteins, for instance phosphorylation near the active site of YFV NS5 MTase or dephosphorylation of the WNV capsid protein for encapsidation, was also proposed to modulate phases of the life cycle [6, 15]. Based on the list of the host factors described above and in Table 4.1, it is possible to hypothesize that binding cellular proteins will also play important roles in deciding the fate of the viral RNAs. For instance, competition between binding of PABP, required for translation and DDX6, required for packaging to the DENV-2 3′DB structures could influence the balance between these two processes and direct the viral RNAs towards infectious virion production. Binding of several factors to the 3′SL (translation activator NFKB2 and translation repressor YBX1, or pro-replication SSB, ERI3, NF90 or EEF1A) could similarly direct the viral RNA into antigenome synthesis, genome synthesis or translation. Competition between viral and cellular factors can also be considered and a mechanism potentially influencing genome packaging was recently described for the related Flaviviridae hepatitis C virus (HCV). Modification of the viral RNA at specific sites with N-6 methyladenosine (m^6A) by the host enzymes METTL3 and METTL4 reduced production of viral particles, presumably because competition between the m^6A-binding protein YTHDF and the viral capsid protein prevents genome encapsidation [43]. While conserved m^6A sites were identified in the genomes of DENV, ZIKV and YFV, the relevance of these processes for flaviviruses is yet to be investigated.

4.2.3 Flaviviral RNAs Are a Target of Host Innate Defenses

In addition to using host factors to enhance replication, flaviviral RNAs must also avoid a large numbers of cellular sensors evolved to detect and destroy foreign pathogenic RNAs. In this area, it appears that the predominant strategy for flaviviral RNAs is hiding. Capping and 2′-O methylation of the viral genomes mimics that of cellular mRNAs and prevents detection by innate immune defenses. Indeed, it was demonstrated that replication of WNV deficient in 2′-O MTase activity was suppressed by the action of interferon-stimulated proteins from the IFIT family [22, 107]. Sequestration of dsRNA replication intermediates, which are usually not present in host cells, in the membranes of the RC was also shown to shelter them and reduce their accessibility to antiviral proteins [46, 82].

In spite of this, some antiviral sensors and effectors are able to detect flaviviral RNAs and restrict their replication. Pathogen recognition receptors (PRRs) TLR3, RIG-I (DDX58) and MDA-5 (IFIH1) can recognize dsRNAs and in turn activate complex innate immune defense programs that target the virus at multiple stages of its life cycle [20, 21, 69, 87, 88, 111]. Effectors

of the antiviral response, particularly endonucleases, can also directly influence replication by degrading viral RNAs. RNaseL can cleave WNV RNA but has no effect on DENV-2 or YFV replication, whereas JEV and DENV-2 RNAs could be readily degraded by MCPIP1 (ZC3H12A) [70, 101]. This indicates that as for proviral factors, antiviral proteins can have different specificities within the flavivirus family.

4.2.4 Alternative NS1 Translation Influence Pathogenicity

Not only RNA structures and their interaction with host proteins can influence the fate of flaviviral RNA in infected cells, they can also affect virus pathogenicity at the organism level. In neurotropic flaviviruses such as JEV, WNV and KUNV, it was demonstrated that the presence of a pseudoknot structure preceeded by a slippery heptanucleotide motif at the start of the NS2A-coding sequence could lead to ribosomal framsehifting, resulting in the production of an alternative NS1 protein [35, 80]. This NS1′ differs from NS1 as it contains 30 nucleotides from an alternative NS2A ORF fused to the original protein. Strikingly, mutations abrogating NS1′ production had no effect on viral replication in mammalian cells but significantly reduced neuroinvasiveness of KUNV [72, 80]. While the precise mechanisms involved in the effect of NS1′ on viral tropism are yet to be determined, this indicates that RNA structures in the flaviviral ORF can play a critical role in viral pathogenesis.

4.3 Flaviviral Non-coding RNAs Are Determinant for Viral Pathogenicity

Although most studies on have historically focused on the role of sequences and structures in the flaviviral genome in viral replication, the discovery in recent years of multiple species of viral-derived non-coding RNAs (ncRNAs) has opened a new and wide field of study in flavivirus biology. Indeed, these ncRNAs have emerged as important regulators of the interactions between the virus and the host, influencing pathogenicity, immune responses and even epidemic potential. These findings are reviewed in this section.

4.3.1 Non-coding RNAs in Flavivirus-Infected Cells

To date, three types of viral non-coding RNAs have been identified in flavivirus-infected cells: defective interfering genomes (DIGs), viral small RNAs (vsRNAs) and the subgenomic flaviviral RNA (sfRNA).

DIGs are heterogenous RNAs derived from the viral genome, but with large deletions in the ORF [53, 67, 91]. They are proposed to arise from intramolecular template switching during RNA replication, with the RdRp jumping sections of the ORF and producing incomplete genomic RNAs. While the function of DIGs during infection remains unclear, they can be packaged into viral particles that have been detected at a close to 1:1 ratio with infectious viruses in the blood of patients [67]. Therefore, DIGs are likely to influence the outcome of infection. DIGs could act by increasing activation of immune responses through PRRs, which was shown in cell culture but not validated in animal models [109]. On the contrary, DIGs could hypothetically act as a decoy for the host antibodies, decreasing the effective occupancy of infectious viruses and allowing them to escape neutralization. Although not mutually exclusive, the relevance of these hypotheses remains to be tested.

Even more elusive and controversial are the vsRNAs, a denomination that encompasses 10–30 nucleotide-long flavivirus-derived RNAs, including micro RNAs (miRNAs) and silencing RNAs (siRNAs). vsRNAs are processed by the ancient antiviral RNA interference machinery and in most cases inhibit (although miRNAs, can sometimes upregulate) expression of complementary sequences [19]. This has been proposed to lead to inhibition of viral replication, especially in arthropod cells where RNAi remains the most prominent antiviral mechanism [99]. Conversely, flavivirus-derived vsRNAs have been detected mostly in mosquito cells with little

to no expression in mammalian cells [65, 89, 102]. Flaviviruses have also been proposed to hijack these mechanisms of regulation of gene expression. Through production of a viral miRNA miR-1 derived from the 3′UTR and sfRNA in mosquito cells, KUNV can upregulate the expression of the host protein GATA4 that acts as a pro-viral factor [49]. The relevance and conservation of these mechanisms has been widely questioned and will require more studies.

Last, the sfRNA is the best-characterized flavivirus non-coding RNA. The sfRNA is a 0.3–0.7 kb, nuclease resistant, highly structured RNA produced by all members of the flavivirus genus [26, 73, 93]. Strikingly, it is the most abundant viral RNA in the cytoplasm of flavivirus-infected cells, present in several fold excess over the genomic RNA [7, 78, 93]. The sfRNA appears concurrently with RNA replication and is produced by incomplete degradation of genomes by the cellular exonuclease XRN1, which stalls on xrRNA structures in the 3′VR [13, 14, 37, 106]. In spite of its high concentration, the resulting product is surprisingly relatively dispensable for viral replication, as KUNV, YFV and DENV-2 mutants deficient in sfRNA production only show mild to no impairment in their replicative ability [37, 104, 106]. However, sfRNA production is absolutely required for cytopathogenicity and interferes with various cellular processes to ensure viral fitness (Fig. 4.3); (Table 4.2).

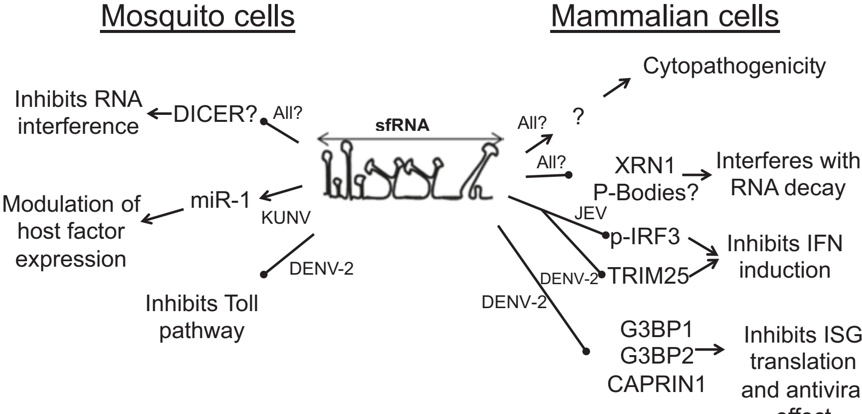


Fig. 4.3 Cellular functions of the non-coding sfRNA

Table 4.2 Flaviviral coding and non-coding RNAs in infected cells

RNA	Size	Abundance	Localization	Function
Genome (+)RNA	11 kb	1	Cytoplasm, replication complexes, virions	Vector of genetic information
Antigenome (−)RNA	11 kb	0.1	Replication complexes	Intermediate template for genome synthesis
Defective interfering genomes (DIGs)	0.5-10 kb	~1	Cytoplasm, replication complexes, virions	Unknown
Subgenomic flaviviral RNA (sfRNA)	0.3–0.7 kb	>1–10[a]	Cytoplasm	Pathogenicity, interference with host response
Viral small RNAs (vsRNA)	10-30 nt	Unknown, but higher in mosquito than mammalian cells	Cytoplasm	Modulation of viral and host gene expression

[a]The relative abundance of the sfRNA varies significantly depending on the virus and some variation may be due to different methods for measuring sfRNA levels

4.3.2 The sfRNA Interferes with Host RNA Metabolism

With the sfRNA being the most abundant viral RNA, and possibly the most abundant RNA in infected cells, it is not surprising that the sfRNA interferes with several host RNA metabolism pathways. The sfRNA is processed by the 5′-3′-exonuclease XRN1, an enzyme required for normal mRNA decay and is able to subsequently inhibit XRN1 activity, possibly by remaining associated to it through the highly structured region that blocks its progression [14, 85, 106]. This XRN1 sequestration by the sfRNA leads to accumulation of uncapped but undegraded cellular mRNA in the cytoplasm of the infected cells [85]. Although the consequences of this accumulation are unclear, it was proposed that it could interfere with cellular processes requiring rapid mRNA turnover, such as immune responses. In addition to XRN1, many other P-Body proteins bind to flaviviviral RNAs and relocate to sites of viral replication, disrupting these structures and likely their function in RNA decay in infected cells [11, 30]. Therefore, it is possible that a global effect on the mRNA degradation machinery of the sfRNA participates in dysregulation of normal cellular functions.

Another important aspect of RNA metabolism is the regulation of gene expression by RNA interference (RNAi). RNAi in insects remains one of the major antiviral mechanisms against flaviviruses [99]. Viral RNAs are processed by the enzyme DICER, which cleaves dsRNA, and short ~20 nucleotides fragments are loaded into the RNA-induced silencing complex (RISC), leading to inhibition of expression of complementary viral sequences and downregulation of viral replication. Interestingly, DENV-1 and WNV sfRNA were reportedly able to inhibit *in vitro* RNA processing by DICER, suggesting that the sfRNA could act as a viral RNAi suppressor [86, 103]. Supporting this hypothesis, RNAi pathways were disrupted in WNV-infected cells and this phenomenon could be reproduced by overexpression of sfRNA but not any viral protein and WNV sfRNA was required for virus transmission by the mosquito [42, 86, 103]. Although the detection of vsRNA produced by processing of the viral RNA by the RNAi machinery indicates that this inhibition is not complete, this provides an explanation as to why flaviviruses are able to establish persistent infections in arthropods in spite of this potent antiviral mechanism.

4.3.3 The sfRNA Is a Critical Regulator of Immune Responses and Influence Epidemic Potential

The most characterized effect of the sfRNA is its role as a suppressor of the interferon type I (IFN) response – the most potent antiviral mechanism in mammalian cells. Interestingly several different mechanisms of action targeting this same pathway have been described in the literature and while not mutually exclusive, they might not be conserved among even closely related viruses.

In vitro and *in vivo* experiments have confirmed that flaviviruses unable to produce sfRNA are impaired in IFN competent, but not IFN incompetent systems, establishing the sfRNA as a potent IFN antagonist [7, 12, 26, 78, 104]. KUNV sfRNA-deficient viruses were rescued to nearly wild-type levels in IRF3-/- IRF7-/- cells and mice unable to respond to IFNs and by co-treatment with IFNAR neutralizing antibodies, indicating that these mutant viruses are more sensitive to the antiviral effects of IFNs [104]. In the case of KUNV, this did not appear to be linked to direct inhibition of the IFN-induced antiviral effector RNaseL by the sfRNA. One alternative strategy by which the sfRNA could protect the virus against the antiviral activity of IFNs was described for DENV-2. DENV-2 sfRNA binds to the host stress granule proteins G3BP1, G3BP2 and CAPRIN1, which are usually required for induction of antiviral IFN-stimulated genes (ISGs). Sequestration of these proteins by the sfRNA impairs the expression of ISGs and attenuates the effects on IFNs on viral replication. Like for the

inhibition of mRNA decay described earlier, the sfRNA therefore acts as a decoy, soaking up host proteins required for normal IFN responses [7]. Surprisingly, the binding of G3BP1, G3BP2 and CAPRIN1 was not conserved for DENV-3, YFV or KUNV sfRNAs, indicating that related viruses must use related but different strategies to antagonize IFN action [8].

In addition to inhibiting the action of IFNs, the sfRNA was also shown to impair the production of IFNs in infected cells. Preliminary evidence came with the observation that JEV sfRNA could inhibit phosphorylation of IRF3, therefore preventing its action as transcriptional activator of the IFN promoter [12]. ZIKV and DENV-2 sfRNA were also demonstrated to interfere with IFN expression [26, 78]. For DENV-2 sfRNA the mechanism involves binding and inactivation of TRIM25 [78]. TRIM25 is an E3 ubiquitin ligase that polyubiquitinates RIG-I to enhance its signaling capacities in order to amplify IFN signaling. Sequestration and inhibition of TRIM25 by the sfRNA could downregulate expression of IFNs by infected cells, therefore reducing IFN-induced inhibition of viral replication in the infected cell as well as establishment of the antiviral state in neighboring cells. In addition, since IFNs are also involved in priming antibody and T cell responses, such mechanisms could have critical repercussions on virus pathogenesis and adaptive immunity. Interestingly, the mechanism involving TRIM25 evolved in a subset of DENV-2 strains isolated during an epidemic in Puerto Rico in 494, after only a handful of point mutations in the 3′VR. The mutations allowed more efficient TRIM25 binding and evasion of IFN responses, resulting in viruses with greater fitness and a complete replacement of the endemic DENV-2 strains in the region [78]. The mutations also influenced sfRNA abundance in mosquito salivary glands and infection rate, through inhibition of the Toll pathway [96]. This clearly indicates that the sfRNA, as much as other RNA structures and sequences described earlier, can have a decisive role in shaping flavivirus epidemic potential.

4.4 Conclusions

In this chapter, we have described a variety of mechanisms by which flaviviral RNAs affect the outcome of infection: RNA sequences and structures, especially in the 5′ and 3′UTRs tightly regulate different steps of the viral life cycle; interactions with host cellular proteins positively or negatively modulate the efficiency of viral replication, translation and/or and production of infectious particles; finally, non-coding RNAs derived from the viral genome critically influence immune responses, pathogenesis and epidemic potential.

Given this central role in flavivirus biology, it is no surprise that many strategies have been considered to target flaviviral RNAs for therapeutic interventions. One of the oldest approach used oligonucleotides with exact complementarity with the viral genome to induce disruption of secondary structures, inhibition of gene expression and/or RNA degradation through RNA interference [48, 122]. Although promising results were obtained *in vitro*, this strategy is difficult to apply for therapeutic use given the variability and rapid evolution of RNA sequences in the flavivirus genome.

Inhibition of host factors and host factors/viral RNA interactions required for flaviviral replication could be an alternative, given that host factors do not mutate as rapidly as viral RNAs. However, this approach has not been tested so far as a therapeutic option since none of the host factors discovered were known drug targets. In addition, the requirements for host RNA-binding proteins in flaviviral replication also appear to vary widely between related viruses or strains of the same virus [5, 7, 58, 59, 101, 105]. This indicates that flaviviruses might be able to use host factors with redundant activities, or rapidly switch between host factors that provide them similar benefits. Because no conserved, unique and obligatory flavivirus host factor has been discovered to date, this makes them less attractive targets for drug development.

Perhaps one of the most promising approaches has been the use of viral RNAs for prophylactic interventions. Incorporating the knowledge

gained on the role of RNA structures in the interaction with the host immune system, two types of live attenuated vaccine candidates have been rationally designed. One is a DENV-2 deficient in 2′O-methyltransferase activity, resulting in better detection of viral RNAs and activation of immune defenses [68]. The other one is a KUNV deficient in sfRNA production, therefore with reduced ability to counteract IFN responses [37, 104]. Both showed great promise by inducing protective immune responses in animal models and are undergoing further clinical evaluation.

While the critical role of viral RNAs in flavivirus replication have opened exciting opportunities for developing new therapeutic interventions, clearly more studies will be needed to fully understand the implications of many observations described in this chapter. Notably, the conservation of mechanisms and host protein interactions between closely related viruses is unclear and further investigations could inform on their relevance in flavivirus biology. In addition, while it is clear that changes as small as single nucleotide mutations can significantly affect viral fitness, delineating between the effects of RNA sequence, structures or host factor binding is challenging because of the interconnection of all stages of the viral life cycle. Therefore, many hurdles need to be overcome to fully understand some of the mechanisms of action described in this chapter. Finally, almost all studies have focused on the role of the short 5′ and 3′UTRs in viral replication and host interaction. As with the example of alternative NS1′ in a subset of flaviviruses, it is highly likely that more RNA sequences and structures in the ~10 kb ORF will add to the complexity of flaviviral RNA-host interactions.

References

1. Akiyama BM, Laurence HM, Massey AR, Costantino DA, Xie X, Yang Y, Shi PY, Nix JC, Beckham JD, Kieft JS (2016) Zika virus produces noncoding RNAs using a multi-pseudoknot structure that confounds a cellular exonuclease. Science 354(6316):1148–1152. https://doi.org/10.1126/science.aah3963
2. Alvarez DE, De Lella Ezcurra AL, Fucito S, Gamarnik AV (2005) Role of RNA structures present at the 3′UTR of dengue virus on translation, RNA synthesis, and viral replication. Virology 339(2):200–212. https://doi.org/10.1016/j.virol.2005.06.009
3. Alvarez DE, Lodeiro MF, Luduena SJ, Pietrasanta LI, Gamarnik AV (2005) Long-range RNA-RNA interactions circularize the dengue virus genome. J Virol 79(11):6631–6643. https://doi.org/10.1128/JVI.79.11.6631-6643.2005
4. Alvarez DE, Filomatori CV, Gamarnik AV (2008) Functional analysis of dengue virus cyclization sequences located at the 5′ and 3′UTRs. Virology 375(1):223–235. https://doi.org/10.1016/j.virol.2008.01.014
5. Anwar A, Leong KM, Ng ML, Chu JJ, Garcia-Blanco MA (2009) The polypyrimidine tract-binding protein is required for efficient dengue virus propagation and associates with the viral replication machinery. J Biol Chem 284(25):17021–17029. https://doi.org/10.1074/jbc.M109.006239
6. Bhattacharya D, Hoover S, Falk SP, Weisblum B, Vestling M, Striker R (2008) Phosphorylation of yellow fever virus NS5 alters methyltransferase activity. Virology 380(2):276–284. https://doi.org/10.1016/j.virol.2008.07.013
7. Bidet K, Dadlani D, Garcia-Blanco MA (2014) G3BP1, G3BP2 and CAPRIN1 are required for translation of interferon stimulated mRNAs and are targeted by a dengue virus non-coding RNA. PLoS Pathog 10(7):e1004242. https://doi.org/10.1371/journal.ppat.1004242
8. Bidet K, Garcia-Blanco MA (2014) Flaviviral RNAs: weapons and targets in the war between virus and host. Biochem J 462(2):215–230. https://doi.org/10.1042/BJ20140456
9. Campos RK, Garcia-Blanco MA, Bradrick SS (2017). Roles of pro-viral host factors in mosquito-borne Flavivirus infections. Curr Top Microbiol Immunol https://doi.org/10.1007/82_2017_26
10. Campos RK, Wong B, Xie X, Lu YF, Shi PY, Pompon J, Garcia-Blanco MA, Bradrick SS (2017). RPLP1 and RPLP2 are essential Flavivirus host factors that promote early viral protein accumulation. J Virol 91(4) https://doi.org/10.1128/JVI.01706-16
11. Chahar HS, Chen S, Manjunath N (2013) P-body components LSM1, GW182, DDX3, DDX6 and XRN1 are recruited to WNV replication sites and positively regulate viral replication. Virology 436(1):1–7. https://doi.org/10.1016/j.virol.2012.09.041
12. Chang RY, Hsu TW, Chen YL, Liu SF, Tsai YJ, Lin YT, Chen YS, Fan YH (2013) Japanese encephalitis virus non-coding RNA inhibits activation of interferon by blocking nuclear translocation of interferon regulatory factor 3. Vet Microbiol 166(1–2):11–21. https://doi.org/10.1016/j.vetmic.2013.04.026
13. Chapman EG, Costantino DA, Rabe JL, Moon SL, Wilusz J, Nix JC, Kieft JS (2014) The structural basis of pathogenic subgenomic flavivirus RNA (sfRNA) production. Science 344(6181):307–310. https://doi.org/10.1126/science.1250897

14. Chapman EG, Moon SL, Wilusz J, Kieft JS (2014) RNA structures that resist degradation by Xrn1 produce a pathogenic Dengue virus RNA. eLife 3:e01892. https://doi.org/10.7554/eLife.01892
15. Cheong YK, Ng ML (2011) Dephosphorylation of West Nile virus capsid protein enhances the processes of nucleocapsid assembly. Microbes Infec Inst Pasteur 13(1):76–84. https://doi.org/10.1016/j.micinf.2010.10.014
16. Chien HL, Liao CL, Lin YL (2011) FUSE binding protein 1 interacts with untranslated regions of Japanese encephalitis virus RNA and negatively regulates viral replication. J Virol 85(10):4698–4706. https://doi.org/10.1128/JVI.01950-10
17. Clyde K, Barrera J, Harris E (2008) The capsid-coding region hairpin element (cHP) is a critical determinant of dengue virus and West Nile virus RNA synthesis. Virology 379(2):314–323. https://doi.org/10.1016/j.virol.2008.06.034
18. Clyde K, Harris E (2006) RNA secondary structure in the coding region of dengue virus type 2 directs translation start codon selection and is required for viral replication. J Virol 80(5):2170–2182. https://doi.org/10.1128/JVI.80.5.2170-2182.2006
19. Cullen BR (2011) Viruses and microRNAs: RISCy interactions with serious consequences. Genes Dev 25(18):1881–1894. https://doi.org/10.1101/gad.17352611
20. da Conceicao TM, Rust NM, Berbel AC, Martins NB, do Nascimento Santos CA, Da Poian AT, de Arruda LB (2013) Essential role of RIG-I in the activation of endothelial cells by dengue virus. Virology 435(2):281–292. https://doi.org/10.1016/j.virol.2012.09.038
21. Daffis S, Samuel MA, Suthar MS, Gale M Jr, Diamond MS (2008) Toll-like receptor 3 has a protective role against West Nile virus infection. J Virol 82(21):10349–10358. https://doi.org/10.1128/JVI.00935-08
22. Daffis S, Szretter KJ, Schriewer J, Li J, Youn S, Errett J, Lin TY, Schneller S, Zust R, Dong H, Thiel V, Sen GC, Fensterl V, Klimstra WB, Pierson TC, Buller RM, Gale M Jr, Shi PY, Diamond MS (2010) 2′-O methylation of the viral mRNA cap evades host restriction by IFIT family members. Nature 468(7322):452–456. https://doi.org/10.1038/nature09489
23. Davis WG, Blackwell JL, Shi PY, Brinton MA (2007) Interaction between the cellular protein eEF1A and the 3′-terminal stem-loop of West Nile virus genomic RNA facilitates viral minus-strand RNA synthesis. J Virol 81(18):10172–10187. https://doi.org/10.1128/JVI.00531-07
24. de Borba L, Villordo SM, Iglesias NG, Filomatori CV, Gebhard LG, Gamarnik AV (2015) Overlapping local and long-range RNA-RNA interactions modulate dengue virus genome cyclization and replication. J Virol 89(6):3430–3437. https://doi.org/10.1128/JVI.02677-14
25. Dechtawewat T, Songprakhon P, Limjindaporn T, Puttikhunt C, Kasinrerk W, Saitornuang S, Yenchitsomanus PT, Noisakran S (2015) Role of human heterogeneous nuclear ribonucleoprotein C1/C2 in dengue virus replication. Virol J 12:14. https://doi.org/10.1186/s12985-014-0219-7
26. Donald CL, Brennan B, Cumberworth SL, Rezelj VV, Clark JJ, Cordeiro MT, Freitas de Oliveira Franca R, Pena LJ, Wilkie GS, Da Silva Filipe A, Davis C, Hughes J, Varjak M, Selinger M, Zuvanov L, Owsianka AM, Patel AH, McLauchlan J, Lindenbach BD, Fall G, Sall AA, Biek R, Rehwinkel J, Schnettler E, Kohl A (2016) Full genome sequence and sfRNA interferon antagonist activity of Zika virus from Recife, Brazil. PLoS Negl Trop Dis 10(10):e0005048. https://doi.org/10.1371/journal.pntd.0005048
27. Dong H, Zhang B, Shi PY (2008) Terminal structures of West Nile virus genomic RNA and their interactions with viral NS5 protein. Virology 381(1):123–135. https://doi.org/10.1016/j.virol.2008.07.040
28. Dong Y, Yang J, Ye W, Wang Y, Miao Y, Ding T, Xiang C, Lei Y, Xu Z (2015) LSm1 binds to the dengue virus RNA 3′ UTR and is a positive regulator of dengue virus replication. Int J Mol Med 35(6):1683–1689. https://doi.org/10.3892/ijmm.2015.2169
29. Edgil D, Polacek C, Harris E (2006) Dengue virus utilizes a novel strategy for translation initiation when cap-dependent translation is inhibited. J Virol 80(6):2976–2986. https://doi.org/10.1128/JVI.80.6.2976-2986.2006
30. Emara MM, Brinton MA (2007) Interaction of TIA-1/TIAR with West Nile and dengue virus products in infected cells interferes with stress granule formation and processing body assembly. Proc Natl Acad Sci U S A 104(21):9041–9046. https://doi.org/10.1073/pnas.0703348104
31. Emara MM, Liu H, Davis WG, Brinton MA (2008) Mutation of mapped TIA-1/TIAR binding sites in the 3′ terminal stem-loop of West Nile virus minus-strand RNA in an infectious clone negatively affects genomic RNA amplification. J Virol 82(21):10657–10670. https://doi.org/10.1128/JVI.00991-08
32. Filomatori CV, Carballeda JM, Villordo SM, Aguirre S, Pallares HM, Maestre AM, Sanchez-Vargas I, Blair CD, Fabri C, Morales MA, Fernandez-Sesma A, Gamarnik AV (2017) Dengue virus genomic variation associated with mosquito adaptation defines the pattern of viral non-coding RNAs and fitness in human cells. PLoS Pathog 13(3):e1006265. https://doi.org/10.1371/journal.ppat.1006265
33. Filomatori CV, Iglesias NG, Villordo SM, Alvarez DE, Gamarnik AV (2011) RNA sequences and structures required for the recruitment and activity of the dengue virus polymerase. J Biol Chem 286(9):6929–6939. https://doi.org/10.1074/jbc.M110.162289
34. Filomatori CV, Lodeiro MF, Alvarez DE, Samsa MM, Pietrasanta L, Gamarnik AV (2006) A 5′ RNA element promotes dengue virus RNA synthesis on

a circular genome. Genes Dev 20(16):2238–2249. https://doi.org/10.1101/gad.1444206

35. Firth AE, Atkins JF (2009) A conserved predicted pseudoknot in the NS2A-encoding sequence of West Nile and Japanese encephalitis flaviviruses suggests NS1′ may derive from ribosomal frameshifting. Virol J 6:14. https://doi.org/10.1186/1743-422X-6-14
36. Friebe P, Harris E (2010) Interplay of RNA elements in the dengue virus 5′ and 3′ ends required for viral RNA replication. J Virol 84(12):6103–6118. https://doi.org/10.1128/JVI.02042-09
37. Funk A, Truong K, Nagasaki T, Torres S, Floden N, Balmori Melian E, Edmonds J, Dong H, Shi PY, Khromykh AA (2010) RNA structures required for production of subgenomic flavivirus RNA. J Virol 84(21):11407–11417. https://doi.org/10.1128/JVI.01159-10
38. Garcia-Blanco MA, Vasudevan SG, Bradrick SS, Nicchitta C (2016) Flavivirus RNA transactions from viral entry to genome replication. Antivir Res 134:244–249. https://doi.org/10.1016/j.antiviral.2016.09.010
39. Garcia-Montalvo BM, Medina F, del Angel RM (2004) La protein binds to NS5 and NS3 and to the 5′ and 3′ ends of dengue 4 virus RNA. Virus Res 102(2):141–150. https://doi.org/10.1016/j.virusres.2004.01.024
40. Gebhard LG, Filomatori CV, Gamarnik AV (2011) Functional RNA elements in the dengue virus genome. Virus 3(9):1739–1756. https://doi.org/10.3390/v3091739
41. Gillespie LK, Hoenen A, Morgan G, Mackenzie JM (2010) The endoplasmic reticulum provides the membrane platform for biogenesis of the flavivirus replication complex. J Virol 84(20):10438–10447. https://doi.org/10.1128/JVI.00986-10
42. Goertz GP, Fros JJ, Miesen P, Vogels CB, van der Bent ML, Geertsema C, Koenraadt CJ, van Rij RP, van Oers MM, Pijlman GP (2016) Noncoding subgenomic Flavivirus RNA is processed by the mosquito RNA interference machinery and determines West Nile virus transmission by Culex pipiens mosquitoes. J Virol 90(22):10145–10159. https://doi.org/10.1128/JVI.00930-16
43. Gokhale NS, McIntyre AB, McFadden MJ, Roder AE, Kennedy EM, Gandara JA, Hopcraft SE, Quicke KM, Vazquez C, Willer J, Ilkayeva OR, Law BA, Holley CL, Garcia-Blanco MA, Evans MJ, Suthar MS, Bradrick SS, Mason CE, Horner SM (2016) N6-methyladenosine in flaviviridae viral RNA genomes regulates infection. Cell Host Microbe. https://doi.org/10.1016/j.chom.2016.09.015
44. Gomila RC, Martin GW, Gehrke L (2011) NF90 binds the dengue virus RNA 3′ terminus and is a positive regulator of dengue virus replication. PLoS One 6(2):e16687. https://doi.org/10.1371/journal.pone.0016687
45. Groat-Carmona AM, Orozco S, Friebe P, Payne A, Kramer L, Harris E (2012) A novel coding-region RNA element modulates infectious dengue virus particle production in both mammalian and mosquito cells and regulates viral replication in Aedes aegypti mosquitoes. Virology 432(2):511–526. https://doi.org/10.1016/j.virol.2012.06.028
46. Hoenen A, Liu W, Kochs G, Khromykh AA, Mackenzie JM (2007) West Nile virus-induced cytoplasmic membrane structures provide partial protection against the interferon-induced antiviral MxA protein. J Gen Virol 88(Pt 11):3013–3017. https://doi.org/10.1099/vir.0.83125-0
47. Holden KL, Harris E (2004) Enhancement of dengue virus translation: role of the 3′ untranslated region and the terminal 3′ stem-loop domain. Virology 329(1):119–133. https://doi.org/10.1016/j.virol.2004.08.004
48. Holden KL, Stein DA, Pierson TC, Ahmed AA, Clyde K, Iversen PL, Harris E (2006) Inhibition of dengue virus translation and RNA synthesis by a morpholino oligomer targeted to the top of the terminal 3′ stem-loop structure. Virology 344(2):439–452. https://doi.org/10.1016/j.virol.2005.08.034
49. Hussain M, Torres S, Schnettler E, Funk A, Grundhoff A, Pijlman GP, Khromykh AA, Asgari S (2012) West Nile virus encodes a microRNA-like small RNA in the 3′ untranslated region which up-regulates GATA4 mRNA and facilitates virus replication in mosquito cells. Nucleic Acids Res 40(5):2210–2223. https://doi.org/10.1093/nar/gkr848
50. Issur M, Geiss BJ, Bougie I, Picard-Jean F, Despins S, Mayette J, Hobdey SE, Bisaillon M (2009) The flavivirus NS5 protein is a true RNA guanylyltransferase that catalyzes a two-step reaction to form the RNA cap structure. RNA 15(12):2340–2350. https://doi.org/10.1261/rna.1609709
51. Jiang L, Yao H, Duan X, Lu X, Liu Y (2009) Polypyrimidine tract-binding protein influences negative strand RNA synthesis of dengue virus. Biochem Biophys Res Commun 385(2):187–192. https://doi.org/10.1016/j.bbrc.2009.05.036
52. Jin Z, Deval J, Johnson KA, Swinney DC (2011) Characterization of the elongation complex of dengue virus RNA polymerase: assembly, kinetics of nucleotide incorporation, and fidelity. J Biol Chem 286(3):2067–2077. https://doi.org/10.1074/jbc.M110.162685
53. Juarez-Martinez AB, Vega-Almeida TO, Salas-Benito M, Garcia-Espitia M, De Nova-Ocampo M, Del Angel RM, Salas-Benito JS (2013) Detection and sequencing of defective viral genomes in C6/36 cells persistently infected with dengue virus 2. Arch Virol 158(3):583–599. https://doi.org/10.1007/s00705-012-1525-2
54. Katoh H, Mori Y, Kambara H, Abe T, Fukuhara T, Morita E, Moriishi K, Kamitani W, Matsuura Y (2011) Heterogeneous nuclear ribonucleoprotein A2 participates in the replication of Japanese encephalitis virus through an interaction with viral proteins and RNA. J Virol 85(21):10976–10988. https://doi.org/10.1128/JVI.00846-11

55. Khromykh AA, Meka H, Guyatt KJ, Westaway EG (2001) Essential role of cyclization sequences in flavivirus RNA replication. J Virol 75(14):6719–6728. https://doi.org/10.1128/JVI.75.14.6719-6728.2001
56. Khromykh AA, Varnavski AN, Sedlak PL, Westaway EG (2001) Coupling between replication and packaging of flavivirus RNA: evidence derived from the use of DNA-based full-length cDNA clones of Kunjin virus. J Virol 75(10):4633–4640. https://doi.org/10.1128/JVI.75.10.4633-4640.2001
57. Klema VJ, Ye M, Hindupur A, Teramoto T, Gottipati K, Padmanabhan R, Choi KH (2016) Dengue virus nonstructural protein 5 (NS5) assembles into a dimer with a unique Methyltransferase and polymerase Interface. PLoS Pathog 12(2):e1005451. https://doi.org/10.1371/journal.ppat.1005451
58. Krishnan MN, Ng A, Sukumaran B, Gilfoy FD, Uchil PD, Sultana H, Brass AL, Adametz R, Tsui M, Qian F, Montgomery RR, Lev S, Mason PW, Koski RA, Elledge SJ, Xavier RJ, Agaisse H, Fikrig E (2008) RNA interference screen for human genes associated with West Nile virus infection. Nature 455(7210):242–245. https://doi.org/10.1038/nature07207
59. Le Sommer C, Barrows NJ, Bradrick SS, Pearson JL, Garcia-Blanco MA (2012) G protein-coupled receptor kinase 2 promotes flaviviridae entry and replication. PLoS Negl Trop Dis 6(9):e1820. https://doi.org/10.1371/journal.pntd.0001820
60. Lee AS, Kranzusch PJ, Doudna JA, Cate JH (2016) eIF3d is an mRNA cap-binding protein that is required for specialized translation initiation. Nature 536(7614):96–99. https://doi.org/10.1038/nature18954
61. Lei Y, Huang Y, Zhang H, Yu L, Zhang M, Dayton A (2011) Functional interaction between cellular p100 and the dengue virus 3′ UTR. J Gen Virol 92(Pt 4):796–806. https://doi.org/10.1099/vir.0.028597-0
62. Lescrinier E, Dyubankova N, Nauwelaerts K, Jones R, Herdewijn P (2010) Structure determination of the top-loop of the conserved 3′-terminal secondary structure in the genome of flaviviruses. Chembiochem Eur J Chem Biol 11(10):1404–1412. https://doi.org/10.1002/cbic.200900765
63. Li W, Brinton MA (2001) The 3′ stem loop of the West Nile virus genomic RNA can suppress translation of chimeric mRNAs. Virology 287(1):49–61. https://doi.org/10.1006/viro.2001.1015
64. Li W, Li Y, Kedersha N, Anderson P, Emara M, Swiderek KM, Moreno GT, Brinton MA (2002) Cell proteins TIA-1 and TIAR interact with the 3′ stem-loop of the West Nile virus complementary minus-strand RNA and facilitate virus replication. J Virol 76(23):11989–12000
65. Li SC, Shiau CK, Lin WC (2008) Vir-Mir db: prediction of viral microRNA candidate hairpins. Nucleic Acids Res 36(Database issue):D184–D189. https://doi.org/10.1093/nar/gkm610
66. Li XF, Jiang T, Yu XD, Deng YQ, Zhao H, Zhu QY, Qin ED, Qin CF (2010) RNA elements within the 5′ untranslated region of the West Nile virus genome are critical for RNA synthesis and virus replication. J Gen Virol 91(Pt 5):1218–1223. https://doi.org/10.1099/vir.0.013854-0
67. Li D, Lott WB, Lowry K, Jones A, Thu HM, Aaskov J (2011) Defective interfering viral particles in acute dengue infections. PLoS One 6(4):e19447. https://doi.org/10.1371/journal.pone.0019447
68. Li SH, Dong H, Li XF, Xie X, Zhao H, Deng YQ, Wang XY, Ye Q, Zhu SY, Wang HJ, Zhang B, Leng QB, Zuest R, Qin ED, Qin CF, Shi PY (2013) Rational design of a flavivirus vaccine by abolishing viral RNA 2′-O methylation. J Virol 87(10):5812–5819. https://doi.org/10.1128/JVI.02806-12
69. Liang Z, Wu S, Li Y, He L, Wu M, Jiang L, Feng L, Zhang P, Huang X (2011) Activation of toll-like receptor 3 impairs the dengue virus serotype 2 replication through induction of IFN-beta in cultured hepatoma cells. PLoS One 6(8):e23346. https://doi.org/10.1371/journal.pone.0023346
70. Lin RJ, Chien HL, Lin SY, Chang BL, Yu HP, Tang WC, Lin YL (2013) MCPIP1 ribonuclease exhibits broad-spectrum antiviral effects through viral RNA binding and degradation. Nucleic Acids Res 41(5):3314–3326. https://doi.org/10.1093/nar/gkt019
71. Lindenbach BD (2007) Flaviviridae: the viruses and their replication. In: Knipe DM, Howley PM (eds) Fields virology. Lippincott-Raven, Philadelphia
72. Liu WJ, Wang XJ, Clark DC, Lobigs M, Hall RA, Khromykh AA (2006) A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. J Virol 80(5):2396–2404. https://doi.org/10.1128/JVI.80.5.2396-2404.2006
73. Liu R, Yue L, Li X, Yu X, Zhao H, Jiang Z, Qin E, Qin C (2010) Identification and characterization of small sub-genomic RNAs in dengue 1-4 virus-infected cell cultures and tissues. Biochem Biophys Res Commun 391(1):1099–1103. https://doi.org/10.1016/j.bbrc.2009.12.030
74. Liu ZY, Li XF, Jiang T, Deng YQ, Zhao H, Wang HJ, Ye Q, Zhu SY, Qiu Y, Zhou X, Qin ED, Qin CF (2013) Novel cis-acting element within the capsid-coding region enhances flavivirus viral-RNA replication by regulating genome cyclization. J Virol 87(12):6804–6818. https://doi.org/10.1128/JVI.00243-13
75. Liu Y, Liu H, Zou J, Zhang B, Yuan Z (2014) Dengue virus subgenomic RNA induces apoptosis through the Bcl-2-mediated PI3k/Akt signaling pathway. Virology 448:15–25. https://doi.org/10.1016/j.virol.2013.09.016
76. Liu ZY, Li XF, Jiang T, Deng YQ, Ye Q, Zhao H, Yu JY, Qin CF (2016) Viral RNA switch mediates the dynamic control of flavivirus replicase recruitment by genome cyclization. Elife 5. https://doi.org/10.7554/eLife.17636

77. Lodeiro MF, Filomatori CV, Gamarnik AV (2009) Structural and functional studies of the promoter element for dengue virus RNA replication. J Virol 83(2):993–1008. https://doi.org/10.1128/JVI.01647-08
78. Manokaran G, Finol E, Wang C, Gunaratne J, Bahl J, Ong EZ, Tan HC, Sessions OM, Ward AM, Gubler DJ, Harris E, Garcia-Blanco MA, Ooi EE (2015) Dengue subgenomic RNA binds TRIM25 to inhibit interferon expression for epidemiological fitness. Science 350(6257):217–221. https://doi.org/10.1126/science.aab3369
79. Marceau CD, Puschnik AS, Majzoub K, Ooi YS, Brewer SM, Fuchs G, Swaminathan K, Mata MA, Elias JE, Sarnow P, Carette JE (2016) Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. Nature 535(7610):159–163. https://doi.org/10.1038/nature18631
80. Melian EB, Hinzman E, Nagasaki T, Firth AE, Wills NM, Nouwens AS, Blitvich BJ, Leung J, Funk A, Atkins JF, Hall R, Khromykh AA (2010) NS1 of flaviviruses in the Japanese encephalitis virus serogroup is a product of ribosomal frameshifting and plays a role in viral neuroinvasiveness. J Virol 84(3):1641–1647. https://doi.org/10.1128/JVI.01979-09
81. Miorin L, Maiuri P, Hoenninger VM, Mandl CW, Marcello A (2008) Spatial and temporal organization of tick-borne encephalitis flavivirus replicated RNA in living cells. Virology 379(1):64–77. https://doi.org/10.1016/j.virol.2008.06.025
82. Miorin L, Albornoz A, Baba MM, D'Agaro P, Marcello A (2012) Formation of membrane-defined compartments by tick-borne encephalitis virus contributes to the early delay in interferon signaling. Virus Res 163(2):660–666. https://doi.org/10.1016/j.virusres.2011.11.020
83. Miorin L, Romero-Brey I, Maiuri P, Hoppe S, Krijnse-Locker J, Bartenschlager R, Marcello A (2013) Three-dimensional architecture of tick-borne encephalitis virus replication sites and trafficking of the replicated RNA. J Virol 87(11):6469–6481. https://doi.org/10.1128/JVI.03456-12
84. Mohan PM, Padmanabhan R (1991) Detection of stable secondary structure at the 3′ terminus of dengue virus type 2 RNA. Gene 108(2):185–191
85. Moon SL, Anderson JR, Kumagai Y, Wilusz CJ, Akira S, Khromykh AA, Wilusz J (2012) A noncoding RNA produced by arthropod-borne flaviviruses inhibits the cellular exoribonuclease XRN1 and alters host mRNA stability. RNA 18(11):2029–2040. https://doi.org/10.1261/rna.034330.112
86. Moon SL, Dodd BJ, Brackney DE, Wilusz CJ, Ebel GD, Wilusz J (2015) Flavivirus sfRNA suppresses antiviral RNA interference in cultured cells and mosquitoes and directly interacts with the RNAi machinery. Virology 485:322–329. https://doi.org/10.1016/j.virol.2015.08.009
87. Nasirudeen AM, Wong HH, Thien P, Xu S, Lam KP, Liu DX (2011) RIG-I, MDA5 and TLR3 synergistically play an important role in restriction of dengue virus infection. PLoS Negl Trop Dis 5(1):e926. https://doi.org/10.1371/journal.pntd.0000926
88. Nazmi A, Dutta K, Basu A (2011) RIG-I mediates innate immune response in mouse neurons following Japanese encephalitis virus infection. PLoS One 6(6):e21761. https://doi.org/10.1371/journal.pone.0021761
89. Parameswaran P, Sklan E, Wilkins C, Burgon T, Samuel MA, Lu R, Ansel KM, Heissmeyer V, Einav S, Jackson W, Doukas T, Paranjape S, Polacek C, dos Santos FB, Jalili R, Babrzadeh F, Gharizadeh B, Grimm D, Kay M, Koike S, Sarnow P, Ronaghi M, Ding SW, Harris E, Chow M, Diamond MS, Kirkegaard K, Glenn JS, Fire AZ (2010) Six RNA viruses and forty-one hosts: viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. PLoS Pathog 6(2):e1000764. https://doi.org/10.1371/journal.ppat.1000764
90. Paranjape SM, Harris E (2007) Y box-binding protein-1 binds to the dengue virus 3′-untranslated region and mediates antiviral effects. J Biol Chem 282(42):30497–30508. https://doi.org/10.1074/jbc.M705755200
91. Pesko KN, Fitzpatrick KA, Ryan EM, Shi PY, Zhang B, Lennon NJ, Newman RM, Henn MR, Ebel GD (2012) Internally deleted WNV genomes isolated from exotic birds in New Mexico: function in cells, mosquitoes, and mice. Virology 427(1):10–17. https://doi.org/10.1016/j.virol.2012.01.028
92. Phillips SL, Soderblom EJ, Bradrick SS, Garcia-Blanco MA (2016) Identification of proteins bound to dengue viral RNA in vivo reveals new host proteins important for virus replication. MBio 7(1):e01865-15. https://doi.org/10.1128/mBio.01865-15
93. Pijlman GP, Funk A, Kondratieva N, Leung J, Torres S, van der Aa L, Liu WJ, Palmenberg AC, Shi PY, Hall RA, Khromykh AA (2008) A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity. Cell Host Microbe 4(6):579–591. https://doi.org/10.1016/j.chom.2008.10.007
94. Polacek C, Foley JE, Harris E (2009) Conformational changes in the solution structure of the dengue virus 5′ end in the presence and absence of the 3′ untranslated region. J Virol 83(2):1161–1166. https://doi.org/10.1128/JVI.01362-08
95. Polacek C, Friebe P, Harris E (2009) Poly(a)-binding protein binds to the non-polyadenylated 3′ untranslated region of dengue virus and modulates translation efficiency. J Gen Virol 90(Pt 3):687–692. https://doi.org/10.1099/vir.0.007021-0
96. Pompon J, Manuel M, Ng GK, Wong B, Shan C, Manokaran G, Soto-Acosta R, Bradrick SS, Ooi EE, Misse D, Shi PY, Garcia-Blanco MA (2017) Dengue subgenomic flaviviral RNA disrupts immunity in mosquito salivary glands to increase virus transmission. PLoS Pathog 13(7):e1006535. https://doi.org/10.1371/journal.ppat.1006535

97. Ray D, Shah A, Tilgner M, Guo Y, Zhao Y, Dong H, Deas TS, Zhou Y, Li H, Shi PY (2006) West Nile virus 5′-cap structure is formed by sequential guanine N-7 and ribose 2′-O methylations by nonstructural protein 5. J Virol 80(17):8362–8370. https://doi.org/10.1128/JVI.00814-06
98. Romero TA, Tumban E, Jun J, Lott WB, Hanley KA (2006) Secondary structure of dengue virus type 4 3′ untranslated region: impact of deletion and substitution mutations. J Gen Virol 87(Pt 11):3291–3296. https://doi.org/10.1099/vir.0.82182-0
99. Sanchez-Vargas I, Scott JC, Poole-Smith BK, Franz AW, Barbosa-Solomieu V, Wilusz J, Olson KE, Blair CD (2009) Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito's RNA interference pathway. PLoS Pathog 5(2):e1000299. https://doi.org/10.1371/journal.ppat.1000299
100. Savidis G, McDougall WM, Meraner P, Perreira JM, Portmann JM, Trincucci G, John SP, Aker AM, Renzette N, Robbins DR, Guo Z, Green S, Kowalik TF, Brass AL (2016) Identification of Zika virus and dengue virus dependency factors using functional genomics. Cell Rep 16(1):232–246. https://doi.org/10.1016/j.celrep.2016.06.028
101. Scherbik SV, Paranjape JM, Stockman BM, Silverman RH, Brinton MA (2006) RNase L plays a role in the antiviral response to West Nile virus. J Virol 80(6):2987–2999. https://doi.org/10.1128/JVI.80.6.2987-2999.2006
102. Schirtzinger EE, Andrade CC, Devitt N, Ramaraj T, Jacobi JL, Schilkey F, Hanley KA (2015) Repertoire of virus-derived small RNAs produced by mosquito and mammalian cells in response to dengue virus infection. Virology 476:54–60. https://doi.org/10.1016/j.virol.2014.11.019
103. Schnettler E, Sterken MG, Leung JY, Metz SW, Geertsema C, Goldbach RW, Vlak JM, Kohl A, Khromykh AA, Pijlman GP (2012) Noncoding flavivirus RNA displays RNA interference suppressor activity in insect and mammalian cells. J Virol 86(24):13486–13500. https://doi.org/10.1128/JVI.01104-12
104. Schuessler A, Funk A, Lazear HM, Cooper DA, Torres S, Daffis S, Jha BK, Kumagai Y, Takeuchi O, Hertzog P, Silverman R, Akira S, Barton DJ, Diamond MS, Khromykh AA (2012) West Nile virus non-coding subgenomic RNA contributes to viral evasion of type I interferon-mediated antiviral response. J Virol. https://doi.org/10.1128/JVI.00207-12
105. Sessions OM, Barrows NJ, Souza-Neto JA, Robinson TJ, Hershey CL, Rodgers MA, Ramirez JL, Dimopoulos G, Yang PL, Pearson JL, Garcia-Blanco MA (2009) Discovery of insect and human dengue virus host factors. Nature 458(7241):1047–1050. https://doi.org/10.1038/nature07967
106. Silva PA, Pereira CF, Dalebout TJ, Spaan WJ, Bredenbeek PJ (2010) An RNA pseudoknot is required for production of yellow fever virus subgenomic RNA by the host nuclease XRN1. J Virol 84(21):11395–11406. https://doi.org/10.1128/JVI.01047-10
107. Szretter KJ, Daniels BP, Cho H, Gainey MD, Yokoyama WM, Gale M Jr, Virgin HW, Klein RS, Sen GC, Diamond MS (2012) 2′-O methylation of the viral mRNA cap by West Nile virus evades ifit1-dependent and-independent mechanisms of host restriction in vivo. PLoS Pathog 8(5):e1002698. https://doi.org/10.1371/journal.ppat.1002698
108. Sztuba-Solinska J, Teramoto T, Rausch JW, Shapiro BA, Padmanabhan R, Le Grice SF (2013) Structural complexity of dengue virus untranslated regions: cis-acting RNA motifs and pseudoknot interactions modulating functionality of the viral genome. Nucleic Acids Res 41(9):5075–5089. https://doi.org/10.1093/nar/gkt203
109. Tapia K, Kim WK, Sun Y, Mercado-Lopez X, Dunay E, Wise M, Adu M, Lopez CB (2013) Defective viral genomes arising in vivo provide critical danger signals for the triggering of lung antiviral immunity. PLoS Pathog 9(10):e1003703. https://doi.org/10.1371/journal.ppat.1003703
110. Tilgner M, Shi PY (2004) Structure and function of the 3′ terminal six nucleotides of the west nile virus genome in viral replication. J Virol 78(15):8159–8171. https://doi.org/10.1128/JVI.78.15.8159-8171.2004
111. Tsai YT, Chang SY, Lee CN, Kao CL (2009) Human TLR3 recognizes dengue virus and modulates viral replication in vitro. Cell Microbiol 11(4):604–615. https://doi.org/10.1111/j.1462-5822.2008.01277.x
112. Vashist S, Anantpadma M, Sharma H, Vrati S (2009) La protein binds the predicted loop structures in the 3′ non-coding region of Japanese encephalitis virus genome: role in virus replication. J Gen Virol 90(Pt 6):1343–1352. https://doi.org/10.1099/vir.0.010850-0
113. Vashist S, Bhullar D, Vrati S (2011) La protein can simultaneously bind to both 3′- and 5′-noncoding regions of Japanese encephalitis virus genome. DNA Cell Biol 30(6):339–346. https://doi.org/10.1089/dna.2010.1114
114. Viktorovskaya OV, Greco TM, Cristea IM, Thompson SR (2016) Identification of RNA binding proteins associated with dengue virus RNA in infected cells reveals temporally distinct host factor requirements. PLoS Negl Trop Dis 10(8):e0004921. https://doi.org/10.1371/journal.pntd.0004921
115. Villordo SM, Gamarnik AV (2013) Differential RNA sequence requirement for dengue virus replication in mosquito and mammalian cells. J Virol 87(16):9365–9372. https://doi.org/10.1128/JVI.00567-13
116. Villordo SM, Filomatori CV, Sanchez-Vargas I, Blair CD, Gamarnik AV (2015) Dengue virus RNA structure specialization facilitates host adaptation. PLoS Pathog 11(1):e1004604. https://doi.org/10.1371/journal.ppat.1004604
117. Ward AM, Bidet K, Yinglin A, Ler SG, Hogue K, Blackstock W, Gunaratne J, Garcia-Blanco MA (2011) Quantitative mass spectrometry of DENV-2

RNA-interacting proteins reveals that the DEAD-box RNA helicase DDX6 binds the DB1 and DB2 3′ UTR structures. RNA Biol 8(6):1173–1186. https://doi.org/10.4161/rna.8.6.17836
118. Ward AM, Calvert ME, Read LR, Kang S, Levitt BE, Dimopoulos G, Bradrick SS, Gunaratne J, Garcia-Blanco MA (2016) The Golgi associated ERI3 is a Flavivirus host factor. Sci Rep 6:34379. https://doi.org/10.1038/srep34379
119. Wei Y, Qin C, Jiang T, Li X, Zhao H, Liu Z, Deng Y, Liu R, Chen S, Yu M, Qin E (2009) Translational regulation by the 3′ untranslated region of the dengue type 2 virus genome. Am J Trop Med Hyg 81(5):817–824. https://doi.org/10.4269/ajtmh.2009.08-0595
120. Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CK, Walther P, Fuller SD, Antony C, Krijnse-Locker J, Bartenschlager R (2009) Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host Microbe 5(4):365–375. https://doi.org/10.1016/j.chom.2009.03.007
121. Westaway EG, Mackenzie JM, Kenney MT, Jones MK, Khromykh AA (1997) Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. J Virol 71(9):6650–6661
122. Yoo JS, Kim CM, Kim JH, Kim JY, Oh JW (2009) Inhibition of Japanese encephalitis virus replication by peptide nucleic acids targeting cis-acting elements on the plus- and minus-strands of viral RNA. Antivir Res 82(3):122–133. https://doi.org/10.1016/j.antiviral.2009.02.187
123. You S, Padmanabhan R (1999) A novel in vitro replication system for dengue virus. Initiation of RNA synthesis at the 3′-end of exogenous viral RNA templates requires 5′- and 3′-terminal complementary sequence motifs of the viral RNA. J Biol Chem 274(47):33714–33722
124. You S, Falgout B, Markoff L, Padmanabhan R (2001) In vitro RNA synthesis from exogenous dengue viral RNA templates requires long range interactions between 5′- and 3′-terminal regions that influence RNA structure. J Biol Chem 276(19):15581–15591. https://doi.org/10.1074/jbc.M010923200
125. Yu L, Markoff L (2005) The topology of bulges in the long stem of the flavivirus 3′ stem-loop is a major determinant of RNA replication competence. J Virol 79(4):2309–2324. https://doi.org/10.1128/JVI.79.4.2309-2324.2005
126. Yu L, Nomaguchi M, Padmanabhan R, Markoff L (2008) Specific requirements for elements of the 5′ and 3′ terminal regions in flavivirus RNA synthesis and viral replication. Virology 374(1):170–185. https://doi.org/10.1016/j.virol.2007.12.035
127. Zhang R, Miner JJ, Gorman MJ, Rausch K, Ramage H, White JP, Zuiani A, Zhang P, Fernandez E, Zhang Q, Dowd KA, Pierson TC, Cherry S, Diamond MS (2016) A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. Nature 535(7610):164–168. https://doi.org/10.1038/nature18625

5 The Molecular Specificity of the Human Antibody Response to Dengue Virus Infections

Emily N. Gallichotte, Ralph S. Baric, and Aravinda M. de Silva

Abstract

Dengue viruses (DENV) are mosquito-borne positive sense RNA viruses in the family *Flaviviridae*. The four serotypes of DENV (DENV1, DENV2, DENV3, DENV4) are widely distributed and it is estimated over a third of the world's population is at risk of infection [4]. While the majority of infections are asymptomatic, DENV infection can cause a spectrum of disease, from mild flu-like symptoms, to the more severe DENV hemorrhagic fever and shock syndrome [24]. Over the past 20 years, there have been intense efforts to develop a tetravalent live-attenuated DENV vaccine [36]. The process of vaccine development has been largely empirical, because effective live attenuated vaccines have been developed for other flaviviruses like yellow fever and Japanese encephalitis viruses. However, recent results from phase III live attenuated DENV vaccine efficacy trials are mixed with evidence for efficacy in some populations but not others [20]. In light of unexpected results from DENV vaccine trials, in this chapter we will review recent discoveries about the human antibody response to natural DENV infection and discuss the relevance of this work to understanding vaccine performance.

Keywords

Dengue virus · Dengue vaccines · Neutralizing antibodies · Human antibodies · Dengue epitopes

E. N. Gallichotte · A. M. de Silva (✉)
Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, USA
e-mail: desilva@med.unc.edu

R. S. Baric
Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, USA

Department of Epidemiology, University of North Carolina School of Public Health, Chapel Hill, NC, USA

5.1 DENV Structure

The DENV genome encodes a single open reading frame that is translated into a polyprotein. Viral and host proteases cleave the polyprotein into three structural and seven non-structural viral proteins. The structural envelope protein (E) contains three domains, domain I (EDI), domain II (EDII) and domain III (EDIII) [45]. Two envelope monomers come together in a head-to-tail orientation, forming the E dimer (Fig. 5.1). Three E dimers form the dimer raft, and 30 dimer rafts cover the surface of the DENV virion in icosahedral orientation with both threefold and fivefold axes of symmetry. Domain II contains the hydro-

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_5

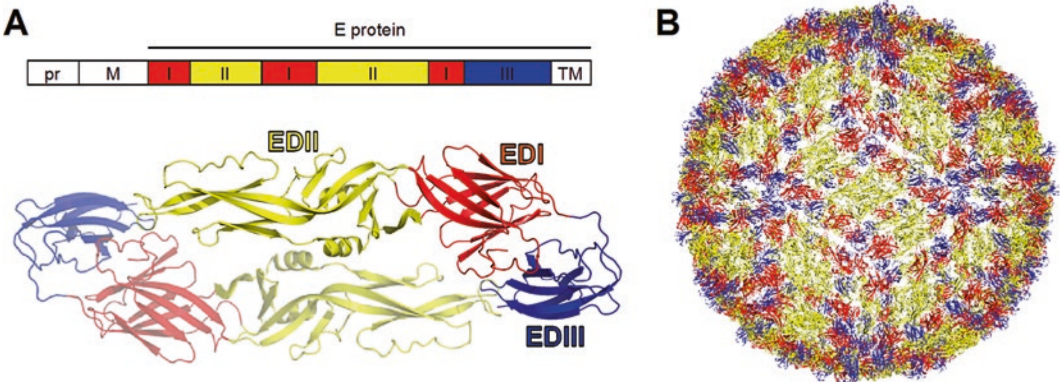


Fig. 5.1 Structure of DENV. (a) Linear schematic of DENV envelope (E) protein. DENV E protein dimer composed of two monomers with domains I, II and III colored in red, yellow and blue respectively. (**b**) DENV virion structure composed of 30 rafts, each containing three E dimers

phobic fusion peptide, which mediates fusion between the virus and host cell membrane. To prevent fusion with the host membrane during egress from infected cells, the pre-membrane (prM) protein covers the fusion loop. As the virus moves through the endosome, pH changes triggers the host protease furin to cleave the prM protein [60]. As the virus is released from cells, cleaved prM dissociates from the virion. This process is inefficient however, leaving a heterogeneous population of fully mature (no prM present), fully immature (containing prM), and partially mature virions [59]. While cell culture grown virus shows a spectrum of maturation states, it is now clear that the overall maturation state of virions can vary between strains and even between different preparations of the same strain [39]. As we discuss later, maturation state can influence the ability of some antibodies to bind and neutralize DENV and other flaviviruses.

5.2 Antibody Response to DENV Infection

The basic kinetics of the DENV specific Ab response, the timing of IgM and IgG Ab development and the timing of neutralizing antibody (Nab) development have been well understood for many years [27, 46, 61]. In brief, individuals with no prior immunity to DENVs mount a primary antibody response that includes a specific IgM response followed by a durable IgG response. The initial IgG response contains different types of antibodies, including serotype cross-reactive neutralizing antibodies, serotype cross-reactive non-neutralizing antibodies, and serotype-specific neutralizing antibodies [6]. The serotype cross-reactive neutralizing antibodies may provide immediate protection to subsequent infection with any of the DENV serotypes, but these antibodies wane over the course of a year. DENV serotype-specific neutralizing antibodies and some cross-reactive poorly neutralizing antibodies are maintained for decades following infection and appear to protect against subsequent re-infection with the same serotype, but do not protect against the other serotypes (Fig. 5.2). Conversely, cross-reactive antibodies not only are non-protective, but can enhance subsequent infection via a mechanism known as antibody dependent enhancement (ADE) whereby non-neutralizing antibodies bind the virus and the antibody-virus complex is taken up by cells via FC-receptor mediated endocytosis [25]. Although ADE is poorly understood, the response is important in natural infection and vaccine development but will not be discussed in this review. Readers are recommended to refer to these earlier reviews for additional information on ADE and DENV [23, 25, 26].

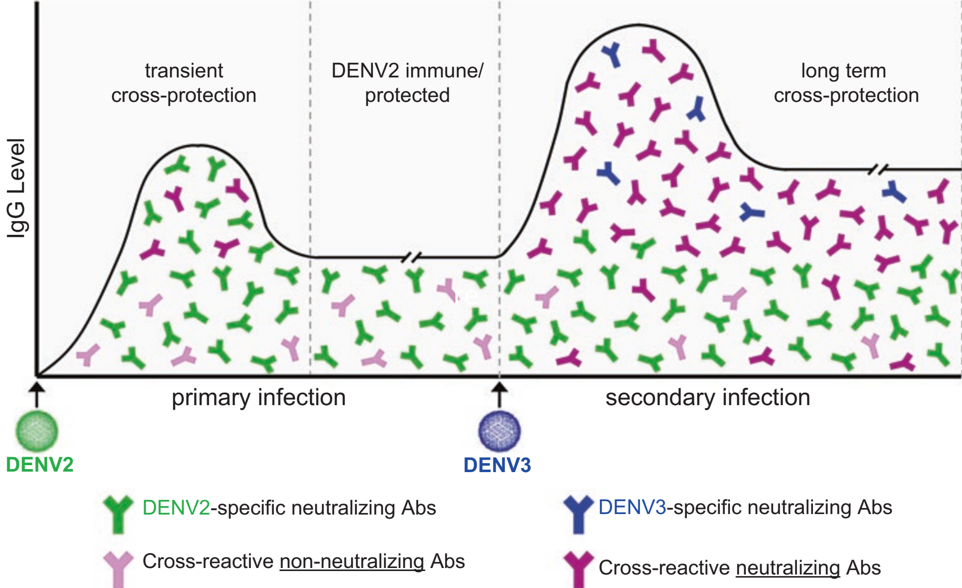


Fig. 5.2 Antibody response following DENV infection. Following primary DENV2 infection, there is an IgG response composed of neutralizing DENV2 serotype-specific antibodies, a transient population of cross-reactive neutralizing antibodies, and long-lived cross-reactive non-neutralizing antibodies. After a secondary infection, in this case with DENV3, the cross-reactive non-neutralizing antibodies become strongly neutralizing. It is also possible to generate a new population of neutralizing serotype-specific antibodies to the second infecting serotype

5.3 Methods to Study the Molecular Specificity of Human Antibodies to DENVs

A variety of techniques have been used to map the viral epitopes targeted by polyclonal antibodies in human immune sera and monoclonal antibodies (MAbs) isolated from dengue patients (Fig. 5.3). Traditionally, to study DENV-specific MAbs, peripheral blood B-cells from DENV immune donors are transformed and clones secreting DENV-reactive MAbs are fused with myeloma cell lines to generate human hybridomas producing the MAb of interest [34, 48]. Recent advances in single-cell-sequencing has allowed individual IgG heavy and light chains from the same cell to be sequenced, cloned and recombinantly expressed [44, 49]. The properties and specificity of these MAbs can then be determined using binding assays to recombinant DENV proteins (rE and rEDIII) and whole virions, and neutralization assays, as well as by solving high-resolution structures of the MAb bound to viral antigen. Once a putative MAb epitope has been identified, recombinant viruses with point mutations at the region of interest can be used to confirm and further refine the epitope. Importantly, these studies have revealed that most DENV neutralizing epitopes are created by presentation of discontinuous residues that are brought together in tertiary and quaternary structures. Additionally, our group has shown that the discontinuous residues that comprise these complex epitopes can be transplanted to a different serotype to generate chimeric DENVs that encode neutralizing epitopes from multiple DENV serotypes, and which can be used to map and confirm the binding and neutralization epitopes of individuals MAbs [17, 38].

Polyclonal sera contains a complex mixture of DENV-specific IgG antibodies, those that are neutralizing or non-neutralizing, and those that are specific to a serotype or cross-reactive to multiple serotypes (Fig. 5.2). Depletion assays can be used to determine the percentage of neutralizing sero-

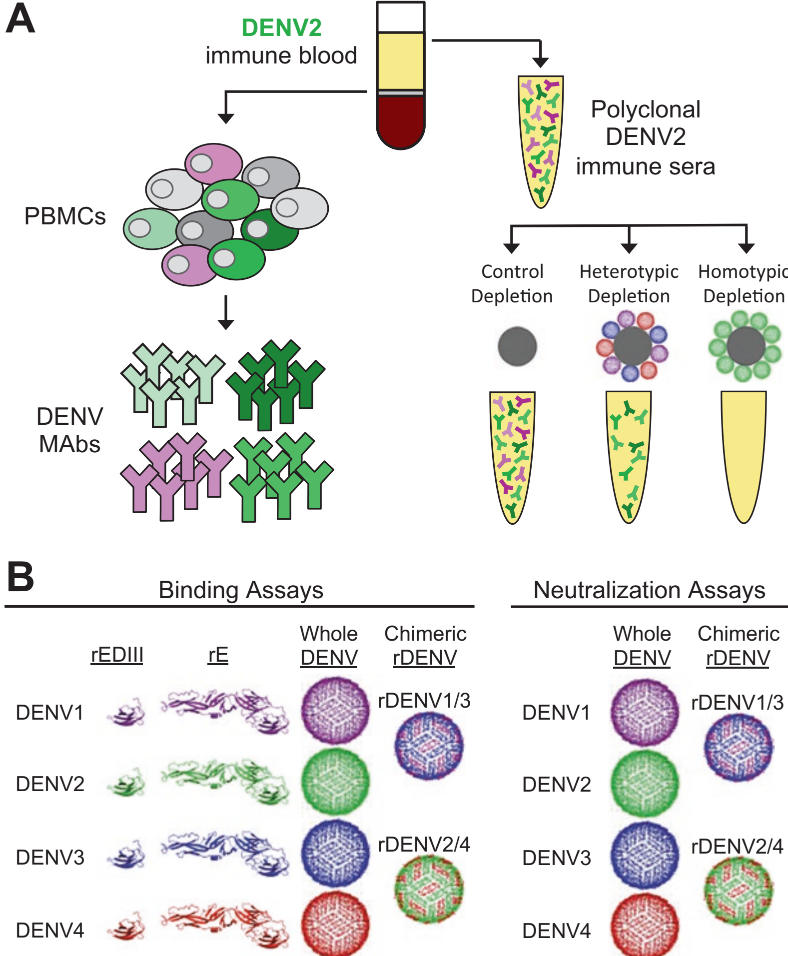


Fig. 5.3 Methods to dissect DENV antibody response. (**a**) Human DENV antibodies can be studied using a variety of approaches. PBMCs from a DENV immune donor can be EBV-transformed to generate MAb producing hybridomas, or antibody DNA sequences can be single-cell sequenced, cloned and recombinant expressed to generate MAbs. DENV polyclonal immune sera can be depleted of different populations of antibodies using beads coated with DENV antigens to determine the relative importance and neutralization capacity of these different populations. For example, a DENV2 immune sera containing polyclonal Abs (PAbs) can be depleted of all DENV cross-reactive antibodies by incubating with beads adsorbed with DENV1, DENV3, and DENV4 antigen, leaving only DENV2 serotype-specific antibodies remaining (heterotypic depletion). Conversely, all DENV antibodies can be depleted using DENV2 antigen (homotypic antigen). (**b**) To map the binding and neutralizing epitopes of these MAbs and PAbs, they can be evaluated for their ability to bind recombinant E domain III (rEDIII), recombinant E (rE), whole DENV, and chimeric viruses containing transplanted epitopes of multiple DENV serotypes (e.g. rDENV1/3 contains epitopes from both DENV1 and DENV3). These MAbs and PAbs can also be evaluated for their ability to neutralize these DENV and chimeric rDENV

type-specific antibodies to neutralizing cross-reactive antibodies (Fig. 5.3a). To remove cross-reactive Abs, primary infection sera can be incubated with beads coated with a heterologous serotype (e.g. a primary DENV2 sera can be incubated with DENV1/DENV3/DENV4-coated beads). Cross-reactive Abs will bind to the virus on the beads and be pelleted out, leaving only DENV2 serotype-specific Abs. Neutralization assays using depleted sera allow one to calculate the fraction of neutralization due to serotype-specific Abs, relative to the total neutralization coming from both serotype-specific and cross-reactive Abs [30, 41, 43]. These depletion techniques, in addition to use of epitope transplant chimeric rDENVs described above, has allowed us to study the amount of polyclonal antibodies targeting epitopes represented by individual MAbs [17, 41].

5.4 Molecular Specificity of Neutralizing MAbs from Primary Cases

The most striking feature of primary DENV infections is the rapid clearance of the virus and the maintenance of serotype-specific neutralizing Abs in the serum in most individuals for decades if not longer. Recent studies have only begun to define the molecular specificity of human B-cells and antibodies responsible for durable type-specific neutralization and protection. The envelope protein is the major antigenic protein and the majority of DENV-specific antibodies target E [45]. Traditionally human monoclonal antibodies (MAbs) have been screened based on their ability to bind recombinant envelope monomeric protein (rE). This has biased our study of MAbs to those that recognize epitopes contained within a single E protein. Several groups have recently used intact dengue virions as antigens in MAb screens [8, 11]. These studies have identified antibodies from each serotype that recognize unique conformations of the E monomer on the viral surface or quaternary structure epitopes that span different E proteins (dimers and rafts) on the viral envelope (Fig. 5.4). Additionally, it has been found that while antibodies using simple epitopes can be neutralizing, it is the antibodies recognizing complex epitopes that are ultimately responsible for polyclonal neutralization [9, 17, 57]. Antibodies recognizing quaternary epitopes are not unique to DENV; West Nile Virus (WNV) and Zika Virus (ZIKV) infection have also been shown to generate human MAbs recognizing similar complex epitopes [28, 31, 52, 58].

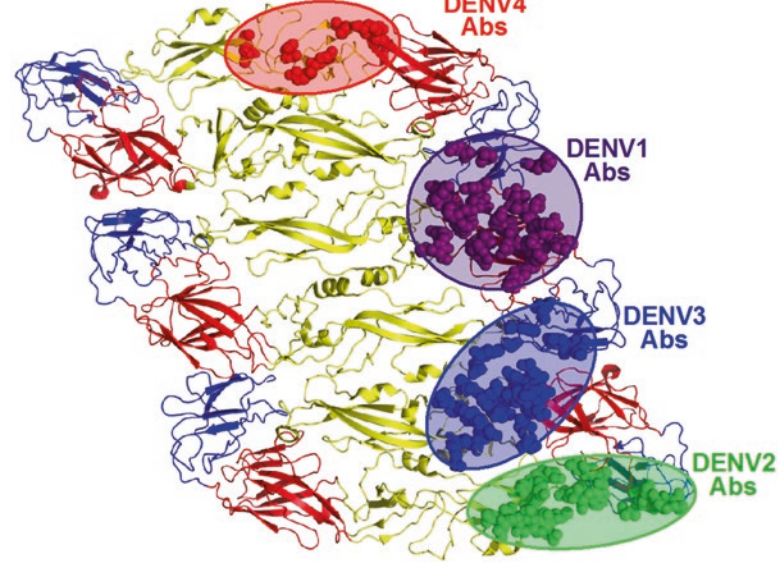


Fig. 5.4 Epitopes recognized by DENV serotype-specific human neutralizing MAbs. Serotype-specific neutralizing human MAbs isolated from primary infections recognize different quaternary structure epitopes displayed on the viral envelope. Note that many MAb footprints span different E molecules

5.5 Differences in Neutralizing MAb Epitopes Across Serotypes

While the E protein is structurally similar between DENV serotypes (~80% conservation of amino acids), the location of type-specific epitopes targeted by human antibodies appear to be different between serotypes (Fig. 5.4). Unlike anti-DENV mouse MAbs that predominantly target EDIII [19, 53], many human MAbs recognize EDI, EDII, and the EDI/II hinge region. For example, DENV1 type-specific human MAbs 1F4 and 14C10 recognize epitopes centered on EDI [14, 54]. The 14C10 epitope includes amino acids on EDI and EDIII on the adjacent dimer. Interestingly, the DENV1 14C10 epitope is quite similar to an epitope on WNV recognized by human MAb CR4354 [31]. The DENV3 MAb 5J7 targets an epitope centered around the EDI/II hinge region and the footprint of this epitope includes amino acids from three different E molecules within a single raft [16]. Recent work has identified human DENV4 MAbs that target epitopes near the EDI/II hinge although further studies are required to precisely map the DENV4 epitopes [41]. Interestingly, DENV2 MAbs appear to use an epitope distinct to the EDI/EDII region, instead centering on EDIII [15, 17]. Our understanding of immunodominant epitopes for each serotype is informed by only a handful of monoclonal antibodies from a few immune individuals. To fully define the boundaries of the polyclonal neutralizing epitopes against each serotype, additional antibodies from more individuals will need to be studied.

5.6 Cryptic Epitopes

The majority of human epitopes studied are present on the surface of the intact virion. Some studies have identified mouse MAbs that target cryptic epitopes not readily accessible on the surface of the virus. However, at elevated temperature E proteins on the viral surface can flex/move and these cryptic epitopes are transiently displayed, allowing antibody binding and neutralization [13]. Recent studies suggest that there are antibodies present in human immune sera that also target these cryptic epitopes, potentially allowing the virus to be neutralized when it is under specific conditions exposing these epitopes [13]. Further studies are needed to evaluate the importance of cryptic epitopes in human antibody neutralization and protective immunity.

5.7 Other Flaviviruses – Zika Virus MAbs

With the emergence of Zika virus (ZIKV), approaches developed for DENV have been extended to isolate MAbs and map the human antibody response to ZIKV [28, 52, 58]. Multiple groups have generated human ZIKV MAbs. Similarly to DENV, the strongest neutralizing MAbs target quaternary epitopes only present on the intact ZIKV virion. These quaternary ZIKV epitopes are similar to previously identified quaternary DENV epitopes that are centered around the EDI/II hinge region, span across E monomers within the dimer, or span across dimers [28, 52, 58].

5.8 Mapping the Molecular Specificity of the Polyclonal Serum Neutralizing Antibody Response

While MAbs are isolated or generated from memory B-cells, circulating polyclonal antibodies come from plasma cells [33]. The memory B-cell derived human MAbs can be used as tools to interrogate the properties and specificity of the more complex polyclonal serum antibody response (Fig. 5.5). Work by multiple groups have shown that individual MAbs can be representative of the anti-DENV B-cell repertoire, polyclonal Abs from the same individual, and polyclonal Abs across other naturally infected and vaccinated individuals, confirming the importance of studying individual monoclonals [17, 22, 41]. Importantly, depletion assays have revealed that after primary DENV infections, the majority of polyclonal neutralization comes from serotype-specific antibodies, not

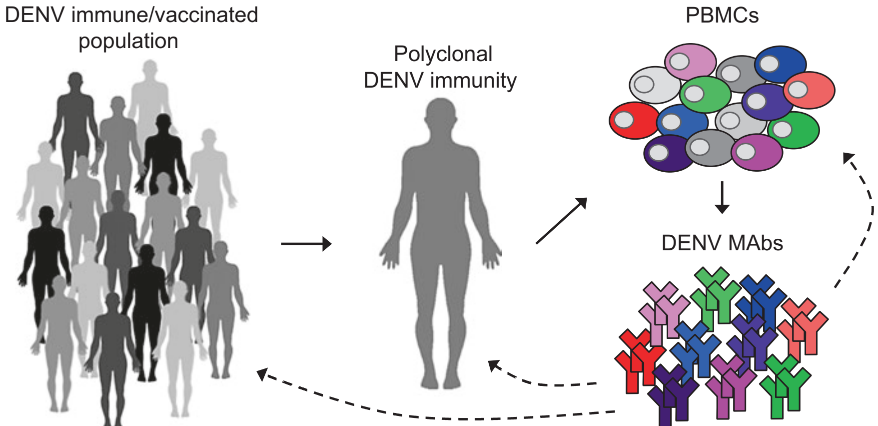


Fig. 5.5 From MAbs to polyclonal serum Abs. Complex host generic diversity, exposure history, and immune differences can make it challenging to study DENV polyclonal antibody responses across a population. Studying DENV antibody immunity in a single individual can simplify these analyses, however there is still the polyclonal nature of the adaptive immune response. Conversely, we can characterize the properties of individual MAbs from DENV immune donors. Information learned from MAbs can then be used to inform study of the B-cell repertoire from that, and other donors. Additionally, it can be determined whether the individual MAbs represent the polyclonal antibodies in that donor, and in a larger DENV immune population

cross-reactive ones [30, 41, 43]. Additionally, we have found that epitopes defined by individual MAbs that are complex and quaternary, are representative of the polyclonal epitopes targeted by neutralizing serotype-specific antibodies [9, 57]. With the rapid emergence of ZIKV, similar techniques as described above were applied to dissecting the antibody response to ZIKV infection. Multiple groups have found that strongly neutralizing ZIKV MAbs target complex quaternary epitopes [28, 52, 58]. Additional work using depletion assays, has identified that primary ZIKV infections can results in ZIKV specific Abs, despite populations of Abs that cross-neutralize DENV [5].

5.9 Molecular Specificity of Neutralizing Antibodies Following Secondary DENV Infection

Individuals experiencing secondary DENV infections with a new serotype develop a neutralizing and protective antibody response that is fundamentally different from a primary infection-induced response. People with known sequential infections with two different DENV serotypes have type-specific antibodies to serotypes of infection and a new population of durable serotype-cross neutralizing antibodies that are also effective against serotypes not encountered by the person [7]. Human cohort studies in dengue-endemic countries have also established that tertiary infections are nearly always mild or inapparent, implicating a protective role for these broadly cross-neutralizing antibodies that develop after a second DENV infection [42]. Figure 5.6 presents a model to explain the evolving antibody response following sequential DENV infections with different serotypes. The model is based on the premise that low affinity DENV cross-reactive memory B-cells derived from primary infections undergo antibody somatic hypermutation and each subsequent DENV exposure selects and expands rare affinity matured clones with greater neutralization breadth and potency [43]. The model is supported by recent studies demonstrating that serotype cross-reactive antibodies derived from secondary infections had stronger neutralization potencies and higher binding avidities than those derived from patients with primary infections [10, 37, 55, 56, 62].

While we know a lot about epitopes targeted by DENV serotype-specific neutralizing and pro-

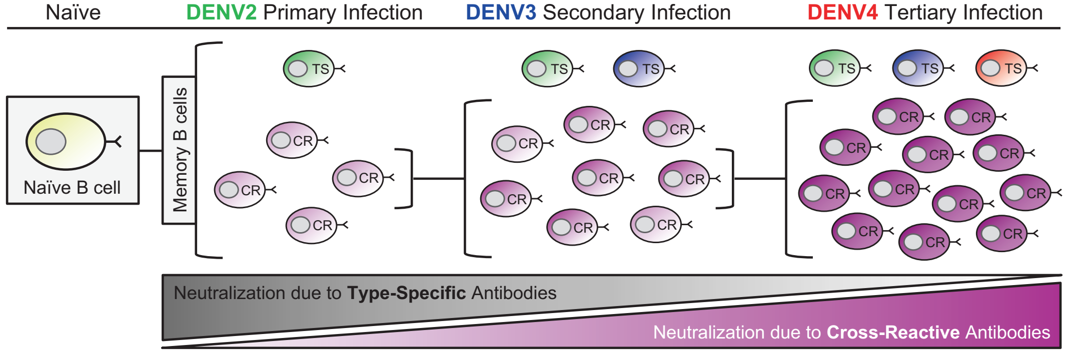


Fig. 5.6 Model of B-cell maturation following sequential DENV infections. With each successive DENV infection, the ratio of serotype-specific (TS) and cross-reactive (CR) antibodies that contribute to DENV neutralization changes. During a primary infection (DENV2 in this example), dengue-specific naïve B-cells are activated and these cells give rise to both memory B-cells (MBCs) and antibody secreting long lived plasma cells (LLPCs). This primary response is dominated MBC and LLPCs clones producing low affinity, weakly neutralizing serotype CR antibodies. The primary response also contains rare MBC and LLPCs producing TS antibodies that strongly neutralize DENV2. Following a secondary infection with a new serotype (DENV3 in this example), the overall DENV-specific B-cell response will be dominated by the activation and expansion of DENV2 and 3 cross-reactive MBCs induced by the primary infection. MBCs producing CR antibodies that bind to the second infecting serotype with high affinity will be preferentially activated. These activated cells will reenter germinal centers and undergo further rounds of somatic hypermutation. CR B-cells with high affinity for the second serotype will be selectively expanded to give rise to cross-reactive MBC and LLPCs that strongly cross-neutralize multiple serotypes. In Fig. 5.6., this increase in affinity and neutralization is depicted by an increase in the color gradient (light pink to bright pink) of CR B-cells. Following a tertiary infection (DENV4 in this example), this process is repeated again and results in a population of CR MBCs and LLPCs that dominate the neutralizing antibody response. While the B-cell clones producing TS strongly neutralizing antibodies are also likely to be maintained through each successive round of infection, the TS response will account for only a small fraction of the total neutralizing response

tective antibodies, less is known about the targets of durable serotype-cross neutralizing antibodies. Several cross-neutralizing human MAbs that bind to an epitope near the bc-loop on domain II of the E protein monomer have been recently described (Fig. 5.7) [50, 55]. Another class of serotype cross-reactive and strongly cross-neutralizing MAbs, which bind to quaternary epitopes on the E homodimer, was recently isolated from acute-phase plasmablasts in the peripheral blood of secondary DENV cases [11, 47]. These MAbs, which have been designated E dimer epitope (EDE) antibodies, bind to epitopes that span domains I or III of one monomer and domain II of the adjacent monomer (Fig. 5.7). It is unclear if the strongly cross-neutralizing MAbs isolated from acute-phase plasmablasts are maintained as MBCs and LLPCs and responsible for the durable cross-neutralizing antibodies observed in people after recovery from secondary infections. Additionally, there are still unknowns regarding if the order of infecting serotypes is important for the epitopes of strongly cross-neutralizing MAbs. The molecular mechanisms leading to the evolution of cross-neutralizing antibodies from the memory B-cell pool from a primary infection are also unclear.

5.10 NS1 and prM MAbs

While E is the major antigenic protein of DENV, antibodies are also generated targeting the viral proteins NS1 and prM. The host sees prM protein

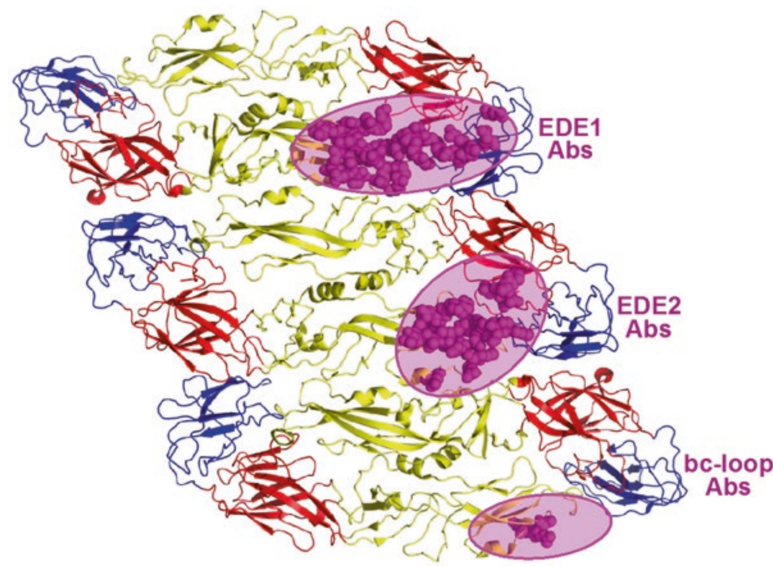


Fig. 5.7 EDE and other cross-reactive epitopes. Envelope dimer epitope 1 (EDE1) targets EDIII of one monomer and spans over the fusion loop region of EDII of the neighboring monomer. EDE2 uses a similar epitope, but is shifted to also expand into EDI of the first monomer. Another class of cross-reactive antibodies targets the highly conserved bc-loop region of EDII

in several configurations. As mature viruses are released from infected cells, prM protein dissociates from the virus and is released as an antigen. Additionally, immature viruses have prM present on their surface, allowing the immune system to recognize them as part of the virus. prM antibodies are predominantly non-neutralizing enhancing antibodies; they allow non-infectious immature viruses to be taken up into cells via FC-receptor-mediated endocytosis [51].

DENV NS1 protein has many roles depending on its interactions and location [1, 40]. NS1 can exist as a monomer, dimer or hexamer, and is important in viral RNA replication, viral assembly and release, and immune evasion. NS1 is secreted from infected cells primarily as a hexamer, which can bind to endothelial cells, triggering hyperpermeability, suggesting a role in the vascular leakage seen in severe DENV disease [3]. Clinically, levels of circulating NS1 are correlated with disease severity [2, 35]. People infected with DENV make antibodies directed against NS1, but it is unclear if these are an important part of the protective immune response, or are merely a consequence of high levels of circulating viral antigen [40].

5.11 Mechanisms of Neutralization

MAbs can neutralize viruses through a variety of mechanisms. MAbs have been shown to neutralize DENV by blocking attachment to host cell receptors, binding directly to the fusion-loop, binding across E proteins preventing conformational changes required for fusion, as well as via opsonization. Anti-DENV MAbs have been shown to neutralize using many of these mechanisms [15, 16, 29, 47]. DENV maturation state (amount of prM present) and virus breathing are important factors for virus neutralization. A fully immature virus (i.e. 180 copies of prM present) is non-infectious, and therefore cannot be neutralized, but a partially mature virus, can still be infectious [12]. Alternatively, under certain temperature conditions, some DENV strains can undergo reversible conformational changes where the E proteins expand and contract analogous to “breathing”. These expansion and contraction changes can reveal or hide epitopes, limiting neutralization by antibodies recognizing these epitopes to specific conditions [13, 18, 32, 63]. While DENV maturation and “breathing”

have been studied in cell culture systems, the importance of these phenomenon in natural infection, and therefore the potential impact on antibody neutralization, is not well understood.

5.12 Implications for Evaluating Antibodies to DENV Live Attenuated Vaccines (LAVs)

Recently we have learned important lessons from DENV tetravalent vaccine clinical trials. The leading tetravalent vaccine had variable efficacy depending on DENV serotype and vaccinated population [20]. The vaccine had higher efficacy in DENV-primed individuals compared to DENV naïve individuals who received the vaccine, establishing the impact of immunological memory on vaccine performance [21]. The population with the greatest need for a DENV vaccine is young children, the majority of whom will be DENV-naïve at vaccination. As discussed above, in people exposed to primary natural DENV infections, the neutralizing and protective antibody response is dominated by type-specific antibodies to quaternary epitopes. Therefore, in this population the success of tetravalent vaccination is likely to require balanced replication of the four vaccine viruses leading to type-specific antibodies that target quaternary epitopes in each serotype.

As discussed above, secondary DENV infections result in activation of memory B-cells and development and expansion of cross-reactive antibodies that broadly neutralize multiple DENV serotypes, driven by the sequential infection and robust replication of two different serotypes of DENV [43]. A similar mechanism is likely to be responsible for the superior performance of tetravalent LAVs in DENV-primed individuals. In a subject with pre-existing DENV-specific MBCs, even unbalanced replication of one or two vaccine components is likely to activate MBCs and expand somatically mutated higher-affinity cross-reactive clones with capacity to broadly neutralize multiple serotypes.

Immune correlates of protection and vaccine efficacy are urgently needed. For the leading DENV vaccine, the mere presence of *in vitro* neutralizing antibodies was not sufficient for protection because many individuals experienced breakthrough infections despite having neutralizing antibodies to the breakthrough serotype [21]. The lessons we have learned from natural infections studies about the molecular specificity of human antibodies to DENV infection may also lead to more robust correlates of vaccine efficacy than mere levels of total neutralizing antibodies [36]. Certainly, the reagents and tools are now available to interrogate vaccine responses in a manner similar to that we have described here for natural DENV infections.

Discussion of Chapter 5 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Aravinda de Silva.

Félix Rey: So you will be calling to question the fact that antibodies against Dengue would neutralize Zika?

Aravinda de Silva: No. But what I am saying is that in people who have recovered from Dengue – when they are in the late convalescent stage – they do not have circulating antibodies that neutralize Zika. I think in people who have secondary Dengue, when you isolate antibodies from their plasmablast, you can certainly find monoclonals that cross-neutralize Zika or even cross-protective against Zika, but it looks like they are not persisting into memory.

Félix Rey: How do you know that?

Aravinda de Silva: So in those people who have repeated Dengue infections – when we bleed them 6 months out from their infection, there is no neutralizing antibody against Zika. And I think that even in some of the other studies that are coming out to say that Zika and Dengue cross-neutralize, many of these studies have been done with samples within the

first 3 or 4 weeks of an acute secondary Dengue infection. We know that one of the hallmarks of Dengue is that soon after they recover from Dengue during the convalescence period, there are very high levels of cross-neutralizing antibodies. This is even the case with primary Dengue infection, where we get a lot of cross-neutralizing antibodies during the convalescence period. But that is transient and it goes down and the response becomes more monotypic.

Paul Young: Can I just explore that further, because we have known that for a very long time. Why does the cross-neutralizing activity go down yet the serotype-specificity stays on. What is driving it?

Aravinda de Silva: So one of the obvious things is that IgM plays a role in cross-neutralization. But the second possibility is that there is an extrafollicular reaction. These cells are activated but they don't get into the germinal centers and differenciate into plasmablast. They make a transcient antibody response but the cells do not persist. So a lot of the cross-neutralizing antibody is coming from extrafollicular reactions.

Paul Young: But why? I'm still a little confused. But I understand that's why it happened. But why are those selectively lost?

Aravinda de Silva: Yes. That's a good question. What is it about those epitopes that are getting lost, why are type-specific ones being maintained?

George Gao: Can we have a big picture for those three domains [of the envelope protein]? Which domain contributes the most to neutralizing antibodies? Can we say that now?

Aravinda de Silva: I think you have to really ask that question in the context of primary infection. In someone who has only had Dengue once or Zika once and no other flavivirus exposures, then what epitope is responsible for durable neutralization? We find in these cases there are defined epitopes responsible and they are the quaternary structure type-specific epitopes. But in someone with repeated infections – at least repeated Dengue infections – it could be ADE antibodies, it could be other antibodies that we haven't discovered. But after natural infection, I don't think that there is evidence that there are these long-lasting memory responses that are cross-neutralizing multiple flaviviruses.

Félix Rey: You would say that if it does not bind recombinant E protein, it has to bind some super-organization between dimers or something, but the recombinant E is monomeric unless you have it at a very high concentration.

Aravinda de Silva: Yes I agree that it could be binding dimers because the recombinant E protein test would not pick dimers.

References

1. Amorim JH, Alves RP, Boscardin SB, Ferreira LC (2014) The dengue virus non-structural 1 protein: risks and benefits. Virus Res 181:53–60
2. Avirutnan P, Punyadee N, Noisakran S, Komoltri C, Thiemmeca S, Auethavornanan K, Jairungsri A, Kanlaya R, Tangthawornchaikul N, Puttikhunt C, Pattanakitsakul SN, Yenchitsomanus PT, Mongkolsapaya J, Kasinrerk W, Sittisombut N, Husmann M, Blettner M, Vasanawathana S, Bhakdi S, Malasit P (2006) Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J Infect Dis 193:1078–1088
3. Beatty PR, Puerta-Guardo H, Killingbeck SS, Glasner DR, Hopkins K, Harris E (2015) Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. Sci Transl Med 7:304ra141
4. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI (2013) The global distribution and burden of dengue. Nature 496:504–507
5. Collins MH, Mcgowan E, Jadi R, Young E, Lopez CA, Baric RS, Lazear HM, De Silva AM (2017) Lack of durable cross-neutralizing antibodies against Zika virus from dengue virus infection. Emerg Infect Dis 23:773–781
6. Coloma J, Harris E (2015) Broad and strong: the ultimate antibody to dengue virus. Nat Immunol 16:135–137
7. Corbett KS, Katzelnick L, Tissera H, Amerasinghe A, De Silva AD, De Silva AM (2015) Preexisting neutralizing antibody responses distinguish clinically inapparent and apparent dengue virus infections in a Sri Lankan pediatric cohort. J Infect Dis 211:590–599

8. De Alwis R, Beltramello M, Messer WB, Sukupolvi-Petty S, Wahala WM, Kraus A, Olivarez NP, Pham Q, Brien JD, Tsai WY, Wang WK, Halstead S, Kliks S, Diamond MS, Baric R, Lanzavecchia A, Sallusto F, De Silva AM (2011) In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection. PLoS Negl Trop Dis 5:e1188
9. De Alwis R, Smith SA, Olivarez NP, Messer WB, Huynh JP, Wahala WM, White LJ, Diamond MS, Baric RS, Crowe JE Jr, De Silva AM (2012) Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. Proc Natl Acad Sci U S A 109:7439–7444
10. De Souza VA, Tateno AF, Oliveira RR, Domingues RB, Araujo ES, Kuster GW, Pannuti CS (2007) Sensitivity and specificity of three ELISA-based assays for discriminating primary from secondary acute dengue virus infection. J Clin Virol 39:230–233
11. Dejnirattisai W, Wongwiwat W, Supasa S, Zhang X, Dai X, Rouvinski A, Jumnainsong A, Edwards C, Quyen NT, Duangchinda T, Grimes JM, Tsai WY, Lai CY, Wang WK, Malasit P, Farrar J, Simmons CP, Zhou ZH, Rey FA, Mongkolsapaya J, Screaton GR (2015) A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. Nat Immunol 16:170–177
12. Dowd KA, Mukherjee S, Kuhn RJ, Pierson TC (2014) Combined effects of the structural heterogeneity and dynamics of flaviviruses on antibody recognition. J Virol 88:11726–11737
13. Dowd KA, Demaso CR, Pierson TC (2015) Genotypic differences in dengue virus neutralization are explained by a single amino acid mutation that modulates virus breathing. MBio 6:e01559–e01515
14. Fibriansah G, Tan JL, Smith SA, De Alwis AR, Ng TS, Kostyuchenko VA, Ibarra KD, Wang JQ, Harris E, De Silva A, Crowe JE, Lok SM (2014) A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface. Embo Molecular Medicine 6:358–371
15. Fibriansah G, Ibarra KD, Ng TS, Smith SA, Tan JL, Lim XN, Ooi JS, Kostyuchenko VA, Wang J, De Silva AM, Harris E, Crowe JE, Lok SM (2015a) DENGUE VIRUS. Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers. Science 349:88–91
16. Fibriansah G, Tan JL, Smith SA, De Alwis R, Ng TS, Kostyuchenko VA, Jadi RS, Kukkaro P, De Silva AM, Crowe JE, Lok SM (2015b) A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins. Nat Commun 6:6341
17. Gallichotte EN, Widman DG, Yount BL, Wahala WM, Durbin A, Whitehead S, Sariol CA, Crowe JE, De Silva AM, Baric RS (2015) A new quaternary structure epitope on dengue virus serotype 2 is the target of durable type-specific neutralizing antibodies. MBio 6:e01461–e01415
18. Goo L, Vanblargan LA, Dowd KA, Diamond MS, Pierson TC (2017) A single mutation in the envelope protein modulates flavivirus antigenicity, stability, and pathogenesis. PLoS Pathog 13:e1006178
19. Gromowski GD, Barrett AD (2007) Characterization of an antigenic site that contains a dominant, type-specific neutralization determinant on the envelope protein domain III (ED3) of dengue 2 virus. Virology 366:349–360
20. Guy B, Jackson N (2016) Dengue vaccine: hypotheses to understand CYD-TDV-induced protection. Nat Rev Microbiol 14:45–54
21. Hadinegoro SR, Arredondo-Garcia JL, Capeding MR, Deseda C, Chotpitayasunondh T, Dietze R, Ismail HI, Reynales H, Limkittikul K, Rivera-Medina DM, Tran HN, Bouckenooghe A, Chansinghakul D, Cortes M, Fanouillere K, Forrat R, Frago C, Gailhardou S, Jackson N, Noriega F, Plennevaux E, Wartel TA, Zambrano B, Saville M, CYD-TDV Dengue Vaccine Working Group (2015) Efficacy and long-term safety of a dengue vaccine in regions of endemic disease. N Engl J Med 373:1195–1206
22. Hadjilaou A, Green AM, Coloma J, Harris E (2015) Single-cell analysis of B cell/antibody cross-reactivity using a novel multicolor FluoroSpot assay. J Immunol 195:3490–3496
23. Halstead SB (2003) Neutralization and antibody-dependent enhancement of dengue viruses. Adv Virus Res 60:421–467
24. Halstead SB (2007) Dengue. Lancet 370:1644–1652
25. Halstead SB (2014) Dengue antibody-dependent enhancement: knowns and unknowns. Microbiol Spectr 2
26. Halstead SB (2015) Pathogenesis of dengue: Dawn of a new era. F1000Res 4
27. Halstead SB, Rojanasuphot S, Sangkawibha N (1983) Original antigenic sin in dengue. Am J Trop Med Hyg 32:154–156
28. Hasan SS, Miller A, Sapparapu G, Fernandez E, Klose T, Long F, Fokine A, Porta JC, Jiang W, Diamond MS, Crowe JE Jr, Kuhn RJ, Rossmann MG (2017) A human antibody against Zika virus crosslinks the E protein to prevent infection. Nat Commun 8:14722
29. He RT, Innis BL, Nisalak A, Usawattanakul W, Wang S, Kalayanarooj S, Anderson R (1995) Antibodies that block virus attachment to Vero cells are a major component of the human neutralizing antibody response against dengue virus type 2. J Med Virol 45:451–461
30. Henein S, Swanstrom J, Byers AM, Moser JM, Shaik SF, Bonaparte M, Jackson N, Guy B, Baric R, De Silva AM (2017) Dissecting antibodies induced by a chimeric yellow fever-dengue, live-attenuated, tetravalent dengue vaccine (CYD-TDV) in naive and dengue-exposed individuals. J Infect Dis 215:351–358
31. Kaufmann B, Vogt MR, Goudsmit J, Holdaway HA, Aksyuk AA, Chipman PR, Kuhn RJ, Diamond MS, Rossmann MG (2010) Neutralization of West Nile virus by cross-linking of its surface proteins with fab fragments of the human monoclonal antibody CR4354. Proc Natl Acad Sci U S A 107:18950–18955

32. Kuhn RJ, Dowd KA, Beth Post C, Pierson TC (2015) Shake, rattle, and roll: impact of the dynamics of flavivirus particles on their interactions with the host. Virology 479–480:508–517
33. Kurosaki T, Kometani K, Ise W (2015) Memory B cells. Nat Rev Immunol 15:149–159
34. Kwakkenbos MJ, Diehl SA, Yasuda E, Bakker AQ, Van Geelen CM, Lukens MV, Van Bleek GM, Widjojoatmodjo MN, Bogers WM, Mei H, Radbruch A, Scheeren FA, Spits H, Beaumont T (2010) Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. Nat Med 16:123–128
35. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S, Green S, Vaughn DW, Nisalak A, Ennis FA, Rothman AL (2002) High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J Infect Dis 186:1165–1168
36. Liu Y, Liu J, Cheng G (2016) Vaccines and immunization strategies for dengue prevention. Emerg Microbes Infect 5:e77
37. Matheus S, Deparis X, Labeau B, Lelarge J, Morvan J, Dussart P (2005) Discrimination between primary and secondary dengue virus infection by an immunoglobulin G avidity test using a single acute-phase serum sample. J Clin Microbiol 43:2793–2797
38. Messer WB, Yount BL, Royal SR, De Alwis R, Widman DG, Smith SA, Crowe JE Jr, Pfaff JM, Kahle KM, Doranz BJ, Ibarra KD, Harris E, De Silva AM, Baric RS (2016) Functional transplant of a dengue virus serotype 3 (DENV3)-specific human monoclonal antibody epitope into DENV1. J Virol 90:5090–5097
39. Mukherjee S, Sirohi D, Dowd KA, Chen Z, Diamond MS, Kuhn RJ, Pierson TC (2016) Enhancing dengue virus maturation using a stable furin over-expressing cell line. Virology 497:33–40
40. Muller DA, Young PR (2013) The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. Antivir Res 98:192–208
41. Nivarthi UK, Kose N, Sapparapu G, Widman D, Gallichotte E, Pfaff JM, Doranz BJ, Weiskopf D, Sette A, Durbin AP, Whitehead SS, Baric R, Crowe JEJ, De Silva AM (2017) Mapping the human memory B cell and serum neutralizing antibody responses to dengue virus serotype 4 infection and vaccination. J Virol 91:e02041–e02016
42. Olkowski S, Forshey BM, Morrison AC, Rocha C, Vilcarromero S, Halsey ES, Kochel TJ, Scott TW, Stoddard ST (2013) Reduced risk of disease during postsecondary dengue virus infections. J Infect Dis 208:1026–1033
43. Patel B, Longo P, Miley MJ, Montoya M, Harris E, De Silva AM (2017) Dissecting the human serum antibody response to secondary dengue virus infections. PLoS Negl Trop Dis 11:e0005554
44. Robinson WH (2015) Sequencing the functional antibody repertoire—diagnostic and therapeutic discovery. Nat Rev Rheumatol 11:171–182
45. Roehrig JT (2003) Antigenic structure of flavivirus proteins. Adv Virus Res 59:141–175
46. Rothman AL (2011) Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat Rev Immunol 11:532–543
47. Rouvinski A, Guardado-Calvo P, Barba-Spaeth G, Duquerroy S, Vaney MC, Kikuti CM, Navarro Sanchez ME, Dejnirattisai W, Wongwiwat W, Haouz A, Girard-Blanc C, Petres S, Shepard WE, Despres P, Arenzana-Seisdedos F, Dussart P, Mongkolsapaya J, Screaton GR, Rey FA (2015) Recognition determinants of broadly neutralizing human antibodies against dengue viruses. Nature 520:109–113
48. Smith SA, Crowe JE Jr (2015) Use of human Hybridoma technology to isolate human monoclonal antibodies. Microbiol Spectr 3, AID-0027-2014
49. Smith K, Garman L, Wrammert J, Zheng NY, Capra JD, Ahmed R, Wilson PC (2009) Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. Nat Protoc 4:372–384
50. Smith SA, De Alwis AR, Kose N, Harris E, Ibarra KD, Kahle KM, Pfaff JM, Xiang X, Doranz BJ, De Silva AM, Austin SK, Sukupolvi-Petty S, Diamond MS, Crowe JE Jr (2013) The potent and broadly neutralizing human dengue virus-specific monoclonal antibody 1C19 reveals a unique cross-reactive epitope on the bc loop of domain II of the envelope protein. MBio 4:e00873–e00813
51. Smith SA, Nivarthi UK, De Alwis R, Kose N, Sapparapu G, Bombardi R, Kahle KM, Pfaff JM, Lieberman S, Doranz BJ, De Silva AM, Crowe JE Jr (2015) Dengue virus prM-specific human monoclonal antibodies with virus replication-enhancing properties recognize a single Immunodominant antigenic site. J Virol 90:780–789
52. Stettler K, Beltramello M, Espinosa DA, Graham V, Cassotta A, Bianchi S, Vanzetta F, Minola A, Jaconi S, Mele F, Foglierini M, Pedotti M, Simonelli L, Dowall S, Atkinson B, Percivalle E, Simmons CP, Varani L, Blum J, Baldanti F, Cameroni E, Hewson R, Harris E, Lanzavecchia A, Sallusto F, Corti D (2016) Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. Science 353:823–826
53. Sukupolvi-Petty S, Austin SK, Purtha WE, Oliphant T, Nybakken GE, Schlesinger JJ, Roehrig JT, Gromowski GD, Barrett AD, Fremont DH, Diamond MS (2007) Type- and subcomplex-specific neutralizing antibodies against domain III of dengue virus type 2 envelope protein recognize adjacent epitopes. J Virol 81:12816–12826
54. Teoh EP, Kukkaro P, Teo EW, Lim AP, Tan TT, Yip A, Schul W, Aung M, Kostyuchenko VA, Leo YS, Chan SH, Smith KG, Chan AH, Zou G, Ooi EE, Kemeny DM, Tan GK, Ng JK, Ng ML, Alonso S, Fisher D, Shi PY, Hanson BJ, Lok SM, Macary PA (2012) The structural basis for serotype-specific neutralization of

dengue virus by a human antibody. Sci Transl Med 4:139ra83

55. Tsai WY, Lai CY, Wu YC, Lin HE, Edwards C, Jumnainsong A, Kliks S, Halstead S, Mongkolsapaya J, Screaton GR, Wang WK (2013) High-avidity and potently neutralizing cross-reactive human monoclonal antibodies derived from secondary dengue virus infection. J Virol 87:12562–12575
56. Tsai WY, Durbin A, Tsai JJ, Hsieh SC, Whitehead S, Wang WK (2015) Complexity of neutralizing antibodies against multiple dengue virus serotypes after heterotypic immunization and secondary infection revealed by in-depth analysis of cross-reactive antibodies. J Virol 89:7348–7362
57. Wahala WM, Kraus AA, Haymore LB, Accavitti-Loper MA, De Silva AM (2009) Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody. Virology 392:103–113
58. Wang Q, Yang H, Liu X, Dai L, Ma T, Qi J, Wong G, Peng R, Liu S, Li J, Li S, Song J, Liu J, He J, Yuan H, Xiong Y, Liao Y, Li J, Yang J, Tong Z, Griffin BD, Bi Y, Liang M, Xu X, Qin C, Cheng G, Zhang X, Wang P, Qiu X, Kobinger G, Shi Y, Yan J, Gao GF (2016) Molecular determinants of human neutralizing antibodies isolated from a patient infected with Zika virus. Sci Transl Med 8:369ra179
59. Yu IM, Zhang W, Holdaway HA, Li L, Kostyuchenko VA, Chipman PR, Kuhn RJ, Rossmann MG, Chen J (2008) Structure of the immature dengue virus at low pH primes proteolytic maturation. Science 319:1834–1837
60. Yu IM, Holdaway HA, Chipman PR, Kuhn RJ, Rossmann MG, Chen J (2009) Association of the pr peptides with dengue virus at acidic pH blocks membrane fusion. J Virol 83:12101–12107
61. Zompi S, Harris E (2013) Original antigenic sin in dengue revisited. Proc Natl Acad Sci U S A 110:8761–8762
62. Zompi S, Montoya M, Pohl MO, Balmaseda A, Harris E (2012) Dominant cross-reactive B cell response during secondary acute dengue virus infection in humans. PLoS Negl Trop Dis 6:e1568
63. Zybert IA, Van Der Ende-Metselaar H, Wilschut J, Smit JM (2008) Functional importance of dengue virus maturation: infectious properties of immature virions. J Gen Virol 89:3047–3051

Check for updates

6 Structures of Zika Virus E & NS1: Relations with Virus Infection and Host Immune Responses

Yi Shi, Lianpan Dai, Hao Song, and George F. Gao

Abstract

Zika virus (ZIKV), first discovered in the Zika forest in Uganda in 1947 was understudied until the recent explosive epidemic in several South American countries where it has become strongly associated with congenital birth defects leading to severe cranial malformations and neurological conditions. The increase in number of case of microcephaly in newborn children associated with ZIKV infection triggered the World Health Organization to declare the epidemic as a Public Health Emergency of International Concern in February of 2016. ZIKV is a member of the flavivirus genus and is transmitted by *Aedes aegypti* mosquitoes, however in the current epidemic clear evidence is emerging to suggest the virus can be sexually transmitted from human to human. The differences in epidemiology and manifestations of ZIKV infection during these outbreaks have prompted researchers to investigate mechanisms of dissemination, pathogenesis, and host immune response which contributes significantly to the control of the virus infection. The E and NS1 proteins of ZIKV are the major targets for neutralizing and protective antibodies. In this chapter, we mainly focus on recent research on the crystal structures of the ZIKV E and NS1 proteins, and their relations with virus infection and immune responses. These studies will be helpful to develop novel therapeutics and vaccines for protection and control of ZIKV infection.

Keywords

Crystal structure · Zika virus · Neutralizing antibodies · NS1 · E protein

Y. Shi (✉)
CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China
e-mail: shiyi@im.ac.cn

L. Dai · H. Song
Research Network of Immunity and Health (RNIH), Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing, China

G. F. Gao (✉)
CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

Research Network of Immunity and Health (RNIH), Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing, China
e-mail: gaof@im.ac.cn

Zika virus (ZIKV), a *Flaviviridae* family member transmitted to humans by mosquitoes, has emerged as a major health concern during 2015 [12]. ZIKV infection of pregnant women can cause congenital malformations including fetal and newborn micro-

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_6

cephaly and serious neurological complications, such as Guillain–Barré syndrome [13, 27, 40, 41, 62]. In addition to transmission by mosquitoes, ZIKV may establish long-term persistent infections [38] and be transmitted by sexual activities [23, 24, 45] or vertically from mother to fetus [10, 27]. Also, ZIKV was detected in human semen and spermatozoa [39], and ZIKV infections of male adult mice can cause testicular and epididymal damage, leading to cell death and destruction of the seminiferous tubules [30, 37]. These facts pose new challenges for controlling outbreaks caused by this virus. Yet, currently there is no available drug approved to treat or prevent ZIKV infection. In order to develop specific medical countermeasures, more studies are needed to understand the pathogenesis of ZIKV.

Like other flaviviruses, such as dengue virus (DENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV), ZIKV is an enveloped, single-stranded, positive-sense RNA virus. The RNA genome of ZIKV is directly translated into a long polyprotein in the cytoplasm of the infected cells. The polyprotein is further co-translationally and post-translationally cleaved and processed by host and viral proteases into three structural proteins (precursor membrane (prM) protein, envelope (E) protein, capsid (C) protein), which form the virus particle, and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) that perform essential functions in genome replication, polyprotein processing and manipulation of cellular processes for viral advantage.

Antibody-mediated humoral immunity contributes significantly to the control of ZIKV infection. The E and NS1 proteins are the major targets for neutralizing and protective antibodies. In this chapter, we mainly focus on the structures of the E and NS1 proteins, and their relations with virus infection and immune responses.

6.1 ZIKV E Protein and Its Role in the Viral Life Cycle

The flavivirus E proteins belong to the class-II fusion proteins, which lie flat on the virus surface in the form of antiparallel homodimers [44]. Each E monomer basically consists of three domains with folds consisting mainly of β-sheets: the central β-barrel domain I (DI), the elongated finger-like domain II (DII) with a hydrophobic fusion loop (FL) at its tip, and the C-terminal IgC-like domain III (DIII) (Fig. 6.1a). The E protein of ZIKV shows a pre-fusion structure similar to all other flavivirus E proteins of known structure [21]. 90 E dimers pack tightly on the virus envelope with a ‘herringbone’ arrangement, supporting the icosahedral symmetry [49]. During virus infection, the E-dimers bind to – as yet unidentified – cellular receptors and are internalized into the endosome of the target cell. There, the acidic-pH environment triggers a transition at the virus surface [43]: E-dimers are thought to

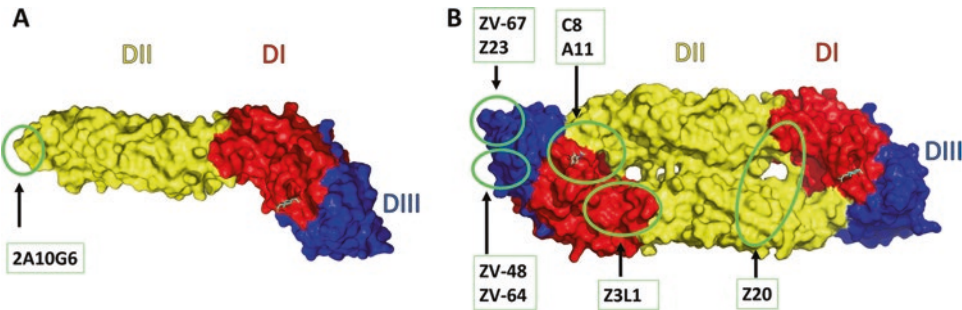


Fig. 6.1 Schematic demonstration of eptiopes for different neutralizing antibodies on ZIKV E protein. The ZIKV E monomer (**a**) and dimer (**b**) are shown as the top view. DI, DII and DIII contained in each E subunit are colored as red, yellow and blue, respectively. Glycans in 150-loops are shown as cyan sticks. The epitopes for their corresponding neutralizing antibodies are marked by green circles

dissociate into monomers with structural rearrangement that permit the fusion loops to insert into the membrane of the target cell. Subsequently, the E-monomers reassociate into fusogenic trimers and pull together the membranes of cell and virion to complete the virus fusion [3, 52, 53]. At a late stage of the viral life cycle, the newly assembled progenies enter the endoplasmic reticulum (ER) lumen forming immature virions [61]. 60 trimeric protrusions of the E and prM heterodimers are displayed as a spiky surface of the virus. The immature virions subsequently undergo maturation, where prM is cleaved by the host-cell protease furin in the Trans-Golgi network (TGN) and the pr peptide of the prM protein dissociates from the virion upon release into the neutral-pH extracellular space. In the mature virus the E protein are further rearranged into E homodimer forming a smooth surface. However, the process of virus maturation is often incomplete, and the uncleaved prM will lead to the formation of spiky prM-E protrusions on the virus surface, resulting in immature virions or partially immature virions (mosaic particles) [61].

6.2 Structural Basis of Neutralizing Antibodies against ZIKV

Protective antibodies prevent flavivirus infection by neutralization or Fcγ receptor and complement-mediated phagocytosis (ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxity) [56]. The neutralizing monoclonal antibodies (MAbs) function by blocking viral attachment or interfering with virus fusion [36] at post-attachment steps [32, 33]. Of note, flavivirus-reactive MAbs are known to cause antibody-dependent enhancement (ADE) of virus infection when their neutralization activities are weak or local concentrations are insufficient [9]. Consequently, a high concentration of MAbs with high neutralization potency is required for antibody-mediated protection against flaviviruses.

To date, a number of neutralizing and protective MAbs against ZIKV infection have been reported [7, 21, 25, 47, 51, 57, 63]. Structural studies of MAbs bound to ZIKV-E protein by X-ray crystallography or cryo-electron microscopy (cryo-EM) provide platforms to dissect the molecular basis of antibody-mediated virus protection [22].

The first MAb reported to react with ZIKV-E was 2A10G6, a broadly protective antibody of flaviviruses as previously identified [21, 26]. 2A10G6 is a murine MAb and can neutralize all four serotypes of DENV as well as WNV and YFV with potency at nanomolar concentrations [26]. The epitope of this MAb is mapped to the ^{98}DRXW^{101} motif of the E protein, a region conserved throughout the flaviviruses that is involved in the fusion of the cellular and viral membranes. Because of the conservation of this FL in sequence and structure, MAbs recognizing this region are usually cross-reactive. 2A10G6 binds to ZIKV soluble E protein (sE) with high affinity, but unexpectedly, shows weaker neutralization potency against ZIKV compared to DENV and other tested flaviviruses (100-fold lower neutralization potency). Based on structural characterization, 2A10G6 engages the distal end of DII of ZIKV sE, covering key residues in the FL, including W101 and F108 [21] (Fig. 6.1a). The binding sites in the FL for MAbs are usually occluded in the prefusion E-dimer of flaviviruses. Thereby, virion 'breathing' is required for the recognition of this epitope in the FL by 2A10G6. 'Breathing' describes the conformational fluctuation of E proteins of the virus particle which consequently alters the antigenic surface for MAb binding [29, 34, 43]. Increasing the temperature has been demonstrated to enhance the exposure of the cryptic epitope of DENV by virus 'breathing'. Interestingly, ZIKV has been shown to feature a thermally more stable structure compared to DENV [29, 34]. Accordingly, the FL- targeting MAbs, including 2A10G6, which efficiently neutralize DENV, show low or no neutralizing activities against ZIKV [7, 21, 25]. Besides, FL-specific MAbs can recognize epitopes on immature or partly immature virions, also known as mosaic particles [16], in which the FL of the E protein is exposed on the protrusion of prM heterodimers. Despite weak neutralization activity, 2A10G6 exerts a partially protective effect *in vivo*. This

protection is likely due to other effector functional mechanisms, possibly involving Fcγ receptor-mediated or complement-dependent effectors [56]. In summary, the FL-targeting MAbs with weak neutralizing potency are connected with a higher risk of ADE, especially for ZIKV treatment, which should be further studied in the near future.

Subsequently, two neutralizing antibodies, C8 and A11, have been shown to be effective against ZIKV infection [7]. C8 and A11 are human MAbs that broadly neutralize all four serotypes of DENV and ZIKV, and they are defined as E-dimer-dependent (EDE) MAbs binding to the quaternary-structure-dependent epitopes in the conserved region of DENV and ZIKV, including the b strand, the ij loop and the FL in DII, the 150-loop in DI, and the A strand in DIII [7, 46] (Fig. 6.1b). They are potent neutralizers for the DENV/ZIKV super-serogroup.

How about ZIKV-specific neutralizing antibodies? Recently, our group [57] described three ZIKV-specific neutralizing antibodies, Z20, Z23 and Z3L1, that were isolated from a ZIKV-infected patient. All three antibodies can provide complete protection against ZIKV infection in immunodeficient mice [57]. For the Z20-sE complex, structural studies have revealed that its epitope includes the a, b, d, i, and j strands, and the bc, hi and ij loops in DII of one sE, as well as the fg and aBI_0 loops from DII of the adjacent subunit (Fig. 6.1b). A cryo-EM structural study shows that Z23 mainly binds to DIII of one E monomer, and can cross-react with two E-dimers in the dimer-dimer interface (Fig. 6.1b). 120 Z23 Fragments of antigen binding were observed to bind to the ZIKV particle, indicating that its epitope overlaps with that of ZV-67 [57, 63]. As to Z3L1, it has been demonstrated to bind to the DI and DI-DII hinge region on the top surface of the sE dimer (Fig. 6.1b).

Meanwhile, four ZIKV-specific murine neutralizing MAbs targeting DIII have been reported, including ZV-48, ZV-54, ZV-64 and ZV-67 [63]. ZV-54 and ZV-67 are more efficient in ZIKV neutralization with a broader spectrum, compared to ZV-48 and ZV-64. Further structural and competition binding studies revealed that ZV-48 and ZV-64 share an overlapping epitope in the C-C′ loop, while ZV-54 and ZV-67 bind to the lateral ridge (Fig. 6.1b). *In vivo* passive transfer studies revealed the protective activities of DIII-lateral ridge specific neutralizing MAbs (ZV-54 and ZV-67) in a mouse model of ZIKV infection [63]. The idea that E-DIII-targeting antibodies are mainly ZIKV-specific was confirmed in a study that characterized memory lymphocytes from ZIKV-infected patients [51]. The structural basis of this observation needs to be studied in the future.

6.3 NS1 Structures and Functional Implications

Among the non-structural proteins, NS1, which possesses multiple functions in the viral life cycle including viral replication, immune evasion and pathogenesis, is the most enigmatic protein of the flaviviruses [4]. NS1 is a glycoprotein, and the glycosylation of NS1 is important for efficient secretion, virulence and viral replication. The glycosylated ~50-kDa NS1 exists as a membrane-associated homodimer after translocation into the endoplasmic reticulum (ER) lumen, and plays an essential role in viral genome replication. Infected cells also secrete NS1 into the extracellular space as a hexameric lipoprotein particle (sNS1). The sNS1 lipoprotein is involved in immune evasion and pathogenesis by interacting with components from both the host innate and adaptive immune systems, as well as other host factors [6, 19]. NS1 also represents the major antigenic marker for viral infection [60]. The molecular mechanisms are relatively-well established for DENV and WNV [1, 28], but little was known about the NS1 of ZIKV, until recent publications reported crystal structures of the ZIKV NS1 protein [11, 50, 58]. The structures show special properties of ZIKV NS1, and help learn more about this pathogenicity factor with its many faces [31]. The overall structure of the ZIKV NS1 protein is very similar to the DENV2 and WNV NS1 structures, with the same protein fold and domain arrangement [11, 50, 58]. All of them form a homodimer, and each monomer has

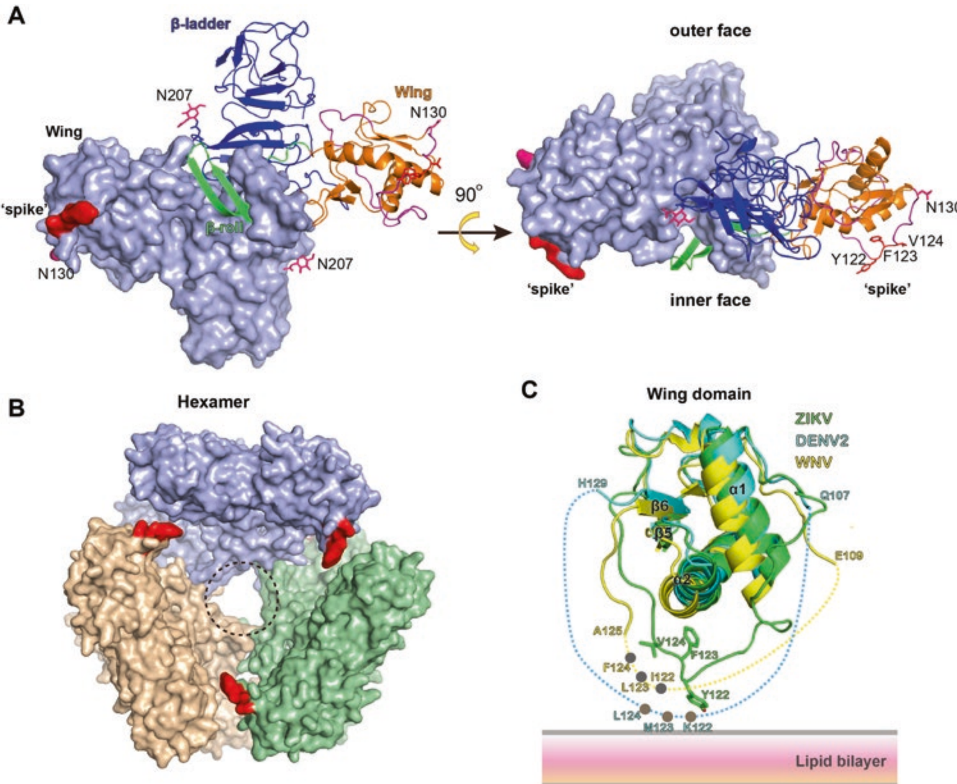


Fig. 6.2 Structure of ZIKV NS1 protein and the newly-identified 'spike' region. (**a**) Overall structure of the dimeric ZIKV NS1 protein. The NS1 monomer can be divided into three major domains, the β-roll domain, the wing domain and the β-ladder domain. N-linked glycosylation sites are shown as sticks. The newly-defined 'spike' region in the wing domain is colored in red. (**b**) Model of the NS1 hexamer. The 'spike' region is predicted to be involved in the formation of the NS1 hexamer. The hydrophobic hole is indicated by dotted lines. (**c**) Comparison of the 'spike' regions from DENV, WSV and ZIKV NS1 proteins. The hydrophobic 'spike' region is related to membrane association. The disordered "spike" regions in the DENV2 and WNV NS1 structures are indicated by dashed lines

three domains, the β-hairpin domain, the wing domain, and the β-ladder domain (Fig. 6.2a). For ZIKV NS1, the N-terminal β-hairpin domain (residues 1–30) undergoes a mini-domain-swap with the β-hairpin domain of the other protomer, yielding a β-roll dimerization domain. The wing domain (residues 31–181) consists of three subdomains: an α/β subdomain (residues 38–151), a long intertwined loop between the β5 and β6 strands (residues 91–130) and a discontinuous connector subdomain (residues 31–37 and 152–180) connecting the wing domain to the central β-roll and β-ladder domains. The C-terminal β-ladder domains (residues 182–352) from two NS1 monomers form a long β-sheet with 20 β strands arranged like the rungs of a ladder. On the opposite side of the ladder surface, an irregular surface is formed by a complex arrangement of loop structures including a long "spaghetti loop". There are two potential N-linked glycosylation sites (N130 and N207) in NS1, which are highly conserved for different flavivirus.

The NS1 dimer has two faces: the inner face and the outer face. The β-ladder defines the inner face along with the β-roll domain and the second half of the intertwined loop of the wing domain, and on the other side, there is the loop surface formed by the "spaghetti loop" and the first half of the intertwined loop of the wing domain. On the inner face of the dimer, the β-roll, the connector subdomain, and the second half of the intertwined loop of the wing create a discontinuous

linear hydrophobic protrusion with a markedly hydrophobic surface that is the prime candidate for membrane interaction. The dimer outer face is polar and contains the N130-glycosylation site. In the NS1 hexamer, three dimers assemble with the polar outer faces pointing outward and the hydrophobic inner faces pointing inward, so that they can interact with lipid molecules in the sNS1 lipoprotein particle (Fig. 6.2b).

The ZIKV NS1 structures allow the first visualization of the second half of the intertwined loop in the wing domain, which is disordered in previously-reported DENV2 and WNV NS1 crystal structures. This long loop (containing hydrophobic residues Y122, F123, V124) forms an extended hydrophobic protrusion ("spike") together with the β-roll and the connector subdomain, and is most likely involved in associating with the membrane surface. Especially, residues 122–124 of the intertwined loop are always hydrophobic or positively charged in different flaviviruses, irrespective of amino-acid variations, which is beneficial for the membrane association (such as K122, M123, L124 in DENV2 and I122, L123, F124 in WNV) (Fig. 6.2c).

From the conservation analysis of NS1 proteins among different flaviviruses, the most conserved surfaces are on the β-roll region and the C-terminal tip of the central β-ladder. The outer face of the wing domain is the most variable region. The ZIKV NS1 structure reveals unique electrostatic potentials compared with the available NS1 structures of DENV2 and WNV [1]. One predominant feature lies in the loop surface of the β-ladder domain [50]. The DENV2 NS1 structure displays a positively charged surface in the central region of the loop surface, whereas the WNV NS1 structure has a negatively charged central region. For ZIKV, the loop surface exhibits a composite surface containing both a positively and negatively charged central region and displays negative charges toward the two distal ends. In addition, in the inner face with the β-roll domain, only ZIKV displays a negatively charged surface, whereas DENV2 and WNV display surfaces of neutral charge. Moreover, ZIKV NS1 displays a positively charged surface in the tip region of the outer face of the wing domain, whereas the WNV NS1 structure has a negatively-charged region here, and the DENV2 NS1 structure contains both a positively- and a negatively-charged region.

The model of the ZIKV sNS1 hexamer, built on the basis of the previously reported DENV2 NS1 hexamer [1], reveals a hydrophobic hole surrounded by the inner faces of three NS1 dimers, containing the β-roll domain and the intertwined loop "spike". By contrast, the outer faces can be easily bound by host factors and reactive antibodies.

The high level of secreted NS1 in the blood of flavivirus-infected individuals during early infection has made NS1 a primary biomarker in disease diagnosis [2]. More recently, a panel of monoclonal antibodies from ZIKV-infected patients was isolated, and most of the antibodies were ZIKV-specific, and memory T cells against NS1 proteins were poorly cross-reactive, even in donors pre-exposed to DENV, indicating that the ZIKV NS1 protein can induce a prominent ZIKV-specific immune response [51]. The results of these authors support our data showing that the ZIKV NS1 structure has unique surface characteristics, and it will be instructional instructive for developing a serological diagnostic tool targeting ZIKV NS1. Indeed Eroimmun AG has developed a Zik-NS1 specific detection test (see Chap. 3) that could become widely used in the clinical setting. In addition, NS1 is a potential vaccine candidate against flavivirus infection [4]. Immunization of mice with DENV NS1 protects them from lethal DENV challenge [48]. However, NS1 cannot only elicit protective antibodies, but also auto-antibodies. These auto-antibodies can induce severe forms of dengue known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [15, 17]. A wide range of host proteins present on platelets and endothelial cells have been identified as cross-reactive targets by NS1-induced auto-antibodies [15, 17, 18, 35, 54, 55, 59]. DENV2 sNS1 itself has also been shown to disrupt endothelial cell monolayer integrity and activate innate immune cells partially induced by interaction between sNS1 and Toll-like receptor 4 (TLR4) [8, 14, 42]. The outer face of the NS1 structure has been suggested to play a

crucial role in interactions of NS1 with host factors and antibodies [5, 19, 20, 28].

In summary, compared to other flavivirus NS1 molecules, ZIKV NS1 displays a variable surface and divergent electrostatic potential that may result in altered binding properties to host factors and protective antibodies. The unique surface characteristics of ZIKV NS1 may be related to ZIKV neurotropism and may help the virus cross the blood-brain barrier, the blood-placenta barrier, the blood-eye barrier and the blood-testis barrier. It could also be exploited in the development of novel therapeutics and diagnostic tools for ZIKV infection [51].

Discussion of Chapter 6 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Drs. Gao and Shi.

Félix Rey: When you selected the antibodies, were you using the Zika virus?

George Gao: We have had one imported case, which is the first imported case of Zika infection [in China]. We cannot label the whole virus so we only label the His-tagged E protein for antibody selection.

Félix Rey: You said that your E protein was monomeric. So why are you surprised that you got many fusion-loop antibodies, if it is monomeric?

George Gao: I agree that in the monomeric form you expose the fusion loop but it is possible based on the structure that it could be covered like you see in a dimer.

Félix Rey: But your selection process is not picking those, that are at the interface.

George Gao: If it is not that immunogenic as you are expecting, then they will not be selected and we persistently see only the fusion loop antibodies.

Félix Rey: In Gavin Screaton's screen, he had seven patients and the response was binary: From some patients, he only got ADE (enhancing antibodies) and from others he only got fusion loop antibodies. But he used whole viruses for selection.

George Gao: I agree. Our selection processes may be biased.

Aravinda de Silva: I think, we have had a lot of discussion about fusion loop antibodies and protection. And I think we are all interpreting that data. So after primary Dengue virus infection, people make a lot of fusion loop antibodies. And we know that they are not protective, because if they were protective, you would not get an infection with a different serotype, because fusion loop antibodies are broadly crossreacting. What that tells me is that *in vivo*, in people, when you have been exposed to a different serotype fusion loop, that antibodies are not protective. And that might mean that *in vivo,* the fusion loop is not exposed in the viruses that go on to mediate infection. And I think one other thing that we need to think about is when we think about virus breathing and exposure of fusion loops, how the lab-adapted strains of viruses that we work with are compared with clinical strains. And we know very little about the maturation state flexibility of the virions that circulate in people. So despite the abundance of fusion loop antibodies, there is no evidence in people that they are protective. That is different from the ADE-type antibodies, which clearly do. I do not think you can call those fusion loop antibodies, even though they are binding next to the fusion loop, they are binding to domain II, but they are not really binding to the fusion loop.

George Gao: OK this could mean where the antibody actually binds is an important aspect but it may be minor. We use the soluble E protein of the virus for selection but end with an antibody that bind as if the protein is a dimer.

Félix Rey: You know that the Dengue virus surface is very heterogeneous with immature patches. And you have like 60% uncleaved prM on the virion surface. So in all of those, when prM is in complex with the E protein, the fusion loop is on the side. Pr is not covering the fusion loop and it is accessible to the

antibodies. I think that gives rise to the immunogenicity. I mean most of those fusion loop antibodies come from the partially immature virus.

George Gao: We isolated at least one antibody, targeting the inner side, not on the surface of the domain I. How can we get this type of antibody that binds very strongly, of course it is non-neutralizing, but the fact is that you can isolate from our selection procedure, a new antibody that cover the inner side. I don't see the connection with immature conformation.

Félix Rey: That is probably because you always have some misfolded particles somewhere in the mix of your protein, even against internal proteins you can get antibodies.

Paul Young: Have you done any lipid membrane modeling studies yet, with the Zika NS1?

Yi Shi: No, we are working on it. We have just designed mutant proteins to see whether this region is important. We are now doing the lipid floating experiment which is a membrane-association technique.

Subhash Vasudevan: Have you compared your Brazilian strain with the MR766 strain in the region where you think the loop structures are different? Did you compare the sequences?

Yi Shi: You mean the sequence that is in the spike region?

Subhash Vasudevan: Yes. There must be some differences between the strains.

Yi Shi: No, not yet but there must be some differences.

Aravinda de Silva: So you have suggested that the protein must have a role in helping the virus to cross the placenta. For Dengue, it is really endothelial cells that are affected by NS1. And for the placenta, the barrier is not really the endothelial cells. Because the layer between maternal and fetal blood, is the trophoblast and it forms the barrier between the mother and the baby. So if NS1 has an effect there, it has to be not on endothelial cells but on the cell that really separate the maternal and the fetal compartment in the placenta and that is the trophoblast.

Yi Shi: Maybe I think, our proposal of NS1 helping the virus to get into the placenta is just a hypothesis. As you know, the NS1 has many effectors and there are a lot of factors that with which it can interact. It may have some function in the placenta.

Aravinda de Silva: I think, it is an interesting hypothesis. What I am suggesting is to look at the layers of trophoblast in the placenta and not in the endothelial cells.

References

1. Akey DL, Brown WC, Dutta S, Konwerski J, Jose J, Jurkiw TJ, DelProposto J, Ogata CM, Skiniotis G, Kuhn RJ, Smith JL (2014) Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system. Science 343:881–885
2. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M (2002) Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 40:376–381
3. Allison SL, Schalich J, Stiasny K, Mandl CW, Kunz C, Heinz FX (1995) Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. J Virol 69:695–700
4. Amorim JH, Alves RP, Boscardin SB, Ferreira LC (2014) The dengue virus non-structural 1 protein: risks and benefits. Virus Res 181:53–60
5. Avirutnan P, Zhang L, Punyadee N, Manuyakorn A, Puttikhunt C, Kasinrerk W, Malasit P, Atkinson JP, Diamond MS (2007) Secreted NS1 of dengue virus attaches to the surface of cells via interactions with heparan sulfate and chondroitin sulfate E. PLoS Pathog 3:e183
6. Avirutnan P, Fuchs A, Hauhart RE, Somnuke P, Youn S, Diamond MS, Atkinson JP (2010) Antagonism of the complement component C4 by flavivirus nonstructural protein NS1. J Exp Med 207:793–806
7. Barba-Spaeth G, Dejnirattisai W, Rouvinski A, Vaney MC, Medits I, Sharma A, Simon-Loriere E, Sakuntabhai A, Cao-Lormeau VM, Haouz A, England P, Stiasny K, Mongkolsapaya J, Heinz FX, Screaton GR, Rey FA (2016) Structural basis of potent Zika-dengue virus antibody cross-neutralization. Nature 536:48–53
8. Beatty PR, Puerta-Guardo H, Killingbeck SS, Glasner DR, Hopkins K, Harris E (2015) Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. Sci Transl Med 7:304ra141
9. Beltramello M, Williams KL, Simmons CP, Macagno A, Simonelli L, Quyen NT, Sukupolvi-Petty S,

Navarro-Sanchez E, Young PR, de Silva AM, Rey FA, Varani L, Whitehead SS, Diamond MS, Harris E, Lanzavecchia A, Sallusto F (2010) The human immune response to dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. Cell Host Microbe 8:271–283

10. Brasil P, Pereira JP Jr, Raja Gabaglia C, Damasceno L, Wakimoto M, Ribeiro Nogueira RM, Carvalho de Sequeira P, Machado Siqueira A, Abreu de Carvalho LM, Cotrim da Cunha D, Calvet GA, Neves ES, Moreira ME, Rodrigues Baiao AE, Nassar de Carvalho PR, Janzen C, Valderramos SG, Cherry JD, Bispo de Filippis AM, Nielsen-Saines K (2016) Zika virus infection in pregnant women in Rio de Janeiro. N Engl J Med 375:2321–2334
11. Brown WC, Akey DL, Konwerski JR, Tarrasch JT, Skiniotis G, Kuhn RJ, Smith JL (2016) Extended surface for membrane association in Zika virus NS1 structure. Nat Struct Mol Biol 23:865–867
12. Calvet GA, Santos FB, Sequeira PC (2016) Zika virus infection: epidemiology, clinical manifestations and diagnosis. Curr Opin Infect Dis 29:459–466
13. Carteaux G, Maquart M, Bedet A, Contou D, Brugières P, Fourati S, Cleret de Langavant L, de Broucker T, Brun-Buisson C, Leparc-Goffart I, Mekontso Dessap A (2016) Zika virus associated with meningoencephalitis. N Engl J Med 374:1595–1596
14. Chen J, Ng MM, Chu JJ (2015) Activation of TLR2 and TLR6 by dengue NS1 protein and its implications in the immunopathogenesis of dengue virus infection. PLoS Pathog 11:e1005053
15. Cheng HJ, Lin CF, Lei HY, Liu HS, Yeh TM, Luo YH, Lin YS (2009) Proteomic analysis of endothelial cell autoantigens recognized by anti-dengue virus nonstructural protein 1 antibodies. Exp Biol Med (Maywood, NJ) 234:63–73
16. Cherrier MV, Kaufmann B, Nybakken GE, Lok SM, Warren JT, Chen BR, Nelson CA, Kostyuchenko VA, Holdaway HA, Chipman PR, Kuhn RJ, Diamond MS, Rossmann MG, Fremont DH (2009) Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody. EMBO J 28:3269–3276
17. Chuang YC, Lei HY, Lin YS, Liu HS, Wu HL, Yeh TM (2011) Dengue virus-induced autoantibodies bind to plasminogen and enhance its activation. J Immunol 187:6483–6490
18. Chuang YC, Lin YS, Liu HS, Yeh TM (2014) Molecular mimicry between dengue virus and coagulation factors induces antibodies to inhibit thrombin activity and enhance fibrinolysis. J Virol 88:13759–13768
19. Chung KM, Liszewski MK, Nybakken G, Davis AE, Townsend RR, Fremont DH, Atkinson JP, Diamond MS (2006a) West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. Proc Natl Acad Sci U S A 103:19111–19116
20. Chung KM, Nybakken GE, Thompson BS, Engle MJ, Marri A, Fremont DH, Diamond MS (2006b) Antibodies against West Nile virus nonstructural protein NS1 prevent lethal infection through fc gamma receptor-dependent and -independent mechanisms. J Virol 80:1340–1351
21. Dai L, Song J, Lu X, Deng YQ, Musyoki AM, Cheng H, Zhang Y, Yuan Y, Song H, Haywood J, Xiao H, Yan J, Shi Y, Qin CF, Qi J, Gao GF (2016a) Structures of the Zika virus envelope protein and its complex with a flavivirus broadly protective antibody. Cell Host Microbe 19:696–704
22. Dai L, Wang Q, Qi J, Shi Y, Yan J, Gao GF (2016b) Molecular basis of antibody-mediated neutralization and protection against flavivirus. IUBMB Life 68:783–791
23. Davidson A, Slavinski S, Komoto K, Rakeman J, Weiss D (2016) Suspected female-to-male sexual transmission of Zika virus – New York city, 2016. Morb Mort Weekly Rep (MMWR) 65:716–717
24. Deckard DT, Chung WM, Brooks JT, Smith JC, Woldai S, Hennessey M, Kwit N, Mead P (2016) Male-to-male sexual transmission of Zika virus—Texas, January 2016. Morb Mort Weekly Rep (MMWR) 65:372–374
25. Dejnirattisai W, Supasa P, Wongwiwat W, Rouvinski A, Barba-Spaeth G, Duangchinda T, Sakuntabhai A, Cao-Lormeau VM, Malasit P, Rey FA, Mongkolsapaya J, Screaton GR (2016) Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. Nat Immun 17:1102–1108
26. Deng YQ, Dai JX, Ji GH, Jiang T, Wang HJ, Yang HO, Tan WL, Liu R, Yu M, Ge BX, Zhu QY, Qin ED, Guo YJ, Qin CF (2011) A broadly flavivirus cross-neutralizing monoclonal antibody that recognizes a novel epitope within the fusion loop of E protein. PLoS One 6:e16059
27. Driggers RW, Ho CY, Korhonen EM, Kuivanen S, Jaaskelainen AJ, Smura T, Rosenberg A, Hill DA, DeBiasi RL, Vezina G, Timofeev J, Rodriguez FJ, Levanov L, Razak J, Iyengar P, Hennenfent A, Kennedy R, Lanciotti R, du Plessis A, Vapalahti O (2016) Zika virus infection with prolonged maternal viremia and fetal brain abnormalities. N Engl J Med 374:2142–2151
28. Edeling MA, Diamond MS, Fremont DH (2014) Structural basis of flavivirus NS1 assembly and antibody recognition. Proc Natl Acad Sci U S A 111:4285–4290
29. Fibriansah G, Ng TS, Kostyuchenko VA, Lee J, Lee S, Wang J, Lok SM (2013) Structural changes in dengue virus when exposed to a temperature of 37 °C. J Virol 87(13):7585–7592
30. Govero J, Esakky P, Scheaffer SM, Fernandez E, Drury A, Platt DJ, Gorman MJ, Richner JM, Caine EA, Salazar V, Moley KH, Diamond MS (2016) Zika virus infection damages the testes in mice. Nature 540:438–442

31. Hilgenfeld R (2016) Zika virus NS1, a pathogenicity factor with many faces. EMBO J 35:2631–2633
32. Kaufmann B, Chipman PR, Holdaway HA, Johnson S, Fremont DH, Kuhn RJ, Diamond MS, Rossmann MG (2009) Capturing a flavivirus pre-fusion intermediate. PLoS Pathog 5:e1000672
33. Kaufmann B, Vogt MR, Goudsmit J, Holdaway HA, Aksyuk AA, Chipman PR, Kuhn RJ, Diamond MS, Rossmann MG (2010) Neutralization of West Nile virus by cross-linking of its surface proteins with fab fragments of the human monoclonal antibody CR4354. Proc Natl Acad Sci U S A 107:18950–18955
34. Kostyuchenko VA, Lim EX, Zhang S, Fibriansah G, Ng TS, Ooi JS, Shi J, Lok SM (2016) Structure of the thermally stable Zika virus. Nature 533:425–428
35. Liu IJ, Chiu CY, Chen YC, Wu HC (2011) Molecular mimicry of human endothelial cell antigen by autoantibodies to nonstructural protein 1 of dengue virus. J Biol Chem 286:9726–9736
36. Lok SM, Kostyuchenko V, Nybakken GE, Holdaway HA, Battisti AJ, Sukupolvi-Petty S, Sedlak D, Fremont DH, Chipman PR, Roehrig JT, Diamond MS, Kuhn RJ, Rossmann MG (2008) Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. Nat Struct Mol Biol 15:312–317
37. Ma W, Li S, Ma S, Jia L, Zhang F, Zhang Y, Zhang J, Wong G, Zhang S, Lu X, Liu M, Yan J, Li W, Qin C, Han D, Qin C, Wang N, Li X, Gao GF (2016) Zika virus causes testis damage and leads to male infertility in mice. Cell 167:1511–1524
38. Mansuy JM, Dutertre M, Mengelle C, Fourcade C, Marchou B, Delobel P, Izopet J, Martin-Blondel G (2016a) Zika virus: high infectious viral load in semen, a new sexually transmitted pathogen? Lancet Infect Dis 16:405
39. Mansuy JM, Suberbielle E, Chapuy-Regaud S, Mengelle C, Bujan L, Marchou B, Delobel P, Gonzalez-Dunia D, Malnou CE, Izopet J, Martin-Blondel G (2016b) Zika virus in semen and spermatozoa. Lancet Infect Dis 16:1106–1107
40. Mecharles S, Herrmann C, Poullain P, Tran TH, Deschamps N, Mathon G, Landais A, Breurec S, Lannuzel A (2016) Acute myelitis due to Zika virus infection. Lancet 387:1481
41. Mlakar J, Korva M, Tul N, Popović M, Poljšak-Prijatelj M, Mraz J, Kolenc M, Resman Rus K, Vesnaver Vipotnik T, Fabjan Vodušek V, Vizjak A, Pižem J, Petrovec M, Avšič Županc T (2016) Zika virus associated with microcephaly. N Engl J Med 374:951–958
42. Modhiran N, Watterson D, Muller DA, Panetta AK, Sester DP, Liu L, Hume DA, Stacey KJ, Young PR (2015) Dengue virus NS1 protein activates cells via toll-like receptor 4 and disrupts endothelial cell monolayer integrity. Sci Transl Med 7:304ra142
43. Modis Y, Ogata S, Clements D, Harrison SC (2004) Structure of the dengue virus envelope protein after membrane fusion. Nature 427:313–319
44. Mukhopadhyay S, Kuhn RJ, Rossmann MG (2005) A structural perspective of the flavivirus life cycle. Nat Rev Microbiol 3:13–22
45. Prisant N, Bujan L, Benichou H, Hayot PH, Pavili L, Lurel S, Herrmann C, Janky E, Joguet G (2016) Zika virus in the female genital tract. Lancet Infect Dis 16:1000–1001
46. Rouvinski A, Guardado-Calvo P, Barba-Spaeth G, Duquerroy S, Vaney MC, Kikuti CM, Navarro Sanchez ME, Dejnirattisai W, Wongwiwat W, Haouz A, Girard-Blanc C, Petres S, Shepard WE, Despres P, Arenzana-Seisdedos F, Dussart P, Mongkolsapaya J, Screaton GR, Rey FA (2015) Recognition determinants of broadly neutralizing human antibodies against dengue viruses. Nature 520:109–113
47. Sapparapu G, Fernandez E, Kose N, Cao B, Fox JM, Bombardi RG, Zhao H, Nelson CA, Bryan AL, Barnes T, Davidson E, Mysorekar IU, Fremont DH, Doranz BJ, Diamond MS, Crowe JE (2016) Neutralizing human antibodies prevent Zika virus replication and fetal disease in mice. Nature 540:443–447
48. Schlesinger JJ, Brandriss MW, Walsh EE (1987) Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus nonstructural glycoprotein NS1. J Gen Virol 68:853–857
49. Sirohi D, Chen Z, Sun L, Klose T, Pierson TC, Rossmann MG, Kuhn RJ (2016) The 3.8 Å resolution cryo-EM structure of Zika virus. Science 352:467–470
50. Song H, Qi J, Haywood J, Shi Y, Gao GF (2016) Zika virus NS1 structure reveals diversity of electrostatic surfaces among flaviviruses. Nat Struct Mol Biol 23:456–458
51. Stettler K, Beltramello M, Espinosa DA, Graham V, Cassotta A, Bianchi S, Vanzetta F, Minola A, Jaconi S, Mele F, Foglierini M, Pedotti M, Simonelli L, Dowall S, Atkinson B, Percivalle E, Simmons CP, Varani L, Blum J, Baldanti F, Cameroni E, Hewson R, Harris E, Lanzavecchia A, Sallusto F, Corti D (2016) Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. Science 353:823–826
52. Stiasny K, Allison SL, Marchler-Bauer A, Kunz C, Heinz FX (1996) Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. J Virol 70:8142–8147
53. Stiasny K, Bressanelli S, Lepault J, Rey FA, Heinz FX (2004) Characterization of a membrane-associated trimeric low-pH-induced form of the class II viral fusion protein E from tick-borne encephalitis virus and its crystallization. J Virol 78:3178–3183
54. Sun DS, King CC, Huang HS, Shih YL, Lee CC, Tsai WJ, Yu CC, Chang HH (2007) Antiplatelet autoantibodies elicited by dengue virus non-structural protein 1 cause thrombocytopenia and mortality in mice. J Thromb Haemost 5:2291–2299
55. Sun DS, Chang YC, Lien TS, King CC, Shih YL, Huang HS, Wang TY, Li CR, Lee CC, Hsu PN, Chang HH (2015) Endothelial cell sensitization by death receptor fractions of an anti-dengue nonstructural pro-

tein 1 antibody induced plasma leakage, coagulopathy, and mortality in mice. J Immunol 195:2743–2753
56. Vogt MR, Dowd KA, Engle M, Tesh RB, Johnson S, Pierson TC, Diamond MS (2011) Poorly neutralizing cross-reactive antibodies against the fusion loop of West Nile virus envelope protein protect *in vivo* via Fcgamma receptor and complement-dependent effector mechanisms. J Virol 85:11567–11580
57. Wang Q, Yang H, Liu X, Dai L, Ma T, Qi J, Wong G, Peng R, Liu S, Li J, Li S, Song J, Liu J, He J, Yuan H, Xiong Y, Liao Y, Li J, Yang J, Tong Z, Griffin DB, Bi Y, Liang M, Xu X, Qin C, Cheng G, Wang P, Qiu X, Kobinger G, Shi Y, Yan J, Gao GF (2016) Human neutralizing antibodies isolated from a Zika patient and their molecular determinants. Sci Transl Med 8:369ra179
58. Xu X, Song H, Qi J, Liu Y, Wang H, Su C, Shi Y, Gao GF (2016) Contribution of intertwined loop to membrane association revealed by Zika virus full-length NS1 structure. EMBO J 35:2170–2178
59. Yin Y, Jiang L, Fang D, Jiang L, Zhou J (2013) Differentially expressed genes of human microvascular endothelial cells in response to anti-dengue virus NS1 antibodies by suppression subtractive hybridization. Viral Immunol 26:185–191
60. Young PR, Hilditch PA, Bletchly C, Halloran W (2000) An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol 38:1053–1057
61. Yu IM, Zhang W, Holdaway HA, Li L, Kostyuchenko VA, Chipman PR, Kuhn RJ, Rossmann MG, Chen J (2008) Structure of the immature dengue virus at low pH primes proteolytic maturation. Science 319:1834–1837
62. Zhang Y, Chen W, Wong G, Bi Y, Yan J, Sun Y, Chen E, Yan H, Lou X, Mao H, Xia S, Gao GF, Shi W, Chen Z (2016) Highly diversified Zika viruses imported to China, 2016. Prot Cell 7:461–464
63. Zhao H, Fernandez E, Dowd KA, Speer SD, Platt DJ, Gorman MJ, Govero J, Nelson CA, Pierson TC, Diamond MS, Fremont DH (2016) Structural basis of zika virus-specific antibody protection. Cell 166:1016–1027

7 Plugging the Leak in Dengue Shock

Daniel Watterson, Naphak Modhiran, David A. Muller, Katryn J. Stacey, and Paul R. Young

Abstract

Recent structural and functional advances provide fresh insight into the biology of the dengue virus non-structural protein, NS1 and suggest new avenues of research. The work of our lab and others have shown that the secreted, hexameric form of NS1 has a systemic toxic effect, inducing inflammatory cytokines and acting directly on endothelial cells to produce the hallmark of dengue disease, vascular leak. We also demonstrated that NS1 exerts its toxic activity through recognition by the innate immune receptor TLR4, mimicking the bacterial endotoxin LPS. This monograph covers the background underpinning these new findings and discusses new avenues for antiviral and vaccine intervention.

Keywords

Dengue NS1 · Secreted NS1(sNS1) · Dengue diseases · Dengue associated vascular leak · Dengue NS1 induced toxicity

D. Watterson (✉) · N. Modhiran · D. A. Muller · K. J. Stacey · P. R. Young (✉)
Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia
e-mail: d.watterson@uq.edu.au; p.young@uq.edu.au

7.1 Introduction

First described as a soluble complement fixing (SCF) antigen more than four decades ago [10, 90, 102], the dengue NS1 protein remains a focus of both basic and translational research. A potential driving role for SCF (NS1) in disease pathogenesis was postulated early on, in light of the observed association between high levels of complement consumption and the more severe form of dengue disease, dengue shock syndrome (DSS) [9].

Subsequent investigation into the interplay between dengue virus (DENV) and complement yielded further evidence that immune complexes, whether virion or NS1 derived, potentiate disease severity [89, 103, 110]. Indeed, the extent and interactions between NS1 and the complement pathway have since been expanded considerably, however a clear paradigm of the role NS1 plays in disease progression is only now just emerging.

First observed at the protein level as a 46 kDa species [101], it is now known that NS1 comes in a variety of flavors, with distinct populations incorporating alternate post-translational modifications and quaternary structures that traffic to different locations in and from the infected cell. The much-anticipated recent elucidation of the crystal structure of NS1 has shed new light on these alternate NS1 species and opened new avenues for diagnostic, antiviral and vaccine development [2, 30]. In parallel, our recent dem-

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_7

onstration that the DENV NS1 engages TLR4, with the consequent induction of cytokine production and vascular permeability, provides the first mechanistic explanation to link NS1 with disease severity [7, 69]. These findings place NS1 as a central player in DENV pathology and provide the blueprints for new treatment and prevention strategies. We recently reviewed the extensive literature that describes NS1 biology [74, 123]. Building on these comprehensive reviews, this monograph will provide a brief overview of NS1 molecular biology, and give an update on the state of NS1 research in light of the recent functional and structural advancements.

7.1.1 NS1 Expression, Processing and Post-translational Modification

Early events in the flavivirus lifecycle, including viral entry, nucleocapsid uncoating and translation have been well documented [81]. Following nucleocapsid disassembly, the viral RNA is released into the cytoplasm and relocates to the ER. Once engaged with the membrane-bound ribosomal machinery, the single open reading frame encoded by the positive sense flaviviral genome serves as a template for the first round of translation into the viral polyprotein which is co-translationally cleaved by viral and host proteases. During translation, NS1 is translocated into the lumen of the ER via a signal peptide encoded by the C terminal residues within the upstream flavivirus envelope (E) gene [33]. Within the lumen, the N-terminus of NS1 is released after processing by resident host signal peptidase [77]. Cleavage of the NS1/NS2A junction also occurs within the ER lumen, however the identity of the protease responsible is not yet known and remains the topic of ongoing research.

The NS1 protomer contains 12 cysteines that pair to form 6 disulfide bonds that mediate intra-domain stabilization. Reflecting the importance of these residues, all 12 cysteines are conserved across the mammalian and avian flaviviurses, and 10 of the 12 are also found in the insect only flavivirus NS1 where sequence identity to NS1 from avian or mammalian flaviviruses is minimal. The inter-residue linkages were first assessed via mass spectrometry [117], and have been further clarified in the resolved crystal structures for West Nile virus (WNV) and DENV NS1.

While still within the ER, the NS1 monomer dimerises and acquires a partially hydrophobic nature. Atomic structures of the DENV and WNV NS1 dimer have revealed a hydrophobic undercarriage comprised of the N-terminal region of NS1. The first ~20 amino acids of each monomer within the dimer form an interlocking beta-roll domain, which contributes substantially to the dimeric interface. Together with a hydrophobic protrusion located approximately 150 amino acids downstream, these domains are proposed to insert into the lumenal membrane and account for the close association between NS1 and lipids. In addition to this hydrophobic interface we had earlier identified the incorporation of a glycosylphosphatidylinositol (GPI) moiety within a sub-population of NS1 [48, 76]. The importance of the GPI addition remains unclear, and has been the subject of subsequent investigation [76].

Concurrent with protomer folding and proteolytic processing within the lumen, N-linked glycan addition within NS1 is mediated by the oligosaccharyl transferase complex [122]. An atypical property for a non-structural viral protein, the presence of glycans was one of the first features of interest identified within NS1, drawing the attention of early researchers [121]. Sequence analysis first revealed 2–3 putative N-linked glycosylation sites for the flavivirus NS1, depending on the virus analyzed [87, 113]. It is likely these differences represent variations on an immune evasion strategy, however they may also play a role in receptor binding and cellular activation. After the addition of high-mannose carbohydrate moieties within the OTC, NS1 separates into three distinct populations; a significant fraction is incorporated in the viral replication complex and associated vesicle packets (VPs) [51, 62, 63, 67], a second minor population is trafficked to the plasma membrane [96, 124] and the third is secreted into the extracellular milieu (sNS1, Fig. 7.1b) [22, 23, 35]. The secreted form is first trafficked via the Golgi

where exposed carbohydrate moieties are trimmed and processed to more complex forms by resident glycotransferases and glycosidases.

It is of note that all known flaviviruses, including insect only viruses, incorporate a glycosylation site at Asn 207, while there are additional sites at Asn 130 for DENV1–4, YFV, JEV; Asn 130 and Asn 175 for WNV, Saint Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus (MVEV); and Asn 85 and Asn 223 for the tick-borne viruses Tick-bourne encepthalitis virus (TBEV) and Looping ill virus (LIV). These differences correlate with processing status, as Asn207 remains unprocessed in mammalian cells [82] and although solvent exposed in the resolved crystal structures (Fig. 7.1a), the carbohydrate moiety points downward towards the proposed position of the lumenal membrane (or inwards towards the interface of the hexamer) potentially excluding the activity of the glycoprocessing machinery. Indeed, glycosylation abolishing mutations at this site dramatically affect NS1 oligomer assembly and secretion [21, 84, 104], highlighting a critical structural role for this modification. Conversely, sites at Asn 130 and Asn 175 are processed to a complex carbohydrate form within mammalian cells in both natural infection and recombinant expression settings [24].

7.1.2 The sNS1 Lipoparticle

Concomitant with glycan processing and entry into the secretory pathway, NS1 transitions from a membrane associated dimer into a hexameric lipo-particle (Fig. 7.1b). The exact mechanics involved in the transition between dimer to hexamer are still unknown, however the recent NS1 crystal structures contain some tantalizing clues. An atomic structure for NS1 has been a long-sought goal of the flaviviral research community, however attempts to crystalize NS1 were met with significant difficulty. Progress towards a structural model for NS1 was made with the release of two low-resolution, single-particle based reconstructions of hexameric NS1 [39, 73]. Both studies revealed an open barrel like structure with three asymmetrically aligned densities likely to represent the NS1 dimer (Fig. 7.1a). Analysis of the secreted particle using mass spectrometry and thin layer chromatography revealed the particle contains a significant lipid component, with triglycerides, mono- and diacylglycerols, cholesterol esters and phospholipids among the various lipid species identified (Fig. 7.1b) [39]. This lipid cargo is thought to fill the interior of the barrel like structure, where tight packing would allow the estimated ~70 individual lipid molecules to fill the interior volume of the hexamer. Similar lipidic profiles were obtained for NS1 recovered from DENV1 infected vero cells and recombinant DENV2 NS1 produced in *Drosophila* Schneider 2 cells (S2). Taken together with the apparent stoichiometric quantities of lipid species within the NS1 hexamer it appears the NS1 cargo manifest is tightly regulated, and possibly mediated by structural interactions. The discrete nature of the cargo also suggests avenues for antiviral intervention, as alteration in the lipids available to NS1 can be expected to alter both hexamer formation and downstream functions.

7.1.3 NS1 Structure

It is probable that the lipid component presented the major obstacle to the generation of high-quality NS1 crystals for X-ray diffraction studies. In their recent publication of the atomic structure of NS1, Akey and colleagues were able to overcome these difficulties by obtaining lipid-free NS1 using detergent solubilization followed by reformation of soluble hexamers using sequential size exclusion chromatographic steps [2]. Utilizing this method for recombinant NS1 derived from both WNV and DENV2 based constructs, crystal structures were resolved that revealed a striking similarity in fold between the dimeric form of the two viral proteins, a finding in marked contrast to their significant sequence divergence (ca. RMSD 0.845 Å, 54.7% sequence identity). Intriguingly, two forms of WNV NS1 were resolved, assuming either perfect hexameric symmetry with a central cavity open on both ends (closed form), or with C3 symmetry and splayed open to reveal a single, much wider opening at

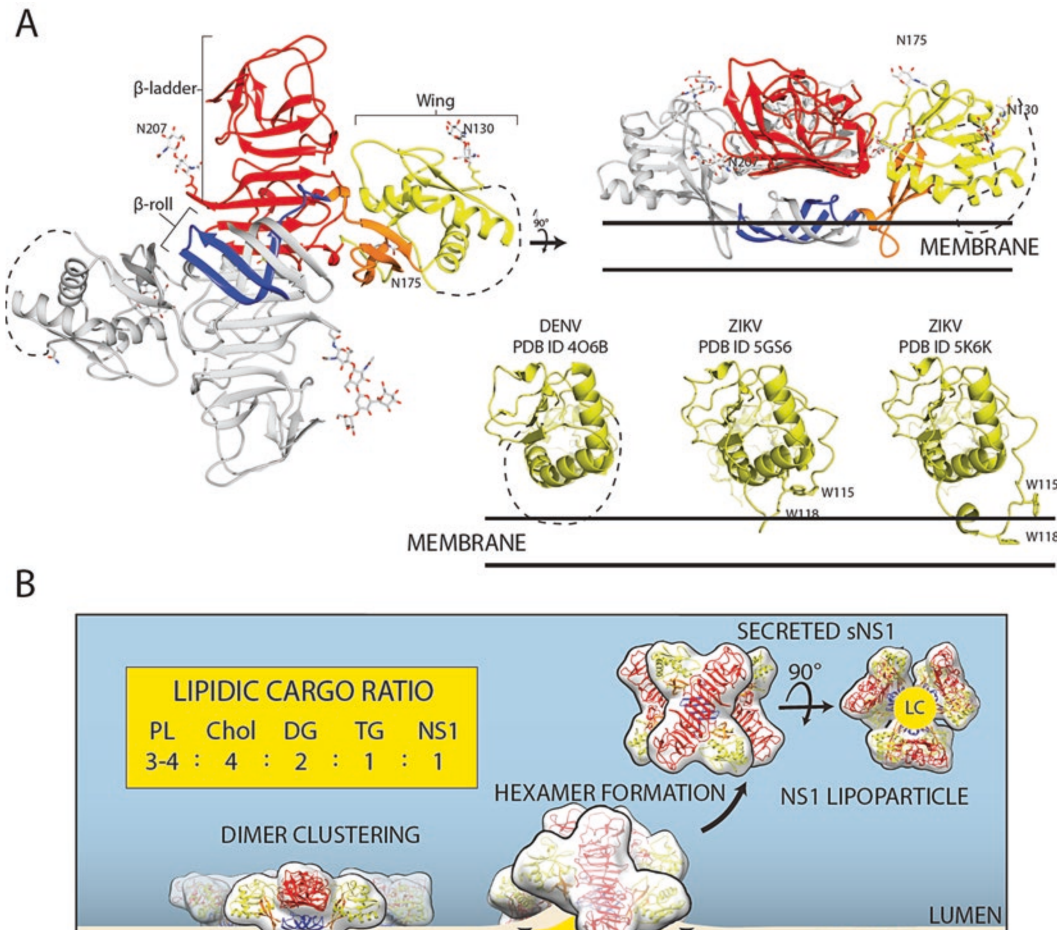


Fig. 7.1 NS1 protein structure and hexamer formation. (**a**) Domain arrangement within the NS1 dimer. Domains of only one NS1 subunit are colored for clarity. Indicated domains include the wing domain (yellow), the central β-ladder (red) and the hydrophobic β-roll and connecting domain (blue and orange). N-linked glycans that were resolved in the crystal structure are indicated. A 90° rotation reveals the proposed membrane topology, with the hydrophobic undercarriage inserting into the cellular membrane. Insets show newly resolved structure of the flexible wing domain loop in the ZIKV NS1 structures. (**b**) Within the golgi of the infected cell, NS1 dimers associate on cholesterol lipid microdomains to form a hexamer. The assembled hexameric sNS1 progresses through the secretory pathway and is released into the bloodstream. The ratio of lipid components (phospholipid, PL; Cholesterol, Chol; Di- and Tri-glycerides, DG and TG) to a single NS1 molecule are as indicated

one end of the hexamer (open form). These forms may be analogous to the cryo-EM (D3 symmetry) and negative stain reconstructions (C3 symmetry) previously reported [39, 73].

The two crystal conformations observed for WNV NS1 and the two distinct EM single-particle reconstructions, both suggest dynamic movement between the dimers, with the open form possibly representing a structural intermediate that is formed during the process of hexamer formation. The equimolar distribution of triglycerides to NS1 monomers within the secreted particle lead Gutsche et al. to suggest a model where NS1 dimers are able to specifically "pinch off" a subset of lipids from neutral lipid rich proto-lipid droplets (LDs) within the ER [39]. Thus, 3 dimers may rearrange into an organized array and interact with specific lipid subpopulations before lifting up and closing to encase their lipid cargo in the form of the mature hexameric lipo-particle (Fig. 7.1b). The elucidation of bound detergent moieties within the open crystal form of WNV NS1 at the interdimeric boundary also supports this model. This process would be dependent on both the immediate NS1 concentration and the local lipidic environment.

Within the quaternary organization of the hexamer, the atomic details revealed by the crystal structures identified motifs within the NS1 dimer that are likely to play key structural roles in lipid association and hexamer formation. NS1 is arranged into three distinct domains; a hydrophobic β-roll (aa 1–29) which, together with a downstream loop, appears as a hydrophobic protrusion from the cross-like structure of the dimer which is formed by a central β-ladder domain and a globular α/ β domain with a RIG-I like fold (Fig. 7.1A). Within the dimer, the two α/β domains project laterally from either sides of the elongated central β-ladder and have been dubbed the "wing" domain. This organization imparts an amphipathic like overall structure to the dimer, with the inner hydrophobic face comprised of the β-roll and associated loop structure proposed to intercalate with the lumenal facing leaflet of the ER membrane.

Interestingly, NS1 in its lipid free state was observed to bind to and remodel cholesterol rich liposomes, in a process that might mimic the native insertion into the lumenal membrane. This property provides a potential avenue for investigating NS1-lipid interactions, and was explored by Akey et al. in the first structure-based investigation of NS1 function. Using site-directed mutagenesis the researchers probed these potential interactions through the insertion of charged amino acids in place of conserved hydrophobic residues within the exposed loop (Fig. 7.1a, insets). Remarkably, no differences in liposome remodeling relative to wild-type protein were observed. Conversely, a reduction in viral replication efficiency was observed when these same mutations were incorporated into an infectious clone, leading the researchers to suggest that this loop may be involved in other roles within the viral life cycle such as interaction with the hydrophobic viral cofactors of the replication complex.

However, in a subsequent commentary [3] it was noted that these same mutations resulted in enhanced secretion of NS1, which could impact on replication efficiency through skewing the ratio of intracellular vs. extracellular NS1 levels. Of particular note, the enhanced secreted NS1 derived from these mutant constructs was dimeric in nature, demonstrating that hexamerization is not a strict requirement for secretion, at least in a recombinant insect cell system. This result reiterates the critical nature of this exposed loop, which although conserved, can adopt different conformations as was observed between the two crystal forms of WNV NS1. These subtle differences in orientation may underlie the lipid loading of the NS1 hexamer, where the exposed hydrophobic residues potentiate incorporation of the lipidic cargo and repositioning of the loop facilitates the dimer to hexamer transitions.

Missing from the initial crystal structures was the exposed loop between the β5 and β6 strands within the wing domain, which was unresolved in both DENV and WNV structures. This loop is a major antigenic site and is well conserved between the mammalian and avian flaviviruses. Recent work has suggested this region may play an important role in mediating NS1 interaction with the envelope protein, thus tethering the replication complex with virion formation [92]. Interestingly, this loop was resolved in subse-

quent reports of the ZIKV NS1 structure [11, 126]. Here, two independent groups reported the crystallization of dimeric NS1 and although the secondary structure of the loop differs between the two structures, the overall orientation is similar and reveals that this region wraps almost underneath the wing domain (Fig. 7.1a, lower panels). This orientation has two potential implications for NS1 biology. First it suggests that this region may provide additional membrane contacts for mNS1. Second, fitting of the ZIKV dimer structure to the hexameric DENV structure puts this region at the inter-dimer interface, where it may interact with the lipidic cargo and potentially stabilize the hexamer form.

The closed hexameric crystallographic form of DENV and WNV NS1 appears to match well with the reported cryo-EM reconstruction of native NS1, suggesting it is an accurate representation of the quaternary organization within the mature hexameric lipo-particle secreted from infected cells. Although the ZIKV NS1 was crystalized from a purified dimeric form, the authors also reported a hexameric form which failed to crystalize [11]. However, negative TEM analysis suggested that this hexamer adopted a similar open barrel form to that observed for DENV and WNV NS1. This is a surprising finding given the lack of lipid in the crystal structure form. Indeed, there is limited to no inter-dimer contact within the crystallographic hexamer and it is expected that the lipid cargo contributes substantially to the structural integrity of the hexamer form. Despite the very similar dimeric architecture between DENV and WNV NS1, close comparison of the hexameric structures reveals significant differences in the inter-dimer contacts. In fact, there are no resolved residues within bonding distance within the WNV hexamer (form 2 or closed) and only one residue (K11) within the resolved DENV hexameric structure that is within bond forming distance. However, the two residues at the tip of the DENV extended loop (F162 and G163) were not resolved and may contribute to inter-dimer contact, potentially through pi-pi interactions with the DENV specific W9 and F162. Although remarkable, these findings sit well with a previous study from Youn et al. [130] that suggested a role for an N-terminal motif in NS1 secretion [128]. Here the researchers demonstrated that the exchange of residues between WNV and DENV at this site (RQ10NK) resulted in an enhanced secretion within a WNV infectious clone, reminiscent of the native DENV phenotype. As discussed above, the newly resolved wing loop structure may provide hexamer stabilizing inter-dimer contacts and account for the lack of interactions observed in the WNV and DENV hexamers. However, the fact that this loop has only been resolved in the dimeric form does not accord with such a role. In addition, the loop is placed at the asymmetric unit interface in both structures and may be stabilized in a non-native form due to crystal packing. Thus, further structural and functional work is required to reveal the role this loop plays in the native, lipid-filled sNS1 hexamer.

7.1.4 sNS1 Role in Pathology

Perhaps the most notable aspect of NS1 is the high level of secreted protein found circulating in the blood of flavivirus infected individuals. It appears that the levels of secreted NS1 differs significantly between flaviviruses, although there is limited reliable *in vivo* data to allow accurate sNS1 quantification outside of DENV infection. The levels of sNS1 in DENV are substantial, with levels in excess of 10 μg/ml reported [4]. The high level of NS1 in the bloodstream, which is present early during infection, has made NS1 a primary biomarker in disease diagnosis. The timing and level of sNS1 within DENV has also lead many researchers to propose a direct role for sNS1 in disease pathology.

Until recently, most evidence pointed towards a secondary role for NS1 in pathology, mediated through auto-immunity and complement interactions. However, in two recent and complementary studies from our laboratory and that of Eva Harris, NS1 treatment alone was demonstrated to elicit the pathological hallmarks of dengue disease, endothelial leak and inflammatory cytokine activation [7, 69]. Using an animal model of disease, the work of Beatty et al. observed increased disease symptoms with the co-administration of

recombinant NS1 together with virus. Going further, they revealed that the intravenous delivery of NS1 to naive animals produced observable morbidity, and was accompanied by cytokine induction and endothelial barrier breakdown. Maximal effects were recorded 3 days post administration mirroring the lag in disease severity and viral replication kinetics that is observed in humans.

Complementing these findings, work in our laboratory revealed the molecular mechanisms underpinning the activity of sNS1. Using knock out cell lines and reconstituted *in vitro* receptor systems we established that sNS1 is recognized by the pathogen-associated molecular pattern (PAMP) receptor TLR4. TLR4 is required for both monocyte activation (Fig. 7.2, panel 1) and direct NS1-endothelial interactions (Fig. 7.2, panel 2). This activity mimics that of the endotoxin LPS and when examined from this perspective there are striking similarities between the manifestations of severe dengue disease and those of septic shock. Unlike sepsis however, the aforementioned lag time between disease onset and severe symptom development provides a window of opportunity for the application of NS1/TLR4 targeted therapeutics.

Interestingly, an alternative mechanistic hypothesis for NS1 activity was put forward in a

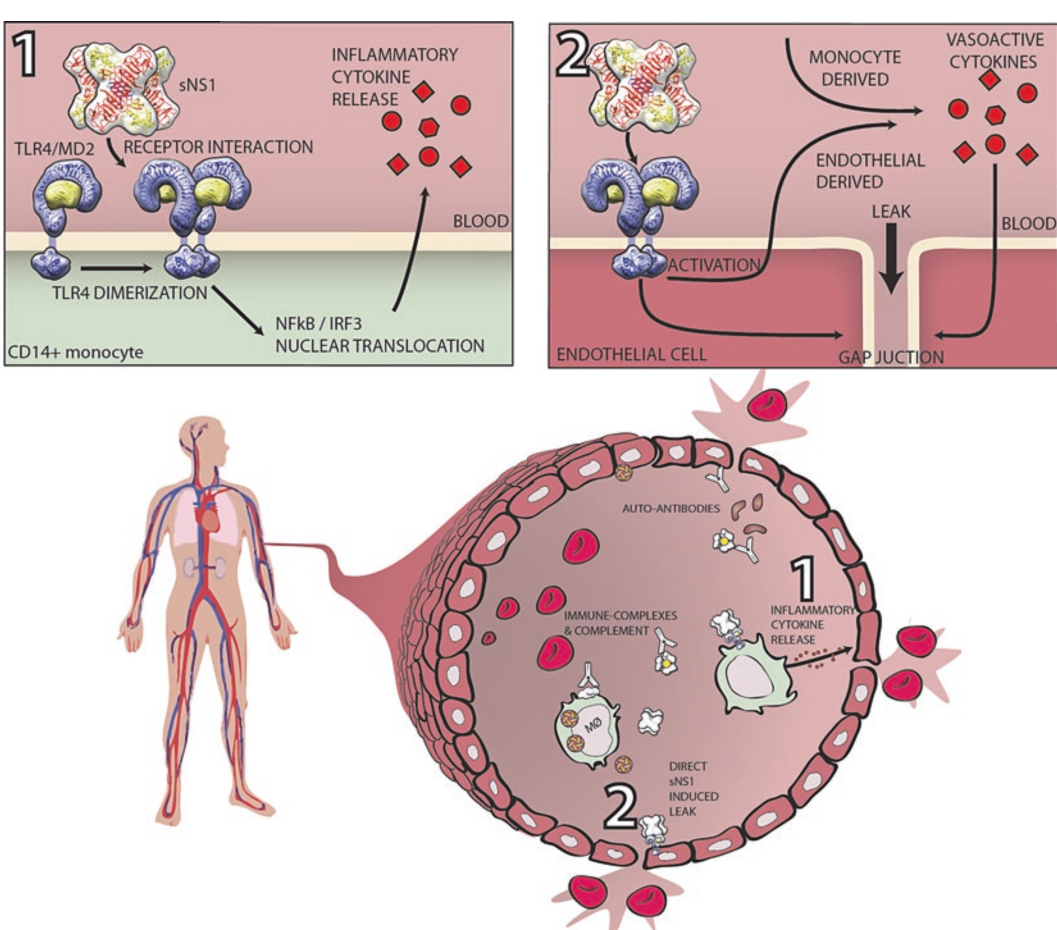


Fig. 7.2 The many functions for extracellular sNS1. (*1*) sNS1 interacts with the cell surface receptor TLR4 on the surface of CD14+ monocytes, inducing cellular activation and the release of inflammatory cytokines. (*2*) sNS1 activates endothelial cells directly and causes loss of endothelial barrier integrity through a TLR4 dependent pathway. Inflammatory cytokines released from sNS1 stimulated monocytes can also lead to endothelial damage and leak

paper from Chen et al. [14]. In this work, the authors proposed that NS1 triggers an inflammatory response through a TLR2 and TLR6 dependent pathway. In an effort to resolve the disparities with our findings we investigated both possibilities through a direct comparison of the activity of recombinant hexameric NS1 derived from insect cells (as used in our previous work) and the *Escherichia coli*-derived recombinant NS1 preparation used by Chen et al. Using mouse gene knockout macrophages and antibodies blocking TLR function in human peripheral blood mononuclear cells, we demonstrated that eukaryotically derived NS1 induces cytokine production via TLR4 but not TLR2/6, while the prokaryotically derived recombinant NS1 appeared highly contaminated by several microbial TLR ligands and activated cells through multiple pathways [70]. Furthermore, we showed that the prokaryotically expressed NS1 was monomeric, and therefore present in a non-native conformation. This finding was not unexpected, as many studies of recombinant NS1 expression in *E. coli* systems report a misfolded NS1 product that is retained in inclusion bodies and extensive refolding is required to obtain native-like protein. To date, only the C terminal domain has been conclusively validated to adopt a native-like conformation after refolding [30, 105]. Notably, Chen et al. also showed newborn Tlr6−/− mice had improved survival upon infection with high doses of DENV compared to wild type mice, thus TLR6 is a contributor in dengue pathology in this model. However, no direct association between TLR6 and NS1 can be made and the authors observations regarding TLR6 dependent toxicity of NS1 injected into newborn mice is rendered moot considering our findings regarding the presence of several inflammatory microbial contaminants in the NS1 preparations used.

In our original publication, we provided a range of different approaches aimed to exclude LPS as the source of TLR4-stimulating activity in our NS1 preparation [69]. However, the exact nature of the NS1-TLR4 interaction remains an open question. It is tempting to speculate that the lipidic cargo carried within the barrel-like sNS1 hexamer plays a direct role in receptor engagement and downstream signaling. Indeed, TLR4 activation by a variety of lipidic agonists has now been established and is proposed to play a role in the activation of TLR4 in influenza pathogenesis [44, 100]. It is also conceivable that trace amounts of LPS within the bloodstream could be bound and concentrated on NS1, with NS1 becoming a delivery agent for LPS to the TLR4 receptor complex. Indeed, it is worth noting that circulating LPS levels become further elevated in DENV infection, possibly via gut leakiness, and may play a role in pathology [114]. However, our observations of NS1 activity *in vitro*, including increased induction of human compared with mouse cytokine mRNAs relative to LPS, and the greater effectiveness of NS1 than LPS in the disruption of endothelial monolayers, support an LPS-independent mechanism. Importantly, TLR4 antagonists are sufficient to completely abrogate NS1 function in both cellular activation and endothelial permeability assays *in vitro*, and the TLR4 antagonist LPS-RS prevented vascular leak in DENV-infected mice [69, 70]. Thus, irrespective of a direct role for LPS, the potential for therapeutic reduction of pathology with TLR4 antagonists remains a promising avenue of research.

7.1.5 sNS1 and Endothelial Barrier Function

The recognition of NS1 as a direct mediator of endothelial permeability provides a new paradigm to view DENV induced vascular leak. Previously, a number of *in vitro* studies have reported direct viral infection of endothelial cells, leading to apoptosis [27, 56, 65, 115]. However, these *in vitro* findings did not correlate with *in vivo* observations. For example, although several histopathological studies have observed viral particles within the endothelial cells of multiple organs [6, 20, 50, 75, 83, 91], no evidence of active viral replication has been observed. These findings are therefore most likely due to endothelial cell mediated antigen uptake, rather than productive infection of endothelial cells *in vivo* [50]. Furthermore, autopsy reports of fatal DENV infections have indicated increasing capillary permeability with

no distinct morphological damage to capillary endothelium [5, 8, 86]. With no substantial evidence of direct infection or cell death of endothelial cells *in vivo*, researchers turned their attention to DENV modulation of capillary permeability through alternative pathways; inter-endothelial junction and glycocalyx layer.

7.1.6 NS1 and the Glycocalyx

Under normal conditions, the vasculature system is tightly regulated by two semi-permeable barriers consisting of endothelial cells and their associated surface glycocalyx layer. The endothelial layer is highly associated with inter-endothelial junctions, with a complex network of cellular connections mediated by a set of junctional proteins that form the adherens, tight, and gap junctions. Early DENV studies proposed that vasoactive mediators are responsible for increasing vascular permeability, a logical consequence of the elevated levels of pro-inflammatory cytokines/chemokines consistently observed in severe dengue patients [43, 54]. Concordantly, *in vitro* analysis of endothelial layer function demonstrated increasing permeability as well as loss of tight junction proteins in response to several of these pro-inflammatory mediators, in particular TNF-α [127, 129]. Prior to the recognition of NS1 activity, these factors were proposed to be a product of viral infection of monocyte/macrophages and endothelial cells [13], and T cell activation [71]. However, although some cytokines are observed at high levels in severe patients (DHF/DSS) when compared to dengue fever (DF), the levels do not correlate with disease kinetics [41]. In addition to cytokines, platelet activating factor (PAF), has recently been found to be associated with disease severity, and a possible mechanism proposed through the loss of junction protein, ZO-1 [49]. Nevertheless, these factors alone are insufficient to account for DENV specific vascular leak phenomenon, as they are also elevated in other viral infections [64]. The discovery of a direct role for NS1 in endothelial barrier dysfunction by ourselves and others therefore provided a solution to this missing piece of the DENV puzzle and revealed a new avenue for DENV research [7, 15, 69].

In a follow-up study, Puerta-Guardo et al. probed the effects of DENV NS1 on the endothelial glycocalyx. Interestingly, they noted a dose-dependent NS1-induced loss of sialic acid from endothelial cell surface. In addition, exposure to NS1 mediated shedding of heparin sulphate, presumably though activation of cathepsin L. These effects were shown to be mediated by NS1-induced expression of sialidase and heparanase [85]. These effects are in agreement with a TLR4 dependent pathway, as bacterial LPS has been reported to cause glycocalyx degradation from lung endothelium by activation of heparanase in both a murine model of sepsis and acute respiratory syndrome [98, 120]. Given these findings it will be of interest to determine whether the effects are at play within the vasculature system of infected humans. However, detection of structural changes within the glycocalyx layer are technically challenging and may prevent detailed *in vivo* characterization.

Of note, inhibitors of neuraminidases and glycocalyx remodelling protein were shown to completely abolish NS1-induced endothelial monolayer permeability [69, 85]. While these discoveries open other avenues for therapeutic intervention, it is not yet clear that these pathways represent an independent mechanism or are downstream effects of the NS1-TLR4 interaction. Interestingly, the cleaved product of heparin sulphate, a main component of glygocalyx, has been previously reported to trigger the release of pro-inflammatory cytokines via a TLR4-dependent mechanism [38]. Further investigation into these intriguing phenomena at the molecular level will no doubt provide deeper understanding of disease pathogenesis.

7.1.7 NS1 as a Vaccine Target

The work of Beatty et al. illustrated the potential for rational vaccine design, using NS1 anti-serum and monoclonal antibodies to inhibit NS1 function *in vitro* and *in vivo*. Although there is precedent within the literature for NS1 use in vaccine

design [17, 97], these findings, when combined with the recent NS1 structural data, provide the framework for novel vaccine approaches which could potentially block NS1 function without the off-target effects observed in the case of NS1 auto-antibodies or the complications arising from virion specific antibody dependent enhancement (ADE) of infection. This potential is of particular relevance given the challenge of developing an effective DENV vaccine.

The first DENV vaccine (Dengvaxia, Sanofi Pasteur) was licensed in December 2015 in Mexico. It has since been licensed in several other countries, but in two human trials showed only 60% efficacy [41]. Furthermore, during clinical trials it became apparent that prior exposure to dengue appeared to be necessary in order to prime an effective vaccine immune response. Consequently, use of this vaccine is restricted to individuals older than 9 years and to endemic countries where there is existing immunity. Relevant for future trials, the Dengvaxia clinical trials also showed that neutralizing antibody levels did not provide a correlate for protection. These disappointing findings now prompt a rethink of current vaccine strategies. Recognizing the driving role of NS1 in disease pathology, vaccines with a focus on NS1 responses are among a range of new approaches now under development.

Although the mechanism underlying NS1 mediated pathology has only recently been revealed, anti-NS1 mediated immunity was documented over three decades earlier. These seminal studies demonstrated that the passive transfer of anti-NS1 monoclonal antibodies [38, 40, 93, 94] or active NS1 vaccination resulted in solid protection from lethal virus challenge [34, 94]. These early experiments identified a correlation between complement consumption and protection [34, 93–95]. This suggested a mechanism of protection via complement-mediated lysis of cells displaying NS1 on the surface. Since these initial experiments it has been found that anti-NS1 antibodies can protect independent of complement through phagocytosis and subsequent clearance of infected cells [17, 18, 28]. Given the potential for NS1 based vaccine strategies to avoid the potential risks of antibody dependent enhancement, they have been delivered as subunit [7, 12, 37, 42, 55, 95, 118], live recombinant vaccinia virus [34], defective recombinant adenovirus [46, 47, 111], naked DNA [19, 57, 112, 125] and peptide formulations [116]. While these approaches have been successful at generating anti-NS1 antibody mediated protection in multiple model systems (mouse and monkey) and for many of the flaviviruses (DENV, WNV, ZIKV, TBEV, YFV, JEV) it has also been broadly observed that some anti-NS1 antibodies can actually enhance disease.

Antibodies targeting NS1 have also been identified as binding to a range of cellular components such as extracellular matrix, blood clotting and integrin/adhesion proteins, platelets as well as to ATP synthase B chain, protein disulphide isomerise, vimentin and heat shock protein on endothelial cells [16, 31, 32, 45, 58–61, 107]. While these antibodies have been proposed to contribute to severe disease there has been little comment on disease recovery during convalescence in the presence of the on-going circulation of these proposed damaging auto-antibodies [72]. Amino acid residues 311–330 located within the β ladder of NS1 have been identified as sharing a sequence motif with a number of cellular components (ATP synthase B chain, PDI, vimentin and Heat shock protein 60) [16]. Given the problematic self-reactive antibodies recognize the C-terminal half of NS1, an approach using a truncated NS1 recombinant lacking amino acids 271–352 has been trialed and was shown to produce antibodies capable of recognizing full length NS1 but without a host-reactive antibody response [118]. This study found that passive transfer of antibodies abolished dengue virus-induced macrophage skin infiltration and localised skin haemorrhage, in mice challenged intradermally. While these results are promising, the protective efficacy of truncated NS1 remains to be tested in a true infectious challenge model.

It is likely that NS1 vaccines will find eventual application in a multivalent approach that includes

the structural proteins of the virion. With the recent recognition of the highly neutralizing E dimer epitope (EDE) specific antibodies [26], researchers are now developing epitope focused E vaccines that promote EDE responses and negate non-neutralizing, ADE enhancing responses [88]. Likewise, with the newly available NS1 atomic structure and insights into NS1 driven pathology, researchers can now apply a rational approach to next generation NS1 vaccine design.

7.1.8 NS1-TLR4 Targeted Therapeutics

Beyond vaccination, mechanistic understanding of NS1 function provides a promising therapeutic strategy. Our work validated this approach using both TLR4 blocking antibodies and antagonists to inhibit NS1 activity within *in vitro* experimental models of monocyte activation and endothelial leak. Encouragingly, we were able to translate these findings to an *in vivo* model, where a reduction in the levels of leak in dengue infected mice was observed following treatment with LPS-RS, a naturally occurring antagonist of TLR4 signaling. Due to the pressing need for anti-sepsis strategies, there exists a number of potential treatments that could be re-purposed for the dengue setting. These include compounds that have entered or completed phase III trials such as the Lipid A analogue E5564 and TAK-242, which block TLR4 signalling [99, 100]. Other candidates that have progressed to phase II trials include another LPS analogue, E5531 [52], and AV411, a phosphodiesterase inhibitor [36]. Beyond these established examples there are also a range of inhibitors from various classes in preclinical trials which may prove useful in dengue treatment [1, 25, 29, 53, 68, 78–80, 106, 119]. Successful translation of these will require a robust development pathway, using *in vitro* assessment of potential NS1 activity to facilitate QSAR before moving to animal models of disease and eventually human trials.

In contrast, many of the established TLR4 antagonists have the potential to move directly to clinical trials. However, the previous trials of celgosovir and the anti-inflammatory corticosteroid prednisolone provide a cautionary tale for DENV antiviral development [66, 108, 109]. Ideal markers of disease severity, in particular capillary leak, are not readily available for clinical assessment. It will also be of great interest weather TLR4 antagonists can reduce overall disease symptoms, or are best suited to preventing severe DENV. Preclinical testing of such compounds in appropriate mouse models will be essential to guide human application.

7.2 Conclusions

The recent structural details revealed by crystallographic and electron microscopy approaches have been complemented by the discovery of a key role that NS1 plays in disease development. Acting as a systemic toxin, secreted NS1 exerts its pathological activity through interaction with the innate immune receptor TLR4. This discovery provides a mechanistic paradigm to complement the many decades of work within the field, which consistently hinted at a potential driving role for NS1 in DENV pathogenesis. Importantly, the finding that TLR4 antagonists block leak *in vivo* offers a promising new avenue for antiviral therapies. The next steps will be to explore the possibility of repurposing clinically tested TLR4 inhibitors for DENV treatment. In parallel, recognition and mechanistic understanding of NS1 induced pathology also offers new solutions for DENV vaccine development. However, despite the clear translational opportunities, NS1 mediated pathology still remains poorly understood within the context of the viral lifecycle. Future work to clarify the role of the newly recognized systemic NS1 functions within the broader framework of virus replication and transmission lies ahead. The enigma that is NS1 remains, for now.

Discussion of Chapter 7 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Paul Young.

Subhash Vasudevan: Which Virus strains did you use for the mouse challenge experiment?

Paul Young: We have done two principle studies, multiple times. S221 which is the Shresta mouse-adapted strain in AG129. And also an East Timor clinical strain, not mouse-adapted for DENV1.

George Gao: Have you compared the dimer versus the hexamer in your experiment?

Paul Young: You can't use the dimer form in tissue culture experiments. The dimer in a non-detergent enviroment would aggregate. That was one of the biggest issues in terms of trying to crystallize it. It was crystallized in the end in the presence of detergent, so you can break down the hexamer into a dimer, but then it aggregates again when you put it into a normal detergent-free, *in vivo* enviroment.

George Gao: So then the question is that both Janet Smith and my group have published NS1 dimer structures. We think it is a dimer.

Paul Young: Yes, you got the Zika NS1 dimer but I would assume there is detergent in the crystallization buffer.

George Gao: But can you purify it?

Paul Young: If you purify it in the context of detergent you get the dimer form. We routinely purify it as a hexamer in the absence of detergent. So what I am saying is: you can purify it in its dimer form, and can maintain it as a dimer when there is detergent present. But if you put it into an *in vivo* system, if you take away the detergent it aggregates because of the exposed hydrophobic domains.

Norbert Heinrich: This may be a naive question, but seeing as you showed that NS1 triggers IL6 release from PBMC, obviously the next step would be CRP (C-reactive protein) production in the liver. Do you know whether that is clinically relevant following the conclusion that we can not use CRP to discriminate viral from bacterial infections in Dengue?

Paul Young: That could be true. Hepatomegaly is a significant clinical feature for Dengue patients. So we have seen that is happening and CRP is shown to be elevated in Dengue patients.

Norbert Heinrich: And likewise would you think the same is true for the other Flaviviruses and their NS1?

Paul Young: I never try to extrapolate from one flavivirus to another Aravinda. Hepatomegaly is a feature of other flaviviruses and maybe it is for yellow fever?

Aravinda de Silva: Yes. I do not know.

Paul Young: Yes I do not know either. It is possible.

Aravinda de Silva: So how do you explain the reduced viremia which is a very surprising result?

Paul Young: We were also surprised. What we do know – there has been some literature, and we have confirmed it ourselves – is that if you add NS1 to macrophages that have been infected you will increase viral infection. We think that it is simply because the activated profile of the macrophages are a much better enviroment for the virus to grow. So you can imagine that knocking down that effect might have some impact, but we didn't expect to knock it back completely. I can imagine that some drop in viral load, but not completely. We are currently trying to investigate the mechanism.

Subhash Vasudevan: And you see this 48 hours post-infection which is spectacular.

Paul Young: Yes and also the fact that treatment at day zero would be exactly the wrong thing to be doing.

Shi Yi: Do you have any direct binding data for toll-like receptor 4 and NS1?

Paul Young: We have co-precipitation data. What we are obviously trying to do is to get a co-crystal structure. That is going to be a huge challenge, but we are doing the co-precipitation studies and have shown that it will co-precipitate when you combine them.

References

1. Akashi S, Ogata H, Kirikae F, Kirikae T, Kawasaki K, Nishijima M, Shimazu R, Nagai Y, Fukudome K, Kimoto M, Miyake K (2000) Regulatory roles for CD14 and phosphatidylinositol in the signaling via toll-like receptor 4-MD-2. Biochem Biophys Res Commun 268(1):172–177. https://doi.org/10.1006/bbrc.2000.2089
2. Akey DL, Brown WC, Dutta S, Konwerski J, Jose J, Jurkiw TJ, DelProposto J, Ogata CM, Skiniotis G, Kuhn RJ, Smith JL (2014) Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system. Science 343(6173):881–885. https://doi.org/10.1126/science.1247749
3. Akey DL, Brown WC, Jose J, Kuhn RJ, Smith JL (2015) Structure-guided insights on the role of NS1 in flavivirus infection. BioEssays 37(5):489–494. https://doi.org/10.1002/bies.201400182
4. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M (2002) Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 40(2):376–381
5. Aye KS, Charngkaew K, Win N, Wai KZ, Moe K, Punyadee N, Thiemmeca S, Suttitheptumrong A, Sukpanichnant S, Prida M, Halstead SB (2014) Pathologic highlights of dengue hemorrhagic fever in 13 autopsy cases from Myanmar. Hum Pathol 45(6):1221–1233. https://doi.org/10.1016/j.humpath.2014.01.022
6. Balsitis SJ, Coloma J, Castro G, Alava A, Flores D, McKerrow JH, Beatty PR, Harris E (2009) Tropism of dengue virus in mice and humans defined by viral nonstructural protein 3-specific immunostaining. Am J Trop Med Hyg 80(3):416–424
7. Beatty PR, Puerta-Guardo H, Killingbeck SS, Glasner DR, Hopkins K, Harris E (2015) Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. Sci Transl Med 7(304):304ra141. https://doi.org/10.1126/scitranslmed.aaa3787
8. Bhamarapravati N, Tuchinda P, Boonyapaknavik V (1967) Pathology of Thailand haemorrhagic fever: a study of 100 autopsy cases. Ann Trop Med Parasitol 61(4):500–510
9. Bokisch VA, Top FH Jr, Russell PK, Dixon FJ, Muller-Eberhard HJ (1973) The potential pathogenic role of complement in dengue hemorrhagic shock syndrome. N Engl J Med 289(19):996–1000. https://doi.org/10.1056/nejm197311082891902
10. Brandt WE, Chiewslip D, Harris DL, Russell PK (1970) Partial purification and characterization of a dengue virus soluble complement-fixing antigen. J Immunol 105(6):1565–1568
11. Brown WC, Akey DL, Konwerski JR, Tarrasch JT, Skiniotis G, Kuhn RJ, Smith JL (2016) Extended surface for membrane association in Zika virus NS1 structure. Nat Struct Mol Biol 23(9):865–867. https://doi.org/10.1038/nsmb.3268. http://www.nature.com/nsmb/journal/v23/n9/abs/nsmb.3268.html#supplementary-information
12. Calvert AE, Huang CY, Kinney RM, Roehrig JT (2006) Non-structural proteins of dengue 2 virus offer limited protection to interferon-deficient mice after dengue 2 virus challenge. J Gen Virol 87(Pt 2):339–346
13. Carr JM, Hocking H, Bunting K, Wright PJ, Davidson A, Gamble J, Burrell CJ, Li P (2003) Supernatants from dengue virus type-2 infected macrophages induce permeability changes in endothelial cell monolayers. J Med Virol 69(4):521–528. https://doi.org/10.1002/jmv.10340
14. Chen J, Ng MM, Chu JJ (2015) Activation of TLR2 and TLR6 by dengue NS1 protein and its implications in the Immunopathogenesis of dengue virus infection. PLoS Pathog 11(7):e1005053. https://doi.org/10.1371/journal.ppat.1005053
15. Chen HR, Chuang YC, Lin YS, Liu HS, Liu CC, Perng GC, Yeh TM (2016) Dengue virus nonstructural protein 1 induces vascular leakage through macrophage migration inhibitory factor and autophagy. Plos Negl Trop Dis 10(7) ARTN e0004828 151371/journal.pntd.0004828
16. Cheng HJ, Lin CF, Lei HY, Liu HS, Yeh TM, Luo YH, Lin YS (2009) Proteomic analysis of endothelial cell autoantigens recognized by anti-dengue virus nonstructural protein 1 antibodies. Exp Biol Med 234(1):63–73
17. Chung KM, Nybakken GE, Thompson BS, Engle MJ, Marri A, Fremont DH, Diamond MS (2006) Antibodies against West Nile virus nonstructural protein NS1 prevent lethal infection through fc gamma receptor-dependent and -independent mechanisms. J Virol 80(3):1340–1351. https://doi.org/10.1128/jvi.80.3.1340-1351.2006
18. Chung KM, Thompson BS, Fremont DH, Diamond MS (2007) Antibody recognition of cell surface-associated NS1 triggers Fc-gamma receptor-mediated phagocytosis and clearance of West Nile virus-infected cells. J Virol 81(17):9551–9555. doi:JVI.00879-07 [pii] 10.1128/JVI.00879-07
19. Costa SM, Azevedo AS, Paes MV, Sarges FS, Freire MS, Alves AM (2007) DNA vaccines against dengue virus based on the ns1 gene: the influence of different signal sequences on the protein expression and its correlation to the immune response elicited in mice. Virology 358(2):413–423
20. Couvelard A, Marianneau P, Bedel C, Drouet MT, Vachon F, Henin D, Deubel V (1999) Report of a fatal case of dengue infection with hepatitis: demonstration of dengue antigens in hepatocytes and liver apoptosis. Hum Pathol 30(9):1106–1110
21. Crabtree MB, Kinney RM, Miller BR (2005) Deglycosylation of the NS1 protein of dengue 2 virus, strain 16681: construction and characterization of mutant viruses. Arch Virol 150(4):771–786. https://doi.org/10.1007/s00705-004-0430-8

22. Crooks AJ, Lee JM, Dowsett AB, Stephenson JR (1990) Purification and analysis of infectious virions and native non-structural antigens from cells infected with tick-borne encephalitis virus. J Chromatogr 502(1):59–68
23. Crooks AJ, Lee JM, Easterbrook LM, Timofeev AV, Stephenson JR (1994) The NS1 protein of tick-borne encephalitis virus forms multimeric species upon secretion from the host cell. J Gen Virol 75(Pt 12):3453–3460. https://doi.org/10.1099/0022-1317-75-12-3453
24. Dalgarno L, Trent DW, Strauss JH, Rice CM (1986) Partial nucleotide sequence of the Murray Valley encephalitis virus genome. Comparison of the encoded polypeptides with yellow fever virus structural and non-structural proteins. J Mol Biol 187(3):309–323. doi: 0022-2836(86)90435-3 [pii]
25. Daubeuf B, Mathison J, Spiller S, Hugues S, Herren S, Ferlin W, Kosco-Vilbois M, Wagner H, Kirschning CJ, Ulevitch R, Elson G (2007) TLR4/MD-2 monoclonal antibody therapy affords protection in experimental models of septic shock. J Immunol 179(9):6107–6114
26. Dejnirattisai W, Wongwiwat W, Supasa S, Zhang X, Dai X, Rouvinski A, Jumnainsong A, Edwards C, Quyen NTH, Duangchinda T, Grimes JM, Tsai WY, Lai CY, Wang WK, Malasit P, Farrar J, Simmons CP, Zhou ZH, Rey FA, Mongkolsapaya J, Screaton GR (2015) A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. Nat Immunol 16(2):170–177. https://doi.org/10.1038/ni.3058
27. Dhawan R, Khanna M, Chaturvedi UC, Mathur A (1990) Effect of dengue virus-induced cytotoxin on capillary permeability. J Exp Pathol (Oxford) 71(1):83–88
28. Diamond MS, Pierson TC, Fremont DH (2008) The structural immunology of antibody protection against West Nile virus. Immunol Rev 225:212–225
29. Dunn-Siegrist I, Leger O, Daubeuf B, Poitevin Y, Depis F, Herren S, Kosco-Vilbois M, Dean Y, Pugin J, Elson G (2007) Pivotal involvement of Fcgamma receptor IIA in the neutralization of lipopolysaccharide signaling via a potent novel anti-TLR4 monoclonal antibody 15C1. J Biol Chem 282(48):34817–34827. https://doi.org/10.1074/jbc.M706440200
30. Edeling MA, Diamond MS, Fremont DH (2014) Structural basis of Flavivirus NS1 assembly and antibody recognition. Proc Natl Acad Sci U S A 111(11):4285–4290. https://doi.org/10.1073/pnas.1322036111
31. Falconar AK (1997) The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 142(5):897–916
32. Falconar AK (2007) Antibody responses are generated to immunodominant ELK/KLE-type motifs on the nonstructural-1 glycoprotein during live dengue virus infections in mice and humans: implications for diagnosis, pathogenesis, and vaccine design. Clin Vaccine Immunol 14(5):493–504
33. Falgout B, Chanock R, Lai CJ (1989) Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. J Virol 63(5):1852–1860
34. Falgout B, Bray M, Schlesinger JJ, Lai CJ (1990) Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. J Virol 64(9):4356–4363
35. Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V (1999) Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J Virol 73(7):6104–6110
36. Fort MM, Mozaffarian A, Stover AG, Correia Jda S, Johnson DA, Crane RT, Ulevitch RJ, Persing DH, Bielefeldt-Ohmann H, Probst P, Jeffery E, Fling SP, Hershberg RM (2005) A synthetic TLR4 antagonist has anti-inflammatory effects in two murine models of inflammatory bowel disease. J Immunol 174(10):6416–6423
37. Gibson CA, Schlesinger JJ, Barrett AD (1988) Prospects for a virus non-structural protein as a subunit vaccine. Vaccine 6(1):7–9
38. Goodall KJ, Poon IK, Phipps S, Hulett MD (2014) Soluble heparan sulfate fragments generated by heparanase trigger the release of pro-inflammatory cytokines through TLR-4. https://doi.org/10.1371/journal.pone.0109596. PMID: 25295599
39. Gould EA, Buckley A, Barrett AD, Cammack N (1986) Neutralizing (54K) and non-neutralizing (54K and 48K) monoclonal antibodies against structural and non-structural yellow fever virus proteins confer immunity in mice. J Gen Virol 67(Pt 3):591–595
40. Gutsche I, Coulibaly F, Voss JE, Salmon J, d'Alayer J, Ermonval M, Larquet E, Charneau P, Krey T, Megret F, Guittet E, Rey FA, Flamand M (2011) Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high-density lipoprotein. Proc Natl Acad Sci U S A 108(19):8003–8008. https://doi.org/10.1073/pnas.1017338108
41. Hadinegoro SR, Arredondo-Garcia JL, Capeding MR, Deseda C, Chotpitayasunondh T et al (2015) Efficacy and long-term safety of a dengue vaccine in regions of endemic disease. N Engl J Med 373:1195–1206. pmid:26214039
42. Henchal EA, Henchal LS, Schlesinger JJ (1988) Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol 69(Pt 8):2101–2107
43. Hernandez SID, Puerta-Guardo HN, Aguilar HF, Mateos SG, Martinez IL, Ortiz-Navarrete V, Ludert JE, del Angel RM (2016) Primary dengue virus infections induce differential cytokine production in Mexican patients. Memorias Do Instituto

Oswaldo Cruz 111(3):161–U79. https://doi.org/10.1590/0074-02760150359
44. Hertz T, Beatty PR, MacMillen Z, Killingbeck SS, Wang C, Harris E (2017) Antibody epitopes identified in critical regions of dengue virus nonstructural 1 protein in mouse vaccination and natural human infections. J Immunol 198(10):4025–4035. https://doi.org/10.4049/jimmunol.1700029
45. Hung NT, Lei HY, Lan NT, Lin YS, Huang KJ, Lien LB, Lin CF, Yeh TM, Ha DQ, Huong VTQ, Chen LC, Huang JH, My LT, Liu CC, Halstead SB (2004) Dengue hemorrhagic fever in infants: a study of clinical and cytokine profiles. J Infect Dis 189(2):221–232. https://doi.org/10.1086/380762
46. Imai Y, Kuba K, Neely GG, Yaghubian-Malhami R, Perkmann T, van Loo G, Ermolaeva M, Veldhuizen R, Leung YH, Wang H, Liu H, Sun Y, Pasparakis M, Kopf M, Mech C, Bavari S, Peiris JS, Slutsky AS, Akira S, Hultqvist M, Holmdahl R, Nicholls J, Jiang C, Binder CJ, Penninger JM (2008) Identification of oxidative stress and toll-like receptor 4 signaling as a key pathway of acute lung injury. Cell 133(2):235–249. https://doi.org/10.1016/j.cell.2008.02.043
47. Immenschuh S, Rahayu P, Bayat B, Saragih H, Rachman A, Santoso S (2013) Antibodies against dengue virus nonstructural protein-1 induce heme oxygenase-1 via a redox-dependent pathway in human endothelial cells. Free Radic Biol Med 54:85–92. https://doi.org/10.1016/j.freeradbiomed.2012.10.551
48. Jacobs SC, Stephenson JR, Wilkinson GW (1992) High-level expression of the tick-borne encephalitis virus NS1 protein by using an adenovirus-based vector: protection elicited in a murine model. J Virol 66(4):2086–2095
49. Jacobs SC, Stephenson JR, Wilkinson GW (1994) Protection elicited by a replication-defective adenovirus vector expressing the tick-borne encephalitis virus non-structural glycoprotein NS1. J Gen Virol 75(Pt 9):2399–2402
50. Jacobs MG, Robinson PJ, Bletchly C, Mackenzie JM, Young PR (2000) Dengue virus nonstructural protein 1 is expressed in a glycosyl-phosphatidylinositol-linked form that is capable of signal transduction. FASEB J 14(11):1603–1610
51. Jeewandara KC, Gomes L, Gutowska-Owsiak D, Waithe D, Paranavitane SA, Shyamali NA, Ogg GS, Malavige GN (2015) Platelet activating factor contributes to vascular leak in acute dengue infection. Allergy 70:138–138
52. Jessie K, Fong MY, Devi S, Lam SK, Wong KT (2004) Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis 189(8):1411–1418. https://doi.org/10.1086/383043
53. Khromykh AA, Sedlak PL, Guyatt KJ, Hall RA, Westaway EG (1999) Efficient trans-complementation of the flavivirus kunjin NS5 protein but not of the NS1 protein requires its coexpression with other components of the viral replicase. J Virol 73(12):10272–10280
54. Kobayashi S, Kawata T, Kimura A, Miyamoto K, Katayama K, Yamatsu I, Rossignol DP, Christ WJ, Kishi Y (1998) Suppression of murine endotoxin response by E5531, a novel synthetic lipid A antagonist. Antimicrob Agents Chemother 42(11):2824–2829
55. Kuronuma K, Mitsuzawa H, Takeda K, Nishitani C, Chan ED, Kuroki Y, Nakamura M, Voelker DR (2009) Anionic pulmonary surfactant phospholipids inhibit inflammatory responses from alveolar macrophages and U937 cells by binding the lipopolysaccharide-interacting proteins CD14 and MD-2. J Biol Chem 284(38):25488–25500. https://doi.org/10.1074/jbc.M109.040832
56. La Braga E, Moura P, Pinto LM, Ignacio SR, Oliveira MJC, Cordeiro MT, Kubelka CF (2001) Detection of circulant tumor necrosis factor-alpha, soluble tumor necrosis factor p75 and interferon-gamma in Brazilian patients with dengue fever and dengue hemorrhagic fever. Memorias Do Instituto Oswaldo Cruz 96(2):229–232
57. Lai YC, Chuang YC, Liu CC, Ho TS, Lin YS, Anderson R, Yeh TM (2017) Antibodies against modified NS1 wing domain peptide protect against dengue virus infection. Sci Rep 7(1):6975. https://doi.org/10.1038/s41598-017-07308-3
58. Limonta D, Capo V, Torres G, Perez AB, Guzman MG (2007) Apoptosis in tissues from fatal dengue shock syndrome. J Clin Virol 40(1):50–54. https://doi.org/10.1016/j.jcv.2007.04.024
59. Lin YL, Chen LK, Liao CL, Yeh CT, Ma SH, Chen JL, Huang YL, Chen SS, Chiang HY (1998) DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. J Virol 72(1):191–200
60. Lin CF, Lei HY, Liu CC, Liu HS, Yeh TM, Wang ST, Yang TI, Sheu FC, Kuo CF, Lin YS (2001) Generation of IgM anti-platelet autoantibody in dengue patients. J Med Virol 63(2):143–149
61. Lin CF, Lei HY, Shiau AL, Liu HS, Yeh TM, Chen SH, Liu CC, Chiu SC, Lin YS (2002) Endothelial cell apoptosis induced by antibodies against dengue virus nonstructural protein 1 via production of nitric oxide. J Immunol 169(2):657–664
62. Lin CF, Lei HY, Shiau AL, Liu CC, Liu HS, Yeh TM, Chen SH, Lin YS (2003) Antibodies from dengue patient sera cross-react with endothelial cells and induce damage. J Med Virol 69(1):82–90
63. Lin CF, Wan SW, Cheng HJ, Lei HY, Lin YS (2006) Autoimmune pathogenesis in dengue virus infection. Viral Immunol 19(2):127–132
64. Lindenbach BD, Rice CM (1997) Trans-complementation of yellow fever virus NS1 reveals a role in early RNA replication. J Virol 71(12):9608–9617
65. Lindenbach BD, Rice CM (1999) Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. J Virol 73(6):4611–4621
66. Liu Q, Zhou YH, Yang ZQ (2016) The cytokine storm of severe influenza and development of immu-

nomodulatory therapy. Cell Mol Immunol 13(1):3–10. https://doi.org/10.1038/cmi.2015.74
67. Long X, Li Y, Qi Y, Xu J, Wang Z, Zhang X, Zhang D, Zhang L, Huang J (2013) XAF1 contributes to dengue virus-induced apoptosis in vascular endothelial cells. FASEB J 27(3):1062–1073. https://doi.org/10.1096/fj.12-213967
68. Low JG, Sung C, Wijaya L, Wei Y, Rathore AP, Watanabe S, Tan BH, Toh L, Chua LT, Hou Y, Chow A, Howe S, Chan WK, Tan KH, Chung JS, Cherng BP, Lye DC, Tambayah PA, Ng LC, Connolly J, Hibberd ML, Leo YS, Cheung YB, Ooi EE, Vasudevan SG (2014) Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): a phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial. Lancet Infect Dis 14(8):706–715. https://doi.org/10.1016/s1473-3099(14)70730-3
69. Mackenzie JM, Jones MK, Young PR (1996) Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology 220(1):232–240. https://doi.org/10.1006/viro.1996.0307
70. Mizuno T, Kurotani T, Komatsu Y, Kawanokuchi J, Kato H, Mitsuma N, Suzumura A (2004) Neuroprotective role of phosphodiesterase inhibitor ibudilast on neuronal cell death induced by activated microglia. Neuropharmacology 46(3):404–411. https://doi.org/10.1016/j.neuropharm.2003.09.009
71. Modhiran N, Watterson D, Muller DA, Panetta AK, Sester DP, Liu L, Hume DA, Stacey KJ, Young PR (2015) Dengue virus NS1 protein activates cells via toll-like receptor 4 and disrupts endothelial cell monolayer integrity. Sci Transl Med 7(304):304ra142. https://doi.org/10.1126/scitranslmed.aaa3863
72. Modhiran N, Watterson D, Blumenthal A, Baxter AG, Young PR, Stacey KJ (2017) Dengue virus NS1 protein activates immune cells via TLR4 but not TLR2 or TLR6. Immunol Cell Biol 95(5):491–495. https://doi.org/10.1038/icb.2017.5
73. Mongkolsapaya J, Duangchinda T, Dejnirattisai W, Vasanawathana S, Avirutnan P, Jairungsri A, Khemnu N, Tangthawornchaikul N, Chotiyarnwong P, Sae-Jang K, Koch M, Jones Y, McMichael A, Xu X, Malasit P, Screaton G (2006) T cell responses in dengue hemorrhagic fever: are cross-reactive T cells suboptimal? J Immunol 176(6):3821–3829. doi:176/6/3821 [pii]
74. Muller DA, Young PR (2013) The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. Antivir Res 98(2):192–208. https://doi.org/10.1016/j.antiviral.2013.03.008
75. Muller DA, Landsberg MJ, Bletchly C, Rothnagel R, Waddington L, Hankamer B, Young PR (2012) Structure of the dengue virus glycoprotein non-structural protein 1 by electron microscopy and single-particle analysis. J Gen Virol 93(Pt 4):771–779. https://doi.org/10.1099/vir.0.039321-0
76. Muller-Eberhard HJ, Dixon FJ, Tuchinda P, Bukkavesa S, Suvatte V (1973) Pathogenetic mechanisms in dengue haemorrhagic fever: report of an international collaborative study. Bull World Health Organ 48(1):117–133
77. Neri M, Othman SM, Cantatore S, De Carlo D, Pomara C, Riezzo I, Turillazzi E, Fineschi V (2012) Sudden infant death in an 8-month-old baby with dengue virus infection: searching for virus in post-mortem tissues by immunohistochemistry and western blotting. Pediatr Infect Dis J 31(8):878–880. https://doi.org/10.1097/INF.0b013e31825c4a08
78. Noisakran S, Dechtawewat T, Rinkaewkan P, Puttikhunt C, Kanjanahaluethai A, Kasinrerk W, Sittisombut N, Malasit P (2007) Characterization of dengue virus NS1 stably expressed in 293T cell lines. J Virol Methods 142(1–2):67–80. https://doi.org/10.1016/j.jviromet.2007.01.008
79. Nowak T, Farber PM, Wengler G, Wengler G (1989) Analyses of the terminal sequences of West Nile virus structural proteins and of the in vitro translation of these proteins allow the proposal of a complete scheme of the proteolytic cleavages involved in their synthesis. Virology 169(2):365–376
80. Numata M, Chu HW, Dakhama A, Voelker DR (2010) Pulmonary surfactant phosphatidylglycerol inhibits respiratory syncytial virus-induced inflammation and infection. Proc Natl Acad Sci U S A 107(1):320–325. https://doi.org/10.1073/pnas.0909361107
81. Numata M, Kandasamy P, Nagashima Y, Posey J, Hartshorn K, Woodland D, Voelker DR (2012) Phosphatidylglycerol suppresses influenza a virus infection. Am J Respir Cell Mol Biol 46(4):479–487. https://doi.org/10.1165/rcmb.2011-0194OC
82. Numata M, Nagashima Y, Moore ML, Berry KZ, Chan M, Kandasamy P, Peebles RS Jr, Murphy RC, Voelker DR (2013) Phosphatidylglycerol provides short-term prophylaxis against respiratory syncytial virus infection. J Lipid Res 54(8):2133–2143. https://doi.org/10.1194/jlr.M037077
83. Pierson TC, Kielian M (2013) Flaviviruses: braking the entering. Curr Opin Virol 3(1):3–12. https://doi.org/10.1016/j.coviro.2012.12.001
84. Post PR, Carvalho R, Galler R (1991) Glycosylation and secretion of yellow fever virus nonstructural protein NS1. Virus Res 18(2–3):291–302
85. Povoa TF, Alves AM, Oliveira CA, Nuovo GJ, Chagas VL, Paes MV (2014) The pathology of severe dengue in multiple organs of human fatal cases: histopathology, ultrastructure and virus replication. PLoS One 9(4):e83386. https://doi.org/10.1371/journal.pone.0083386
86. Pryor MJ, Wright PJ (1994) Glycosylation mutants of dengue virus NS1 protein. J Gen Virol 75(Pt 5):1183–1187
87. Puerta-Guardo H, Glasner DR, Harris E (2016) Dengue virus NS1 disrupts the endothelial Glycocalyx, leading to Hyperpermeability. PLoS Pathog 12(7):e1005738. https://doi.org/10.1371/journal.ppat.1005738
88. Rathi KR, Arora MM, Sahai K, Tripathi S, Singh SP, Raman DK, Anand KB (2013) Autopsy findings

in fatal dengue haemorrhagic fever – 06 cases. Med J Armed Forces India 69(3):254–259. https://doi.org/10.1016/j.mjafi.2012.08.021
89. Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH (1985) Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science 229(4715):726–733
90. Rouvinski A, Dejnirattisai W, Guardado-Calvo P, Vaney M-C, Sharma A, Duquerroy S, Supasa P, Wongwiwat W, Haouz A, Barba-Spaeth G, Mongkolsapaya J, Rey FA, Screaton GR (2017) Covalently linked dengue virus envelope glycoprotein dimers reduce exposure of the immunodominant fusion loop epitope. Nature 8:15411. https://doi.org/10.1038/ncomms15411. https://www.nature.com/articles/ncomms15411#supplementary-information
91. Ruangjirachuporn W, Boonpucknavig S, Nimmanitya S (1979) Circulating immune complexes in serum from patients with dengue haemorrhagic fever. Clin Exp Immunol 36(1):46–53
92. Russell PK, Chiewsilp D, Brandt WE (1970) Immunoprecipitation analysis of soluble complement-fixing antigens of dengue viruses. J Immunol 105(4):838–845
93. Salgado DM, Eltit JM, Mansfield K, Panqueba C, Castro D, Vega MR, Xhaja K, Schmidt D, Martin KJ, Allen PD, Rodriguez JA, Dinsmore JH, Lopez JR, Bosch I (2010) Heart and skeletal muscle are targets of dengue virus infection. Pediatr Infect Dis J 29(3):238–242. https://doi.org/10.1097/INF.0b013e3181bc3c5b
94. Scaturro P, Cortese M, Chatel-Chaix L, Fischl W, Bartenschlager R (2015) Dengue virus non-structural protein 1 modulates infectious particle production via interaction with the structural proteins. PLoS Pathog 11(11):e1005277. https://doi.org/10.1371/journal.ppat.1005277
95. Schlesinger JJ, Brandriss MW, Walsh EE (1985) Protection against 17D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp48 and by active immunization with gp48. J Immunol 135(4):2805–2809
96. Schlesinger JJ, Brandriss MW, Cropp CB, Monath TP (1986) Protection against yellow fever in monkeys by immunization with yellow fever virus nonstructural protein NS1. J Virol 60(3):1153–1155
97. Schlesinger JJ, Brandriss MW, Walsh EE (1987) Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. J Gen Virol 68(Pt 3):853–857
98. Schlesinger JJ, Brandriss MW, Putnak JR, Walsh EE (1990) Cell surface expression of yellow fever virus non-structural glycoprotein NS1: consequences of interaction with antibody. J Gen Virol 71(Pt 3):593–599. https://doi.org/10.1099/0022-1317-71-3-593
99. Schlesinger JJ, Foltzer M, Chapman S (1993) The fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. Virology 192(1):132–141. https://doi.org/10.1006/viro.1993.1015
100. Schmidt EP, Yang Y, Janssen WJ, Gandjeva A, Perez MJ, Barthel L, Zemans RL, Bowman JC, Koyanagi DE, Yunt ZX, Smith LP, Cheng SS, Overdier KH, Thompson KR, Geraci MW, Douglas IS, Pearse DB, Tuder RM (2012) The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. Nat Med 18(8):1217–1223. https://doi.org/10.1038/nm.2843
101. Sha T, Sunamoto M, Kitazaki T, Sato J, Ii M, Iizawa Y (2007) Therapeutic effects of TAK-242, a novel selective toll-like receptor 4 signal transduction inhibitor, in mouse endotoxin shock model. Eur J Pharmacol 571(2–3):231–239. https://doi.org/10.1016/j.ejphar.2007.06.027
102. Shirey KA, Lai W, Scott AJ, Lipsky M, Mistry P, Pletneva LM, Karp CL, McAlees J, Gioannini TL, Weiss J, Chen WH, Ernst RK, Rossignol DP, Gusovsky F, Blanco JC, Vogel SN (2013) The TLR4 antagonist Eritoran protects mice from lethal influenza infection. Nature 497(7450):498–502. https://doi.org/10.1038/nature12118
103. Smith GW, Wright PJ (1985) Synthesis of proteins and glycoproteins in dengue type 2 virus-infected vero and Aedes albopictus cells. J Gen Virol 66(Pt 3):559–571
104. Smith TJ, Brandt WE, Swanson JL, McCown JM, Buescher EL (1970) Physical and biological properties of dengue-2 virus and associated antigens. J Virol 5(4):524–532
105. Sobel AT, Bokisch VA, Müller-Eberhard HJ (1975) C1q deviation test for the detection of immune complexes, aggregates of IgG, and bacterial products in human serum. J Exp Med 142(1):139–150. https://doi.org/10.1084/jem.142.1.139
106. Somnuke P, Hauhart RE, Atkinson JP, Diamond MS, Avirutnan P (2011) N-linked glycosylation of dengue virus NS1 protein modulates secretion, cell-surface expression, hexamer stability, and interactions with human complement. Virology 413(2):253–264. https://doi.org/10.1016/j.virol.2011.02.022
107. Song H, Qi J, Haywood J, Shi Y, Gao GF (2016) Zika virus NS1 structure reveals diversity of electrostatic surfaces among flaviviruses. Nat Struct Mol Biol 23(5):456–458. https://doi.org/10.1038/nsmb.3213. http://www.nature.com/nsmb/journal/v23/n5/abs/nsmb.3213.html#supplementary-information
108. Spiller S, Elson G, Ferstl R, Dreher S, Mueller T, Freudenberg M, Daubeuf B, Wagner H, Kirschning CJ (2008) TLR4-induced IFN-gamma production increases TLR2 sensitivity and drives gram-negative sepsis in mice. J Exp Med 205(8):1747–1754. https://doi.org/10.1084/jem.20071990
109. Sun DS, King CC, Huang HS, Shih YL, Lee CC, Tsai WJ, Yu CC, Chang HH (2007) Antiplatelet autoantibodies elicited by dengue virus non-structural protein 1 cause thrombocytopenia and mortality in mice. J Thromb Haemost 5(11):2291–2299

110. Sung C, Wei Y, Watanabe S, Lee HS, Khoo YM, Fan L, Rathore AP, Chan KW, Choy MM, Kamaraj US, Sessions OM, Aw P, de Sessions PF, Lee B, Connolly JE, Hibberd ML, Vijaykrishna D, Wijaya L, Ooi EE, Low JG, Vasudevan SG (2016) Extended evaluation of Virological, immunological and pharmacokinetic endpoints of CELADEN: a randomized, placebo-controlled trial of Celgosivir in dengue fever patients. PLoS Negl Trop Dis 10(8):e0004851. https://doi.org/10.1371/journal.pntd.0004851
111. Tam DT, Ngoc TV, Tien NT, Kieu NT, Thuy TT, Thanh LT, Tam CT, Truong NT, Dung NT, Qui PT, Hien TT, Farrar JJ, Simmons CP, Wolbers M, Wills BA (2012) Effects of short-course oral corticosteroid therapy in early dengue infection in Vietnamese patients: a randomized, placebo-controlled trial. Clin Infect Dis 55(9):1216–1224. https://doi.org/10.1093/cid/cis655
112. Theofilopoulos AN, Wilson CB, Dixon FJ (1976) The Raji cell radioimmune assay for detecting immune complexes in human sera. J Clin Investig 57(1):169–182
113. Timofeev AV, Ozherelkov SV, Pronin AV, Deeva AV, Karganova GG, Elbert LB, Stephenson JR (1998) Immunological basis for protection in a murine model of tick-borne encephalitis by a recombinant adenovirus carrying the gene encoding the NS1 non-structural protein. J Gen Virol 79(Pt 4):689–695
114. Timofeev AV, Butenko VM, Stephenson JR (2004) Genetic vaccination of mice with plasmids encoding the NS1 non-structural protein from tick-borne encephalitis virus and dengue 2 virus. Virus Genes 28(1):85–97
115. Trent DW, Kinney RM, Johnson BJ, Vorndam AV, Grant JA, Deubel V, Rice CM, Hahn C (1987) Partial nucleotide sequence of St. Louis encephalitis virus RNA: structural proteins, NS1, ns2a, and ns2b. Virology 156(2):293–304
116. van de Weg CA, Koraka P, van Gorp EC, Mairuhu AT, Supriatna M, Soemantri A, van de Vijver DA, Osterhaus AD, Martina BE (2012) Lipopolysaccharide levels are elevated in dengue virus infected patients and correlate with disease severity. J Clin Virol 53(1):38–42. https://doi.org/10.1016/j.jcv.2011.09.028
117. Vasquez Ochoa M, Garcia Cordero J, Gutierrez Castaneda B, Santos Argumedo L, Villegas Sepulveda N, Cedillo Barron L (2009) A clinical isolate of dengue virus and its proteins induce apoptosis in HMEC-1 cells: a possible implication in pathogenesis. Arch Virol 154(6):919–928. https://doi.org/10.1007/s00705-009-0396-7
118. Volpina OM, Volkova TD, Koroev DO, Ivanov VT, Ozherelkov SV, Khoretonenko MV, Vorovitch MF, Stephenson JR, Timofeev AV (2005) A synthetic peptide based on the NS1 non-structural protein of tick-borne encephalitis virus induces a protective immune response against fatal encephalitis in an experimental animal model. Virus Res 112(1–2):95–99
119. Wallis TP, Huang CY, Nimkar SB, Young PR, Gorman JJ (2004) Determination of the disulfide bond arrangement of dengue virus NS1 protein. J Biol Chem 279(20):20729–20741. https://doi.org/10.1074/jbc.M312907200
120. Wan SW, Lu YT, Huang CH, Lin CF, Anderson R, Liu HS, Yeh TM, Yen YT, Wu-Hsieh BA, Lin YS (2014) Protection against dengue virus infection in mice by administration of antibodies against modified nonstructural protein 1. PLoS One 9(3):e92495. https://doi.org/10.1371/journal.pone.0092495
121. Wang R, Stephens J, Lacy MJ (2003) Characterization of monoclonal antibody HTA125 with specificity for human TLR4. Hybrid Hybridomics 22(6):357–365. https://doi.org/10.1089/153685903771797057
122. Wang L, Huang X, Kong G, Xu H, Li J, Hao D, Wang T, Han S, Han C, Sun Y, Liu X, Wang X (2016) Ulinastatin attenuates pulmonary endothelial glycocalyx damage and inhibits endothelial heparanase activity in LPS-induced ARDS. Biochem Biophys Res Commun 478(2):669–675. https://doi.org/10.1016/j.bbrc.2016.08.005
123. Watterson D, Modhiran N, Young PR (2016) The many faces of the flavivirus NS1 protein offer a multitude of options for inhibitor design. Antivir Res 130: 7–18.
124. Westaway EG (1975) The proteins of Murray Valley encephalitis virus. J Gen Virol 27(3):293–292
125. Winkler G, Randolph VB, Cleaves GR, Ryan TE, Stollar V (1988) Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. Virology 162(1):187–196
126. Winkler G, Maxwell SE, Ruemmler C, Stollar V (1989) Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. Virology 171(1):302–305
127. Wu SF, Liao CL, Lin YL, Yeh CT, Chen LK, Huang YF, Chou HY, Huang JL, Shaio MF, Sytwu HK (2003) Evaluation of protective efficacy and immune mechanisms of using a non-structural protein NS1 in DNA vaccine against dengue 2 virus in mice. Vaccine 21(25–26):3919–3929
128. Xu X, Song H, Qi J, Liu Y, Wang H, Su C, Shi Y, Gao GF (2016) Contribution of intertwined loop to membrane association revealed by Zika virus full-length NS1 structure. Embo J 35(20):2170–2178. https://doi.org/10.15252/embj.201695290
129. Ye D, Ma I, Ma TY (2006) Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier. Am J Physiol Gastrointest Liver Physiol 290(3):G496–G504. https://doi.org/10.1152/ajpgi.00318.2005
130. Youn S, Cho H, Fremont DH, Diamond MS (2010) A short N-terminal peptide motif on flavivirus nonstructural protein NS1 modulates cellular targeting and immune recognition. J Virol 84(18):9516–9532. https://doi.org/10.1128/jvi.00775-10

Check for updates

8 Viral Entry and NS1 as Potential Antiviral Drug Targets

Aravinda M. de Silva, Félix A. Rey, Paul R. Young, Rolf Hilgenfeld, and Subhash G. Vasudevan

Abstract

A general discussion on viral entry and NS1 as potential drug targets was held at the Tofo Advanced Study Week (TASW) on Emerging Viral Diseases in September 2016. The opportunities and gaps for developing therapeutic countermeasures, to take advantage of the high-resolution cryo-electron microscopy structures of dengue and Zika viruses as well as the novel features of NS1 revealed by the 3D structures, were deliberated.

Keywords

Flavivirus entry mechanism · Therapeutic antibodies · Entry inhibitors · NS1 pathogenicity · NS1 delipidation

Subhash Vasudevan: To open up the general discussion on drug targets, perhaps a good place to start will be virus entry. The structure of the virus that Félix Rey presented and the assays and the functional tests for virus neutralization that Aravinda de Silva presented set the stage for this discussion. What are the opportunities with entry as a potential target for inhibitors?

Aravinda de Silva: The process of the actual entry-mechanism of flaviviruses is still quite mysterious. We have the textbook view that the virus binds to a yet unknown entry receptor. We know quite a bit about attachment factors but we still don't know what is the entry receptor. And then, magically, it gets to the

---

A general discussion on viral entry and NS1 as potential drug targets in *Dengue and Zika: Control and Antiviral Treatment Strategies held at Praia do Tofo, Mozambique.*
This discussion was transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Drs. Aravinda de Silva, Félix Rey, Paul Young, Rolf Hilgenfeld and Subhash G. Vasudevan. For further information please contact hilgenfeld@biochem.uni-luebeck.de or subhash.vasudevan@duke-nus.edu.sg.

A. M. de Silva
Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, USA
e-mail: desilva@med.unc.edu

F. A. Rey
Institut Pasteur, Structural Virology Unit, CNRS UMR3569, 25-28 rue du Dr. Roux, 75015 Paris, France
e-mail: felix.rey@pasteur.fr

P. R. Young
Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia
e-mail: p.young@uq.edu.au

R. Hilgenfeld (✉)
Institute of Biochemistry, University of Lübeck, Lübeck, Germany
e-mail: hilgenfeld@biochem.uni-luebeck.de

S. G. Vasudevan (✉)
Emerging Infectious Diseases Program, Duke–NUS Medical School Singapore, Singapore, Singapore
e-mail: subhash.vasudevan@duke-nus.edu.sg

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_8

endosome, where pH is low and the virus fuses with the host membrane. It is probably more complicated and people have started looking at other viruses in more detail. In the case of Ebola virus, there might actually be a fusion receptor in the endosome. One of the things, as Subhash said, is exploring the antibodies used for vaccine development assays as tools for probing entry. And so let us get to the more fundamental question: We know that for many viruses the actual level of virus-specific antibody is predicitve of protection, presumably, because antibodies coat the viral surface and block attachment. But as we know for dengue, overall levels of virus-binding antibodies are not predicitve of protection. The sub-population of antibodies that have been implicated in potent neutralization and durable protection appear to block a step after viral attachment, perhaps fusion. Although it makes sense that quaternary epitopes recognized by human antibodies can block the fusogenic process where E protein dimers rearrange into trimers, there is little direct experimental evidence to support this hypothesis. Targeting this quaternary switch would be a good entry target but I think there is a lot more to be done to get to this point, though I'm not an expert on viral entry; I think there are others here who have thought about it more. So this would be a good point to discuss. In any case, experiments where we let the virus attach to the surface of cells, and then add these quaternary antibodies demonstrate that these antibodies can block infection by targeting a step after attachment. What we observe is that there is equal effect whether you add the antibody before or after the virus binds to cells suggesting that the main mechanism is not through blocking the attachment to the surface. There is additional data in this respect from the work of Mike Diamond and colleagues, with some of the mouse antibodies, where the neutralizing antibodies bind to virions and get taken into the endosomes with the neutralizing antibodies. I think there is still a lot of work needed using biochemical and other approaches towards understanding this process.

Félix Rey: I think the topic of entry is very broad, as Aravinda said. What are the receptors? What are the receptors that bind to virion proteins? There are receptors like DC-SIGN that bind to the glycans on the virion, and then there are papers showing that the TIM & TAM familiy of receptors that bind directly to the negatively charged phosphatidylserine (PS) lipids on the virion. What this is saying is that the membrane composition is important, as newly formed flavivirus particles initally bud into the ER lumen, so they acquire a piece of the ER membrane. PS lipids are present in the ER membrane, but not in the plasma membrane, where the presence of PS signals apoptosis, and PS as an "eat me" signal for phagocytosis by specialized cells carrying "eat me" receptors such as TIM and TAM/AxL. In the case of Ebola virus, which finds its way to the endosome there is a fusion receptor involved. In Ebola virus you cannot just lower the pH to get the particle to fuse with the liposomes. In flaviviruses, if you have the right anionic lipid the drop in pH will be enough to drive fusion with liposomes, for instance. But to enter a cell, it is not only the pH but also the compartment that has the right lipid. Jolanda Smit has done some nice tracking experiments with a very particular attenuated dengue virus strain (PR159-S1), I think, because she was not allowed to use a low passage clinical isolate. But the caveat is that the attenuated virus she used may use a different pathway – different from the one used by clinical strains for entry. But the other thing that we want to allude to is the mechanism by which the most potently neutralizing antibodies neutralize the virus particle. There are several aspects to consider: one of the most important is that if the stoichiometry threshold for neutralization is not reached, i.e., if there is not enough bound antibody per particle, the virus particle will still be endocytosed and will fuse in the endosome and infect the cells. And one problem is that the fusion loop antibodies, which can bind only upon breathing of the E dimer, will require time to reach the neutralizing threshold, and in the meantime, they may facilitate entry via $F_{C\gamma}$-receptors, causing

ADE. Then if you have these mosaic particles that have prM on the one side and mature smooth particle on the other, the former are spiky because of these prM molecules, the antibodies against the fusion loop would bind on that side and would leave the other side free so that the virus can fuse through the smooth patch. This is one of the aspects that is not conducive to neutralizaion, but to enhancement. This leads to the question of what should be used as an immunogen in order to develop a vaccine. There is the strategy of the tetravalent vaccine from Sanofi. They use a chimera with yellow fever virus background with prM/E from the four different dengue viruses. Some people say this is not a good vaccine, because they do not have the right T-cell epitopes that are mostly on the non-structural proteins. Possibly that is one thing that has not been fully explored. Another thing is that they noticed that it usually protects well against serotypes 3 and 4, but is less protective against serotype 1 and even more poorly protective against serotype 2. In the view of several people – we have not done this ourselves – this vaccine's performance correlates with virus breathing. There are signs that Dengue 2 is the one that has most breathing. Dengue 1 has less breathing than Dengue 2, but still quite significant. While Dengue 3 and Dengue 4 apparently, have least breathing. However this will depend on the strain and what cells were used to grow the virus strains etc. Also as Aravinda mentioned, there could be single mutations on the envelope, that can lead to very different properties of neutralization by antibodies. In summary there are many things to master to really understand what you could do to elicit antibodies that will efficiently block virus entry. Concerning entry, the other thing that could be used is small molecule antivirals. We know that the E protein has to be in a metastable conformation in order to induce fusion as it has to change from the dimer to a trimer. The dimer has to be metastable for it to be functional, and if we can find compounds that specifically bind to the E dimer and stabilize it, such that it will remain a dimer and will not change to trimer, and thereby block fusion. There are reports in the literature that researchers at Novartis had targeted the E protein, but I think that did not work out.

Paul Young: Just to extend that, not only can you find compounds that bind to the dimer and restrain it from transition to a fusogenic trimer, but you could identify compounds that trigger fusion prematurely so that they are no longer infectious. One of the big differences between strains, let alone between subtypes, is the pH at which they transition. It is not just a single pH, but varies from just below 7 down to 5.5. As a consequence, the virus fuses in different compartments of the endosome. It could fuse in the early or the late endosome, depending on how low the pH needs to be before transition occurs. There is quite a lot of variation. Coming back to the point of viruses getting into the endosome in the presence of the antibody. James Porterfield published a paper in the early 80s in *Nature* that showed West Nile virus getting into cells and into the endosome with attached antibodies and showed that the neutralizing effect of the antibody was at that late fusion stage. So we have known this for some time. But I think those quaternary interacting antibodies is the key for neutralization. I also want to get back to something I mentioned in the earlier talk on the mechanism of NS1 protection. It is true, not just for non-structural proteins, but also for E that antibodies may introduce protection through ADCC [antibody-dependent cellular cytotoxicity] and not necessarily just neutralization.

Subhash Vasudevan: Félix, do you expect the monoclonal antibodies that you are developing, as therapeutic antibodies to coat the entire virus? How many antibodies should the virus bind?

Félix Rey: It was also shown by Ted Pearson that flavivirus neutralization works by a "multiple hit" mode, following the "antibody coating" model proposed earlier by Dennis Burton, which poses that you need a minimum stoichiometry for neutralization. I think you need to have at least 30 antibodies bound to the particle for neutralization. So in principle, there

could be 180. But if you have 30 equally distributed antibodies it would perhaps suffice. There is a caveat here, because you could have 90 copies only on one side of the particle. Because that is where the fusion loop is exposed. Then the other side is not blocked. What you need is an even blocking of the mosaic particle.

Aravinda de Silva: The number of 30 is shown for West Nile virus, but I think the number, in my opinion, will really depend on the actual antibodies and mechanisms by which it neutralizes. What is clear is that you have to get above a certain threshold number. Only one antibody binding is not going to work. I guess there are examples, maybe, in the literature – some virus like polio where binding of antibody to the virus particle may destabilize the virus surface – but that is not the case for Dengue. I think to determine the number of antibodies that is needed to neutralize and block fusion, we should consider how many E molecules are actually participating in the fusion reaction, in the fusion pore. I think that Steve Harrison has done some work and has come up with some idea about how many molecules are actually a part of the fusion pore, but I do not know that literature very well. The other important thing to touch upon is that the actual entry pathway and intracellular compartments may be different when the virus gets in via ADE versus a traditional cell surface receptor. I think this is an area worth exploring further and people call it intrinsic ADE. The idea here is that a cell infected via the ADE pathway evade, cellular antiviral responses more efficiently than virions entering via a traditional receptor. So, antibody-enhanced entry can lead to a greater number of infected cells as well as individual infected cells releasing more infectious virions. There is also the issue of mature versus immature virions. We always thought that immature virions are not infectious and Jolanda Smit has done some nice work to show that these immature virions can be infectious under some conditions. Her data indicates that immature virions can get into the endosome. And that furin in the endosome can actually cleave prM and mature those virions. We have observed that furin inhibitors can block the entry of immature virions, whereas the entry of mature virions is not blocked by furin inhibitors. So we always talk about maturation on the way out of the cell, but we also need to consider how virus maturation state influences entry into the cell.

Subhash Vasudevan: I guess we can talk a lot more about the complexity of entry, but in the interest of time, we should perhaps move this discussion along to NS1. So over the past decade, a lot of work from several key players and also from Paul Young's laboratory, as we heard in the presentation earlier, suggest that NS1 could be a direct antiviral target. So Paul, do you want to kick off and add something to what you already said?

Paul Young: Not really. I think I've said it all [sniggers from the audience]. There are a number of NS1-mediated effects we have already discussed but I think there will be more to talk about in the future, given that NS1 has been shown to engage with a wide range of host molecules. I think many of them are likely to be acting in synergy to exacerbate immune responses, sending some infected individuals down a pathogenic pathway. The anamnestic antibody response during the acute stage of secondary infections and the resulting early formation of immune complexes offers another activation pathway. There are all these different layers that seem to be in play, adding complexity, synergizing with each other to enhance that activation effect. There have been many subtle differences discovered between the endothelial membrane and the monocyte system regarding the effect that NS1 is having. Hopefully we can understand and tease out the pathway in the near future, but there are multiple pathways for aggravating that activity.

Félix Rey: Also through the lipids that NS1 transports within the core of the secreted hexameric form. So that is another factor that can contribute to the effect.

Paul Young: Absolutely! We are delipidating NS1 at the moment. It is a challenging task to delipidate and keep it as a functional hexameric unit but it can be done. The crystallography of NS1 suggests that it is possible. We made a point to mention in our paper that we have not dismissed the possibility that the lipid component may be involved in activation. Early studies suggest otherwise but I think they are probably playing a role somewhere.

Siew Pheng Lim: So in terms of intervention with respect to NS1: Where do you think we should target? The partners of NS1? Or antibodies with NS1?

Paul Young: This is a good question. It is not only what you target, but temporally, when you target, as animal experiments show. When we first tried the sepsis drug in the animal model on day zero, at the same time as virus infection, we saw a worse outcome. So clearly, the timing of treatment is critical and might prove challenging to achieve in clinical trials. A worse outcome at the time of infection suggests that innate immune responses are important in dampening down the early infection. But the fact that it had such a positive effect 2 days post infection, indicates we may have a window of opportunity provided appropriate early diagnostic detection is done. Whether we use a TLR4 antagonist, such I have shown here or perhaps a blocker of a partner like CD14, or a blocker of NS1 itself all remain possible. We are also setting up screening assays to identify small compound inhibitor drugs of NS1 activation. As Eva Harris has shown, antibodies to NS1 can also block the activity. So an antibody response elicited to NS1 may be an important co-deliverable to any vaccine in the future. It's worth noting that the Sanofi vaccine not only does not deliver a full array of dengue relevant T-cell epitopes but it also does not express dengue NS1. The absence of that specific protective immune response may have played a role in the vaccine's poorer than expected performance. More than two decades ago, when we were all looking at NS1 as a putative vaccine, as an approach to get away from ADE, and the only observation of the NS1 antibodies delivering passive protection was in mice that NS1 gave protection that offered an opportunity to go forward. It was not pursued further because it was too difficult to actually tease apart how that protecion was actually working. So the ADCC was mentioned as a primary component. But as Jack Schlezenger showed, there are clearly components outside of the ADCC pathway of protection that we could not understand. And if we look at this data now, maybe the additional component was simply the blocking of NS1 activation of cell populations within mice. So, multiple targets and you have to be very careful about timing of treatment. Maybe one other thing to mention here. There is a focus in particular on disease at the severe end of the spectrum, shock and endothelial leak. But if you think about the major symptoms of the milder dengue fever, including severe joint pain it is possible that these may simply be the consequence of an over-robust pro-inflammatory cytokine response. So I think that medication that dampens the inflammatory cytokine response, even in dengue fever infection, might have some beneficial effects.

Anuja Mathew: Are there any parts of NS1 that are known to be more immunodominant and recognized by antibodies? Andrew Falconar has used peptides and found some antibodies that block.

Paul Young: Andrew was my PhD student and we worked together on this. So yes, we did look at mice, rabbits, and humans and saw different profiles, different epitopes. Many other groups have also subsequently examined this question and we now have a comprehensive picture of the epitope topography of NS1. There are common cross-reactive epitopes and there are a number of dominant epitopes. One of these is the loop structure at the end of the wing domain. Antibodies to this epitope cross-react with all four dengue virus serotypes and a number of other flaviviruses. We have representative antibodies from the majority of identified epitopes and we have looked at their

ability to block this activation effect. In short, some do and some do not.

George Gao: So what is the correlation for the primary infection and the secondary infection for the NS1 antibody response curves? Do you have anything on that?

Paul Young: We have looked at serum samples from primary and secondary infected patients using immunoblotting, to examine the profile of antigen specificity. Serum from patients in the acute stage of primary infection recognises E with some recognising both E and prM. Very rarely do you see an anti-NS1 response in primary infections except in late convalescence. But if you look at secondary infected patients, NS1 responses are strong, one assumes just like with ADE, it is the memory response to the NS1 seen in the primary infection (original antigenic sin). I would suggest that those secondary anti-NS1 responses are low-affinity responses to the incoming acute NS1. And I think that has a role to play as well as low-affinity immune complexes.

George Gao: Because everybody is talking about using E antibody and NS1 antibody for therapeutics: Do you think that the NS1 antibody alone would work?

Paul Young: Probably not. But a combined approach with neutralizing antibodies may have some benefits. It should be noted that there have also been reports of anti-NS1 antibodies enhancing disease, because of a mimicry with epitope domains present on endothelial cells. Those antibodies responsible have been identified as binding to the C-terminal end of NS1. It has been suggested that truncated NS1 could be used to induce protective antibodies without enhancing disease. Induction of a protective anti-E and NS1 response, that supresses infection early, on is probably the path to go.

George Gao: So far, how many NS1-targeting antibodies and for how many flavivirus members has protection been observed?

Paul Young: West Nile is one.

George Gao: What else?

Paul Young: Dengue, Japanese encephalitis virus. There are a number of studies that were done back in the 90s and all of them essentially show that an NS1-induced response could protect.

Aravinda de Silva: I think this is the place where we should take a lesson from what we have learned about antibody responses to E protein. Nearly all of the mapping of NS1 antibodies were done with mouse antibodies. And I think that it is critical that we start to map the human antibody responses to NS1 from different flaviviruses. We need to define plasmablast and memory B-cell responses to dengue NS1 after primary and secondary infections. The current antibody screens that most groups use do not pick up NS1 antibodies because the antigens used are E protein or virions. So I think, for all of us working in this area, it is time to start including NS1 antigen in our screens to start to understand human response and how it varies across serotypes and between individuals and in primary versus secondary infection.

Paul Young: Agreed.

Subhash Vasudevan: To your question, Siew Pheng, I thought more to the replication complex and the potential role of NS1 and its interaction with other proteins in the context of viral replication. Is there any merit in targeting specifically NS1 through interrupting interactions in the replication complex?

Paul Young: We need to know how NS1 is actually engaged in the replication complex, and that is a long-standing task that we have not really been able to resolve. There is engagement with a number of non-structural proteins: NS4A and 4B, and 2A. But a number of mutagenesis studies have not made that really clear. But if we find out what those biophysical engagements are, we can potentially interfere with them.

Suzanne Kaptein: I agree with you that NS1 for sure is a very interesting target. However it is rather curious to see that during all these screening campaigns that have been going on – so I am only refering to antiviral campaigns based on cell-based infection, not for NS1 as such – we find hits against NS3, NS5, E, NS4, but never against NS1.

Paul Young: I think targeting the pathogenic role of NS1 in human infection may be more tractable. Targeting the replication role of NS1 is much more difficult, given where it is and the fact we still do not understand its functional role in the replication complex. It is buried under a number of membrane compartments, from the plasma cell membrane to the membranes of the replication complex. The challenge is actually getting something to the site of activity. That is probably why nothing is showing up in the screens.

Subhash Vasudevan: The challenge may be getting the right type of screening assay to ask that question. For yellow fever virus it was reported that NS1 and NS4A interaction is important for replication.

Katja Fink: Also Ralf Bartenschlager's lab showed the interaction between NS1 and E protein. I mean that was kind of surprising, because I think he proposed NS1 might facilitate the packaging of the virus particles. Could that also have an impact, or explain how anti-NS1 treatment could be used or how anti-NS1 antibodies would work? Because I still do not understand actually how anti-NS1 antibodies can be protective. You mentioned ADCC. There is not much NS1 on infected cells?

Paul Young: There is actually quite a bit of NS1 on infected cells, particularly macrophages. Its certainly on the surface and it is in a dimeric form, which you can show by crosslinking. That is the reason why Bartenschlager's lab suggests that it might be the wing domain region of the NS1 protein that was engaged with E protein. They showed you can co-immunoprecipitate E and NS1 from infected cells. So inhibiting that particular interaction may have some beneficial effect. I am trying to remember the data. They did some mutational work, where they abrogated infectivity substantially.

Check for updates

9 The Dengue Virus Replication Complex: From RNA Replication to Protein-Protein Interactions to Evasion of Innate Immunity

Julien Lescar, Sherryl Soh, Le Tian Lee, Subhash G. Vasudevan, Congbao Kang, and Siew Pheng Lim

Abstract

Viruses from the Flavivirus family are the causative agents of dengue fever, Zika, Japanese encephalitis, West Nile encephalitis or Yellow fever and constitute major or emerging public health problems. A better understanding of the flavivirus replication cycle is likely to offer new opportunities for the design of antiviral therapies to treat severe conditions provoked by these viruses, but it should also help reveal fundamental biological mechanisms of the host cell. During virus replication, RNA synthesis is mediated by a dynamic and membrane-bound multi-protein assembly, named the replication complex (RC). The RC is composed of both viral and host-cell proteins that assemble within vesicles composed of the endoplasmic reticulum membrane, near the nucleus. At the heart of the flavivirus RC lies NS4B, a viral integral membrane protein that plays a role in virulence and in down-regulating the innate immune response. NS4B binds to the NS2B-NS3 protease-helicase, which itself interacts with the NS5 methyltransferase polymerase. We present an overview of recent structural and functional data that augment our understanding of how viral RNA is replicated by dengue virus. We focus on structural data that illuminate the various roles played by proteins NS2B-NS3, NS4B and NS5. By participating in viral RNA cap methylation, the NS5 methyltransferase enables the virus to escape the host cell innate immune response. We present the molecular basis for this activity. We summarize what we know about the network of interactions established by NS2B-NS3, NS4B and NS5 (their

J. Lescar (✉)
Nanyang Institute of Structural Biology, Singapore, Singapore

School of Biological Sciences, Nanyang Technological University, Singapore, Singapore
e-mail: julien@ntu.edu.sg

S. Soh
Novartis Institute for Tropical Diseases, Singapore, Singapore

Nanyang Institute of Structural Biology, Singapore, Singapore

L. T. Lee
Nanyang Institute of Structural Biology, Singapore, Singapore

S. G. Vasudevan
Emerging Infectious Diseases Program, Duke-NUS Medical School Singapore, Singapore, Singapore

C. Kang
Experimental Therapeutics Center, Singapore, Singapore

S. P. Lim
Denka Life Innovation Pte Ltd., Singapore, Singapore

Novartis Institute for Tropical Diseases, Singapore, Singapore
e-mail: siewpheng-lim@denka.com.sg

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_9

"interactome"). This leads to a working model that is captured in the form of a rather naïve "cartoon", which we hope will be refined towards an atomic model in the near future.

Keywords
Flavivirus · Replication complex · Protein-protein interactions · Innate immunity evasion · Antiviral drug discovery

9.1 Introduction

Flaviviruses such as Yellow Fever Virus (YFV), Dengue virus (DENV), West-Nile virus (WNV), Japanese Encephalitis Virus (JEV) and the recently re-emerging Zika virus (ZIKV) represent significant public-health problems. The four DENV serotypes cause hundreds of thousands cases of the severe haemorrhagic form of the disease every year and a large and increasing proportion of the world population is at risk (http://www.who.int/topics/dengue/en/). The recently marketed Sanofi tetravalent vaccine (CYD-TDV) fails to confer good protection against serotype 2 and is not recommended for children younger than 11 years old, which unfortunately represents a significant limitation of its applicability [15, 56]. While vaccination remains in principle the best way to protect large populations against infectious diseases and has indeed been extremely successful to control YFV [63], specific antiviral drugs to treat the various conditions caused by flaviviruses, such as DENV, ZIKV, WNV, YFV and JEV would also be desirable, because they would significantly expand our prophylactic and therapeutic options [29, 43]. For instance despite the availability of a very efficient YFV vaccine [63] for almost 80 years, between 30,000 and 60,000 deaths are caused every year by infections with this virus and an important outbreak was reported this year in in Angola, Uganda and Democratic Republic of the Congo, Africa. Outbreak management currently relies on controlling the mosquito vector population via the release of mosquitoes infected by the bacterium *Wolbachia* (see Chapter by Scott O'Neill and colleagues). Plus-strand RNA virus replication occurs in association with cytoplasmic host-cell membranes, where viral and host-cell factors cooperate within an organelle-like factory called replication complex (RC) (see refs. [3, 13, 44, 49, 64]). DENV is a well-studied enveloped virus with a positive-strand RNA genome that also serves as a prototype to understand the flavivirus structure and its replication cycle. Upon attachment to a susceptible cell, DENV viral particles about 50 nm in diameter [26] are internalized in vesicles and transported to endosomes, where major conformational changes in the envelope protein E triggered by low pH [4], lead to the release of the ribonucleocapsid into the cytoplasm. The coding region of the capped single-strand RNA genome which contains also untranslated regions (UTR) both at its 5′ and 3′ ends, is then translated into a viral polypeptide and processed into ten proteins following maturation by host proteases and also by the NS2B-NS3 viral protease (Fig. 9.1). The N-terminal part of the viral polyprotein contains three structural proteins C, prM and E and the C-terminal part seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (Fig. 9.1). Viral NS proteins and host factors assemble to form a membrane-bound RC that functions like a molecular factory by orchestrating viral RNA replication [36, 37]. Following RNA synthesis, newly copied RNA molecules are either recycled for translation and replication or alternatively, extruded from the vesicle for packaging into nascent virions. Importantly, several NS proteins of the flavivirus RC including NS2B-NS3, NS4B and NS5 constitute validated drug targets because they play crucial functions during viral replication. Today however, a major impediment in developing drugs targeting the flavivirus RC is that both its morphology and exact composition, the interplay between its molecular constituents at various stages of the replication cycle, as well as the precise molecular mechanisms for viral RNA replication are still largely elusive. One could say that the field in flavivirus research is lagging about 5–10 years behind the

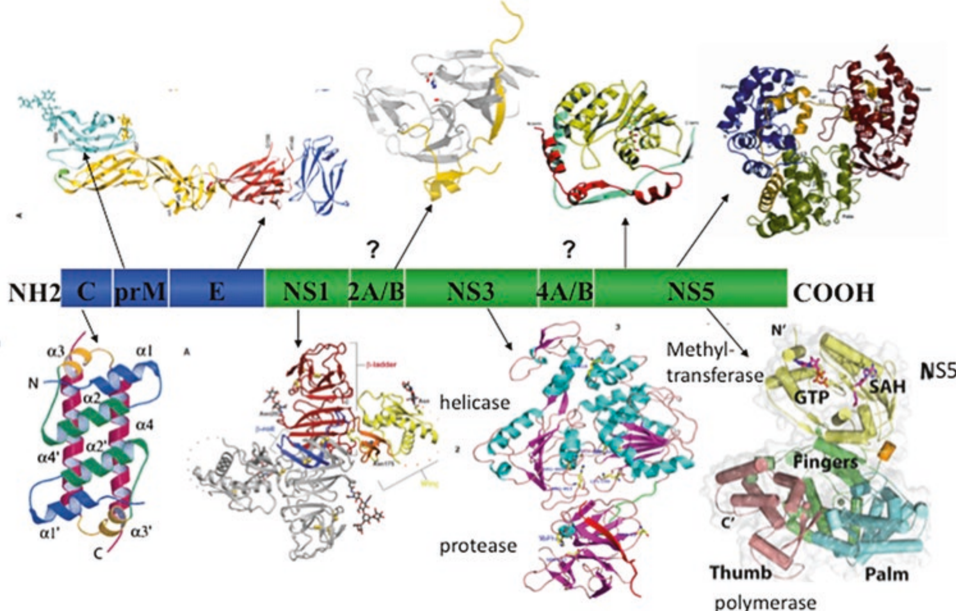


Fig. 9.1 **The flavivirus polyprotein.** Shown here is a gallery of structures determined for DENV, available from the PDB at www.rcsb.org. References to the original work (and respective authors) are either included in the text or can be found in the PDB. The polyprotein is depicted as blue rectangles for the three structural proteins C (capsid), Pr and M Membrane protein, E, envelope protein and as green rectangles for the seven non structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5, expressed during the intracellular phase of the virus replication cycle. Question marks indicate NS proteins for which no high-resolution structure is available, but NMR data detailing their secondary structures and topology is available for NS2A (25), NS2B (28) and NS4B (29–30, 32) (see text)

field of HCV research. In this respect the emergence of viruses like ZIKV is likely to provide the momentum and the level of investment needed to fill this gap.

9.2 The Flavivirus Replication Complex

Over the last decade, several individual protein components of the RC were characterized both at the functional and structural level and also as potential antiviral drug targets [36, 37, 50]. Moreover, several human and insect proteins putatively linked to DENV replication were recently identified using proteomics and RNAi approaches [23, 58]. An interesting –although ambitious- challenge ahead is to gain a better basic understanding of the flavivirus RC as a whole, in order to picture at the atomic level molecular interactions that orchestrate the various steps of replication: The RC coordinates steps of viral cycle by spatial segregation of replicating RNA from ribosomes and from capsids undergoing assembly. In this process, an intricate and dynamic network of various RNA-RNA, RNA-protein and RNA-protein interactions must be established in the context of the physical barrier provided by ER membrane (Fig. 9.2). An immediate benefit for the replicating flavivirus is the protection that such ER perinuclear membrane vesicles confer against host cell nucleases and also the avoidance of innate immunity sensors such as RIG-I and IFIT family members, that can detect dsRNA molecules [12]. In addition, there is an increasing appreciation that intermolecular interactions between members of the RC can regulate their enzymatic activities. Catalytic activities of multifunctional proteins like the NS2B-NS3 protease NTPase/helicase or the NS5

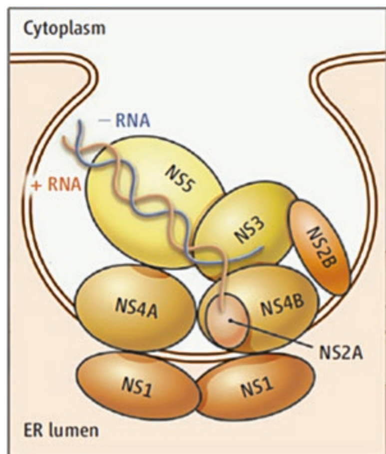


Fig. 9.2 The flavivirus replication complex. Schematic depiction of the membrane bound flavivirus replication complex inserted in a protective vesicle. The figure is not drawn to scale as many RCs are likely to be present in each vesicule, see for instance reference 6. Moreover, the multimerization proposed for several NS proteins such as NS4B (54) or NS5 (70) is ignored in this schematic representation. (Adapted From Pei-Yong Shi Science. 2014 Feb 21;343(6173):849–50)

methyltransferase-polymerase appear to be different in the context of the full-length proteins compared to isolated recombinant enzymatic domains [40–42]. Further modulation of enzymatic activities is expected in the context of the membrane bound RC. This suggests the existence of an extensive network of "cross-talks" where both inter-domain and intermolecular interactions regulate the various enzymatic activities harbored by each protein domain. Today, we are just beginning to understand the molecular basis of these "cross-talks", thanks to a combination of structural and functional studies and also by using infectious clones and replicon systems to perform site-directed mutagenesis informed by structural data.

Here we briefly describe ongoing work that used DENV RC as model. An interesting, yet still largely open question, pertains to whether the DENV RC is representative of all other flaviviruses, or alternatively, whether divergence in evolution has produced various answers in terms of the architecture and dynamics of say the RC from JEV, ZIKV or even between the various DENV serotypes. Initial indications given by recent 3D structures determination of the full-length NS5 protein from JEV, DENV3 and ZIKV (see PDB code 5TFR) indeed suggest variations in the architecture of their respective RC introduced during evolution. However, some clustering during the divergent evolution of flaviviruses appears likely as outlined below taking the example of the full-length NS5 protein. One immediate task therefore consists in identifying a common ancestral RC "core" shared between various flaviviruses. Another task is to check whether a simple phylogenetic analysis suffices to assign one particular RC to one group of closely related flaviviruses. In our lab, we have focused our efforts on DENV and on mapping the interactions ("interactome") of three proteins NS2B-NS3, NS4B and NS5. These proteins play either key enzymatic roles or act as "a hub" within the flavivirus RC like NS4B -an integral membrane protein with also a role in virulence and down-regulation of the host innate immune response. NS4B binds to the NS2B-NS3 protease-helicase which itself interacts with the NS5 methyl-transferase-polymerase and we ask how are these molecular interfaces formed and what impact they have on the activities of the individual proteins?

Like other (+) strand RNA viruses such as HCV [53], DENV RNA replication is performed in close association with remodeled cell membranes inside a vesicular like compartment (Fig. 9.2). The morphology and activity of the DENV RC and TBEV was studied by purifying membrane fractions from cells transfected with replicon RNA. Such isolated RCs are able to synthesize RNA and the RdRp activity that produces dsRNA intermediates is protected from nuclease and protease digestion and also concealed from the innate immune system via vesicular structures that were recently described using elegant electron tomographic reconstructions [46, 68].

Our understanding of flavivirus NS proteins, particularly in their membrane-bound environment, has been hampered by the lack of robust *in vitro* expression systems and by the inherent difficulties associated with the study of integral membrane proteins. Thus, for many years, much of what was known in terms of atomic structures

has made use of soluble fragments of viral replicative enzymes expressed in *E. coli* [50] (Fig. 9.1). However, growing evidence suggests that it is the interplay between viral enzymes, host cofactors and viral RNA that orchestrates temporally and spatially the virus RNA genome replication [17]. Thus, despite their crucial roles for viral genome replication, relatively little structural information has been available for NS2A, NS2B, NS4A, NS4B, that are integral membrane proteins (Fig. 9.1). This has recently changed and we now have a clearer picture for the topology of membrane-associated proteins NS1 [1], NS2A [73], NS2B [8, 21, 31, 32] and NS4B [31–34] by various structural and biochemical techniques including NMR and X-ray crystallography. We also have more information about how these integral membrane proteins interact with members of the RC, how they might affect membrane structure and also the assembly of new viral particles [27, 30]. In a recent study on NS2B from JEV [33, 34], the trans-membrane region of NS2B from JEV was suggested to contribute both to viral RNA replication and to the formation of new virus particles. Moreover the interaction between NS2B and NS2A may participate in modulating viral assembly [33, 34]. Remarkably, NMR structural studies performed on NS4B reconstituted in micelles [33, 34] have largely confirmed pioneering work on NS4B that was performed in the mid 2000s by the Bartenschlager group, using serial truncation and localization/labelling experiments (in order to detect ER vs cytoplasmic exposure) [45]. As said earlier, cell biology and electron tomography studies have provided *in situ* pictures of the complete flavivirus RC highlighting membrane rearrangements into vesicles and spherules (Fig. 9.2). However, to understand the RC at the atomic level, one must now combine these relatively low-resolution images of the RC derived by tomography with higher resolution data derived from X-ray crystallography or NMR and this task remains a major challenge. Compound screening campaigns have identified potent DENV [67] and YFV [14] inhibitors targeting NS4B. How these inhibitors interact (eventually only through a "genetic interaction") with NS4B remains elusive in the absence of supporting biochemical and structural data. Thus structural studies of the integral membranes proteins from DENV and other flaviviruses will certainly provide crucial information required to understand their roles in membrane remodelling, in viral replication and viral particle assembly and also for the design of specific inhibitors with antiviral activity [36, 37].

9.3 NS2B and NS2B-NS3

NS2B-NS3 is one of the most conserved flavivirus enzymes. For instance the ZIKV NS2B-NS3 protease was found to bear high structural similarity to the homologous enzymes from DENV and WNV [51]. NS2B-NS3 possesses protease, helicase and RNA triphosphatase activities stimulated by ssRNA binding [40, 41]. The N-terminal domain of NS3 (NS3pro) is important for viral maturation because it cleaves the viral polyprotein at the junctions between NS2A/2B, NS2B/3, NS3/4A and NS4B/5. NS2B is a small (14 kDa) protein with four hydrophobic transmembrane (TM) domains formed by helices α1 (G4 to L19), α2 (L25 to M41), α3 (N90-G105) and α4 (P112-T125). A central hydrophilic region (residues 42–90), projects into the cytoplasm and acts as a necessary cofactor for the protease N-terminal domain of NS3 (NS3pro). In addition to its membrane anchoring cofactor NS2B, NS3pro from DENV has an exposed hydrophobic turn formed by residues "$G_{29}LFG_{32}$", which is also likely to contribute to membrane association of the protein [42]. The helicase domain of NS3 (NS3hel) is involved in viral RNA replication together with the RNA-dependent RNA polymerase-NS5 [28, 76] with which it interacts [61, 80]. The linker between the NS3pro and NS3hel domains was reported to confer overall inter-domain flexibility and a switch between two conformational states was proposed to accompany the transition between polyprotein proteolysis and RNA replication [42]. However, the conformation of NS3 in the context of other RC proteins including the complete NS2B protein, NS4B and NS5 remains

largely unknown. Likewise the exact role NS3 itself plays in viral replication is not clearly defined: besides its role in cap synthesis through hydrolysis of the gamma phosphate of GTP, NS3hel could participate in viral genome replication by unwinding dsRNA replication intermediates. The interaction between NS3 and NS4B is essential for dissociating the helicase from single-stranded RNA, which modulates viral replication [65]. Interestingly Dengue virus non-structural protein 3 was found to redistributes fatty acid synthase to sites of viral replication and increases fatty acid synthesis, a result in line with the requirement for lipid membrane reorganization in the context of the RC [19].

9.4 The NS4B Protein

Both NS4A and NS4B have been implicated in membrane rearrangements and RNA replication. NS4A associates with the membrane via four TM domains and has a C-terminal "2 K fragment" that serves as a signal sequence for the translocation of the adjacent NS4B protein into the ER lumen. An N-terminal amphipathic helix in DENV NS4A mediates oligomerization and is essential for replication [60]. NS4B confers virulence through enhancing viral synthesis in a mouse model of DENV infection [18]. Likewise, NS4B from WNV is involved in virulence and a mutation resulting in an attenuated virus was identified [69]. NS4B is also implicated in the inhibition of alpha/beta interferon signalling [47] and in the regulation of immune response mediators (cytokines) associated with haemorrhagic fever [22, 52, 70]. Remarkably, NS4B influences the helicase activity of NS3 presumably via a direct protein-protein interaction [65]. NS4B was found to colocalize with NS3 and with double stranded RNA, which is an intermediate in viral RNA replication [81, 82]. A topology model initially proposed for NS4B on the basis of truncation and cytoplasmic vs ER localization experiments [45] was later essentially confirmed by NMR studies, leading however to a more precise assignment of secondary structure elements [33, 34].

For crystallization trials and interaction studies, the NS4B protein was expressed in *Escherichia coli*, reconstituted in dodecyl maltoside (DDM) detergent micelles, and purified to >95% homogeneity [33, 34]. Proper folding was initially checked via CD spectroscopy indicating the presence of a majority of alpha-helical structures as expected from secondary structure sequence prediction (Le Tian Lee et al., unpublished data). *In vitro*, the recombinant NS4B protein forms dimers, as shown by gel filtration, chemical cross-linking, and multi-angle light scattering [81]. The dimeric form of NS4B could also be detected when the protein was expressed in cells as well as in cells infected with DENV2 [81]. Mutagenesis showed that the cytosplasmic loop spanning amino acids 129–165 and the C-terminal region extending from amino acids 166–248 are important contributors driving NS4B dimerization.

We raised two mouse mAbs against the recombinant NS4B protein from DENV2 [74]. These mAbs named 10-3-7 and 44-4-7 were characterized in terms of their cross-reactivity towards other DENV serotypes and their epitopes were mapped to the available topology model for NS4B. While mAb 10-3-7 appears strictly specific for NS4B from DENV2, mAb 44-4-7 is particularly interesting as it cross-reacts with NS4B from the four DENV serotypes and also with ZIKV NS4B (Jia Huan et al., unpublished results). Using overlapping peptides spanning the whole NS4B sequence, we found that mAb 44-4-7 binds to the cytoplasmic loop of NS4B [74]). We expressed in *E. coli* a scFv construct containing the variable regions of mAb 44-4-7, that recapitulates binding of the parent mAb. Besides possible applications in pull-down experiments using RC extracted from infected cells, this scFv was used as a vehicle to promote the formation of well-ordered 3D crystals to increase hydrophilic surface and improve lattice order [20, 24]. However, a caveat is that the NS4B protein used for eliciting mAbs might have been presented to the mouse immune system in a partially denatured state. This could explain the lack of success in crystallizing the NS4B protein we have encountered so far. Therefore, the struc-

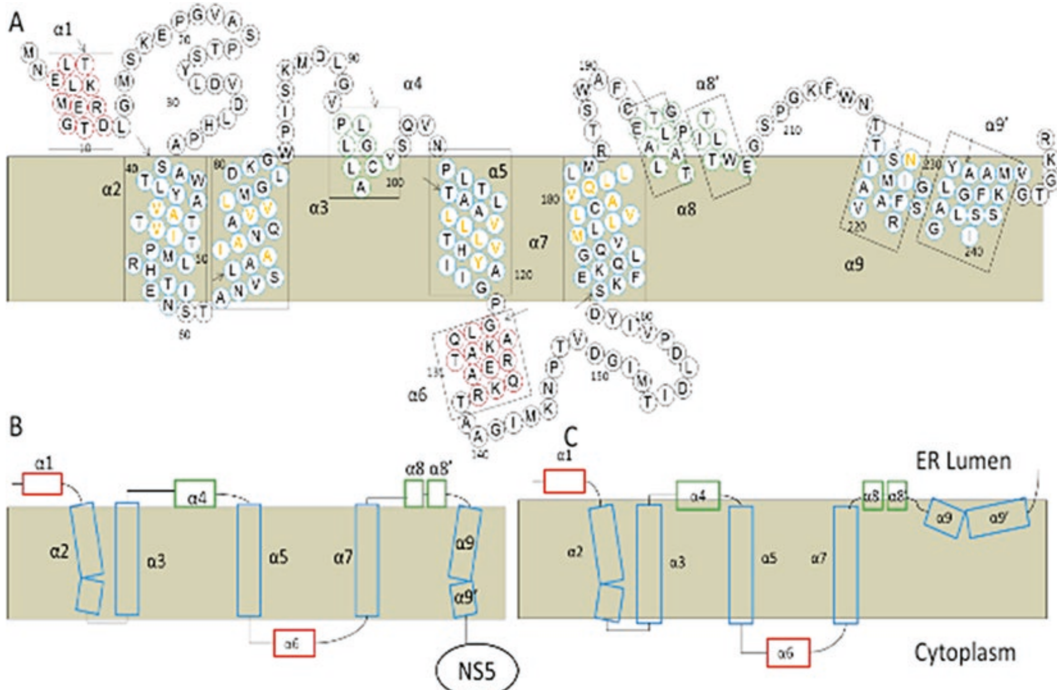


Fig. 9.3 **Membrane topology of NS4B.** (**a**). The topology of NS4B is represented sequence based on PRE and H–D exchange experiments. Water-soluble residues are highlighted in red, membrane interacting residues in green and residues protected from exposure to gadolinium are in blue. Residues highlighted in brown are protected from H–D exchanges. (**b**, **c**) The NS4B topology varies depending on the presence (**b**) or absence (**c**) of NS5 (that is before or after cleavage of the flavivirus polyprotein at the NS4B-NS5 junction. (Adapted from Ref. [[30]])

tural study of NS4B from DENV3 was carried out using NMR in LMPG detergent micelles [33, 34]. This work revealed the presence of a total of eleven helices with five potential TM regions (Fig. 9.3). This work has given the first detailed secondary structure assignment for this important viral protein: NS4B was found to be the site of escape mutations when DENV or YFV are grown in the presence of several antiviral inhibitors [72]. However, a direct molecular interaction between small molecule inhibitors and NS4B has not been clearly demonstrated so far and the observed evolutive pressure on NS4B brought about by incubating the virus with these drugs could be due to indirect interactions [72]. Rather surprisingly, some data suggesting a genetic and physical interaction between nonstructural proteins NS1 and NS4B with a role in modulating replication of WNV was also presented [77]. This observation brings some alteration to the currently accepted topology model in which NS1 is exposed to the lumen side of the ER whilst NS4B is exposed to the cytoplasmic side (Fig. 9.2).

9.5 Mapping the Interactions Between NS2B-NS3 and NS4B

Remarkably, NS4B regulates the helicase activity of NS3 by modulating NS4B affinity for RNA. We therefore attempted to detect a direct protein-protein interaction between these two proteins and to map their interactions on the respective proteins. Co-immuno-precipitation and *in situ* proximity ligation assay confirmed that NS3 co-localizes with NS4B in both DENV-infected cells and cells that co-expressed both proteins [82]. We then expressed the full-length NS3 protein and also its helicase and protease domains separately. Surface plasmon resonance showed that the helicase domain of NS3 alone

was able to bind NS4B with µM affinity whilst the protease domain of NS3 gave much weaker interactions. We then took advantage of the possibility of expressing subdomains of the helicase region of NS3 spanning its subdomains 1, 2 and 2 + 3 respectively. Surface plasmon resonance demonstrated that subdomains 2 and 3 of the NS3 helicase region had the highest affinity for NS4B. Remarkably, we found that the predicted cytoplasmic loop of NS4B could be expressed in *E. coli* as a soluble protein. This recombinant protein is flexible, with a tendency to form a three-turn α-helix and two short beta-strands. The cytoplasmic loop of NS4B was found to be required for binding NS3, giving support to the topology model that places this segment of the protein in the cytoplasm, and therefore accessible for interaction with other members of the flavivirus RC such as NS3 and NS5. A weak interaction in the high micromolar range was shown between peptides spanning the NS4B cytoplasmic loop and the NS3 helicase domain. Using nuclear magnetic resonance (NMR), we found that upon binding to the NS3 helicase, 12 amino acids within the cytoplasmic loop of NS4B exhibited line broadening, suggesting a participation in the interaction with NS3. Sequence alignment showed that four of these twelve residues are strictly conserved across different flaviviruses. Individual mutation of these residues in the context of a DENV infectious clone showed that three residues (Q134, G140, and N144) of the four evolutionarily conserved NS4B residues are essential for DENV replication. Interestingly, these results are in agreement with a genetic complementation studies of NS4B using a replication independent expression system: Q134 from the cytosolic loop was found to be a critical determinant for NS4B-NS3 interaction and an Alanine substitution at this site completely abrogated the interaction between the two proteins and remarkably also DENV RNA replication [6]. Taken together, these results highlight the importance of some key protein-protein interactions within the RC for virus replication. In this respect, molecular interactions between both NS2B-NS3 with NS4B and between NS2B-NS3 and NS5 (via K330 from the NS5 polymerase domain) appear to play a crucial role for replication, despite a minimal impact on the individual enzymatic activities [80]. Thus, the mapping of the NS3/NS4B-interacting regions described in these two papers [6, 82] could be exploited for a compound-screening assay with the hope to design inhibitors that disrupt protein-protein interfaces for antiviral therapy [75].

9.6 RNA Recognition by the Methyltransferase Domain of NS5

Capping of the DENV RNA genome is an essential structural modification that protects the viral RNA from degradation by 5′ exoribonucleases. This modification ensures efficient expression of viral proteins and by disguising the viral RNA as a host cell mRNA, this “Trojan horse” strategy allows escape from the host innate immune response [12]. The flavivirus nonstructural protein 5 (NS5) (Mr = 105 kDa) possesses two RNA methyltransferase activities at its N-terminal region [35]. These activities are important for capping the virus RNA genome. The methyltransfer reactions are thought to occur sequentially using the strictly conserved flavivirus 5′ RNA sequence starting with “5’AG” as substrate (GpppAG-RNA), leading to the formation of the 5′ RNA cap: G_0pppAG RNA → m^7G0pppAG-RNA (“cap-0”) → m^7G_0pppAm2′-O-G-RNA (“cap-1”) [78, 79].

Using X-ray crystallography, we elucidated how viral RNA is specifically recognized and methylated by determining the crystal structure of a ternary complex comprising the full-length NS5 protein from DENV, an 8-mer cap-0 viral RNA substrate bearing the authentic DENV genomic sequence (5′-m7G_0pppA1G2U3U-4G5U6U7–3′), and S-adenosyl- L-homocysteine (SAH), the by-product of the methylation reaction [9, 78, 79]. Interestingly, many attempts by several groups including ours using the isolated recombinant methyltransferase domain of NS5 were unsuccessful in providing a complex relevant to methylation. Only when using the full-length NS5 protein could a relevant complex with

RNA be obtained. This suggests important differences in the energetic of RNA binding between the full-length proteins and the isolated individual domains. The structure provided for the first time a molecular basis for specific adenosine 2′-O-methylation in the flavivirus family. It also explained in molecular terms a wealth of mutagenesis studies targeting the K61-D146-K180-E216 enzymatic tetrad used for 2'O methylation, as well as residues lining the RNA binding groove. Remarkably, the RNA substrate was found to be positioned such that the 2′-O atom of residue A1 lies next to the sulfur atom of SAH and adjacent to the K180 side chain from the "K61-D146-K180-E216" enzymatic motif, poised to accept a methyl group from a SAM methyl donor. Basically, the crystal structure explains the specific recognition of the flavivirus RNA 5′ cap by NS5 and the strict requirement for an adenosine at position 1 and the preference for a guanosine at position 2 for steric reasons.

9.7 The Full-Length NS5 Protein

NS5 is the largest protein component within the flavivirus RC. NS5 plays key enzymatic roles through its N-terminal methyltransferase (MTase) and C-terminal RNA-dependent-RNA polymerase (RdRp) domains, and these two enzymatic activities constitute major targets for the design of antivirals. Crystal structures of the full length NS5 from JEV [39] and from DENV3 [78, 79] have been determined using X-ray crystallography. Moreover, small angle X-ray scattering (SAXS) solution studies have complemented these crystallographic studies [5, 7, 57]. We determined a crystal structure of the full-length NS5 protein from Dengue virus serotype 3 (DENV3) at a resolution of 2.3 Å in the presence of bound SAH and GTP [78, 79]. Although the overall molecular shape of NS5 from DENV3 resembles that of NS5 from Japanese Encephalitis Virus (JEV), the relative orientation between the MTase and RdRp domains differs between the two structures (Fig. 9.4). This observation (which was confirmed by another group who crystallized the DENV NS5 protein in a different crystal form where the protein forms crystallographic dimers, but where NS5 displays the same interdomain arrangement [25]) can be interpreted in essentially two mutually non exclusive ways: (i) it could suggest the existence of a set of discrete stable molecular conformations that may be required for NS5 function and crystallization has trapped one of these discrete possible conformations. (ii) it could also suggest that the full-length NS5 proteins from various flaviviruses have diverged during evolution such that their interdomain interfaces are not identical, leading to a variety of "cross-talks" or allosteric interactions between their two enzymatic domains (Fig. 9.4). While the inter-domain region is mostly disordered in NS5 from JEV, the NS5 structure from DENV3 reveals a well-ordered linker region comprising a short 3_{10} helix that may act as a swivel. Solution Hydrogen/Deuterium Exchange Mass Spectrometry (HDX-MS) analysis revealed an increased mobility of the thumb subdomain of RdRp in the context of the full length NS5 protein. This observation correlated well with the analysis of the crystallographic temperature factors of DENV3 NS5. Site-directed mutagenesis targeting the mostly polar interface between the MTase and RdRp domains of DENV3 NS5 led to the identification of several evolutionarily conserved residues that are important for viral replication. This suggested that inter-domain cross-talk in NS5 regulates virus replication. A possible evolutionary pathway for the full length NS5 protein is summarized in Fig. 9.4: the MTase domain and RdRp domain probably originally existed as two separate smaller proteins. These two proto-domains became linked together to form the NS5 protein from an ancestral Flavivirus through gene fusion. This fusion promoted co-localization of both enzymatic activities and increased the effective concentration of the proteins with respect to each other (middle panel of Fig. 9.4). Following further divergent evolution, NS5 acquired different adaptive mutations giving rise to the full length NS5 protein structures now observed for various viruses such as DENV, JEV and also ZIKV (see PDB code) (Fig. 9.4). Thus

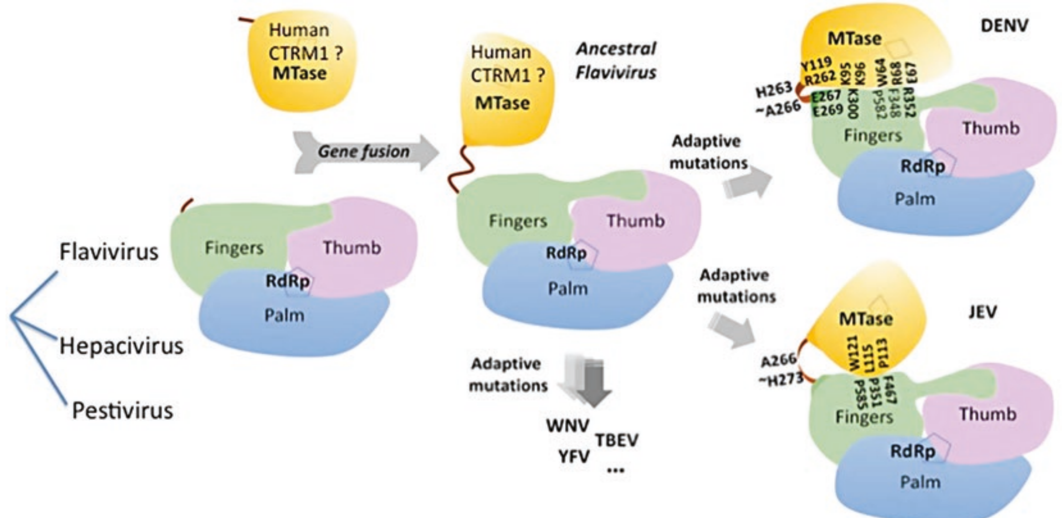


Fig. 9.4 A model for the divergent evolution of Flaviviridae and the flavivirus full length NS5 protein. Methyltransferase (MTase) domain of NS5 is in yellow, the polymerase (RdRp) fingers in green, palm in blue, thumb in salmon. The linker region 3_{10} helix (residues 263–266) between the two domains is in orange. Active sites for MTase and RdRp are labelled with dotted tetragon and pentagon respectively. Linker residues and interface residues are labeled. (see text) Modified from reference 66

NS5 proteins from DENV on one hand, and JEV and ZIKV on the other hand, appear to have different conformations and different allosteric mechanisms, in which the MTase and RdRp domain cross-talk to each other through unique interfaces specific to either DENV or JEV/ZIKV. Remarkably, a phylogenetic analysis of various flaviviruses correlates with the evolutionary hypothesis presented in this section: the four DENV serotypes cluster in a separate branch, whilst JEV, WNV and ZIKV originate from the same node suggesting a common NS5 full length structure for these three viruses.

9.8 A "Cartoon Model" of the Interactions between NS5, NS2B-NS3 and NS4B

The NS3 protease-helicase plays a central role in viral replication by interacting both with the integral membrane protein NS4B and with NS5, a soluble protein consisting in two domains. Moreover, both NS2B-NS3 and NS5 interact with RNA. Interestingly, the RNA triphosphatase activity of NS3 implies that NS3 must interact with the 5′ end of the RNA genome before AND after RNA synthesis. This conundrum might be resolved by the presence of cyclization sequences identified in the genome. The interaction between NS3 and NS5 was mapped to the NLS region of the NS5 protein with residue K330 crucial for the interaction and to the helicase domain of NS3 [65]. We summarize in Fig. 9.5 what we know about these interactions. Disrupting either NS3–NS5, NS3–NS4B or NS3–NS2B interactions constitute possible approaches to identify compounds with antiviral activity.

9.9 Conclusions

Work by several groups over more than 20 years have led to our current understanding of RNA replication by flaviviruses. While a vaccine against DENV has already reached the market, there is a consensus that this vaccine can be improved in terms of safety and the protection it provides to large populations affected by

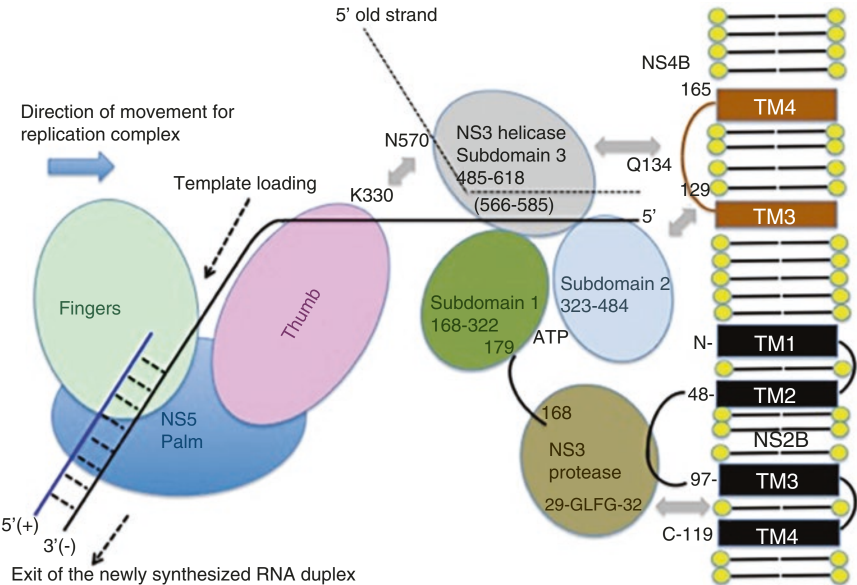


Fig. 9.5 The current model for NS2B-NS3-NS4B interactions and RNA + strand synthesis
Cartoon model of the membrane-bound NS2B–NS3 protein and its interactions with NS4B and NS5. NS3pro is colored in brown, NS3hel in green, cyan (RecA like subdomains 1 and 2, and ssRNA is depicted as a black line. NS5 and NS4B, are drawn across the ER membrane and their intermolecular interfaces are depicted schematically with the subdomain and residues involved in the interactions labeled

DENV. Other vaccines from Takeda and other manufacturers are also making their way to the market. Immunotherapeutic strategies have also been proposed [55] but it remains to be seen how practical these could be given the relatively small window available for intervention and the associated cost inherent to such strategies. Likewise, it also remains to be seen how effective will antiviral strategies be to treat such acute diseases. Target-based strategies have produced promising compounds against the NS5 polymerase domain that in our opinion should be developed further [38, 48]. Unfortunately, the financial investment to bring these compounds to the market is very high and is testing the patience of funding agencies, government bodies and private pharmas alike, as shown recently by the closure of the Novartis Institute for Tropical Diseases in Singapore, who has pioneered this approach against DENV. In this respect it would be interesting to compare the funding invested in DENV research compared to the cost associated with the development of efficient drugs to treat HCV like sofosbuvir [59]. Aside from practical vaccinology and antiviral drug discovery aspects, one fascinating aspect of flaviviruses is their ability to down-regulate the innate immune response of the host [2, 11, 16] with only a very limited set of proteins, providing opportunities to dissect the set of interactions between viral and host immune proteins in more details. In this respect the many interactions established by NS5 including with the importin/exportin system of the host cell appears also worth to study [62] (see Chapter by Subhash G. Vasudevan and colleagues), keeping in mind the existence of divergent groups that is started to be revealed between DENV on one hand and ZIKV, WNV, JEV on the other hand (Fig. 9.4). A further challenge remaining is to provide a more complete description of the interactome underlying the formation and functioning of the RC within membrane bilayers, a challenging “multi-scale” task that is likely to use a combination of structural and biochemical techniques ranging from Cryo-EM tomography to nano-discs to mass spectrometry aided by replicon and infectious clones systems [10, 54, 66, 71].

Acknowledgements This work was supported by grant NRF2016NRF-CRP001-063 to the laboratory of JL. We acknowledge the contributions of many coworkers (some now PIs active in the field) whose names are listed in the references. We also apologize for omitting some relevant primary litterature due to space limitations.

References

1. Akey DL et al (2014) Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system. Science 343:881–885
2. Ashour J, Laurent-Rolle M, Shi PY, García-Sastre A (2009) NS5 of dengue virus mediates STAT2 binding and degradation. J Virol 83(11):5408–5418
3. Belov GA, van Kuppeveld FJM (2012) (+) RNA viruses rewire cellular pathways to build replication organelles. Current Opin Virol 2:740–747
4. Bressanelli S, Stiasny K, Allison S, Stura EA, Duquerroy S, Lescar J, Heinz FX, Rey FA (2004) Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. EMBO J 23:728–738
5. Bussetta C, Choi KH (2012) Dengue virus nonstructural protein 5 adopts multiple conformations in solution. Biochemistry 51:5921–5931
6. Chatel-Chaix L, Fischl W, Scaturro P, Cortese M, Kallis S, Bartenschlager M, Fischer B, Bartenschlager R (2015) A combined genetic-proteomic approach identifies residues within dengue virus NS4B critical for interaction with NS3 and viral replication. J Virol 89(14):7170–7186
7. Choi KH, Morais M (2016) Use of small-angle X-ray scattering to investigate the structure and function of dengue virus NS3 and NS5. Methods Mol Biol 1138:241–252
8. Choksupmanee O, Hodge K, Katzenmeier G, Chimnaronk S (2012) Structural platform for the autolytic activity of an intact NS2B-NS3 protease complex from dengue virus. Biochemistry 51:2840–2851
9. Chung KY, Dong H, Chao A, Shi P-Y, Lescar J, Lim SP (2010) Higher catalytic efficiency of N7 methylation is responsible for processive N7 and 2'-O methyltranferase activity in dengue virus. Virology 402:52–60
10. Cyrklaff M et al (2013) Cryo-electron tomography of vaccinia virus. PNAS 102:2772–2777
11. Dalrymple NA, Cimica V, Mackow ER (2015) Dengue virus NS proteins inhibit RIG-I/MAVS signaling by blocking TBK1/IRF3 phosphorylation: dengue virus serotype 1 NS4A is a unique interferon-regulating virulence determinant. MBio 6(2015 May):e00553
12. Decroly E, Ferron F, Lescar J, Canard B (2012) Conventional and unconventional mechanisms for capping of virus mRNA. Nat Rev Microbiol 10:51–65
13. Den Boon JA, Ahlquist P (2010) Organelle-like membrane compartmentalization of positive strand RNA virus replication factories. Ann Rev Microbiol 64:241–256
14. Fang G et al (2016) A novel benzodiazepine compound inhibits yellow fever virus infection by specifically targeting NS4B protein. J Virol 90(23):10774–10788
15. Ferguson NM, Rodríguez-Barraquer I, Dorigatti I, Mier-Y-Teran-Romero L, Laydon DJ, Cummings DA (2016) Benefits and risks of the Sanofi-Pasteur dengue vaccine: Modeling optimal deployment. Science 353(6303):1033–1036
16. Fernandez-Garcia M-D, Mazzon M, Jacobs M, Amara A (2009) Pathogenesis of flaviviruses: using and abusing the host cell. Cell Host Microbe 5:318–328
17. Fischl W & Bartenschlager R (2011) Exploitation of cellular pathways by Dengue virus 14, 470–475
18. Grant D, Tan GK, Qing M, Ng JKW, Yip A, Zou G, Xie X, Yuan Z, Schreiber MJ, Schul W, Shi P-Y, Alonso S (2011) A single amino-acid in non-structural protein NS4B confers virulence to dengue virus in AG129 mice though enhancement of viral synthesis. J Virol 85:775–7787
19. Heaton NS, Perera R, Berger KL, Khadka S, Lacount DJ, Kuhn RJ, Randall G (2010) Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases fatty acid synthesis. PNAS 107:17345–17350
20. Hino T, Iwata S, Murata T. (2013) Generation of functional antibodies for mammalian membrane protein crystallography. Curr Opin Struct Biol. Aug;23(4):563–8.
21. Huang Q, Li Q, Joy J, Chen AS, Ruiz-Carrillo D, Hill J, Lescar J, Kang C (2013) Lyso-myristoyl phosphatidylcholine micelles sustain the activity of dengue non structural protein 3 protease domain fused with the full-length NS2B. Protein Expr Purif 92:156–162
22. Kelley JF, Kaufusi PH, Volper EM, Nerurkar VR (2011) Mutation of dengue virus NS4B in monocytes enhances production of haemorrhagic fever associated chemokines and cytokines. Virology 418:27–39
23. Khadka S et al (2011) A Physical Interaction Network of Dengue Virus and Human Proteins. Molecular & Cellular Proteomics 10 (12) 1–16 https://doi.org/10.1074/mcp.M111.012187-1
24. Kim AR, Dobransky T, Rylett RJ, Shilton BH. (2005) Surface-entropy reduction used in the crystallization of human choline acetyltransferase. Acta Crystallogr D Biol Crystallogr. Sep;61(Pt 9):1306–10
25. Klema VJ, Ye M, Hindupur A, Teramoto T, Gottipati K, Padmanabhan R, Choi KH (2015) Dengue virus nonstructural protein 5 (NS5) assembles into a dimer with a unique methyltransferase and polymerase interface. Plos Pathogens 12(2):e1005451. https://doi.org/10.1371/journal.ppat.1005451
26. Kuhn et al (2002) Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 108:717–725
27. Kummerer BM, Rice CM (2002) Mutations in the yellow fever virus non-structural protein NS2A selectively block production of infectious particles. J Virol 76:4773–4784
28. Lescar J, Canard B (2009) RNA-dependent RNA polymerases from flaviviruses and *picornaviridae*. Curr Opin in Struct Biol 19:759–767

29. Lescar J, Luo D, Xu T, Lim SP, Canard B, Vasudevan SG (2008) Towards the design of antiviral inhibitors against flaviviruses: the case for the multifunctional NS3 protein from dengue virus as a target. Antivir Res 80:94–101
30. Leung JY, Piljman GP, Kondratieva N, Hyde J, Mackenzie JM, Khromykh AA (2008) Role of non structural protein NS2A in flavivirus assembly. J Virol 82:4731–4474
31. Li Y, Li Q, Wong YL, Liew LS, Kang C (2015) Membrane topology of NS2B of dengue virus revealed by NMR spectroscopy. Biochim Biophys Acta 1848(10 Pt A):2244–2252. https://doi.org/10.1016/j.bbamem.2015.06.010
32. Li Y, Kim YM, Zou J, Wang QY, Shovanlal Gayen S, Wong YL, Lee LT, Xie X, Huang Q, Lescar J, Shi PY, Kang C (2015) Secondary structure and membrane topology of dengue virus NS4B N-terminal 125 amino acids. Biochim Biophys Acta 1848(12):3150–3157
33. Li Y, Wong YL, Lee MY, Li Q, Wang QY, Lescar J, Shi PY, Kang CB (2016) Secondary structure and membrane topology of the full length dengue NS4B in micelles. Angew Chem Int Ed 55:12068–12072
34. Li XD, Deng CL, Ye HQ, Zhang HL, Zhang QY, Chen DD, Zhang PT, Shi PY, Yuan ZM, Zhang B (2016) Transmembrane domains of NS2B contribute to both viral RNA replication and particle formation in Japanese encephalitis virus. J Virol 90(12):5735–5749. https://doi.org/10.1128/JVI.00340-16
35. Lim SP, Wen D, Yap TL, Yan CK, Lescar J, Vasudevan SG (2008) A scintillation proximity assay for dengue virus NS5 2'O-methyltransferase: kinetic and inhibition analysis. Antivir Res 80:360–369
36. Lim SP, Wang Q-Y, Noble CG, Chen Y-L, Dong H, Zou B, Yokokawa F, Nilar S, Smith P, Beer D, Lescar J, Shi P-Y (2013) Ten years of dengue drug discovery: progress and prospects. Antivir Res 100:500–519
37. Lim SP, Hong J, Seh CC, Liew CW, Davidson AD, Chua LS, Chandrasekaran R, Cornvik T, Shi P-Y, Lescar J (2013) A crystal structure of the dengue virus NS5 polymerase delineates inter-domain amino acids residues that enhance its thermostability and *de novo* initiation activities. J Biol Chem 288:31105–31114
38. Lim SP, Noble CG, Seh CC, Soh TS, El Sahili A, Chan GK, Lescar J, Arora R, Benson T, Nilar S Manjunatha U, Wan KF, Dong H, Xie X, Shi PY, Yokokawa F. (2016) Potent allosteric dengue virus NS5 polymerase inhibitors: mechanism of action and resistance profiling. PLoS Pathog. 12(8):e1005737. doi: https://doi.org/10.1371/journal.ppat.1005737.
39. Lu G, Gong P (2013) Crystal structure of the full-length Japanese encephalitis virus NS5 reveals a conserved methyltransferase-polymerase interface. PLoS Pathog 9(8):e1003549
40. Luo D, Xu T, Hunke C, Gruber G, Vasudevan SG, Lescar J (2008) Crystal structure of the NS3 protease-helicase from dengue virus. J Virol 82:173–183
41. Luo D, Xu T, Watson RP, Scherer-Becker D, Sampath A, Jahnke W, Yeong SS, Wang CH, Lim SP, Strongin A, Vasudevan SG, Lescar J (2008) Insights into RNA unwinding and ATP hydrolysis by the flavivirus NS3 protein. EMBO J 27:3209–3219
42. Luo D, Wei N, Doan D, Paradkar P, Chong Y, Davidson A, Kotaka M, Lescar J, Vasudevan SG (2010) Flexibility between the protease and helicase domains of the dengue virus NS3 protein conferred by the linker region and its functional implications. J Biol Chem 285:18817–18827
43. Malet H, Massé N, Selisko B, Romette J-L, Alvarez K, Guillemot J-C, Tolou H, Yap TL, Vasudevan SG, Lescar J, Canard B (2008) The flavivirus polymerase as a target for drug discovery. Antivir Res 80:23–35
44. Miller S, Krinjse-Locker J (2008) Modification of intracellular membrane structures for virus replication. Nat Rev Microbiol 6:363–374
45. Miller S, Sparacio S, Bartenschlager R (2006) Subcellular localization and membrane topology of the dengue virus type 2 non-structural protein 4B. J Biol Chem 281(13):8854–8863
46. Miorin L, Romero-Brey I, Maiuri P, Hoppe S, Krinjse-Locker J, Bartenschlager R, Marcello A (2013) Three-dimensional architecture of tick-borne encephalitis virus replication sites and trafficking of the replicated RNA. J Virol 87:6469–6481
47. Munoz Jordan JL, Laurent-Rolle M, Ashour J, Martinez-Sobrido L, Ashok M, Lipkin WI, A G-S (2005) Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J Virol 79:8004–8013
48. Noble CG, Lim SP, Chen Y-L, Liew CW, Yap L, Lescar J, Shi P-Y (2013) Conformational flexibility of the dengue virus RNA-dependent RNA polymerase revealed by a complex with an inhibitor. J Virol 87:5291–5295
49. Paul D, Bartenschlager R (2013) Architecture and biogenesis of plus-strand RNA virus replication factories. World J Virol 2(2):32–48
50. Perera R, Kuhn RJ (2008) Structural proteomics of dengue virus. Curr Opin Microbiol 11(4):369–377. https://doi.org/10.1016/j.mib.2008.06.004. Epub 2008 Jul 31. Review. PMID:18644250
51. Phoo WW, Li Y, Zhang Z, Lee MY, Loh YR, Tan YB, Ng EY, Lescar J, Kang CB, Luo D (2016) Structure of the NS2B-NS3 protease form Zika virus caught after self- cleavage. Nat Commun 7:13410. https://doi.org/10.1038/ncomms13410
52. Puig-Basagoiti F, Tilgner M, Bennett CJ, Zhou Y, Munoz-Jordan JL, Garcia-Sastre A, Bernard KA, Shi P-Y (2007) A mouse cell adapted NS4B mutation attenuates West-Nile virus RNA synthesis. Virology 361:229–241
53. Quinkert D, Bartenschlager R, Lohmann V (2005) Quantitative analysis of the hepatitis C virus replication complex. J Virol 79:13594–13605
54. Ritchie TK, Grinkova YV, Bayburt TH, Denisov IG, Zolnerciks JK, Atkins WM, Sligar SG (2009) Reconstitution of membrane proteins in phospholipid bilayer nanodiscs. Methods Enzymol 464:211–231
55. Robinson L, Tharakaraman K, Rowley K, Costa VV, Chan KR, Wong YH, Ong LC, Tan HC, Koch

T, Cain D, Kirloskar R, Viswanathan K, Liew CW, Tissire H, Ramakrishnan B, Myette J, Babcock GJ, Sasisekharan V, Alonso S, Chen J, Lescar J, Shriver Z, Ooi EE, Sasisekharan R (2015) Structure-guided design of an anti-dengue antibody directed to a non-immunodominant epitope. Cell 162:493–504
56. Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, Suvannadabba S, Jiwariyavej V, Dulyachai W, Pengsaa K, Wartel TA, Moureau A, Saville M, Bouckenooghe A, Viviani S, Tornieporth NG, Lang J (2012) Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomized, controlled phase 2b trial. Lancet 380(9853):1559–1567
57. Saw WG, Tria G, Grüber A, Subramanian Manimekalai MS, Zhao Y, Chandramohan A, Srinivasan Anand G, Matsui T, Weiss TM, Vasudevan SG, Grüber G (2015) Structural insight and flexible features of NS5 proteins from all four serotypes of dengue virus in solution. Acta Crystallogr D Biol Crystallogr 71(Pt 11):2309–2327
58. Sessions OM et al (2009) Discovery of insect and human dengue virus host factors. Nature 458:1047–1050
59. Sofia MJ (2016) Enter Sofosbuvir: the path to curing HCV. Cell 167(1):25–29
60. Stern O, Hung YF, Valdau O, Yaffe Y, Harris E, Hoffmann S, Willbold D, Sklan EH (2013) An N-terminal amphipathic helix in dengue virus nonstructural protein 4A mediates oligomerization and is essential for replication. J Virol 87:4080–4085
61. Tay M, Saw WG, Zhao YQ, Chan WK, Singh D, Chong Y, Forwood JK, Ooi EE, Gruber G, Lescar J, Luo D, Vasudevan SG (2015) The C-terminal 50 amino acid residues of dengue NS3 protein are important for NS3-NS5 interaction and viral replication. J Biol Chem 290:2379–2394
62. Tay MYF, Smith K, Ng IHW, Chan KWK, Zhao Y, Lescar J, Luo D, Jans DA, Forwood JK, Vasudevan SG (2016) The C-terminal 18 amino acid region of dengue virus NS5 regulates its subcellular localization and contains a conserved arginine residue essential for Infectious Virus Production. PLoS Pathog 12(9):e1005886. https://doi.org/10.1371/journal.ppat.1005886
63. Theiler M, Smith HH (1937) The use of yellow fever virus modified by in vitro cultivation for human immunization. J Exp Med 65:787–800
64. Uchil PD, Satchidanandam V (2003) Architecture of the flaviviral replication complex. J Biol Chem 278:24388–24398
65. Umareddy I, Chao A, Sampath A, Gu F, Vasudevan SG (2006) Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. J Gen Virol 87(Pt 9):2605–2614
66. Waltzthoeni T, Leitner A, Stengel F, Aebersold R (2013) Mass spectrometry supported determination of protein complex structure. Current Opin Struct Biol 23:252–260
67. Wang QY, Dong H, Zou B, Karuna R, Wan KF, Zou J, Susila A, Xu H, Ding M, Chan WL, Gu F, Seah PG, Liu W, Lakshminarayana SB, Kang C, Lescar J, Blasco F, Smith P, Shi P-Y (2015) Discovery of dengue virus NS4B inhibitors. J Virol 89:8233–8244
68. Welsch S et al (2009) Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host & Microbes 5:365–375
69. Wicker J, Whiteman MC, Beasley DWC, Davis CT, Zhang S, Schneider BS, Higgs S, Kinney RM, Barrett ADT (2006) A single amino-acid substitution in the central portion of the West-Nile virus NS4B protein confers a highly attenuated phenotype in mice. Virology 349:245–253
70. Wicker JA, Whiteman MC, Beasley DWC, Davis CT, McGee CE, Lee C-JC, Higgs S, Kinney RM, CYH H, Barrett ADT (2012) Mutational analysis of the West-Nile virus NS4B protein. Virology 426:22–33
71. Xia Y, Yu H, Jansen R, Seringhaus M, Baxter S, Greenbaum D, Zhao H, Gerstein M (2004) Analyzing cellular biochemistry in terms of molecular networks. Annu Rev Biochem 73:1051–1087
72. Xie X, Wang QY, Xu HY, Qing M, Kramer L, Yuan Z, Shi PY (2011) Inhibition of dengue virus by targeting viral NS4B protein. J Virol 85(21):11183–11195
73. Xie X, Gayen S, Kang C, Yuan Z, Shi PY (2013) Membrane topology and function of dengue virus NS2A protein. J Virol 87(8):4609–4622
74. Xie X, Zou J, Wang Q-Y, Noble CG, Lescar J, Shi P-Y (2014) Generation and characterization of two mouse monoclonal antibodies against NS4B protein of dengue virus. Virology 450–451:250–257
75. Xie X, Zou J, Wang QY, Shi PY (2015) Targeting dengue virus NS4B protein for drug discovery. Antivir Res 118:39–45. https://doi.org/10.1016/j.antiviral.2015.03.007
76. Yap TL, Xu T, Chen YL, Malet H, Egloff M-P, Canard B, Vasudevan SG, Lescar J (2007) The crystal structure of the dengue virus RNA-dependent RNA polymerase at 1.85 Å resolution. J Virol 81:4753–4765
77. Youn S, McCune BT, Edeling MA, Fremont DH, Cristea IM, Diamond MS (2012) Evidence for a genetic and physical interaction between nonstructural proteins NS1 and NS4B that modulate replication of West Nile virus. J Virol 86:7360–7371
78. Zhao Y, Soh S, Chung KY, Lim SP, Swaminathan K, Vasudevan SG, Shi PY, Lescar J, Luo D (2015) Molecular basis for viral RNA recognition and 2'O ribose methylation by the dengue virus NS5 protein. Proc Natl Acad Sci U S A 112(48):14834–14839

79. Zhao Y, Soh S, Zheng J, Chan KWK, Phoo WW, Swaminathan K, Cornvik TC, Lim SP, Shi PY, Lescar J, Vasudevan SG, Luo D (2015) A crystal structure of the dengue virus NS5 protein reveals a novel inter-domain interface essential for protein flexibility and virus replication. PLoS Pathog 11(3):e1004682. https://doi.org/10.1371/journal.ppat.1004682
80. Zou G, Chen Y-L, Dong H, Lim CC, Yap LJ, Yau YH, Geifman Shochat S, Lescar J, Shi P-Y (2011) Functional analysis of two cavities in flavivirus NS5 polymerase. J Biol Chem 286:14362–14372
81. Zou J, Xie X, Lee LT, Chandrasekaran R, Reynaud A, Yap L, Wang Q-Y, Dong HP, Kang C, Yuan Z, Lescar J, Shi P-Y (2014) Dimerization of flavivirus NS4B protein. J Virol 88:3379–3391
82. Zou J, Lee LT, Wang QY, Xie X, Lu S, Yau YH, Yuan Z, Geifman Shochat S, Kang C, Lescar J, Shi P-Y (2015) Mapping the interactions between the NS4B and NS3 proteins of dengue virus. J Virol 89:3471–3483

10 The Structure of the Zika Virus Protease, NS2B/NS3^{pro}

Rolf Hilgenfeld, Jian Lei, and Linlin Zhang

Abstract

In this chapter, we first briefly review the history of Zika virus (ZIKV) over the past 70 years since its discovery. We then focus on the ZIKV NS2B/NS3 protease, a major potential target for anti-ZIKV therapeutics. We describe the structure of the complex between Zika virus NS2B-NS3 protease and a peptide boronic-acid inhibitor that we determined in early 2016. We then review other structural studies on the Zika virus protease, which have been published in the past few months. Three different types of construct for the protease have been investigated by X-ray crystallography and NMR spectroscopy: the traditional "linked" construct comprising the NS2B cofactor, a $Gly_4SerGly_4$ linker, and the NS3^{pro} chain; a construct where the linker has been replaced by Lys-Thr-Gly-Lys-Arg, which leads to autocleavage; and the bimolecular "unlinked" protease consisting of the NS2B cofactor segment and NS3^{pro}. In complex with an inhibitor, the protease adopts a closed, "active" conformation with the NS2B chain wrapped around the NS3^{pro} and contributing to the S2 pocket. In the ligand-free state, the $Gly_4SerGly_4$-linked enzyme adopts an open or relaxed conformation, with the C-terminal half of the NS2B cofactor highly flexible and disordered. Very surprisingly, however, the "unlinked", bimolecular protease has been reported to adopt the closed conformation in the crystal, even though, apparently, no peptide was bound to the substrate-binding site. The $Gly_4SerGly_4$-linked enzyme has been used successfully in drug discovery efforts.

Keywords

Zika virus history · Zika virus disease · congenital Zika syndrome · Zika virus protease · boronic-acid inhibitor · Zika virus protease dimerization · X-ray crystallography · NMR spectroscopy · anti-Zika virus drug discovery

R. Hilgenfeld (✉) · L. Zhang
Institute of Biochemistry, Center for Structural and Cell Biology in Medicine, University of Lübeck, Ratzeburger Allee 160, 23562 Lübeck, Germany

German Center for Infection Research (DZIF), Hamburg – Lübeck – Borstel – Riems Site, University of Lübeck, Lübeck, Germany
e-mail: hilgenfeld@biochem.uni-luebeck.de

J. Lei
Institute of Biochemistry, Center for Structural and Cell Biology in Medicine, University of Lübeck, Ratzeburger Allee 160, 23562 Lübeck, Germany

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_10

10.1 Introduction

10.1.1 History of Zika Virus Discoveries and Outbreaks

Zika virus (ZIKV) is a mosquito-borne flavivirus related to other members of the genus, such as Dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV), and Japanese encephalitis virus (JEV). The virus was discovered in 1947 in a sentinel monkey in the Zika forest of Uganda [10]. In these early studies, a pronounced neurotropism of the virus in mice was noted, and antibodies were found in monkeys and humans living near the area of first discovery of the virus [11]. Serological evidence for ZIKV infection of humans was obtained in the 1950s in Mozambique ([19]; see Chap. 2 of this volume). The first human ZIKV infection was described by Simpson [49], who after a self-experiment (or a laboratory accident?) reported headache, maculopapular rash all over the body, low-grade fever, and slight malaise. Along with arthralgia and conjunctivitis, these symptoms remain common for ZIKV infections today. The majority of ZIKV cases are either asymptomatic or connected to relatively benign disease.

In 1953, Smithburn tested blood samples collected in Malaysia for protective antibodies against ZIKV and concluded that "*it seems likely that Zika virus occurs in both Malaya and Borneo, but there is a possibility that some of the instances of protection against this agent may be due to infection with a heterologous agent which is antigenically related*" [51]. This was the first report on ZIKV in Asia. In 1967, ZIKV was isolated from *Aedes aegypti* mosquitoes in Malaysia [32]. In subsequent years, serological evidence for human ZIKV infection was obtained in several East, Central, and West African countries as well as in India and South-East Asia (see, e.g., [38, 39]). Further, ZIKV has been isolated from humans in Nigeria [12, 13, 33] and other West African countries; caution is advised with some of the earlier reports from this region, because the virus studied was probably the highly related Spondweni virus, which could easily be mistaken for ZIKV [1].

Whereas the number of reported human cases of ZIKV disease stayed very small (<20) until 2006, the first outbreak that deserved this designation occurred in 2007 on Yap Island (Micronesia) in the Pacific Ocean, where 73% of the population was infected. This outbreak was characterized by rash, arthralgia, and conjunctivitis [17]. Subsequently, the virus was introduced in other islands of the Pacific, in particular those of French Polynesia, where a major outbreak in 2013–2014 affected 28,000 people (11% of the population) [34, 35]. Here again, patients presented with fever, maculopapular rash, arthralgia, and conjunctivitis [35]. Also, during and after the outbreak in French Polynesia, neurological symptoms such as Guillain–Barré syndrome (GBS) were reported for the first time to be a consequence of ZIKV infection [4].

In 2014, ZIKV reached the Easter Islands (Chile) [44, 53]. Following this, many cases of ZIKV infections were reported from the Northeast of Brazil in early 2015 [5]. Several South American countries reported autochthonous cases of ZIKV infection, which subsequently culminated in the massive 2015–2016 outbreak in South and Central America. It is estimated that the number of cases in Brazil amounted to at least 220,000 in 2015 [2]; other authors estimated the number of infected people in the Americas as 1–1.5 million [1]. At a time when the epidemic was declining in the Americas, at the end of August, 2016, more than 450 cases of ZIKV infection were recorded in an unrelated outbreak in Singapore [50].

10.1.2 Congenital Zika Syndrome and Zika Virus Transmission

Only during the recent outbreak in Latin America was a correlation between microcephaly of newborns and ZIKV infection of pregnant women observed [42], and retrospective analysis of the ZIKV outbreak in French Polynesia (2013–2014) retrieved the same phenomenon [6]. Along with microcephaly, additional neonatal malformations caused by ZIKV infection of the mother and detected during and after the outbreak in the

Americas, such as ocular abnormalities, deafness, central nervous system lesions (e.g., calcifications), and developmental retardation, are now collectively called “congenital Zika virus syndrome” (CZS; [1]).

Mosquitoes of *Aedes spp*. were identified as a vector of ZIKV early on [15]. The main vector is *Aedes aegypti*, the mosquito that also transmits dengue virus, Chikungunya virus, and – in urban settings – yellow fever virus, so that it is no surprise that ZIKV is commonly found in regions where at least the two former are present as well. However, different from other flaviviruses, ZIKV has also been shown to spread by mother-to-fetus transmission, by blood transfusion, and by sexual activities (see [1], for a review). Long-term persistence of ZIKV in the male and female genitourinary tract has been demonstrated; thus, high levels of virus can be found in sperm months after the resolution of symptoms [36]. It has been shown in male mice that the presence of ZIKV can lead to damage of the testes and infertility [30]; so far, it is unclear whether this observation is of relevance for humans.

10.1.3 Genomics and Proteomics of Zika Virus

Two major lineages of ZIKV have been described: an African strain and an Asian strain. The outbreak strains in French Polynesia and in the Americas were closely related to the Asian lineage. The ZIKV strain involved in the 2016 Singapore outbreak corresponded to a distinct branch from the traditional Asian strains and was clearly different from the strains causing the epidemic in the Americas [50].

The ~11-kb RNA genome of flaviviruses is directly translated into a single large polyprotein (with >3000 amino-acid residues) that is cotranslationally incorporated into the membrane of the endoplasmic reticulum (ER; Fig. 10.1). Here, the polyprotein is processed to yield three structural proteins – capsid (C), pre-membrane (prM), and envelope (E) – that form the new virus particle, and 7 non-structural proteins – NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 – that are mainly involved in RNA replication but also in counteracting the innate immune system of the host cell and in virus-particle formation. Polyprotein processing is performed by the host-cell signalase on the lumenal side of the ER membrane and by the viral NS2B/NS3 protease on its cytoplasmic side (Fig. 10.1). Subsequently, the pr peptide is cleaved off the prM protein in the *trans*-Golgi network by the host-cell protease furin.

Non-structural protein 3 (NS3) of ZIKV is a multifunctional enzyme; it has protease, helicase, nucleoside triphosphatase (NTPase), and RNA triphosphatase activities. The protease domain (NS3pro) comprises residues 1–170, i.e. it resides in the N-terminal third of the NS3 protein, whereas the remaining activities are connected

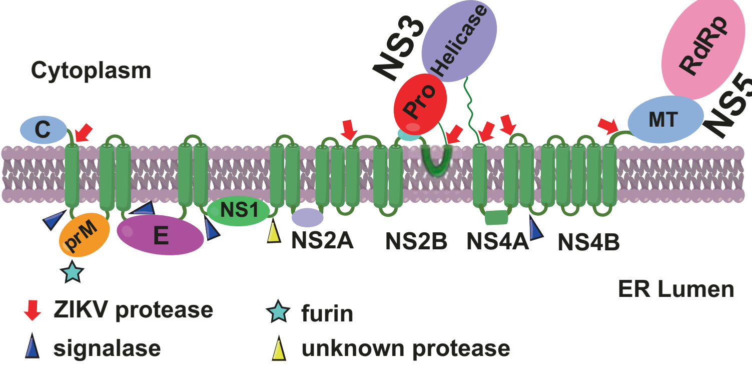


Fig. 10.1 Topology diagram of the ZIKV polyprotein incorporated into the ER membrane. The cleavage sites for viral and host-cell proteases are indicated. *C* capsid protein, *prM* pre-membrane protein, *E* envelope protein, *NS* non-structural protein, *Pro* protease, *MT* methyltransferase, *RdRp* RNA-dependent RNA polymerase

with the helicase domain (residues 180–617). The NS3^{pro} is a serine protease of the chymotrypsin type, with the classical catalytic triad Ser135 – His51 – Asp75. Proper folding and catalytic activity of the NS3^{pro} domain require NS2B as a cofactor. Similar to other flaviviruses [9], ZIKV NS2B is tightly associated with the ER membrane and thus provides the membrane anchor for NS3 (Fig. 10.1). It features three domains, among which the N- and C-terminal ones comprise several transmembrane helices, whereas the central segment (residues 45–96) is hydrophilic. It is this latter region that acts as the cofactor for the NS3^{pro}.

Previous studies have revealed that the flavivirus NS2B/NS3^{pro} cleaves the polyprotein C-terminal to dibasic sites such as Arg-Arg and Lys-Arg [24, 43, 55]. ZIKV NS3^{pro} exhibits a similar cleavage preference ([14, 46]; see below).

10.2 Structure of the Zika Virus NS2B-NS3^{pro} in Complex with a Boronate Inhibitor

Leung et al. [23] showed for DENV2 NS2B/NS3^{pro} that the hydrophilic segment of NS2B comprising residues 49–95 is sufficient to activate the NS3 protease, when it is linked to the NS3^{pro} N-terminus *via* an artificial $Gly_4SerGly_4$ linker. Over the years, this design very much facilitated functional and structural studies on the flavivirus proteases; the linker itself is highly flexible and does not show up in the electron-density map for any flavivirus protease. It is this "linked form" what we used in our studies of the ZIKV NS2B-NS3^{pro} (of a Brazilian outbreak strain). In July, 2016, we reported the first crystal structure of this enzyme ([22], PDB entry 5LC0). We had cocrystallized the enzyme with a capped dipeptide boronic-acid inhibitor (cn-716; benzoyl-4-aminomethylphenylalanyl-arginyl boronic acid; see Fig. 10.2) synthesized by the group of C.D. Klein ([37]; see also Chap. 13 of this volume). This inhibitor reversibly deactivates the recombinant ZIKV NS2B-NS3 protease with IC_{50} = 0.25 ± 0.02 μM and inhibition constant (K_i) = 0.040 ± 0.006 μM (in the presence of 20% glycerol; [22]).

The structure reveals the canonical chymotrypsin fold for NS3^{pro}, with the β-strand 52*-57* of NS2B (the asterisk, *, indicates a residue from NS2B) inserted into the N-terminal β-barrel of the protease domain (Fig. 10.3a). The structure is in the closed conformation, with residues 74*–86* of the C-terminal part of $NS2B_{49^*\text{-}95^*}$ forming a β-hairpin involved in shaping the S2 subsite of the protease. The electrophilic boron atom in the warhead of the inhibitor accepts a 1.60-Å covalent bond from the Oγ of Ser135, the catalytic nucleophile of the NS3 protease (Fig. 10.3b). Moreover, the boronic acid moiety forms a six-membered cyclic diester with glycerol (which we had present in all steps of enzyme preparation and crystallization) (Fig. 10.4). The formation of

Fig. 10.2 Structural formula of the boronic-acid compound cn-716 [37]

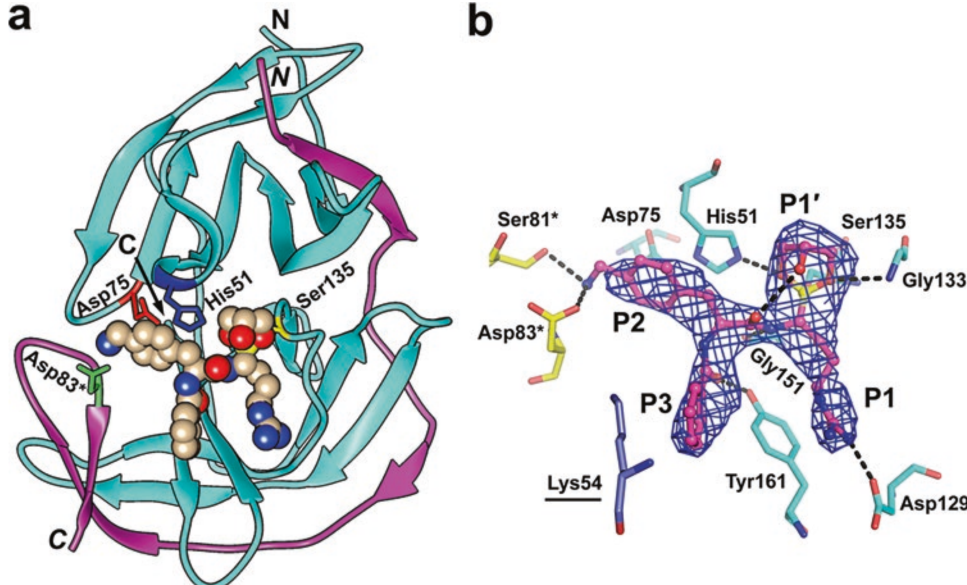


Fig. 10.3 (**a**) Structure (cartoon presentation) of the ZIKV NS2B-NS3pro in complex with the boronic-acid inhibitor, cn-716. The NS2B chain is depicted in purple, the NS3 chain in light blue. N- and C-termini are labelled (in *italics* for NS2B). The inhibitor is shown in the space-filling mode, with carbon atoms in wheat, oxygen in red, nitrogen in blue, boron in yellow. Residues of the catalytic triad, Ser135, His51, and Asp75 are indicated, as is Asp83* of NS2B. (**b**) Interactions between the boronic-acid inhibitor cn-716 and the ZIKV NS2B-NS3 protease. Hydrogen bonds are indicated by dashed lines. Carbon atoms of NS2B and NS3 are yellow and light blue, resp., nitrogen atoms are blue, and oxygen atoms are red. Carbon atoms of the inhibitor are orange, and the boron is yellow. The F_o-F_c electron density for the inhibitor is shown (contoured at 2.5σ). Lys54 from the other monomer in the dimer interacts with cn-716 and is indicated by underlined "Lys54".
Images adapted from Lei et al., Science 353, 503–505 (2016). Image (**a**) prepared by using Chimera [40], whereas image (**b**) was prepared with Pymol (Schrödinger; http://www.pymol.org/)

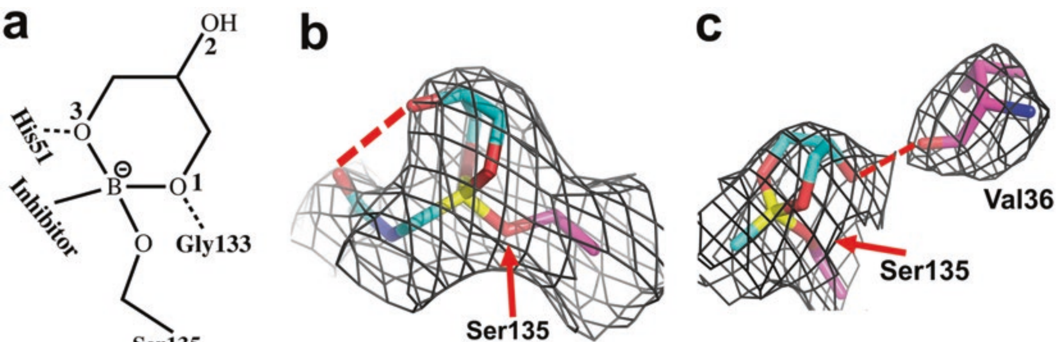


Fig. 10.4 The 6-membered boronate created by ester formation between the OH groups of the boronic acid and the terminal hydroxyl groups of glycerol. (**a**) Structural formula. (**b**, **c**) F_o–F_c difference electron density (contoured at 2.5σ) for the cyclic diester in molecule A (**b**) and molecule B (**c**) The boron atom is yellow, carbon atoms of the inhibitor and of the protein are light blue and orange, resp. Oxygen atoms are red and nitrogens are dark blue. Hydrogen bonds are indicated by dashed red lines. (Figure adapted from Lei et al., Science 353, 503–505 (2016). Images (**b**) and (**c**) prepared by using Pymol (Schrödinger; http://www.pymol.org/))

5- or 6-membered cyclic diesters between boronic acid and diols or triols is a well-established reaction; in case of the ZIKV NS2B-NS3^{pro} structure, a 6-membered ring is formed *via* reaction with the terminal hydroxyl groups of glycerol, whereas in our structure of the same inhibitor (cn-716) with the WNV protease [37], we observe a 5-membered ring formed as a result of reaction between the boronic-acid OH groups and a terminal as well as the central hydroxyl group of glycerol. In the complex with the ZIKV protease, the 6-membered boronate ring is either in a boat-like (molecule A in the asymmetric unit) or in a somewhat twisted half-chair conformation (molecule B) and neatly occupies the S1′ pocket of the enzyme, a site so far rarely addressed by synthetic inhibitors of flavivirus proteases. While the potency of the inhibitor is nearly unchanged (IC_{50} = 0.20 ± 0.02 μM) in the absence of glycerol [22], we note that boronic ester formation with larger, more hydrophobic diols or triols will likely lead to increased membrane permeability of this type of compound. In the cytosol, the diester would be hydrolyzed, releasing the boronic acid; thus, the cyclic boronates can be considered prodrugs. The remaining free central hydroxyl group of glycerol is in an axial position relative to the 6-membered ring; it makes a hydrogen bond with the carbonyl oxygen of the P2 residue of the inhibitor in molecule A, but interacts with the carbonyl oxygen of Val36 in molecule B (Fig. 10.4b, c).

The Arg residue in the P1 position of the inhibitor forms a salt-bridge with Asp129 and its guanidinium group stacks against Tyr161 of the protease (Fig. 10.3b), interactions similarly seen in inhibitor complexes of other flaviviral proteases (see, e.g., [16]). Importantly, the amino group of the 4-aminomethylphenylalanine moiety in the P2 position of cn-716 is involved in a salt-bridge with Asp83* of the NS2B chain. The latter residue is Ser or Thr in the proteases of DENV serotypes 1–4, and Asn in WNV, i.e. unable to form an ion-pair interaction with the positively charged P2 residues of substrates or inhibitors. As we have shown by changing Asp83* of ZIKV NS2B for Asn, the ion-pair interaction is partially responsible for the high catalytic activity of ZIKV NS2B-NS3^{pro} towards the fluorogenic substrate Bz-Nle-Lys-Lys-Arg-AMC, compared to other flavivirus proteases [22]. The N-cap of cn-716, benzoyl (Bz), which can be considered the P3 residue, does not occupy a specific pocket. It weakly interacts with NS3 residue Lys54 of molecule B (Fig. 10.3b) in the NS2B-NS3^{pro} dimer that has been crystallized (see below).

In this structure, an unusual tight dimer was observed, with a head-to-tail organization of the monomers, i.e. the N-terminal β-barrel of molecule A interacts with the C-terminal β-barrel of molecule B, and *vice versa* (Fig. 10.5a). This is facilitated by a high surface complementarity between these domains (Fig. 10.5b). In the dimer, the substrate-binding pockets as well as the inhibitors from the two monomers are facing each other. Although there are some weak contacts between the two inhibitor molecules in the dimer (Fig. 10.5c), its formation is not induced by the inhibitor. In our structure of the West Nile virus NS2B-NS3 protease in complex with the same inhibitor, cn-716, we only found a monomer in the crystal [37]. In fact, such a tight dimer has not been reported for other flavivirus proteases, as they appear to lack the high surface complementarity between the N-terminal domain and the C-terminal domain.

Fig. 10.5 (continued) electron density. The putative NS2B helices inserted into the ER membrane are shown in dark blue and green for molecule A and B, resp. The hydrophobic segments (Val101*–Val125* of each monomer) are probably too short to form two transmembrane helices each; hence, we have sketched an α-helix for each protomer that is embedded in the ER membrane Images (**a**, **b**, and **d**) prepared by using Pymol (Schrödinger; http://www.pymol.org/). Images (**a**), (**b**) adapted from Lei et al., Science 353, 503–505 (2016). Image (**c**) incl. legend taken from the online supplementary material to Lei et al., Science 353, 503–505 (2017) (www.sciencemag.org/content/353/6298/503/suppl/ DC1)

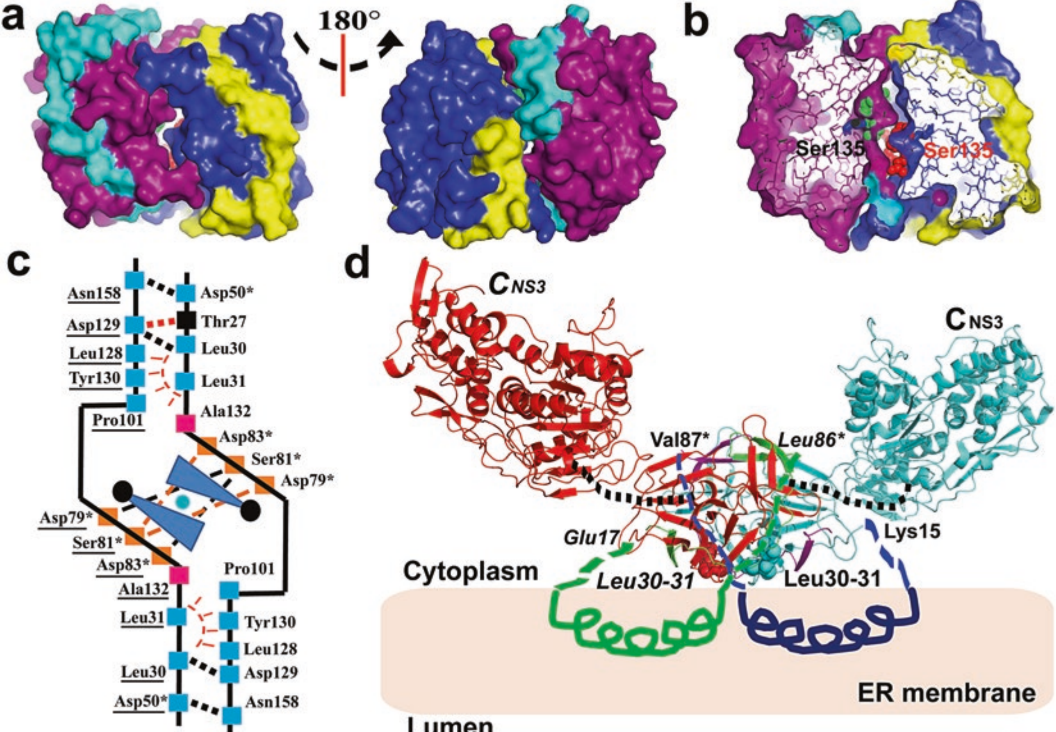


Fig. 10.5 The "tight" dimer observed in the crystal structure of the ZIKV NS2B-NS3pro in complex with the boronate inhibitor [22]. (**a**) Front view and back view. The surfaces of NS2B and NS3 are yellow and dark blue, resp., in molecule A, and light blue and purple, resp., in molecule B. Note the surface complementarity of the monomers in this non-crystallographic dimer with quasi twofold symmetry. (**b**) A slice through the interior of the dimer, showing the catalytic Ser135 side-chains covalently bound to the inhibitor molecules. The color code is the same as in (**a**). The inhibitor molecules are colored red and green. (**c**) Schematic drawing of the interactions across the monomer – monomer interface. Amino-acid residues from the NS2B chains are labeled with an asterisk (*). Residues of molecule B (left) have their labels underlined. The dimerization interface comprises a total of 9 hydrogen bonds (dashed lines) between the two monomers and a few hydrophobic interactions (red dashed arcs with radiating spokes). The inhibitor molecules are indicated by blue arrows, with the warhead symbolized as a dark ball. A water molecule located close to the non-crystallographic axis of symmetry is indicated by a light-blue sphere. This water is part of a hydrogen-bonding network connecting across the interface the side-chains of Asp79*, Ser81*, and Asp83* of each of the NS2B polypeptides, with the Ser81* side-chains of molecules A and B approaching each other closely. The NS2B-NS3 proteases of WNV and DENV-2 both carry an aspartate residue in this position, which would give rise to repulsion at physiological pH and probably prevent formation of the dimer. The dipeptide boronate inhibitor molecules also contribute to dimer formation via a hydrophobic contact between the benzoyl cap and the methylene groups (Cγ and Cδ atoms) of Lys54 of the opposing protomer. (**d**). The dimer of the ZIKV NS2B-NS3pro seen in the crystal [22] would be compatible with the helicase part of NS3 and with the interaction with the ER membrane. The model of full-length ZIKV NS3 has been composed of the crystal structures of the ZIKV NS3 protease ([22]; PDB entry 5LC0) and the ZIKV helicase ([52]; PDB entry 5JMT) superimposed onto the structure of the full-length DENV4 NS3 ([29]; PDB entry 2WHX). In this hypothetical dimer, NS2B and NS3 of molecule A are shown in purple and light blue, resp., whereas NS2B and NS3 of molecule B are displayed in green and red, resp.. Leu 30 and Leu31 ("Leu30-31") of NS3pro (in *italics* for molecule B) interact with the ER membrane. They are shown in light-blue and red spheres for the two molecules. The C-terminus of each NS3 molecule is marked (in *italics* for molecule B). Black dashed lines: 7 residues between NS3pro and NS3hel of each monomer missing in this hypothetical model; blue dashed lines indicate the total of 32 residues (molecule A) missing in this model between NS2B residue Val87* and the putative α-helix located to the membrane as well as the residues between this helix and the first residue (Lys15) from the N-terminus of NS3pro that is defined by electron density; green dashed lines indicate the total of 35 residues (molecule B) missing in this model between NS2B residue *Leu86** and the putative α-helix located to the membrane as well as the residues between this helix and the first residue (*Glu17*) from the N-terminus of NS3pro that is defined by

The tight dimer of the ZIKV protease has openings at both sides, allowing the access of substrates or inhibitors to the active center upon some "breathing". We did not detect this dimer in solutions of the enzyme:boronic-acid inhibitor complex up to a concentration of 133 μM (although we recently observed indications for its existence in solutions of the Cys80Ser/Cys143Ser double mutant of the ligand-free protease). In any case, the large buried surface (~1240 Å^2, inhibitor not included) and the high shape complementarity imply that this dimer possibly has biological relevance. It is conceivable that it is a good model for protease assemblies existing at the high local concentrations of the enzyme at the ER membrane. In fact, our crystallographic model of the dimer offers space for the helicase domain of each monomer and is also compatible with the association of NS2B with the ER membrane (Fig. 10.5d).

In the crystal, the tight dimers are loosely connected with each other *via* a disulfide bond between Cys143 of molecule A and the same residue of molecule B of a neighboring dimer, and *vice versa*. This way, the tight dimers form disulfide-linked polymers in the crystal lattice (Fig. 10.6). Interestingly, while this disulfide bond is necessary for crystallization (the Cys80Ser/Cys143Ser mutant does not form crystals), it is broken within a few seconds of diffraction data collection due to the X-irradiation. This does not affect the structure of the tight dimer.

10.3 Other Crystal Structures of the Zika Virus Protease

Later in 2016, Luo and colleagues reported a 1.8-Å crystal structure of ZIKV NS2B-NS3^{pro} using a novel construct (designated "eZiPro"), in which the NS2B$_{45*-96*}$ chain is connected to the NS3^{pro} *via* the five C-terminal residues (Lys-Thr-Gly-Lys-Arg) of the full-length NS2B protein ([41]; PDB entry 5GJ4). During overexpression and purification of this protein, the NS2B-NS3^{pro} junction is subject to autocleavage, giving rise to separated NS2B and NS3^{pro} chains. Different from the Gly$_4$SerGly$_4$-linked NS2B-NS3^{pro} ([22]; designated "gZiPro" by [41]), two pairs of NS2B/NS3^{pro} homodimers were observed per asymmetric unit of the crystal. The structure adopts a closed conformation as found in the boronate inhibitor complex, with the NS2B fragment wrapping around the NS3^{pro}. Binding of the L-shaped Thr-Gly-Lys-Arg C-terminal tetrapeptide of NS2B in the substrate-binding site, with Arg in the S1 subsite and Lys in S2, induces the closed conformation of the protease. The catalytic activity is relatively low towards the substrate Bz-Nle-Lys-Arg-Arg-AMC because of competition with the Thr-Gly-Lys-Arg tetrapeptide of the NS2B C-terminus in the substrate-binding site.

Subsequently, the crystal structure of an unlinked ZIKV NS2B/NS3^{pro} lacking the C-terminal tetrapeptide of NS2B (designated "bZiPro") was reported at 1.58 Å resolution by the same group ([57]; PDB entry 5GPI). Although the enzyme is described as being devoid of bound substrate or product peptides, all four of the unlinked ZIKV NS2B/NS3^{pro} molecules in the asymmetric unit surprisingly adopt the closed conformation, with the NS2B fragment fully wrapped around NS3^{pro}. In the "linked" flavivirus protease, this form is normally only present in structures of complexes with substrates or inhibitors. In the Zhang et al. [57] structure, three of the four NS2B/NS3^{pro} molecules in the asymmetric unit are described as ligand-free enzyme, whereas one NS2B/NS3^{pro} is bound to the N-terminal Lys14-Lys15-Gly16-Glu17 tetrapeptide segment of a neighboring NS3^{pro}, with the peptide located in the substrate-binding site in a reverse orientation, compared to common peptidic inhibitors. According to the authors, the crystal structure is highly similar to the structure of the Gly$_4$SerGly$_4$-linked ZIKV NS2B-NS3^{pro} in complex with the boronate inhibitor [22] and the cleaved Lys-Thr-Gly-Lys-Arg-linked NS2B-NS3^{pro} [41], with RMSD values of 0.52 Å and 0.45 Å, respectively, for Cα atoms. In addition, a crystal structure of unlinked ZIKV NS2B/NS3^{pro} in complex with a dipeptide aldehyde (Ac-Lys-Arg-CHO) was determined by Li et al. ([26]; PDB entry 5H6V). The structure adopts the closed conformation, which is similar to other structures determined

Fig. 10.6 Structure of the disulfide-linked polymer of tight dimers in crystals of the ZIKV NS2B-NS3pro complex with the boronate inhibitor. In each tight dimer, molecules A and B are shown in light blue and purple, resp.. The corresponding inhibitors are displayed as red and dark blue spheres. Residues Cys143 forming the disulfide bonds are shown in yellow spheres. (Figure prepared by using Pymol (Schrödinger; http://www.pymol.org/))

earlier, with RMSD values for Cα atoms of 0.50 Å ([22]; PDB entry 5LC0), 0.20 Å ([57]; PDB entry 5H4I), and 0.39 Å ([41]; PDB entry 5GJ4), respectively. The inhibitor forms a covalent bond with Ser135 of the catalytic triad, and the Arg in P1 and Lys in P2 interact with the target protein *via* several hydrogen bonds. Interestingly, a dimer was not observed in this complex structure. ^{1}H-^{15}N HSQC (heteronuclear single-quantum coherence) NMR spectra indicate that in solution, the aldehyde inhibitor also binds to the active site and can stabilize the protein structure in the closed conformation.

Usually, flavivirus NS2B-NS3 proteases adopt an open conformation in the absence of inhibitors or substrates. In this conformation, the N-terminal β-strand of the hydrophilic NS2B segment still integrates into the N-terminal β-barrel of NS3pro, but much of the NS2B chain beyond residue 60* is highly flexible and often not defined by electron density in the crystal structures. This feature was also reported by Yang and colleagues ([8]; PDB entry 5GXJ) for the unliganded $Gly_4SerGly_4$-linked ZIKV NS2B-NS3pro at a resolution of 2.6 Å. In stark contrast to the structure of the unlinked free enzyme reported by Zhang et al. [57], the C-terminal part of the NS2B chain is largely invisible in the electron density map. Subsequently, Lee et al. [21] also determined a crystal structure of the free "linked" ZIKV NS2B-NS3pro enzyme at 3.1 Å resolution (PDB entry 5T1V). In both the Chen et al. [8] and Lee et al. [21] structures, the C-terminal portion of the NS3pro polypeptide (beyond residue 152) is oriented away from the substrate-binding site and instead makes hydrophobic interactions with the linker between the two NS3pro β-barrels (Fig. 10.7a). In the closed conformation, this segment forms the β-hairpin β-EIIb/β-FII, which participates through hydrophobic and π...π interactions in shaping the S1 subsite for accommodating the P1-Arg residue of the inhibitor (Fig. 10.7b) [22]. Chen et al. [8] call the conformation of the ligand-free ZIKV NS2B-NS3pro "relaxed" and Lee et al. [21] "pre-open" (the RMSD between the two structures is 0.33 Å for the Cα atoms; [21]). Possibly, the C-terminal region of NS3pro acts as a switch between the "active-closed conformation" and the "inactive-relaxed conformation" of the ZIKV NS2B-NS3pro. Such a switch has not been described for the NS2B-NS3 proteases of DENV or WNV, but in the JEV enzyme, a similar observation ([54]; PDB entry 4R8T) can be made. Potentially, this feature may offer novel opportunities for discovering inhibitors that block the transition between the open and the closed conformation of the enzyme.

Coincidentally, a dimer is found in the asymmetric unit in both of the ligand-free "linked" protease structures, with the catalytic sites of the two monomers facing each other [8, 21], similar to the "tight dimer" found by us in the boronate inhibitor complex [22]. However, the surface buried upon dimerization is considerably smaller (~800 Å^2) compared to the structure of the boronate

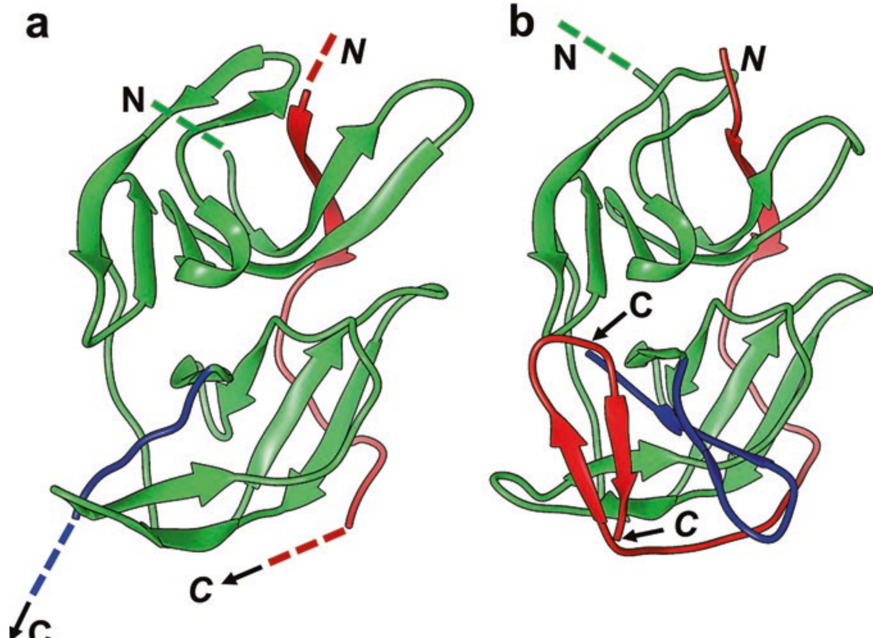


Fig. 10.7 Structure of free "linked" ZIKV NS2B-NS3^{pro} [8] (**a**) in comparison with the closed "linked" structure [22] (**b**): The NS2B chain is shown in red, the NS3 chain in green, except for its C-terminal region (beyond residue 152), which is colored dark blue to highlight the different conformation in the open, ligand-free form (**a**) and the closed, ligand-bound form (**b**) of the protease. Figure prepared by Chimera [40]

complex dimer. Nevertheless, this observation is another indication that the unique dimer observed by us for the ZIKV NS2B-NS3^{pro} is not induced by inhibitor binding.

10.4 NMR Studies of the Zika Virus Protease

In addition to the extensive studies of the ZIKV NS2B-/NS3^{pro} by X-ray crystallography, several groups also investigated the conformations of different constructs in solution by nuclear magnetic resonance (NMR). Phoo et al. [41] recorded ^{1}H-^{15}N spectra for the three different constructs of the inhibitor-free protease mentioned above, i.e. eZiPro (NS2B$_{45*-96*}$-Lys-Thr-Gly-Lys-Arg-NS3^{pro}), gZiPro (NS2B$_{45*-96*}$-Gly$_4$SerGly$_4$-NS3^{pro}), and bZiPro, "unlinked" NS2B/NS3^{pro}). These three constructs share a similar overall fold, but with many chemical shifts differing between the eZiPro and bZiPro proteases. Residues near the active site display sharper signals in the eZiPro, indicating that the C-terminal Thr-Gly-Lys-Arg tetrapeptide of NS2B predominantly occupies the substrate-binding site as seen in the crystal structure. In the absence of substrate or inhibitor in the binding site, line-broadening of the residues located in the protease active site is caused by exchanges with the environment in the "unlinked" bZiPro.

A remarkable difference between the ZIKV NS2B-NS3^{pro} and the proteases of DENV and WNV concerns the affinity for the bovine pancreatic trypsin inhibitor (BPTI, aprotinin). In NMR studies with the three different ZIKV constructs mentioned above [41], only bZiPro shows obvious chemical shift changes upon addition of BPTI. Therefore, the C-terminal Thr-Gly-Lys-Arg of eZiPro or the Gly$_4$SerGly$_4$ linker in gZiPro may compete with the binding of BPTI to the substrate-binding site of the protease. Similar observations were made by Roy et al. [45]. The dispersed cross peaks in the ^{1}H-^{15}N HSQC spectra for bZiPro [57] demonstrate that this "unlinked" protease construct is well-folded and

features a secondary structure similar to that of the crystal structure presented by the same group. On the other hand, Roy et al. [45] reported complete disorder for the C-terminal region of NS2B (residues 73*-100*) in the solution structure of the ligand-free, unlinked protease, supporting the predominance of the open conformation in the free two-chain ZIKV NS2B-NS3[pro]. Mahawaththa et al. [31] studied the conformation in solution of a $Gly_4SerGly_4$-linked ZIKV NS2B-NS3[pro] construct highly similar to the one we used for our crystallographic work [22]. The authors showed that upon titration of the enzyme with the boronic-acid compound cn-716 [37], new cross-peaks of the NS2B chain appear that exhibit similar chemical shifts as observed for eZiPro [41], implying that the $Gly_4SerGly_4$-linked construct adopts the closed conformation in solution upon binding the high-affinity inhibitor. Li et al. [25] also assigned the cross-peaks in the ^1H-^{15}N HSQC spectrum of the $Gly_4SerGly_4$-linked ZIKV NS2B-NS3[pro] in the absence of inhibitor and found the peaks corresponding to the β-hairpin of NS2B (residues 74*–86*) missing, revealing the open conformation, in agreement with the crystallographic results of Chen et al. [8] and Lee et al. [21]. Furthermore, the ^1H-^{15}N HSQC and NOE data suggested that the peptide Ac-Lys-Arg binds to the substrate-binding site and helps the protease adopt the closed conformation. As the chemical shifts of residues close to the active site (e.g., His51) show distinct differences between the $Gly_4SerGly_4$-linked (gZiPro) and unlinked (bZiPro) ZIKV NS2B-/NS3[pro] constructs, these authors proposed that the flexible linker may influence the substrate-binding site in solution.

10.5 Design of and Screening for Inhibitors of the ZIKV Protease

With the ZIKV NS2B-NS3 protease being an attractive drug target, several groups engaged in inhibitor discovery efforts against this enzyme [3, 7, 20, 21, 27, 28, 37, 45–48, 56]. For example, Yuan et al. [56] employed virtual screening against the crystal structure of the $Gly_4SerGly_4$-linked protease [22] and identified the FDA-approved drug novobiocin as an inhibitor. The survival rate of novobiocin-treated ZIKV-infected mice was 100%, *vs.* 0% among untreated mice. Novobiocin-treated mice exhibited lower mean viral loads in blood and tissue and less histopathological damage than the control group. Notably, novobiocin has been approved for usage in pregnant women.

Investigation of the cleavage preference of ZIKV NS2B-NS3[pro] can generate valuable information for the design and improvement of peptidomimetic inhibitors against this drug target. Gruba et al. [14] and Rut et al. [46] presented extensive studies of the cleavage preferences of the ZIKV NS2B-NS3[pro] by using peptide libraries with natural (Gruba et al.) and natural as well as non-natural (Rut et al.) amino acids. Rut et al. [46] showed that the S1 pocket of the enzyme has a strong preference for *L*-Arg(Me) and *L*-Arg, whereas the most preferred amino-acid residue for the P2 position is ornithine (*L*-Orn). Among the proteinogenic amino acids, *L*-Lys or *L*-Arg were also found to be suitable, but substantially less so [46]. The most preferred P3 residue is *L*-Lys, but less dominantly so compared to P1 and P2. The S4 site exhibits very broad specificity according to Rut et al., whereas Gruba et al. claimed that it has a strong preference towards Val. The bulky hydrophobic N-terminal 2-aminobenzoic acid cap used in the latter study might influence the binding specificity at the P4 position. Based on their extensive study of the substrate specificity of ZIKV NS2B-NS3[pro], Rut et al. designed a peptidomimetic inhibitor with a phosphonate warhead, *D*-Arg−Lys−Orn−$Arg^P(OPh)_2$, that exhibits superb inhibitory activity *in vitro*.

10.6 Conclusions

In summary, structures for multiple constructs of the ZIKV NS2B/NS3 protease, in either the “open” or the “closed” conformation, were reported within 1 year since the WHO declared the association of ZIKV with microcephaly a Public Health Emergency of International

Concern (PHEIC) [18]. The swift response of structural biologists to this emergency established the ZIKV protease as an attractive target for the discovery of antiviral drugs. It is to be hoped that the efforts towards this goal that are now underway in many research laboratories, may eventually yield efficient and safe anti-Zika virus drugs or, even better, pan-flavivirus therapeutics.

Discussion of Chapter 10 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Rolf Hilgenfeld.

Christoph Nitsche: I have just one comment: you showed this nice cartoon that the protease upon inhibitor or substrate binding goes from the apo or the open form to the closed form; however, I think that is not totally clear yet. From the crystallographers, we see open forms if there is no inhibitor, and we see closed forms if there is an inhibitor present. However, in NMR studies we see, regardless whether there is an inhibitor present or not, that the majority is in the closed form and there is only some in the open form as a minority conformer; so we do not know this yet.

Rolf Hilgenfeld: I completely agree with that. And there is an ongoing debate on the relevance of the open conformation. I only know that we find the same open conformation in different crystal forms, even with different flavivirus proteases. Although we also find, for instance with Dengue-1, another type of open conformation. But some of these open forms can be superimposed quite well, also for the NS2B part. I don't know, but I realize that in the NMR-studies, you mostly see the closed conformation.

Paul Young: Just a brief comment on the open and the closed structures. We actually looked at the closed structure of a complex with an inhibitor or a substrate. That structure implies, that given the overhanging nature of the co-factor, upon engagement with the substrate, it actually might be there. So whether it is completely overhanging and flexible with those hydrophobic parts on the other side that you see in the crystal structure or not, is a moot point. I think there has to be flexibility to some degree to allow substrate in. There is no other way of imagining that. But the subtlety of that movement is a matter of debate.

What I wanted to ask you about that dimer, however, is whether you have done any modeling of the full-length NS3 structure. Would the helicase be in the way?

Rolf Hilgenfeld: The helicase would not be in the way. The helicase would be compatible with that dimer. Full-length flavivirus NS3 probably exists in more than one conformation, and these may represent different functional states. We modelled dimeric full-length ZIKV NS3 in both the conformation seen in DENV-4 NS3 ([29]; as shown in Fig. 10.5d) and the one found in Murray Valley Encephalitis virus NS3 (work by Assenberg et al., 2009), and in either, the helicase domain would be compatible with the quasi-symmetric protease dimer that we see in our crystal structure.

Paul Young: So is there any evidence in the literature for dimeric NS3 in the cell?

Rolf Hilgenfeld: Not really, but don't forget that we work with quite an artificial system. We are lacking the membrane and we are lacking the hydrophobic portions of NS2B as you know all too well. It could well be that in the cellular situation, these hydrophobic parts even promote the dimerization, of which we only see a glimpse here.

Paul Young: Sure.

Subhash Vasudevan: Could you break this dimer by introducing single mutations? Is there any contribution in the dimer interface that you could interupt?

Rolf Hilgenfeld: Yes, I think there is a lot of opportunity to do that. We haven't done it yet, but there is quite pronounced contacts, for instance that hydrophobic hook which, as has been pointed out by Julien Lescar before, maybe involved in membrane binding by NS3; in case of Zika virus, these residues are Leu30 and Leu31. These residues really fit into a hole on the opposing monomer, but they still offer enough exposed hydrophobic surface for interaction with the ER membrane. We have a head-to-tail arrangement of this dimer – the head of monomer 1 interacts with the tail of monomer 2, and *vice versa*. There is also a total of nine hydrogen bonds across the monomer – monomer interface. So yes, there is an opportunity to play with this interface. We also had to investigate whether this dimer is only formed in the presence of this type of inhibitor, since it looks as if the inhibitor kind of contributes to dimer formation. But we determined the crystal structure of West Nile virus NS2B-NS3pro with the same inhibitor, cn-716, and there, we only see a monomer in the crystal.

Subhash Vasudevan: Your inhibitor is a very interesting inhibitor, a dipeptide but then you have the contribution of the glycerol that probably enhances the interaction. Have you made a mimic?

Rolf Hilgenfeld: Not yet, but we are of course thinking of reacting the boronic acid with longer and more hydrophobic triols and diols that form the same type of boronic ester. And then we would have a vehicle for getting the drug as a prodrug into the cell, through the membrane. The membrane permeability of these boronic acids themselves is not very good. But the modification with longer-chain triols or diols may help to get the product through the membrane, and then the diester would be hydrolyzed inside the cell. Of course, we still have a specificity problem, the specificity of these compounds for the flavivirus proteases has to be improved.

References

1. Aliota MT, Bassit L, Bradrick SS, Cox B, Garcia-Blanco MA, Gavegnano C, Friedrich TC, Golos TG, Griffin DE, Haddow AD, Kallas EG, Kitron U, Lecuit M, Magnani DM, Marrs C, Mercer N, McSweegan E, Ng LFP, O'Connor DH, Osorio JE, Ribeiro GS, Ricciardi M, Rossi SL, Saade G, Schinazi RF, Schott-Lerner GO, Shan C, Shi PY, Watkins DI, Vasilakis N, Weaver SC (2017) Zika in the Americas, year 2: what have we learned? What gaps remain? A report from the Global Virus Network. Antivir Res 144:223–246
2. Baud D, Gubler DJ, Schaub B, Lanteri MC, Musso D (2017) An update on Zika-virus infection. Lancet 390:2099–2109
3. Brecher M, Li Z, Liu B, Zhang J, Koetzner CA, Alifarag A, Jones SA, Lin Q, Kramer LD, Li H (2017) A conformational switch high-throughput screening assay and allosteric inhibition of the flavivirus NS2B-NS3 protease. PLoS Pathog 13:e1006411
4. Cao-Lormeau VM, Blake A, Mons S, Lastere S, Roche C, Vanhomwegen J, Dub T, Baudouin L, Teissier A, Larre P, Vial AL, Decam C, Choumet V, Halstead SK, Willison HJ, Musset L, Manuguerra JC, Despres P, Fournier E, Mallet HP, Musso D, Fontanet A, Neil J, Ghawche F (2016) Guillain-Barré syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet 387:1531–1539
5. Cardoso CW, Paploski IA, Kikuti M, Rodrigues MS, Silva MM, Campos GS, Sardi SI, Kitron U, Reis MG, Ribeiro GS (2015) Outbreak of exanthematous illness associated with Zika, Chikungunya, and Dengue viruses, Salvador, Brazil. Emerg Infect Dis 21:2274–2276
6. Cauchemez S, Besnard M, Bompard P, Dub T, Guillemette-Artur P, Eyrolle-Guignot D, Salje H, van Kerkhove MD, Abadie V, Garel C, Fontanet A, Mallet HP (2016) Association between Zika virus and microcephaly in French Polynesia, 2013–15: a retrospective study. Lancet 387:2125–2132
7. Chan JF, Chik KK, Yuan S, Yip CC, Zhu Z, Tee KM, Tsang JO, Chan CC, Poon VK, Lu G, Zhang AJ, Lai KK, Chan KH, Kao RY, Yuen KY (2017) Novel antiviral activity and mechanism of bromocriptine as a Zika virus NS2B-NS3 protease inhibitor. Antivir Res 141:29–37
8. Chen X, Yang K, Wu C, Chen C, Hu C, Buzovetsky O, Wang Z, Ji X, Xiong Y, Yang H (2016) Mechanisms of activation and inhibition of Zika virus NS2B-NS3 protease. Cell Res 26:1260–1263
9. Clum S, Ebner KE, Padmanabhan R (1997) Cotranslational membrane insertion of the serine proteinase precursor NS2B-NS3Pro of dengue virus type 2 is required for efficient *in vitro* processing and is mediated through the hydrophobic regions of NS2B. J Biol Chem 272:30715–30723
10. Dick GW (1952) Zika virus. II. Pathogenicity and physical properties. Trans R Soc Trop Med Hyg 46:521–534

11. Dick GWA, Kitchen SF, Haddow AJ (1952) Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg 46:509–520
12. Fagbami A (1977) Epidemiological investigations on arbovirus infections at Igbo-Ora, Nigeria. Trop Geogr Med 29:187–191
13. Fagbami AH (1979) Zika virus infections in Nigeria: virological and seroepidemiological investigations in Oyo state. J Hyg (Lond) 83:213–219
14. Gruba N, Rodriguez Martinez JI, Grzywa R, Wysocka M, Skorenski M, Burmistrz M, Lecka M, Lesner A, Sienczyk M, Pyrc K (2016) Substrate profiling of Zika virus NS2B-NS3 protease. FEBS Lett 590:3459–3468
15. Haddow AJ, Williams MC, Woodall JP, Simpson DI, Goma LK (1964) Twelve isolations of Zika virus from *Aedes (Stegomyia) africanus (Theobald)* taken in and above a Uganda forest. Bull World Health Organ 31:57–69
16. Hammamy MZ, Haase C, Hammami M, Hilgenfeld R, Steinmetzer T (2013) Development and characterization of new peptidomimetic inhibitors of the West Nile virus NS2B-NS3 protease. ChemMedChem 8:231–241
17. Hayes EB (2009) Zika virus outside Africa. Emerg Infect Dis 15:1347–1350
18. Heymann DL, Hodgson A, Sall AA, Freedman DO, Staples JE, Althabe F, Baruah K, Mahmud G, Kandun N, Vasconcelos PF, Bino S, Menon KU (2016) Zika virus and microcephaly: why is this situation a PHEIC? Lancet 387:719–721
19. Kokernot RH, Smithburn KC, Gandara AF, Mc'Intosh BM, Heymann CS (1960) Neutralization tests with sera of inhabitants of Mozambique against certain virus strains isolated in Africa and transmitted by arthropods. Anais Inst Med Trop 17:201–230. *(in Portuguese)*
20. Kuiper BD, Slater K, Spellmon N, Holcomb J, Medapureddy P, Muzzarelli KM, Yang Z, Ovadia R, Amblard F, Kovari IA, Schinazi RF, Kovari LC (2017) Increased activity of unlinked Zika virus NS2B/NS3 protease compared to linked Zika virus protease. Biochem Biophys Res Commun 492:668–673
21. Lee H, Ren J, Nocadello S, Rice AJ, Ojeda I, Light S, Minasov G, Vargas J, Nagarathnam D, Anderson WF, Johnson ME (2017) Identification of novel small molecule inhibitors against NS2B/NS3 serine protease from Zika virus. Antivir Res 139:49–58
22. Lei J, Hansen G, Nitsche C, Klein CD, Zhang L, Hilgenfeld R (2016) Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor. Science 353:503–505
23. Leung D, Schroder K, White H, Fang NX, Stoermer MJ, Abbenante G, Martin JL, Young PR, Fairlie DP (2001) Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors. J Biol Chem 276:45762–45771
24. Li J, Lim SP, Beer D, Patel V, Wen D, Tumanut C, Tully DC, Williams JA, Jiricek J, Priestle JP, Harris JL, Vasudevan SG (2005) Functional profiling of recombinant NS3 proteases from all four serotypes of dengue virus using tetrapeptide and octapeptide substrate libraries. J Biol Chem 280:28766–28774
25. Li Y, Phoo WW, Loh YR, Zhang Z, Ng EY, Wang W, Keller TH, Luo D, Kang C (2017) Structural characterization of the linked NS2B-NS3 protease of Zika virus. FEBS Lett 591:2338–2347
26. Li Y, Zhang Z, Phoo WW, Loh YR, Wang W, Liu S, Chen MW, Hung AW, Keller TH, Luo D, Kang C (2017) Structural dynamics of Zika virus NS2B-NS3 protease binding to dipeptide inhibitors. Structure 25:1242–1250
27. Li Z, Brecher M, Deng YQ, Zhang J, Sakamuru S, Liu B, Huang R, Koetzner CA, Allen CA, Jones SA, Chen H, Zhang NN, Tian M, Gao F, Lin Q, Banavali N, Zhou J, Boles N, Xia M, Kramer LD, Qin CF, Li H (2017) Existing drugs as broad-spectrum and potent inhibitors for Zika virus by targeting NS2B-NS3 interaction. Cell Res 27:1046–1064
28. Lim HJ, Nguyen TT, Kim NM, Park JS, Jang TS, Kim D (2017) Inhibitory effect of flavonoids against NS2B-NS3 protease of Zika virus and their structure activity relationship. Biotechnol Lett 39:415–421
29. Luo D, Wei N, Doan DN, Paradkar PN, Chong Y, Davidson AD, Kotaka M, Lescar J, Vasudevan SG (2010) Flexibility between the protease and helicase domains of the dengue virus NS3 protein conferred by the linker region and its functional implications. J Biol Chem 285:18817–18827
30. Ma W, Li S, Ma S, Jia L, Zhang F, Zhang Y, Zhang J, Wong G, Zhang S, Lu X, Liu M, Yan J, Li W, Qin C, Han D, Qin C, Wang N, Li X, Gao GF (2016) Zika virus causes testis damage and leads to male infertility in mice. Cell 167:1511–1524
31. Mahawaththa MC, Pearce BJG, Szabo M, Graham B, Klein CD, Nitsche C, Otting G (2017) Solution conformations of a linked construct of the Zika virus NS2B-NS3 protease. Antivir Res 142:141–147
32. Marchette NJ, Garcia R, Rudnick A (1969) Isolation of Zika virus from *Aedes aegypti* mosquitoes in Malaysia. Am J Trop Med Hyg 18:411–415
33. Moore DL, Causey OR, Carey DE, Reddy S, Cooke AR, Akinkugbe FM, David-West TS, Kemp GE (1975) Arthropod-borne viral infections of man in Nigeria, 1964–1970. Ann Trop Med Parasitol 69:49–64
34. Musso D (2015) Zika virus transmission from French polynesia to Brazil. Emerg Infect Dis 21:1887
35. Musso D, Nilles EJ, Cao-Lormeau VM (2014) Rapid spread of emerging Zika virus in the Pacific area. Clin Microbiol Infect 20:O595–O596
36. Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau VM (2015) Potential sexual transmission of Zika virus. Emerg Infect Dis 21:359–361
37. Nitsche C, Zhang L, Weigel LF, Schilz J, Graf D, Bartenschlager R, Hilgenfeld R, Klein CD (2017) Peptide-boronic acid inhibitors of flaviviral proteases: Medicinal chemistry and structural biology. J Med Chem 60:511–516

38. Olson JG, Ksiazek TG, Suhandiman T (1981) Zika virus, a cause of fever in Central Java, Indonesia. Trans R Soc Trop Med Hyg 75:389–393
39. Olson JG, Ksiazek TG, Gubler DJ, Lubis SI, Simanjuntak G, Lee VH, Nalim S, Juslis K, See R (1983) A survey for arboviral antibodies in sera of humans and animals in Lombok, Republic of Indonesia. Ann Trop Med Parasitol 77:131–137
40. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF Chimera – a visualization system for exploratory research and analysis. J Comput Chem 25:1605–1612
41. Phoo WW, Li Y, Zhang Z, Lee MY, Loh YR, Tan YB, Ng EY, Lescar J, Kang C, Luo D (2016) Structure of the NS2B-NS3 protease from Zika virus after self-cleavage. Nat Commun 7:13410
42. Rasmussen SA, Jamieson DJ, Honein MA, Petersen LR (2016). Zika virus and birth defects – reviewing the evidence for causality. N Engl J Med 374:1981–1987
43. Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH (1985) Nucleotide sequence of yellow fever virus, implications for flavivirus gene expression and evolution. Science 229:726–733
44. Roth A, Mercier A, Lepers C, Hoy D, Duituturaga S, Benyon E, Guillaumot L, Souares Y (2014) Concurrent outbreaks of dengue, chikungunya and Zika virus infections – an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012–2014. Euro Surveill 19:pii: 20929
45. Roy A, Lim L, Srivastava S, Lu Y, Song J (2017) Solution conformations of Zika NS2B-NS3pro and its inhibition by natural products from edible plants. PLoS One 12:e0180632
46. Rut W, Zhang L, Kasperkiewicz P, Poreba M, Hilgenfeld R, Drag M (2017) Extended substrate specificity and first potent irreversible inhibitor/activity-based probe design for Zika virus NS2B-NS3 protease. Antiviral Res 139:88–94
47. Sahoo M, Jena L, Daf S, Kumar S (2016) Virtual screening for potential inhibitors of NS3 protein of Zika virus. Genomics Inform 14:104–111
48. Shiryaev SA, Farhy C, Pinto A, Huang CT, Simonetti N, Ngono AE, Dewing A, Shresta S, Pinkerton AB, Cieplak P, Strongin AY, Terskikh AV (2017) Characterization of the Zika virus two-component NS2B-NS3 protease and structure-assisted identification of allosteric small-molecule antagonists. Antivir Res 143:218–229
49. Simpson DI (1964) Zika virus in man. Trans R Soc Trop Med Hyg 58:335–338
50. Singapore Zika Study Group (2017) Outbreak of Zika virus infection in Singapore: an epidemiological, entomological, virological, and clinical analysis. Lancet Infect Dis 17:813–821
51. Smithburn KC (1954) Neutralizing antibodies against arthropod-borne viruses in the sera of long-time residents of Malaya and Borneo. Am J Hyg 59:157–163
52. Tian H, Ji X, Yang X, Xie W, Yang K, Chen C, Wu C, Chi H, Mu Z, Wang Z, Yang H (2016) The crystal structure of Zika virus helicase: basis for antiviral drug design. Protein Cell 7:450–454
53. Tognarelli J, Ulloa S, Villagra E, Lagos J, Aguayo C, Fasce R, Parra B, Mora J, Becerra N, Lagos N, Vera L, Olivares B, Vilches M, Fernández J (2016) A report on the outbreak of Zika virus on Easter Island, South Pacific, 2014. Arch Virol 151:665–668
54. Weinert T, Olieric V, Waltersperger S, Panepucci E, Chen L, Zhang H, Zhou D, Rose J, Ebihara A, Kuramitsu S, Li D, Howe N, Schnapp G, Pautsch A, Bargsten K, Prota AE, Surana P, Kottur J, Nair DT, Basilico F, Cecatiello V, Pasqualato S, Boland A, Weichenrieder O, Wang BC, Steinmetz MO, Caffrey M, Wang M (2015) Fast native-SAD phasing for routine macromolecular structure determination. Nat Methods 12:131–133
55. Wengler G, Czaya G, Farber PM, Hegemann JH (1991) *In vitro* synthesis of West Nile virus proteins indicates that the amino-terminal segment of the NS3 protein contains the active centre of the protease which cleaves the viral polyprotein after multiple basic amino acids. J Gen Virol 72:851–858
56. Yuan S, Chan JF, den-Haan H, Chik KK, Zhang AJ, Chan CC, Poon VK, Yip CC, Mak WW, Zhu Z, Zou Z, Tee KM, Cai JP, Chan KH, de la Pena J, Perez-Sanchez H, Ceron-Carrasco JP, Yuen KY (2017) Structure-based discovery of clinically approved drugs as Zika virus NS2B-NS3 protease inhibitors that potently inhibit Zika virus infection *in vitro* and *in vivo*. Antivir Res 145:33–43
57. Zhang Z, Li Y, Loh YR, Phoo WW, Hung AW, Kang C, Luo D (2016) Crystal structure of unlinked NS2B-NS3 protease from Zika virus. Science 354:1597–1600

11

The Transactions of NS3 and NS5 in Flaviviral RNA Replication

Moon Y. F. Tay and Subhash G. Vasudevan

Abstract

Dengue virus (DENV) replication occurs in virus-induced vesicles that contain the replication complex (RC) where viral RNA, viral proteins and host proteins participate in RNA-RNA, RNA-protein and protein-protein interactions to ensure viral genome synthesis. However, the details of the multitude of interactions involved in the biogenesis of the infectious virion are not fully understood. In this review, we will focus on the interaction between non-structural (NS) proteins NS3 and NS5, as well as their interactions with viral RNA and briefly also the interaction of NS5 with the host nuclear transport receptor protein importin-α. The multifunctional NS3 protease/helicase and NS5 methyltransferase (MTase)/RNA-dependent RNA polymerase (RdRp) contain all the enzymatic activities required to synthesize the viral RNA genome. The success stories of drug discovery and development with Hepatitis C virus (HCV), a member of the Flaviviridae family, has led to the view that DENV NS3 and NS5 may be attractive antiviral drug targets. However, more than 10 years of intensive research effort by Novatis has revealed that they are not "low hanging fruits" and therefore, the search for potent directly acting antivirals (DAAs) remains a pipeline goal for several medium to large drug discovery enterprises. The effort to discover DAAs for DENV has been boosted by the epidemic outbreak of the closely related flavivirus member – Zika virus (ZIKV). Because the viral RNA replication occurs within a molecular machine that is composed several viral and host proteins, much interest has turned to characterising functionally essential protein-protein interactions in order to identify potential allosteric inhibitor binding sites within the RC.

Keywords

NS5 RNA-dependent RNA polymerase · Dengue virus infectious clone · NS3 helicase · Site-directed mutagenesis · NS3 and NS5 antibodies

M. Y. F. Tay
Nanyang Technological University Food Technology Centre (NAFTEC), Nanyang Technological University (NTU), Singapore, Singapore

S. G. Vasudevan (✉)
Emerging Infectious Diseases Program, Duke-NUS Medical School Singapore, Singapore, Singapore
e-mail: subhash.vasudevan@duke-nus.edu.sg

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_11

11.1 Introduction

11.1.1 Flavivirus RNA Replication by Multifunctional Enzymes NS3 and NS5

The ~11 kb positive-sense single-stranded RNA genome of the four Dengue virus (DENV) serotypes share around 70% sequence identity and contain a 5'type-1 cap, as well as 5′- and 3′-untranslated regions (UTR) that flank a single open reading frame (ORF) [40]. The ORF is translated by host machinery into polyprotein consisting of ~3300 amino acids, which is processed by host and viral encoded protease into three structural proteins (capsid (C), premembrane protein (prM) and envelope protein (E)) and seven nonstructural proteins (NS; NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). DENV replication occurs within virus-induced vesicles in the perinuclear region of infected cells in a replication complex (RC) which is composed of viral RNA, viral proteins and host proteins. The efficient replication of viral RNA within the RC molecular machine is crucially dependent on many important RNA-RNA, RNA-protein and protein-protein interactions [13, 73]. By electron microscopy and tomography, virus-induced vesicles have been shown to be derived from the invagination of ER membrane [78] and contain all NS proteins and double-stranded RNA (dsRNA) [13, 73, 78]. The newly synthesized viral genomic RNA (gRNA) is encapsulated by the capsid protein and surrounded by a host derived lipid bilayer containing the membrane and envelop proteins [40].

Protein-protein interaction between several viral NS proteins of RC have been described, including that of DENV NS2B and NS3 [57]; NS4A and NS4B [95]; NS1 and NS4B [1, 94]; NS3 and NS4B [74, 93]; as well as NS3 and NS5 [9, 29, 46, 51]. *In vitro* binding studies on Kunjin virus proteins showed that NS2A could bind to NS3, NS5 and 3'UTR, while NS4A could bind strongly to itself. NS3 and NS5 were also shown to bind to 3'UTR [45]. Studies on Yellow Fever virus (YFV) and DENV proteins demonstrated a genetic interaction between NS4A with NS1 and NS4B, respectively [39, 67]. By using confocal microscopy, fluorescence resonance energy transfer assay and biological fluorescence complementation in conjunction with ectopically expressed WNV proteins, it was shown that NS2A, NS2B, NS4A and NS4B co-localized with ER membrane protein calnexin, but not NS1, NS3 or NS5 [84]. These studies also showed interactions between (1) NS2A and NS4A, (2) NS2B and NS2A, NS4A or NS4B, (3) NS2B and NS3, as well as (4) NS3 and NS5. Interestingly, they showed that the interactions between NS2A, NS4A and NS4B with NS3 only occurred when NS2B was present [84]. No protein-protein interaction was found between NS1 and the other NS protein, but fluorescence resonance energy transfer data suggested the formation of NS1 homodimer [84], which is the consistent with the observation that intracellular NS1 is predominately dimeric [1, 23, 53, 54]. Further functional evidence that supports interaction between NS proteins has been demonstrated by the modulation of NS3 enzymatic/functional activity by NS4A [65], NS4B [74] and NS5 [15, 83]. Recent structural studies have shown that DENV NS5 can form dimers [31] and also a yeast three-hybrid study points to interaction of DENV 3'UTR with the RdRp [25]. Collectively, these studies have led to the proposed model of interaction between the NS proteins in the RC (Fig. 11.1) and this review will focus on the transactions of

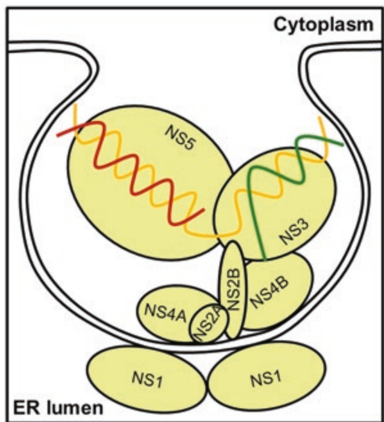


Fig. 11.1 Proposed model of flavivirus replication complex [54, 64]

NS3 and NS5, as well as the potential biological implications of NS5 dimerization and 3'UTR binding to RdRp domain [71].

11.1.2 NS3

NS3 is a multifunctional protein and it is the second largest (69 kDa, 618 amino acids) viral protein [40]. It contains a N-terminal protease domain [36], and a C-terminal domain that has helicase, RNA-stimulated nucleoside triphosphatase (NTPase), and RNA 5′-triphosphatase (RTPase) activities [8, 15, 60, 79, 80]. The DENV NS3 protease, works in concert with its NS2B cofactor and is responsible for cleaving the viral polyprotein at NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B and NS4B/NS5 junctions [5, 10–12, 59, 60, 85, 86]. The NS2B- NS3 protease also cleaves the ER anchored form of C (anchC) at the cytoplasmic side [3, 4, 41]. The NS3 helicase domain, together with NS5 RNA-dependent RNA polymerase (RdRp) and methyltransferase (MTase) activities, is involved in viral RNA replication and type 1 cap formation, respectively. During viral RNA replication, the helicase activity is thought to be required for unwinding of dsRNA and/or secondary structure of single-stranded RNA, whereas the NTPase activity provides the energy for helicase unwinding activity. The first committed step in type 1 RNA cap structure formation, involves the NS3 RTPase activity that is responsible for removing the γ-phosphate from 5'triphosphorylated RNA (also see later for details on subsequent steps by NS5) [77, 80]. In infected cells, NS3 co-localises with dsRNA and NS4B proteins in the perinuclear region [78] and it was shown to interact with both NS4B [74] and hypophorylated NS5 [29]; these findings are consistent with the role of NS3 in viral RNA replication in RC. Apart from its interaction with viral proteins, NS3 has also been shown to interact with the host fatty acid synthase (FAS); this interaction is mediated by the protease domain and through this interaction, it enhances the activity of FAS at sites of viral replication to possibly establish or expand the membranous network for viral replication [24].

The N-terminal domain of NS3 (residues 1–168) resembles a trypsin-like serine protease that contains the catalytic triad composed of His-53, Asp-77 and Ser-138 at its active site. For it to be a functional protease, NS3 has to interact with the hydrophilic region of NS2B, residues 49–95 [35]. Without $NS2B_{49–95}$, NS3 expresses as an insoluble protein. Soluble active form of NS3 protease for enzymatic characterization, inhibitor screening and structural studies was expressed as a bifunctional fusion construct with $NS2B_{49–95}$ that was connected by flexible glycine linker (G_4SG_4) to the protease domain [35, 37]. The crystal structure of DENV protease revealed a chymotrypsin-like fold with two β-barrels and each barrel is formed by six β-strands; the barrel interface contains the catalytic triad. The N-terminal part of NS2B from residues 49–66 contribute a β-strand to the N-terminal β-barrel of NS3 protease domain. In the presence of inhibitor, the C-terminal region of NS2B (residues 67–95) form a conserved β-turn hairpin that wraps around the C-terminal β-barrel of NS3 to form a "closed" conformation and this allows the β-turn hairpin to form part of the S2 and S3 pockets in the substrate binding site to interact directly with substrate/inhibitor [2, 20, 56]. Hence, this collectively implies that NS2B residues 49–95 are needed for proper folding and catalytic activity of NS3. One vexing issue that has attracted much commentary in several publications is the question of the "open" and "closed" conformations of NS2B's interaction with NS3 protease. This question was directly addressed in WNV NS2B-NS3 protease that is known to be more stable than other flavivirus proteases, by nuclear magnetic resonance (NMR) spectroscopy studies that used specific isotope labels and nuclear "spin" labels and it was shown that the "closed" conformations forms the predominant species in solution [66]. The "closed" conformation of NS2B-NS3 protease is critical for structure-directed drug discovery and this is discussed in more detail in Chaps. 10 and 13 in this monograph and the ZIKV protease is proving to be an interesting target and may provide the pathway for the development of a pan-flaviviral inhibitor [87].

The C-terminal domain of NS3 is a multifunctional protein (residues 180–618) belonging to superfamily 2 (SF2) class of helicases that contains the DEAH sequence motif [22]. NS3 helicase (NS3H) contains three subdomains and the crystal structure revealed the RecA-like fold of subdomain I and II [80]. The ATPase and RTPase activities are found in domains I and II while the single-stranded RNA binding tunnel is formed by the base of subdomain III, which separates it from subdomains I and II. NS3 helicase activity was shown to be ATP- and Mg^{2+}-dependent [8] and its interaction with NS4B promotes its dissociation from single-stranded RNA, which subsequently enhances its helicase activity [74]. Its NTPase and RTPase activities were also found to be Mg^{2+}-dependent and both activities could be stimulated by NS5 [15, 83]. Biochemical and mutagenesis studies suggest that NTPase and RTPase may share a common active site [7]. Similarly, mutations affecting helicase activity also reduced RTPase activity [8], suggesting that the helicase, NTPase and RTPase activities may be linked.

By linking NS2B residues 49–66 via G_4SG_4 to NS3 (also known as $NS2B_{18}NS3$), a soluble full-length protein was expressed, purified and crystallized. Using this strategy, the structure of DENV4 full-length NS3 revealed an elongated conformation with the protease domain connected by a natural linker spanning residues to 169–179 to the helicase domain [44]. The NS3 protein structures showed that the protease domain can adopt a different orientation relative to the helicase (protease domain rotated by ~161° with reference to the helicase domain) owing to flexibility of the natural linker that has evolved to an optimum length to confer flexibility to full-length NS3 protein [43]. The two conformations of NS3 protein probably reflects the different conformations that may be adopted for it to engage in the various protein-protein and protein-RNA interactions during polyprotein processing and RNA replication.

11.1.3 NS5

NS5 is the largest (103 kDa, 900 amino acids) and most conserved protein among all viral proteins [40]. Its N-terminal domain contains MTase [17, 18, 33] and putative GTase activities [27] that are involved in type 1 cap formation, whereas its C-terminal harbours RdRp activity that is responsible for viral RNA replication with NS3 [81]. In the infected cell, DENV2 NS5 is known to interact with NS3 [29] and also to shuttle between the cytoplasm and nucleus [9, 34, 61, 62]. NS5 was shown to be a potent TNF-α, IL-6 and IL-8 inducer [30, 50], and its IL-8 inducing ability may correlate with NS5 nuclear localization and infectious virus production [61, 62]. It was demonstrated that DENV2 NS5 can bind to STAT2 to promote UBR4-mediated STAT2 degradation by bridging STAT2 and UBR4, an E3 ligase that recognizes and degrades proteins, to collectively lead to inhibition of type I interferon signalling [6, 49, 52].

NS5 MTase has two binding sites: an S-adenosyl-L-methionine (SAM) binding site and a RNA cap site for binding to guanosine of the cap structure [18]. Its MTase domain possesses two methylation activities: guanine-N7 [33] and nucleoside 2′-O methylation [17]. The current model for the formation of type 1 cap structure assumes four sequential steps: [1] the RTPase activity of NS3 removes 5′ γ-phosphate from newly synthesized RNA [8, 80], [2] the newly identified GTPase activity of NS5 may cap the RNA 5′-diphosphate end with guanosine monophosphate (GMP) through a 5′ to 5′ phosphodiester bond [27], [3] the guanine-N7 methylation activity of NS5 transfers a methyl group to the N7 position of the guanine moiety to form a cap 0 structure ($^{7me}G_{ppp}A$-RNA) [91] and lastly, [4] the 2′-O methylation activity of NS5 catalyses methylation at the ribose 2′-*O* position of adenosine to form type 1 cap structure ($^{7me}G_{ppp}A_{2'\text{-}O\text{-me}}$-RNA) [17]. Time course experiment showed that N7-methylation occurs before

2'O-methylation [63] and both use SAM as methyl donor for methylation [55]. Through mutagenesis study, the N7-methyaltion was found to be important for virus replication [91]. Recently, a 2'-O MTase mutant virus (^{216}E➔A in DENV1 and ^{217}E➔A in DENV2) was shown to replicate like wildtype (WT) in cell culture but poorly in mice and rhesus monkeys, and able to induce protection in rhesus monkeys during homologous challenge. These studies show the potential value of using 2'-O MTase mutant virus as a rationally designed vaccine [94].

The RdRp domain of DENV3 has the classical "cupped right hand" architecture with fingers, palm and thumb subdomains that have been described for various flaviviruses [38, 42, 47, 81]. The βNLS and α/βNLS (reside in residues 320–405), which were originally thought to be an interdomain linker region between MTase and RdRp [17], were found to be an integral part of the RdRp domain and are distributed between the fingers and thumb subdomains. The highly conserved palm domain contains four out of the six conserved sequence motifs that are important for NTP binding and catalysis, including the GDD catalytic active site. Its active site is encircled by several loops to form a tunnel that can direct the RNA template strand to the active site [81]. The thumb subdomain contains the most structurally variable elements among the known polymerase structures that help to shape the RNA template tunnel and possibly regulate the entry and exit of template into active site, and also the priming loop that is involved in *de novo* initiation [81]. Functional mutagenesis study of two conserved cavities of thumb subdomain led to the identification of residues K328, Y859 and I863 that are crucial for *de novo* initiation but not elongation, and also residue K330 that is involved in NS3-NS5 interaction [92].

Recently, the structure of DENV3 full-length NS5 was solved and it revealed a compact structure with extensive interaction between the MTase and RdRp domains [89], in agreement with the comprehensive biochemical studies carried out by Potisopon and colleagues [58]. The structure also revealed for the first time the molecular basis for specific viral RNA recognition of the conserved 5′-AG dinucleotide found in all flaviviruses and also the mechanism for 2'O ribose methylation [90]. The DENV3 NS5 structure differed in relative orientation of the MTase and RdRp (MTase domain rotated by 105° with reference to the RdRp domain) when compared to extended conformation of Japanese Encephalitis virus (JEV) full-length NS5 [42], despite the overall similarity in shape. The differences in the length, sequence and structure of linker, as well as the molecular nature of MTase-RdRp interaction interface between DENV3 and JEV NS5s may reflect the different conformations that are being adopted by the protein with regard to its various functional roles during virus life cycle (see also Chaps. 9 and 14 in this monograph).

11.1.4 NS3-NS5 Interaction Is Required for Coordinated Positive- and Negative-Strand RNA Synthesis

Previous biochemical studies using yeast two-hybrid assays have narrowed down the region of interaction between NS3 and NS5 to residues 303–618 and residues 320–368, respectively [9, 28, 75]. Mutation in NS5 residue K330 to alanine in an infectious clone abolished viral replication through disruption of NS3-NS5 interaction. However, biochemical studies showed that the *in vitro* RdRp activity of NS5 in the K330A mutant remained intact [92]. The interaction region on NS3 that is involved in NS3-NS5 interaction was fine-mapped to residues 566–585 by competitive NS3-NS5 interaction ELISA, peptide-phage ELISA and truncated NS3 protein using novel anti-NS3 antibodies [70]. Specifically, the overlapping peptides spanning residues 566–580 and 571–585 of NS3 inhibited the $NS2B_{18}NS3$ interaction with RdRp in the ELISA-based interaction assay. Sequence alignment of residues 566–585 of NS3 show a high degree of conservation amongst flaviviruses (Fig. 11.2). Competition ELISA with truncated NS3 proteins showed that wild-type $NS3_{566–585}$ inhibited $NS2B_{18}NS3$-NS5 interaction in a concentration dependent manner.

```
        566  570                585
DENV2   GIKNNQILEENVE-VEIWTKE
DENV3   GERNNQILEENMD-VEIWTKE
DENV1   GERNNQVLEENMD-VEIWTKE
DENV4   GERNNQILEENME-VEIWTRE
WNV     GPRTNTILEDNNE-VEVITKL
JEV     GPRTNAILEDNTE-VEIVTRM
MVE     GPRSNIILEDNNE-VEIITRI
YF      GPEEHEILNDSGETVKCRAPG
```

Fig. 11.2 Sequence conservation of NS3 around residue N570. Sequence alignment of NS3 residues 566–585 of DENV and other flaviviruses show N570 is conserved (highlighted and bold. The GenBank accession numbers are as follows: DENV2 (AF038403), DENV1 (U88535), DENV3 (M93130), DENV4 (AF326573), Yellow Fever virus (YFV; X15062), Japanese Encephalitis virus (JEV; M55506), Murray Valley Encephalitis virus (MVEV; AF161266) and West Nile virus (WNV; M12294). The numbering of residues is based on DENV2 protein sequence [70]

On the other hand, $NS3_{566–585(N570A)}$ peptide carrying a single mutation or unrelated peptides did not show any reduction in $NS2B_{18}$NS3-NS5 interaction. The NTPase activity of $NS3_{566–585(N570A)}$ was identical to the wild-type protein.

Virological support for the importance of the NS3 N570 through introducing the NS3:N570A mutation into a DENV2 infectious complementary DNA (cDNA) clone and comparing it to one carrying the NS5:K330A mutation [70]. Similar to NS5:K330A mutant, NS3:N570A mutant did not produce any infectious virus [92], however it was able to synthesize low amount of viral RNA and viral proteins. This lends further weight to the critical role of NS3 N570 interaction between NS3 and NS5. Robust biochemical assays that can screen for compounds that block the interaction may be valuable for the discovery of potent inhibitors [51, 68, 70].

Interestingly, the WT and mutant infectious clones also provided insights into the viral RNA replication process through real-time RT-PCR investigation of negative- and positive-strand RNA synthesis. The WT RNA transfected cells show synchronized synthesis of negative- and positive-strand RNA, with an excess of positive- to negative-strand RNA as seen with DENV infected cells [72]. The NS5:K330A mutant was completely inactive since the transfected positive-strand RNA degraded over time and the negative-strand RNA that was detected corresponded to background levels. Strikingly, the NS3:N570A mutant virus showed fairly robust negative-strand synthesis from transfected RNA template, somewhat analogous to early events during DENV RNA replication in an infected cell. The weakened or abolished interaction between NS3 and NS5 in NS3:N570A mutant does not support the replication of new positive-strand RNA from 6 to 24 h when compared to WT virus. Although the NS5:K330A mutant was enzymatically active *in vitro*, it was not able to produce any negative-strand RNA when compared to NS3:N570A mutant. This indicates that the coordinated synthesis of negative- and positive-strand RNA requires a functional RC with optimum protein interaction affinities between NS3 and NS5. The interaction region is conserved in almost all flaviviruses except that in YFV NS3 which has a histidine in place of asparagine at position 570 (Fig. 11.2). Interestingly, in YFV NS5 the position 330 is occupied by a tyrosine residue instead of lysine [70]. The development of a pan-flaviviral NS3-NS5 interaction inhibitor will need to take this into account (Fig. 11.2).

11.1.5 Proof of Concept for Developing Inhibitors of NS3-NS5 Interaction

To further validate the protein-protein interaction (PPI) target, the peptide sequences of the interaction region between NS3 and NS5 were either synthesized as a fusion product with cell penetrating peptide (penetratin) or supplied as a complex with penetratin in conjunction with scrambled peptide to show that the disruption of PPI decreases viral RNA synthesis. This paves the way to examine stapled-peptides approach [76] to inhibit NS3-NS5 PPI, as well as other small molecule screening approaches. Another

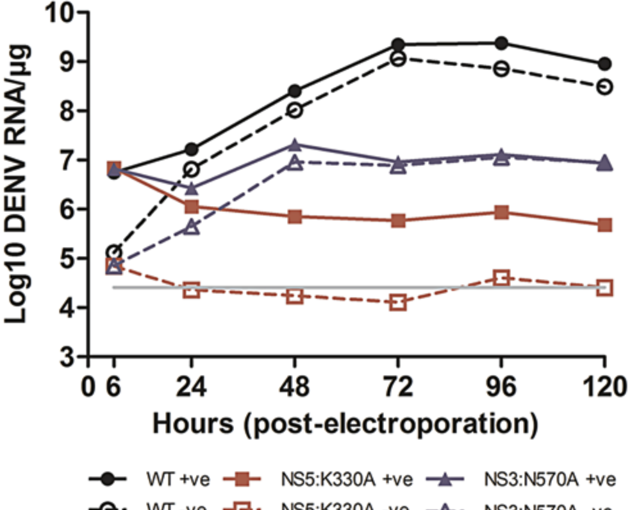


Fig. 11.3 Dynamics of viral RNA replication in BHK-21 cells transfected with DENV2 WT, NS5:K330A or NS3:N570A RNAs monitored over the course of 5-days. BHK-21 cells were electroporated with 10 mg of genomic-length RNA and infected cells were harvested daily, for 5 days. Extracted RNA from infected cells was used to quantify the amount of negative and positive-strand synthesis by real-time RT-PCR. Absolute copy numbers of both strands in log scale per µg of RNA used for real-time RT-PCR were plotted; data are shown as the mean ± SD of duplicate from one independent experiment. The grey line indicates the limit of detection of the negative-strand from transfected RNA. (Adapted from [70])

attractive proposition from this study is that mutants such as NS3:N570 may serve as potential mRNA-mediated vaccine since our study shows that low level translation of viral proteins can be achieved with residues that are selected on the basis of structure and function.

11.1.6 NS5 Interaction with the Nuclear Import Receptor Importin-α and Its Implication for Dengue Virus 2 RNA Synthesis

Hyperphosphorylation and transport to the nucleus is an unexpected feature of DENV2 NS5, which catalyses viral RNA synthesis in the cytoplasm of infected cells [29]. Inspection of the NS5 sequence suggested a bipartite nuclear localization signal (NLS) existed between residues 369–405. This sequence was sufficient to target bacterial β-galactosidase, a normally nuclear excluded reporter protein, to the nuclear compartment [21]. Further studies suggested that NS5 shuttles between the cytoplasm and nucleus [9, 34, 61, 62], while contradicting data was reported by Kumar et al. [34] on the relevance of nuclear localization to viral RNA replication. Furthermore, the elucidation of the 3D structure of the RdRp showed that the NLS was not a separate domain and the cellular role of NS5 targeting to the nucleus needed further studies. To address the question of NS5 sub-cellular localization of the four serotypes in the context of DENV infection, a naïve human Fab-phage library was screened to identify a serotype cross-reactive, NS5 specific antibody fragment (5R3). This fragment was converted into a full-length human IgG and expressed in HEK293T cells [88]. The cross-reactive 5R3 antibody detected NS5 from DENV2, 3 and 4 in the nucleus while DENV1 NS5, which shares more than 70% sequence identity is distributed mostly in the cytoplasm (Fig. 11.4). The same sub-cellular distribution pattern detected with 5R3 antibody following DENV infection was also observed for recombi-

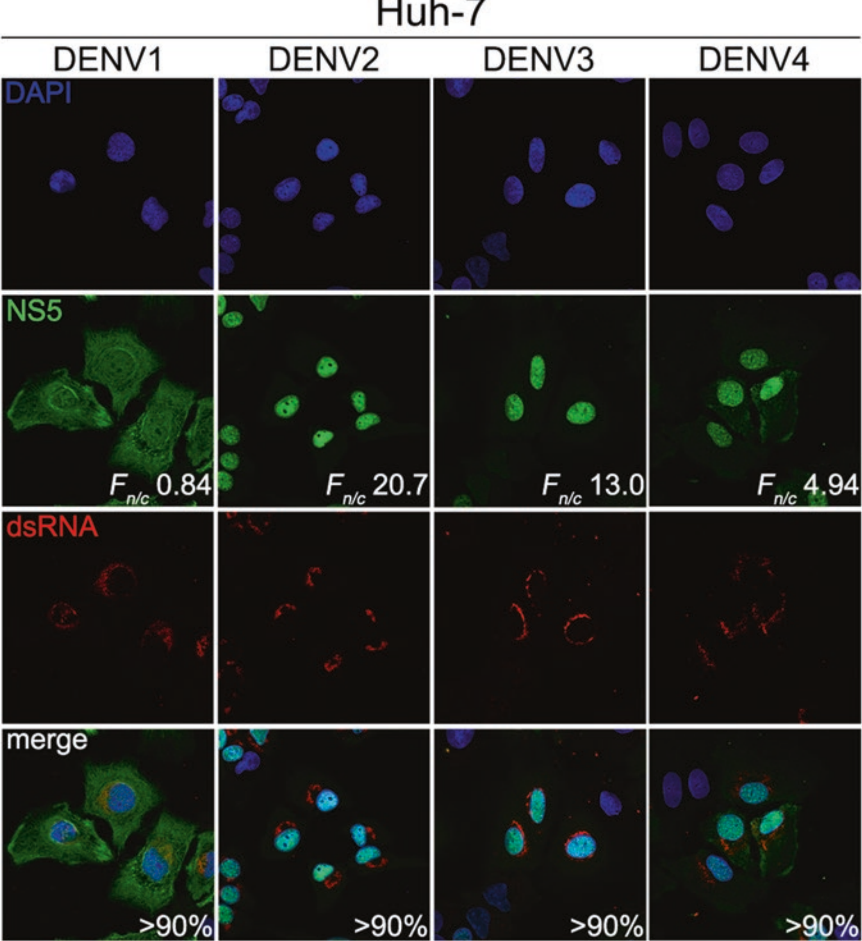


Fig. 11.4 Subcellular localization of NS5 in DENV1-4 infected cells and sequence alignment of DENV1 and 2 NS5. (A) Huh-7 cells were infected with DENV1-4 at MOI 10 and the infected cells (>90%) were analysed for presence of NS5 (green) and dsRNA (red) by IFA at 24 h post-infection. Digitized images were captured by Zeiss LSM 710 upright confocal microscope by 63× oil immersion lens. Image analysis was performed on digitized images of NS5 staining with ImageJ software [14] to determine nuclear to cytoplasmic fluorescence ratio ($F_{n/c}$) as done previously [34, 61, 62, 69]

nant GFP-NS5 of DENV1 and 2. These constructs permitted comprehensive domain and motif shuffling to identify the sequence element responsible for the cytoplasmic localization of DENV1 NS5 since the protein contained an almost identical NLS to DENV2 NS5 in the region between 369 and 405 [71].

Surprisingly the extensive swapping of GFP-NS5 gene fragments of DENV1 and 2 [71] showed for the first time that a functional monopartite NLS was present within C-terminal 18 residues ($Cter_{18}$) of DENV2 NS5, residues 883–900 that was sufficient to direct the protein to the nucleus [71]. This finding is in general agreement with the observation that classical NLS is usually found at either at N- or C-terminal end of nuclear cargo proteins so that they are accessible to nuclear transport factors [48] and it is also predicted by the cNLSMapper program [32]. The available crystal structures of various flavivirus NS5 did not include the $Cter_{18}$ [38, 42, 47, 81, 89], probably due to the dynamics of the region. Indeed, the high quality crystals that resulted in the resolution of the first full-length

structure of DENV3 NS5 [89] was only obtained upon the truncation of the last 5 amino acids of DENV3 NS5.

Although DENV2 NS5 is targeted to the nucleus by the NLS within $Cter_{18}$, it was still unclear why the same region of DENV1 NS5, which also contains a $Cter_{18}$ sequence that resembles a NLS was localised predominantly in the cytoplasm. The GST-NS5 $Cter_{18}$ of DENV1–4 proteins binding studies with recombinant importin-α (Impα) showed that the tightest binding was observed for DENV2 and 3 followed by DENV1, and DENV4 NS5 $Cter_{18}$ was the poorest binder. Crystal structures of $Cter_{18}$-Impα complex for DENV2 and 3 were solved to 2.2 Å resolution. In the case of DENV4 $Cter_{18}$, although the cNLSmapper [32] program predicts an NLS sequence, it is probably regulated by phosphorylation and therefore, only observable in the context of infection that DENV4 NS5 is nuclear-localised [71]. The DENV1 $Cter_{18}$ has almost identical Impα binding determinants but it is predominant in the cytoplasm. Comparison of residues immediately N-terminal to NLS binding determinants suggested that the proline residue at position 884 for DENV2 and 3 is required for presenting the NLS to Impα (Table 11.1) for efficient nuclear transport [71].

Biochemical assays with DENV3 NS5 carrying mutations in the C-terminal NLS (K887A/R888A; R890A/R891A; R888A, R888E or R888K) or $Cter_{18}$ truncations did not impact on the RdRp activity [71]. It was therefore expected that introduction of these mutations into a DENV2 cDNA clone should not impact viral RNA replication. However, the transfection of mutant RNA in comparison to WT showed that the K887A/R888A double mutant and the R888A, E or K single mutant showed no plaques. Strikingly, the P884T mutant replicated to similar level as WT but its NS5 was predominantly in the cytoplasm. Since the mutation of a single residue (P884T) redirected DENV2 NS5 mostly to the cytoplasm without impacting its cellular infectivity, it may be assumed that nuclear localization of NS5 may not be required for viral RNA replication but the abolished or diminished replication of the $Cter_{18}$ monopartite NLS mutants implies the opposite.

The confocal microscopy images of immunofluorescence assay (IFA) shows that >90% of NS5 is in the nucleus and only a small fraction that is needed for dsRNA synthesis is detected in the perinuclear region. The NS5 P884T mutant shows that the mislocalised NS5 is distributed throughout the cytoplasm, however the level and pattern of distribution of dsRNA is similar to WT. The double-mutant R890A/R891A was attenuated, showing lower infectivity and predominantly cytoplasmic NS5. Remarkably, the R888A or R888E mutants were not viable but R888K showed a small-plaque phenotype, and in the cells that were infected it was shown to be predominantly in the nucleus and dsRNA was detected in the perinuclear region as with WT. The impact of mislocalisation of NS5 on the modulation of host pathways has not been studied with these mutants. One possible interpretation from the reverse genetics studies and images in Fig. 11.5 is that in order for viral RNA replication to occur in a coordinated fashion within the RC, it may be necessary to limit the level of NS5 the perinuclear region where it is detected. It is conceivable that the $Cter_{18}$ of DENV1–4 NS5s may have undergone adaptive evolution [89] to engage specific cellular proteins to shuttle the excess NS5s away from the replication factories. Another peculiarity is that R888, which is within

Table 11.1 Alignment of DENV1-4 $Cter_{18}$ region containing the monopartite NLS

NS5 NLS region	Residues	NLS binding determinants					Residues
		P1	P2	P3	P4	P5	
Dengue 1[a]	880-DYMTS	M	K	R	F	K	NES-892
Dengue 2	881-DYMPS	M	K	R	F	R	REE-893
Dengue 3	881-DYMPS	M	K	R	F	R	KEE-893
Dengue 4[a]	882-DYMPV	M	K	R	Y	S	APS-894

[a]Structures not determined. Alignment based on crystal structures for DENV2 and 3 with Impα

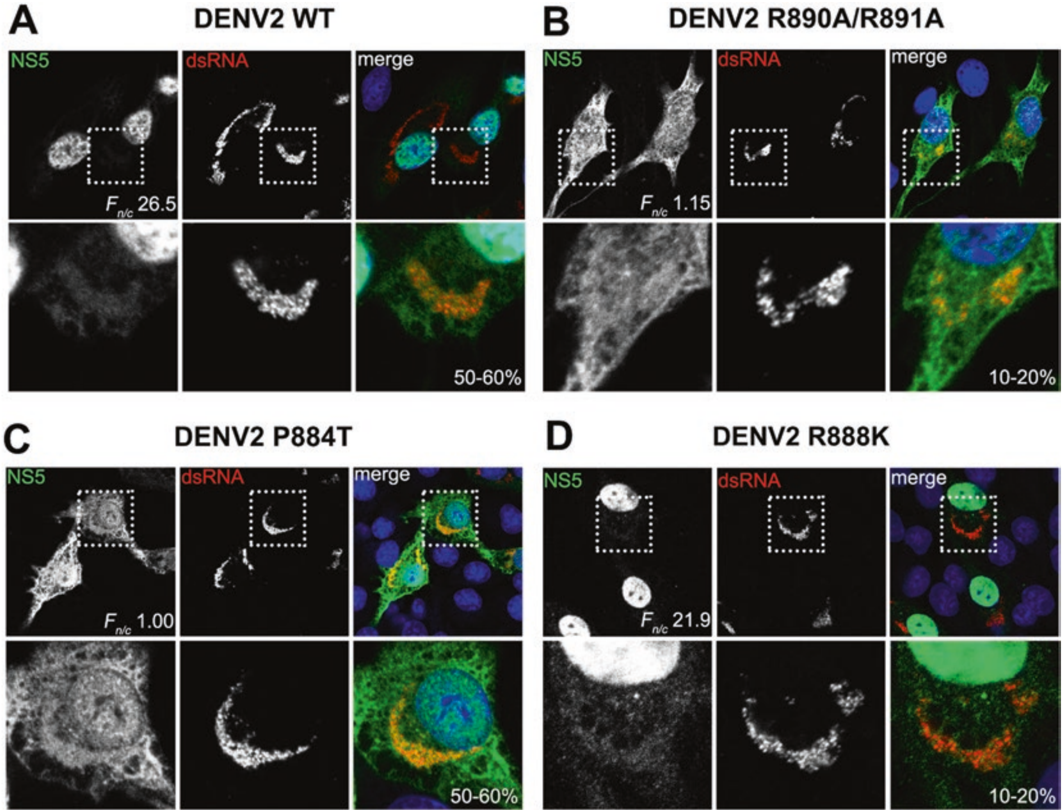


Fig. 11.5 BHK-21 cells transfected with (**a**) DENV2 WT, (**b**) DENV2 R890A/R891A (**c**) DENV2 P884T and (**d**) DENV2 R888K analysed for presence of NS5 (green) and dsRNA (red) by IFA on day 3 post-transfection. The % infectivity is indicated for WT and mutant viruses. The nuclear to cytoplasmic fluorescence ratio ($F_{n/c}$) of NS5 was obtained using Image J software as done previously [34, 61, 62, 69]. Insets: zoom-in views of the dotted boxed regions

the NLS region, appears to play a seminal role in viral RNA replication in the infected cell that is not revealed by its *in vitro* enzyme activity. The replication of R888K mutant virus is attenuated but the NS5 is in the nucleus. Residue R888 is conserved in NS5 of all four DENV serotypes and also completely conserved in all flaviviruses, including ZIKA. Together, these imply that R888 could have a previously unknown function in RNA replication and it was postulated that it may have a role in *de novo* RNA initiation (Fig. 11.6).

Support for this notion is indirect and it is based on the studies by Klema and colleagues who reported DENV3 NS5 structure carrying a six-residue priming loop deletion (^{795}WSIHAH^{800}) that crystallized as two different types of dimers within a single unit cell [31]. The so-called "type 2 dimers" could resolve the C-terminal region because residues R890 to G897 from one monomer formed an α-helix that interacted with the MTase domain of second NS5 molecule. In these "type 2 dimer" R888 interacts with the hydroxyl group of Y838 within the same monomer, which is also completely conserved in all flaviviruses. It is likely that critical interactions for *de novo* priming are correctly positioned when R888 forms a hydrogen bond with Y838 when NS5 oligomerizes during early stages of the infection. The survival of the virus is dependent on successful translation of the genomic RNA that is released from the infecting virion and on the copying of the genomic RNA to form a negative-strand RNA that is used as template for new genomic RNA synthesis. It is possible that once sufficient negative-strand RNA is made, NS5 interacts with NS3 to carry out further co-

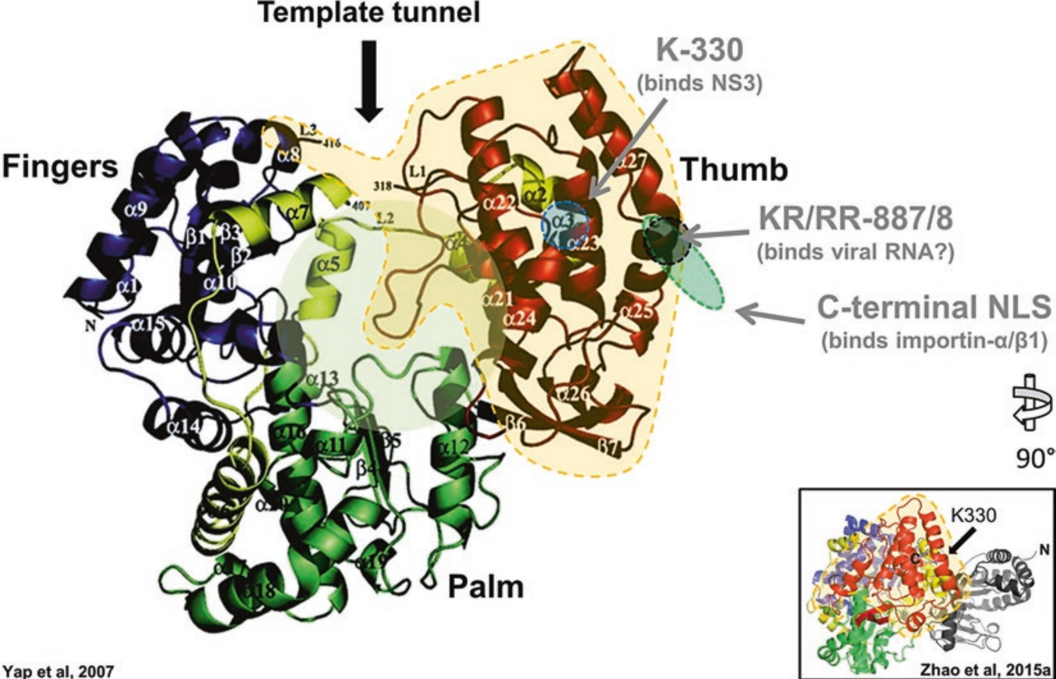


Fig. 11.6 Structure of DENV3 RdRp. Ribbon representation of DENV3 NS5 RdRp (residues 273–900, PDB: 2J7U, [81]) and full-length (in inset, residues 6–895, PDB: 4V0Q [89]) structures. The fingers, palm and thumb subdomains and residues 320–405 are coloured blue, yellow, green and red, respectively. The MTase is shaded in light green. The secondary structures (α-helix and β-strand) and protein termini (N and C) are labelled as described in [81]. The region that is missing in the structure is also defined by the boundary numbers. The template entry tunnel is arrowed. The priming looing is circled in grey. The α/βNLS and C-terminal NLS are circled in purple and green, respectively; C-terminal NLS is missing in the structure and the position is to denote its location in the structure. NS5 K330 that is important for NS3-NS5 interaction reside in α3 and it is circled in blue. The regions on NS5 RdRp that were shown [89] by Hydrogen/Deuterium Exchange Mass Spectrometry to be more flexible as compared to other parts of the protein are circled in orange, which include residues 735–748, 781–809, 812–850 and 853–895 from thumb subdomain and residues 311–340 from fingers subdomain. *Inset*: The different regions are coloured as per described above, with the following exception: the MTase is coloured in grey and an arrow is used to indicate the position of K330. (This figure is adapted from Yap et al. [81] and Zhao et al. [89])

ordinated synthesis of genomic RNA for packaging. As shown in Fig. 11.3 the N570A mutation in NS3 permits negative-strand RNA synthesis in the early stages of infection but the NS3-NS5 interaction is weakened and this prevents further new synthesis of positive-strand genomic RNA required for packaging the infectious virion [70]. Based on this, it can be hypothesized that the accumulation of negative-strand RNA in the first 3–12 h post infection requires NS5 oligomerization. At the later stages, the increased NS5 concentration may be a liability for concerted RNA synthesis and different strategies may be used by flaviviruses to shunt the excess NS5 away from the RC to different subcellular locations using sequence features on the exposed C-terminal region to either the nucleus or throughout the cytoplasm, away from the RC (Fig. 11.4). The crucial role of Y838 in replication initiation was independently verified by Hodge and colleagues [25] through yeast three hybrid study that explored all possible interactions between RNA genome and DENV RdRp, as well as site directed mutagenesis studies in a DENV replicon to show that the 3’UTR panhandle structure interacts near the thumb subdomain of RdRp in a region that contains several positively charged residues and also including the conserved Y838. Based on RNA binding and mutational studies, the authors suggested that the stacking interaction between

the sidechain of Y838 and the RNA base may be required for replication initiation.

11.1.7 Summary

In the crystal structure of full-length DENV3 NS5, the MTase is placed above the fingers subdomian, and the MTase and RdRp domains interact at two contact areas via residues from the MTase domain (residues 63–69, 95–96 and 252–256), the linker region (residues 262–273) and the fingers subdomain from the RdRp domain to create an interaction interface that is unique to DENV [89]. The interaction of NS5 with NS3 or importin-α has been shown to be near the thumb subdomain of the RdRp domain of NS5, which was shown to be more flexible by hydrogen/deuterium exchange mass spectrometry [89]. The flexibility of the thumb subdomain may enable it to adopt different conformations to interact with different viral proteins and host proteins during the course of RNA replication. The DENV3 NS5 RdRp structure showed that NS5 residues 320–405, which includes the α/βNLS are an integral part of the RdRp domain, and are distributed within α2-α7 helices (βNLS in α2-α5 and α/βNLS in α6-α7 (circled in purple)) [81]. The α2-α4 helices (residues 323–341) are part of the thumb subdomain (coloured in red); α5 and α7 helices (residues 349–355 and 397–405) are found at the finger tips, between the fingers (coloured in blue) and thumb subdomains and α6 helix (residues 367–386) is close to the palm subdomain (coloured in green). Given the involvement and/or close proximity of these helices to the three subdomains, it may not be surprising if mutations [26, 34, 61] or truncations [62, 81] in residues 320–405 destabilize NS5 protein structure. This can conceivably result in a pleiotropic effect on NS5 activity, nuclear localization and RNA replication. Interestingly, the C-terminal NLS within $Cter_{18}$ is also within the thumb subdomain (not visible in the structure, circled in green) and is believed to make a crucial interaction with Y838 to inaugurate *de novo* RNA synthesis. Mutational studies by Gamarnik and coworkers [26] on basic residues of NS5 showed that dibasic residues ^{840}KR (located within helix α26) on thumb subdomain were lethal to virus when mutated to alanine, despite an intact polymerase activity [26]. Similarly ^{887}KR (especially R888) may have the ability to bind RNA and may have a key role in *de novo* initiation. Coincidentally, several viral proteins have been reported to have their functional NLS sequence overlapping with RNA binding domain [16, 19, 82]. Based on the importance of the K887/R888 in this study and its location in thumb subdomain, it is tempting to hypothesize that K887/R888 may be involved in binding to both RNA and importin-α/β1 and it is likely that the loss of binding to RNA resulted in absence of virus replication. Taken together, the thumb subdomain may hold the key to controlling the localization of NS5 through interacting with either NS3 and/or viral RNA, and possibly host factors to stay in the cytoplasm for viral RNA replication or importin-α/β1 to enter the nucleus for it elusive and yet intriguing role. The thumb subdomain of NS5 is a hotspot for viral and viral-host protein interactions as well as protein-RNA interactions, making it an ideal target for drug development.

Acknowledgments We thank our past and present collaborators David A. Jans, Jade K. Forwood, Andrew Brooks, Magnus Johansson, Andrew Davidson, Julien Lescar, Dahai Luo, Yap T.L., Siew Pheng Lim, Nicole J. Moreland, Indira Umareddy, Aruna Sampath, Yongqian Zhao, Ivan H.W. Ng, Kitti W.K. Chan, Wuan Geok Saw, Gerhard Gruber, Crystall Swarbrick, Shu Ann Chan, Kate Smith, Gottfried Otting, Zheng Yin, Li Shang and Rolf Hilgenfeld for their important contributions to understanding of flavivirus NS proteins. The research on NS3 and NS5 interactions of flaviviruses in the Vasudevan lab is supported by National Medical Research Council (NMRC/CBRG/0103/2016) and National Research Foundation (NRF-CRP17-2017-04) in Singapore.

Discussion of Chapter 11 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Subhash Vasudevan.

Félix Rey: Very nice story! What did you make of the other NLSs that were postulated before? I'm referring to the NLS in the folded part of RdRp domain.

Subhash Vasudevan: Right! You're talking about what we call the conventional α/βNLS between residues 369 and 405. These are sequences that were actually idenified based on work that was carried out with peptide fragments fused to β-galactosidase or to GFP. However, for DENV, these do not appear to be functional in the context of the full-length NS5 in the cell-based assesments. I think the caveat is that one really has to look at localization in the context of full-length NS5. Presentation of the NLS is very important as we show in our current work with the P884T mutation. But having said that, I still believe that the thumb subdomain region of NS5 where the α/βNLS in the folded part occurs is very dynamic and it's possible that in the context of some viruses like ZIKV NS5 for instance, it may be important. What I haven´t discussed here is that ZIKV NS5 is also localized to the nucleus much more strongly than some of the DENVs – and moreover the NS5 forms distinct nuclear structures. Why these structures form is not known but the C-terminal part of Zika virus NS5 does not contain a NLS like what we have described for DENV NS5. And there also doesn't appear to be any kind of potential phosphorylation regulation as we have suggested for DENV 4 C-terminal NLS. I should add that this suggestion still needs to be verified experimentally.

Paul Young: I know you don't know the answer, but can you speculate on why at least the vast majority of NS5 actually shows up in the nucleus? And what's the chronology of that? How early in an infection does NS5 transfer? Is it late in infection? Is it during when you are expecting most replication?

Subhash Vasudevan: DENV2 NS5 and ZIKV NS5 start to go into the nucleus as early as about 12 hours post infection. And what is the role? While the NS5 P884T mutant virus (in DENV) suggested that it is replicating like a wild-type even though it is mostly localized to the cytoplasm. We have not clearly investigated what the downstream effect could be of the mislocalization – I think, as Julien Lescar pointed out in his talk, these NS5s may have evolved to engage different host factors a spart of their pathogenesis mechanism. It is evident when you compare JEV NS5 structure with our DENV 3 NS5. The DENV 3 NS5 seems to form this very nice compact structure that shows extensive interaction between the methyltransferase and the RdRp domains, whereas in the JEV NS5, there is not much of that. It is possible that these might interact with host proteins that could modulate events that results in the varying pathogenesis mechanisms but more work needs to be done in this area. {Note: Two publications since the meeting have shown that NS5 can cause splice variations that could modulate genes involved in innate immune pathway or sequester host nuclear transport receptors so that cellular transport required for normal host innate immune response is affected.}

David Jans: So if I can also add a little bit. I think the simplest model at the moment would be that clearly the C-terminus plays a big role in localization, but also I think the conventional α/βNLS in the folded part does as well. All the evidence would suggest that both are important.

Félix Rey: So you think that the folded NLS of NS5 is exposed?

David Jans: I think that is quite possible and if you have the situation between dimers and monomers of NS5, a lot of things can happen. Don't forget, and this is something that even I hardly mention in talks, that there is also an export signal present in DENV NS5. And if you inhibit nuclear export, NS5 level is increased in the nucleus by a lot. So we think that this is true for all of the DENV serotypes – even DENV1 NS5. Clearly NS5 is cycling all the time in a cell. And if you inhibit one or the other you can change where it goes. So it's dyamic. And so i think probably we should be thinking that the dynamics may be the key thing anyway for what it does in the nucleus or in the cytosol for that matter.

Ok the reverse genetics experiments are quite crystalline and the P884T has some in the cytoplasm and the virus still grow and so on. But I just want to raise a potential caveat to something that we hadn't thought about – the dynamics of the nuclear import/export process. So the reverse genetics is done in DENV2?

Subhash Vasudevan: Yes.

David Jans: So not in DENV1 or DENV3. The crystal structures of full-length NS5 were from DENV3, right?

Subhash Vasudevan: But DENV2 NS5 is the one that is in the nucleus and so is DENV3 NS5.

David Jans: We also have to admit that we're limited by the crystal structures. So that's DENV3 we have the full-length NS5 structure of. We have the reverse genetic side of DENV2. And maybe we should do some reverse genetics in DENV1? I think that the 884 mutation to convert threonine into proline would be quite interesting to do. Also what we want really is the crystal structure of the full-length NS5 bound to importin-α. If we had the full-length, we'd all be happy and Félix would know what's going on.

Subhash Vasudevan: Agree that the mutation should be done in DENV1 infectious clone and also in DENV4.

References

1. Akey DL, Brown WC, Dutta S et al (2014) Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system. Science 343:881–885
2. Aleshin AE, Shiryaev SA, Strongin AY et al (2007) Structural evidence for regulation and specificity of flaviviral proteases and evolution of the Flaviviridae fold. Protein Sci 16:795–806
3. Amberg SM, Nestorowicz A, McCourt DW et al (1994) NS2B-3 proteinase-mediated processing in the yellow fever virus structural region: in vitro and in vivo studies. J Virol 68:3794–3802
4. Amberg SM, Rice CM (1999) Mutagenesis of the NS2B-NS3-mediated cleavage site in the flavivirus capsid protein demonstrates a requirement for coordinated processing. J Virol 73:8083–8094
5. Arias CF, Preugschat F, Strauss JH (1993) Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. Virology 193:888–899
6. Ashour J, Laurent-Rolle M, Shi PY et al (2009) NS5 of dengue virus mediates STAT2 binding and degradation. J Virol 83:5408–5418
7. Bartelma G, Padmanabhan R (2002) Expression, purification, and characterization of the RNA 5′-triphosphatase activity of dengue virus type 2 nonstructural protein 3. Virology 299:122–132
8. Benarroch D, Selisko B, Locatelli GA et al (2004) The RNA helicase, nucleotide 5′-triphosphatase, and RNA 5′-triphosphatase activities of Dengue virus protein NS3 are Mg2+−dependent and require a functional Walker B motif in the helicase catalytic core. Virology 328:208–218
9. Brooks AJ, Johansson M, John AV et al (2002) The interdomain region of dengue NS5 protein that binds to the viral helicase NS3 contains independently functional importin beta 1 and importin alpha/beta-recognized nuclear localization signals. J Biol Chem 277:36399–36407
10. Cahour A, Falgout B, Lai CJ (1992) Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. J Virol 66:1535–1542
11. Chambers TJ, Grakoui A, Rice CM (1991) Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. J Virol 65:6042–6050
12. Chambers TJ, Hahn CS, Galler R et al (1990) Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44:649–688
13. Chen CJ, Kuo MD, Chien LJ et al (1997) RNA-protein interactions: Involvement of NS3, NS5, and 3′ noncoding regions of Japanese encephalitis virus genomic RNA. J Virol 71:3466–3473
14. Collins TJ (2007) ImageJ for microscopy. BioTechniques 43:25–30
15. Cui T, Sugrue RJ, Xu Q et al (1998) Recombinant dengue virus type 1 NS3 protein exhibits specific viral RNA binding and NTPase activity regulated by the NS5 protein. Virology 246:409–417
16. Donnelly M, Verhagen J, Elliott G (2007) RNA binding by the herpes simplex virus type 1 nucleocytoplasmic shuttling protein UL47 is mediated by an N-terminal arginine-rich domain that also functions as its nuclear localization signal. J Virol 81:2283–2296
17. Egloff MP, Benarroch D, Selisko B et al (2002) An RNA cap (nucleoside-2′-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. EMBO J 21:2757–2768
18. Egloff MP, Decroly E, Malet H et al (2007) Structural and functional analysis of methylation and 5′-RNA sequence requirements of short capped RNAs by the

methyltransferase domain of dengue virus NS5. J Mol Biol 372:723–736
19. Elster C, Larsen K, Gagnon J et al (1997) Influenza virus M1 protein binds to RNA through its nuclear localization signal. J Gen Virol 78(Pt 7):1589–1596
20. Erbel P, Schiering N, D'Arcy A et al (2006) Structural basis for the activation of flaviviral NS3 proteases from Dengue and West Nile virus. Nat Struct Mol Biol 13:372–373
21. Forwood JK, Brooks A, Briggs LJ, Jans DA, Vasudevan SG (1999) The 37 amino acid linker between the putative methyltransferase and polymerase domains of dengue virus NS5 protein contains a functional nuclear localization signal. Biochem Biophys Res Comm 257:731–737
22. Gorbalenya AE, Donchenko AP, Koonin EV et al (1989) N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases. Nucleic Acids Res 17:3889–3897
23. Gutsche I, Coulibaly F, Voss JE et al (2011) Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high-density lipoprotein. Proc Natl Acad Sci U S A 108:8003–8008
24. Heaton NS, Perera R, Berger KL et al (2010) Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. Proc Natl Acad Sci U S A 107:17345–17350
25. Hodge K, Tunghirun C, Kamkaew M et al (2016) Identification of a conserved RdRp-RNA interface required for flaviviral replication. J Biol Chem 291:17437–17449
26. Iglesias NG, Filomatori CV, Gamarnik AV (2011) The F1 motif of dengue virus polymerase NS5 is involved in promoter-dependent RNA synthesis. J Virol 85:5745–5756
27. Issur M, Geiss BJ, Bougie I et al (2009) The flavivirus NS5 protein is a true RNA guanylyltransferase that catalyzes a two-step reaction to form the RNA cap structure. RNA 15:2340–2350
28. Johansson M, Brooks AJ, Jans DA et al (2001) A small region of the dengue virus-encoded RNA-dependent RNA polymerase, NS5, confers interaction with both the nuclear transport receptor importin-beta and the viral helicase, NS3. J Gen Virol 82:735–745
29. Kapoor M, Zhang LW, Ramachandra M et al (1995) Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. J Biol Chem 270:19100–19106
30. Kelley JF, Kaufusi PH, Volper EM et al (2011) Maturation of dengue virus nonstructural protein 4B in monocytes enhances production of dengue hemorrhagic fever-associated chemokines and cytokines. Virology 418:27–39
31. Klema VJ, Ye M, Hindupur A et al (2016) Dengue Virus Nonstructural Protein 5 (NS5) Assembles into a Dimer with a Unique Methyltransferase and Polymerase Interface. PLoS Pathog 12:e1005451
32. Kosugi S, Hasebe M, Tomita M et al (2009) Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs. Proc Natl Acad Sci U S A 106:10171–10176
33. Kroschewski H, Lim SP, Butcher RE et al (2008) Mutagenesis of the dengue virus type 2 NS5 methyltransferase domain. J Biol Chem 283:19410–19421
34. Kumar A, Buhler S, Selisko B et al (2013) Nuclear localization of dengue virus nonstructural protein 5 does not strictly correlate with efficient viral RNA replication and inhibition of type I interferon signaling. J Virol 87:4545–4557
35. Leung D, Schroder K, White H et al (2001) Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors. J Biol Chem 276:45762–45771
36. Li HT, Clum S, You SH et al (1999) The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of Dengue virus type 2 NS3 converge within a region of 20 amino acids. J Virol 73:3108–3116
37. Li J, Lim SP, Beer D et al (2005) Functional profiling of recombinant NS3 proteases from all four serotypes of dengue virus using tetrapeptide and octapeptide substrate libraries. J Biol Chem 280:28766–28774
38. Lim SP, Koh JH, Seh CC et al (2013) A crystal structure of the dengue virus non-structural protein 5 (NS5) polymerase delineates interdomain amino acid residues that enhance its thermostability and de novo initiation activities. J Biol Chem 288:31105–31114
39. Lindenbach BD, Rice CM (1999) Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. J Virol 73:4611–4621
40. Lindenbach BD, Thiel H, Rice CM (2007) Flaviviridae: the viruses and their replication. In: Fields virology, 5th edn. Wolters kluwer/Lippincott Williams & Wilkins, Philadelphia, pp 1101–1152
41. Lobigs M (1993) Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. Proc Natl Acad Sci U S A 90:6218–6222
42. Lu G, Gong P (2013) Crystal Structure of the full-length Japanese encephalitis virus NS5 reveals a conserved methyltransferase-polymerase interface. PLoS Pathog 9:e1003549
43. Luo D, Wei N, Doan DN et al (2010) Flexibility between the protease and helicase domains of the dengue virus NS3 protein conferred by the linker region and its functional implications. J Biol Chem 285:18817–18827
44. Luo D, Xu T, Hunke C et al (2008) Crystal structure of the NS3 protease-helicase from dengue virus. J Virol 82:173–183
45. Mackenzie JM, Khromykh AA, Jones MK et al (1998) Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology 245:203–215

46. Mairiang D, Zhang H, Sodja A et al (2013) Identification of new protein interactions between dengue fever virus and its hosts, human and mosquito. PLoS One 8:e53535
47. Malet H, Egloff MP, Selisko B et al (2007) Crystal structure of the RNA polymerase domain of the West Nile virus non-structural protein 5. J Biol Chem 282:10678–10689
48. Marfori M, Lonhienne TG, Forwood JK et al (2012) Structural basis of high-affinity nuclear localization signal interactions with importin-alpha. Traffic 13:532–548
49. Mazzon M, Jones M, Davidson A et al (2009) Dengue virus NS5 inhibits interferon-alpha signaling by blocking signal transducer and activator of transcription 2 phosphorylation. J Infect Dis 200:1261–1270
50. Medin CL, Fitzgerald KA, Rothman AL (2005) Dengue virus nonstructural protein NS5 induces interleukin-8 transcription and secretion. J Virol 79:11053–11061
51. Moreland NJ, Tay MY, Lim E et al (2012) Monoclonal antibodies against dengue NS2B and NS3 proteins for the study of protein interactions in the flaviviral replication complex. J Virol Methods 179:97–103
52. Morrison J, Laurent-Rolle M, Maestre AM et al (2013) Dengue virus co-opts UBR4 to degrade STAT2 and antagonize type I interferon signaling. PLoS Pathog 9:e1003265
53. Muller DA, Landsberg MJ, Bletchly C et al (2012) Structure of the dengue virus glycoprotein non-structural protein 1 by electron microscopy and single-particle analysis. J Gen Virol 93:771–779
54. Muller DA, Young PR (2013) The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. Antivir Res 98:192–208
55. Noble CG, Chen YL, Dong H et al (2010) Strategies for development of Dengue virus inhibitors. Antivir Res 85:450–462
56. Noble CG, Seh CC, Chao AT et al (2012) Ligand-bound structures of the dengue virus protease reveal the active conformation. J Virol 86:438–446
57. Phong WY, Moreland NJ, Lim SP et al (2011) Dengue protease activity: the structural integrity and interaction of NS2B with NS3 protease and its potential as a drug target. Biosci Rep 31:399–409
58. Potisopon S, Priet S, Collet A et al (2014) The methyltransferase domain of dengue virus protein NS5 ensures efficient RNA synthesis initiation and elongation by the polymerase domain. Nucleic Acids Res 42:11642–11656
59. Preugschat F, Strauss JH (1991) Processing of nonstructural proteins NS4A and NS4B of dengue 2 virus in vitro and in vivo. Virology 185:689–697
60. Preugschat F, Yao CW, Strauss JH (1990) In vitro processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. J Virol 64:4364–4374
61. Pryor MJ, Rawlinson SM, Butcher RE et al (2007) Nuclear localization of dengue virus nonstructural protein 5 through its importin alpha/beta-recognized nuclear localization sequences is integral to viral infection. Traffic 8:795–807
62. Rawlinson SM, Pryor MJ, Wright PJ et al (2009) CRM1-mediated nuclear export of dengue virus RNA polymerase NS5 modulates interleukin-8 induction and virus production. J Biol Chem 284:15589–15597
63. Selisko B, Peyrane FF, Canard B et al (2010) Biochemical characterization of the (nucleoside-2′O)-methyltransferase activity of dengue virus protein NS5 using purified capped RNA oligonucleotides (7Me)GpppAC(n) and GpppAC(n). J Gen Virol 91:112–121
64. Shi PY (2014) Structural biology. Unraveling a flavivirus enigma. Science 343:849–850
65. Shiryaev SA, Chernov AV, Aleshin AE et al (2009) NS4A regulates the ATPase activity of the NS3 helicase: a novel cofactor role of the non-structural protein NS4A from West Nile virus. J Gen Virol 90:2081–2085
66. Su XC, Ozawa K, Qi R et al (2009) NMR analysis of the dynamic exchange of the NS2B cofactor between open and closed conformations of the West Nile virus NS2B-NS3 protease. PLoS Negl Trop Dis 3:e561
67. Tajima S, Takasaki T, Kurane I (2011) Restoration of replication-defective dengue type 1 virus bearing mutations in the N-terminal cytoplasmic portion of NS4A by additional mutations in NS4B. Arch Virol 156:63–69
68. Takahashi H, Takahashi C, Moreland NJ et al (2012) Establishment of a robust dengue virus NS3-NS5 binding assay for identification of protein-protein interaction inhibitors. Antivir Res 96:305–314
69. Tay MY, Fraser JE, Chan WK et al (2013) Nuclear localization of dengue virus (DENV) 1-4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin. Antivir Res 99:301–306
70. Tay MY, Saw WG, Zhao Y et al (2014) The C-terminal 50 amino acid residues of Dengue NS3 protein are important for NS3-NS5 interaction and viral replication. J Biol Chem 290:2379–2394
71. Tay MY, Smith K, Ng IH et al (2016) The C-terminal 18 amino acid region of Dengue virus NS5 regulates its subcellular localization and contains a conserved arginine residue essential for infectious virus production. PLoS Pathog 12:e1005886
72. Tomassini JE, Boots E, Gan L et al (2003) An in vitro Flaviviridae replicase system capable of authentic RNA replication. Virology 313:274–285
73. Uchil PD, Satchidanandam V (2003) Architecture of the flaviviral replication complex. Protease, nuclease, and detergents reveal encasement within double-layered membrane compartments. J Biol Chem 278:24388–24398
74. Umareddy I, Chao A, Sampath A et al (2006) Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. J Gen Virol 87:2605–2614
75. Vasudevan SG, Johansson M, Brooks AJ et al (2001) Characterisation of inter- and intra-molecular interactions of the dengue virus RNA dependent RNA

polymerase as potential drug targets. Farmaco 56:33–36
76. Verdine GL, Hilinski GJ (2012) Stapled peptides for intracellular drug targets. Methods Enzymol 503:3–33
77. Wang CC, Huang ZS, Chiang PL et al (2009) Analysis of the nucleoside triphosphatase, RNA triphosphatase, and unwinding activities of the helicase domain of dengue virus NS3 protein. FEBS Lett 583:691–696
78. Welsch S, Miller S, Romero-Brey I et al (2009) Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host Microbe 5:365–375
79. Wengler G (1993) The NS 3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. Virology 197:265–273
80. Xu T, Sampath A, Chao A et al (2005) Structure of the Dengue virus helicase/nucleoside triphosphatase catalytic domain at a resolution of 2.4 A. J Virol 79:10278–10288
81. Yap TL, Xu T, Chen YL et al (2007) Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution. J Virol 81:4753–4765
82. Yik JH, Chen R, Pezda AC et al (2004) A human immunodeficiency virus type 1 Tat-like arginine-rich RNA-binding domain is essential for HEXIM1 to inhibit RNA polymerase II transcription through 7SK snRNA-mediated inactivation of P-TEFb. Mol Cell Biol 24:5094–5105
83. Yon C, Teramoto T, Mueller N et al (2005) Modulation of the nucleoside triphosphatase/RNA helicase and 5′-RNA triphosphatase activities of Dengue virus type 2 nonstructural protein 3 (NS3) by interaction with NS5, the RNA-dependent RNA polymerase. J Biol Chem 280:27412–27419
84. Yu L, Takeda K, Markoff L (2013) Protein-protein interactions among West Nile non-structural proteins and transmembrane complex formation in mammalian cells. Virology 446:365–377
85. Yusof R, Clum S, Wetzel M et al (2000) Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids in vitro. J Biol Chem 275:9963–9969
86. Zhang L, Mohan PM, Padmanabhan R (1992) Processing and localization of Dengue virus type 2 polyprotein precursor NS3-NS4A-NS4B-NS5. J Virol 66:7549–7554
87. Zhang Z, Li Y, Loh YR et al (2016) Crystal structure of unlinked NS2B-NS3 protease from Zika virus. Science 354:1597–1600
88. Zhao Y, Moreland NJ, Tay MY et al (2014) Identification and molecular characterization of human antibody fragments specific for dengue NS5 protein. Virus Res 179:225–230
89. Zhao Y, Soh S, Zheng J et al (2015a) A crystal structure of the dengue virus NS5 protein reveals a novel inter-domain interface essential for protein flexibility and virus replication. PLoS Pathog 11:e1004682
90. Zhao Y, Soh TS, Lim SP et al (2015b) Molecular basis for specific viral RNA recognition and 2′-O-ribose methylation by the dengue virus nonstructural protein 5 (NS5). Proc Natl Acad Sci U S A 112:14834–14839
91. Zhou Y, Ray D, Zhao Y et al (2007) Structure and function of flavivirus NS5 methyltransferase. J Virol 81:3891–3903
92. Zou G, Chen YL, Dong H et al (2011) Functional analysis of two cavities in flavivirus NS5 polymerase. J Biol Chem 286:14362–14372
93. Zou J, Lee LT, Wang QY et al (2015a) Mapping the interactions between the NS4B and NS3 proteins of dengue virus. J Virol 89:3471–3483
94. Zou J, Xie X, Lee le T et al (2014) Dimerization of flavivirus NS4B protein. J Virol 88:3379–3391
95. Zou J, Xie X, Wang QY et al (2015b) Characterization of dengue virus NS4A and NS4B protein interaction. J Virol 89:3455–3470
96. Zust R, Dong H, Li XF et al (2013) Rational design of a live attenuated dengue vaccine: 2′-o-methyltransferase mutants are highly attenuated and immunogenic in mice and macaques. PLoS Pathog 9:e1003521

Check for updates

12 Establishment and Application of Flavivirus Replicons

Beate M. Kümmerer

Abstract

Dengue virus (DENV) and Zika virus (ZIKV) are enveloped, positive-strand RNA viruses belonging to the genus Flavivirus in the family *Flaviviridae*. The genome of ~11 kb length encodes one long open reading frame flanked by a 5′ and a 3′ untranslated region (UTR). The 5′ end is capped and the 3′ end lacks a poly(A) tail. The encoded single polyprotein is cleaved co-and posttranslationally by cellular and viral proteases. The first one-third of the genome encodes the structural proteins (C-prM-E), whereas the nonstructural (NS) proteins NS1-NS2A-NS3-NS4A-2K-NS4B-NS5 are encoded by the remaining two-thirds of the genome.

Research on flaviviruses was driven forward by the ability to produce recombinant viruses using reverse genetics technology. It is known that the purified RNA of flaviviruses is per se infectious, which allows initiation of a complete viral life cycle by transfecting the genomic RNA into susceptible cells. In 1989, the first infectious flavivirus RNA was transcribed from full-length cDNA templates of yellow fever virus (YFV) facilitating molecular genetic analyses of this virus. In addition to the production of infectious recombinant viruses, reverse genetics can also be used to establish non-infectious replicons. Replicons contain an in-frame deletion in the structural protein genes but still encode all nonstructural proteins and contain the UTRs necessary to mediate efficient replication, a factor that enables their analyses under Biosafety Level (BSL) 1 conditions. This is particularly important since many flaviviruses are BSL3 agents.

The review will cover strategies for generating flavivirus replicons, including the establishment of bacteriophage (T7 or SP6) promoter-driven constructs as well as cytomegalovirus (CMV) promoter-driven constructs. Furthermore, different reporter replicons or replicons expressing selectable marker proteins will be outlined using examples of their application to answer basic questions of the flavivirus replication cycle, to select and test antiviral compounds or to produce virus replicon particles. The establishment and application of flavivirus replicons will further be exemplified by my own data using an established YFV reporter replicon to study the role of YFV NS2A in the viral life cycle. In addition, we established a reporter replicon of a novel insect-specific flavivirus, namely Niénokoué virus (NIEV), to define the barrier(s) involved in host range restriction.

B. M. Kümmerer (✉)
Institute of Virology, University of Bonn Medical Centre, Bonn, Germany

German Center for Infection Research (DZIF) Partner site Bonn-Cologne, Bonn, Germany
e-mail: kuemmerer@virology-bonn.de

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_12

Keywords

Flavivirus reverse genetics technology · Non-infectious replicons · Selectable marker proteins · Yellow fever virus NS2A protein · Niénokoué virus

12.1 Introduction

The genus Flavivirus in the family Flaviviridae comprises over 50 species including medically important viruses like yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV) or Japanese encephalitis virus (JEV). Also Zika virus (ZIKV), which recently emerged in the South Americas belongs to the genus flavivirus.

The ability to reconstruct and modify flaviviral genomes opens up a variety of applications in the field of these important RNA viruses. Many of these viruses cause important human diseases like dengue fever, yellow fever, Zika virus disease or Japanese encephalitis. Hence, reverse genetic tools like flavivirus replicons are of utmost importance to better understand the flavivirus life cycle, but also in terms of applied science like antiviral screening, development of vaccines or diagnostic assays.

The genome of flaviviruses is a positive-sense RNA of about 11 kb in length that is capped at the 5′ end but lacks a poly(A) tail. The RNA contains one open reading frame (ORF) which is flanked by a 5′ and a 3′ untranslated region (UTR). The ORF encodes one large polyprotein, which is cleaved by host cell and viral proteases to release the mature viral proteins. The structural proteins (capsid, C; precursor membrane protein, prM; envelope, E) are encoded from the 5′-terminal part of the genome, whereas the remaining two-thirds encode the nonstructural proteins. The order of the proteins in the polyprotein is NH_2-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5-COOH. Whereas most cleavages in the structural protein region are mediated by cell-derived signalases, the majority of cleavages in the nonstructural protein region are mediated by the virus encoded NS2B-3 serine protease. The central role in viral RNA replication is played by the RNA-dependent RNA polymerase NS5 (see Lindenbach et al. [17] for detailed description of polyprotein processing and functions of flaviviral proteins).

12.2 Establishment of Flavivirus Replicons

Reverse genetics encompasses the introduction of mutations into the genome of an organism and the subsequent analyses of the impact of the mutations on the phenotype. Since mutations can only be introduced on the level of DNA, the genomes of RNA viruses need to be first reverse transcribed. The resulting complementary DNA (cDNA), which can be mutagenized, is cloned into a plasmid e.g. under control of a bacteriophage T7 or SP6 RNA polymerase promoter [14, 30]. The latter permits the generation of RNA via *in vitro* transcription from the mutagenized cDNA using T7 or SP6 RNA polymerase, respectively. In the case of positive strand RNA viruses the viral genome is per se infectious, hence, electroporation of RNA transcribed *in vitro* from a flavivirus cDNA clone can initiate a viral life cycle resulting in the production of recombinant virus. In contrast, subgenomic replicons possess all genetic elements necessary for self-replication, but lack the structural genes necessary for the production of progeny virus (Fig. 12.1). In general, flavivirus replicons mostly exhibit a large in frame deletion encompassing the C-prM-E region. However, at least the first 20 codons of the C protein are necessary since they contain important elements necessary for efficient RNA replication (*cis*-acting elements) [4, 13]. Furthermore, the 3′ terminal sequence of the E gene (24–30 codons) needs to be retained, since it encodes the signal sequence for the downstream NS1 protein [10, 13].

As mentioned, the viral replicon sequence can be cloned into a plasmid under control of a bacteriophage promoter like the T7 or SP6 RNA polymerase promoter [10, 13, 29]. These promoters mediate the start of the *in vitro* transcribed RNA. To obtain the authentic 3′ terminus of the

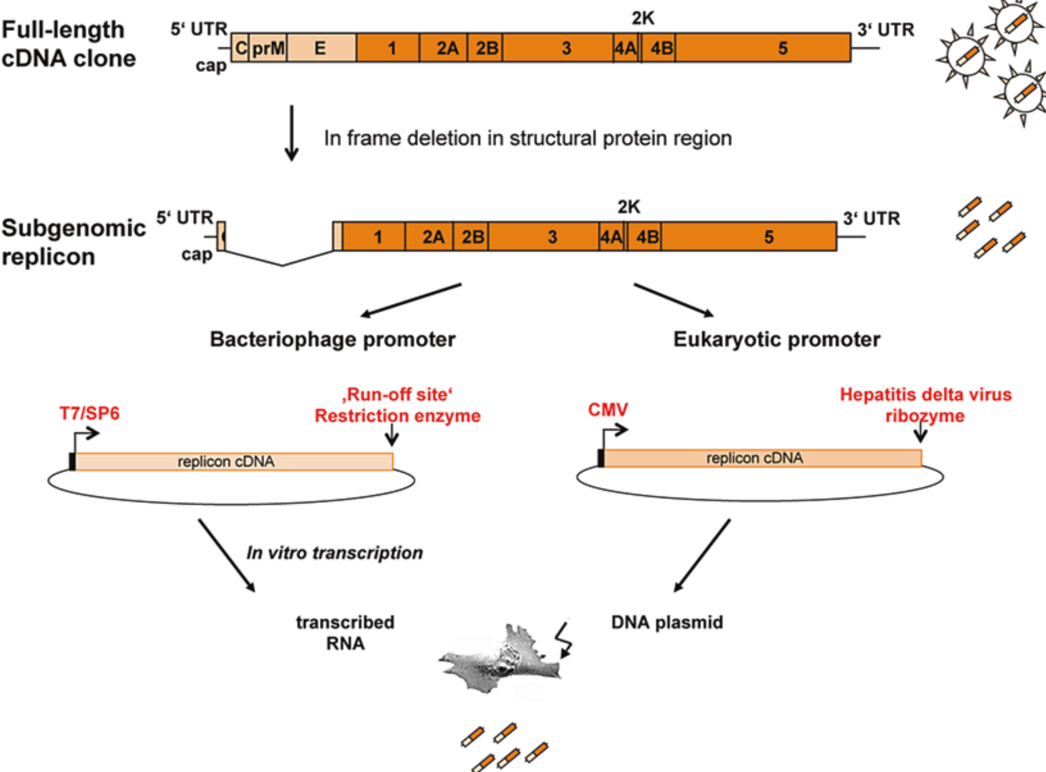


Fig. 12.1 Schematic presentation of the sequences encoded by a flavivirus full-length cDNA clone and a flavivirus subgenomic replicon construct. While the full-length clone allows recovering infectious virus, the subgenomic replicon only mediates replication of the subgenomic RNA without virus production. The replicon sequences can either be cloned under control of a T7 or SP6 bacteriophage promoter or a eukaryotic cytomegalovirus (CMV) promoter. In the first case, linearization at the 3′ end of the viral genome with a unique restriction enzyme mediates production of the viral 3′ end after run-off *in vitro* transcription. Transfection of the obtained RNA into eukaryotic cells results in replication of the flavivirus subgenomic replicon. In contrast, the CMV-driven construct allows transfection of the DNA plasmid. In this case, the viral 3′ end is generated by a hepatitis delta virus ribozyme inserted directly after the viral 3′ end upon transcription of the viral sequence in the nucleus. Light orange: structural proteins; dark orange: nonstructural proteins NS1–NS5. UTR, untranslated region; C, capsid; prM, precursor membrane protein; E, envelope protein

viral genome, a singular restriction enzyme site is inserted into the plasmid just downstream of the 3′ end of the viral genome. The plasmid linearized at this site is then used for *in vitro* run-off transcription, in which the T7 or SP6 RNA polymerase falls off the template at the linearized site. This results in *in vitro* transcribed RNA ending with the 3′ end of the viral genome, which upon transfection into eukaryotic cells results in replication of the subgenomic RNA (Fig. 12.1).

Alternatively, the viral sequence can be cloned under control of an eukaryotic promoter like the cytomegalovirus (CMV) promoter [12]. In this case transcription of the viral subgenomic RNA occurs in the nucleus of the cell and production of the authentic 3′ end of the viral genome has to be mediated by a hepatitis delta virus ribozyme cloned downstream of the viral genome. The establishment of CMV promoter-driven replicon constructs allows direct transfection of plasmid DNA (Fig. 12.1). However, one needs to be aware that flaviviruses replicate in the cytoplasm and the viral genomes might contain splice sites that interfere with the production of transcripts when using the CMV promoter-driven approach. Nevertheless, both strategies have been pursued successfully for

several flaviviruses, including WNV and its close relative Kunjin virus (KUNV), the YFV, DENV, JEV, tick-borne encephalitis virus (TBEV) and ZIKV [2, 5, 10, 12, 16, 21, 22, 29, 35].

12.3 Applications of Flavivirus Replicons

Subgenomic flavivirus replicons find their application in several research fields. Besides analyses of flavivirus replication, testing of antiviral compounds, heterologous gene expression, chimeric vaccine production or the production of virus replicon particles (VRPs) are described. Furthermore, subgenomic replicons have recently been used for host range restriction studies.

12.3.1 Analyses of RNA Replication

Many flaviviruses, like WNV, JEV and DENV are classified as BSL3 agents. Hence, working with these viruses require a BSL3 facility, which can be a costly undertaking. In contrast, flavivirus replicons do not produce infectious particles and therefore can be handled under BSL1 and BSL2 conditions making them a convenient tool to analyse flavivirus RNA replication.

To be able to easily follow the replication of subgenomic flavivirus replicons, reporter protein genes can be introduced. Common reporter proteins that are used allow either luminometric detection (e.g. Renilla, Firefly or Gaussia luciferase) or fluorescent detection (e.g. green fluorescent protein (GFP) or mCherry) of RNA replication. In many cases the reporter protein gene is introduced in place of the deleted structural protein region. An ubiquitin coding sequence can be fused upstream of the reporter gene [11, 34]. This allows production of the N-terminus of the reporter protein via cleavage of cellular ubiquitin hydrolases. To release the reporter protein at its C-terminus from the polyprotein, a foot-and-mouth disease virus (FMDV) 2A autoprotease is inserted downstream of the reporter [10, 34]. The FMDV 2A autoprotease mediates cleavage at the fusion site to the C-terminal E protein domain, which serves as a signal sequence for the subsequent NS1 protein (Fig. 12.2a). Alternatively, introduction of the reporter gene has been described via construction of bicistronic replicons that allow introduction of a stop codon at the 3′ terminus of the reporter gene [10]. The reporter protein is still expressed from the 5′ terminal region of the genome and translation initiation of the nonstructural proteins is then mediated by an internal ribosomal entry site (IRES) derived from the encephalomyocarditis virus (Fig. 12.2a). Alternatively, an IRES-driven reporter gene cassette can be introduced after the ORF in the 3′ UTR of the viral genome [10, 31] (Fig. 12.2a). The transfection of such flavivirus reporter replicons enables transient expression of the reporter proteins. Since the level of reporter gene expression correlates with the levels of accumulated replicon RNA, measurement of the reporter protein allows the follow up of the replication efficiency of the replicon. Therefore, such subgenomic reporter replicons represent a great tool to answer basic questions of the flavivirus replication cycle. This includes the functional analysis of single flavivirus nonstructural proteins by determining the impact of specific mutations on the replication efficacy. Using a YFV reporter replicon, comparative replication analyses have, for example, been performed for a set of NS2A charged-to-alanine scanning mutants, which were also analysed based on an infectious full-length clone [34]. Those studies demonstrated that certain NS2A mutants which affect the production of infectious particles are still able to replicate to wild type levels while other NS2A mutations also affect RNA replication. This indicates a role of NS2A both in infectious particle production as well as RNA replication. However, for certain studies the use of self-limiting replicons might also be disadvantageous, e.g. when second-site mutations shall be selected that are able to compensate a defect in infectious particle production as was the case in our NS2A charged-to-alanine scanning analyses [34].

Besides mutations in nonstructural proteins, determinants of the UTRs important for RNA translation and replication were analysed using flavivirus replicons. For example a DENV

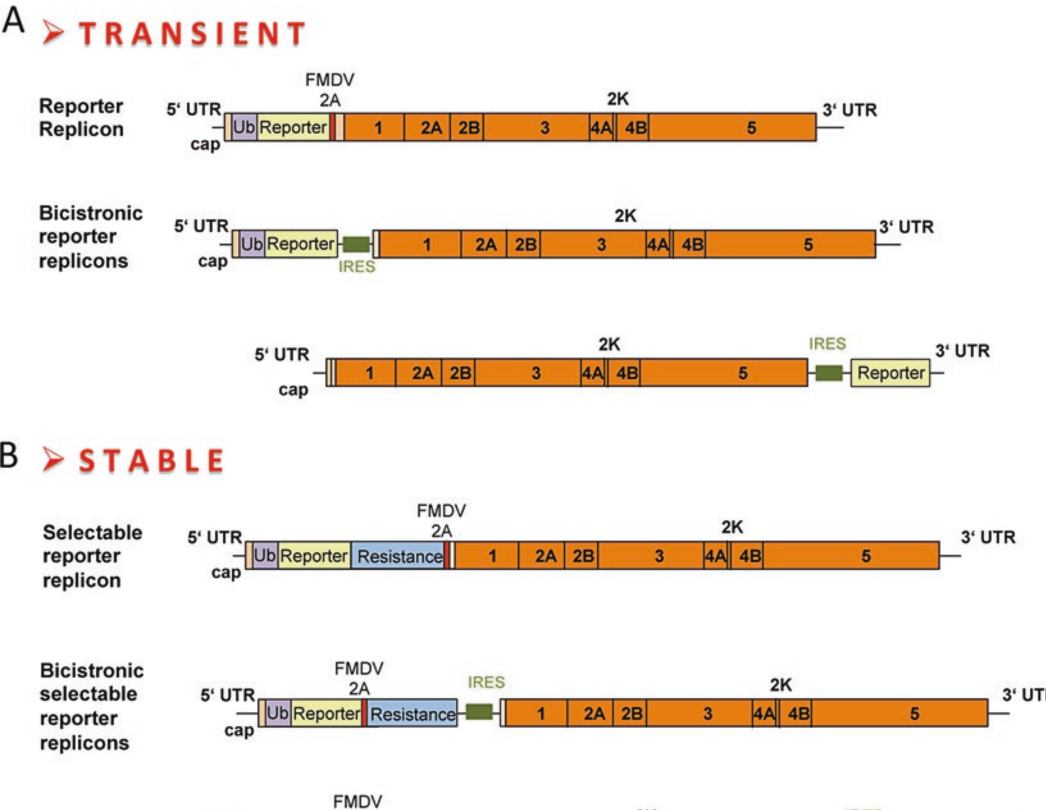


Fig. 12.2 Schematic presentation of constructs for (**a**) transient or (**b**) stable expression of subgenomic flavivirus reporter replicons. Flavivirus replicons can either be established as mono- or bicistronic variants. In case of bicistronic variants translation of the second open reading frame is mediated by an internal ribosomal entry side (IRES). Vectors for the generation of stable flavivirus replicon cell lines express a resistance gene for selection. Ub, ubiquitin; FMDV2A, foot-and-mouth disease virus 2A; UTR, untranslated region

replicon was used to determine the role of RNA structures in the 3′ UTR on DENV translation, RNA synthesis and viral replication [1].

12.3.2 Testing of Antiviral Compounds

Although transient replication of subgenomic reporter replicons can be used to test antiviral compounds affecting RNA replication, establishment of a cell line stably harbouring the replicon largely facilitates high-throughput antiviral screening. To this end, insertions of resistance genes into the flavivirus replicons have been described. Commonly used resistance genes are the puromycin N-acetyl-transferase (pac) or a neomycin phosphotransferase (neo) gene. Both monocistronic as well as bicistronic replicons have been described that combine the expression of a reporter and a resistance gene allowing stable expression of the reporter protein by the flavivirus replicon (Fig. 12.2b). Dependent on the cytopathic nature of the flavivirus replicon in the cell type used, selection under the drug might be accompanied by accumulation of mutations mediating a noncytopathic phenotype. Antiviral screening has been performed using a pool of the selected cell clones. Alternatively, individual cell clones can be characterized and expanded. The use of stable cell lines that contain persistently replicating reporter replicons for high-throughput

antiviral screening has been described for several flaviviruses including WNV, DENV and ZIKV [15, 18, 35, 37]. However, stable subgenomic replicon cell lines only allow the discovery of drugs affecting RNA replication but not viral entry or assembly/release.

12.3.3 Heterologous Gene Expression

Besides insertion of reporter proteins and selection markers as already mentioned above, flavivirus replicons have also been used to express other foreign genes of interest. This is especially so for the noncytophatic KUNV replicon, which was used as a vector for gene expression using both the T7 and CMV-promoter driven approaches. The foreign proteins are C-terminally fused to an FMDV-2A protease sequence to allow release of the viral polyprotein [33]. Cleavage at the N-terminus of the foreign protein was either also mediated by an FMDV-2A protease or by introduction of an ubiquitin sequence, which allows cleavage by cellular ubiquitin hydrolases [2, 6, 26]. Alternatively, the foreign proteins have been produced as fusion products retaining the N-terminal 20 amino acids of the flavivirus C protein [33]. Expressed foreign proteins of interest included for example the glycoprotein G of vesicular stomatitis virus, the Core and NS3 proteins of hepatitis C virus, the HIV-1 Gag antigen or the Ebolavirus glycoprotein GP [6, 24, 26, 33]. In the initial analyses, protein expression served as proof of principle for the use of replicons as a vector. Subsequent studies expressed specific viral proteins or epitopes as potential vaccine candidates. In the latter case, the replicons were often packaged into virus replicon particles (VRPs) to allow better delivery by providing the structural proteins in *trans*. This will be outlaid in more detail below.

12.3.4 Virus Replicon Particles

As mentioned, flavivirus replicons can be packaged to yield VRPs by providing the structural proteins in *trans*. In contrast to replicons, virus replicon particles allow performing studies that involve viral entry. Furthermore, VRPs represent single round infectious particles (SRPs), which are especially advantageous for studies on flaviviruses that are classified as BSL3 agents.

Different ways have been described to mediate expression of the structural proteins in *trans*. Among those are the establishment of tetracycline inducible cell lines, alphavirus replicons or lentiviral expression vectors expressing C, prM and E [7, 10, 27, 28]. The structural proteins can either be expressed from one template as C-prM-E expressing cassette [7, 10] or divided among two constructs, one expressing the mature capsid protein and the other expressing prM-E proceeded by the capsid anchor, which also functions as signal sequence of prM [27]. As mentioned, VRPs are often used for vaccine studies, either against the respective flavivirus itself [3, 8, 32] or against members of other virus families for which they express specific antigens. Examples for the latter case include vaccine studies against HIV, Ebola virus or enterovirus-71 [9, 24, 26]. Using the packaged flavivirus replicons allows delivery of the gene of interest via an infection process rather than a transfection process without resulting in a spread of infection.

In addition, VRPs allow screening of antiviral compounds that interfere with viral entry and/or RNA replication where inhibition of entry or RNA replication will result in reduced levels of the reporter protein. Use of VRPs in antiviral screening has been applied to different flaviviruses including JEV, WNV, and DENV [19, 23, 25].

Since the envelope proteins are primary targets for the generation of neutralizing antibodies, VRPs find their application also in neutralization assays. In case of reporter replicon containing VRPs, the

level of neutralization can be determined as a function of reporter gene expression with neutralizing antibodies reducing the level of the reporter protein. VRP based neutralization assays have been described for several flaviviruses including ZIKV, DENV, and TBE [20, 36, 38].

12.3.5 Host Restriction Studies

The genus Flavivirus contains arthropod-borne viruses infecting vertebrates as well as insect-specific viruses whose host range is restricted to insects. These different host ranges raised the question at which level(s) the insect-specific viruses are blocked with regard to infection of vertebrates. As flavivirus replicons are delivered into cells by transfection, the entry step is circumvented. This allows direct analysis of the level of RNA translation and replication independent of the entry step. In the case of Niénokoué virus (NIEV), an insect-specific virus isolated from mosquitoes in Côte d'Ivoire, a reporter replicon expressing Renilla luciferase was established to analyse RNA translation and replication in vertebrate cells [11]. While efficient RNA replication of the NIEV replicon was observed in insect cells, electroporation into vertebrate cells did not result in RNA replication. In contrast, initial translation of the incoming RNA also occurred in vertebrate cells as proven by the increasing Renilla levels early after electroporation. Flavivirus replicons therefore represent a useful tool to dissect different steps of the viral life cycle in different hosts.

12.3.6 Conclusion

As reverse genetic tools, flavivirus replicons represent a major breakthrough. Their applications are widespread, spanning from vaccine and diagnostic development to antiviral research and can be transferred to other new and re-emerging flaviviruses.

Discussion of Chapter 12 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Beate Mareike Kümmerer.

Rolf Hilgenfeld: I would like to know, why it is important to be able to cleave out your reporter protein by proteases. Is it really necessary to do this? And the second question would be: Do you make sure in every case that the luciferase activity from your reporter protein is proportional to RNA synthesis?

Beate Kümmerer: Actually, at the N-terminal part, some people do not include the ubiquitin signal sequence for cleavage and they just have a certain number of codons from the capsid protein attached to the reporter. This is not a problem, you can always try it and it may also depend on what reporter you are using. I think for FMDV-2A after the reporter gene, this is necessary, because it is important to mediate cleavage before the signal sequence of NS1. But at the N-terminus, some people leave out the ubiquitin and just make this fusion. The second question was whether it is always proportional. That is basically the case, because each RNA that is produced also gets translated and then the more RNA you have, the more translation you have. This is known and you can measure this.

David Jans: When you do the second-site mutation selection experiment in cell culture you wait a long time. Do you get the mutation in other genes?

Beate Kümmerer: Sometimes no, sometimes yes. For one NS2A mutant we did get one in NS3 actually.

David Jans: In that sort of experiments do you analyze them further to characterize the double mutants?

Beate Kümmerer: So actually, for the NS2A-NS3 story that has already been published, we did some co-immunoprecipitation studies and cloned these genes and their mutants to see whether they interact.

Aravinda de Silva: I have a question about the rescue system. When you express the structural proteins in *trans*, and when you recover those single-round infectious virions, how efficient is that rescue compared to the wild-type virus? In other words: Is assembly the same, when you express the structural genes in *trans,* compared to when everything is expressed off one large RNA molecule?

Beate Kümmerer: The things that I have covered in my talk are from the published literature that I have reviewed. So we have not done too much in this area ourself. However we have done one construct where we actually have the capsid until the NS3 protease in one open reading frame for expression in *trans*. Thereby we have the protease that cleaves also the capsid protein, because you need to get rid of the anchor region to get good packaging. I know from publications that people say it is difficult to get VLPs. If you express it, you have the transmembrane-domain in the E protein that may misfold and it is not so efficient and you have to play around with conditions to express it. But these were not things that we did in the lab, but more like a review of what people did from the literature.

Aravinda de Silva: I understand that at least for dengue, the systems are **very** inefficient and I am also concerned that when the structure has been done on those virions, then it is actually based on the rescue, thus, how similar are they to the wild-type virus?

Beate Kümmerer: This is probably what Félix Rey knows better: The structures of the VRPs.

Félix Rey: I do not think anyone has done this. VLPs, just prM and E-protein, were studied in tick-borne encephalitis virus. And they were extremely heterogeneous and not a good system to start with.

Aravinda de Silva: Those were VLPs, just consisting of prM and E protein?

Félix Rey: Just recombinant [proteins], exactly, but those virus replicon particles (VRPs) that you mentioned would probably have RNA. What we studied did not have RNA.

References

1. Alvarez DE, De Lella Ezcurra AL, Fucito S, Gamarnik AV (2005) Role of RNA structures present at the 3′UTR of Dengue virus on translation, RNA synthesis, and viral replication. Virology 339:200–212
2. Anraku I, Harvey TJ, Linedale R, Gardner J, Harrich D, Suhrbier A, Khromykh AA (2002) Kunjin virus replicon vaccine vectors induce protective CD8+ T-cell immunity. J Virol 76:3791–3799
3. Cao F, Li XF, Yu XD, Deng YQ, Jiang T, Zhu QY, Qin ED, Qin CF (2011) A DNA-based West Nile virus replicon elicits humoral and cellular immune responses in mice. J Virol Methods 178:87–93
4. Corver J, Lenches E, Smith K, Robison RA, Sando T, Strauss EG, Strauss JH (2003) Fine mapping of a cis-acting sequence element in yellow fever virus RNA that is required for RNA replication and cyclization. J Virol 77:2265–2270
5. Gehrke R, Ecker M, Aberle SW, Allison SL, Heinz FX, Mandl CW (2003) Incorporation of tick-borne encephalitis virus replicons into virus-like particles by a packaging cell line. J Virol 77:8924–8933
6. Harvey TJ, Anraku I, Linedale R, Harrich D, Mackenzie J, Suhrbier A, Khromykh AA (2003) Kunjin virus replicon vectors for human immunodeficiency virus vaccine development. J Virol 77:7796–7803
7. Harvey TJ, Liu WJ, Wang XJ, Linedale R, Jacobs M, Davidson A, Le TT, Anraku I, Suhrbier A, Shi PY, Khromykh AA (2004) Tetracycline-inducible packaging cell line for production of flavivirus replicon particles. J Virol 78:531–538
8. Huang Y, Liu S, Yang P, Wang C, Du Y, Yu W, Sun Z (2012) Replicon-based Japanese encephalitis virus vaccines elicit immune response in mice. J Virol Methods 179:217–225
9. Huang YT, Liao JT, Yen LC, Chang YK, Lin YL, Liao CL (2015) Japanese encephalitis virus replicon-based vaccine expressing enterovirus-71 epitope confers dual protection from lethal challenges. J Biomed Sci 22:74
10. Jones CT, Patkar CG, Kuhn RJ (2005) Construction and applications of yellow fever virus replicons. Virology 331:247–259
11. Junglen S, Korries M, Grasse W, Wieseler J, Kopp A, Hermanns K, Leon-Juarez M, Drosten C, Kümmerer BM (2017) Host range restriction of insect-specific Flaviviruses occurs at several levels of the viral life cycle. mSphere 2:e00375–e00316

12. Khromykh AA, Varnavski AN, Sedlak PL, Westaway EG (2001) Coupling between replication and packaging of flavivirus RNA: evidence derived from the use of DNA-based full-length cDNA clones of Kunjin virus. J Virol 75:4633–4640
13. Khromykh AA, Westaway EG (1997) Subgenomic replicons of the flavivirus Kunjin: construction and applications. J Virol 71:1497–1505
14. Lai CJ, Zhao BT, Hori H, Bray M (1991) Infectious RNA transcribed from stably cloned full-length cDNA of dengue type 4 virus. Proc Natl Acad Sci U S A 88:5139–5143
15. Leardkamolkarn V, Sirigulpanit W (2012) Establishment of a stable cell line coexpressing Dengue virus-2 and green fluorescent protein for screening of antiviral compounds. J Biomol Screen 17:283–292
16. Li SH, Li XF, Zhao H, Deng YQ, Yu XD, Zhu SY, Jiang T, Ye Q, Qin ED, Qin CF (2013) Development and characterization of the replicon system of Japanese encephalitis live vaccine virus SA14-14-2. Virol J 10:64
17. Lindenbach BD, Murrey CL, Thiel H-J, Rice CM (2013) Flaviviridae. In: Knipe DM, Howley PM (eds) Fields virology, 6th edn. Lippincott Williams & Wilkons, Philadelphia, pp 712–746
18. Lo MK, Tilgner M, Shi PY (2003) Potential high-throughput assay for screening inhibitors of West Nile virus replication. J Virol 77:12901–12906
19. Lu CY, Hour MJ, Wang CY, Huang SH, Mu WX, Chang YC, Lin CW (2017) Single-round infectious particle antiviral screening assays for the Japanese Encephalitis virus. Virus 9:76
20. Mukherjee S, Pierson TC, Dowd KA (2014) Pseudo-infectious reporter virus particles for measuring antibody-mediated neutralization and enhancement of dengue virus infection. Methods Mol Biol 1138:75–97
21. Pang X, Zhang M, Dayton AI (2001) Development of Dengue virus type 2 replicons capable of prolonged expression in host cells. BMC Microbiol 1:18
22. Pierson TC, Sanchez MD, Puffer BA, Ahmed AA, Geiss BJ, Valentine LE, Altamura LA, Diamond MS, Doms RW (2006) A rapid and quantitative assay for measuring antibody-mediated neutralization of West Nile virus infection. Virology 346:53–65
23. Puig-Basagoiti F, Deas TS, Ren P, Tilgner M, Ferguson DM, Shi PY (2005) High-throughput assays using a luciferase-expressing replicon, virus-like particles, and full-length virus for West Nile virus drug discovery. Antimicrob Agents Chemother 49:4980–4988
24. Pyankov OV, Bodnev SA, Pyankova OG, Solodkyi VV, Pyankov SA, Setoh YX, Volchkova VA, Suhrbier A, Volchkov VV, Agafonov AA, Khromykh AA (2015) A Kunjin Replicon virus-like particle vaccine provides protection against Ebola virus infection in nonhuman primates. J Infect Dis 212(Suppl 2):S368–S371
25. Qing M, Liu W, Yuan Z, Gu F, Shi PY (2010) A high-throughput assay using dengue-1 virus-like particles for drug discovery. Antivir Res 86:163–171
26. Reynard O, Mokhonov V, Mokhonova E, Leung J, Page A, Mateo M, Pyankova O, Georges-Courbot MC, Raoul H, Khromykh AA, Volchkov VE (2011) Kunjin virus replicon-based vaccines expressing Ebola virus glycoprotein GP protect the guinea pig against lethal Ebola virus infection. J Infect Dis 204(Suppl 3):S1060–S1065
27. Scaturro P, Trist IM, Paul D, Kumar A, Acosta EG, Byrd CM, Jordan R, Brancale A, Bartenschlager R (2014) Characterization of the mode of action of a potent dengue virus capsid inhibitor. J Virol 88:11540–11555
28. Scholle F, Girard YA, Zhao Q, Higgs S, Mason PW (2004) trans-Packaged West Nile virus-like particles: infectious properties in vitro and in infected mosquito vectors. J Virol 78:11605–11614
29. Shi PY, Tilgner M, Lo MK (2002) Construction and characterization of subgenomic replicons of New York strain of West Nile virus. Virology 296:219–233
30. Sumiyoshi H, Tignor GH, Shope RE (1995) Characterization of a highly attenuated Japanese encephalitis virus generated from molecularly cloned cDNA. J Infect Dis 171:1144–1151
31. Suzuki R, de Borba L, Duarte dos Santos CN, Mason PW (2007) Construction of an infectious cDNA clone for a Brazilian prototype strain of dengue virus type 1: characterization of a temperature-sensitive mutation in NS1. Virology 362:374–383
32. Suzuki R, Ishikawa T, Konishi E, Matsuda M, Watashi K, Aizaki H, Takasaki T, Wakita T (2014) Production of single-round infectious chimeric flaviviruses with DNA-based Japanese encephalitis virus replicon. J Gen Virol 95:60–65
33. Varnavski AN, Khromykh AA (1999) Noncytopathic flavivirus replicon RNA-based system for expression and delivery of heterologous genes. Virology 255:366–375
34. Vossmann S, Wieseler J, Kerber R, Kümmerer BM (2015) A basic cluster in the N terminus of yellow fever virus NS2A contributes to infectious particle production. J Virol 89:4951–4965
35. Xie X, Zou J, Shan C, Yang Y, Kum DB, Dallmeier K, Neyts J, Shi PY (2016) Zika Virus Replicons for Drug Discovery. EBioMedicine 12:156–160
36. Yamanaka A, Moi ML, Takasaki T, Kurane I, Matsuda M, Suzuki R, Konishi E (2017) Utility of Japanese encephalitis virus subgenomic replicon-based single-round infectious particles as antigens in neutralization tests for Zika virus and three other flaviviruses. J Virol Methods 243:164–171
37. Yang CC, Hu HS, Wu RH, Wu SH, Lee SJ, Jiaang WT, Chern JH, Huang ZS, Wu HN, Chang CM, Yueh A (2014) A novel Dengue virus inhibitor, BP13944, discovered by high-throughput screening with dengue virus replicon cells selects for resistance in the viral NS2B/NS3 protease. Antimicrob Agents Chemother 58:110–119
38. Yoshii K, Ikawa A, Chiba Y, Omori Y, Maeda J, Murata R, Kariwa H, Takashima I (2009) Establishment of a neutralization test involving reporter gene-expressing virus- like particles of Tick-borne encephalitis virus. J Virol Methods 161:173–176

13 Strategies Towards Protease Inhibitors for Emerging Flaviviruses

Christoph Nitsche

Abstract

Infections with flaviviruses are a continuing public health threat. In addition to vaccine development and vector control, the search for antiviral agents that alleviate symptoms in patients are of considerable interest. Among others, the flaviviral protease NS2B-NS3 is a promising drug target to inhibit viral replication. Flaviviral proteases share a high degree of structural similarity and substrate-recognition profile, which may facilitate a strategy towards development of pan-flaviviral protease inhibitors. However, the success of various drug discovery attempts during the last decade has been limited by the nature of the viral enzyme as well as a lack of robust structural templates. Small-molecular, structurally diverse protease inhibitors have been reported to reach affinities in the lower micromolar range. Peptide-based, substrate-derived compounds are often nanomolar inhibitors, however, with highly compromised drug-likeness. With some exceptions, the antiviral cellular activity of most of the reported compounds have been patchy and insufficient for further development. Recent progress has been made in the elucidation of inhibitor binding using different structural methods. This will hopefully lead to more rational attempts for the identification of various lead compounds that may be successful in cellular assays, animal models and ultimately translated to patients.

Keywords

Protease · Inhibitor · Peptides · Small-molecular · Flavivirus · Dengue · West Nile · Zika

C. Nitsche (✉)
Research School of Chemistry, The Australian National University, Canberra, Australia
e-mail: christoph.nitsche@anu.edu.au

13.1 The Need for Antivirals Against Flaviviruses

The inhibition of viral enzymes plays an outstanding role in antiviral therapy, especially in cases where vaccines and vector control are not suffieciently robust. Recent progress in flaviviral vaccine development faces crucial challenges with unpredictable outcomes. Four dengue virus (DENV) where there are four serotypes, the serotype-related cross reactivity with human antibodies has been one of the main difficulties. DENV serotypes, are widely spread over the tropical and subtropical countries around the world and have become endemic in more than 100 countries within the last five decades. Antibody depen-

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_13

dent enhancement (ADE) during secondary infection after a previous infection with a different serotype can cause severe life-threatening symptoms, such as dengue haemorrhagic fever or shock syndrome. For this reason vaccines that simultaneously create pronounced immunity against all four serotypes are needed. Since late 2015, the first vaccine CYD (Dengvaxia), with significantly varying efficiency among the four dengue serotypes, has been approved in several countries. However, recent studies have demonstrated that the effect of cross reactivity is not limited to the four known dengue serotypes. Immunity to dengue can cause antibody-dependent enhancement of Zika virus infections and potentially increases viremia and severity of the disease [7, 13, 39]. Consequently, it cannot be ruled out that treatment with dengue vaccine may increase the chance of a later enhanced Zika virus infection. Due to such unexpected phenomena, the extensive search for effective antiviral drugs should be promoted in addition to vaccine development. One important approach aims to inhibit viral protease activity, as successfully demonstrated for chronic diseases such as hepatitis C or AIDS, where, among others, inhibitors of HCV and HIV proteases are established in modern combination therapy.

13.2 Flaviviral Proteases as Drug Targets

Due to limited druggability, all campaigns towards clinically relevant protease inhibitors for emerging flaviviruses have so far been unsuccessful. However, since the flaviviral proteases share a high degree of similarity in shape, substrate recognition and catalytic function, a successful drug development process may lead to pan-flaviviral protease inhibitors, which could be used against several globally challenging infectious diseases, such as dengue, West Nile or Zika. Therefore, the present chapter aims to briefly summarize the progress that has been made and tries to derive strategies for future attempts focusing on the protease. Within the last decade, a particular focus has been on the proteases of dengue and West Nile viruses. The highlights for these two most prominent examples will be discussed in this chapter. For a more comprehensive and detailed analysis regarding anti-infectives for these two viruses the interested reader is kindly referred to recent reviews [4, 27, 28, 33]. The chapter will also highlight recent campaigns for the identification of suitable inhibitors for Zika virus protease. Outcomes from all these attempts will inform general perspectives for a more efficient and hopefully successful search for drug-like protease inhibitors for flaviviruses.

13.3 Function and Structure of Flaviviral Proteases

Flaviviruses consist of a single-stranded positive-sense RNA genome, which is translated into a single polyprotein by the host cell's ribosomal system. The polyprotein comprises three structural (C, prM, E) and eight non-structural (NS) proteins, which have to be released from the polyprotein after selective protease cleavage [9, 26, 33]. This essential posttranslational procession is executed by host and flaviviral proteases at the membrane of the endoplasmatic reticulum. The viral protease complex comprises a protease unit, located at the N-terminal part of NS3 and requires a hydrophilic core fragment of the membrane-associated protein NS2B as cofactor for catalytic activity. Highly conserved residues S135, His51 and Asp75 assemble the catalytic triad. The flaviviral serine NS2B-NS3 endoproteases show a common tendency to cleave peptidic backbones after two basic residues. However, detailed substrate preferences as well as catalytic efficiency vary among different flaviviruses.

Several crystal structures of dengue and West Nile proteases have been reported during the last decade. The main deviations are related to the role of the cofactor NS2B. Some structures reported NS2B to be disordered (referred as open or inactive form) whereas others resolved the cofactor domain wrapped around the active site of NS3 (referred as closed or active form). NMR studies indicate that, regardless of the presence or absence of ligands, the closed conformation is

predominant [12, 47]. The importance of NS2B towards correct folding of the disordered NS3 domain has also been demonstrated [16]. Unfortunately, only a limited number of co-crystal structures with ligands or inhibitors are available (as a reliable basis for rational drug design). None of those comprise a small-molecular drug-like inhibitor. The first X-ray crystal structures of dengue and West Nile virus proteases became available in 2006 [14]. In case of West Nile virus this structure showed the catalytically active closed conformation with a substrate-derived tetrapeptidic aldehyde inhibitor covalently bound to Ser135 (pdb code: 2FP7). In case of dengue serotype 2 only an inactive open protease form without ligand could be crystallized (pdb code: 2FOM). Although inadequate, the latter structure was used as basis for several drug discovery campaigns until a closed and active form of dengue protease serotype 3 with the same tetrapeptidic aldehyde inhibitor became available in 2012 (pdb code: 3U1I) [37].

Recently, the first crystal structure of Zika virus protease in the active form with a boronate inhibitor, which suits a reasonable model for rational drug discovery campaigns, could be solved (pdb code: 5LC0) [25]. A second crystal structure without inhibitor revealed the open form (apo) of the enzyme with missing resolution for the C-terminal part of NS2B (pdb code: 5GXJ) [11]. A significantly divergent conformation was also obtained for an NS3 loop region between residues 152 and 167, which contributes to the S_1 shape. The obvious deviations between these initial crystal structures suggested a conformational activation upon substrate or inhibitor binding. Additional crystallographic and in-solution experiments were necessary to analyse these results in more detail. Since, several new crystal structures and NMR studies in presence and absence of ligands, covering ‘pre-open’ (pdb code: 5T1V), open and closed conformations have been reported [24, 30, 38, 56]. They discovered, in contrast to previous studies with dengue and West Nile protease, a more delicate dependence of open and closed conformations from the construct that was used to fuse NS2B and NS3. The artificial covalently linked construct gZiPro only adopts the closed conformation in presence of substrate-like ligands, but can also bind inhibitors in the open conformation [30]. A construct with an autocleavage site between NS2B and NS3 (eZiPro) showed the closed conformation in the crystal structure (pdb code: 5GJ4), but NMR relaxation data indicated high mobility of NS2B in solution [38]. Apparently, a C-terminal tetrapeptide of NS2B that was found to occupy the active site could not maintain the closed state in solution. A construct without covalent linkage (bZiPro) displayed the closed conformation in solution and in the single crystal (pdb code: 5GPI) [56]. This construct could even be used to capture the structure of a fragment hit in the closed state after being soaked into the crystal (pdb code: 5H4I) [56]. Consequently, bZiPro represents the most suitable construct for compound screening in addition to gZiPro, which may be superior to identify compounds that are able to bind to the open conformation or supposedly perturb the interaction between NS2B and NS3.

13.4 Inhibitors of Flaviviral Proteases

In case of dengue protease, which by far has been the most prominent and extensively studied example, several screening campaigns and related inhibitor development approaches were not able to identify promising lead compounds during the last decade [33]. The main reasons for failure have been a lack of structural basis, a relatively flat binding site and a particular focus on often not rationally designed small-molecular compounds. Although viral proteases recognize peptidic substrates, only the minority of studies dealt with peptide-based inhibitors, which have shown to be the only class of compounds that can reach sufficient inhibition in the nanomolar concentration range. However, in contrast to the HIV and HCV success stories, the preference for two permanently charged basic side chains in flaviviral substrates complicates the development of drug-like peptide-derived inhibitors with sufficient bioavailability and antiviral activity in cell culture and animal models (Table 13.1).

Table 13.1 Biochemical and cellular activities of selected flaviviral protease inhibitors discussed in this chapter

	Dengue virus [μM][a]		West Nile virus [μM]		Zika virus [μM]	
Compound	Biochemical[b]	Cellular	Biochemical[b]	Cellular	Biochemical[b]	Cellular
1	$IC_{50} = 1.1$	Inactive				
2	$K_i = 2.0$		$K_i = 4.6$			
3	$IC_{50} = 2.0$	$EC_{50} = 59.5$ $CC_{50} = 135$	$IC_{50} = 8.7$	$EC_{50} = 42.4$ $CC_{50} = 135$		
4	$IC_{50} = 2.2$					
5	$IC_{50} = 1.0$	$EC_{50} = 0.8$ $CC_{50} > 10$				
6	$IC_{50} = 0.5$					
7	$IC_{50} = 15.4$	$EC_{50} = 0.17$ $CC_{50} = 29.3$				
8	$IC_{50} = 1.2$	$EC_{50} = 39.4$ $CC_{50} > 100$				
9	$IC_{50} = 8.5$		$IC_{50} = 0.11$			
10	$IC_{50} = 2.8$	$EC_{50} = 40$ $CC_{50} = 213$	$IC_{50} = 0.26$	$EC_{50} = 42.3$ $CC_{50} = 213$	$IC_{50} = 1.1$	
11	$IC_{50} > 10$	$EC_{50} = 81.5$ $CC_{50} = 236$	$IC_{50} = 0.44$	$EC_{50} = 17$ $CC_{50} = 236$	$IC_{50} > 10$	
12			$IC_{50} = 1.1$			
13					$K_i = 9.5$	
14	Inactive	$EC_{50} = 0.8$ $CC_{50} = 54$			$IC_{50} = 21.6$	$EC_{50} = 13.0$ $CC_{50} > 40$
15	$IC_{50} > 10$	$EC_{50} > 100$ $CC_{50} = 257$	$IC_{50} = 0.74$	$EC_{50} = 107$ $CC_{50} = 257$	$IC_{50} = 0.82$	$EC_{50} \sim 50$ $CC_{50} = 257$
16	$K_i = 0.051$	$EC_{50} = 30$ $CC_{50} > 100$	$K_i = 0.082$	$EC_{50} = 38$ $CC_{50} > 100$	$K_i = 0.040$	
17	$K_i = 0.078$	$EC_{50} = 19$ $CC_{50} > 100$	$K_i = 0.16$	$EC_{50} > 50$ $CC_{50} > 100$	$IC_{50} = 2.1$	
18	$K_i = 0.012$	$EC_{50} = 20$ $CC_{50} > 100$	$K_i = 0.039$	$EC_{50} = 23$ $CC_{50} > 100$		
19	$IC_{50} = 0.028$	$EC_{50} = 7.1$ $CC_{50} > 100$	$IC_{50} = 0.12$		$IC_{50} = 1.0$	
20	$IC_{50} = 0.18$	$EC_{50} = 3.4$ $CC_{50} = 100$	$IC_{50} = 0.56$	$EC_{50} = 15.6$ $CC_{50} > 100$		

[a]Activities have been reported for various serotypes. The serotype with the best activity results is reported here
[b]If reported activities vary by method or report, the lowest (best) value is shown

13.5 Small-Molecular Dengue Virus Protease Inhibitors

Approximately 40 approaches towards small-molecular non-peptide-derived inhibitors have been reported in the literature during the last decade. Unfortunately, only a minority of them provided cellular data to confirm that the compounds also achieve proper antiviral activity in cells and are not cytotoxic. A remarkable number of compounds with broad structural variety that are able to inhibit dengue (and West Nile) protease in the concentration range between 25 and 100 μM have been identified. Further ligand derivatizations often only led to limited improvements (maximal affinities in the one-digit micromolar range). Examples are rare where a small structural change causes a pronounced impact on affinity (activity cliff). Consequently, no studies could provide small-molecular non-peptidic compounds that are able to bind to dengue protease in the desirable lower nanomolar range.

Compound **1** resulted from a campaign including high-throughput screening, scaffold optimization and subsequent derivatization [6]. This compound has an IC_{50} of 1.1 μM against dengue

serotype 2 and decreased potency towards the other serotypes. Specific competitive binding was confirmed by several orthogonal methods; however, despite strong efforts no further optimization of this compound class, which also lacked antiviral activity in cell culture, could be achieved [28]. Compound **2** was identified from a computational screening approach [20] with a K_i value of 2.0 μM (serotype 2). Guanidine groups are crucial for sufficient activity, indicating a likely electrostatic interaction with residues in the S_1 or S_2 pocket of the protease. A covalent interaction of the activated ester bond with Ser135 is possibly, but has not yet been confirmed experimentally. Compound **3** is supposedly an allosteric inhibitor, identified from a virtual screening of compounds, actually aiming at West Nile virus protease, which may inhibit the interactions between NS3 and NS2B [42]. It shows an IC_{50} value of 2.0 μM, some basic antiviral activity in cells and limited cytotoxicity. A structurally similar but larger derivative **10** (Fig. 13.2) identified from the same campaign showed only slightly lower affinity (IC_{50} = 2.8 μM), but improved cellular data (EC_{50} = 40 μM). From a series of thiadiazoloacrylamides, compound **4** showed best activity with an IC_{50} of 2.2 μM (K_d = 2.1 μM) [29]. In correlation with various previously studied compound series the SAR between derivatives remained remarkably flat.

For compound **5**, IC_{50} values of up to 1.0 μM (serotype 3) from a biochemical and 3.2 μM from a cell-based protease assay have been reported [51]. The compound shows cellular antiviral activity in the same range (EC_{50} = 0.8 μM) but also cytotoxic effects at concentrations above 10 μM. The most active small-molecular non-peptidic inhibitor reported so far for dengue protease is compound **6**. It is one of the few examples with affinity significantly lower than 1 μM with an IC_{50} value of 0.5 μM for serotype 2 [22]. It is also one of the few examples that aim at targeting the catalytically active serine by a covalent interaction. This could be proven by mass spectrometry and may be the key towards small ligands of high affinity. After reaction, the biphenyl-3-carboxylate remains bound to the protease and blocks all further substrate procession until ester hydrolysis may restore the protease activity again.

Another high-throughput screen identified amphiphilic compound **7** with only moderate activity in the biochemical assay (IC_{50} = 15.4 μM) [52]. However, cellular assays revealed one of the highest reported antiviral activities in cells for any discovered small-molecular dengue protease inhibitor (EC_{50} = 0.17 μM). Resistance breeding experiments suggested an inhibition of interactions between the NS2B and NS3 domains. The remarkable discrepancy between biochemical and cellular activity may, however, indicate that

Fig. 13.1 Small-molecular inhibitors of dengue virus protease, which either reached highest affinity in biochemical or phenotypic assays

the protease is not the only target of this compound. An independent screening campaign based on a dengue replicon assay identified a structurally similar compound with related biochemical and virological results [53].

Recently, further derivatizations (including rigidification) of previously published methionine-proline anilides [57] towards non-peptidic analogues revealed compound **8** as small-molecular dengue protease inhibitor with pronounced affinity (IC_{50} = 1.2 μM) [50]. Selective interaction with the protease is supported by the inactivity of a stereoisomer of **8**. In combination, both SARs indicate that the two aromatic nitro substituents are necessary for proper affinity. Although this functional group is highly questionable in terms of drug-likeness, the compound showed no cytotoxic effects at the highest assayed concentration of 100 μM and was proven to inhibit viral replication in cell culture (EC_{50} = 39.4 μM).

Tolcapone, tannic acid and suramin have been reported as hits from a high-throughput screening with K_i values significantly below 1 μM [1]. Tannic acid (K_i = 0.34 μM) showed also exceptional activity in a viral plaque assay with an EC_{50} value of 0.084 μM and only limited cytotoxic effects. However, with a molecular mass of 1700 Da and a polyphenolic structure, this compound would usually not be considered as a suitable lead in drug discovery.

13.6 Small-Molecular West Nile Virus Protease Inhibitors

Most of the remarks and conclusions that have been made for the development of dengue virus protease inhibitors can be transferred to the closely related West Nile virus protease. In fact, only a limited number of studies directly aimed at identifying West Nile virus protease inhibitors. However, often West Nile protease activity was additionally assessed within dengue protease inhibitor campaigns. Compound **2** (Fig. 13.1) for example was found to be notably active against West Nile virus protease (K_i = 4.6 μM), although the computational screening approach based on a homology model of dengue protease. The highest affinities that could be reached with small-molecular compounds were often up to one order of magnitude better compared to dengue protease.

Analogues of compound **6** (Fig. 13.1) have been studied for West Nile virus protease before they were evaluated for dengue protease [18, 45]. For derivate **9** promising IC_{50} values between 0.11 and 0.16 μM have been reported (only 8.5 μM for dengue protease) [18, 22]. These covalently binding pyrazole esters can be considered as the class of small-molecular compounds with the highest reported affinities and ligand efficiencies for dengue and West Nile virus proteases. However, due to general high reactivity,

Fig. 13.2 Selected examples of small-molecular compounds that reached highest inhibition activities against West Nile virus protease

their chemical stability even in the usual assay buffer is limited [45]. Compounds **10** and **11** were identified from the same virtual screening campaign as compound **3** with IC_{50} values of 0.26 and 0.44 µM as well as moderate antiviral activity in cells with EC_{50} values of 42 and 17 µM, respectively [42]. From a high-throughput screening a compound comprising an 8-hydroxyquinoline scaffold was identified as a West Nile virus protease inhibitor with promising activity in cell culture (EC_{50} = 1.4 µM) [31]. Further derivatizations of this compound class produced **12** with an IC_{50} value of 1.1 µM [15].

13.7 Small-Molecular Zika Virus Protease Inhibitors

Inspired by previous campaigns, initial progress has been made in the discovery of the first small-molecular inhibitors of Zika virus protease. Several compounds that emerged from dengue or West Nile virus protease screenings, such as **10** and **11,** also showed inhibition against Zika virus protease [43]. Compound **13** (K_i = 9.5 µM) is an example of a series of lead compounds that have been recycled from an HCV protease high-throughput screening campaign [24]. The dopamine antagonist bromocriptine (**14**), which has previously been reported to inhibit viral replication for all four dengue serotypes [19], also reduces Zika virus replication in cell culture [8]. In contrast to dengue, the Zika virus protease could be confirmed as potential target of bromocriptine (IC_{50} = 21.6 µM). Compound **15**, which was previously reported to be active against West Nile (IC_{50} = 0.74 µM) but inactive against dengue protease (at concentrations lower than 10 µM), was found to be also a promising Zika protease inhibitor (IC_{50} = 0.82 µM), especially from the perspective of ligand efficiency (molecular weight = 190 Da). It inhibited viral replication in cell culture (EC_{50} ~ 50 µM) and could reduce the level of circulating Zika viruses in mice [43]. Although these data are promising, apart from docking studies, which suggest binding close to the active site and interference with NS2B, structural data that would facilitate a hit-to-lead campaign are missing (Fig. 13.3).

13.8 Peptide-Derived Inhibitors of Dengue, West Nile and Zika Virus Proteases

Substrate-based peptidic inhibitors of dengue and West Nile virus proteases have been studied quite extensively during the earlier attempts. They usually consist of a substrate segment comprising at least two basic side chain residues and may additionally be featured with a C-terminal electrophilic warhead, most often an aldehyde moiety, for covalent binding to Ser135. They often cannot be considered as drug-like leads due to their unsuitable pharmacokinetic and physiochemical properties, such as bioavailability, specificity and plasma stability. Notably, these peptides could reach higher affinity towards West Nile than dengue virus protease. For oligo-D-arginines affinities up to 1 nM have been reported in case of West Nile virus [44]. Peptidomimetics with N-terminal dichloro-substituted phenylacetyl groups and C-terminal arginine mimetics reached IC_{50} values of up to 0.12 µM [17]. Peptides with an additional possibility for covalent interaction with Ser135 were able to generate affinities of up

Fig. 13.3 Small-molecular inhibitors of Zika virus protease that resulted from previous campaigns for related viruses

to 9 nM (K_i) for the tripeptidic aldehyde phenacetyl-Lys-Lys-Arg-H [21, 41, 46]. Although this compound showed serum stability, cell permeability and antiviral activity (EC_{50} = 1.6 μM) no further studies towards more drug-like derivatives have been reported [46].

In case of dengue virus protease, studies with simple substrate-derived peptides reached K_i values of only up to 0.3 μM [40]. Recently, cyclic peptides comprising unnatural amino acids were found to be active in cell culture (EC_{50} = 2.0 μM) [48]. Substrate-like peptides containing C-terminal aldehydes were less active in case of dengue protease with a K_i of 1.5 μM for the most active derivative Bz-Lys-Arg-Arg-H [54]. However, alternatively studied electrophiles such as the trifluoromethylketone in Bz-Nle-Lys-Arg-Arg-CF_3 (K_i = 0.85 μM) or a boronic acid function in a similar analogue Bz-Nle-Lys-Arg-Arg-$B(OH)_2$ (K_i = 0.043 μM) showed increased activity [55].

Recent studies have shown that even very small peptide-derived compounds can exhibit extraordinary binding affinities, if they are combined with such a boronic acid moiety, which forms a boronate with the catalytically active residue S135 [36]. These compounds may not only offer a route towards more drug-like small-molecular derivatives, they have also become valuable tools in structural biology to elucidate inhibitor-protease interactions, which will hopefully illuminate the way towards a more structure-based drug design. Although, the drug-likeness of this compound class is limited, a significant reduction of viral titers for West Nile and dengue viruses in cell culture could be observed. Compound **16** showed pronounced pan-flaviviral protease affinity with K_i values of 51, 82 and 40 nM for dengue, West Nile and Zika virus proteases, respectively. This derivative was co-crystalized with the proteases of Zika [25] and West Nile [36] viruses. Derivative **17** was used to demonstrate a novel NMR-based approach to identify the binding mode of tightly binding inhibitor molecules towards dengue virus protease from sertotype 2 [10]. The *tert*-butyl moiety in **17** appears as a sharp and isolated signal in proton NMR spectra. Using the power of paramagnetic NMR spectroscopy [34] combined with NOEs, the positions of the *tert*-butyl group and aromatic protons in close proximity could be predicted in relation to the 3D structure of the protein. Very recently, compounds **16** and **17** have been used to study the conformational flexibility of the NS2B cofactor of Zika protease in solution [30] (Fig. 13.4).

Finally, over the last 5 years the stepwise elaboration of non-covalently binding tripeptidic inhibitors, comprising two basic side chains, has been reported regularly [2, 3, 5, 32, 35, 49]. The optimization focused so far only on enzymatic inhibition in biochemical assays, limiting the drug-likeness and pharmacokinetic properties of this compound class. However, with a K_i value of 12 nM, **18** is the compound of highest affinity towards dengue serotype 2 protease reported so far [3]. It also shows remarkable affinity against West Nile virus protease with a K_i of 39 nM. Due to limited permeability, the antiviral cellular activity is only moderate with EC_{50} values of 20 and 23 μM for dengue and West Nile viruses, respectively. Recently, the highly related derivative **19** was analysed against Zika protease [23]. It showed reduced inhibition potential for Zika (IC_{50} = 1.0 μM) compared to previous reported results for dengue (IC_{50} = 0.028 μM) and West Nile proteases (IC_{50} = 0.12 μM) [3, 23]. Increased cellular activity was found for analogue **20** with slightly improved permeability (dengue: EC_{50} = 3.4 μM; West Nile: EC_{50} = 15.6 μM) [3]. However, the affinity of **20** towards the proteases dropped significantly compared to **18** or **19** with IC_{50} values of 176 and 557 nM for dengue and West Nile proteases, respectively. Structural evidences, such as NMR or crystallographic data, for the binding mode of this compound class are unfortunately missing. This information would

Fig. 13.4 Recently published high-affinity peptide-derived inhibitors of flaviviral proteases

be very valuable to rationalize the pronounced SAR.

13.9 Perspectives Towards Zika Virus Protease Inhibitors

All compounds that have recently been described to inhibit Zika protease, either small molecules or peptides, originated from previous drug discovery campaigns for related viruses. The co-crystal structures of compound **16** [25] and the fragment benzimidazol-1-ylmethanol [56] in the active form present a unique chance for rationality in the upcoming drug discovery attempts. This opportunity was not available during a long period of early dengue protease inhibitor investigations. The main lessons for Zika learned from a decade of challenging dengue protease research are: to balance the focus between small-molecular and substrate-derived inhibitors; to take advantage of the virtue of covalence in enzyme inhibition; and to generate as much structural information as possible.

The latter aspect becomes obvious by taking a closer look into the co-crystal structure of Zika virus protease with compound **16**. In contrast to other flaviviral proteases, such as those from dengue or West Nile viruses, Zika comprises a negatively charged aspartate residue in position 83 of the cofactor domain NS2B. The West Nile protease contains a structurally very similar, although uncharged, asparagine residue in that position. Dengue protease serotypes have either a serine or threonine in that position. Without any structural information this situation may not have led to any special attention for drug development purposes with Zika virus protease. However, from the crystal structure it turned out that this NS2B aspartate residue is responsible for a salt-bridge formation with the aminomethyl-phenyl moiety (P_2) of compound **16** leading to an extra tight binding of inhibitors and substrates. As the possibilities for tight interactions of inhibitors with flaviviral proteases are usually limited, this information is highly relevant for further drug design campaigns.

13.10 Conclusion

Although alternative flaviviral proteins (e.g. NS1 and NS5) as well as virus-host interactions have become attractive drug targets, the NS2B-NS3 protease is still of considerable interest. However, the progress towards drug-like compounds with promising intracellular activity was limited. It may take another decade until the first selective flaviviral protease inhibitor that convincingly works in an animal model will become available. The main challenges are the rather flat binding sites, the absence of product inhibition (in contrast to HCV) and the strong recognition preference for basic moieties, which highly complicates the development of compounds with high affinity and a desirable ADME profile.

As new methods and technologies have also become available during the last decade of struggling inhibitor development, they may open the way for alternative strategies. Many of the earlier attempts (especially with small-molecular compounds) were pursued without any reasonable structural basis. Co-crystal structures of flaviviral proteases with small-molecular ligands are still remarkably rare compared to other drug discovery campaigns. Technologies that do not rely on protease crystals, such as modern NMR, can help to elucidate the binding mode of inhibitor candi-

dates. In this context, recently emerging fragment-based screening approaches in combination with NMR, which have so far been neglected for flaviviral proteases, may also revitalise the drug discovery process. The identification of new small scaffolds with only weak affinity and their consequent rational elaboration into drug-like inhibitors may be superior compared to previous approaches.

In addition to new strategies regarding the identification and optimization of lead compounds, new approaches regarding cellular assays will be required to address the present delay or total lack of cellular data. Some initial progress on intracellular protease assays has been made, but further advance in this area, also concerning imaging techniques to track potential drug candidates within the cell, is necessary.

Note

Since the 'Tofo Advanced Study Week on Arboviruses' significant progress has been achieved particularly in the field of Zika virus protease. These recent results are reflected in this chapter, although they were not content of the conference presentation and discussion.

Acknowledgements Funding by the Alexander von Humboldt Foundation is gratefully acknowledged.

References

1. Balasubramanian A, Manzano M, Teramoto T, Pilankatta R, Padmanabhan R (2016) High-throughput screening for the identification of small-molecule inhibitors of the flaviviral protease. Antivir Res 134:6–16
2. Bastos Lima A, Behnam MA, El Sherif Y, Nitsche C, Vechi SM, Klein CD (2015) Dual inhibitors of the dengue and West Nile virus NS2B-NS3 proteases: synthesis, biological evaluation and docking studies of novel peptide-hybrids. Bioorg Med Chem 23(17):5748–5755
3. Behnam MA, Graf D, Bartenschlager R, Zlotos DP, Klein CD (2015) Discovery of nanomolar dengue and West Nile virus protease inhibitors containing a 4-benzyloxyphenylglycine residue. J Med Chem 58(23):9354–9370
4. Behnam MA, Nitsche C, Boldescu V, Klein CD (2016) The medicinal chemistry of dengue virus. J Med Chem 59(12):5622–5649
5. Behnam MA, Nitsche C, Vechi SM, Klein CD (2014) C-terminal residue optimization and fragment merging: discovery of a potent peptide-hybrid inhibitor of dengue protease. ACS Med Chem Lett 5(9):1037–1042
6. Bodenreider C, Beer D, Keller TH, Sonntag S, Wen D, Yap L, Yau YH, Shochat SG, Huang D, Zhou T, Caflisch A, Su X-C, Ozawa K, Otting G, Vasudevan SG, Lescar J, Lim SP (2009) A fluorescence quenching assay to discriminate between specific and nonspecific inhibitors of dengue virus protease. Anal Biochem 395(2):195–204
7. Castanha PMS, Nascimento EJM, Braga C, Cordeiro MT, de Carvalho OV, de Mendonça LR, Azevedo EAN, França RFO, Dhalia R, Marques ETA (2017) Dengue virus–specific antibodies enhance Brazilian Zika virus infection. J Infect Dis 215(5):781–785
8. Chan JF-W, Chik KK-H, Yuan S, Yip CC-Y, Zhu Z, Tee K-M, Tsang JO-L, Chan CC-S, Poon VK-M, Lu G, Zhang AJ, Lai K-K, Chan K-H, Kao RY-T, Yuen K-Y (2017) Novel antiviral activity and mechanism of bromocriptine as a Zika virus NS2B-NS3 protease inhibitor. Antivir Res 141:29–37
9. Chappell KJ, Stoermer MJ, Fairlie DP, Young PR (2008) West Nile virus NS2B/NS3 protease as an antiviral target. Curr Med Chem 15(27):2771–2784
10. Chen WN, Nitsche C, Pilla KB, Graham B, Huber T, Klein CD, Otting G (2016) Sensitive NMR approach for determining the binding mode of tightly binding ligand molecules to protein targets. J Am Chem Soc 138(13):4539–4546
11. Chen X, Yang K, Wu C, Chen C, Hu C, Buzovetsky O, Wang Z, Ji X, Xiong Y, Yang H (2016) Mechanisms of activation and inhibition of Zika virus NS2B-NS3 protease. Cell Res 26(11):1260–1263
12. de la Cruz L, Chen WN, Graham B, Otting G (2014) Binding mode of the activity-modulating C-terminal segment of NS2B to NS3 in the dengue virus NS2B-NS3 protease. FEBS J 281(6):1517–1533
13. Dejnirattisai W, Supasa P, Wongwiwat W, Rouvinski A, Barba-Spaeth G, Duangchinda T, Sakuntabhai A, Cao-Lormeau VM, Malasit P, Rey FA, Mongkolsapaya J, Screaton GR (2016) Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with Zika virus. Nat Immunol 17(9):1102–1108
14. Erbel P, Schiering N, D'Arcy A, Renatus M, Kroemer M, Lim SP, Yin Z, Keller TH, Vasudevan SG, Hommel U (2006) Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus. Nat Struct Mol Biol 13(4):372–373
15. Ezgimen M, Lai H, Mueller NH, Lee K, Cuny G, Ostrov DA, Padmanabhan R (2012) Characterization of the 8-hydroxyquinoline scaffold for inhibitors of West Nile virus serine protease. Antivir Res 94(1):18–24
16. Gupta G, Lim L, Song J (2015) NMR and MD studies reveal that the isolated dengue NS3 protease is an intrinsically disordered chymotrypsin fold which

absolutely requests NS2B for correct folding and functional dynamics. PLoS One 10(8):e0134823
17. Hammamy MZ, Haase C, Hammami M, Hilgenfeld R, Steinmetzer T (2013) Development and characterization of new peptidomimetic inhibitors of the West Nile virus NS2B-NS3 protease. ChemMedChem 8(2):231–241
18. Johnston PA, Phillips J, Shun TY, Shinde S, Lazo JS, Huryn DM, Myers MC, Ratnikov BI, Smith JW, Su Y, Dahl R, Cosford ND, Shiryaev SA, Strongin AY (2007) HTS identifies novel and specific uncompetitive inhibitors of the two-component NS2B-NS3 proteinase of West Nile virus. Assay Drug Dev Technol 5(6):737–750
19. Kato F, Ishida Y, Oishi S, Fujii N, Watanabe S, Vasudevan SG, Tajima S, Takasaki T, Suzuki Y, Ichiyama K, Yamamoto N, Yoshii K, Takashima I, Kobayashi T, Miura T, Igarashi T, Hishiki T (2016) Novel antiviral activity of bromocriptine against dengue virus replication. Antivir Res 131:141–147
20. Knehans T, Schuller A, Doan DN, Nacro K, Hill J, Guntert P, Madhusudhan MS, Weil T, Vasudevan SG (2011) Structure-guided fragment-based in silico drug design of dengue protease inhibitors. J Comput Aided Mol Des 25(3):263–274
21. Knox JE, Ma NL, Yin Z, Patel SJ, Wang WL, Chan WL, Ranga Rao KR, Wang G, Ngew X, Patel V, Beer D, Lim SP, Vasudevan SG, Keller TH (2006) Peptide inhibitors of West Nile NS3 protease: SAR study of tetrapeptide aldehyde inhibitors. J Med Chem 49(22):6585–6590
22. Koh-Stenta X, Joy J, Wang SF, Kwek PZ, Wee JL, Wan KF, Gayen S, Chen AS, Kang C, Lee MA, Poulsen A, Vasudevan SG, Hill J, Nacro K (2015) Identification of covalent active site inhibitors of dengue virus protease. Drug Des Devel Ther 9:6389–6399
23. Kuiper BD, Slater K, Spellmon N, Holcomb J, Medapureddy P, Muzzarelli KM, Yang Z, Ovadia R, Amblard F, Kovari IA, Schinazi RF, Kovari LC (2017) Increased activity of unlinked Zika virus NS2B/NS3 protease compared to linked Zika virus protease. Biochem Biophys Res Commun 492:668–673.
24. Lee H, Ren J, Nocadello S, Rice AJ, Ojeda I, Light S, Minasov G, Vargas J, Nagarathnam D, Anderson WF, Johnson ME (2017) Identification of novel small molecule inhibitors against NS2B/NS3 serine protease from Zika virus. Antivir Res 139:49–58
25. Lei J, Hansen G, Nitsche C, Klein CD, Zhang L, Hilgenfeld R (2016) Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor. Science 353(6298):503–505
26. Lescar J, Luo D, Xu T, Sampath A, Lim SP, Canard B, Vasudevan SG (2008) Towards the design of antiviral inhibitors against flaviviruses: the case for the multifunctional NS3 protein from dengue virus as a target. Antivir Res 80(2):94–101
27. Lim SP, Shi PY (2013) West Nile virus drug discovery. Virus 5(12):2977–3006
28. Lim SP, Wang QY, Noble CG, Chen YL, Dong H, Zou B, Yokokawa F, Nilar S, Smith P, Beer D, Lescar J, Shi PY (2013) Ten years of dengue drug discovery: progress and prospects. Antivir Res 100(2):500–519
29. Liu H, Wu R, Sun Y, Ye Y, Chen J, Luo X, Shen X, Liu H (2014) Identification of novel thiadiazoloacrylamide analogues as inhibitors of dengue-2 virus NS2B/NS3 protease. Bioorg Med Chem 22(22):6344–6352
30. Mahawaththa MC, Pearce BJG, Szabo M, Graham B, Klein CD, Nitsche C, Otting G (2017) Solution conformations of a linked construct of the Zika virus NS2B-NS3 protease. Antivir Res 142:141–147
31. Mueller NH, Pattabiraman N, Ansarah-Sobrinho C, Viswanathan P, Pierson TC, Padmanabhan R (2008) Identification and biochemical characterization of small-molecule inhibitors of West Nile virus serine protease by a high-throughput screen. Antimicrob Agents Chemother 52(9):3385–3393
32. Nitsche C, Behnam MA, Steuer C, Klein CD (2012) Retro peptide-hybrids as selective inhibitors of the dengue virus NS2B-NS3 protease. Antivir Res 94(1):72–79
33. Nitsche C, Holloway S, Schirmeister T, Klein CD (2014) Biochemistry and medicinal chemistry of the dengue virus protease. Chem Rev 114(22):11348–11381
34. Nitsche C, Otting G (2017) Pseudocontact shifts in biomolecular NMR using paramagnetic metal tags. Prog Nucl Magn Reson Spectrosc 98–99:20–49
35. Nitsche C, Schreier VN, Behnam MA, Kumar A, Bartenschlager R, Klein CD (2013) Thiazolidinone-peptide hybrids as dengue virus protease inhibitors with antiviral activity in cell culture. J Med Chem 56(21):8389–8403
36. Nitsche C, Zhang L, Weigel LF, Schilz J, Graf D, Bartenschlager R, Hilgenfeld R, Klein CD (2017) Peptide–boronic acid inhibitors of flaviviral proteases: medicinal chemistry and structural biology. J Med Chem 60(1):511–516
37. Noble CG, Seh CC, Chao AT, Shi PY (2012) Ligand-bound structures of the dengue virus protease reveal the active conformation. J Virol 86(1):438–446
38. Phoo WW, Li Y, Zhang Z, Lee MY, Loh YR, Tan YB, Ng EY, Lescar J, Kang C, Luo D (2016) Structure of the NS2B-NS3 protease from Zika virus after self-cleavage. Nat Commun 7:13410
39. Priyamvada L, Quicke KM, Hudson WH, Onlamoon N, Sewatanon J, Edupuganti S, Pattanapanyasat K, Chokephaibulkit K, Mulligan MJ, Wilson PC, Ahmed R, Suthar MS, Wrammert J (2016) Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus. Proc Natl Acad Sci U S A 113(28):7852–7857
40. Prusis P, Junaid M, Petrovska R, Yahorava S, Yahorau A, Katzenmeier G, Lapins M, Wikberg JES (2013) Design and evaluation of substrate-based octapeptide and non substrate-based tetrapeptide inhibitors of dengue virus NS2B–NS3 proteases. Biochem Biophys Res Commun 434(4):767–772
41. Schüller A, Yin Z, Brian Chia CS, Doan DN, Kim HK, Shang L, Loh TP, Hill J, Vasudevan SG (2011)

Tripeptide inhibitors of dengue and West Nile virus NS2B-NS3 protease. Antivir Res 92(1):96–101
42. Shiryaev SA, Cheltsov AV, Gawlik K, Ratnikov BI, Strongin AY (2011) Virtual ligand screening of the National Cancer Institute (NCI) compound library leads to the allosteric inhibitory scaffolds of the West Nile virus NS3 proteinase. Assay Drug Dev Technol 9(1):69–78
43. Shiryaev SA, Farhy C, Pinto A, Huang CT, Simonetti N, Ngono AE, Dewing A, Shresta S, Pinkerton AB, Cieplak P, Strongin AY, Terskikh AV (2017) Characterization of the Zika virus two-component NS2B-NS3 protease and structure-assisted identification of allosteric small-molecule antagonists. Antivir Res 143:218–229
44. Shiryaev SA, Ratnikov BI, Chekanov AV, Sikora S, Rozanov DV, Godzik A, Wang J, Smith JW, Huang Z, Lindberg I, Samuel MA, Diamond MS, Strongin AY (2006) Cleavage targets and the D-arginine-based inhibitors of the West Nile virus NS3 processing proteinase. Biochem J 393(Pt 2):503–511
45. Sidique S, Shiryaev SA, Ratnikov BI, Herath A, Su Y, Strongin AY, Cosford ND (2009) Structure-activity relationship and improved hydrolytic stability of pyrazole derivatives that are allosteric inhibitors of West Nile virus NS2B-NS3 proteinase. Bioorg Med Chem Lett 19(19):5773–5777
46. Stoermer MJ, Chappell KJ, Liebscher S, Jensen CM, Gan CH, Gupta PK, Xu WJ, Young PR, Fairlie DP (2008) Potent cationic inhibitors of West Nile virus NS2B/NS3 protease with serum stability, cell permeability and antiviral activity. J Med Chem 51(18):5714–5721
47. Su XC, Ozawa K, Qi R, Vasudevan SG, Lim SP, Otting G (2009) NMR analysis of the dynamic exchange of the NS2B cofactor between open and closed conformations of the West Nile virus NS2B-NS3 protease. PLoS Negl Trop Dis 3(12):e561
48. Takagi Y, Matsui K, Nobori H, Maeda H, Sato A, Kurosu T, Orba Y, Sawa H, Hattori K, Higashino K, Numata Y, Yoshida Y (2017) Discovery of novel cyclic peptide inhibitors of dengue virus NS2B-NS3 protease with antiviral activity. Bioorg Med Chem Lett 27:3586–3590.
49. Weigel LF, Nitsche C, Graf D, Bartenschlager R, Klein CD (2015) Phenylalanine and phenylglycine analogues as arginine mimetics in dengue protease inhibitors. J Med Chem 58(19):7719–7733
50. Weng Z, Shao X, Graf D, Wang C, Klein CD, Wang J, Zhou GC (2016) Identification of fused bicyclic derivatives of pyrrolidine and imidazolidinone as dengue virus-2 NS2B-NS3 protease inhibitors. Eur J Med Chem 125:751–759
51. Wu H, Bock S, Snitko M, Berger T, Weidner T, Holloway S, Kanitz M, Diederich WE, Steuber H, Walter C, Hofmann D, Weissbrich B, Spannaus R, Acosta EG, Bartenschlager R, Engels B, Schirmeister T, Bodem J (2015) Novel dengue virus NS2B/NS3 protease inhibitors. Antimicrob Agents Chemother 59(2):1100–1109
52. Yang CC, Hsieh YC, Lee SJ, Wu SH, Liao CL, Tsao CH, Chao YS, Chern JH, Wu CP, Yueh A (2011) Novel dengue virus-specific NS2B/NS3 protease inhibitor, BP2109, discovered by a high-throughput screening assay. Antimicrob Agents Chemother 55(1):229–238
53. Yang CC, Hu HS, Wu RH, Wu SH, Lee SJ, Jiaang WT, Chern JH, Huang ZS, Wu HN, Chang CM, Yueh A (2014) A novel dengue virus inhibitor, BP13944, discovered by high-throughput screening with dengue virus replicon cells selects for resistance in the viral NS2B/NS3 protease. Antimicrob Agents Chemother 58(1):110–119
54. Yin Z, Patel SJ, Wang W-L, Chan W-L, Ranga Rao KR, Wang G, Ngew X, Patel V, Beer D, Knox JE, Ma NL, Ehrhardt C, Lim SP, Vasudevan SG, Keller TH (2006) Peptide inhibitors of dengue virus NS3 protease. Part 2: SAR study of tetrapeptide aldehyde inhibitors. Bioorg Med Chem Lett 16(1):40–43
55. Yin Z, Patel SJ, Wang W-L, Wang G, Chan W-L, Rao KRR, Alam J, Jeyaraj DA, Ngew X, Patel V, Beer D, Lim SP, Vasudevan SG, Keller TH (2006) Peptide inhibitors of dengue virus NS3 protease. Part 1: warhead. Bioorg Med Chem Lett 16(1):36–39
56. Zhang Z, Li Y, Loh YR, Phoo WW, Hung AW, Kang C, Luo D (2016) Crystal structure of unlinked NS2B-NS3 protease from Zika virus. Science 354(6319):1597–1600
57. Zhou GC, Weng Z, Shao X, Liu F, Nie X, Liu J, Wang D, Wang C, Guo K (2013) Discovery and SAR studies of methionine-proline anilides as dengue virus NS2B-NS3 protease inhibitors. Bioorg Med Chem Lett 23(24):6549–6554

14

Discovery of Potent Non-nucleoside Inhibitors of Dengue Viral RNA-Dependent RNA Polymerase from Fragment Screening and Structure-Guided Design

Siew Pheng Lim, Christian G. Noble, Shahul Nilar, Pei-Yong Shi, and Fumiaki Yokokawa

Abstract

Flavivirus NS5 RNA-dependent RNA polymerase (RdRp) is an important drug target. Whilst a number of allosteric inhibitors have been described for Hepatitis C virus RdRp, few have been described for DENV RdRp. In addition, compound screening campaigns have not yielded suitable leads for this enzyme. Using fragment-based screening via X-ray crystallography, we identified a biphenyl acetic acid fragment that binds to a novel pocket of the dengue virus (DENV) RdRp, in the thumb/palm interface, close to its active site (termed "N pocket"). Structure-guided optimization yielded nanomolar inhibitors of the RdRp *de novo* initiation activity, with low micromolar EC_{50} in DENV cell-based assays. Compound-resistant DENV replicons exhibited amino acid mutations that mapped to the N pocket. This is the first report of a class of pan-serotype and cell-active DENV RdRp inhibitors and provides a significant opportunity for rational design of novel therapeutics against this proven antiviral target.

Keywords

Flaviviruses · Dengue virus · RNA dependent RNA polymerase · Anti-viral · Drug discovery · Rational design · X-ray crystallography · Mechanism of inhibition · Resistance phenotype

S. P. Lim (✉) · C. G. Noble · F. Yokokawa
Novartis Institute for Tropical Diseases, Singapore, Singapore
e-mail: siewpheng-lim@denka.com.sg

S. Nilar
Global Blood Therapeutics, South San Francisco, CA, USA

Novartis Institute for Tropical Diseases, Singapore, Singapore

P.-Y. Shi
Novartis Institute for Tropical Diseases, Singapore, Singapore

Department of Biochemistry & Molecular Biology, Sealy Center for Structural Biology & Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_14

14.1 Introduction

Flavivirus non-structural protein 5 (NS5) is the largest protein encoded by the viral genome. It is also the most highly conserved, with approximately 67-82% amino-acid sequence identity between the four serotypes of dengue virus (DENV-1 to -4). The *N*-terminal region comprises an RNA methyltransferase (MTase) domain [9] that has both *N*7 and 2′-*O* methyltransferase activities [4, 6, 12, 22]. Together, they are responsible for type 1 cap structure (m7GpppAm) formation at the 5′ end of the viral genome. The *C*-terminal two-thirds of NS5 harbors an RNA-dependent RNA polymerase domain (RdRp; [8]) which performs *de novo* RNA synthesis from the 3′ end of the viral template [3]. These two domains are linked via a short amino acid sequence of low conservation and may play a role in regulating RdRp activity [13, 21].

Crystal structures of the DENV and other flavivirus MTase and RdRp domains and the full length NS5 protein, have been solved. The overall architecture of the polymerase resembles a right hand with fingers, palm, and thumb subdomains, with a fully-encircled active site. In addition, they are well-characterized, structurally and functionally. For more detailed information on these aspects, the readers are encouraged to refer to excellent reviews [1, 3, 5, 14, 21] as well as Chaps. 8, 9, and 19 in this monograph.

Recently, a class of pyridobenzothiazole compounds that inhibit DENV RdRp and DENV infection at low micromolar potencies was reported [23]. This class of compounds binds in a similar site as NITD107, in the DENV RdRp a RNA tunnel [19]. Herein, we describe a class of acyl sulfonamide-thiophene compounds that has nanomolar inhibitory potency against the *de novo* initiation activities of DENV1-4 RdRp [18] and also exhibit EC_{50} values of 1–2 micromolar in DENV-infected cells [15, 20, 26].

14.2 Identification of N-Pocket Binding Fragment by DENV RdRp Fragment-Based Screening

14.2.1 Fragment-Based Screening Via DENV RdRp Crystallography

A total of 1408 compounds obtained from the Novartis in-house fragment library were screened with DENV3 RdRp, in pools of 8, totaling 176 separate pools. DENV3 RdRp crystals were first crystallized in conditions as previously described [19] and soaked for 4–6 h in drops containing compound mixtures in crystallization buffer and 10% DMSO, giving a final concentration per compound of 625 μM. The crystals were then frozen in liquid nitrogen after cryo-protection in the same crystallization solution supplemented with 10% glycerol and 10% compound/DMSO. The X-ray diffraction data were collected on beam-line X10SA (PXII) at the Swiss Light Source, integrated using MOSFLM [11] and scaled using SCALA, part of the CCP4 suite [24]. The structures were refined using REFMAC5 [17] starting from the deposited DENV-3 RdRp structure (PDB code 4HHJ) [19]. Useful X-ray diffraction data was collected for 145 out of 176 pools (with resolution better than 2.5 Å). Only one dataset, which was solved to 2.0 Å resolution, showed difference density that could be interpreted as a single contiguous compound. Models for the eight possible fragments from the pool were built into the density and only compound JF-31-MG46 fitted the data satisfactorily. To confirm that the single compound bound in the pocket, the DENV-3 RdRp was co-crystallized with the pure compound and the structure was solved to 2.05 Å (Fig. 14.1a; PDB 5F3T). The co-crystal structure showed that the single compound bound at the same site and orientation with full occupancy. Specifically, it bound in a novel pocket between the thumb and palm sub-domains of the RdRp and the priming

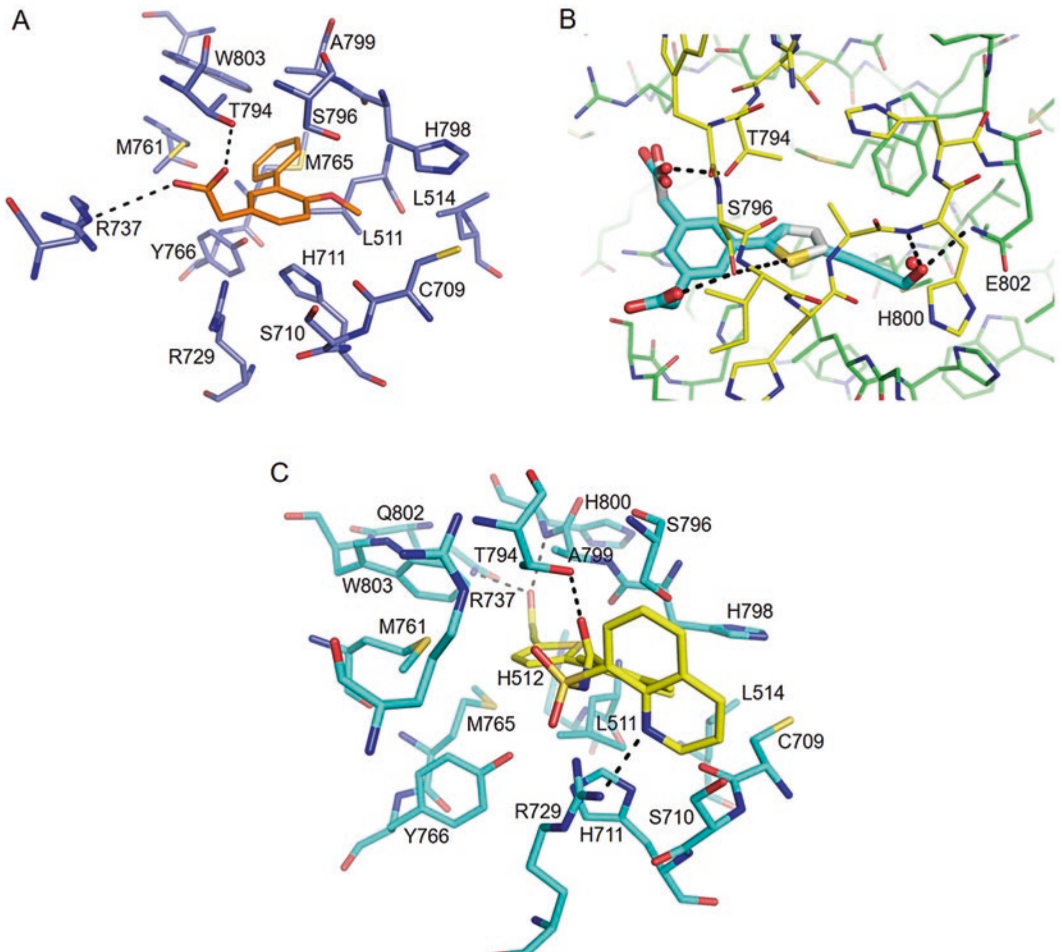


Fig. 14.1 Crystal structure of N pocket inhibitors and the DENV-3 RdRp. Binding of JF-31-MG46 (orange, **a**), compounds **10** (grey, **b**) and **15** (cyan, **b**) and compound 29 (yellow, **c**) in the N pocket of DENV-3 RdRp domain are shown as sticks. Amino acid residues lining the N pocket are colored in purple (**a**), cyan (**c**), or green (palm and thumb subdomains, **b**) and yellow (priming loop, **b**). Individual residues are labelled according to their numbering in the DENV3 polymerase and H-bond interactions are indicated with dashed lines

loop. This site is designed the "N" pocket and is similar to the Site III (Palm I) in HCV ([3]; See also Summary and Conclusions).

Additional information on this Section can be found in [20].

14.2.2 Compound JF-31-MG46 and DENV RdRp N Pocket

Compound JF-31-MG46, **3** is a biphenyl acetic acid fragment, and forms interactions with several amino acid residues in the DENV RdRp N pocket which are highly conserved across the four DENV serotypes, and in other flaviviruses such as WNV, YFV, and JEV (Fig.14.1a; PDB 5F3T). Its outer phenyl is sandwiched between Ser796 on one side and Tyr766 and His711 on the other. The carboxyl group on this ring, interacts with the side chain OH of Thr794, and also forms a water-mediated interaction with Arg737. The other substituent on the ring, the methoxy group, is surrounded by residues Cys709, Leu514, His798, and Leu511. The inner phenyl occupies

the back of the pocket lined with several hydrophobic residues, including Trp803, Met761, Met765, Ala799, and Leu511, with Ser710 located at the mouth of the pocket. Surface-plasmon resonance (SPR) analyses indicated that JF-31-MG46 binds to DENV-3 RdRp with *Kd* of 209 μM and with threefold lower binding affinity for DENV-4 RdRp (*Kd* = 610 μM). No binding was detected by isothermal calorimetry, presumably due to the lack of sufficient sensitivity at the maximum compound solubility. Compound JF-31-MG46 inhibited the *de novo* initiation activities of DENV-1-4 with IC_{50} values ranging from 734 to 796 μM and is consistent with the affinities measured by SPR. The calculated ligand efficiency [10] for compound JF-31-MG46 is 0.24.

Additional information on this Section can be found in Lim et al. [15] and [20].

14.3 Structure-Guided Inhibitor Optimization of N-Pocket Inhibitors

14.3.1 Improving Inhibitory Property of N-pocket Inhibitors

Based on the X-ray crystallography finding that a related analog, **4** (IC_{50} = 769 μM, SPR-*K*d > 200 μM, LE 0.26; PDB 5HNO) also bound in the same pocket but in the opposite orientation of the carboxylic acid moiety compared to compound JF-31-MG, the two fragments were merged, to generate the bis-acid, **5.** This resulted in stronger binding in the N-pocket and better inhibition (IC_{50} = 177 μM, ITC-K_d = 154 μM; LE = 0.26; PDB 5HMW). The initial optimization efforts focused on exploring the SAR of the inner benzene ring of **5**. Substitution with 2′-thiophene ring, **10,** provided a tenfold increase in potency (IC_{50} = 15 μM, ITC-K_d = 28 μM). This higher affinity is likely due to non-covalent interaction of the sulfur of the thiophene with the oxygen of the OH side-chain of Ser796 (PDB 5HMX). Computational studies detected a water molecule in the back of the DENV N pocket, which forms H-bond interactions with residues Gln802 and His800. Design strategies to improve inhibitor potency by contacting this water molecule were not fruitful. An alternate approach was conducted to displace the water molecule. A propargyl alcohol extension from the 2-thiophene ring provided **15** (IC_{50} = 1.7 μM, ITC-K_d = 1.4 μM; LE = 0.35), with a 100-fold increase in potency as compared to **5**. Co-crystallization of **15** with the NS5 RdRp domain confirmed that the propargyl alcohol filled the narrow cavity and displaced the water molecule to form the H-bond interactions with the side chain of Gln802 and the backbone of His800 as predicted (Fig. 14.1b; PDB 5HMY). Compound **15** displayed inhibitory activity against all four serotypes with IC_{50} values of 0.3 to 2.2 μM.

Additional information on this Section can be found in Lim et al. [15] and [26].

14.3.2 Improving Cellular Permeability of N-Pocket Inhibitors

Poor membrane permeability rendered compound **15** to be inactive in DENV-infected cells, mostly due to the presence of the negatively charged bis-carboxylic acids. Efforts to identify carboxylic bioisoteres lead to the acylsulfonamide moiety which dramatically improved their potencies and binding affinities in the biochemical and biophysical analyses (compounds **23–29i**; Fig. 14.1c; Table 14.1). Crystal structure of DENV-3 RdRp with **23** revealed that the acylsulfonamide formed H-bond interactions with the side chains of Thr794 and Arg729 and the backbone of Trp795 (PDB 5HMZ). The methyl group of the acylsulfonamide moiety was exposed to the solvent space, providing the opportunity to modify the overall physicochemical property of inhibitor without interfering with its affinity to the enzyme. Changing the methyl to benzene sulfonamide **25** (which increased its lipophilicity, whilst retaining similar acidity (pKa) and permeability as ***23***), provided weak cellular activity (EC_{50} 18-41 μM). Replacing the 6-methyl group of the methoxy benzene ring of **25** with another electron-donating methoxy group, improved cell

Table 14.1 Inhibitory and physio-chemical profiles of acid bioisoster analogs 23–29i

23, 25-29i

Cpd	R_1	R_2	R_3	DV4 IC_{50} (μM)[a]	DV4 Kd (μM)[b]	EC_{50} (mM, A547 cells)[c]				logD (pH 7.4)	pKa	Caco-2[d] (cm/s × 10^{-6})
						DV1	DV2	DV3	DV4			
23	Me	Me	OMe	0.31	0.12	>50	>50	>50	>50	0.8	4.0	0.5
25	Ph	Me	OMe	0.17 ± 0.11	nd	31	34	18	41	1.7	3.8	0.5
26	Ph	OMe	OMe	0.25 ± 0.13	0.09	15	36	7.2	14	1.5	4.8	10.8
26i	3-MeOPh	Me	OMe	0.11 ± 0.06	0.05	15.2	26.9 ± 8.8	16.8	20.4 ± 3.5	1.3	nd	nd
27	3-MeOPh	OMe	OMe	0.17 ± 0.10	0.07	1.8 ± 0.1	2.3 ± 0.5	1.8 ± 0.5	1.8 ± 0.2	1.6	4.7	3.91
28	3-MeOPh	OMe	Cl	0.14	0.01	6.8	13	5.5	7.0	0.9	3.8	nd
29		Me	OMe	0.023 ± 0.001	0.007	6.3 ± 0.9	14.1 ± 3.5	3.8 ± 0.7	10.2 ± 3.4	1.5	4.4	2.57
29i		OMe	OMe	0.074 ± 0.031	0.01	2.1	6.2 ± 1.2	1.3 ± 0.01	2.7 ± 0.3	1.6	nd	nd

Enzyme IC_{50} values and DENV cell based EC_{50} values were determined as described Lim et al. [15]. K_d values were determined by SPR as described in Noble et al. [20]. Apical to basal permeability at pH 7.4. DV1 = DENV-1, DV2 = DENV-2, DV3 = DENV-3, DV4 = DENV-4

permeability of **26** (by reducing ionization of the acylsulfonamide) and led to modest improvement in cellular potency (EC_{50} = 7.2–36 μM). The most potent anti-dengue activity was obtained with the 3-methoxyphenyl sulfonamide derivative, ***27***, which displayed EC_{50} values of low micromolar against all four serotypes (EC_{50} 1.8–2.3 μM). The exact cause of the significant improvement in the cellular potency of **27** is not known, as it has poorer permeability compared to **26**. A related analog, **26i**, which contains a single methyoxy group on the central benzene ring, compared to two methoxy substituents on **27**, exhibited significantly poorer cellular activity than the latter (EC_{50} = 15-27 μM). The additional methoxy group in ***27*** may allow the formation of an intra-hydrogen bond with the N-atom of the sulfonamide linker to facilitate better cell permeability. The same observation applies to the 8-quinolyl sulfonamide derivatives **29** and **29i** (EC_{50} = 4–14 μM and 2–6 μM respectively). Inhibitors **26–29i** were inactive against HCV replicon and human rhinoviruses (EC_{50} values >25 μM and >50 μM, respectively). No cytotoxicity was observed in five different cell lines tested (EC_{50} > 50 μM).

Additional information on this Section can be found in Lim et al. [15] and [26].

14.4 Profiling Mechanism of Action of N-Pocket Inhibitors

14.4.1 Mechanism Studies of Inhibitors in Biochemical Assays

In the standard assay format for DENV *dn*I FAPA assay, N pocket compounds **15**, **27** and **29** were first exposed to enzyme alone followed by reaction initiations with ssRNA template and NTPs. Order-of-reagent addition experiments in which compounds were exposed to pre-formed enzyme-ssRNA complexes, followed by reaction initiation with NTPs, did not result in significant changes in their IC_{50} values (Table 14.2). Compounds exposed to elongated enzyme-dsRNA complexes, in which the active site was occupied by the ssRNA template and newly synthesized short RNA products AGAA or AGAACC reduced their inhibitory potencies by 8–15-fold. These findings suggest that the N-pocket underwent conformational changes when the DENV polymerase entered into the elongation phase, which reduced the binding affinities of the compounds. Compounds also demonstrated 10–23-folds weaker inhibitory properties in the DENV elongation FAPA assay compared to the standard *dn*I assay. Overall, the data indicate that N pocket inhibitors primarily inhibit DENV polymerase *de novo* initiation activity. Kinetics studies using Lineweaver-Burk plots further revealed that both **15** and **29** have uncompetitive inhibition profiles with respect to the viral ssRNA template. On the other hand, they display mixed non/uncompetitive inhibition with respect to GTP.

Additional information on this Section can be found in Lim et al. [15].

14.4.2 Biological Relevance of N-pocket for DENV Replication

Alanine mutations of RdRp residues interacting with **27** (PDB 5K5M, 5I3P and 5JJS) or **29** (PDB 5I3Q and 5JJR) and as well as residues lining the N-pocket were generated in the recombinant NS5 protein and the DENV replicon. Alanine mutations mostly negatively impacted DENV polymerase *de novo* initiation (particularly for residues S710 and R737), and have less influence on elongation activity. In agreement with the biochemical data, N pocket mutant DENV replicon R737A (as well as R729A mutant replicon) was non-replicative. R729A RdRp exhibited about 30–40% of both *dn*I and elongation activities whilst *dn*I activity of R737A was completely abolished. Mutant replicons Y766A, and W803A were also non-replicative, despite showing 55-98% *de novo* initiation and elongation activities *in vitro*. Mutant replicons with T794A and S796A with comparable levels of *in vitro* polymerase activities as WT NS5 polymerase, were weakly replicative. Overall, the N pocket confor-

Table 14.2 Inhibitory and binding properties of DENV polymerase N-pocket compounds

Experiments with DV4 NS5		Compounds			
		3′dGTP	15	27	29
de novo IC_{50} (μM); order of addition experiments [fold change]	Enzyme + compound	0.79 ± 0.20	1.66 ± 0.35	0.172 ± 0.097	0.023 ± 0.001
	[Enzyme + RNA] + compound	0.44 ± 0.02	1.93 ± 0.77 [1.1X]	0.20 ± 0.07 [0.84X]	0.073 ± 0.02 [3.2X]
	[Enzyme + RNA + ATP + GTP] + compound	0.74 ± 0.26	12.9 ± 4.0 [7.8X]	2.2 ± 1.91 [9.3X]	0.338 ± 0.12 [14.7X]
	[Enzyme + RNA + ATP + GTP + ATTO-CTP] + compound	0.71 ± 0.20	13.3 ± 2.6 [8X]	1.89 ± 1.56 [8X]	0.239 [10.4X]
Elongation IC_{50} (μM)	Enzyme + compound	0.43 ± 0.29	16.2 ± 4.7 [9.8X]	5.46 ± 2.14 [23X]	0.427 ± 0.013 [18.6X]

Order-of-addition experiments were performed with DENV4 FL NS5 *de novo* initiation FAPA assay as described Lim et al. [15]. IC_{50} values were averaged from ≥3 independent experiments with compound **15** and **27**, and from at least one experiment for **29**. IC_{50} values obtained from elongation assays were averaged from >3 independent experiments for all three compounds. DV4 = DENV-4

mation observed in the inhibitor-bound crystal structures is likely to correspond to the structural state adopted by the DENV RdRp during *de novo* initiation and they play important functions for virus replication.

Additional information on this Section can be found in Lim et al. [15] and [20].

14.4.3 Resistant DENV Replicons Raised to N-Pocket Inhibitors

We propagated stable DENV-2-NGC EGFP replicon cells in 20 μM of **29** (≈1X EC_{90} value) for 5 weeks, and then increased the compound concentration to 25 μM. Two separate compound-resistant replicon clones harbored the same nucleotide change in NS5 (GA<u>A</u> → GA<u>C</u>), resulting in Glu802Asp mutation (note that residue 802 is Glu in DENV2-NGC and Gln in DENV3 RdRp). A third clone contained another nucleotide change in NS5 (<u>C</u>TG → <u>G</u>TG), resulting in Leu511Val mutation. A fourth clone contained a mixed profile in NS5, in the same position, with both the WT nucleotide (G) as well as mutation to C nucleotide present (GTG → G/CTG), giving rise to partial Leu511Val mutation. Similar efforts to raise resistant cells by exposure to high concentrations of **27** (14–20 μM; ≈2X EC_{90} value) were not successful. Stable DENV-2-NGC replicon cells were exposed to increasing concentrations of **27**, starting from 1.5 μM (≈0.5X EC_{50}), with media change every 2–3 days. After about 6 weeks, cells kept in 28 μM of **27** propagated robustly, at similar rates to WT DENV-2 replicon cells. RNA sequenced from the cells contained a partial Glu802Asp mutation profile (GAA → GAA/T). The crystal structures of DENV-3-RdRp bound to **29** (PDB 5I3Q, 5JJR) shows that the polar side chain of residue Gln802 (Glu802 in DENV2) hydrogens bond with the hydroxyl group of the propargyl alcohol of **29**. E802D mutation results in the shortening of the amino acid side-chain by one methyl group and is likely to disrupt this H-bond formation. Residue Leu511 (in DENV-2 and -3) forms van der Waals interactions with the thiophene ring of **29**. The loss of a methyl group from Val511 mutation, would weaken the interaction with the thiophene ring. Overall, these mutations lower the binding affinity of **29** in the N-pocket and verify that **27** and **29** inhibit DENV replication in cells by binding to the N-pocket in the DENV polymerase.

Additional information on this Section can be found in Lim et al. [15].

14.4.4 Impact of Resistance Mutations on Compound Inhibition

We generated recombinant NS5 mutant proteins with N-pocket amino acid changes in DENV-2 (L511V and E802D) and DENV-4 (L512V and Q803N) and tested them against **27** and **29** (Table 14.3). Both compounds were significantly less active against mutant enzymes than WT protein in the *dn*I FAPA assay. Compared WT protein, IC_{50} values of **29** declined by 4–12-folds in DENV-2 and DENV-4, single mutants, and by 52–133-folds in double mutant enzymes. Likewise for **27**, inhibitory potency fell by 5–14-folds in DENV-2 single mutants and by 88-folds in the double mutant. A complete loss of inhibitory activity (IC_{50} > 20 μM) was observed in DENV-4 single and double mutants. Furthermore, these compounds were less effective in stabilizing the mutant enzymes compared to the corresponding WT proteins [15]. Similar single and double (L511V and E802D) amino acid changes were also introduced into the DENV-2 (strain NGC) replicon and its infectious full length virus genome to examine their effects on the potencies of N pocket inhibitors (Table 14.3). Compared to WT replicon and virus, EC_{50} value of **29** was reduced by 3–6-folds in single and double mutant DENV-2 replicons and by 5–6-folds in virus mutants. On the other hand, EC_{50} values of **27** were reduced only by 2–4-folds in mutant DENV-2 replicons and viruses. The observed weaker EC_{50} shifts for **27** are puzzling as its binding mode is similar to **29** and involves non-covalent interaction of the thiophene ring with L511 and H-bond formation between the propargyl alcohol and E802D. Additional **27**-resistant DENV-2 EGFP-replicons shifted the EC_{50} values

Table 14.3 Inhibitory profiles of N-pocket compounds in DENV polymerase and DENV2 replicon and infectious virus harboring resistant phenotype amino acid changes

		IC_{50} (fold change compared to WT), μM		DENV2 NGC	EC_{50} (μM) (fold increase compared to WT)			
		27	*29*		Replicon		Virus	
DENV2-NGC FL NS5	**WT**	0.173	0.036		**27**	**29**	**27**	**29**
	L511V	2.548 (14.7X)	0.352 (9.9X)					
	E802D	0.936 (5.4X)	0.148 (4.2X)	**Wild type**	7.83	6.70	0.87	1.11
	L511V/ E802D	15.21 (88X)	1.85 (52X)	**L512V**	25.86 (3.3X)	32.32 (4.8X)	2.2 (2.5X)	6 (5.4X)
DENV4 FL NS5	**WT**	0.134	0.033	**E802D**	12.77 (1.6X)	21.39 (3.2X)	1.42 (1.6X)	5.8 (5.2X)
	L512V	>20 (>100X)	0.28 (8.7X)	**L512V/E802D**	28.42 (3.6X)	38.76 (5.8X)	2.32 (2.6X)	7.01 (6.4X)
	Q803N	>20 (>100X)	0.38 (11.7X)	**Wild type passaged in DMSO**	2.46	3.55	nd	nd
	L512V/ Q803N	>20 (>100X)	4.37 (133X)	**Compound 29-resistant**	41.7 (17X)	34 (9.6X)	nd	nd

n.d= not done

IC_{50} values from DENV-2 and DENV-4 *de novo* initiation FAPA assays were obtained from dose response testing as described in Lim et al. [15]. EC_{50} values were obtained from BHK-21 cells electroporated with WT and mutant DENV-2 replicons or infected with WT and mutant DENV2 viruses as described in Lim et al. [15]. DV2 = DENV-2, DV4 = DENV-4

of **27** and **29** shifted by 17- and 10-folds, respectively, in these cells, compared to control cells raised in DMSO. Full replicon genome sequence analyses revealed secondary mutations present in NS5 methyl-transferase and NS4B in the **27**-resistant replicon cells, suggesting that N pocket inhibitors also affected the replication complex formation.

Additional information on this Section can be found in Lim et al. [15].

14.5 Summary and Conclusions

From fragment-based screening by X-ray crystallography with the DENV-3 apo-RdRp protein [19, 25], we identified a novel allosteric pocket at the DENV-3 RdRp thumb and palm interface [15, 20, 26]. This pocket which we term the "N pocket" is located near the priming loop (aa782-809) and is lined by residues highly conserved across DENV-1-4, as well as in other flaviviruses. Alanine mutagenesis studies indicate that this pocket is important for NS5 polymerase *de novo* initiation activity and virus replication. N-pocket inhibitors generated by rational design potently inhibited DENV-1-4 polymerase *de novo* initiation activities and virus replication in various cell types. They have a mixed non−/uncompetitive inhibition profile and bind with strong affinity to recombinant apo-enzyme as well as FL NS5 from DENV replicon cell lysates [15].

Resistant DENV raised against N pocket inhibitors harbored amino acid mutations (L511 V and E802D; DENV-2 numbering) that mapped to the N-pocket and reduced compound potencies in DENV RdRp enzyme and DENV cell-based assays. Based on results from order-of-reagent addition experiments, N pocket inhibitors block the enzyme *de novo* initiation activity better than the elongation activity. Compound potencies are reduced when the enzyme is pre-occupied with newly synthesized duplex RNA, and not by single-stranded viral RNA. Presumably, retraction of the priming loop (aa782-809) from the active site during enzyme elongation alters the conformation of the N-pocket, leading to

weaker binding affinities of the RdRp for the compounds.

DENV N-pocket compounds resemble HCV polymerase Site III (palm 1) non-nucleoside inhibitors [2]. The latter inhibitors bind at the interface of the HCV polymerase thumb and palm subdomains, with one side comprising the "primer grip" and the opposite side formed by the β-hairpin loop (equivalent to the priming loop in DENV RdRp). Inhibitor binding is promoted by interactions with both sides, in particular, with Y448 from the β-loop. This locks the HCV RdRp thumb subdomain in a conformation that prevents *de novo* initiation. Dasabuvir (ABT 333), a Site III inhibitor, has recently been approved for HCV therapy in combination with NS3/4A protease and NS5 inhibitors [7].

There is, however, no equivalent of the HCV RdRp primer grip wall for DENV N-pocket. In addition, unlike HCV RdRp where the C-terminal loop penetrates the active site and participates in enzyme activity, the C-terminal end of flavivirus RdRp is disordered in most reported crystal structures. We speculate that the absence of both regions prevent formation of additional contacts with inhibitors, and is the reason for the weaker binding affinities of N-pocket compounds, compared to HCV site III inhibitors. The availability of a co-crystal structure of DENV RdRp with RNA would hopefully, facilitate new design strategies to further enhance inhibitor binding affinity and block elongation activity.

High clearance was observed for acyl-sulfonamide propargyl alcohol compounds *in vitro* which rendered them unsuitable for mouse efficacy studies. To develop N-pocket inhibitors, more stable functionalities with better pharmacokinetic properties, that retain key hydrogen bond interactions, are required.

Finally, compounds **27** and **29** were inactive when tested on the WNV replicon cell-based assay. Previous comparisons revealed that the WNV RdRp priming loop is closer to the *i*-1 site, and prevents formation of a similar N-pocket [16]. Whilst DENV N-pocket residues are mostly conserved across the flavivirus family, residues 799-802, which accommodate the propargyl alcohol arm, are more divergent. This may explain the lack of activity of **27** and **29** on WNV. Interestingly, residues 799-802 are more similar amongst JEV, MVEV WNV, YFV and ZIKV compared to DENV-1-4. In this light, it may not be plausible to develop pan-active N-pocket inhibitors that work on all flaviviruses. Rather, designing N-pocket inhibitors that specifically target different subgroups of the flavivirus family may be a more attainable goal.

Discussion of Chapter 14 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Siew Pheng Lim.

Paul Young: Great work! A perfect example of a great collaboration between biology and chemistry. The work you describe seems to have hit a little bit the end of the road. You have identified and derived reasonably active sompounds but they do not appear to be effectively bioavailable. So what's the next step?

Siew Pheng Lim: So this is only one part of the story. Our goal is to design compounds that extend out of the pocket. In the parlance of chemists in the team these extension from the pocket are done to find the so-called sweet spot to further improve the potency. But despite a lot of effort, we did not find another sweet spot. I also talked a little about some similarity the N-pocket shares with HCV palm 1 site. However HCV palm 1 pocket has a another wall contributed by the C-terminal part of the protein which allows those inhibitors to bindto both sides. In the case of Dengue virus polymerase we appear to have only one wall, we are trying to find a way to reach a second wall and that has not been successful so far.

David Jans: I just wanted to know what EC_{50} you have been happy with. Several of the compounds have EC_{50} of around 3 micromolar. Do you think that would be potent enough?

In other words what should be the target for an effective drug?

Siew Pheng Lim: If I compare against the phenotypic screens that my colleagues do, they typically find inhibitors that are double-digit nanomolar in EC_{50}. There they have a different challenge because they have issues with solubility and clearance as the compounds are too greasy. So I think for us if we can hit below one micromolar, We set ourselves a benchmark of less than one micromolar which we consider a good threshold. So you can see we have not quite reach that point yet.

Subhash Vasudevan: In the case of HCV palm 1, the compound that targeted a similar pocket actually got to the clinic implying that it is possible to develop a non-nucleosidic inhibitor. The pocket that you are looking is also truly a hotspot for Dengue and other flaviviruses. So the question I guess is do we give up on that area based on your experience so far?

Siew Pheng Lim: For now, this work has been put on hold, and we published the work so that other researchers in the field can expand on the effort and take a fresh look at approaches to target this important pocket. The search may include using other flaviviruses and find something we have not yet found to extend the chemistry. As David Jans alluded yesterday, we primarily work with DENV3 NS5 RdRp for which we have structural data. So it would be nice to have structural studies on other serotypes and other flaviviruses to see if something maybe different.

Subhash Vasudevan: But your data clearly shows that Dengue 2 and Dengue 4 give broadly similar results. In the discovery process if you are trying to do DENV1 to 4 separately then that would be a huge challenge. But NS5 is a really important target and since the non-nucleoside approach is not quite ready yet for pan-serotype inhibitor, what about the nucleoside aproach?

Siew Pheng Lim: Nucleoside approach is still ongoing. We started with KAB-344, the adenine analog (also known as NITD008) that eventually failed in the two-week animal toxicity studies and could not be progressed further. Since then we are working on other scaffolds. I think the issue with nucleosides is the unpredictable toxicity. To mitigate this we have implemented additional cytotoxicity assays trying to weed out toxicity early in the flowchart, such as mitochondrial assays –longer cytotoxicity assays – to capture those compounds that are potentially cytotoxic before we go into two-week toxicity studies in rats and dogs. Through this process, we have a few candidates that we are evaluating. The challenge with nucleoside inhibitors is the potentially lengthy scale up synthesis which can be expensive – so the way forward remains to be seen.

References

1. Bollati M, Alvarez K, Assenberg R, Baronti C, Canard B, Cook S, Coutard B, Decroly E, de Lamballerie X, Gould EA, Grard G, Grimes JM, Hilgenfeld R, Jansson AM, Malet H, Mancini EJ, Mastrangelo E, Mattevi A, Milani M, Moureau G, Neyts J, Owens RJ, Ren J, Selisko B, Speroni S, Steuber H, Stuart DI, Unge T, Bolognesi M (2010) Structure and functionality in flavivirus NS-proteins: perspectives for drug design. Antivir Res 87:125–148
2. Caillet-Saguy C, Simister PC, Bressanelli S (2011) An objective assessment of conformational variability in complexes of hepatitis C virus polymerase with non-nucleoside inhibitors. J Mol Biol 414:370–384
3. Caillet-Saguy C, Lim SP, Shi P-Y, Lescar J, Bressanelli S (2014) Polymerases of hepatitis C viruses and flaviviruses: structural and mechanistic insights and drug development. Antivir Res 105:8–16
4. Chung KY, Dong H, Chao AT, Shi P-Y, Lescar J, Lim SP (2010) Higher catalytic efficiency of N-7-methylation is responsible for processive N-7 and 2′-O methyltransferase activity in dengue virus. Virology 402:52–60
5. Davidson AD (2009) Chapter 2. New insights into flavivirus nonstructural protein 5. Adv Virus Res 74:41–101
6. Egloff MP, Benarroch D, Selisko B, Romette JL, Canard B (2002) An RNA cap (nucleoside-2′-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. EMBO J 21:2757–2768
7. Flisiak R, Janczewska E, Wawrzynowicz-Syczewska M, Jaroszewicz J, Zarębska-Michaluk D, Nazzal K, Bolewska B, Bialkowska J, Berak H, Fleischer-Stępniewska K, Tomasiewicz K, Karwowska K, Rostkowska K, Piekarska A, Tronina O, Madej G,

Garlicki A, Lucejko M, Pisula A, Karpińska E, Kryczka W, Wiercińska-Drapało A, Mozer-Lisewska I, Jabłkowski M, Horban A, Knysz B, Tudrujek M, Halota W, Simon K (2016) Real-world effectiveness and safety of ombitasvir/paritaprevir/ritonavir ± dasabuvir ± ribavirin in hepatitis C: AMBER study. Aliment Pharmacol Ther 44(9):946–956

8. Koonin EV (1991) The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J Gen Virol 72:2197–2206
9. Koonin EV (1993) Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus. J Gen Virol 74:733–740
10. Kuntz ID, Chen K, Sharp KA, Kollman PA (1999) The maximal affinity of ligands. Proc Natl Acad Sci U S A 96(18):9997–10002
11. Leslie AGW (1992) Recent changes to the MOSFLM package for processing film and image plate data. Joint CCP4-ESF-EAMCB newsletter on protein crystallography, no. 26
12. Lim SP, Wen D, Yap TL, Yan CK, Lescar J, Vasudevan SG (2008) A scintillation proximity assay for dengue virus NS5 2′-O-methyltransferase-kinetic and inhibition analyses. Antivir Res 80:360–369
13. Lim SP, Koh JH, Seh CC, Liew CW, Davidson AD, Chua LS, Chandrasekaran R, Cornvik TC, Shi PY, Lescar J (2013) A crystal structure of the dengue virus non-structural protein 5 (NS5) polymerase delineates interdomain amino acid residues that enhance its thermostability and de novo initiation activities. J Biol Chem 288(43):31105–31114
14. Lim SP, Noble CG, Shi PY (2015) The dengue virus NS5 protein as a target for drug discovery. Antivir Res 119:57–67
15. Lim SP, Noble CG, Seh CC, Soh TS, El Sahili A, Chan GK, Lescar J, Arora R, Benson T, Nilar S, Manjunatha U, Wan KF, Dong H, Xie X, Shi PY, Yokokawa F (2016) Potent Allosteric Dengue Virus NS5 Polymerase Inhibitors: Mechanism of Action and Resistance Profiling. PLoS Pathog 12(8):e1005737
16. Malet H, Massé N, Selisko B, Romette JL, Alvarez K, Guillemot JC et al (2008) The flavivirus polymerase as a target for drug discovery. Antivir Res 80:23–35
17. Murshudov GN, Skubak P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, Winn MD, Long F, Vagin AA (2011) REFMAC5 for the refinement of macromolecular crystal structures. Acta Crystallogr D Biol Crystallogr 67:355–367
18. Niyomrattanakit P, Wan KF, Chung KY, Abas SN, Seh CC, Dong H et al (2015) Stabilization of dengue virus polymerase in de novo initiation assay provides advantages for compound screening. Antivir Res 119:36–46
19. Noble CG, Lim SP, Chen YL, Liew CW, Yap L, Lescar J, Shi PY (2013) Conformational flexibility of the dengue virus RNA-dependent RNA polymerase revealed by a complex with an inhibitor. J Virol 87:5291–5295
20. Noble CG, Lim SP, Arora R, Yokokawa F, Nilar S, Seh CC, Wright SK, Benson TE, Smith PW, Shi PY (2016) A conserved pocket in the dengue virus polymerase identified through fragment-based screening. J Biol Chem 291(16):8541–8548
21. Potisopon S, Priet S, Collet A, Decroly E, Canard B, Selisko B (2014) The methyltransferase domain of dengue virus protein NS5 ensures efficient RNA synthesis initiation and elongation by the polymerase domain. Nucleic Acids Res 42:11642–11656
22. Ray D, Shah A, Tilgner M, Guo Y, Zhao Y, Dong H, Deas TS, Zhou Y, Li H, Shi PY (2006) West Nile virus 5′-cap structure is formed by sequential guanine N-7 and ribose 2′-O methylations by nonstructural protein 5. J Virol 80:8362–8370
23. Tarantino D, Cannalire R, Mastrangelo E, Croci R, Querat G, Barreca ML, Bolognesi M, Manfroni G, Cecchetti V, Milani M (2016) Targeting flavivirus RNA dependent RNA polymerase through a pyridobenzothiazole inhibitor. Antivir Res 134:226–235
24. Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB, Leslie AG, McCoy A, McNicholas SJ, Murshudov GN, Pannu NS, Potterton EA, Powell HR, Read RJ, Vagin A, Wilson KS (2011) Overview of the CCP4 suite and current developments. Acta Crystallogr D Biol Crystallogr 67:235–242
25. Yap TL, Xu T, Chen YL, Malet H, Egloff MP, Canard B et al (2007) Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution. J Virol 81:4753–4765
26. Yokokawa F, Shahul N, Noble CG, Lim SP, Rao R, Tania S et al (2016) Discovery of potent non-nucleoside inhibitors of dengue viral RNA-dependent RNA polymerase from a fragment hit using structure-based drug design. J Med Chem 59:3935–3952
27. Zhao Y, Soh S, Zheng J, Chan KWK, Phoo WW, Lee CC et al (2015) A crystal structure of the dengue virus NS5 protein reveals a novel inter-domain interface essential for protein flexibility and virus replication. PLoS Pathog 11(3):e1004682

Nucleocytoplasmic Trafficking of Dengue Non-structural Protein 5 as a Target for Antivirals

15

David A. Jans and Alexander J. Martin

Abstract

Signal-dependent movement of proteins into and out of the nucleus through the importin superfamily of transporters is central to the replication of many viruses in infected cells, including RNA viruses such as the flavivirus Dengue virus (DENV). DENV non-structural protein 5 (NS5) traffics into and out of the host cell nucleus/nucleolus, being observed in the nucleus, although to differing extents, very early in infection in the case of all 4 DENV serotypes; with results from both reverse genetics and inhibitor studies indicating that this trafficking is critical to DENV infection. Knowledge of the transporters and targeting signals responsible for nuclear trafficking of NS5 has enabled inhibitors of DENV NS5 nuclear import to be identified using a novel screening/counterscreen approach. *N*-(4-hydroxyphenyl) retinamide (4-HPR) is of particular interest as a specific, non-toxic inhibitor able to protect against infection by all four serotypes of DENV, as well as the severe, antibody-enhanced form of DENV infection, in a lethal mouse model. Since 4-HPR can also inhibit DENV-related flaviviruses of medical significance such as West Nile Virus and Zika virus, it is of great interest for future commercialisation. Targeting nucleocytoplasmic trafficking of flavivirus proteins promises to be a powerful strategy to counter flaviviruses, for which the development of protective vaccines has thus far proven problematic.

Keywords

Dengue virus · DENV · Nucleus · Nuclear trafficking · Nucleocytoplasmic trafficking · Antiviral · 4-HPR

15.1 Dengue Virus; Key Role of Non-structural Protein 5

15.1.1 The Threat of Dengue Virus

Dengue virus (DENV), of which there are four immunologically distinct serotypes (DENV 1–4), is the causative agent of dengue fever and its more severe, antibody-dependent enhanced (ADE), dengue haemorrhagic fever (DHF) form [2, 52, 54]. Dengue fever is a mosquito-borne infection, and with the success of the *Aedes aegypti* vector in colonising urban environments, two thirds of the world population are at risk. Although there are approximately 96 million

D. A. Jans (✉) · A. J. Martin
Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Australia
e-mail: david.jans@monash.edu

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_15

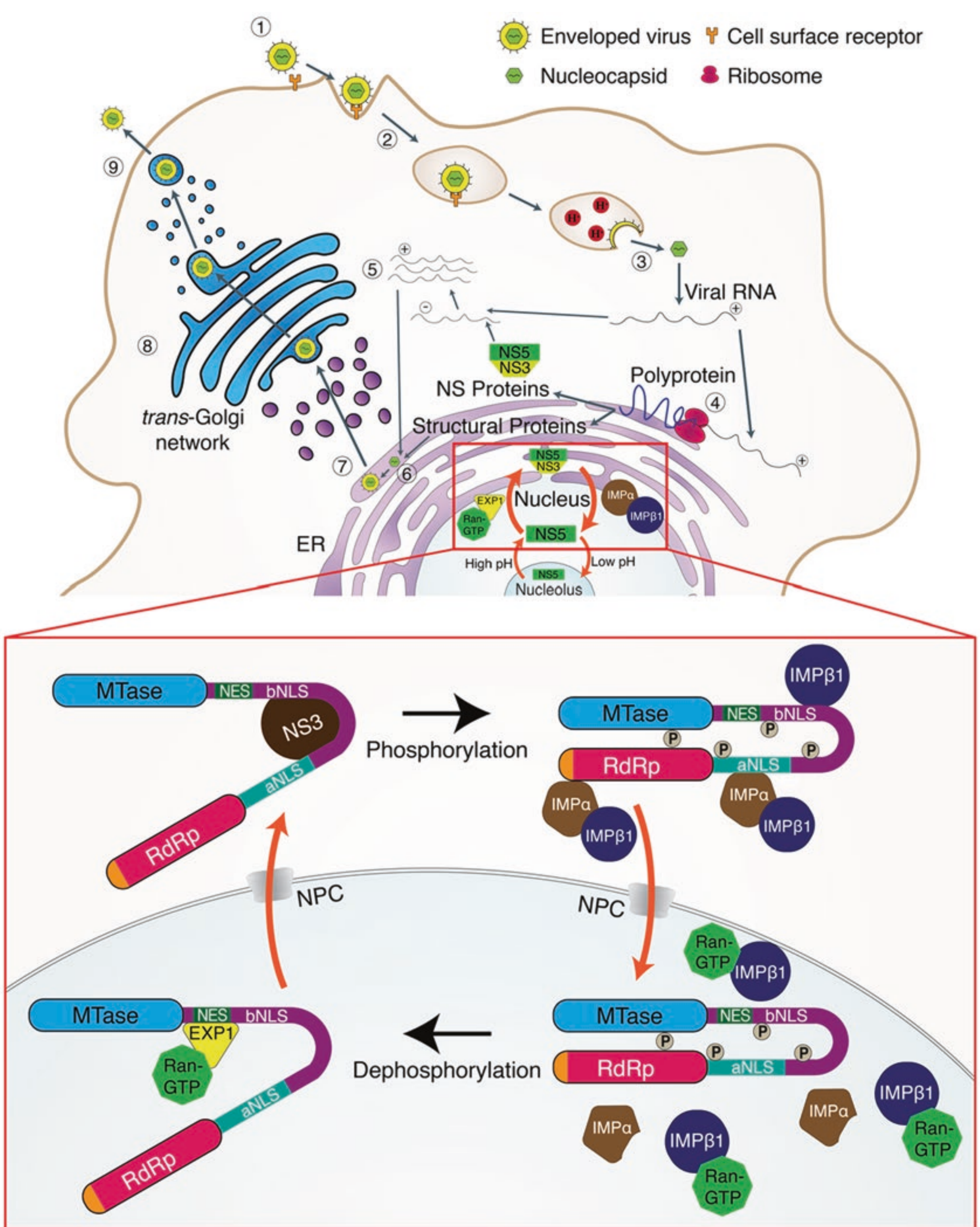


Fig. 15.1 Schematic of the DENV infectious cycle and NS5 nucleocytoplasmic trafficking. (Top). Similar to most flaviviruses, the E protein of DENV facilitates virion binding to cell-surface receptors (*1*), followed by virion entry via clathrin-mediated endocytosis (*2*). Endosomal acidification (denoted by H^+ in the schematic) subsequently results in viral fusion and release of the viral nucleocapsid (*3*). Translation and genome replication are tightly linked, where the positive-sense viral RNA is translated by ribosomes at the rough ER (*4*), forming a viral polyprotein with sections embedded within the ER membrane, cleaved by host and viral proteases into 3 structural and 7 non-structural (NS) proteins [35, 36]. The NS proteins, and NS3 (helicase) and NS5 (replicase) in particular, bind the +-sense viral RNA and form the replication complex, which synthesises the negative-sense strand RNA, which is in turn used to synthesise more +-sense RNA (*5*); replication largely occurs in

symptomatic cases leading to 500,000 hospitalizations and >20,000 deaths annually, there is no approved antiviral agent currently available to treat DENV infection [14, 21, 22, 52, 54, 67]. Additionally, the shortcomings of the recently approved polyvalent DENV vaccine [7, 9, 61] raise concerns regarding the viability of a vaccine-led eradication strategy [11].

The development of inhibitors and vaccines to DENV is complicated by the fact that previous infection with one DENV serotype can predispose an individual to severe infection with the other serotypes through ADE [11, 21, 22]. Thus, although 75% or more of the estimated incidence of 400 million DENV infections per year worldwide are not symptomatic, each case is of potential importance in terms of predisposing to severe ADE infection in the future. ADE is a particular concern for vaccine approaches, since immunity against one but not all serotypes could, rather than protect against infection, lead to increased likelihood of severe, life-threatening infection, depending on the DENV serotype carried by the mosquito vector. Clearly in this context, new therapeutics for treatment and prevention of DENV infection in all its forms are urgently required.

15.1.2 DENV Life Cycle

DENV is a member of the *Flavivirus* genus, related to other viruses of medical significance, including West Nile Virus (WNV), Zika Virus (ZIKV), Yellow Fever Virus (YFV) and Japanese Encephalitis Virus (JEV). It possesses a single-stranded, positive (+)-sense RNA genome that is translated as a single polyprotein prior to cleavage/processing into three structural and seven non-structural (NS) proteins [46]. Figure 15.1 (top) shows the main steps of the infectious cycle of DENV, which is typical for most flaviviruses. Upon envelope protein (E) conferring binding to host cell-surface receptors, virion entry occurs via clathrin-mediated endocytosis. Endosomal acidification follows to effect viral fusion and release of the viral nucleocapsid. The free +-sense viral RNA is then translated by ribosomes at the rough ER, resulting in a viral polyprotein, which is subsequently cleaved by host and viral proteases into three structural and seven non-structural (NS) proteins [35, 36]. The NS proteins, including NS3 (helicase) and NS5 (RNA polymerase), bind the +-sense viral RNA and form the key components of the replication complex, which synthesises the negative (−)-sense RNA, which is in turn used to synthesise more +-sense RNA, replication occurring in virus-induced membrane vesicles ("vesicle packets" – VPs) derived from the ER and Golgi [10, 40, 58]. The +-sense RNA is exported from the VPs to bind the C protein and form the nucleocapsid, which localises to the rough ER and binds viral E and prM proteins. The resulting immature virion buds off the ER, acquiring its lipid envelope, and is transported through the trans-Golgi network, where the virion matures, and prM is cleaved into pr and M. The mature virion is then released from the host cell by exocytosis to spread to and infect other cells of the host and/or be ingested by the mosquito vector.

Fig. 15.1 (continued) virus-induced membrane vesicles ("vesicle packets" – VPs – not shown) derived from the ER and Golgi [10, 40, 58]. The +-sense RNA is exported from the VPs to bind the C protein and form the nucleocapsid (*6*), which localises to the rough ER and binds viral E and prM proteins. The resulting immature virion buds off the ER, acquiring its lipid envelope (*7*), and being transported through the trans-Golgi network (*8*), where the virion matures, and prM is cleaved into pr and M. The mature virion is then released from the host cell by exocytosis (*9*). Non-phosphorylated NS5 is found together with NS3 in the cytoplasm in the viral replication complex, where it performs its canonical replicative functions, but is also found in the nucleus and nucleolus (enhanced by low pH conditions) in the infected cell [15, 28], where its role is to suppress the host cell antiviral response (e.g. [43, 51, 55]). The ***enlargement*** highlights NS5 nucleocytoplasmic trafficking, where NS5 transport into and out of the nucleus through the nuclear pore complex (NPC) is largely mediated by the Importin (Imp) α/β1 heterodimer and Exp1, respectively [51, 55], and likely to be modulated by NS5 hyperphosphorylation [28]

15.1.3 DENV Non-structural Protein 5 Trafficks into the Nucleus and Nucleolus of Infected Cells

The most conserved coding sequence across flaviviruses is that of the replicase NS5, spanning RNA polymerase (RdRp) and methyltransferase (MTase) domains. Together with the NS3 helicase, NS5 is primarily responsible for DENV replication, which, as for most RNA viruses, takes place entirely in the cytoplasm [3], in virus-induced Golgi- and endoplasmic reticulum-derived-membrane vesicles (see Fig. 15.1 top). Despite its critical role in replication in the cytoplasm, NS5 is known to be predominantly located inside the nucleus throughout infection (Fig. 15.1 enlargement; see Fig. 15.2), where it is thought to play a role in suppression of the host anti-viral response [28, 43, 51, 54, 55]. Suppression of interleukin 8 production, for example, has been shown to be dependent on NS5 nuclear localisation in infected cells [43, 51, 55], with other transcriptional effects noted for cell cycle regulators (unpublished), which presumably relates to arrest of the infected cell in the stage of the cell cycle which is optimal for virus replication (see [25, 27]). Kapoor et al. [28] showed that non-phosphorylated NS5 is predominantly the form of NS5 found together with NS3 in the cytoplasm in the viral replication complex, whereas NS5 in the nucleus in the infected cell is in a hyperphosphorylated state, the clear implication being that post-translational modification regulates NS5 nucleocytoplasmic trafficking, with phosphorylation favouring nuclear accumulation.

Initial localisation studies were focussed largely on DENV2 NS5 [13, 28], but it has more recently become clear that NS5 from all 4 DENV serotypes can localise in the nucleus, although to differing extents [24, 56], with comparable observations for WNV, Zika Virus (manuscript in preparation), YFV [5] and JEV [12]. NS5 from DENV3 is strongly nuclear, in similar fashion to NS5 from DENV2 (see Fig. 15.2), whilst NS5 from DENV1 and 4 is more cytoplasmic (see Fig. 15.2; not shown) [24, 56]. Treatment of cells with a specific inhibitor of nuclear export (leptomycin B – LMB) increases the localisation of NS5 from all DENV serotypes significantly [55, 56], demonstrating that NS5 cycles between nucleus and cytoplasm (see Figs. 15.1 and 15.2). That NS5 alone has the intrinsic trafficking ability (targeting signals) to move between cytoplasm and nucleus is indicated by the fact that, out of the context of infection, ectopically expressed NS5 is nuclear and sensitive to LMB (see Fig. 15.2).

Finally, nuclear NS5 appears to be able to localise dynamically in the nucleolus, a subcompartment of the nucleus in infected cells, as well as when ectopically expressed ([15] – see Fig. 15.2). Low pH appears to drive nucleolar localisation, which may be relevant to acidosis in the severe DHF form of DENV disease. That NS5 nucleolar localisation is of physiological significance is implied by the fact that a reverse genetics-derived mutant DENV that is impaired in NS5 nucleolar trafficking is not viable; although the precise functional role in the nucleolus of NS5 is not yet clear, modulation of ribosome assembly, a key function of the nucleolus, and hence host cell translation appears a possibility. Significantly, this is consistent with the fact that ribosomal protein subunits can be identified in the NS5 interactome, as determined using unbiased screening approaches such as the yeast 2-hybrid system (unpublished).

15.2 Nucleocytoplasmic Trafficking of NS5

15.2.1 The Host Cell Nuclear Trafficking Machinery

Transport into and out of the eukaryotic cell nucleus of molecules >45 kDa requires possession of a specific targeting signal (nuclear localization signal – NLS – and nuclear export sequence – NES – for nuclear import and export respectively) which is recognised by members of the importin (IMP) superfamily of nuclear transport receptors, of which multiple α and β types exist [33, 49]. Nuclear import occurs when an NLS is recognized by either the IMPα/β1

Fig. 15.2 DENV NS5 nuclear/nucleolar localisation in infected or transfected cells. Confocal miscroscopic images are shown for DENV1- or 2-infected cells fixed and stained for NS5 at the indicated time point post infection (p.i.) (left), GFP-tagged DENV1 or 2 NS5 16 hours post-transfection, in the absence or presence of treatment with the Exp1 inhibitor LMB for 3 hours prior to imaging (right) (see [52, 55, 56]). Lower panels highlight NS5 nucleolar localisation in immunostained DENV2 infected cells (left), or colocalisation of GFP-DENV2-NS5 in transfected cells that are costained for the nucleolar marker fibrillarin (see Ref. [15])

heterodimer or IMPβ1 alone or one of the many homologues thereof, followed by translocation through the nuclear envelope-embedded nuclear pore complexes. Once inside the nucleus, binding of RanGTP to the IMPβ dissociates the complex to release the cargo within the nucleoplasm to play its nuclear role [33, 47]. Nuclear protein export occurs analogously, whereby a NES in the cargo protein is recognized in the nucleus by specific members of the IMPβ family called exportins (EXPs) (of which CRM-1/XPO1/EXP1 is the best characterized) bound to RanGTP, prior to export to the cytoplasm and dissociation of the complex [50, 63].

15.2.2 Targeting Signals Mediating Nucleocytoplasmic Trafficking of NS5

Nucleocytoplasmic transport of DENV NS5 is regulated, at least in part, by multiple targeting signals within the "interdomain" region between the N-terminal MTase domain and the C-terminal RdRp (see Fig. 15.1, enlargement) [4]. The NS5 interdomain region contains two NLS signals; the aNLS (recognized by IMPα/β1) and the bNLS (recognized by IMPβ1 alone) [4], of which the aNLS appears to be dominant (see [51]). Further, the aNLS has been shown to be neces-

sary and sufficient for DENV2 NS5 nuclear accumulation; i.e. it has been shown to be able to target a large, normally non-nuclear reporter protein into the nucleus out of the context of NS5 (see [4, 13]; see however [57]). The interdomain region also contains a CRM-1 recognised NES [55] and the binding site for NS5 interaction with NS3 [34], an essential part of the viral replication complex [2, 35, 36, 46]. Mutation of any of these signals has dramatic effects on the viability of the virus [51, 55]. In particular, mutation of 5 lysine (K) residues within the aNLS to alanine (A) results in such severe effects on viral replication that the virus is unable to be recovered. Partial mutation of this region (DENV A2 mutant; KKK387-389AAA) produces a severely impaired virus with dramatically reduced virus growth kinetics ([51]; see also [23]).

Finally, recent work [57] implicates the additional contribution to NS5 nuclear localisation of the C-terminal resides of NS5 (C-NLS – see Fig. 15.3), which can be crystallised as a synthetic peptide with a truncated form of IMPα that is unable to bind IMPβ1; mutation of this C-NLS also appears to impact NS5 nuclear localisation in infected cells and the production of infectious virus, although this C-terminal region of NS5 also appears to be important for dimerisation, which may be important to DENV replication (see [57, 69]). Importantly, while the C-NLS is exposed in crystal structures of full length NS5, in contrast to parts of the interdomain region (e.g. the NES region – see [29]), the possibility of NS5 hyperphosphorylation changing NS5 conformation/masking or unmasking targeting sequences has not been considered experimentally thus far. It should also be remembered that a crystal structure of IMPα bound to a peptide NLS is somewhat distant from the physiologically relevant situation of a full length protein being recognised by the transport competent IMPα/β1 heterodimer in the context of a cell. Clearly, the field would greatly benefit from crystal structures of full length NS5 bound to IMPα (and preferably the IMPα/β1 heterodimer); this would resolve the important question of the nuclear transport competent complex that is relevant to flavivirus biology and cell biology in general. It is certainly striking that the basic residues of the C-NLS are not conserved throughout DENV1–4, or in related flavivirus NS5 sequences, in contrast to the bNLS/aNLS and NES of the interdomain region (see Fig. 15.3).

Taken together, it seems likely that ***the subcellular localisation/nucleocytoplamic trafficking ability of NS5 is a product of the combined activities of the various contributing NLS and NES sequences***. Importantly, these targeting sequences themselves would appear likely to be regulated by phosphorylation (e.g. [28]; see [16, 17, 33]) or potentially other post-translational modifications (e.g. [16, 18]). Our recent work (manuscript in preparation) suggests that phosphorylation within the interdomain region may well be key to switching between nuclear import and export (see also [13]), and hence the driver of cycling between the nucleus and the cytoplasm.

15.2.3 Nucleocytoplasmic Trafficking of NS5 as a Therapeutic Target

15.2.3.1 Reverse Genetics Studies

There is a body of evidence that shows that NS5 nuclear trafficking is essential to DENV infection. Firstly, as indicated above, reverse genetics approaches, where key mutations are incorporated into a DENV infectious clone to test for their effect on viral infection, indicate that mutation of the interdomain NLSs as well as NES, result in attenuation or lethality for DENV ([51, 55]; see also [23]), with comparable results for a mutant virus impairing NS5 nucleolar targeting [15]. Clearly, where viable mutant virus cannot be recovered, it is not possible to conclude that the impact of impaired nucleocytoplasmic trafficking, as opposed to additional functions of NS5, is the basis of lethality *in vivo*. In the case of the DENV A2 mutant (KKK387-389AAA), alluded to above, however, there is a correlation between NS5 nuclear import kinetics and DENV virus production, where the A2 mutant NS5 does accumulate in the nucleus in infected cells, but much more slowly that wild type, and has

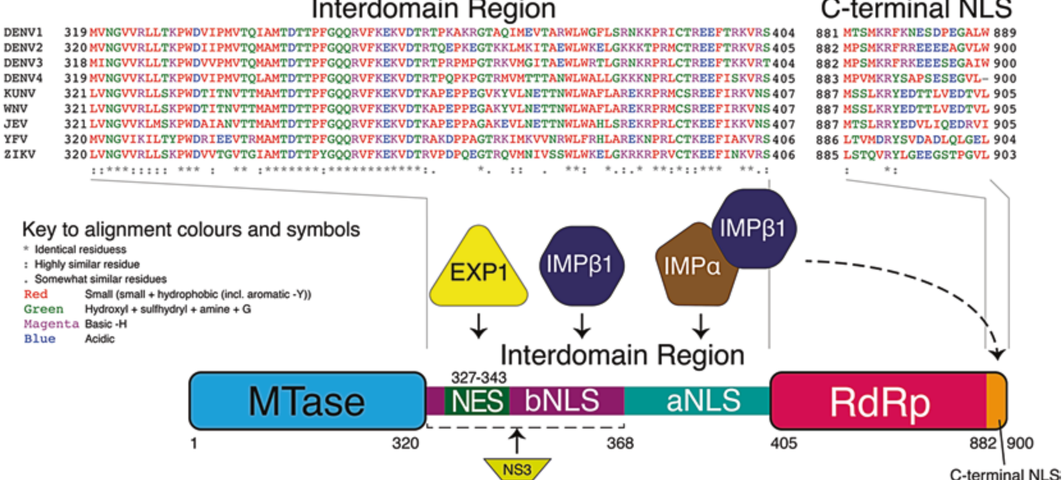


Fig. 15.3 Schematic diagram of the key domains for nuclear trafficking within DENV NS5 aligned to related Flaviviruses. Sequences of the interdomain (left) and C-terminus (right) are shown in the single amino acid letter code. Sequence alignments are for the NS5 proteins from DENV1–4 (GenBank IDs AIU47321.1, AAK67712.1, AHN50411.1, AMP43484.1 respectively) and related flaviviruses including Yellow fever virus (YFV, GenBank NP_041726.1), Zika virus (ZIKV, GenBank YP_002790881.1), Japanese encephalitis virus (JEV, GenBank NP_059434.1), Kunjin virus (KUNV, GenBank AAP78941.1) and West Nile virus (WNV, GenBank ALF00101.1). The symbol '*' below a position indicates identical residues, while ':' indicates conservation of highly similar residues (roughly equal to a score of >0.5 in the Gonnet PAM 250 matrix), and '.' indicates conservation of only somewhat-similar residues (roughly equal to a score of between 0.5 and 0 in the Gonnet PAM 250 matrix). A high degree of sequence identity is observed within the NS5 interdomain regions of the viruses, while the C-terminal NLS is less-well conservation between viruses. The schematic below shows the NS5 domains, with the S-adenosylmethyltransferase (MTase) domain within the N-terminal end, separated from the C-terminal RNA-dependent RNA polymerase (RdRp) by the interdomain region. The interdomain region contains two nuclear localisation sequences (NLS) and one nuclear export sequence (NES), and their proposed importin (IMP) and exportin (Exp) binding partners shown [51, 55]. The recently described C-terminal NLS [57] is also shown. The viral NS3 protein is thought to bind within the NS5 bNLS region [34], with K^{330} absolutely required [70]. Although the key residues for nucleolar targeting are known to include L^{165}/L^{167}/ V^{168} [15], the minimal nucleolar targeting element of DENV2 NS5 has not yet been defined, and so homology alignment around this region of NS5 is not presented

concomitantly reduced virus growth kinetics [51]. Clearly, the fact that the A2 virus can replicate *in vivo* implies that effects of the mutation on replicative functions of NS5/protein stability *etc.* are unlikely; in short, all results thus far from *in vitro* studies, transfected cell studies and infected cell studies give no hint whatsoever that there could be an impact of the A2 mutation on NS5 protein stability, interaction with NS3 or replicase activity ([51]; see however [57]).

Several other studies suggest that the situation may be more complex, with mutations in the interdomain region ("mutant 397–398") reducing NS5 nuclear accumulation, but not impacting virus growth kinetics significantly, according to Kumar et al. [31]. Comparable results have been reported for various C-NLS region mutations ([57], and not shown). In all cases, it is difficult to conclude definitively that a low but functionally important level of NS5 nucleocytoplasmic trafficking may not be not occurring; analysis in the presence of LMB has not been performed for these mutated derivatives. As evident for DENV1 and 4, NS5 is largely cytoplasmic at steady state, but LMB treatment elevates the degree of nuclear accumulation (e.g. see Fig. 15.2; [56]), clearly demonstrating that NS5 is trafficking dynamically between nucleus and cytoplasm. That the low levels of DENV1/4 NS5 nuclear trafficking are functionally significant is implied by the fact that the growth of DENV1/4, like that of DENV2/3, is impacted significantly by nuclear

import inhibitors (e.g. the NS5-specific nuclear import inhibitor *N*(4-hydroxyphenyl) retinamide or 4-HPR – see Sect. 15.2.3.2 below) ([14, 56]; manuscript in preparation). Thus, testing reverse genetics-generated mutant DENV virus for susceptibility to 4-HPR may well be a definitive method to demonstrating that NS5 nucleocytoplasmic trafficking is not required for efficient DENV infection. Taken together, the results from reverse genetics studies are consistent with nucleocytoplasmic trafficking of NS5 being integral to efficient infection/virus production.

15.2.3.2 Host Gene Screens

Several studies have performed genome-wide screens using siRNAs to assess which host genes impact flavivirus production (e.g. [30], for WNV as well as DENV). Such studies are limited in terms of the extent of the gene knockdown and its potential cell toxicity, and the lack of validation of given targets, and hence are rather inconclusive in terms of whether they implicate host cell factors such as IMPs/EXPs as contributors to infection. Hanneman et al. [24] used targeted siRNAs to assess the role of specific IMPs in DENV infection, with results clearly implicating IMPβ1 (*KPNB1* gene) as a host cell factor contributing to DENV infection. Knockdown of IMPα1 (*KPNA2* gene) was not found to impact DENV infection, but since there are multiple distinct IMPα isoforms that work with IMPβ1 to mediate nuclear import of classical NLS (aNLS)-containing cargoes such as NS5), it cannot be excluded that one of the other IMPαs can complement the absence of normal levels of IMPα1. CRISPR knockout screens in cell culture systems (e.g [41, 68].) have revealed some effect of *KPNA2* or *KPNB1* knockout in assay systems for multiple flavivirus members, including DENV, but the caveats of IMPα redundancy overcoming effects of *KPNA2* knockout complicate the interpretation of results, and cytotoxicity due to knockout can also be a confounding factor in CRISPR/Cas9 screens (e.g. *KPNB1* knockout is also known to be embryonic lethal in mouse – [45]); we have indeed found various host cells to be highly sensitive to even IMPβ1 knockdown using siRNA (e.g. [6, 26, 32]).

15.2.3.3 IMP-Targeting Small Molecule Inhibitors

The first nuclear transport inhibitor to be shown to have effects on DENV replication was the EXP1-specific inhibitor LMB [55]. Strikingly, LMB treatment, which inhibits NS5 nuclear export and hence increases NS5 nuclear accumulation, resulted in significantly increased rather than decreased virus production [55]; experiments using Karyopharm new generation EXP1 inhibitors (e.g. [44]; unpublished), or small inhibitory RNAs (siRNAs) to EXP1 resulted in similar effects (data not shown). This strong correlation of the efficiency of virus production with the extent of NS5 nuclear accumulation is further consistent with NS5 nuclear accumulation being an important contributing factor to the DENV infectious cycle/virus production.

The first nuclear import inhibitor to inhibit DENV replication was ivermectin, derived using the approach depicted in Figure 15.4a [64, 65]. Briefly, a chemical library was tested in a high throughput format for the ability to inhibit recognition by IMPα/β1 of a viral protein (in this case, the IMPα/β1-recognised integrase protein from human immunodeficiency virus-1 – HIV-1). As shown in Fig. 15.4a, a counterscreen to identify inhibitors interfering with the screening readout reaction (ALPHAScreen – see [62, 64]), followed by a crossscreen to identify inhibitors that are specific to integrase (by testing for the ability to inhibit binding of IMPα/β1 to another viral protein – in this case, simian virus SV40 large tumor antigen – T-ag), enables rapid differentiation of hit compounds which are either specific to the viral protein in question (see [14, 64]), or "broad spectrum" inhibitors that likely target the host IMPs. Ivermectin was identified in such a screen, and subsequently shown to be able to inhibit a range of viruses that rely on IMPα/β1 for nuclear import of a particular protein that is critical for the infectious cycle; these include DENV1–4 [56, 65], and ZIKV (unpublished). More recently, we were successful in identifying a distinct IMPα interacting small molecule from an analogous screen where DENV2 NS5-IMPα/β1 binding was the target (manuscript in preparation); again, the inhibitor ("GSP") inhibited

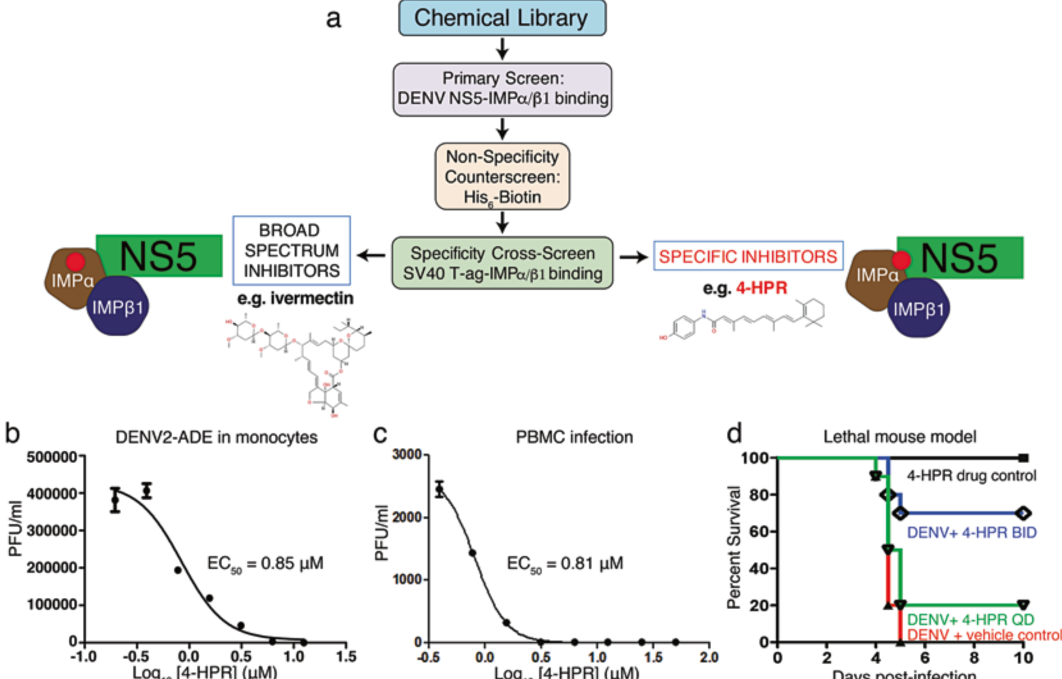


Fig. 15.4 4-HPR as a specific inhibitor of DENV infection. (**a**) The high-throughput screening methodology used to identify inhibitors targeting the host-pathogen interface, including counter- and cross-screening steps. Screening for inhibitors of DENV2 NS5-IMPα/β1 is an example, leading to 4-HPR as potent specific inhibitor (and ivermectin as a broad spectrum inhibitor targeting IMPα). (**b**, **c**) Efficacy of 4-HPR in models of antibody-dependent enhanced (ADE) DENV infection using the THP-1 monocyte cell line and peripheral blood mononuclear cells (PBMCs), respectively. (**d**) 4-HPR protects against lethal ADE-infection in Sv/129 mice (see [14]). QD, daily administration; BID, twice daily administration

infection by DENV1–4. The results both implicate the key role of NS5 nucleocytoplasmic trafficking in the DENV infectious cycle, and confirm that nuclear import of NS5 is a viable target for the development of antivirals.

15.3 Specific Antivirals for DENV and Related Viruses

15.3.1 N-(4-Hydroxyphenyl) Retinamide (4-HPR), a Specific Inhibitor for DENV Targeting the Host-Pathogen Interface

An important step forward in developing new strategies to identifying antivirals has been to target the host-virus interface. As outlined above (Sect. 15.2.3.3; see Fig. 15.4a), screening for specific inhibitors can be fast-tracked using this streamlined screen-counter screen-cross screen approach, used for the first time to identify a specific inhibitor of HIV-1 integrase nuclear import for the first time [64], and subsequently shown to inhibit HIV-1 infection [65]. As indicated, broad spectrum inhibitors that target IMPs and have strong activity as antivirals (such as ivermectin) can be easily identified ([20, 38, 56, 65]; manuscript in preparation), but specific inhibitors that truly target the host-virus interface (see Fig. 15.4a) are highly desirable, to avoid the potentially cytotoxic effects of host-targeted inhibitors such as ivermectin and LMB/Karyopharm EXP1 inhibitors. Agents specifically targeting host enzymes/properties such as celgosovir (host α-glucosidase I inhibitor) and lovastatin (a lipid lowering statin which improves endothelial function) have thus far have proven

ineffective in clinical trials as anti-DENV agents [37, 66], further underlining the low likelihood of success in treating DENV infection with host–acting agents. Strikingly, identifying inhibitors that target the host-pathogen interface, as opposed to the viral component specifically ensures a reduced selective pressure for mutated derivatives of viral pathogens that can circumvent the antiviral agent (e.g. see [39]); as long as the host-pathogen interface that is targeted is absolutely essential for efficient infection, the selection to maintain the integrity of the interface is likely to be strong.

Fraser et al. [14] succeeded in applying the strategy outlined in Figure 15.4a to the NS5-IMPα/β1 interface, identifying N-(4-hydroxyphenyl) retinamide (4-HPR) as a specific inhibitor for DENV. 4-HPR was shown to inhibit various forms of DENV disease, including DENV1–4 and DENV-ADE infection, in various cell models, human peripheral blood mononucleocyte culture (PBMCs), which represent a reasonable model of human infection, and in a lethal animal model [14]. 4-HPR had activity both as a prophylactic, providing some protection when added before infection, as well as a treatment agent, having efficacy even when added up to 24 hours post-infection ([14]; not shown). Finally, 4-HPR was also efficacious in protecting against WNV [14] and ZIKV (unpublished). Excitingly, 4-HPR has an established safety profile, having been used extensively in an oral formulation in humans for various forms of cancer in Phase I-III trials, including oral administration of high doses to children for long periods [8, 19, 42, 53, 59, 60]. Using tablet or lipid-based granular formulations at a dose of 1700 mg/m^2/day, plasma levels as high as 21 μM have been achieved indicating that high levels of 4-HPR can be maintained for long periods in humans without toxic indications, thus meaning that clinically-effective concentrations of 4-HPR are likely to be realistically achieved to combat DENV infection in humans. Clearly, 4-HPR is a very exciting prospect for further development as an anti-DENV agent.

15.3.2 Future Antiviral Development; New Targets and Host-Pathogen Interfaces

As indicated in Sect. 15.3.1, 4-HPR is an exciting prospect for combating DENV infection, with the potential to combat WNV and ZIKV. New derivatives of 4-HPR generated by medicinal chemistry indicate potential improvements in structure-activity relationship testing (unpublished) and suggest that future medicinal chemistry approaches may well yield an even better, potentially more stable/longer-acting antiviral, with more efficacious prophylactic properties, that will of course need testing in clinical trials for safety and efficacy. New screens along the lines of the strategy illustrated in Figure 15.4a could be conducted for other host-pathogen interfaces relevant to DENV such as the NS5:EXP1 interface; even though LMB increases rather than reduces virus production, it is clear that preventing NS5 nuclear export function leads to lethality [55], implying that this could still be a good target, e.g. for agents that stabilize the interaction. Along similar lines, enhancing the binding of host proteins that can act to inhibit nuclear import (negative regulators of nuclear import or NRNIs, such as BRCA1-binding protein 2 – BRAP2 – in the case of SV40 large T-antigen and cytomegalovirus ppUL44 processivity factor – [17]) could be a novel approach, although it has not yet been formally established that NS5 is a target of NRNIs such as BRAP2.

Since phosphorylation of NS5 is known to enhance NS5 nuclear accumulation strongly [28], agents that inhibit specific phosphorylation of NS5 (or enhance phosphatase activity specifically directed at key sites in NS5) could have antiviral effects (manuscript in preparation). Various studies have used inhibitors to limit DENV infection (e.g. [1]). Noppakunmongkolchai et al. [48] used protein kinase C (PKC) inhibitors/activators to monitor alterations to NS5 RdRp phosphorylation, with PKC concluded to be an antiviral agent; phosphorylation of NS5 by

PKC seemed to reduce DENV infection. How exactly these results relate to the observation of nuclear (non-replication complex active) NS5 being hyperphosphorylated [28] is unclear, but certainly of interest for future study. Agents that target host kinases (or phosphatases) directly are likely to lead to cytotoxicity, so using these as therapeutics in the case of flavivirus may not be advisable, but the idea of finding specific inhibitors that can mask particular key phosphorylation sites on NS5 could well be an exciting possibility. A screen of the type shown in Figure 15.4a for compounds targeting recognition of NS5 by a particular kinase at a key site for nuclear targeting could well prove viable as a means to identify new agents with antiviral activity that target the host-NS5 nuclear localisation interface.

Finally, a target that until now seems to have attracted little attention is to try to target the interactions of NS5 with its viral binding partners. In particular, NS3-NS5 binding, clearly essential to DENV replication, would appear to be a virus-virus interface well worthy of high-throughput screening approaches; as for the host-pathogen interface, selection for resistance to inhibitors of NS3-NS5 binding would be unlikely, since the NS3-NS5 interface is so critical to viral replication.

15.4 Conclusion

Although nuclear targeting by DENV NS5 was reported over 22 years ago, and its molecular basis in terms of the host factors responsible 10 years ago, it has only been the last 6 years that have seen this information used to identify inhibitors of the process that have antiviral action. It is to be hoped that this work progresses towards efficacious therapeutics in the clinic, with the novel strategies used to target NS5 nuclear trafficking able to be applied to develop antivirals to tackle the other viruses of global medical significance that have no vaccine or antiviral agent available. It seems likely that agents found to be efficacious towards DENV will be easily transferrable to ZIKV, WNV and related viruses; the extent to which the agents themselves will be transferable to other more distantly related viruses cannot be estimated at this stage, but there seems every reason to believe that nucleocytoplasmic targeting of viral proteins will prove a viable target in almost every case. With a considered focus on strategies to target the host-virus interface, the coming years should prove a fruitful and exciting time in the antiviral field.

Acknowledgements The authors acknowledge the many collaborators over a number of years, with special thanks to Prof. S. G. Vasudevan (Duke NUS) and Prof. J. Forwood (Charles Sturt University) for very important contributions spanning almost 18 years of research. We also acknowledge the support of the National Health and Medical Research Council, Australia for research funding (APP1059137) and senior principal research fellowship (to DAJ; APP1103050).

Discussion of Chapter 15 at *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. David Jans.

Katja Fink: So at least for the mouse model studies with 4-HPR, was that prophylactic treatment or post-infection?

David Jans: It was co-treatment.

Katja Fink: So the next step will be to see whether it works post-infection.

David Jans: Yes, we still need to do all those experiments in mice.

Laura Rivino: So just a thought: If you block NS5 entry in the nucleus, you will have accumulation in the cytoplasm, right? And also, there will be rapid degradation. So would this lead to increased presentation of NS5 peptides and could this lead to excessive T-cell activation? NS5 is one of the main targets of T-cells, but you may not see this in the AG129 mouse model, because it is more impaired in T-cell responses.

David Jans: Yes, we can speculate about that. The way I think about it is at least in the first line, that you will not have the effects on T-cell activation, and that the antiviral response will be much more robust.

Siew Pheng Lim: Does the 4-HPR affect the polymerase activity?

David Jans: No.

Siew Pheng Lim: Have you tried to crystallize this compound with importin-alpha?

David Jans: We have tried that, but failed. It does not crystallize and I don't think it binds 4-HPR. I suppose, it may seem counter-intuitive, but it looks like most of the 4-HPR binding is on NS5 and of course we would like to get a co-crystal of this.

Félix Rey: I am not a specialist in this localization-by-fluorescence assay. So I was wondering what would you expect if NS5 was localized at the outer nuclear membrane, in invaginations where there is replication.

David Jans: So you mean the nuclear envelope?

Félix Rey: Yes, the outer nuclear membrane which is continuous between the ER and the nucleus. If you have invaginations containing replication complexes all around the nuclear membrane, what would you expect to see by fluorescence?

David Jans: You saw the images that we get in an infected cell and I do not see very much fluorescence. But we would be able to see differences for sure depending on how much proteins is there, I suppose. So we are limited always by the amount of protein in the infected cell. If we use the GFP protein, then we can visualize it much better, may be because of the dynamics etc.

Félix Rey: I would expect it to be localized in the cytoplasm, right, where NS5 is supposed to be located for replication.

David Jans: You saw the pictures: There are huge amounts of NS5 in the nucleus.

Félix Rey: You mean in the nucleus and not in the nuclear membrane?

David Jans: Correct. And in the nucleolus as well.

Aruna Sampath: So this is focused on NS5. I just want to make a comment that in one of the screens where you look at the importance of alpha/beta inhibitor, there is potential for broad-spectrum activity. The exportins are actually more prominent, there are molecules with broad-spectrum activity. There are molecules that are actually in clinical trials right now.

David Jans: We are working on them. They certainly seem to work for flu.

Subhash Vasudevan: David, one comment that has to be made is that, as you pointed out in your response to Siew Pheng's question, 4-HPR does not affect RdRp activity. There is still a lot to be learned about how 4-HPR works. One possible scenario is that if you replace NS5 with a host protein that is required in the replication process and that is probably shuttling in and out of the nucleus, you will get the same antiviral response. Two papers have been published for Zika recently, where they picked up the molecule that you discovered, ivermectin, through cell-based infection assays. I guess it is the same probably for most of the flaviviruses – they require this critical unknown host factor that is cycling in and out the nucleus.

David Jans: I don't think we need to go there. These are repurposed drugs, so 4-HPR has been used before for other indications and Ivermectin is the same. But clearly, host factors are required in replication and there is no question about that. Is it that the key thing in the replication complex that is being blocked or is it something else? I think we can speculate on it, but I don't think there is any evidence for it. What is clear is that 4-HPR binds NS5 directly.

References

1. Anwar A, Hosoya T, Leong KM, Onogi H, Okuno Y, Hiramatsu T, Koyama H, Suzuki M, Hagiwara M, Garcia-Blanco MA (2011) The kinase inhibitor SFV785 dislocates dengue virus envelope protein from the replication complex and blocks virus assembly. PLoS One 6:23246
2. Bartenschlager R, Miller S (2008) Molecular aspects of Dengue virus replication. Future Microbiol 3:155–165

3. Bartholomeusz A, Thompson P (1999) Flaviviridae polymerase and RNA replication. J Viral Hepat 6:261–270
4. Brooks AJ et al (2002) The interdomain region of dengue NS5 protein that binds to the viral helicase NS3 contains independently functional importin beta 1 and importin alpha/beta-recognized nuclear localization signals. J Biol Chem 277:36399–36407
5. Buckley A, Gaidamovich S, Turchinskaya A, Gould EA (1992) Monoclonal antibodies identify the NS5 yellow fever virus non-structural protein in the nuclei of infected cells. J Gen Virol 73:1125–1130
6. Caly L, Li H-M, Jans DA (2016) Host factors modulating RSV infection; use of small interfering RNAs to probe functional importance. Methods Mol Biol 1442:93–118
7. Capeding MR, Tran NH, Hadinegoro SRS, Ismail HIHM, Chotpitayasunondh T, Chua MN, Luong CQ, Rusmil K, Wirawan DN, Nallusamy R, Pitisuttithum P (2014) Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. Lancet 384:1358–1365
8. Costa A, Malone W, Perloff M, Buranelli F, Campa T, Dossena G, Magni A, Pizzichetta M, Andreoli C, Del Vecchio M, Formelli F (1989) Tolerability of the synthetic retinoid fenretinide (HPR). Eur J Cancer Clin Oncol 25:805–808
9. Coudeville L, Baurin N, Vergu E (2016) Estimation of parameters related to vaccine efficacy and dengue transmission from two large phase III studies. Vaccine 34:6417–6425
10. Den Boon JA, Diaz A, Ahlquist P (2010) Cytoplasmic viral replication complexes. Cell Host Microbe 8:77–85
11. Dow G, Mora E (2012) The maximum potential market for dengue drugs V 1.0. Antivir Res 96:203–212
12. Edward Z, Takegami T (1993) Localization and functions of Japanese encephalitis virus nonstructural proteins NS3 and NS5 for viral RNA synthesis in the infected cells. Microbiol Immunol 37:239–243
13. Forwood JK, Brooks A, Briggs LJ, Xiao C-Y, Jans DA, Vasudevan SG (1999) The 37 amino acid interdomain of dengue virus NS5 RNA polymerase contains a functional NLS and inhibitory CK2 site. Biochem Biophys Res Commun 257:731–737
14. Fraser JE, Watanabe S, Wang C, Chan KK, Maher B, Lopez-Denman A, Hick C, Wagstaff KM, Sexton P, Mackenzie J, Vasudevan SG, Jans DA (2014) A nuclear transport inhibitor that modulates the UPR and provides in vivo protection against lethal Dengue virus infection. J Infect Dis 210:1780–1791
15. Fraser JE, Rawlinson SM, Heaton S, Jans DA (2016) Dynamic nucleolar targeting of dengue virus polymerase NS5 in response to extracellular pH. J Virol 90:5797–5807
16. Fulcher A, Jans DA (2011) Regulation of nucleocytoplasmic trafficking of viral proteins; an integral role in pathogenesis? Biochim Biophys Acta Mol Cell Res 1813:2176–2190
17. Fulcher AF, Roth DM, Fatima S, Alvisi G, Jans DA (2010) The BRCA-1 binding protein BRAP2 is a novel, negative regulator of nuclear import of viral proteins, dependent on phosphorylation flanking the nuclear localization signal. Fed Am Soc Exp Biol J 24:1454–1466
18. Fulcher AF, Sivakumaran H, Jin H, Rawle DJ, Harrich D, Jans DA (2016) The protein arginine methyltransferase PRMT6 inhibits HIV-1 Tat nucleolar retention. Biochim Biophys Acta, Mol Cell Res 1863:254–262
19. Garaventa A, Luksch R, Piccolo MSL, Cavadini E, Montaldo PG, Pizzitola MR, Boni L, Ponzoni M, Decensi A, De Bernardi B, Bellani FF (2003) Phase I trial and pharmacokinetics of fenretinide in children with neuroblastoma. Clin Cancer Res 9:2032–2039
20. Götz V, Magar L, Dornfeld D, Giese S, Pohlmann A, Höper D, Kong B-W, Jans DA, Beer M, Haller O, Schwemmle M (2016) Influenza A viruses escape from MxA restriction at the expense of efficient nuclear vRNP import. Sci Rep 6:25428
21. Gubler DJ (2002) The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res 33:330–342
22. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, Hunsperger E, Kroeger A, Margolis HS, Martínez E, Nathan MB (2010) Dengue: a continuing global threat. Nat Rev Microbiol 8:S7–16
23. Hanley KA, Lee JJ, Blaney JE Jr, Murphy BR, Whitehead SS (2002) Paired charge-to-alanine mutagenesis of dengue virus type 4 NS5 generates mutants with temperature-sensitive, host range, and mouse attenuation phenotypes. J Virol 76:525–531
24. Hannemann H, Sung PY, Chiu HC, Yousuf A, Bird J, Lim SP, Davidson AD (2013) Serotype-specific differences in dengue virus non-structural protein 5 nuclear localization. J Biol Chem 288:22621–22635
25. Helt A-M, Harris E (2005) S-phase-dependent enhancement of dengue virus 2 replication in mosquito cells, but not in human cells. J Virol 79:13218–13230
26. Hu M-J, Li H-M, Bogoyevitch MA, Jans DA (2017) Mitochondrial protein p32/HAPB1/gc1qr/c1qbp is required for efficient respiratory syncytial virus production. Biochim Biophys Res Commun 489(4):460–465
27. Kannan RP, Hensley LL, Evers LE, Lemon SM, McGivern DR (2011) Hepatitis C virus infection causes cell cycle arrest at the level of initiation of mitosis. J Virol 85:7989–8001
28. Kapoor M, Zhang L, Ramachandra M, Kusukawa J, Ebner KE, Padmanabhan R (1995) Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. J Biol Chem 270:19100–19106
29. Klema VJ, Ye M, Hindupur A, Teramoto T, Gottipati K, Padmanabhan R, Choi KH (2016) Dengue virus nonstructural protein 5 (NS5) assembles into a dimer with a unique methyltransferase and polymerase interface. PLoS Pathog 12:1005451

30. Krishnan MN, Ng A, Sukumaran B, Gilfoy FD, Uchil PD, Sultana H, Brass AL, Adametz R, Tsui M, Qian F, Montgomery RR (2008) RNA interference screen for human genes associated with West Nile virus infection. Nature 455:242–245
31. Kumar A, Bühler S, Selisko B, Davidson A, Mulder K, Canard B, Miller S, Bartenschlager R (2013) Nuclear localization of dengue virus nonstructural protein 5 does not strictly correlate with efficient viral RNA replication and inhibition of type I interferon signaling. J Virol 87:4545–4557
32. Kuusisto H, Jans DA (2015) Hyper-dependence of breast cancer cell types on the nuclear transporter Importin β1. Biochim Biophys Acta, Mol Cell Res 1853:1870–1878
33. Jans DA, Xiao CY, Lam MHC (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? BioEssays 22:532–544
34. Johansson M, Brooks A, Jans DA, Vasudevan SG (2001) A small region of Dengue Virus encoded NS3 confers interaction with both the nuclear transport receptor importin-β and the viral RNA Dependent RNA Polymerase NS5. J Gen Virol 82:735–745
35. Lindenbach BD, Rice CM (2001) Flaviviridae: the viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Strauss SE (eds) Fields virology, vol 1. Lippincott Williams & Willkins, Philadelphia, pp 991–1041
36. Lindenbach BD, Rice CM (2003) Molecular biology of flaviviruses. Adv Virus Res 59:23–61
37. Low JG, Sung C, Wijaya L, Wei Y, Rathore APS, Watanabe S, Tan BH, Toh L, Chua LT, Hou Y, Chow A, Howe S, Chan WK, Tan KH, Chung JS, Cherng BP, Lye DC, Tambayah PA, Ng LC, Connolly J, Hibberd ML, Leo YS, Cheung YB, Ooi EE, Vasudevan SG (2014) Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): a phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial. Lancet Infect Dis 14:706–715
38. Lundberg L, Pinkham C, Baer A, Narayanan A, Wagstaff KM, Jans DA, Kehn-Hall K (2013) Nuclear import and export inhibitors Alter capsid protein distribution in mammalian cells and reduce Venezuelan equine encephalitis virus replication. Antivir Res 100:662–672
39. Lundberg L, Pinkham C, de la Fuente C, Benedict A, Shafagati N, Wagstaff KM, Jans DA, Tamir S, Kehn-Hall K (2016) Selective inhibitor of nuclear export (SINE) compounds Alter new world alphavirus capsid localization and reduce viral replication in mammalian cells. PLoS Negl Trop Dis 10(11):e0005122. https://doi.org/10.1371/journal.pntd.0005122
40. Mackenzie JM, Jones MK, Westaway EG (1997) Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. J Virol 73:9555–9567
41. Marceau CD, Puschnik AS, Majzoub K, Ooi YS, Brewer SM, Fuchs G, Swaminathan K, Mata MA, Elias JE, Sarnow P, Carette JE (2016) Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. Nature 535:159–163
42. Maurer BJ, Kang MH, Villablanca JG, Janeba J, Groshen S, Matthay KK, Sondel PM, Maris JM, Jackson HA, Goodarzian F, Shimada H (2013) Phase I trial of fenretinide delivered orally in a novel organized lipid complex in patients with relapsed/refractory neuroblastoma: a report from the New Approaches to Neuroblastoma Therapy (NANT) consortium. Pediatr Blood Cancer 60:1801–1808
43. Medin CL, Fitzgerald KA, Rothman AL (2005) Dengue virus nonstructural protein NS5 induces interleukin-8 transcription and secretion. J Virol 79:11053–11061
44. Mendonca J, Sharma A, Kim H-S, Hammers H, Meeker A, De Marzo A, Carducci M, Kauffman M, Shacham S, Kachhap S (2014) Selective inhibitors of nuclear export (SINE) as novel therapeutics for prostate cancer. Oncotarget 5:6102–6112
45. Miura K, Yoshinobu K, Imaizumi T, Haruna K, Miyamoto Y, Yoneda Y, Nakagata N, Araki M, Miyakawa T, Yamamura K, Araki K (2006) Impaired expression of importin/karyopherin beta1 leads to post-implantation lethality. Biochem Biophys Res Commun 341:132–138
46. Murray CL, Jones CT, Rice CM (2008) Architects of assembly: roles of Flaviviridae non-structural proteins in virion morphogenesis. Nat Rev Microbiol 6:699–708
47. Nigg EA (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature 386:779–787
48. Noppakunmongkolchai W, Poyomtip T, Jittawuttipoka T, Luplertlop N, Sakuntabhai A, Chimnaronk S, Jirawatnotai S, Tohtong R (2016) Inhibition of protein kinase C promotes dengue virus replication. Virol J 13:35
49. Pemberton LF, Paschal BM (2005) Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic 6:187–198
50. Poon IK, Jans DA (2005) Regulation of nuclear transport: central role in development and transformation? Traffic 6:173–186
51. Pryor MJ, Butcher RE, Rawlinson SM, Barton CL, Wen D, Vasudevan S, Wright PJ, Jans DA, Davidson AD (2007) Nuclear localization of dengue virus nonstructural protein 5 through its importin alpha/beta-recognized nuclear localization sequences is integral to viral infection. Traffic 8:795–807
52. Pryor MJ, Rawlinson SM, Wright PJ, Jans DA (2006) CRM1 dependent nuclear export of dengue virus type-2 NS5. In: Bock G, Goode J (eds) New treatment strategies for dengue and other Flaviviral diseases. Wiley, Chichester, pp 149–160
53. Rao RD, Cobleigh MA, Gray R, Graham ML, Norton L, Martino S, Budd GT, Ingle JN, Wood WC (2011) Phase III double-blind, placebo-controlled, prospective randomized trial of adjuvant tamoxifen vs. tamoxifen and fenretinide in postmenopausal women with positive receptors (EB193): an intergroup trial

coordinated by the Eastern Cooperative Oncology Group. Med Oncol 28:39–47

54. Rawlinson SM, Pryor MJ, Wright PJ, Jans DA (2006) Dengue virus RNA polymerase NS5: a potential therapeutic target? Curr Drug Targets 7:1623–1638
55. Rawlinson SM, Pryor MJ, Wright PJ, Jans DA (2009) CRM1-mediated nuclear export of dengue virus RNA polymerase NS5 modulates interleukin-8 induction and virus production. J Biol Chem 284:15589–15597
56. Tay MYF, Fraser JE, Chan KK, Moreland NJ, Rathore AP, Wang C, Vasudevan SG, Jans DA (2013) Nuclear localization of dengue virus (DENV) 1-4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin. Antivir Res 99:301–306
57. Tay MYF, Smith K, Ng IHW, Chan KWK, Zhao Y, Ooi EE, Lescar J, Luo D, Jans DA, Forwood JK, Vasudevan SG (2016) The C-terminal 18 amino acid region of dengue virus NS5 regulates its subcellular localization and contains a conserved arginine residue essential for infectious virus production. PLoS Pathog 12:1005886
58. Uchil PD, Satchidanandam V (2003) Architecture of the flaviviral replication complex. Protease, nuclease, and detergents reveal encasement within double-layered membrane compartments. J Biol Chem 278:24388–24398
59. Villablanca JG, Krailo MD, Ames MM, Reid JM, Reaman GH, Reynolds CP (2006) Phase I trial of oral fenretinide in children with high-risk solid tumors: a report from the Children's Oncology Group (CCG 09709). J Clin Oncol 24:3423–3430
60. Villablanca JG, London WB, Naranjo A, McGrady P, Ames MM, Reid JM, McGovern RM, Buhrow SA, Jackson H, Stranzinger E, Kitchen BJ (2011) Phase II study of oral capsular 4-hydroxyphenylretinamide (4-HPR/fenretinide) in pediatric patients with refractory or recurrent neuroblastoma: a report from the Children's Oncology Group. Clin Cancer Res 17:6858–6866
61. Villar L, Dayan GH, Arredondo-García JL, Rivera DM, Cunha R, Deseda C, Reynales H, Costa MS, Morales-Ramírez JO, Carrasquilla G, Rey LC (2015) Efficacy of a tetravalent dengue vaccine in children in Latin America. N Engl J Med 372:113–123
62. Wagstaff KM, Jans DA (2006) Intramolecular masking of nuclear localization signals: analysis of importin binding using a novel AlphaScreen-based method. Anal Biochem 348:49–56
63. Wagstaff KM, Jans DA (2009) Importins and beyond: non-conventional nuclear transport mechanisms. Traffic 10:1188–1198
64. Wagstaff KM, Rawlinson SM, Hearps AC, Jans DA (2011) An AlphaScreen(R)-based assay for high-throughput screening for specific inhibitors of nuclear import. J Biomol Screen 16:192–200
65. Wagstaff KM, Sivakumaran H, Heaton SM, Harrich D, Jans DA (2012) Ivermectin is a specific inhibitor of importin alpha/beta-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. Biochem J 443:851–856
66. Whitehorn J, Nguyen CVV, Khanh LP, Kien TH, Quyen TH, Tran NTT, Hang NT, Truong NT, Tai LTH, Huong NTC, Nhon VT, Tram TV, Farrar J, Wobers M, Simmons CP, Wills B (2016) Lovastatin for the treatment of adult patients with dengue: a randomized, double-blind, placebo-controlled trial. Clin Infect Dis 62:468–476
67. World Health Organisation (2009) Dengue guidelines for diagnosis, treatment, prevention and control: new edition. World Health Organization, Geneva
68. Zhang R, Miner JJ, Gorman MJ, Rausch K, Ramage H, White JP, Zuiani A, Zhang P, Fernandez E, Zhang Q, Dowd KA (2016) A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. Nature 535:164–168
69. Zhao Y, Soh TS, Zheng J, Chan KWK, Phoo WW, Lee CC, Tay MY, Swaminathan K, Cornvik TC, Lim SP, Shi PY (2015) A crystal structure of the dengue virus NS5 protein reveals a novel inter-domain interface essential for protein flexibility and virus replication. PLoS Pathog 11:1004682
70. Zou G, Chen YL, Dong H, Lim CC, Yap LJ, Yau YH, Shochat SG, Lescar J, Shi PY (2011) Functional analysis of two cavities in flavivirus NS5 polymerase. J Biol Chem 286:14362–14372

16 Animal Models for Dengue and Zika Vaccine Development

Eduardo Alves dos Santos and Katja Fink

Abstract

The current status of animal models in the study of dengue and Zika are covered in this review. Mouse models deficient in IFN signaling are used to overcome the natural resistance of mice to non-encephalitic flaviviruses. Conditional IFNAR mice and non-human primates (NHP) are useful immuno-competent models. Sterile immunity after dengue vaccination is not observed in NHPs. Placental and fetal development in NHPs is similar to humans, facilitating studies on infection-mediated fetal impairment.

Keywords

Dengue animal models · Zika non-human primate model · Conditional knockout IFNAR mouse model · Non-IFN-receptor-based immuno-compromised mouse models · Humanized mice for dengue and Zika vaccine testing

E. Alves dos Santos · K. Fink (✉)
Singapore Immunology Network, Agency for Science, Technology and Research A*STAR, Singapore, Singapore
e-mail: katja_fink@immunol.a-star.edu.sg

16.1 Introduction

Flaviviruses are the cause of major arthropod-transmitted diseases that affect millions of people globally each year. Clinically most relevant are dengue virus (DENV), yellow fever virus (YFV), west nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV) and, more recently, Zika virus. An efficacious vaccine is available for yellow fever. However, since yellow fever vaccine YFV-17D is a live-attenuated virus it is not suitable for children, the elderly and immuno-compromised. The disease is currently re-emerging in Africa, a situation that has led to renewed efforts in vaccine development for YFV [132]. For JEV and TBEV at least two efficacious vaccines are available [35, 137]. A WNV vaccine is licensed for veterinary use only, while candidates for humans are in phase I or II clinical trials [3]. Major gaps remain for DENV and Zika virus. While Sanofi-Pasteur's Dengvaxia® is available for licensing since 2016 the vaccine is only suitable for highly endemic countries due to its low efficacy in sero-negative individuals [43, 56]. The vaccine is currently also limited to those aged between 9 and 45 years. Vaccines for Zika are in development and the first candidate, a DNA vaccine developed by NIAID, has entered phase I clinical testing [71] (ClinicalTrials.gov, identifier NCT02840487).

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_16

A major hurdle for vaccine development of the non-encephalitic flavivirus DENV has been the absence of relevant animal models. The same caveat now applies to Zika virus. YFV, DENV and Zika virus have evolved to replicate efficiently in mosquitoes and humans, but not in other species. The mechanistic explanation is the capacity of these viruses to specifically block the human interferon response. When mice, and other non-natural hosts are infected, the interferon response is activated effectively and stops virus replication early, resulting in very limited local infection that does not cause disease. In the absence of an animal model it is difficult to predict protection of vaccines, in particular if *in vitro* readouts such as neutralization assays are not established as correlates of protection, and are not standardized across laboratories.

Various animal models for dengue have been tested and they all have advantages and disadvantages. In this review, we will focus on animal models for vaccine development. The models that are established for dengue also seem to be useful for Zika, and a discussion about vaccine development for this recently emerging flavivirus is included.

16.2 Non-human Primate Models

16.2.1 Non-human Primate Models for Dengue Vaccine Testing

Non-human primates (NHP) are natural hosts and reservoirs for YFV, DENV and Zika virus [117, 136]. While YFV causes severe disease in Rhesus macaques [42], DENV infection in NHPs is asymptomatic and NHPs are therefore only of limited use for vaccine development. Nevertheless, viremia can be detected in NHPs and the model has been employed for the testing of all vaccine candidates in advanced development. Green monkeys, rhesus macaques and cynomolgus macaques were most commonly used (reviewed in [113]). All dengue vaccine candidates in phase I-III development have been tested in either Rhesus and/or cynomolgus macaques (Table 16.1). Marmosets have more recently been described as animal models for dengue infection but no advanced vaccine candidates have been tested in marmosets yet [92].

A limitation of NHP models is the availability of reagents to assess the immune response after vaccination. While reagents to assess antibody (Ab) responses are easily available – antibody responses can be quantified by neutralization assay and by ELISA – the detection of specific T cell responses is less established. Few T cell epitopes are described for NHPs [87, 133] and tetramers are not easily available. In fact, surprisingly few NHP studies assessed the T cell response, given that NHPs are immuno-competent and superior to IFN-receptor-deficient mice in this respect. Amongst the reports listed in Table 16.1, Raviprakash et al.' study mentions a low or undetectable T cell response by ELISPOT [106, 108]. Koraka et al. found low and very variable numbers of mostly $CD8^{-}CD3^{+}$ T cell-mediated IFN-γ secretion using bead-sorted cells in ELISPOT [69]. Several groups report ELISA assays to measure IFN-γ, but this approach cannot distinguish whether the measured cytokines were secreted by T cells, NK cells or monocytes (Table 16.1). Other groups might have attempted to measure T cell responses but failed to do so and did not report negative results.

In almost all NHP studies that measured neutralizing antibodies after challenge the animals showed a more or less significant anamnestic antibody response. This means that none of vaccine candidates listed in Table 16.1 induced sterile immunity, defined as complete neutralization of the inoculated virus by antibodies and consequently no priming of pre-existing specific immune memory. In addition to challenges after vaccination, similar anamnestic antibody responses were also observed after infection with wild-type viruses and subsequent homologous challenge [14]. In fact, in a scenario of booster immunization 1 year after the initial vaccination, anamnestic neutralizing antibody responses and the ensuing amplification of specific immune cells can be an advantage [54]. It remains to be established to which extent the generation and maintenance of immune memory can be compared between humans and NHPs. However,

Table 16.1 Reports using NHPs for dengue vaccine testing

Model	Vaccines tested	Readouts	Company/Institute	References
Rhesus macaques[a]	EDIII protein/DNA vaccine	Abs by ELISA, NT50, viremia after challenge	Oregon Health & Science University	[83]
	DEN-80E	NT50 (LiCor-microneutralization) [49] or PRNT50 [31], viremia after challenge	Merck	[31, 49]
	2×EDIII linked to Flagellin	FRNT, Abs by ELISA, viremia after challenge	Merck	[49]
	Purified inactivated DENV (TDEN-PIV) i.m.	MN50, viremia after challenge	GSK	[44]
	Chimeric DENV-JEV SA14-14-2 s.c.	PRNT, viremia after challenge	Beijing Institute of Microbiology and Epidemiology	[75]
	2′O-Methyltransferase mutant DENV	PRNT, viremia after challenge	A*STAR, Novartis Institute of Tropical Diseases	[150]
	Vaxfectin-adjuvanted DNA vaccine	PRNT, T cell ELISPOT after re-stimulation with virus particles, viremia after challenge	Naval Medical Research Center, Silver Spring, MD USA	[102]
	Prime with recE or DNA vaccine, boost with live-attenuated (PDK-passaged)	PRNT, viremia after challenge	Naval Medical Research Center, Silver Spring, MD USA	[122]
	Evaluation of three non-replicating dengue virus type 2 (DENV-2) vaccines: (i) a DNA vaccine containing the prM-E, (ii) a recombinant fusion protein consisting of E protein domain III and maltose-binding protein and (iii) a purified inactivated virus vaccine. DNA vaccine was given i.d., proteins i.m.	Abs by ELISA, PRNT, viremia after challenge	Naval Medical Research Center, Silver Spring, MD USA	[123]
	EDIII fused to P64k from Neisseria Meningitidis	Abs by ELISA, PRNT, viremia after challenge	Pedro Kourí Tropical Medicine Institute and Center for Geneti Engineeering and Biotechnology (CGEB), Cuba	[13]
	prM-E-expressing adenovirus	Abs by ELISA, PRNT, no T cell response could be detected 4 and 8 weeks after second vaccination and all vaccinated animals showed a moderate T cell response when measured 4 weeks after virus challenge	Naval Medical Research Center, Silver Spring, MD USA	[108]
	Chimeric DNA vaccines	Abs by ELISA, PRNT, viremia after challenge	Naval Medical Research Center, Silver Spring, MD USA	[106]

(continued)

Table 16.1 (continued)

Model	Vaccines tested	Readouts	Company/Institute	References
	PDK- and fetal rhesus monkey lung cell-attenuated DENV-1 to 4	PRNT, viremia after challenge	Walter Reed Army Institute of Research WRAIR; same vaccine lot later used in clinical trials	[128]
	17D-D2 yellow fever DENV-2 chimeric virus	PRNT, viremia after challenge	Fiocruz	[47]
	Comparison of various vaccine candidates (PIV, DENV-80E, PDK-passaged live-attenuated virus), all s.c.	PRNT, viremia after challenge; interesting: no sterile immunity for all candidates tested	WRAIR, Hawaii Biotech, GSK	[109]
	Chimeric DENV3/4, DENV-2 and DENV-4 delta30 viruses	Viremia after immunization s.c. and challenge s.c. (for DENV-3/4 only), PRNT	NIH	[15, 16, 57]
	DEN2/4delta 30 chimeric virus	Viremia after immunization s.c., PRNT50	NIH	[139]
	ChimeriVax-DENV1	Attenuation tested after s.c. and i.c. infection	Acambis	[53]
	DENV-1 to 4 passaged in PDK, vaccine lots produced in fetal rhesus monkey diploid cell cultures	Attenuation tested after s.c. immunization, PRNT	WRAIR	[40]
	DENV-2 DNA vaccine	PRNT, viremia after challenge	WRAIR	[105]
	DENV-1 delta30	PRNT, attenuation after immunization s.c.	NIH	[138]
	Chimerivax DENV-1 to -4	Attenuation after immunization, PRNT	Acambis Inc.	[50]
	DEN2mutF with a 3′ mutation that attenuates the virus highly in mosquitoe cells	Attenuation after immunization, PRNT, viremia after challenge	FDA	[82]
	DENV-1 DNA vaccine, i.m. versus i.d.	PRNT, Abs by ELISA, viremia after challenge, PBL stimulation with DENV and IFN-γ ELISA as a readout for T cell activation	Naval Medical Research Center	[107]
	Modified Vaccinia Ankara expressing DENV E protein	PRNT, viremia after challenge	NIAID	[85]
	Chimerivax DENV-2 s.c.	AttenuationPRNT, viremia after challenge	OraVax	[52]
	DENV-2 PIV	PRNT, viremia after challenge	WRAIR	[104]
	Chimeras DEN1/DEN4 and DEN2/DEN4	Attenuation, PRNT, viremia after challenge	NIAID	[20]
	3′mutated DENV-4	Attenuation, PRNT	NIAID	[84]
	baculovirus-dengue type-4 (DEN-4) recombinant-infected cell extracts	PRNT, Viremia after challenge	WRAIR	[41]

(continued)

Table 16.1 (continued)

Model	Vaccines tested	Readouts	Company/Institute	References
	Primary Canine Kidney (PDK)-cell passaged DENV-4	Hemagglutination inhibition antibodies, PRNT, attenuation, viremia after challenge	University of Hawaii	[80]
Cynomolgous Macaques[b]	Tetravalent live-attenuated DENV vaccine on DENV-2 backbone (TDV) s.c. versus i.d. comparison	Peripheral blood transcriptome analysis, MN titers, viremia after challenge	Stanford, Takeda	[126]
	DENV-2 virus-like particles from stably transfected mosquito cells and boost after prime with LAV	PRNT, EDIII ELISA	National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok	[129]
	TDV s.c.	Cellular Immune response: IFN-γ and IL-2 by ELISPOT with DENV-stimulated PBMCs, PRNT, viremia after immunization and after challenge	Inviragen	[94]
	DEN 1 or DEN 2 E proteins fused to the P64k protein from Neisseria meningitidis	PRNT against different genotypes of DENV-1 and -2	Pedro Kourí Tropical Medicine Institute and CGEB	[11]
	ChimeriVax; assess competition between serotypes in tetravalent formulation s.c., injections in the same or two arms, YF naïve or pre-immune	Liquid 50% seroneutralization assay (SN50), viremia after immunization	Sanofi-Pasteur	[54]
	DEN 1 to 4 E proteins fused to the P64k protein from Neisseria meningitidis	Serotype-specific ELISA and HAI titer	Pedro Kourí Tropical Medicine Institute and CGEB	[63]
	DENV-1 P64k protein from Neisseria meningitidis	Serotype-specific ELISA, PRNT, viremia after DENV-1 challenge	Pedro Kourí Tropical Medicine Institute and CGEB	[13]
	Fusion protein of DENV-2 EDIII and P64k protein from Neisseria meningitidis	Anamnestic Abs after challenge by ELISA (IgM and IgG), HAI, PRNT	Pedro Kourí Tropical Medicine Institute and CGEB	[12]
	DENV-1 DNA vaccine priming, DENV-1 prM expressed in Venezuelan equine encephalitis (VEE) virus replicon particles (VRP) for boosting	IgM and IgG by virus particle ELISA, PRNT, T cell ELISPOT after restimulation with purified DENV-1, viremia after challenge	Naval Medical Center	[29]

(continued)

Table 16.1 (continued)

Model	Vaccines tested	Readouts	Company/Institute	References
	PDK-passaged, live-attenuated strains tested in naive and primed monkeys	IgM and IgG ELISA, PRNT, T cell ELISPOT after restimlation with infected, autologous transformed B cells, ELISPOT with magnetically sorted T cells	Erasmus Medical Center, Rotterdam	[69]
	Fusion protein of DENV-2 EDIII and P64k protein from Neisseria meningitidis	IgG by ELISA, PRNT, viremia after challenge	CGEB, Cuba	[58]
	ChimeriVax DENV-1 with one amino acid constitution	Viremia after i.c and s.c. infection, histopathology of brain and spinal cord, PRNT	Acambis Inc.	[53]
	Tetravalent ChimeriVax	Viremia after i.c and s.c. infection, histopathology of brain and spinal cord, PRNT, viremia post challenge	Acambis Inc.	[51]
	DENV-4 recombinant E protein (E4rec) expressed in Pichia pastoris	IgM and IgG ELISA, HAI titer, PRNT, viremia after challenge; Anamnestic Abs after challenge by ELISA (IgM and IgG), HAI, PRNT	Pedro Kourí Tropical Medicine Institute and CGEB,	[55]
	Chimeric DEN2/1, live-attenuated DEN2-PDK53 as backbone	IgM by ELISA, PRNT, viremia after challenge	Mahidol University	[23]
	Live-attenuated DENV-2 and recombinant E protein	PRNT, viremia after challenge	Erasmus Medical Center, Rotterdam	[134]
	DEN-3 PGMK 33 intrathalamically, intraspinally and intramuscularly	PRNT, histopathology	Mahidol University	[5]

i.c. intracranial, *i.d.* intradermal, *i.m.* intramuscular, *s.c.* subcutaneous, *FRNT* focus forming neutralizing titer, *MN50* 50% neutralizing titer measured in a micro-neutralization assay, *PRNT* plaque reduction neutralizing titer, *recE* recombinant E protein

[a]Search-criteria: rhesus macaques AND dengue AND vaccine in Pubmed, 82 hits from 1990 to mid August 2016

[b]Search criteria: cynomolgous macaques AND dengue AND vaccine in Pubmed, 25 hits considering studies from 1990 to mid August 2016

induction of sterile immunity in NHP studies as a criterium for vaccine efficacy seems not realistic since not even natural dengue infection induces sterile immunity in NHPs.

16.2.2 Non-human Primate Models for Zika

The mouse models described for Zika have the same limitations as those for dengue for vaccine development: they are genetically immunocompromised or are rendered immunocompromised temporarily by the administration of anti-IFN-α-receptor (IFNAR) antibodies. Moreover, mouse models only display some of the disease symptoms observed in humans. In contrast to dengue, animal models for Zika should be able to replicate maternofetal infection and the potential virus-induced fetal malformations. This is very challenging in a mouse model since human and mouse fetal development are substantially different [9]. Important to note in the context of a potential antibody-dependent

Table 16.2 NHP models for Zika infection and vaccine testing

Model	Vaccines tested	Readouts	Company/Institute	References
Rhesus macaques[a]	N/A	Virus RT-PCR in plasma, saliva, urine, vaginal fluid, CSF; increase in proliferating NK and T cells, plasmablast quantification, PRNT	University of Wisconsin-Madison	[39]
	N/A	Virus RT-PCR in serum, urine, saliva, lacrimal fluid and in various organs; histology; IgM and IgG ELISA, PRNT, IFN-g and IL-10 ELISPOT of ex-vivo PMBCs	State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing	[76]
	DNA vaccine VRC5283 and VRC5288	Virus RT-PCR after challenge, ELISA, PRNT	NIH	[37]

Search criteria[a]: animal model AND Zika AND vaccine: 10 hits by end Oct 2016

enhancement is the difference in the transmission of maternal antibodies: while in humans the majority of maternal antibodies is acquired by the fetus via the placenta and only small amounts are absorbed by newborns via breastmilk through intestinal uptake after birth, the transport in rodents (mice and rats) occurs mostly post-natal during lactation [97].

To study maternofetal transmission of virus and a potential impact of infection on fetal development Rhesus macaques are a better model than mice due to their similar physiology with human fetal development [39] (Table 16.2). Dudley et al. report a study in which they infected Rhesus macaques with Asian Zika virus to establish a model for Zika virus pathology. Peak virus loads in plasma and saliva were similar for animals infected with 10^6, 10^5 or 10^4 pfu of virus, yet peak viremia occurred later for lower infection doses. Pregnant macaques were infected and evidence of virus in the developing fetuses was tested by amniocenteses. No virus could be detected by the time of the first publication [39]. Interestingly, however, both pregnant monkeys, showed a consistent viremia up to day 29 and 57 after infection, respectively. In a follow-up report, viral RNA was detected in tissues of four out of four macaques delivered by cesarean section at term, indicating that maternofetal virus transmission seems prevalent [90]. Fetuses grew normally apart from showing a below average head circumference during the last month of pregnancy as assessed by ultrasound [90]. None of the newborn macaques had microcephaly. In humans, prolonged viremia has been described for a pregnant woman whose fetus had a high virus load and brain malformations [38]. It remains to be studied whether this is a general phenomenon observed in a majority of infected pregnant women and whether vaccines should be tested for their efficacy in protecting from prolonged viremia in pregnant non-human primates.

16.3 Rodent Models

16.3.1 Genetically Modified Mice for Dengue Vaccine Testing

16.3.1.1 AG129 Mice

Rodents are not natural host for DENV. While DENV infection has been reported in wild type (WT) mice after virus inoculation through the intracerebral (i.c) [73, 74], intravenous (i.v) [28, 61, 118], intradermal (i.d) [27] and intraperitoneal (i.p) [7, 95, 125] route, those mice failed to show clinical signs observed in humans. The lack of a laboratory animal capable to naturally develop the severe forms of DENV infection not only compromises the understanding of the pathogenicity and the development of therapeutic interventions but also hampers the screening and prediction of vaccines efficacy.

In an attempt to overcome the natural resistance of mice against DENV infection is 11 immune-compromised mouse strains have been investigated (Table 16.3). It is known that DENV can inhibit the IFN signal during human infection.

Table 16.3 DENV infection in immune-compromised and wildtype mice and vaccine testing

Mouse model	Deficiency	DENV & inoculation	Features/outcome	Vaccine testing	References
A/J	Lack the complement component C5	M-A DENV-2 PL046 1×10^8 p.f.u/i.v	Thrombocytopenia; Paraplegia. Survive	N/A	[61]
		M-A DENV-2 PL046 1×10^8 p.f.u/i.v	Virus in CNS, Neurodisease, Leukopenia, Elevate Haematocrit.	N/A	[118]
AG129	Lack of Type I and II IFN receptors (129 background)	M-A DENV-2 16681 1×10^6 p.f.u/i.p	Paralysis; Virus in serum, spleen and brain; Death	PDK-53 (attenuated DENV-2). Protective with 2 doses	[66]
		Non M-A DENV-2 PL046 1×10^8 p.f.u/i.v	Neurodisease; Virus in spleen, liver, lymph node and CNS. Death	N/A	[119]
		M-A DENV-2 D2S10 1×10^7 p.f.u/i.v	Vascular leakage; systemic infection. Death	N/A	[120]
		Non M-A DENV-2 TSV01 Up to 1×10^8 p.f.u/i.p	Splenomegaly. Survived	N/A	[115]
		Non M-A DENV-2 D2Y98P 1×10^7 p.f.u/i.p	Systemic infection, vascular leakage, liver and intestinal damage, lymphopenia. Death	N/A	[130]
		M-A DENV1 Mochizuki 1×10^7 p.f.u/i.p	Death.	Multiple chimera vaccines for DENV1, 3 and 4 on DENV-2 PDK53 backbone. (1 dose). Protective.	[60]
		M-A DENV-2 NGC 1×10^3 p.f.u/i.p	Death	DENV-2/ WN-E1-112 and DENV-2-WN-P1 chimeric virus. (2 doses). Limit protection	[24]
		Non M-A DENV4 TVP-376 1×10^7 p.f.u/i.p	Limited mobility; weight loss; leukopenia; liver and spleen damage. Death	N/A	[111]

(continued)

Table 16.3 (continued)

Mouse model	Deficiency	DENV & inoculation	Features/outcome	Vaccine testing	References
		M-A DENV-2 NGC or DENV1 Mochizuki 1×10^6 p.f.u/i.p	Morbidity. Paralysis. Death	Chimeric DENV1, 2,3 and 4 of (E) and (prM) protein on DENV-2 PDK-53 backbone. 2 doses. Protective.	[22]
		M-A DENV4 703 1.5×10^7 p.f.u/i.p	Morbidity. Weight loss. Death	Live attenuated tetravalent vaccine (TDV on DENV-2 or 4 backbone). 1 dose. Protective.	[46]
		Non M-A DENV-2 D2Y98P 1×10^7 p.f.u/i.p or Non M-A DENV1 05K31226 1×10^6 p.f.u/i.p	Death.	Attenuated DENV1 or 2 lacking 2′-O-Mtase. 1 dose. Protectived.	[149]
		Non M-A DENV-2 S221 5×10^8 GE/i.p	Virus in organs and serum. Death.	UV inactivated DENV-2 S221 in Alum. 2 doses. Not protective.	[145]
		Non M-A DENV3 C0360/94 Up to $1 \times 10^{7.5}$ f.f.u/i.p	Virus in spleen, liver and large intestine; leukopenia, thrombocytopenia, vascular leakage. Death	N/A	[112]
A129	Lack of type I IFN receptor (129 background)	M-A DENV-2 D221 Up to 2×10^{12} GE/i.v	Systemic infection. Death	N/A	[103]
		Non M-A DENV4 TVP-376 1×10^7 p.f.u/i.p	Weight loss; virus in serum. Survived	N/A	[111]
IFNAR$^{-/-}$	Lack of type I IFN receptor (C57BL/6 background)	M-A DENV-2 D221 1×10^7 p.f.u/i.v	Virus in liver, small intestine, bone marrow. Death	N/A	[93]
		M-A DENV-2 D221 Up to 1×10^4 GE/i.v	Acute infection. Virus in spleen and kidney. Survived.	N/A	[141]
		Non M-A DENV-2 D2Y98P 5×10^6 p.f.u/i.p	Weight loss. Systemic infection; virus in brain. Death	DENV-2 EDIII-capsid [131] (3 doses). Non protective.	[151]

(continued)

Table 16.3 (continued)

Mouse model	Deficiency	DENV & inoculation	Features/outcome	Vaccine testing	References
Cardif$^{-/-}$	Lack of Cardif (also known as IPS-1; MAVS or VISA) protein. (129 background)	Non M-A DENV-2 S221 1×10^{12} GE/i.v	Virus in serum, spleen, bone narrow, mesenteric and peripheral lymph nodes. Survived.	N/A	[99]
STAT1$^{-/-}$	Lack of STAT1 protein (129 background)	M-A DENV-2 221 $\cong 2 \times 10^{5}$ p.f.u/i.v	Virus in liver, kidney and small intestine. Survived	N/A	[98]
		Non M-A DENV-2 PL046 1×10^{8} p.f.u/i.v	Temporal Viral load in spleen, liver, lymph node and CNS. Survived.	N/A	[121]
		M-A DENV1 Mochizuki 1×10^{8} p.f.u/i.v			
		Non M-A DENV-2 NGC-N 1×10^{5} p.f.u/i.p	Subcutaneous and intestinal haemorrhage; vascular leakage; paralyses. Death.	N/A	[30]
STAT2$^{-/-}$	Lack of STAT2 protein (129 background)	M-A DENV-2 221 $\cong 2 \times 10^{5}$ p.f.u/i.v	Virus in spleen, kidney, liver and small intestine. Survival	N/A	[98]
STAT1$^{-/-}$2$^{-/-}$	Lack of both STAT1 and STAT 2 proteins (129 background)		High virus load in serum, spleen, kidney, liver and small intestine. Non-neurodisease symptoms. Death	N/A	
STAT1$^{-/-}$/ IFNAR$^{-/-}$	Lack of STAT1 and Type I IFN receptor (129 background)		High virus load in serum, spleen, kidney, liver and small intestine. Non-neurodisease symptoms. Death	N/A	
STAT1$^{-/-}$/ IFNGR$^{-/-}$	Lack of STAT1 and Type II IFN receptor (129 background)		Survived	N/A	
LysM-Cre$^{+/-}$ IFNAR$^{-/-}$	Lack of Type I IFN receptor only in macrophages (129 background)	Non M-A DENV-2 D2Y98P 5×10^{6} p.f.u/i.p	Morbidity with some mortality. Virus in kidney, blood, spleen, liver, brain, lung and inguinal lymph nodes.	DENV-2 EDIII-capsid (3 doses). Limit protection.	[151]
		M-A DENV-2 D2S20 1×10^{6} p.f.u/i.v	Morbidity. Virus in serum, liver, spleen and kidney. Survived	N/A	[100]

(continued)

Table 16.3 (continued)

Mouse model	Deficiency	DENV & inoculation	Features/outcome	Vaccine testing	References
CD11c-Cre$^{+/-}$ IFNAR$^{-/-}$	Lack of Type I IFN receptor only in dendritic cells (129 background)	Non M-A DENV-2 D2Y98P 5×10^6 p.f.u/i.p	Morbidity with some mortality. Virus in kidney, blood, spleen, liver, brain, lung and inguinal lymph nodes.	DENV-2 EDIII-capsid (3 doses). Limit protection.	[151]
CD11c$^{+/-}$LysM^{-} Cre$^{+/-}$ IFNAR$^{-/-}$	Lack of Type I IFN receptor only in macrophages and dendritic cell (129 background)	Non M-A DENV-2 D2Y98P 5×10^6 p.f.u/i.p	Virus in kidney, blood, spleen, liver, brain, lung and inguinal lymph nodes. Death	N/A	

Abbreviations: *M-A* mouse adapted, *N/A* Not applicable, *CNS* Central Nervous System (brain and spinal cord), *p.f.u* plaque-forming unit, *f.f.u* focus-forming unit, *GE* genomic equivalent, *(E)* envelop protein, *(prM)* pre-membrane, *2′-O-Mtase* 2′-O-methyltransferase. Virus inoculation route: *i.p* intraperitoneal and *i.v* intravenous

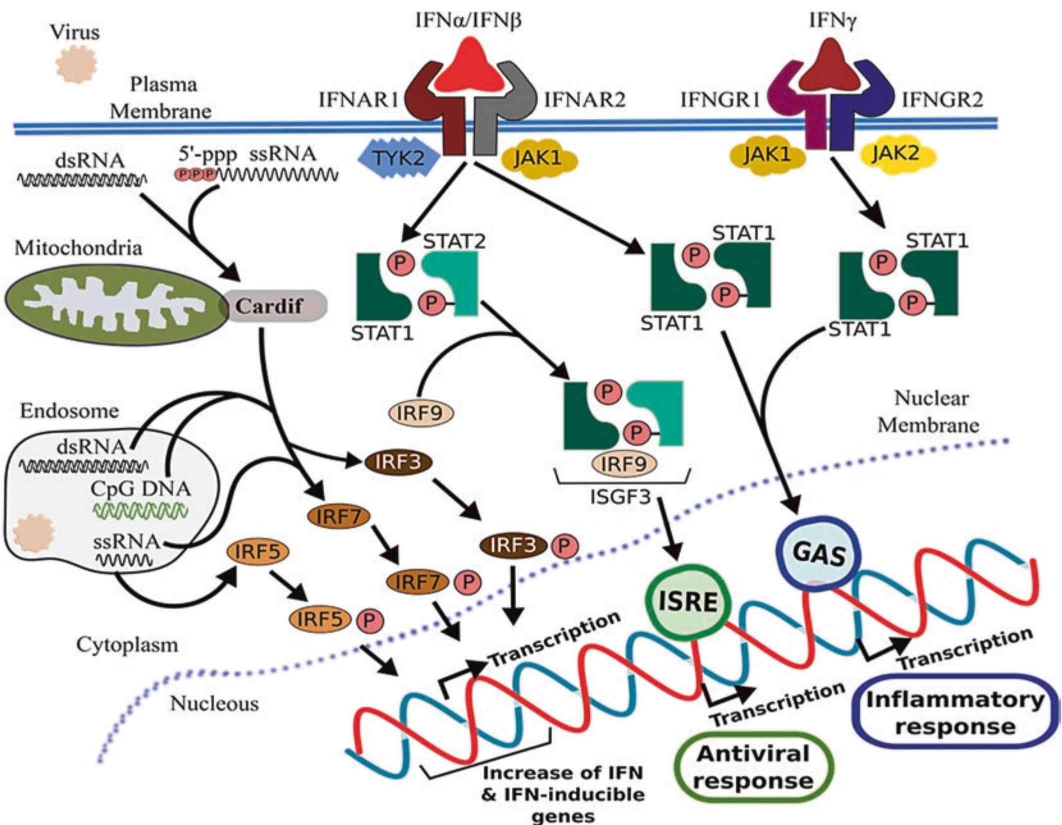


Fig. 16.1 Overview of the canonical type I and II interferon receptor signaling pathways and interferon regulatory factors 3, 5, 7 and 9

However, this inhibition is species-specific and not observed in mice [1, 6, 144]. The activation of the IFN signaling pathway (Fig. 16.1) is an important event for the host inflammatory and antiviral response [62, 101]. Accordingly, mice deficient for the IFN-α/β –receptor (A129 mice) and IFN- α/β and γ-receptors (AG129 mice) on the 129/SV background succumb to

mouse-adapted DENV-strain DENV-2 16681 after i.p challenge regardless of their age [66]. A similar outcome was observed when the i.v route was used to inoculate mouse-adapted DENV-2 PL046 strain [119]. The robust level of DENV replication observed in AG129 mice [66] allows this model to be used for the testing of antiviral drugs [115, 142]. Moreover, depending on the inoculation route and the mouse-adapted DENV-2 strain used, AG129 mice can also present vascular permeability, low platelet count, cytokine storm, increased hematocrit and intestinal hemorrhage [8, 120, 146], which are all signs of severe dengue disease. The use of mouse-adapted DENV-2 strains that are not naturally found in humans for the testing of vaccines can be a controversial issue. AG129 mice are susceptible to DENV-2 clinical isolates injected through the i.p. route, inducing viremia and potentially symptoms similar to the human acute dengue fever with non-lethal outcome [115]. An exception is non-mouse adapted DENV-2 strain D2Y98P, which induces lethal systemic infection in AG129 [130]. As models for the other DENV serotypes, a DENV-3 Thai human isolate C0360/94 [112], non-mouse adapted DENV-4 strains 703-4 [86] and TVP-376 [111] infection were recently successfully established in the AG129 model, resulting in non-neurological lethal disease. The susceptibility of an animal model to all four serotypes of DENV is particularly relevant to prove serotype-specific immunity induced by vaccines and to investigate ADE. AG129 mice, so far, are the most popular animal models for DENV pathogenesis and for the evaluation of therapeutics [26, 147]. However, AG129 is not an ideal model for vaccine studies due their lack of IFN-signal-dependent components of the antiviral immune response. This explains the few published data for DENV vaccine testing in AG129 (Table 16.3): wild-type DENV-2 and chimeric DENV-2/1 vaccines induced humoral responses that protected all mice against subsequent i.p. challenge with DENV-2 NGC or DENV-1 Mochizuki virus [60, 66]; a cDNA vaccine based on non-structural proteins of DENV-2 16681 strain and inactivated DENV failed to protect AG129 despite the generation of antibodies [24, 145], indicating a role for IFN response in DENV vaccine protection. However, live-attenuated chimeric or non-chimeric vaccines were protective in AG129 mice [21, 46, 150].

16.3.1.2 A129 and IFNAR Mice

Contrary to AG129 mice which lack both the Type I and II IFN receptors, A129 (129/Sv background) mice are deficient for IFN Type I receptor. These mice support mouse-adapted DENV-2 replication after i.v. challenge but restrict systemic infection by eliciting a $CD8^+$ T-cell response preventing neurological abnormalities like paralysis [103]. Mice with the same genetic modification on a C57BL/6 background ($IFNAR^{-/-}$) also support mouse-adapted DENV-2 strain S221 (a variant of PL046) replication after i.v. inoculation, yet with non-lethal outcome and, similar to A129, induce a robust $CD8^+$ T-cell response [141]. Non-neuro-pathogenic lethality induced by i.v. inoculation of mouse-adapted DENV-2 strain 220 in $IFNAR^{-/-}$ mice was reported [93], demonstrating the possibility of inducing systemic infection preceding lethality in $IFNAR^{-/-}$ mice. The fact that those models retain an intact IFN-γ signal but are still vulnerable to mouse-adapted DENV infection could be considered an improvement for DENV vaccine mice model over the AG129. However, also the IFN Type 1 receptor has an important role in the host antiviral response (reviewed in [62]). Thus, a global lack of the IFN Type 1 receptor can compromise the evaluation of DENV vaccine efficiency.

Non-IFN-Receptor-Based Immuno-Compromised Models

STAT (Signal Transducer and Activator of Transcription) 1 and STAT2 proteins are downstream components of IFN signaling pathway [77]. $STAT1^{-/-}$ mice infected with DENV-2 NGC strain via i.p. plus i.c. infection exhibited mild paralysis, intestinal hemorrhage and vascular leakage [30], an i.v. challenge with mouse-adapted DENV-2 PL046 resulted in virus replication in multiple tissues in this model [121]. Mouse-adapted DENV-2 S221 injected via the

i.v. route in single deficient mice lacking STAT1$^{-/-}$ or STAT2$^{-/-}$ presented a robust DENV replication but did not succumb to infection while double deficient mice exhibited an early non-neuropathology-related lethality [98]. Caspase Recruitment Domain (CARD) is a protein also known as Cardif, MAVS, VISA or IPS-1 that mediates the early induction of type I IFN in response to RNA virus infection [143]. Cardif-deficient mice present non-lethal systemic infection to DENV-2 S221 after i.v. challenge [99].

Considering the importance of IFNs for vaccine responses, one option for vaccine testing could be different immunocompromised mouse model with a functional IFN signaling. A/J mice, which lack the complement component C5, presented a viremia with lethal outcome when inoculated i.v. with mouse-adapted DENV-2 PL046 [61, 118]. The major disadvantage of this model is the DENV-induced encephalitis as the main cardinal symptom. The involvement of central nervous system (CNS) is a rare condition in human DENV infection [79, 96].

16.3.1.3 Conditional IFNAR Mice

A different approach to overcome the global immunodeficiency presented in mouse models is to selectively delete IFN type 1 receptor in specific individual immune cell types. C57BL/6 mice lacking the IFN type 1 receptor only on CD11c^{+} dendritic cells (CD11c-Cre$^{+/-}$IFNAR$^{-/-}$) or on lysosome M^{+} macrophages and granulocytes (LysM-Cre$^{+/-}$IFNAR$^{-/-}$) were susceptible to non-mouse adapted DENV-2 D2Y98P infection by i.p. route while maintaining global immune competence [151]: a DENV-2 EDIII-capsid subunit vaccine was protective in the conditional models but not in IFNAR mice [81, 131]. Single knock-out conditional IFNAR mice presented systemic infection but were less susceptible than double knock-out CD11c-Cre$^{+/-}$LysM-Cre$^{+/-}$IFNAR$^{-/-}$ mice. LysM-Cre$^{+/-}$ IFNAR$^{-/-}$ also proved to be a suitable model to study ADE using mouse-adapted DENV-2 D2S20 and a non-mouse adapted DENV-3 Thai human isolate through the i.v. route [100]. Conditional mouse models generate a more immuno-competent response compared to global knockout mice, providing a better approach particularly for vaccine testing.

16.4 Genetically Modified Mice as Zika Models

The first attempt to develop a mouse model for Zika virus (ZIKV) infection was conducted in the 1950s. After multiple passages in murine brains, ZIKV induced clinical manifestation in Swiss albino mice resulting in death [33, 34]. The identification of a suitable mouse model to assess vaccine candidates and treatments for ZIKV became urgent after severe complications associated with ZIKV infection were reported in epidemic outbreaks in French Polynesia [25, 91] and Latin America [19, 114, 135]. Following the example of DENV, mice with different deficiencies for IFN signaling have meanwhile been tested to investigate the susceptibility for ZIKV (Table 16.4). Adult AG129 and A129 mice infected with 1×10^5 pfu of the Asian lineage Zika virus strain FSS13025 via the i.p or intradermal (i.d.) route developed viremia and showed weight loss, neurological symptoms and death [110]. A similar outcome was observed when adults A129 were inoculated with 1×10^6 pfu via subcutaneous route with the African lineage MP1751 strain [36]. Lethal models for ZIKV infection were described for AG129 [2, 72] and IFNAR$^{-/-}$ [72] mice below 5 weeks old. The use of young animals with not fully developed immune systems may represent a limitation for vaccine testing. Lethality can be achieved in adult mice with different ZIKV strains in Interferon Regulatory Factor (Irf) 3$^{-/-}$ Irf5$^{-/-}$ Irf7$^{-/-}$ triple knockout mice [72] and AG129 (8 weeks old, i.p. infection with strain H/PF/2013) [2]. Those model are important for the testing of antiviral drugs [148] and to investigate the pathophysiology of ZIKV infection [140].

As at October 2016 only two study reported Zika vaccine testing in mouse models [37, 68]. Meanwhile, several additional studies have been published, notably studies using mouse models to test efficacy. These are a purified inactivated

Table 16.4 Zika virus (ZIKV) infection in immunocompromised mice and vaccine testing

Mouse model	Deficiency	ZIKV/inoculation & mice age	Features/outcome	Vaccination trial	References
AG129	Lack of Type I and II IFN receptors (129 background)	Zika (Asian lineage FSS13025) 1×10^5 p.f.u/i.p or i.d on 3 weeks old mice	Virus in heart, liver, kidney, brain, lung, spleen, muscle and testis. Weight loss, neurologic disease. Death	N/A	[110]
		Zika Dakar strain 41519 1×10^4 p.f.u/s.c on 4 weeks old mice	Weight loss. Morbidity. Death	N/A	[72]
		Zika strain (H/PF/2013) 1×10^5 p.f.u/hind foot or 2×10^5 p.f.u /i.p 3–4 or weeks old mice	Weight loss, lethargy, and neurologic disease. Virus in serum. Death	N/A	[2]
A129	Lack of type I IFN receptor (129 background)	Zika strain MP1751 1×10^6 p.f.u/s.c on 5-6 weeks old mice	Virus in blood, brain, spleen and ovaries. Weight loss. Brain lesions. Death	N/A	[36]
		Zika (Asian lineage FSS13025) 1×10^5 p.f.u/i.p on 3 weeks old mice	Virus in kidney, lung, spleen and muscle. Eventual virus detection on heart, lung, brain and testis. Weight loss, neurologic disease. Death	N/A	[110]
		1×10^5 IFU of FSS13025 or 1×10^6 IFU of PRVABC59 ZIKV, i.p. route	Viremia, survival	10-del, live-attenuated	[116]
		MR 766 and the FSS 13025 ZIKV strains 10^4 PFU s.c.	Viremia, survival	Purified inactivated virus	[127]
IFNAR$^{-/-}$	Lack of type I IFN receptor (C57BL/6 background)	Zika Dakar strains: 41519, 41667 and 41671 1×10^3 p.f.u/s.c on 4 weeks old mice	Weight loss. Neurologic symptoms. Death	N/A	[72]
		1×10^6 plaque-forming units (p.f.u.) of ZIKV PRVABC59 strain by a s.c. route	Viral RNA presence and damage to testes and sperm	DNA vaccine encoding prME	[48]
Irf3$^{-/-}$ Irf5$^{-/-}$ Irf7$^{-/-}$	Lack the Interferon Regulatory Factor 3, 5 and 7 (C57BL/6 background)	Zika strain (H/PF/2013) or (MR 766) 1×10^2 p.f.u/s.c 5-6 weeks old mice	Weight loss; neurologic symptoms. Death	N/A	[72]
C57BL/6	None; wildtype	7 day old mice; Zika strain Dakar 41542 10^5 pfu i.p	Weight loss, death	Adenovirus construct expressing Zika E protein	[68]
C57BL/6 and balb/c	None; wildtype	Adult mice	Antibodies by ELISA and PRNT	DNA vaccine	[37]

Abbreviations: *N/A* Not applicable, *p.f.u* plaque-forming unit, *f.f.u* focus-forming unit. Virus inoculation route, *i.p* intraperitoneal and *s.c* subcutaneous

virus [127], a DNA vaccine [48] and a live-attenuated Zika vaccine [116].

16.4.1 Humanized Mice for Dengue and Zika Vaccine Testing

The concept of using severe combined immunodeficient (SCID) mice capable to sustain human engraftments that allow DENV infection has been extensively reported [4, 10, 32, 45, 64, 65, 70, 78, 88, 89, 124]. These models are known as "humanized" mice and the nature of their immunodeficiency and the type of human cell transplanted has provided a variety of models displaying different degrees of DENV pathology. Nevertheless, they all failed to induce symptoms associated with severe dengue such as hemorrhage and vascular leakage. Humanized mice have been used for dengue antiviral drug testing [45], and have revealed interesting aspects of the humoral response like antibodies class switching after DENV infection [45, 70] and provide interesting models to study the interaction of DENV and human cells *in viv*o. However, there are no reports of DENV vaccine testing in humanized mice, likely due to substantial variation observed in the degree of reconstitution in the mice (batch effect) [10]. Moreover, the murine environment does not correctly mimic the molecular and cellular interactions required by the grafted human cells, resulting in impaired immune functions, in particular in the antibody response [65]. No humanized mice model has been described for ZIKV so far.

16.5 Conclusions

For dengue vaccine testing the following animal model features are required: (i) robust, reproducible viremia, (ii) immuno-competent, (iii) viremia for all four serotypes. It is also desirable that the model shows clinical symptoms that are similar to those observed in dengue patients. Unfortunately, there is no model that fulfills all these criteria (Table 16.5) and the use of a combination of several models is likely the best approach to test and validate potential vaccine candidates.

The same animal model limitations discussed for dengue also apply for Zika. However, Zika vaccines might have to be tested for their capacity to block infection of the placenta and the fetus, which likely requires models that are physiologically similar to the human morphology. Importantly, more clinical studies are needed to establish the effect of Zika on the developing fetus in infected humans. Only once more comprehensive data are available from patients the potential usefulness of mouse models can be confirmed.

Recognition of viral genomic material in the cytoplasm triggering the Cardif signaling pathway and recognition in endosome culminates in the activation of Interferon Regulatory Factors (IRF) 3, 5 and 7, which induces transcription of IFNs and pro-inflammatory cytokines. On engagement, the type I IFN binding to interferon--α/β receptor (IFNAR, composed by IFNAR1 and IFNAR2 subunit) and type II IFN binding to interferon-γ receptor (IFNGR, composed by IFNGR1 and IFNGR2 subunit) activates Janus Kinases 1 (JAK1) and tyrosine kinase 2 (TYK2) for IFNAR and JAK1 and JAK2 for IFNGR. Phosphorylation of the receptor by these kinases recruits the Signal Transducer and Activator of Transcription protein (STAT). The STAT1-STAT1 complexes are formed in response to type I and/or type II IFN, this homo-dimers binds to gamma-activated sequences (GAS) inducing pro-inflammatory genes. The STAT1-STAT2 complexes are formed in response to type I IFN, this heterodimers associate with a third protein: IRF9. The complexes STAT1-STAT2-IRF9 also denominated Interferon-Stimulated Gene Factor 3 (ISFG3) binds to Interferon-Stimulated Response Element (ISRE) sequences to activated classical antiviral genes [18, 59, 62, 67, 101].

Table 16.5 Summary of desirable features for the most commonly used animal models for flavivirus vaccine testing

Model	Adaptive response	Test live-attenuated viruses	Test recombinant vaccines	Viremia	Pathology	Fc expression to assess ADE, effector functions	Study fetal development
AG129	limited	+	–	DENV-1,2,3,4, Zika	+	+/– (no FcgRIIa [17])	+/–
A129, IFNAR	limited	+	–	DENV-2 (DENV-1,3,4), Zika	+	+/– (no FcgRIIa)	+/–
Conditinal IFNAR	yes	+	+	DENV-2, (DENV-1,3,4), Zika (?)	+ (less severe phenotype compared to IFNAR)	+/– (no FcgRIIa)	+/–
NHP (CM and RM)	yes	+	+	DENV-1,2,3,4, Zika	–	+ [17]	+

+: Present in the model
−: Not present in the model
+/−: Not representative of human biology
?: Not tested yet

Discussion of Chapter 16 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.

Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Katja Fink for the presentation of the topic on "Measuring antibody-mediated protection after dengue vaccination with in vitro assays and animal models". However, because animal models are widely used in the study of Dengue and Zika and wanted to present the current status in the monograph we invited Dr. Fink to prepare a review of the topic and provide comprehensive references for the various animal models used by researchers. We however not that the discussion touched on some important points on antibody neutralization that are relevant and have decided to present Dr. Fink's abstract and the discussion.

Measuring Antibody-Mediated Protection After Dengue Vaccination with In Vitro Assays and Animal Models

Katja Fink

Dengue vaccine development and related research has brought a wealth of new knowledge about the relevance of innate and adaptive components of the immune response after dengue virus infection. There is evidence from human challenge studies that dengue neutralizing antibody titers correlate with protection. However, this rule does not seem to be true for prospective natural infection studies, and one reason for conflicting results may be the usage of different assays to measure neutralizing antibodies. In fact, a standard readout to measure a protective immune response is still not defined for dengue. Using examples from our vaccine development and therapeutic antibody studies I will provide an overview of dengue and zika

animal models that are useful to measure protective immune -, and in particular, protective antibody responses.

Joanna Miller: Your skin-derived macrophages were a really nice model to analyze antibody ADE and neutralization. Have you tried maturing monocytes from blood and using them as a model as well?

Katja Fink: No, and the reason for that is first because we had the skin assay already set up and the skin is a really a good source of macrophages and DCs. We also know that in the i*n vitro* differentiation setting, surface markers get down-regulated or upregulated and it is quite difficult to compare this to primary cells. We want to have an assay that closely reflects the situation *in vivo*.

Vijay Dhanasekaran: This is more of a general question in terms of understanding the differences between animal models. Particularly referring to the studies carried out by a Cambridge group (Smith DJ & colleagues) trying to understand the antigenic variation of Dengue viruses, which was published in *Science* last year [2015 Sep 18;349(6254): 1338–43]. They showed a huge difference in polyclonal responses to the same Dengue serotype. And this was much lesser than that exhibited between Dengue serotypes. I was wondering if you had any insight into why they exhibited such a difference and I think that study was done in macaques.

Katja Fink: Did you say that the antigenic variations were studied in macaques? I'm not familar with that work.

Vijay Dhanasekaran: The actual response there was a huge antigenic variation between different strains of the same type. But when compared between types, the differences are much lesser.

Aravinda de Silva: Maybe I can comment on this. The point of the study is that certain strains of Dengue within a serotype are very different from one another with respect to neutralization and that they are closer to another serotype. So that study is looking at the viruses that were outliers. I think we have to be very cautious about how we interpret that study, some of these outlier strains are heavily cell-type passaged strains. As Félix Rey mentioned yesterday, a single mutation within the E protein can change the breathing structure of the viruses and just make them more broadly susceptible to neutralization. So I think, I would'nt draw a conclusion from the published study that in nature serotypes will behave that way. The authors of the study may disagree with me.

References

1. Aguirre S, Maestre AM, Pagni S, Patel JR, Savage T, Gutman D, Maringer K, Bernal-Rubio D, Shabman RS, Simon V, Rodriguez-Madoz JR, Mulder LC, Barber GN, Fernandez-Sesma A (2012) DENV inhibits type I IFN production in infected cells by cleaving human STING. PLoS Pathog 8(10):e1002934. https://doi.org/10.1371/journal.ppat.1002934
2. Aliota MT, Caine EA, Walker EC, Larkin KE, Camacho E, Osorio JE (2016) Characterization of Lethal Zika Virus Infection in AG129 Mice. PLoS Negl Trop Dis 10(4):e0004682. https://doi.org/10.1371/journal.pntd.0004682
3. Amanna IJ, Slifka MK (2014) Current trends in West Nile virus vaccine development. Expert Rev Vaccines 13(5):589–608. https://doi.org/10.1586/14760584.2014.906309
4. An J, Kimura-Kuroda J, Hirabayashi Y, Yasui K (1999) Development of a novel mouse model for dengue virus infection. Virology 263(1):70–77. https://doi.org/10.1006/viro.1999.9887
5. Angsubhakorn S, Yoksan S, Pradermwong A, Nitatpattana N, Sahaphong S, Bhamarapravati N (1994) Dengue-3 (16562) PGMK 33 vaccine: neurovirulence, viremia and immune responses in Macaca fascicularis. Southeast Asian J Trop Med Public Health 25(3):554–559
6. Ashour J, Morrison J, Laurent-Rolle M, Belicha-Villanueva A, Plumlee CR, Bernal-Rubio D, Williams KL, Harris E, Fernandez-Sesma A, Schindler C, Garcia-Sastre A (2010) Mouse STAT2 restricts early dengue virus replication. Cell Host Microbe 8(5):410–421. https://doi.org/10.1016/j.chom.2010.10.007. S1931-3128(10)00344-6 [pii]
7. Atrasheuskaya A, Petzelbauer P, Fredeking TM, Ignatyev G (2003) Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. FEMS Immunol Med Microbiol 35(1):33–42
8. Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, Johnson S, Diamond MS, Beatty PR, Harris E (2010) Lethal antibody enhancement

of dengue disease in mice is prevented by Fc modification. PLoS Pathog 6(2):e1000790. https://doi.org/10.1371/journal.ppat.1000790
9. Becker R (2016) Missing link: animal models to study whether Zika causes birth defects. Nat Med 22(3):225–227. https://doi.org/10.1038/nm0316-225
10. Bente DA, Melkus MW, Garcia JV, Rico-Hesse R (2005) Dengue fever in humanized NOD/SCID mice. J Virol 79(21):13797–13799. https://doi.org/10.1128/JVI.79.21.13797-13799.2005
11. Bernardo L, Fleitas O, Pavon A, Hermida L, Guillen G, Guzman MG (2009) Antibodies induced by dengue virus type 1 and 2 envelope domain III recombinant proteins in monkeys neutralize strains with different genotypes. Clin Vaccine Immunol 16(12):1829–1831. https://doi.org/10.1128/CVI.00191-09
12. Bernardo L, Hermida L, Martin J, Alvarez M, Prado I, Lopez C, Martinez R, Rodriguez-Roche R, Zulueta A, Lazo L, Rosario D, Guillen G, Guzman MG (2008a) Anamnestic antibody response after viral challenge in monkeys immunized with dengue 2 recombinant fusion proteins. Arch Virol 153(5):849–854. https://doi.org/10.1007/s00705-008-0050-9
13. Bernardo L, Izquierdo A, Alvarez M, Rosario D, Prado I, Lopez C, Martinez R, Castro J, Santana E, Hermida L, Guillen G, Guzman MG (2008b) Immunogenicity and protective efficacy of a recombinant fusion protein containing the domain III of the dengue 1 envelope protein in non-human primates. Antiviral Res 80(2):194–199. https://doi.org/10.1016/j.antiviral.2008.06.005
14. Bernardo L, Izquierdo A, Prado I, Rosario D, Alvarez M, Santana E, Castro J, Martinez R, Rodriguez R, Morier L, Guillen G, Guzman MG (2008c) Primary and secondary infections of Macaca fascicularis monkeys with Asian and American genotypes of dengue virus 2. Clin Vaccine Immunol 15(3):439–446. https://doi.org/10.1128/CVI.00208-07
15. Blaney JE Jr, Hanson CT, Firestone CY, Hanley KA, Murphy BR, Whitehead SS (2004a) Genetically modified, live attenuated dengue virus type 3 vaccine candidates. Am J Trop Med Hyg 71(6):811–821
16. Blaney JE Jr, Hanson CT, Hanley KA, Murphy BR, Whitehead SS (2004b) Vaccine candidates derived from a novel infectious cDNA clone of an American genotype dengue virus type 2. BMC Infect Dis 4:39. https://doi.org/10.1186/1471-2334-4-39
17. Bournazos S, DiLillo DJ, Ravetch JV (2015) The role of Fc-FcgammaR interactions in IgG-mediated microbial neutralization. J Exp Med 212(9):1361–1369. https://doi.org/10.1084/jem.20151267
18. Bowie AG, Unterholzner L (2008) Viral evasion and subversion of pattern-recognition receptor signalling. Nat Rev Immunol 8(12):911–922. https://doi.org/10.1038/nri2436
19. Brasil P, Pereira JP Jr, Raja Gabaglia C, Damasceno L, Wakimoto M, Ribeiro Nogueira RM, Carvalho de Sequeira P, Machado Siqueira A, Abreu de Carvalho LM, Cotrim da Cunha D, Calvet GA, Neves ES, Moreira ME, Rodrigues Baiao AE, Nassar de Carvalho PR, Janzen C, Valderramos SG, Cherry JD, Bispo de Filippis AM, Nielsen-Saines K (2016) Zika Virus infection in pregnant women in Rio de Janeiro – preliminary report. N Engl J Med. https://doi.org/10.1056/NEJMoa1602412
20. Bray M, Men R, Lai CJ (1996) Monkeys immunized with intertypic chimeric dengue viruses are protected against wild-type virus challenge. J Virol 70(6):4162–4166
21. Brewoo JN, Kinney RM, Powell TD, Arguello JJ, Silengo SJ, Partidos CD, Huang CY, Stinchcomb DT, Osorio JE (2011) Immunogenicity and efficacy of chimeric dengue vaccine (DENVax) formulations in interferon-deficient AG129 mice. Vaccine. https://doi.org/10.1016/j.vaccine.2011.11.072
22. Brewoo JN, Kinney RM, Powell TD, Arguello JJ, Silengo SJ, Partidos CD, Huang CY, Stinchcomb DT, Osorio JE (2012) Immunogenicity and efficacy of chimeric dengue vaccine (DENVax) formulations in interferon-deficient AG129 mice. Vaccine 30(8):1513–1520. https://doi.org/10.1016/j.vaccine.2011.11.072
23. Butrapet S, Rabablert J, Angsubhakorn S, Wiriyarat W, Huang C, Kinney R, Punyim S, Bhamarapravati N (2002) Chimeric dengue type 2/type 1 viruses induce immune responses in cynomolgus monkeys. Southeast Asian J Trop Med Public Health 33(3):589–599
24. Calvert AE, Huang CY, Kinney RM, Roehrig JT (2006) Non-structural proteins of dengue 2 virus offer limited protection to interferon-deficient mice after dengue 2 virus challenge. J Gen Virol 87(Pt 2):339–346. https://doi.org/10.1099/vir.0.81256-0
25. Cao-Lormeau VM, Blake A, Mons S, Lastere S, Roche C, Vanhomwegen J, Dub T, Baudouin L, Teissier A, Larre P, Vial AL, Decam C, Choumet V, Halstead SK, Willison HJ, Musset L, Manuguerra JC, Despres P, Fournier E, Mallet HP, Musso D, Fontanet A, Neil J, Ghawche F (2016) Guillain-Barre syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet 387(10027):1531–1539. https://doi.org/10.1016/S0140-6736(16)00562-6
26. Chan KW, Watanabe S, Kavishna R, Alonso S, Vasudevan SG (2015) Animal models for studying dengue pathogenesis and therapy. Antiviral Res 123:5–14. https://doi.org/10.1016/j.antiviral.2015.08.013
27. Chen HC, Hofman FM, Kung JT, Lin YD, Wu-Hsieh BA (2007a) Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage. J Virol 81(11):5518–5526
28. Chen HC, Lai SY, Sung JM, Lee SH, Lin YC, Wang WK, Chen YC, Kao CL, King CC, Wu-Hsieh BA (2004) Lymphocyte activation and hepatic cellular infiltration in immunocompetent mice infected by dengue virus. J Med Virol 73(3):419–431
29. Chen L, Ewing D, Subramanian H, Block K, Rayner J, Alterson KD, Sedegah M, Hayes C, Porter K, Raviprakash K (2007b) A heterologous DNA prime-

Venezuelan equine encephalitis virus replicon particle boost dengue vaccine regimen affords complete protection from virus challenge in cynomolgus macaques. J Virol 81(21):11634–11639. https://doi.org/10.1128/JVI.00996-07

30. Chen ST, Lin YL, Huang MT, Wu MF, Cheng SC, Lei HY, Lee CK, Chiou TW, Wong CH, Hsieh SL (2008) CLEC5A is critical for dengue-virus-induced lethal disease. Nature 453(7195):672–676
31. Clements DE, Coller BA, Lieberman MM, Ogata S, Wang G, Harada KE, Putnak JR, Ivy JM, McDonell M, Bignami GS, Peters ID, Leung J, Weeks-Levy C, Nakano ET, Humphreys T (2010) Development of a recombinant tetravalent dengue virus vaccine: immunogenicity and efficacy studies in mice and monkeys. Vaccine 28(15):2705–2715. https://doi.org/10.1016/j.vaccine. S0264-410X(10)00054-X [pii]
32. Cox J, Mota J, Sukupolvi-Petty S, Diamond MS, Rico-Hesse R (2012) Mosquito bite delivery of dengue virus enhances immunogenicity and pathogenesis in humanized mice. J Virol 86(14):7637–7649. https://doi.org/10.1128/JVI.00534-12
33. Dick GW (1952) Zika virus. II. Pathogenicity and physical properties. Trans R Soc Trop Med Hyg 46(5):521–534
34. Dick GW, Kitchen SF, Haddow AJ (1952) Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg 46(5):509–520
35. Domnich A, Panatto D, Arbuzova EK, Signori A, Avio U, Gasparini R, Amicizia D (2014) Immunogenicity against Far Eastern and Siberian subtypes of tick-borne encephalitis (TBE) virus elicited by the currently available vaccines based on the European subtype: systematic review and meta-analysis. Hum Vaccin Immunother 10(10):2819–2833. https://doi.org/10.4161/hv.29984
36. Dowall SD, Graham VA, Rayner E, Atkinson B, Hall G, Watson RJ, Bosworth A, Bonney LC, Kitchen S, Hewson R (2016) A susceptible mouse model for Zika Virus infection. PLoS Negl Trop Dis 10(5):e0004658. https://doi.org/10.1371/journal.pntd.0004658
37. Dowd KA, Ko SY, Morabito KM, Yang ES, Pelc RS, DeMaso CR, Castilho LR, Abbink P, Boyd M, Nityanandam R, Gordon DN, Gallagher JR, Chen X, Todd JP, Tsybovsky Y, Harris A, Huang YS, Higgs S, Vanlandingham DL, Andersen H, Lewis MG, De La Barrera R, Eckels KH, Jarman RG, Nason MC, Barouch DH, Roederer M, Kong WP, Mascola JR, Pierson TC, Graham BS (2016) Rapid development of a DNA vaccine for Zika virus. Science 354(6309):237–240. https://doi.org/10.1126/science.aai9137
38. Driggers RW, Ho CY, Korhonen EM, Kuivanen S, Jaaskelainen AJ, Smura T, Rosenberg A, Hill DA, DeBiasi RL, Vezina G, Timofeev J, Rodriguez FJ, Levanov L, Razak J, Iyengar P, Hennenfent A, Kennedy R, Lanciotti R, du Plessis A, Vapalahti O (2016) Zika virus infection with prolonged maternal viremia and fetal brain abnormalities. N Engl J Med 374(22):2142–2151. https://doi.org/10.1056/NEJMoa1601824
39. Dudley DM, Aliota MT, Mohr EL, Weiler AM, Lehrer-Brey G, Weisgrau KL, Mohns MS, Breitbach ME, Rasheed MN, Newman CM, Gellerup DD, Moncla LH, Post J, Schultz-Darken N, Schotzko ML, Hayes JM, Eudailey JA, Moody MA, Permar SR, O'Connor SL, Rakasz EG, Simmons HA, Capuano S, Golos TG, Osorio JE, Friedrich TC, O'Connor DH (2016) A rhesus macaque model of Asian-lineage Zika virus infection. Nat Commun 7:12204. https://doi.org/10.1038/ncomms12204
40. Eckels KH, Dubois DR, Putnak R, Vaughn DW, Innis BL, Henchal EA, Hoke CH Jr (2003) Modification of dengue virus strains by passage in primary dog kidney cells: preparation of candidate vaccines and immunization of monkeys. Am J Trop Med Hyg 69(6 Suppl):12–16
41. Eckels KH, Dubois DR, Summers PL, Schlesinger JJ, Shelly M, Cohen S, Zhang YM, Lai CJ, Kurane I, Rothman A et al (1994) Immunization of monkeys with baculovirus-dengue type-4 recombinants containing envelope and nonstructural proteins: evidence of priming and partial protection. Am J Trop Med Hyg 50(4):472–478
42. Engelmann F, Josset L, Girke T, Park B, Barron A, Dewane J, Hammarlund E, Lewis A, Axthelm MK, Slifka MK, Messaoudi I (2014) Pathophysiologic and transcriptomic analyses of viscerotropic yellow fever in a rhesus macaque model. PLoS Negl Trop Dis 8(11):e3295. https://doi.org/10.1371/journal.pntd.0003295
43. WHO Strategic Advisory Group of Experts (2016) Dengue vaccine. Wkly Epidemiol Rec 91(21):282–284
44. Fernandez S, Thomas SJ, De La Barrera R, Im-Erbsin R, Jarman RG, Baras B, Toussaint JF, Mossman S, Innis BL, Schmidt A, Malice MP, Festraets P, Warter L, Putnak JR, Eckels KH (2015) An adjuvanted, tetravalent dengue virus purified inactivated vaccine candidate induces long-lasting and protective antibody responses against dengue challenge in rhesus macaques. Am J Trop Med Hyg 92(4):698–708. https://doi.org/10.4269/ajtmh.14-0268
45. Frias-Staheli N, Dorner M, Marukian S, Billerbeck E, Labitt RN, Rice CM, Ploss A (2014) Utility of humanized BLT mice for analysis of dengue virus infection and antiviral drug testing. J Virol 88(4):2205–2218. https://doi.org/10.1128/JVI.03085-13
46. Fuchs J, Chu H, O'Day P, Pyles R, Bourne N, Das SC, Milligan GN, Barrett AD, Partidos CD, Osorio JE (2014) Investigating the efficacy of monovalent and tetravalent dengue vaccine formulations against DENV-4 challenge in AG129 mice. Vaccine 32(48):6537–6543. https://doi.org/10.1016/j.vaccine.2014.08.087
47. Galler R, Marchevsky RS, Caride E, Almeida LF, Yamamura AM, Jabor AV, Motta MC, Bonaldo MC, Coutinho ES, Freire MS (2005) Attenuation and immunogenicity of recombinant yellow

fever 17D-dengue type 2 virus for rhesus monkeys. Braz J Med Biol Res 38(12):1835–1846. S0100-879X2005001200012
48. Girschick HJ, Grammer AC, Nanki T, Mayo M, Lipsky PE (2001) RAG1 and RAG2 expression by B cell subsets from human tonsil and peripheral blood. J Immunol 166(1):377–386
49. Govindarajan D, Meschino S, Guan L, Clements DE, ter Meulen JH, Casimiro DR, Coller BA, Bett AJ (2015) Preclinical development of a dengue tetravalent recombinant subunit vaccine: immunogenicity and protective efficacy in nonhuman primates. Vaccine 33(33):4105–4116. https://doi.org/10.1016/j.vaccine.2015.06.067
50. Guirakhoo F, Pugachev K, Arroyo J, Miller C, Zhang ZX, Weltzin R, Georgakopoulos K, Catalan J, Ocran S, Draper K, Monath TP (2002) Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. Virology 298(1):146–159. https://doi.org/10.1006/viro.2002.1462. S0042682202914625 [pii]
51. Guirakhoo F, Pugachev K, Zhang Z, Myers G, Levenbook I, Draper K, Lang J, Ocran S, Mitchell F, Parsons M, Brown N, Brandler S, Fournier C, Barrere B, Rizvi F, Travassos A, Nichols R, Trent D, Monath T (2004a) Safety and efficacy of chimeric yellow fever-dengue virus tetravalent vaccine formulations in nonhuman primates. J Virol 78(9):4761–4775
52. Guirakhoo F, Weltzin R, Chambers TJ, Zhang ZX, Soike K, Ratterree M, Arroyo J, Georgakopoulos K, Catalan J, Monath TP (2000) Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. J Virol 74(12):5477–5485
53. Guirakhoo F, Zhang Z, Myers G, Johnson BW, Pugachev K, Nichols R, Brown N, Levenbook I, Draper K, Cyrek S, Lang J, Fournier C, Barrere B, Delagrave S, Monath TP (2004b) A single amino acid substitution in the envelope protein of chimeric yellow fever-dengue 1 vaccine virus reduces neurovirulence for suckling mice and viremia/viscerotropism for monkeys. J Virol 78(18):9998–10008. https://doi.org/10.1128/JVI.78.18.9998-10008.2004. 78/18/9998 [pii]
54. Guy B, Barban V, Mantel N, Aguirre M, Gulia S, Pontvianne J, Jourdier TM, Ramirez L, Gregoire V, Charnay C, Burdin N, Dumas R, Lang J (2009) Evaluation of interferences between dengue vaccine serotypes in a monkey model. Am J Trop Med Hyg 80(2):302–311
55. Guzman MG, Rodriguez R, Rodriguez R, Hermida L, Alvarez M, Lazo L, Mune M, Rosario D, Valdes K, Vazquez S, Martinez R, Serrano T, Paez J, Espinosa R, Pumariega T, Guillen G (2003) Induction of neutralizing antibodies and partial protection from viral challenge in Macaca fascicularis immunized with recombinant dengue 4 virus envelope glycoprotein expressed in Pichia pastoris. Am J Trop Med Hyg 69(2):129–134
56. Hadinegoro SR, Arredondo-Garcia JL, Capeding MR, Deseda C, Chotpitayasunondh T, Dietze R, Hj Muhammad HI, Ismail HR, Limkittikul K, Rivera-Medina DM, Tran HN, Bouckenooghe A, Chansinghakul D, Cortes M, Fanouillere K, Forrat R, Frago C, Gailhardou S, Jackson N, Noriega F, Plennevaux E, Wartel TA, Zambrano B, Saville M, Cyd-Tdv Dengue Vaccine Working Group (2015) Efficacy and long-term safety of a dengue vaccine in regions of endemic disease. N Engl J Med 373(13):1195–1206. https://doi.org/10.1056/NEJMoa1506223
57. Hanley KA, Manlucu LR, Manipon GG, Hanson CT, Whitehead SS, Murphy BR, Blaney JE Jr (2004) Introduction of mutations into the non-structural genes or 3′ untranslated region of an attenuated dengue virus type 4 vaccine candidate further decreases replication in rhesus monkeys while retaining protective immunity. Vaccine 22(25-26):3440–3448. https://doi.org/10.1016/j.vaccine.2004.02.031
58. Hermida L, Bernardo L, Martin J, Alvarez M, Prado I, Lopez C, Sierra Bde L, Martinez R, Rodriguez R, Zulueta A, Perez AB, Lazo L, Rosario D, Guillen G, Guzman MG (2006) A recombinant fusion protein containing the domain III of the dengue-2 envelope protein is immunogenic and protective in nonhuman primates. Vaccine 24(16):3165–3171. https://doi.org/10.1016/j.vaccine.2006.01.036
59. Honda K, Taniguchi T (2006) IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 6(9):644–658. https://doi.org/10.1038/nri1900
60. Huang CY, Butrapet S, Tsuchiya KR, Bhamarapravati N, Gubler DJ, Kinney RM (2003) Dengue 2 PDK-53 virus as a chimeric carrier for tetravalent dengue vaccine development. J Virol 77(21):11436–11447
61. Huang KJ, Li SY, Chen SC, Liu HS, Lin YS, Yeh TM, Liu CC, Lei HY (2000) Manifestation of thrombocytopenia in dengue-2-virus-infected mice. J Gen Virol 81(Pt 9):2177–2182. https://doi.org/10.1099/0022-1317-81-9-2177
62. Ivashkiv LB, Donlin LT (2014) Regulation of type I interferon responses. Nat Rev Immunol 14(1):36–49. https://doi.org/10.1038/nri3581
63. Izquierdo A, Bernardo L, Martin J, Santana E, Hermida L, Guillen G, Guzman MG (2008) Serotype-specificity of recombinant fusion proteins containing domain III of dengue virus. Virus Res 138(1-2):135–138. https://doi.org/10.1016/j.virusres.2008.08.008
64. Jaiswal S, Pazoles P, Woda M, Shultz LD, Greiner DL, Brehm MA, Mathew A (2012) Enhanced humoral and HLA-A2-restricted dengue virus-specific T-cell responses in humanized BLT NSG mice. Immunology 136(3):334–343. https://doi.org/10.1111/j.1365-2567.2012.03585.x

65. Jaiswal S, Pearson T, Friberg H, Shultz LD, Greiner DL, Rothman AL, Mathew A (2009) Dengue virus infection and virus-specific HLA-A2 restricted immune responses in humanized NOD-scid IL2rgammanull mice. PLoS One 4(10):e7251. https://doi.org/10.1371/journal.pone.0007251
66. Johnson AJ, Roehrig JT (1999) New mouse model for dengue virus vaccine testing. J Virol 73(1):783–786
67. Kariko K, Buckstein M, Ni H, Weissman D (2005) Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 23(2):165–175. https://doi.org/10.1016/j.immuni.2005.06.008
68. Kim E, Erdos G, Huang S, Kenniston T, Falo LD Jr, Gambotto A (2016) Preventative vaccines for Zika Virus outbreak: preliminary evaluation. EBioMedicine. https://doi.org/10.1016/j.ebiom.2016.09.028
69. Koraka P, Benton S, van Amerongen G, Stittelaar KJ, Osterhaus AD (2007) Efficacy of a live attenuated tetravalent candidate dengue vaccine in naive and previously infected cynomolgus macaques. Vaccine 25(29):5409–5416. https://doi.org/10.1016/j.vaccine.2007.04.079
70. Kuruvilla JG, Troyer RM, Devi S, Akkina R (2007) Dengue virus infection and immune response in humanized RAG2(-/-)gamma(c)(-/-) (RAG-hu) mice. Virology 369(1):143–152
71. Larocca RA, Abbink P, Peron JP, Zanotto PM, Iampietro MJ, Badamchi-Zadeh A, Boyd M, Ng'ang'a D, Kirilova M, Nityanandam R, Mercado NB, Li Z, Moseley ET, Bricault CA, Borducchi EN, Giglio PB, Jetton D, Neubauer G, Nkolola JP, Maxfield LF, De La Barrera RA, Jarman RG, Eckels KH, Michael NL, Thomas SJ, Barouch DH (2016) Vaccine protection against Zika virus from Brazil. Nature 536(7617):474–478. https://doi.org/10.1038/nature18952
72. Lazear HM, Govero J, Smith AM, Platt DJ, Fernandez E, Miner JJ, Diamond MS (2016) A mouse model of Zika Virus Pathogenesis. Cell Host Microbe 19(5):720–730. https://doi.org/10.1016/j.chom.2016.03.010
73. Lee YR, Hu HY, Kuo SH, Lei HY, Lin YS, Yeh TM, Liu CC, Liu HS (2013) Dengue virus infection induces autophagy: an in vivo study. J Biomed Sci 20:65. https://doi.org/10.1186/1423-0127-20-65
74. Lee YR, Huang KJ, Lei HY, Chen SH, Lin YS, Yeh TM, Liu HS (2005) Suckling mice were used to detect infectious dengue-2 viruses by intracerebral injection of the full-length RNA transcript. Intervirology 48(2-3):161–166. https://doi.org/10.1159/000081744
75. Li XF, Deng YQ, Yang HQ, Zhao H, Jiang T, Yu XD, Li SH, Ye Q, Zhu SY, Wang HJ, Zhang Y, Ma J, Yu YX, Liu ZY, Li YH, Qin ED, Shi PY, Qin CF (2013) A chimeric dengue virus vaccine using Japanese encephalitis virus vaccine strain SA14-14-2 as backbone is immunogenic and protective against either parental virus in mice and nonhuman primates. J Virol 87(24):13694–13705. https://doi.org/10.1128/JVI.00931-13
76. Li XF, Dong HL, Huang XY, Qiu YF, Wang HJ, Deng YQ, Zhang NN, Ye Q, Zhao H, Liu ZY, Fan H, An XP, Sun SH, Gao B, Fa YZ, Tong YG, Zhang FC, Gao GF, Cao WC, Shi PY, Qin CF (2016) Characterization of a 2016 clinical isolate of Zika Virus in Non-human Primates. EBioMedicine 12:170–177. https://doi.org/10.1016/j.ebiom.2016.09.022
77. Li X, Leung S, Qureshi S, Darnell JE Jr, Stark GR (1996) Formation of STAT1-STAT2 heterodimers and their role in the activation of IRF-1 gene transcription by interferon-alpha. J Biol Chem 271(10):5790–5794
78. Lin YL, Liao CL, Chen LK, Yeh CT, Liu CI, Ma SH, Huang YY, Huang YL, Kao CL, King CC (1998) Study of Dengue virus infection in SCID mice engrafted with human K562 cells. J Virol 72(12):9729–9737
79. Lum LC, Lam SK, Choy YS, George R, Harun F (1996) Dengue encephalitis: a true entity? Am J Trop Med Hyg 54(3):256–259
80. Marchette NJ, Dubois DR, Larsen LK, Summers PL, Kraiselburd EG, Gubler DJ, Eckels KH (1990) Preparation of an attenuated dengue 4 (341750 Carib) virus vaccine. I. pre-clinical studies. Am J Trop Med Hyg 43(2):212–218
81. Marcos E, Gil L, Lazo L, Izquierdo A, Brown E, Suzarte E, Valdes I, Garcia A, Mendez L, Guzman MG, Guillen G, Hermida L (2013) Purified and highly aggregated chimeric protein DIIIC-2 induces a functional immune response in mice against dengue 2 virus. Arch Virol 158(1):225–230. https://doi.org/10.1007/s00705-012-1471-z
82. Markoff L, Pang X, Houng Hs HS, Falgout B, Olsen R, Jones E, Polo S (2002) Derivation and characterization of a dengue type 1 host range-restricted mutant virus that is attenuated and highly immunogenic in monkeys. J Virol 76(7):3318–3328
83. McBurney SP, Sunshine JE, Gabriel S, Huynh JP, Sutton WF, Fuller DH, Haigwood NL, Messer WB (2016) Evaluation of protection induced by a dengue virus serotype 2 envelope domain III protein scaffold/DNA vaccine in non-human primates. Vaccine 34(30):3500–3507. https://doi.org/10.1016/j.vaccine.2016.03.108
84. Men R, Bray M, Clark D, Chanock RM, Lai CJ (1996) Dengue type 4 virus mutants containing deletions in the 3′ noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. J Virol 70(6):3930–3937
85. Men R, Wyatt L, Tokimatsu I, Arakaki S, Shameem G, Elkins R, Chanock R, Moss B, Lai CJ (2000) Immunization of rhesus monkeys with a recombinant of modified vaccinia virus Ankara expressing a truncated envelope glycoprotein of dengue type 2 virus induced resistance to dengue type 2 virus challenge. Vaccine 18(27):3113–3122

86. Milligan GN, Sarathy VV, Infante E, Li L, Campbell GA, Beatty PR, Harris E, Barrett AD, Bourne N (2015) A dengue virus type 4 model of disseminated Lethal Infection in AG129 Mice. PLoS One 10(5):e0125476. https://doi.org/10.1371/journal.pone.0125476
87. Mladinich KM, Piaskowski SM, Rudersdorf R, Eernisse CM, Weisgrau KL, Martins MA, Furlott JR, Partidos CD, Brewoo JN, Osorio JE, Wilson NA, Rakasz EG, Watkins DI (2012) Dengue virus-specific CD4+ and CD8+ T lymphocytes target NS1, NS3 and NS5 in infected Indian rhesus macaques. Immunogenetics 64(2):111–121. https://doi.org/10.1007/s00251-011-0566-0
88. Mota J, Rico-Hesse R (2009) Humanized mice show clinical signs of dengue fever according to infecting virus genotype. J Virol 83(17):8638–8645. https://doi.org/10.1128/JVI.00581-09
89. Mota J, Rico-Hesse R (2011) Dengue virus tropism in humanized mice recapitulates human dengue fever. PLoS One 6(6):e20762. https://doi.org/10.1371/journal.pone.0020762
90. Nguyen SM, Antony KM, Dudley DM, Kohn S, Simmons HA, Wolfe B, Salamat MS, Teixeira LBC, Wiepz GJ, Thoong TH, Aliota MT, Weiler AM, Barry GL, Weisgrau KL, Vosler LJ, Mohns MS, Breitbach ME, Stewart LM, Rasheed MN, Newman CM, Graham ME, Wieben OE, Turski PA, Johnson KM, Post J, Hayes JM, Schultz-Darken N, Schotzko ML, Eudailey JA, Permar SR, Rakasz EG, Mohr EL, Capuano S 3rd, Tarantal AF, Osorio JE, O'Connor SL, Friedrich TC, O'Connor DH, Golos TG (2017) Highly efficient maternal-fetal Zika virus transmission in pregnant rhesus macaques. PLoS Pathog 13(5):e1006378. https://doi.org/10.1371/journal.ppat.1006378
91. Oehler E, Watrin L, Larre P, Leparc-Goffart I, Lastere S, Valour F, Baudouin L, Mallet H, Musso D, Ghawche F (2014) Zika virus infection complicated by Guillain-Barre syndrome--case report, French Polynesia, December 2013. Euro Surveill 19(9)
92. Omatsu T, Moi ML, Hirayama T, Takasaki T, Nakamura S, Tajima S, Ito M, Yoshida T, Saito A, Katakai Y, Akari H, Kurane I (2011) Common marmoset (Callithrix jacchus) as a primate model of dengue virus infection: development of high levels of viraemia and demonstration of protective immunity. J Gen Virol 92(Pt 10):2272–2280. https://doi.org/10.1099/vir.0.031229-0
93. Orozco S, Schmid MA, Parameswaran P, Lachica R, Henn MR, Beatty R, Harris E (2012) Characterization of a model of lethal dengue virus 2 infection in C57BL/6 mice deficient in the alpha/beta interferon receptor. J Gen Virol 93(Pt 10):2152–2157. https://doi.org/10.1099/vir.0.045088-0
94. Osorio JE, Brewoo JN, Silengo SJ, Arguello J, Moldovan IR, Tary-Lehmann M, Powell TD, Livengood JA, Kinney RM, Huang CY, Stinchcomb DT (2011) Efficacy of a tetravalent chimeric dengue vaccine (DENVax) in Cynomolgus macaques. Am J Trop Med Hyg 84(6):978–987. https://doi.org/10.4269/ajtmh.2011.10-0592
95. Paes MV, Pinhao AT, Barreto DF, Costa SM, Oliveira MP, Nogueira AC, Takiya CM, Farias-Filho JC, Schatzmayr HG, Alves AM, Barth OM (2005) Liver injury and viremia in mice infected with dengue-2 virus. Virology 338(2):236–246. https://doi.org/10.1016/j.virol.2005.04.042
96. Patey O, Ollivaud L, Breuil J, Lafaix C (1993) Unusual neurologic manifestations occurring during dengue fever infection. Am J Trop Med Hyg 48(6):793–802
97. Pentsuk N, van der Laan JW (2009) An interspecies comparison of placental antibody transfer: new insights into developmental toxicity testing of monoclonal antibodies. Birth Defects Res B Dev Reprod Toxicol 86(4):328–344. https://doi.org/10.1002/bdrb.20201
98. Perry ST, Buck MD, Lada SM, Schindler C, Shresta S (2011) STAT2 mediates innate immunity to Dengue virus in the absence of STAT1 via the type I interferon receptor. PLoS Pathog 7(2):e1001297. https://doi.org/10.1371/journal.ppat.1001297
99. Perry ST, Prestwood TR, Lada SM, Benedict CA, Shresta S (2009) Cardif-mediated signaling controls the initial innate response to dengue virus in vivo. J Virol 83(16):8276–8281. https://doi.org/10.1128/JVI.00365-09
100. Pinto AK, Brien JD, Lam CY, Johnson S, Chiang C, Hiscott J, Sarathy VV, Barrett AD, Shresta S, Diamond MS (2015) Defining new therapeutics using a more immunocompetent mouse model of antibody-enhanced Dengue Virus infection. MBio 6(5):e01316–e01315. https://doi.org/10.1128/mBio.01316-15
101. Platanias LC (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 5(5):375–386. https://doi.org/10.1038/nri1604
102. Porter KR, Ewing D, Chen L, Wu SJ, Hayes CG, Ferrari M, Teneza-Mora N, Raviprakash K (2012) Immunogenicity and protective efficacy of a vaxfectin-adjuvanted tetravalent dengue DNA vaccine. Vaccine 30(2):336–341. https://doi.org/10.1016/j.vaccine.2011.10.085
103. Prestwood TR, Morar MM, Zellweger RM, Miller R, May MM, Yauch LE, Lada SM, Shresta S (2012) Gamma interferon (IFN-gamma) receptor restricts systemic dengue virus replication and prevents paralysis in IFN-alpha/beta receptor-deficient mice. J Virol 86(23):12561–12570. https://doi.org/10.1128/JVI.06743-11
104. Putnak R, Barvir DA, Burrous JM, Dubois DR, D'Andrea VM, Hoke CH, Sadoff JC, Eckels KH (1996) Development of a purified, inactivated, dengue-2 virus vaccine prototype in Vero cells:

immunogenicity and protection in mice and rhesus monkeys. J Infect Dis 174(6):1176–1184
105. Putnak R, Fuller J, VanderZanden L, Innis BL, Vaughn DW (2003) Vaccination of rhesus macaques against dengue-2 virus with a plasmid DNA vaccine encoding the viral pre-membrane and envelope genes. Am J Trop Med Hyg 68(4):469–476
106. Raviprakash K, Apt D, Brinkman A, Skinner C, Yang S, Dawes G, Ewing D, Wu SJ, Bass S, Punnonen J, Porter K (2006) A chimeric tetravalent dengue DNA vaccine elicits neutralizing antibody to all four virus serotypes in rhesus macaques. Virology 353(1):166–173. https://doi.org/10.1016/j.virol.2006.05.005
107. Raviprakash K, Porter KR, Kochel TJ, Ewing D, Simmons M, Phillips I, Murphy GS, Weiss WR, Hayes CG (2000) Dengue virus type 1 DNA vaccine induces protective immune responses in rhesus macaques. J Gen Virol 81(Pt 7):1659–1667. https://doi.org/10.1099/0022-1317-81-7-1659
108. Raviprakash K, Wang D, Ewing D, Holman DH, Block K, Woraratanadharm J, Chen L, Hayes C, Dong JY, Porter K (2008) A tetravalent dengue vaccine based on a complex adenovirus vector provides significant protection in rhesus monkeys against all four serotypes of dengue virus. J Virol 82(14):6927–6934. https://doi.org/10.1128/JVI.02724-07
109. Robert Putnak J, Coller BA, Voss G, Vaughn DW, Clements D, Peters I, Bignami G, Houng HS, Chen RC, Barvir DA, Seriwatana J, Cayphas S, Garcon N, Gheysen D, Kanesa-Thasan N, McDonell M, Humphreys T, Eckels KH, Prieels JP, Innis BL (2005) An evaluation of dengue type-2 inactivated, recombinant subunit, and live-attenuated vaccine candidates in the rhesus macaque model. Vaccine 23(35):4442–4452. https://doi.org/10.1016/j.vaccine.2005.03.042
110. Rossi SL, Tesh RB, Azar SR, Muruato AE, Hanley KA, Auguste AJ, Langsjoen RM, Paessler S, Vasilakis N, Weaver SC (2016) Characterization of a novel murine model to Study Zika Virus. Am J Trop Med Hyg 94(6):1362–1369. https://doi.org/10.4269/ajtmh.16-0111
111. Sarathy VV, Infante E, Li L, Campbell GA, Wang T, Paessler S, Robert Beatty P, Harris E, Milligan GN, Bourne N, Barrett AD (2015a) Characterization of lethal dengue virus type 4 (DENV-4) TVP-376 infection in mice lacking both IFN-alpha/beta and IFN-gamma receptors (AG129) and comparison with the DENV-2 AG129 mouse model. J Gen Virol 96(10):3035–3048. https://doi.org/10.1099/jgv.0.000246
112. Sarathy VV, White M, Li L, Gorder SR, Pyles RB, Campbell GA, Milligan GN, Bourne N, Barrett AD (2015b) A lethal murine infection model for dengue virus 3 in AG129 mice deficient in type I and II interferon receptors leads to systemic disease. J Virol 89(2):1254–1266. https://doi.org/10.1128/JVI.01320-14
113. Sariol CA, White LJ (2014) Utility, limitations, and future of non-human primates for dengue research and vaccine development. Front Immunol 5:452. https://doi.org/10.3389/fimmu.2014.00452
114. Sarno M, Sacramento GA, Khouri R, do Rosario MS, Costa F, Archanjo G, Santos LA, Nery N Jr, Vasilakis N, Ko AI, de Almeida AR (2016) Zika Virus infection and stillbirths: A case of Hydrops Fetalis, Hydranencephaly and Fetal Demise. PLoS Negl Trop Dis 10(2):e0004517. https://doi.org/10.1371/journal.pntd.0004517
115. Schul W, Liu W, Xu HY, Flamand M, Vasudevan SG (2007) A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J Infect Dis 195(5):665–674
116. Shan C, Muruato AE, Nunes BTD, Luo H, Xie X, Medeiros DBA, Wakamiya M, Tesh RB, Barrett AD, Wang T, Weaver SC, Vasconcelos PFC, Rossi SL, Shi PY (2017) A live-attenuated Zika virus vaccine candidate induces sterilizing immunity in mouse models. Nat Med 23(6):763–767. https://doi.org/10.1038/nm.4322
117. Shi P-Y (2012) Molecular Virology and Control of Flaviviruses. Caister Academic Press, Norfolk
118. Shresta S, Kyle JL, Robert Beatty P, Harris E (2004a) Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice. Virology 319(2):262–273
119. Shresta S, Kyle JL, Snider HM, Basavapatna M, Beatty PR, Harris E (2004b) Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. J Virol 78(6):2701–2710
120. Shresta S, Sharar KL, Prigozhin DM, Beatty PR, Harris E (2006) Murine model for dengue virus-induced lethal disease with increased vascular permeability. J Virol 80(20):10208–10217. 80/20/10208 [pii] 1261128/JVI.00062-06
121. Shresta S, Sharar KL, Prigozhin DM, Snider HM, Beatty PR, Harris E (2005) Critical roles for both STAT1-dependent and STAT1-independent pathways in the control of primary dengue virus infection in mice. J Immunol 175(6):3946–3954
122. Simmons M, Burgess T, Lynch J, Putnak R (2010) Protection against dengue virus by non-replicating and live attenuated vaccines used together in a prime boost vaccination strategy. Virology 396(2):280–288. https://doi.org/10.1016/j.virol.2009.10.023
123. Simmons M, Porter KR, Hayes CG, Vaughn DW, Putnak R (2006) Characterization of antibody responses to combinations of a dengue virus type 2 DNA vaccine and two dengue virus type 2 protein vaccines in rhesus macaques. J Virol 80(19):9577–9585. https://doi.org/10.1128/JVI.00284-06
124. Sridharan A, Chen Q, Tang KF, Ooi EE, Hibberd ML, Chen J (2013) Inhibition of megakaryocyte development in the bone marrow underlies dengue virus-induced thrombocytopenia in humanized mice. J Virol 87(21):11648–11658. https://doi.org/10.1128/JVI.01156-13

125. St John AL, Rathore AP, Raghavan B, Ng ML, Abraham SN (2013) Contributions of mast cells and vasoactive products, leukotrienes and chymase, to dengue virus-induced vascular leakage. Elife 2:e00481. https://doi.org/10.7554/eLife.00481
126. Strouts FR, Popper SJ, Partidos CD, Stinchcomb DT, Osorio JE, Relman DA (2016) Early transcriptional signatures of the immune response to a live attenuated Tetravalent Dengue Vaccine candidate in Non-human Primates. PLoS Negl Trop Dis 10(5):e0004731. https://doi.org/10.1371/journal.pntd.0004731
127. Sumathy K, Kulkarni B, Gondu RK, Ponnuru SK, Bonguram N, Eligeti R, Gadiyaram S, Praturi U, Chougule B, Karunakaran L, Ella KM (2017) Protective efficacy of Zika vaccine in AG129 mouse model. Sci Rep 7:46375. https://doi.org/10.1038/srep46375
128. Sun W, Nisalak A, Gettayacamin M, Eckels KH, Putnak JR, Vaughn DW, Innis BL, Thomas SJ, Endy TP (2006) Protection of Rhesus monkeys against dengue virus challenge after tetravalent live attenuated dengue virus vaccination. J Infect Dis 193(12):1658–1665. https://doi.org/10.1086/503372
129. Suphatrakul A, Yasanga T, Keelapang P, Sriburi R, Roytrakul T, Pulmanausahakul R, Utaipat U, Kawilapan Y, Puttikhunt C, Kasinrerk W, Yoksan S, Auewarakul P, Malasit P, Charoensri N, Sittisombut N (2015) Generation and preclinical immunogenicity study of dengue type 2 virus-like particles derived from stably transfected mosquito cells. Vaccine 33(42):5613–5622. https://doi.org/10.1016/j.vaccine.2015.08.090
130. Tan GK, Ng JK, Trasti SL, Schul W, Yip G, Alonso S (2010) A non mouse-adapted dengue virus strain as a new model of severe dengue infection in AG129 mice. PLoS Negl Trop Dis 4(4):e672. https://doi.org/10.1371/journal.pntd.0000672
131. Valdes I, Bernardo L, Gil L, Pavon A, Lazo L, Lopez C, Romero Y, Menendez I, Falcon V, Betancourt L, Martin J, Chinea G, Silva R, Guzman MG, Guillen G, Hermida L (2009) A novel fusion protein domain III-capsid from dengue-2, in a highly aggregated form, induces a functional immune response and protection in mice. Virology 394(2):249–258. https://doi.org/10.1016/j.virol. S0042-6822(09)00517-0 [pii]
132. Vasconcelos PF, Monath TP (2016) Yellow Fever remains a potential threat to public health. Vector Borne Zoonotic Dis 16(8):566–567. https://doi.org/10.1089/vbz.2016.2031
133. Vaughan K, Greenbaum J, Blythe M, Peters B, Sette A (2010) Meta-analysis of all immune epitope data in the Flavivirus genus: inventory of current immune epitope data status in the context of virus immunity and immunopathology. Viral Immunol 23(3):259–284. https://doi.org/10.1089/vim.2010.0006
134. Velzing J, Groen J, Drouet MT, van Amerongen G, Copra C, Osterhaus AD, Deubel V (1999) Induction of protective immunity against Dengue virus type 2: comparison of candidate live attenuated and recombinant vaccines. Vaccine 17(11-12):1312–1320
135. Ventura CV, Maia M, Bravo-Filho V, Gois AL, Belfort R Jr (2016) Zika virus in Brazil and macular atrophy in a child with microcephaly. Lancet 387(10015):228. https://doi.org/10.1016/S0140-6736(16)00006-4
136. Waddell LA, Greig JD (2016) Scoping review of the Zika Virus Literature. PLoS One 11(5):e0156376. https://doi.org/10.1371/journal.pone.0156376
137. Wang SY, Cheng XH, Li JX, Li XY, Zhu FC, Liu P (2015) Comparing the immunogenicity and safety of 3 Japanese encephalitis vaccines in Asia-Pacific area: a systematic review and meta-analysis. Hum Vaccin Immunother 11(6):1418–1425. https://doi.org/10.1080/21645515.2015.1011996
138. Whitehead SS, Falgout B, Hanley KA, Blaney Jr JE Jr, Markoff L, Murphy BR (2003a) A live, attenuated dengue virus type 1 vaccine candidate with a 30-nucleotide deletion in the 3′ untranslated region is highly attenuated and immunogenic in monkeys. J Virol 77(2):1653–1657
139. Whitehead SS, Hanley KA, Blaney JE Jr, Gilmore LE, Elkins WR, Murphy BR (2003b) Substitution of the structural genes of dengue virus type 4 with those of type 2 results in chimeric vaccine candidates which are attenuated for mosquitoes, mice, and rhesus monkeys. Vaccine 21(27-30):4307–4316
140. Wu KY, Zuo GL, Li XF, Ye Q, Deng YQ, Huang XY, Cao WC, Qin CF, Luo ZG (2016) Vertical transmission of Zika virus targeting the radial glial cells affects cortex development of offspring mice. Cell Res 26(6):645–654. https://doi.org/10.1038/cr.2016.58
141. Yauch LE, Zellweger RM, Kotturi MF, Qutubuddin A, Sidney J, Peters B, Prestwood TR, Sette A, Shresta S (2009) A protective role for dengue virus-specific CD8+ T cells. J Immunol 182(8):4865–4873
142. Yin Z, Chen YL, Schul W, Wang QY, Gu F, Duraiswamy J, Kondreddi RR, Niyomrattanakit P, Lakshminarayana SB, Goh A, Xu HY, Liu W, Liu B, Lim JY, Ng CY, Qing M, Lim CC, Yip A, Wang G, Chan WL, Tan HP, Lin K, Zhang B, Zou G, Bernard KA, Garrett C, Beltz K, Dong M, Weaver M, He H, Pichota A, Dartois V, Keller TH, Shi PY (2009) An adenosine nucleoside inhibitor of dengue virus. Proc Natl Acad Sci U S A 106(48):20435–20439. https://doi.org/10.1073/pnas.0907010106
143. Yoneyama M, Fujita T (2007) Function of RIG-I-like receptors in antiviral innate immunity. J Biol Chem 282(21):15315–15318. https://doi.org/10.1074/jbc.R700007200
144. Yu CY, Chang TH, Liang JJ, Chiang RL, Lee YL, Liao CL, Lin YL (2012) Dengue virus targets the adaptor protein MITA to subvert host innate immunity. PLoS Pathog 8(6):e1002780. https://doi.org/10.1371/journal.ppat.1002780
145. Zellweger RM, Eddy WE, Tang WW, Miller R, Shresta S (2014) CD8+ T cells prevent antigen-

induced antibody-dependent enhancement of dengue disease in mice. J Immunol 193(8):4117–4124. https://doi.org/10.4049/jimmunol.1401597
146. Zellweger RM, Prestwood TR, Shresta S (2010) Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. Cell Host Microbe 7(2):128–139. https://doi.org/10.1016/j.chom. S1931-3128(10)00030-2 [pii]
147. Zellweger RM, Shresta S (2014) Mouse models to study dengue virus immunology and pathogenesis. Front Immunol 5:151. https://doi.org/10.3389/fimmu.2014.00151
148. Zmurko J, Marques RE, Schols D, Verbeken E, Kaptein SJ, Neyts J (2016) The viral polymerase inhibitor 7-deaza-2′-c-methyladenosine is a potent inhibitor of in vitro Zika virus replication and delays disease progression in a robust mouse infection model. PLoS Negl Trop Dis 10(5):e0004695. https://doi.org/10.1371/journal.pntd.0004695
149. Zust R, Dong H, Li XF, Chang DC, Zhang B, Balakrishnan T, Toh YX, Jiang T, Li SH, Deng YQ, Ellis BR, Ellis EM, Poidinger M, Zolezzi F, Qin CF, Shi PY, Fink K (2013) Rational design of a live attenuated dengue vaccine: 2′-o-methyltransferase mutants are highly attenuated and immunogenic in mice and macaques. PLoS Pathog 9(8):e1003521. https://doi.org/10.1371/journal.ppat.1003521
150. Züst R, Dong H, Li XF, Chang DC, Zhang B, Balakrishnan T, Toh YX, Jiang T, Li SH, Deng YQ, Ellis BR, Ellis EM, Poidinger M, Zolezzi F, Qin CF, Shi PY, Fink K (2013) Rational design of a live attenuated dengue vaccine: 2′-o-methyltransferase mutants are highly attenuated and immunogenic in mice and macaques. PLoS Pathog 9(8):e1003521. https://doi.org/10.1371/journal.ppat.1003521
151. Zust R, Toh YX, Valdes I, Cerny D, Heinrich J, Hermida L, Marcos E, Guillen G, Kalinke U, Shi PY, Fink K (2014) Type I interferon signals in macrophages and dendritic cells control dengue virus infection: implications for a new mouse model to test dengue vaccines. J Virol 88(13):7276–7285. https://doi.org/10.1128/JVI.03827-13

17 Understanding the Human T Cell Response to Dengue Virus

Laura Rivino

Abstract

Our understanding of how T cells respond to dengue virus has greatly advanced in the last decade but important questions still remain unanswered. Dengue virus infection elicits a broad anti-viral T cell response with NS3, NS4b and NS5 being the main targets for CD8+ T cells, which dominate the response while the structural proteins capsid, envelope and the secreted protein NS1 are the preferential targets for CD4+ T cells. Upon T cell activation during acute dengue infection, dengue-specific T cells acquire expression of the skin-homing marker cutaneous associated antigen (CLA) and they can be found at high frequencies in the skin of infected patients. This suggests that the skin represents an important site for the immuno surveillance of dengue virus. The immunoprotective role of skin-homing dengue-specific T cells, their potential involvement in pathological skin manifestations and their long-term persistence as tissue resident T cells to provide immediate onsite protection are open questions that we are currently investigating. The contribution of pre-existing dengue-specific T cells towards protective immunity and/or immunopathology during secondary dengue infection remains a major knowledge gap. The evidence supporting these opposing outcomes and our current understanding of the characteristics of the human T cell response to dengue virus will be discussed.

Keywords

CD4+ and CD8+ T cells · Original antigenic sin · Skin homing marker · Dengue T cells · Tissue-resident T cells

17.1 Role of T Cell Immunity and "Original Antigenic Sin"

CD4+ and CD8+ T cells are an essential component of protective immunity against viral infections and understanding their role and how they develop is critical for the design of optimal vaccines.

Acute viral infections resulting in viral clearance generally elicit effective anti-viral T cell responses that progress through three phases: a period of activation and expansion of virus-specific T cells, a contraction phase where the majority of effector cells undergo death followed by the establishment and maintenance of a pool of virus-specific memory T cells [35]. These memory T cells, together with other components

L. Rivino (✉)
Program in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore, Singapore
e-mail: laura.rivino@duke-nus.edu.sg

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_17

of the immune system, will confer protection upon re-infection with the same virus. In particular, CD8+ T cells are important for viral clearance as they can directly lyse virus-infected cells through the production of IFN-γ and cytotoxic molecules such as perforin and granzymes. These processes involve the recognition through the T cell receptor (TCR) of viral peptides of 9–10 aminoacids in length, that are presented on the cell surface of antigen presenting cells and virus-infected cells in association with molecules of the human leukocyte antigen (HLA) system. CD4+ T cells recognize longer peptides of 12–15 aminoacids in length and have a more diverse function as they are required to develop a broad and efficient antibody response and they are critical for the generation of both B cell and CD8+ T cell memory responses. In addition, CD4+ T cells can also directly kill virus-infected cells through production of IFN-γ and cytotoxic effector functions in a similar manner to CD8+ T cells.

Successful vaccines such as the vaccinia virus vaccine (which led to the eradication of small pox) and the yellow fever vaccine are known to elicit strong and long-lasting antibody and T cell responses [17, 23]. We believe that the induction of both components of the immune system is key to a successful vaccine. However, designing a vaccine for dengue virus has proved to be particularly challenging because of the co-circulation of four distinct serotypes that share approximately 70% of sequence homology. Thus, individuals living in a dengue-endemic region will be exposed to multiple dengue serotypes during their lifetime. Infection with one dengue serotype will confer life-long protective immunity towards the same serotype but evidence shows that that it increases the risk of DHF/DSS upon secondary infection with another serotype [11]. An explanation for these observations was provided by a mechanism called "antibody dependant enhancement" (ADE) whereby anti-DENV antibodies that are generated during a primary dengue infection will bind to – but poorly neutralize – the secondary infecting serotype due to variations in the peptide sequence between the two viruses. Suboptimal neutralization results in increased uptake of virus-antibody complexes through the FcγR present on antigen presenting cells with subsequent increased viral load and antigen-presentation and enhanced immune activation. In 2003 the group of Gavin Screaton proposed a similar mechanism for T cells whereby during secondary dengue infections the T cell response is dominated by weakly cross-reactive memory T cells specific for the primary infecting virus that have suboptimal anti-viral capacity towards the secondary infecting serotype but secrete pro-inflammatory cytokines such as TNF-α, which contribute to plasma leakage [18]. This process known as "original antigenic sin" was first described in a mice to impair CD8+ T cell recognition and clearance of a secondary-infecting Lymphocytic Choriomeningitis Virus (LCMV) bearing mutations in T cell epitopes [13]. However, subsequent mouse models showed that the presence of cross-reactive T cells could in some cases be beneficial. For example, pre-existing memory cells that are cross-reactive with vaccinia virus can protect mice from an otherwise lethal dose of vaccinia virus [4]. Different mouse models of secondary virus infection have shown that pre-existing cross-reactive T cell immunity can alter the hierarchy of the T cell response to a secondary-infecting virus leading to enhanced or diminished protective immunity and altered immunopathology. There is also evidence of a strong skewing of the T cell repertoire in individuals with secondary DENV infections compared to those with primary infection or in vaccinees receiving a tetravalent live-attenuated dengue vaccine as opposed to the monovalent vaccine [32, 33]. However, the implications of the skewed T cell repertoire for T cell functionality remain unclear (Fig. 17.1).

At a molecular level, because the TCR interacts with only few aminoacids within the 9–10 mer peptide, it can accommodate aminoacid variations providing they do not disrupt the peptide-TCR or the peptide-HLA interactions. However, the result of this variation in terms of avidity of the T cell for the peptide and level of T cell

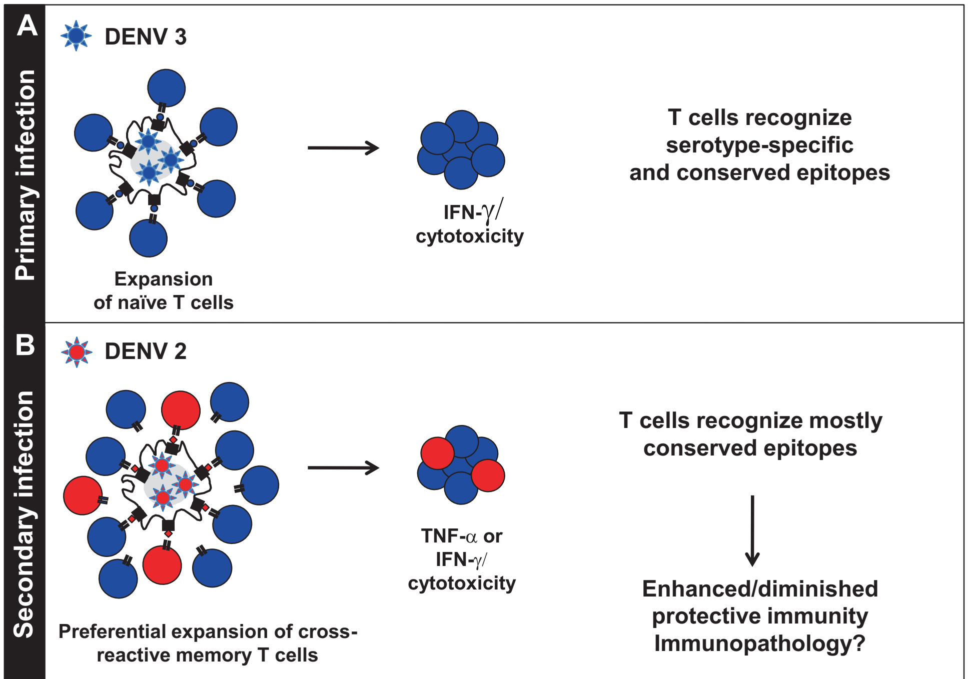


Fig. 17.1 Schematic depicting the process of "original antigenic sin" for T cells during dengue infection and its possible implications for protective immunity and/or immunopathology. (**a**) During a primary infection for example with DENV 3, naïve T cells with high avidity for DENV 3-derived peptides undergo expansion. The resulting dengue-specific T cell pool will comprise of T cells specific for peptides that are unique to DENV 3 (serotype-specific) and those that are shared amongst 2 or more serotypes (conserved). T cells that have high avidity for their cognate antigen undergo optimal TCR triggering which mainly leads to production of IFN-γ and efficient anti-viral function. (**b**) Upon secondary infection for example with DENV 2, pre-existing T cells generated during the primary DENV 3 infection that are cross-reactive to DENV 2 peptides will preferentially expand compared to their naïve counterpart. This occurs because dengue-specific memory cells are present in higher frequencies and have a lower activation threshold compared to their naïve counterparts. As a result, the responding T cell pool will be dominated by cross-reactive T cells specific for conserved epitopes. Some cross-reactive T cells may have a lower avidity for DENV 2 antigens which results in suboptimal TCR triggering and subsequent production of high levels of TNF-α and poor anti-viral efficacy with a possible contribution to immunopathology. However, variable proportions of cross-reactive memory T cells may have high avidity for DENV 2 antigens, will undergo optimal TCR triggering resulting in high levels of IFN-γ and anti-viral capacity. The outcome of the activation of cross-reactive T cells is therefore difficult to predict and could lead to faster and more efficient protective responses or to different degrees of immunopathology. Both scenarios however should result in skewing of the T cell response towards more conserved epitopes

activation is highly variable and difficult to predict. We believe that the complexity of T cell cross-reactivity is at the basis of the contrasting findings reported over the last decade that point to either a protective or a pathologic role of dengue-specific T cells during secondary dengue virus infections [22]. Several studies support the hypotheses proposed in the 2003 study [18], for example dengue-specific T cells are found to be present at higher frequencies in patients with DHF as compared to those with DF [6] and in vitro stimulation of T cells with homologous versus heterologous dengue peptides results in cytokine profiles that are both qualitatively and quantitatively distinct [9, 16, 19]. However, a number of other studies do not confirm these findings [8, 10, 14, 29, 30] and there is now accumulating evidence supporting a protective role of T cell immunity during DENV infection, including data from immune-deficient mouse models

[21, 36, 37]. A prospective study in school children shows that asymptomatic secondary dengue infections correlate with increased levels of DENV-specific T cells compared to symptomatic infection[22]. In healthy adults from dengue endemic regions HLA class I and II molecules associated with decreased susceptibility to severe dengue disease were shown to support memory T cell responses of higher magnitude, suggesting a protective rather than a detrimental role of T cells [31, 34]. Further studies that directly compare the primary versus secondary T cell response to individual DENV serotypes are urgently needed to clarify the role of these cells during infection and to reconcile the contrasting findings reported in the literature. The complex scenario that emerges from the above studies further highlights that it is mandatory for a dengue vaccine to elicit a balanced T cell (as well as antibody) response towards all four DENV serotypes.

17.2 T Cell Responses during Dengue Virus Infection

To better understand the role of T cells during dengue infection we sought to characterize the nature of the T cell response during the course of dengue infection, from acute infection to convalescence. Dengue patients were recruited by the team of Prof Leo Yee Sin at CDC Tan Tock Seng Hospital in Singapore and blood was drawn from the patients at three different time points after diagnosis of dengue infection (days from fever onset: acute day 6–9, post-febrile day 14–21 and convalescent day 60–120). Peripheral blood mononuclear cells (PBMCs) were analyzed at all time points. The first striking observation that we made was that acute dengue infection induces a massive activation and proliferation of CD8+ T cells and a significant but more modest activation and expansion of CD4+ T cells. Activated (HLA-DR+ CD38+) and proliferating (Ki67+ Bcl2-) T cells are found at highest frequencies during acute dengue and represent in some patients up to 70% of all CD8+ T cells (highlighted with a red box in Fig. 17.2a). The proportion of activated and proliferating T cells decreases in the post-febrile and convalescent phases of disease reaching levels comparable to those found in healthy individuals. Representative flow cytometry profiles of the activation and proliferation of CD4+ and CD8+ T cells from a dengue patient are shown in Fig. 17.2a and results from 18 patients are summarized in Fig. 17.2b. Given the large numbers of activated CD8+ T cells observed in the blood of acute dengue patients, it is likely that a proportion of these cells will be bystander-activated and will thus not be specific for DENV. We and others have shown that acute dengue infection is accompanied by the activation and proliferation of CD8+ T cells that are specific for persistent viruses such as human cytomegalovirus or Epstein-Barr virus [25, 26]. These cells may be activated in vivo in a TCR-independent manner through the action of cytokines such as IL-15 that are abundantly produced upon viral infection [26]. However the exact enumeration of dengue-specific T cells is challenging as we rely on functional assays such as IFN-γ production after DENV peptide stimulation which are unable to detect T cells with impaired cytokine-producing capacities and are thus likely to underestimate the number of dengue-specific T cells.

Our knowledge of the kinetics and the protein targets of the dengue-specific T cell response during acute dengue infection has greatly advanced over the last decade. We know that dengue infection elicits a DENV-specific T cell response that peaks around days 8–10 from fever onset [6, 24, 29]. The T cell response is broad as it targets all viral proteins to some extent, with a preferential recognition of the non-structural proteins NS3, NS4b and NS5. Similarly to what occurs in other acute viral infections dengue-specific CD8+ T cells are present at higher frequencies compared to CD4+ T cells. While CD8+ T cell responses preferentially target the non-structural proteins NS3 and NS5, CD4+ T cells mainly target the structural proteins capsid and envelope and the secreted proteins NS1, consistent with virions being the

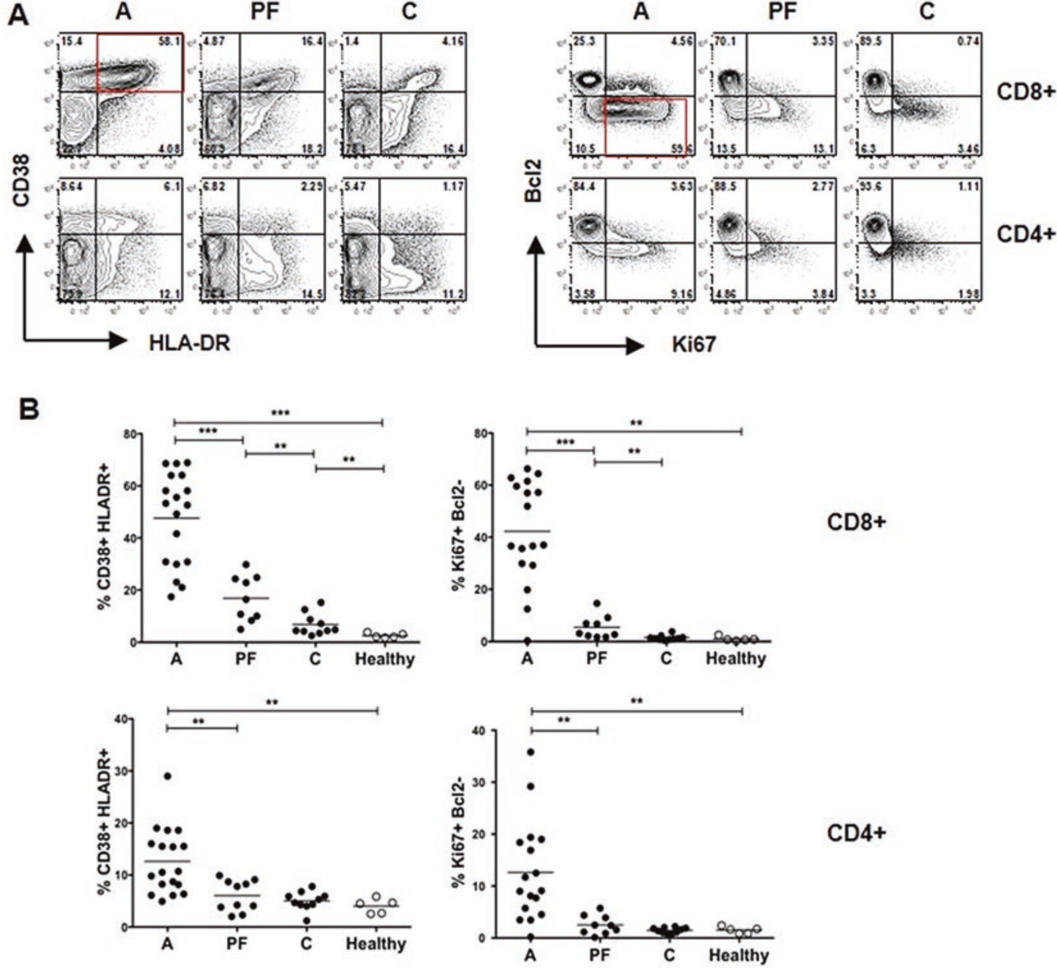


Fig. 17.2 High frequencies of activated and proliferating T cells during acute dengue infection. PBMC samples from dengue patients are analyzed during the acute (*A*), post-febrile and (PF) convalescent (*C*) phases of disease. Live cells are surface stained with antibodies against CD3, CD4, CD8, CD38 and HLA-DR followed by intracellular staining for Ki67 and Bcl2. Isotype controls are used to define negativity for CD38, HLA-DR, Ki67 and Bcl2. (**a**) Activation and proliferation profiles of CD8+ (top panel) and CD4+ T cells (bottom panel) for one representative patient. Activated and proliferating T cells are defined as CD38+ HLA-DR+ and Ki67+ Bcl2- cells, respectively (see red box for CD8+ T cells). (**b**) Results from 18 patients are summarized for CD8+ (top panel) and CD4+ T cells (bottom panel). Statistics are calculated by Mann-Whitney non-parametric test. Values are considered significant when $p < 0.05$

main source of antigen for CD4+ T cells [24]. More recent studies have reported that these patterns of immunodominance vary as a function of the infecting serotype with DENV 3 eliciting CD8+ T cells that target both structural and non-structural proteins while DENV 1, 2 and 4 elicit CD8+ T cells targeting mainly NS3, NS4b and NS5 [32, 33].

17.3 Phenotype and Tissue-Homing of Dengue-Specific T Cells

The identification of a large number of T cell epitopes, the majority of which are CD8+ T cell epitopes, has provided us with tools to better characterize T cells during dengue infection or vaccination. As of September 2017 1613 immu-

nogenic peptides have been deposited in the Immune Epitope Database (http://www.iedb.org/). In particular, the study of CD8+ T cells has been facilitated by the use of peptide-HLA tetramers which are streptavidin-labelled MHC multimers bound to the peptide of interest. These tools have revolutionized the way we can look at T cell immunity as peptide-specific CD8+ T cells can be enumerated and characterized phenotypically directly ex vivo by flow cytometry without relying on a functional read-out [1]. However, the definition of immunodominant epitopes restricted to HLA molecules that are commonly expressed in populations from dengue-endemic regions remains elusive and the selection of T cell epitopes to use for peptide-HLA tetramer analyses of dengue-infected individuals can still be a difficult task.

By using peptide-HLA tetramers for the previously described HLA-A*1101-restricted dengue NS3 27 epitope [18] we addressed the functional and phenotypic characteristics of dengue-specific T cells during the course of dengue infection. We show that during secondary acute dengue infection NS3–27-specific T cells are highly activated and proliferating, produce IFN-γ but not TNF-γ and are able to lyse dengue-infected cells, suggesting that at least in vitro their anti-viral function does not appear to be impaired [25]. To address the capacity of dengue-specific T cells to migrate to different organs and to potentially contribute to immunopathology, we studied the homing receptors present on the surface of these cells. During acute dengue infection NS3 27-specific CD8+ T cells express the skin homing molecule cutaneous lymphocyte-associated antigen (CLA), the chemokine receptors CXCR3 and CCR5 which allow migration to inflamed tissues, but not the gut-homing receptor CCR9. Expression of CLA but not of CCR9 was confirmed for the total population of CD4+ and CD8+ T cells specific for NS3 and NS5 (Fig. 17.3a, b). We show that expression of the skin-homing marker CLA by dengue-specific T cells correlates with their presence in the skin tissue where they could be detected at higher frequencies as compared to peripheral blood [25] (Fig. 17.3c). The exact role of dengue-specific T cells in the skin during acute infection, whether they persist long-term in this site as tissue-resident memory T cells upon resolution of the infection and their possible protective role upon secondary infections is currently under investigation. The expression of CLA on dengue-specific T cells during acute dengue is consistent with their initial activation by skin-derived dendritic cells. Dengue virus enters the body through the skin during the blood meal of an infected mosquito, infects local dendritic cells [3, 28] and travels to skin-draining lymph-nodes where it interacts with virus-specific T cells. Studies have shown that during activation T cells acquire the capacity to migrate to certain tissues by expressing different sets of receptors that serve as "address-codes", the expression of which is determined by the antigen presenting cell and by the local microenvironment [7]. It was shown for example that during T cell priming, tissue-derived antigen presenting cells within cutaneous versus intestinal secondary lymphoid organs imprint the corresponding tissue-specific homing phenotype to the T cell, such that the primed T cells have a predisposition to home back to the skin or gut, respectively [2, 20]. More recently, studies have shown that the route of immunization strongly impacts the migratory capacity and consequently the protective efficacy of T cells upon re-infection, as T cells need to localize to the sites of virus replication in order to exert their function [15, 27]. For example, vaccination with live vaccinia virus was 10^5 times more effective in protecting against viral re-infection if delivered by skin scarification as compared to subcutaneous, intradermal, and intramuscular vaccination [12]. The authors show that immunization by skin scarification was associated with local keratinocyte infection and generation of a long-lived tissue resident CD8+ T cell memory populations. The presence of tissue resident CD8+ T cells was sufficient to protect against re-infection with vaccinia virus and did not require antibodies or recruitment of circulating blood T cells [15]. These considerations highlight an important aspect of T cell immunity that is often not considered in the context of vaccination. We now know that pathogen infection generates

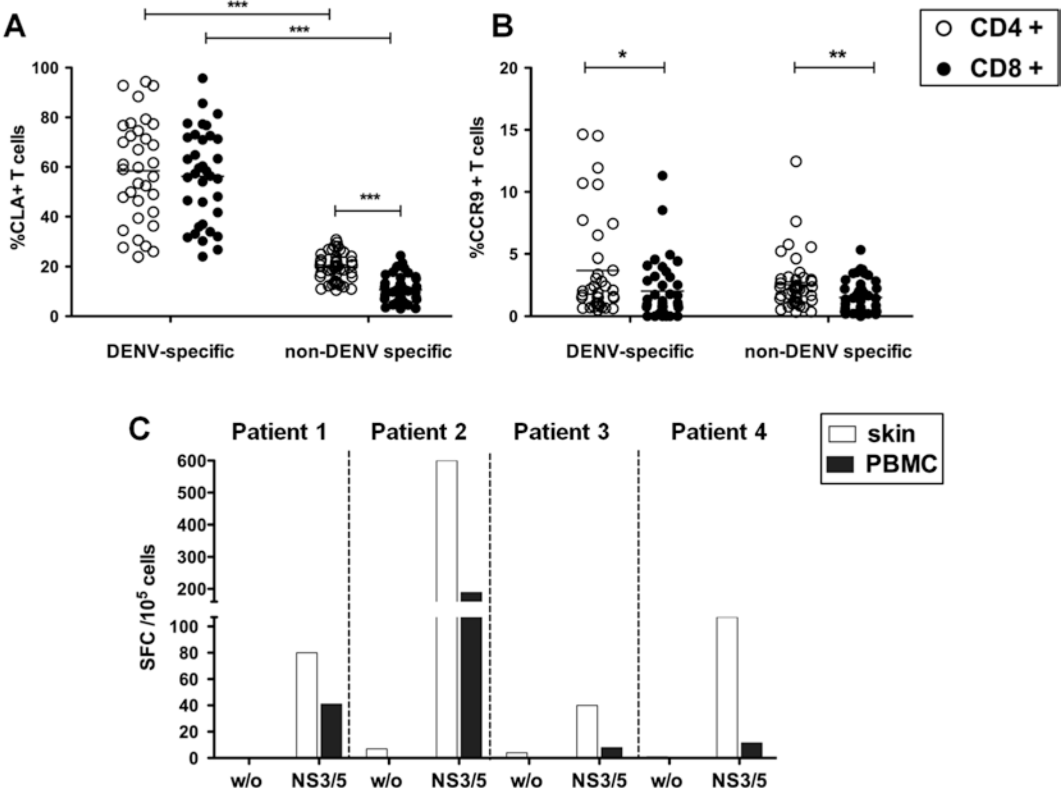


Fig. 17.3 Dengue-specific T cells have skin-tissue migratory properties during acute dengue infection. Expression of the skin-homing receptor CLA (**a**) and the gut-homing receptor CCR9 (**b**) is assessed by flow cytometry on dengue-specific T cells from the peripheral blood of acute dengue patients. PBMCs are briefly stimulated with or without NS3 and NS5 peptides and live cells are stained for expression of CD3, CD4, CD8, CLA and CCR9 followed by intracellular cytokine staining for IFN-γ and TNF-α. Expression of CLA and CCR9 is analysed on cytokine-producing CD4+ and CD8+ T cells (defined as "DENV-specific") or on those that lack cytokine production (defined "non-DENV specific"). The percentage of CLA+ or CCR9+ cells is shown for CD4+ (white circles) and CD8+ T cells (black circles). Isotype controls are used to define negativity for CLA and CCR9 expression. (**c**) The presence of dengue-specific T cells in the skin was investigated by raising skin suction blisters on the forearm of dengue patients. PBMCs or cells obtained from skin suction blisters are stimulated with or without NS3 and NS5 peptides and analysed for production of IFN-γ by ELISPOT. Results obtained from cells derived from the skin (white bar) or the peripheral blood (black bar) are expressed as Spot Forming Cells relative to 10^5 cells (SFC/10^5). Statistics were calculated using a Kruskal-Wallis test, followed by a non-parametric Mann-Whitney test. (Adapted from Rivino et al. Sci Trans Med [25])

populations of non-recirculating tissue-resident memory T cells that persist long-term within tissues such as the skin, lung, gastro-intestinal tract and reproductive tract. These cells have potent effector functions and provide rapid on-site protection in peripheral tissues upon re-infection with previously-encountered pathogens [5]. A better understanding of the localization of dengue-specific memory T cells in dengue-immune individuals and how these cells can be generated in the context of vaccination would greatly aid the design of dengue vaccines with improved protective efficacy.

Acknowledgments This work is supported by a Cooperative Basic Research Grant- New Investigator Grant (CBRG-NIG R-913-301-289-213) awarded by the Singapore National Medical Research Council to Laura Rivino.

Discussion of Chapter 17 in Dengue and Zika: Control and Antiviral Treatment Strategies

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Laura Rivino.

Anuja Mathew: So you found higher frequencies of CD8+ T cells in the skin. Do you have an hypothesis of why you would see a preference for CD8+ or CD4+ T cells?

Laura Rivino: We have preliminary data showing that the dengue-specific T cells that we find in the skin are mostly CD8+ T cells, so we think these cells could account in part for the increase of total CD8+ T cells that we find during acute dengue in the skin. We do not expect this influx of CD8+ T cells to comprise of only dengue-specific T cells. We know, for example from studies performed by us and others that in the blood of acute dengue patients there is also "bystander-activation" of CD8+ T cells that are specific for non-related, persistent viruses such as HCMV and EBV. So the influx of CD8+ T cells that occurs in the skin of acute dengue patients could comprise of both dengue-specific and "bystander-activated" T cells.

This higher frequncies of CD8+ T cells that we observe in the skin of acute dengue patients was not surprising to us since for other skin viral infections, such as Herpex simplex virus, the anti-viral response at the skin level is mainly a CD8+ T cell response.

Katja Fink: The cytokines that you find in the blister fluid are more or less the same as what is observed in the plasma or in the skin?

Laura Rivino: No. So the increase in pro-inflammtory cytokines is more pronounced in the skin compared to the blood. We have done quite a lot of cytokine profiling from the plasma of dengue patents and the increase in pro-inflammatory cytokines at the time points we look at are obvious only for the more severe dengue cases. We generally do not see significant increases of these cytokines in the blood, especially of TNF-α.

Katja Fink: So you compared skin and blood samples from the same patients?

Laura Rivino: Yes.

Aravinda da Silva: Were these cases primary infections?

Laura Rivino: Most of our patients are experiencing secondary infections. We had few primary infections so it's difficult to really make a statement, but when I looked seperately at patients with primary and secondary infection I didn't see a very big difference in terms of immunodominance of the different dengue proteins. The immunodominance was similar in primary and secondary cases and also for different dengue serotypes as well.

Aravinda da Silva: Right, but in terms of seeing the cells so early in the skin by day four of onset of symptoms, it seems very fast for a primary response. Do you see it that fast and in those numbers even in primary infection?

Laura Rivino: We don't have the data yet of which patients were experiencing primary or secondary infection for the skin samples.

References

1. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM (1996) Phenotypic analysis of antigen-specific T lymphocytes. Science 274(5284):94–96
2. Campbell DJ, Butcher EC. (2002) Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. J Exp Med 195(1):135–141. The Rockefeller University Press. https://doi.org/10.1084/jem.20011502
3. Cerny D, Haniffa M, Shin A, Bigliardi P, Tan BK, Lee B, Poidinger M, Tan EY, Ginhoux F, Fink K (2014) Selective susceptibility of human skin antigen presenting cells to productive dengue virus infection (Kuhn RJ (ed)). PLoS Pathogens 10(12):e1004548. Public Library of Science. https://doi.org/10.1371/journal.ppat.1004548
4. Chen HD, Fraire AE, Joris I, Brehm MA, Welsh RM, Selin LK (2001) Memory CD8+ T cells in heterologous antiviral immunity and immunopathology in the lung. Nat Immunol 2(11):1067–1076. https://doi.org/10.1038/ni727

5. Clark RA (2015) Resident memory T cells in human health and disease. Sci Transl Med 7(269)::269rv1–269rv1. American Association for the Advancement of Science. https://doi.org/10.1126/scitranslmed.3010641
6. Duangchinda T, Dejnirattisai W, Vasanawathana S, Limpitikul W, Tangthawornchaikul N, Malasit P, Mongkolsapaya J, Screaton G (2010) Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. Proc Natl Acad Sci 107(39):16922–16927. https://doi.org/10.1073/pnas.1010867107
7. Dudda JC, Simon JC, Martin S (2004) Dendritic cell immunization route determines CD8+ T cell trafficking to inflamed skin: role for tissue microenvironment and dendritic cells in establishment of T cell-homing subsets. J Immunol 172(2):857–863
8. Dung NTP, Duyen HTL, Thuy NTV, Ngoc TV, Chau NVV, Hien TT, Rowland-Jones SL et al (2010) Timing of CD8+ T cell responses in relation to commencement of capillary leakage in children with dengue. J Immunol 184(12):7281–7287. https://doi.org/10.4049/jimmunol.0903262
9. Friberg H, Burns L, Woda M, Kalayanarooj S, Endy TP, Stephens HAF, Green S, Rothman AL, Mathew A (2010, April) Memory CD8+ T cells from naturally acquired primary dengue virus infection are highly cross-reactive. Immunol Cell Biol:1–8. Nature Publishing Group. https://doi.org/10.1038/icb.2010.61
10. Friberg H, Bashyam H, Toyosaki-Maeda T, Potts JA, Greenough T, Kalayanarooj S, Gibbons RV et al (2011) Cross-reactivity and expansion of dengue-specific T cells during acute primary and secondary infections in humans. Sci Rep 1(August). https://doi.org/10.1038/srep00051
11. Halstead SB (2014) Dengue antibody-dependent enhancement: knowns and unknowns. Microbiol Spect 2(6). American Society of Microbiology. https://doi.org/10.1128/microbiolspec.AID-0022-2014
12. Jiang X, Clark RA, Liu L, Wagers AJ, Fuhlbrigge RC, Kupper TS (2012) Skin infection generates non-migratory memory CD8+ TRM cells providing global skin immunity. Nature 483(7388):227–231. https://doi.org/10.1038/nature10851
13. Klenerman P, Zinkernagel RM (1998) Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. Nature 394(6692):482–485. https://doi.org/10.1038/28860
14. Kurane I, Matsutani T, Suzuki R, Takasaki T, Kalayanarooj S, Green S, Rothman AL, Ennis FA (2011) T-Cell Responses to Dengue Virus in Humans. Trop Med Health 39(4 Supplement):S45–S51. https://doi.org/10.2149/tmh.2011-S09
15. Liu L, Zhong Q, Tian T, Dubin K, Athale SK, Kupper TS (2010) Epidermal injury and infection during poxvirus immunization is crucial for the generation of highly protective T cell – mediated immunity. Nat Med 16(2):224–227. Nature Publishing Group. https://doi.org/10.1038/nm.2078
16. Mangada MM, Rothman AL (2005) Altered cytokine responses of dengue-specific CD4+ T cells to heterologous serotypes. J Immunol 175(4):2676–2683
17. Miller JD, van der Most RG, Akondy RS, Glidewell JT, Albott S, Masopust D, Murali-Krishna K et al (2008) Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. Immunity 28(5):710–722. https://doi.org/10.1016/j.immuni.2008.02.020
18. Mongkolsapaya J, Dejnirattisai W, Xu X-n, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, Sawasdivorn S et al (2003) Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med 9(7):921–927. https://doi.org/10.1038/nm887
19. Mongkolsapaya J, Duangchinda T, Dejnirattisai W, Vasanawathana S, Avirutnan P, Jairungsri A, Khemnu N et al (2006) T cell responses in dengue hemorrhagic fever: are cross-reactive T cells suboptimal? J Immunol 176(6):3821–3829
20. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, von Andrian UH (2003) Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nat Publ Group 424(6944):88–93. https://doi.org/10.1038/nature01726
21. Prestwood TR, Morar MM, Zellweger RM, Miller R, May MM, Yauch LE, Lada SM, Shresta S (2012) Gamma interferon (IFN-Γ) receptor restricts systemic dengue virus replication and prevents paralysis in IFN--A/B receptor-deficient mice. J Virol 86(23):12561–12570. American Society for Microbiology. https://doi.org/10.1128/JVI.06743-11
22. Rivino (2016) T cell immunity to dengue virus and implications for vaccine design. Expert Rev Vaccines 15(4):443–453. https://doi.org/10.1586/14760584.2016.1116948
23. Rivino L, Messi M, Jarossay D, Lanzavecchia A, Sallusto F, Geginat J (2004) Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4+ central memory T cells. J Exp Med 200(6):725–735. https://doi.org/10.1084/jem.20040774
24. Rivino L, Kumaran EAP, Jovanovic V, Nadua K, Teo EW, Pang SW, Teo GH et al (2013) Differential targeting of viral components by CD4+ versus CD8+ T lymphocytes in dengue virus infection. J Virol 87(5):2693–2706. https://doi.org/10.1128/JVI.02675-12
25. Rivino L, Kumaran EA, Thein TL, Too CT, Hao Gan VC, Hanson BJ, Wilder-Smith A et al (2015) Virus-specific T lymphocytes home to the skin during natural dengue infection. Sci Transl Med 7(278):278ra35–278ra35. https://doi.org/10.1126/scitranslmed.aaa0526
26. Sandalova, E, Laccabue D, Boni C, Tan AT, Fink K, Ooi EE, Chua R, Shafaeddin Schreve B, Ferrari C, Bertoletti A (2010) Contribution of herpesvirus specific CD8 T cells to anti-viral T cell response in humans (Selin L (ed)). PLoS Pathogens 6(8): e1001051. https://doi.org/10.1371/journal.ppat.1001051

27. Sandoval F, Terme M, Nizard M, Badoual C, Bureau MF, Freyburger L, Clement O et al (2013) Mucosal imprinting of vaccine-induced CD8+ T cells is crucial to inhibit the growth of mucosal tumors. Sci Transl Med 5(172):172ra20–172ra20. https://doi.org/10.1126/scitranslmed.3004888
28. Schmid MA, Harris E (2014) Monocyte recruitment to the dermis and differentiation to dendritic cells increases the targets for dengue virus replication (Richard JK (ed)). PLoS Pathogens 10(12):e1004541. Public Library of Science. https://doi.org/10.1371/journal.ppat.1004541
29. Simmons CP, Dong T, Chau NV, Dung NTP, Chau TNB, Thao LTT, Dung NT, Hien TT, Rowland-Jones S, Farrar J (2005) Early T-cell responses to dengue virus epitopes in Vietnamese adults with secondary dengue virus infections. J Virol 79(9):5665–5675. https://doi.org/10.1128/JVI.79.9.5665-5675.2005
30. Townsley E, Woda M, Thomas SJ, Kalayanarooj S, Gibbons RV, Nisalak A, Srikiatkhachorn A et al (2014) Distinct activation phenotype of a highly conserved novel HLA-B57-restricted epitope during dengue virus infection. Immunology 141(1):27–38. https://doi.org/10.1111/imm.12161
31. Weiskopf, Daniela, Angelo MA, de Azeredo EL, Sidney J, Greenbaum JA, Fernando AN, Broadwater A et al (2013) Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. Proc Nat Acad Sci U S A 110(22):E2046–2053. National Acad Sciences. https://doi.org/10.1073/pnas.1305227110
32. Weiskopf D, Angelo MA, Sidney J, Peters B, Shresta S, Sette A (2014a) Immunodominance changes as a function of the infecting dengue virus serotype and primary versus secondary infection. J Virol 88(19):11383–11394. https://doi.org/10.1128/JVI.01108-14
33. Weiskopf D, Angelo MA, Bangs DJ, Sidney J, Paul S, Peters B, de Silva AD et al (2014b) The human CD8 +T cell responses induced by a live attenuated tetravalent dengue vaccine are directed against highly conserved epitopes (Diamond MS (ed)). J Virol 89(1):120–128. https://doi.org/10.1128/JVI.02129-14
34. Weiskopf D, Bangs DJ, Sidney J, Kolla RV, de Silva AD, de Silva AM, Crotty S, Peters B, Sette A (2015) Dengue virus infection elicits highly polarized CX3CR1 +Cytotoxic CD4 +T cells associated with protective immunity. Proc Natl Acad Sci 112(31):E4256–E4263. https://doi.org/10.1073/pnas.1505956112
35. Wherry EJ, Ahmed R (2004) Memory CD8 T-cell differentiation during viral infection. J Virol 78(11):5535–5545. https://doi.org/10.1128/JVI.78.11.5535-5545.2004
36. Yauch LE, Zellweger RM, Kotturi MF, Qutubuddin A, Sidney J, Peters B, Prestwood TR, Sette A, Shresta S (2009) A protective role for dengue virus-specific CD8+ T cells. J Immunol 182(8):4865–4873. https://doi.org/10.4049/jimmunol.0801974
37. Yauch, Lauren E, Prestwood TR, May MM, Morar MM, Zellweger RM, Peters B, Sette A, Shresta S (2010) CD4+ T cells are not required for the induction of dengue virus-specific CD8+ T cell or antibody responses but contribute to protection after vaccination. J Immunol (Baltimore MD, 1950) 185(9):5405–5416. American Association of Immunologists. https://doi.org/10.4049/jimmunol.1001709.185

18

Regulation and Function of NK and T Cells During Dengue Virus Infection and Vaccination

Anuja Mathew

Abstract

The focus of this review is to discuss findings in the last 10 years that have advanced our understanding of human NK cell responses to dengue virus. We will review recently identified interactions of activating and inhibitory receptors on NK cells with dengue virus, human NK responses to natural dengue infection and highlight possible interactions by which NK cells may shape adaptive immune responses. T cell responses to natural dengue infection will be reviewed by Laura Rivino in Chap. 17. With the advent of numerous dengue vaccine clinical trials, we will also review T and NK cell immune responses to dengue virus vaccination. As our understanding of the diverse functions of NK cell has advanced, it has become increasingly clear that human NK cell responses to viral infections are more complicated than initially recognized.

Keywords

Innate immune system · KIR peptide-MHC interactions · Inhibitory receptors on NK cells · Activationg receptors on NK cells · T-cell response to dengue vaccine

A. Mathew (✉)
Department of Cell and Molecular Biology, Institute for Immunology and Informatics, Providence, RI, USA
e-mail: mathewa@uri.edu

18.1 Introduction

NK cells are large granular lymphocytes that are a component of the innate immune system. NK cells kill target cells spontaneously without the requirement of priming or the expression of major histocompatibility antigens (MHC) on the target cell. NK cells lack the receptor diversity generated by DNA rearrangement found in prototype members of the adaptive immune response, T and B cells. However, similar to T cells, NK cells are able to distinguish healthy cells from virally infected cells. NK cells produce abundant antiviral cytokines, release cytotoxic granules such as granzyme B and trigger the FAS/TRAIL pathway to induce apoptosis of infected cells [35]. In order to mount a response, NK cells have to integrate signals received from many inhibitory and activating receptors to sense the environment and respond. Regulation of NK cell activity is achieved by a balance between these inhibitory and activating signals although signals received by inhibitory receptors tend to be dominant [21]. Many of the initial insights on NK cell function came from studying chronic viral infections in mice such as LCMV and MCMV [7]. The clearest demonstration that NK cells are important to humans stem from the findings that patients with NK cell deficiencies develop severe, recurrent viral infections [6, 54]. Our knowledge of select receptors on human NK cells modulated by viral

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_18

infection and viral peptides that can modulate NK cell function has significantly advanced in the last decade in the context of human viral infections such as HMCV, HIV and HCV [7, 19]. Some of the findings seen in these human chronic viral infections are beginning to be observed for viral infections such as Influenza and Dengue thus opening up new areas of research that will enhance our understanding of how these innate effector cells can control acute viral infections [1, 4, 57, 58, 72]

18.2 Early and Current Observations on NK Cell Responses to Dengue Infections Using Samples from Clinical Cohorts

Human NK cells were shown to lyse dengue virus (DENV)-infected cells to a greater degree compared to uninfected cells over 30 years ago [38]. Antibodies to DENV further augmented the lysis of DENV-infected cells by NK cells. The traditional phenotype used to define human NK cells is based on the absence of CD3 (to exclude T cells) and relative expression of CD56 (neural cell adhesion molecule) and CD16 (low-affinity receptor for the Fc portion of immunoglobulin G). It is now appreciated that CD56 "bright" cells can produce cytokines abundantly but are poorly cytolytic while CD56 "dim" cells (which represent 90% of circulating NK cells) produce moderate cytokines when stimulated but are strongly cytolytic [60]. Using PBMC samples obtained from children in Thailand undergoing severe dengue infection nearly 20 years ago, Green et al. observed increased frequencies of activated NK cells (CD56+ CD69+ cells) in PBMC from patients with severe dengue disease compared to patients with milder disease suggesting that that NK cells are actively involved in the defense against dengue virus [26]. Homchampa et al. found evidence of NK cell cytotoxicity in fresh cells from children with acute dengue and the level of cytotoxicity was related to disease severity [31]. At the time, many of the activating and inhibitory receptors present on NK cells were not identified and the technologies to assess markers on individual NK cells using multiparametric flow cytometry and CyTOF were not available.

In a study published in 2008, frequencies of CD56+ CD69+ NK cells were found to be more activated in hospitalized Vietnamese infants with more severe dengue disease [15]. Recently Petitdemange et al. evaluated NK cell activation in patients from Gabon undergoing acute dengue infection [59]. They examined frequencies of subsets of NK cells and included a number of NK cell receptors NKp30, NKG2A, NKp44 and CD161 which was a significant advance from the early studies where only CD56 expression was used to identify NK cells. Compared to healthy donors, NK cells from patients who had DENV-2 infections expressed significantly less NKp30, NKG2A, and CD161, whereas expression of ILT-2 was increased at the early acute and convalescent time point. Interestingly, frequencies of NKp44+ NK cells were elevated at the early acute time point. Principal component analysis highlighted an association between NKp44 and DENV-2 infections. NKp44 has previously been shown to directly interact with the envelope protein of DENV and WNV and this subset of NK cells and its ligand in target cells is worthy of study in other cohorts [30]. While there have been only a few studies that have looked at NK cell responses in clinical cohorts, the data indicate that subsets of NK cells are involved in the immune responses to natural dengue infection thus warranting further study. NK cell responses in individuals receiving live attenuated tetravalent dengue vaccines have not been examined in any detail. Whether different subsets of NK cells respond to vaccination with live attenuated dengue vaccines versus subunit vaccines and inactivated vaccine antigens are currently unknown. Furthermore, whether the magnitude of NK cell responses is different between vaccination and natural infection has not been explored.

18.3 Activating Receptors on NK Cells

NK cells have a number of receptors that can activate NK cell reactivity (activating receptors) or dampen reactivity (inhibitory receptors). Activating receptors expressed on human NK cells include the C-type lectin receptors NKG2D and NKG2C/E, natural cytotoxicity receptors (NCR) NKp44, NKp30, NKp46, and CD16 (FC-γ-RIII), the low-affinity Fc receptor which mediates antibody dependent cytotoxicity (ADCC) (Fig. 18.1 and Table 18.1). Of the many activating receptors on NK cells and ligands on target cells, there is evidence for interaction of DENV with NKp44, the low-affinity FcR receptor CD16 and ligands for the NKG2D ligand MICB (Fig. 18.1).

In a large genome-wide association study (GWAS) performed in 2011, a single nucleotide polymorphism in MICB (ligand for NKG2D) was found to be a susceptibility locus for severe dengue disease dengue shock syndrome in over 2000 Vietnamese children [37]. In a subsequent study by the same group, MICB was also associated with the less severe form of dengue DF in both children and adults in Vietnam [77]. This genetic susceptibility of a SNP in MICB was confirmed in another study in the Thai population. In Cuban individuals (Guzman), a strong association of DF and DHF with MICA 008 and MICB 008 compared to asymptomatic individu-

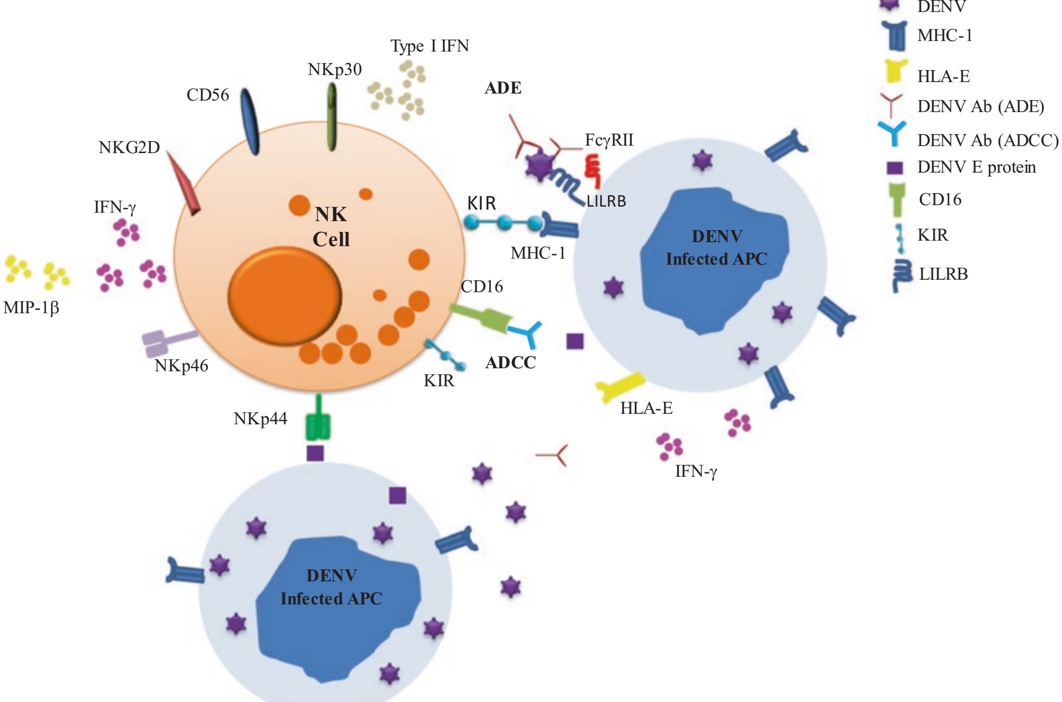


Fig. 18.1 Model of NK cell interaction with virus-infected cells. NK cells can interact with DENV infected target cells through direct and indirect mechanisms. To date, only NKp44 has been shown to directly interact with the E protein of DENV. NK cells can interact with DENV infected APCs by responding to cytokines, interacting with yet to be identified ligands on virus-infected cells and upregulating inhibitory or activating receptors on its surface. This may lead to degranulation and abundant cytokine secretion by NK cells and lysis of virus-infected cells. Alternatively, dominant interaction of inhibitory receptors on NK cells with viral proteins and peptides could dampen responses and represent an immune evasion strategy by DENV. NK cells can also be directed to DENV infected APCs through engagement of CD16 with the Fc receptor of DENV-specific Abs and target cells are lysed through ADCC

Table 18.1 NK cell receptors and ligands

Receptor[a]	Ligand[b]	Function[c]	DENV	Reference
CD16	IgG	Activating		[40]
CD94-NKG2C	HLA-E	Activating		[8, 36],
CD94-NKG2E	HLA-E	Activating		[36]
NKG2D	MICA, MICB, ULBP	Activating	GWAS [37]	[2]
NKp30	Unknown	Activating		[55]
NKp44	Viral hemagglutinins	Activating	DENV protein E [30]	[11, 30]
NKp46	Unknown	Activating		[56]
CD161	LLT1	Activating and inhibitory		[46, 61]
CD38	CD31	Activating		[20, 49, 67]
KIR2DS1	HLA-C	Activating		[52]
KIR2DS2	HLA-C	Activating		[52]
KIR2DS3	HLA-C	Activating		
KIR2DS4	HLA-Cw4	Activating		[25]
KIR2DS5	Unknown	Activating		
KIR3DS1	HLA-Bw4?	Activating		
Cytokine receptors	Type 1 IFN, IL-1,2,12,15,18	Activating		
Cytokine receptors	TGF-β	Inhibitory		
KIR2DL1	HLA-Cw4	Inhibitory		[17, 48]
KIR2DL2	HLA-Cw3	Inhibitory		[17, 48]
KIR2DL3	HLA-Cw3	Inhibitory		[17, 48]
KIR2DL4	HLA-G	Activating? Inhibitory?		[23, 62]
KIR2DL5A/B	Unknown	Inhibitory		
KIR3DL1	HLA-Bw4	Inhibitory	KIR3DL1 and HLA B57 interaction [72]	[73])
KIR3DL2	HLA-B27, HLA-A3, HLA-A11	Inhibitory		[28, 78]
KIR3DL3	HLA-Cw4	Inhibitory		
CD94-NKG2A	HLA-E	Inhibitory		[8]
2B4	CD48	Activating or inhibitory, coreceptor		[42, 9]
LILRB1	HLA class I	Inhibitory	Dengue and ADE [14]	[10]

[a]Receptor present on the surface of NK cells
[b]Ligand(s) bound by the NK cell receptor
[c]Functional effect of receptor signaling in NK cells

als [24]. Interestingly soluble MICB levels were increased in the sera between pre-infection and acute illness among infants with symptomatic primary DENV infections in the Philippines and suggest an immune evasion strategy in dengue viral infection [43]. The data suggest that ligands for the activating receptor NKG2D, MICB may be involved in the immune response against DENV.

A direct interaction has been shown between the NK-activating receptor NKp44 and the DENV and WNV envelope protein [30]. Recombinant NKp44 directly bound purified DENV and WNV envelope proteins. The results suggest that NKp44 expressing NK cells are able to interact directly with the E protein on flavivirus-infected cells.

NK cells express the FcγRIIIA receptor (CD16) that binds the constant (Fc) domain of IgG antibodies. CD16 engagement is a strong activator of NK cell function and allows antigen-specific recruitment of NK responses. Very early on, NK cell-mediated lysis of DENV-2-infected Raji cells was significantly higher when these

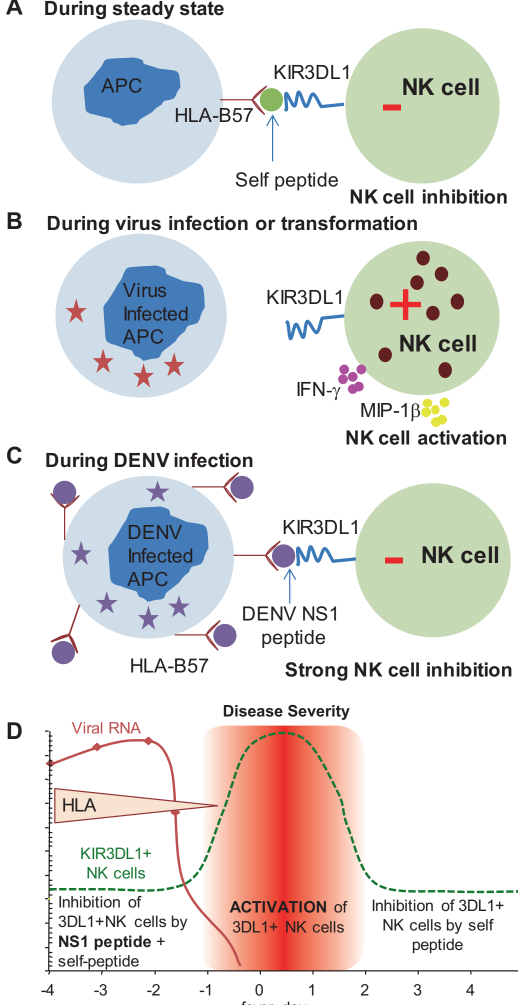


Fig. 18.2 A model of NK-cell inhibition during DENV infection. Specific amino acid changes in the peptides presented by major histocompatibility complex (MHC) class I can impact KIR recognition of peptide/MHC complexes. We hypothesize that the DENV NS1 peptide generated during infection presented by HLA B57 alters the affinity of the inhibitory KIR3DL1 receptor expressed on NK cells for its putative ligand, resulting in the inhibition of NK cells early during viral infection while MHC class I is upregulated. Following the clearance of viremia, HLA-B57 levels return to normal which decreases the inhibitory signal received by KIR3DL1⁺ NK cells. This release of inhibition allows KIR3DL1⁺ NK cells to be activated at fever day 0, coincident with the activation of CD8⁺ T cells

cells were pre-incubated with DENV immune serum through antibody dependent cell-mediated cytotoxicity (ADCC) [38]. More recently efforts have been made to understand in a prospective cohort whether ADCC activity in sera obtained pre-illness in a prospective cohort correlated with subsequent dengue disease severity and viremia levels. A higher level of ADCC activity measured in immune sera before secondary DENV-3 but not secondary DENV-2 infection was associated with lower subsequent viremia levels [41]. These results suggest a protective role for ADCC antibodies in sera obtained from in some children with secondary infection. In vivo, characteristics of both the Abs and NK cells in each individual are likely to impact the function of these Abs. Future studies should compare the contribution of functionally distinct Abs (those that mediate neutralization versus Abs that mediate ADCC) in the sera of individuals who subsequently develop clinical versus subclinical disease.

In order to understand soluble and membrane found factors important for activating NK cells, Lim et al. used DENV-infected monocyte-derived dendritic cells in co-culture with autologous NK cells from healthy donors [44]. The results suggest that a combination of cytokines type I IFNs, TNF-□ secreted by DCs and cell surface receptor–ligand interactions all play some part triggering the anti-dengue response of primary human NK cells while each of these in isolation has a low to minimal response on the activation of human NK cells.

18.4 Inhibitory Receptors on NK Cells

18.4.1 KIRs

A major set of inhibitory receptors important for NK cell licensing is the killer cell immunoglobulin-like receptors (KIRs). The KIR receptors are expressed stochastically, leading to the generation of subpopulations of NK cells within each individual [50]. While only some KIR ligands are known, all are thought to be class I MHC molecules (Table 18.1). Epidemiological studies of immunological events, including responses to infectious diseases, autoimmune diseases, complications with

pregnancy, and tumor responses, have implicated KIR/MHC interactions in protective or pathological roles [5, 34, 32, 39, 53, 63, 68].

In contrast to the T-cell receptor, which recognizes a given viral peptide-MHC complex, KIRs typically recognize subsets of MHC class I molecules with common amino acid motifs in their α1 domains ([13, 33]). However, several studies have shown that inhibitory KIRs are also sensitive to the specific peptides bound by the HLA molecules [12]. In particular, residues 7 and 8 of endogenously derived peptides bound to MHC class I can promote or abrogate binding of KIR. During viral infection, the peptide repertoire changes and a number of viral peptides are presented on MHC molecules for recognition by cytotoxic T lymphocytes. Changes in the endogenous repertoire of peptides can perturb or continue to maintain inhibition of NK cells (Fig. 18.2) depending on how strongly the viral peptide is able to bind and replace the endogenous peptide. Several viral peptides (HIV, HCV, SIV) have been shown to modulate KIR-MHC interactions thus providing a mechanism for the virus to modulate the interaction of the inhibitory KIR with its MHC ligand thus preventing the activation of subsets of NK cells [22, 47]. How changes in the peptide repertoire following viral infection alter NK cell recognition in vivo will depend on the relative amounts of viral peptides being presented, the nature of the peptides produced by infection and the frequency of NK cells expressing the relevant KIR.

18.5 KIR Peptide-MHC Interactions During Dengue Infection

During a study of HLA B57 restricted T cells in acute dengue infection, Townsley et al. found that a T cell tetramer for a CD8+ T cell epitope in the NS1 protein of DENV bound CD56^{dim} NK cells [72]. Using depletion studies and KIR-transfected cell lines, the HLA B57 restricted dengue NS1 tetramer was found to bind the inhibitory receptor KIR3DL1 on NK cells clearly demonstrating an interaction with an inhibitory KIR. Using clinical samples from a long-standing cohort in Thailand the authors further found late activation of NK cells in HLA B57+ individuals. The peak of NK cell activation occurred at the critical time point in people with severe dengue disease which supported the hypothesis that the HLA B57 restricted NS1 peptide can modulate the function of KIR3DL1+ NK cells. The data strongly suggest that subpopulations of NK cells may contribute to disease pathogenesis and enabled the authors to speculate how the NS1 peptide may modulate function during acute dengue infection. At steady state (Fig. 18.1a), the interaction of an HLA B57 presenting endogenous peptides on an antigen presenting cell (APC) with KIR3DL1 maintains an inhibitory signal in NK cells thus preventing lysis of healthy target APCs. During many viral infections, HLA molecules are downregulated which allows NK cells to get activated and lyse virus-infected APCs (Fig. 18.1b). However it is well known that in DENV infection HLA class I molecules are upregulated [79]. We hypothesize that presentation of the NS1 peptide on HLA B57 during the viremic phase in infected individuals maintains the inhibitory interaction with KIR3DL1 (since the tetramer has been shown to bind KIR3DL1) thus delaying the activation of this important subset of NK cells (Fig. 18.1c, d). As viremia decreases, Class I expression decreases and NK cells are able to sense the change in Class I expression, triggering their activation during the critical phase of illness (around fever day 0) (Fig. 18.1d). Our clinical data support our hypothesis but we are yet to show functional alteration of NK cell responses by the NS1 peptide.

We are currently using NK cell sensitive TAP-impaired and TAP-blocked target cells (721.221) transfected with HLA alleles in functional assays. Since self-peptide is able to maintain inhibition, the use of TAP blocked and TAP impaired cell lines HLA transfected cell lines are essential to evaluate whether viral peptides added exogenously such as the DENV NS1 peptide can modulate the function of KIR3DL1+ NK cells. We anticipate that additional KIR/HLA interactions occur which are likely to be modulated by viral peptides during acute dengue.

Petitdemange et al. performed the genotypic analysis of KIR in the combination with their cognate HLA-class I ligands in 73 CHIKV and 55 DENV2 adult cases, compared with 54 healthy individuals [57]. Petitdemange et al. found no evidence of a role for KIR genotypes in patients infected with DENV-2. In contrast, Beltrame *et al.* detected an association between certain KIR genes and their cognate HLA ligands in the context of infection with DENV-3 in Southern Brazil [3, 57]. Differences in population origin and the infecting DENV serotype may explain these disparate results. Petitdemange *et al.* in a subsequent paper assessed NK cell activation by multiparametric flow cytometry in patients with DENV-2, CHIKV or in a few subjects with co-infections [59]. They found different signatures of NK cell responses between the two infections. NK cells in CHIKV infection were activated early and expressed a terminal differentiation pattern with prolonged persistence of NKG2C + CD57+ cells which the authors speculate may contribute to the chronic arthralgia seen in CHIKV infection. DENV-2 infections were associated with an increase in KIR2DL1+ NK cells which recognize HLA-C2. Together, these studies suggest that KIR-MHC interactions are likely to be important during acute dengue infection.

18.6 LILRB

LILRB is an inhibitory receptor present on monocytes, dendritic cells, and NK cells. It interacts with a wide range of MHC Class I molecules and maintains a negative feedback loop to prevent autoimmunity [70]. THE UL-18 protein of HCMV has been shown to bind with higher affinity to LILRB and protect against NK cell recognition in the context of HCMV infection. Recently using dengue-specific antibodies at neutralizing and sub-neutralizing concentrations, mechanisms of antibody-dependent enhancement (ADE) were further characterized in a resistant (THP-1R) and susceptible (THP-1S) subclone of THP-1 cells [14]. In this context, DENV was shown to interact with the inhibitory receptor LILRB. This inhibitory interaction blocked FcγRII signaling and dampened the expression of IFN stimulated genes and enhances DENV replication.

HLA-E is a non-classical MHC molecule that interacts with both activating (CD94C, D, and E) and inhibitory receptors (CD94A). Interaction of HLA-E with the inhibitory receptor NKG2A is of higher affinity compared to the interaction with most known HLA-E/peptide complexes than those transmitting activating signals. A related flavivirus, Japanese Encephalitis virus has been shown to upregulate HLA-E but no work has been published yet for DENV [69].

18.7 The Role of NK Cells in Modulating Adaptive Immune Responses

NK cells have also been implicated in shaping the adaptive response to viral infections in a number of ways including promoting maturation or elimination of DCs, perforin-dependent elimination of $CD8^{+}$ T cells, and cytokine production [64]. Waggoner et al. used the model of lymphocytic choriomeningitis virus (LCMV), to show that NK cells can regulate CD4 T-cell-mediated support for the antiviral CD8 T cells [74, 76]. NK cells have also been shown to be important for long-term CD4+ T cell memory and subsequent antibody responses [18]. The data suggest that NK cells continue to participate in immune modulation well after initial infection when NK cells are traditionally thought to be active. In a previous study of CD8+ T cells by Townsley et al. frequencies of the HLA-B57-restricted epitope, were assessed over the course of acute DENV infection [71]. Given the highly conserved nature of this epitope, we predicted that PBMC from donors with secondary dengue infection would have significantly higher frequencies of B57-$NS1_{26–34}$ CD8+ T cells compared to PBMC from donors with primary dengue infection. While we detected tetramer-positive T cells in all subjects tested, the frequencies in subjects with secondary infections were not higher than in subjects with primary infections, with one exception.

We speculated that an unidentified factor may dampen activation of CD8+ T cells directed at this epitope but had not yet identified that the NS1 peptide presented on HLA-B57 could bind KIR3DL1 an inhibitory receptor on NK cells. Our new findings suggest that NK cells could shape CD8+ T cell responses but given the lack of an authentic animal model that mimics human dengue infection it will be challenging to provide definite proof that inhibitory NK cells can modulate adaptive responses at the epitope level in vivo.

The varying combinations of inhibitory and activating receptors on NK cells and the number of unknown ligands make it difficult to assess changes in absolute frequencies of subsets of NK cells between subjects with mild or severe dengue illness. Furthermore, for meaningful comparisons to be made samples must be collected at multiple points and compared in subjects with mild and severe dengue disease rather than compare responses in all subjects with dengue disease and responses in healthy subjects. Since the hallmark of dengue hemorrhagic fever DHF (severe disease) is plasma leakage, if NK cells are hypothesized to contribute to DHF, then changes in the frequency or function of subsets of NK cells during or prior to defervescence the critical phase of illness must be demonstrated. In vitro assays will need to be performed in order to understand which receptors and ligands interact with virus-infected cells and are important for cytokine production and cytolytic activity in subsets of NK cells following DENV infection as little information exists to date. Basic biological studies of NK cells and multiple studies using clinical samples in chronic viral infections suggest that subsets of NK cells are an important contributor to the overall immune response. We have a partial understanding of how NK cells can regulate dengue immune responses but these findings point to multiple avenues whereby NK cells may control DENV infection and shape adaptive immune responses.

18.8 T-Cell Responses to Dengue Vaccination

T cell immune responses to natural dengue infection will be reviewed by Rivino et al. in Chap. 15. With the advent of a number of dengue tetravalent vaccine trials, T cell responses are being evaluated in vaccinated individuals [66]. These studies indicate that CD4 and CD8 T cells are directed against a number of epitopes across the entire DENV genome. In support of smaller studies performed in individuals receiving monovalent DENV vaccines, the data indicate that non-structural proteins are dominant targets of T cell responses following tetravalent vaccination as well [51]. There are significant differences in the composition of dengue antigens included in each vaccine which are likely to impact the immune response elicited [66]. Prior flavivirus immunity in many volunteers who received dengue vaccines in endemic countries will also influence the magnitude and quality of T cell immune responses. Here, published work to characterize CD4+ and CD8+ T-cell responses to dengue virus antigens and peptides will be reviewed.

The Sanofi Pasteur vaccine is a chimeric Yellow fever virus that expresses the structural components (prM and E) of each of the four serotypes of dengue [65, 66]. In three independent phase 1 clinical trials conducted in Australia and in the USA, Dengue 1–4 and Yellow Fever 17D (YF 17D)-specific CD4 and CD8 cellular responses induced by tetravalent chimeric dengue vaccines (CYD) were analyzed in flavivirus-naive or immune individuals [27]. Significant YF 17D NS3- specific CD8 responses and dengue serotype-specific T helper responses were detected. These responses were dominated by serotype 4 in naive individuals, but a booster vaccination broadened serotype-specific responses. A similar, broader response was seen after primary tetravalent immunization in subjects with pre-existing dengue 1 or 2 immunity caused by prior monovalent live- attenuated dengue vaccination. There was an absence of a Th2 response,

and a more dominant response to IFN-□ compared to TNF-α, for both CD4 and CD8 responses.

CD4 and CD8 T cell responses were assessed before and 28 days after a first and third injection of Sanofi Pasteur live attenuated CYD-TDV and 1 year after the third injection in 80 adolescents and adults enrolled in a phase II trial in Singapore [29]. CD4/IFN-γ and TNF-α responses specific to dengue NS3 were detected before vaccination. Vaccination induced YF-17D-NS3-specific CD8 IFN-γ responses. Using a multiplex analysis of peripheral blood mononuclear cells (PBMC) supernatants after restimulation with each the CYD vaccine viruses, higher levels of IFN-γ were detected compared with TNF-α, and low-level IL-13 levels.

The Takeda vaccine is a live-attenuated tetravalent dengue vaccine (TDV) candidate that consists of an attenuated DENV-2 strain (TDV-2), and 3 chimeric viruses containing the premembrane (prM) and envelope (E) genes of DENV-1, -3, and -4 expressed in the context of the TDV-2 genome (TDV-1, TDV-3, TDV-4, respectively). CD8+ T-cell responses in flavivirus-naive human volunteers vaccinated with 2 doses of the Takeda vaccine 90 days apart via two routes (subcutaneous or intradermal) were assessed using overlapping peptide pools against the NSl, NS3 and NS5 proteins [16]. The TDV vaccine-elicited CD8+ T cell TNF-□ and IFN-□ responses against the nonstructural NS l, NS3, and NS5 proteins of DENV-2. Responses were highest on day 90 after the first dose and were still detectable on 180 days after the second dose. In addition, CD8+ T cells were multifunctional, producing 2 cytokines simultaneously, and cross-reactive to NS proteins of the other 3 DENV serotypes.

For the NIH vaccine, attenuation was achieved by deleting a portion of the 3[1] untranslated region (UTR) of the dengue genome. Longitudinal specimens were collected in flavivirus-naive volunteers and tested for T cell responses against inactivated DENV antigen preparations made in Vero cells following low dose vaccination with a live attenuated DENV-1 candidate vaccine (DEN1L130) from the NIH. CD4+ T cells were found to secrete IFN-γ, TNF-α and IL-2, 3 weeks following exposure to DENV-1 and were detected upto 6 weeks. T cells produced 2 cytokines simultaneously [45]. There was little cross-reactivity in T cell responses.

Weiskopf et al analyzed CD8+ T cell responses in PBMC from healthy naïve volunteers in Vermont who received either monovalent or tetravalent live attenuated DENV vaccine from the NIH [75]. Using peptide pools (9- and 10-mer peptides that were predicted for their binding affinity to 27 major histocompatibility complex (MHC) class I molecules to monovalent and tetravalent DENV vaccines from the NIH. They found broad responses (IFN-□ and TNF-□ responses) to both structural and non-structural proteins were elicited in response to a monovalent vaccine (up to 47 months post-vaccination) while responses to a tetravalent vaccine (up to 12 months post-vaccination) were directed against the non-structural proteins.

Immune responses in vaccinated individuals are continuing to be monitored. How durable T cell responses to any of the attenuated vaccines in clinical trials is of interest as waning immunity to dengue is proposed to increase the risk of severe illness.

Questions

- Are distinct subsets of NK cells elevated in natural DENV infection and do they differ between mild and severe disease, primary or secondary dengue infection?
- Can viral peptides modulate NK cell function by interacting with inhibitory and activating KIRs?
- Can distinct subsets of NK cells be induced following vaccination and how do they differ from natural infection?
- Do preexisting dengue antibodies modulate NK cell responses?
- Are there new ligands on target cells that are important for flavivirus infections?

Discussion of Chapter 18 at Dengue and Zika: Control and Antiviral Treatment Strategies

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Anuja Mathew.

Siew Pheng Lim: Do you see sequence homology of your NS1 peptide with the self derived peptide and also within the sequence of the protein for Dengue?

Anuja Mathew: Definitely, there is some homology between the NS1 peptide and self peptide. There is no strong motif for peptide binding to KIRs, even for self peptides although positions 7 and 8 of bound peptides have been shown to bind KIRs. Our dengue virus peptide does match reasonably. Since self peptide can mediate inhibition, we still have to proove that the dengue peptide can modulate function in vivo.

Katja Fink: Is this a dominant peptide? Do all patients display this dominant NS1 peptide?

Anuja Mathew: So all of the HLA-B57 people that we have tested do. But we have only tested a few. B57 is unfortunately an uncommon allele in Thailand, so our access to samples were limited. If it was on HLA-A11 we had hundreds of samples to test.

Laura Rivino: I just have a question on the first part: when you tested the two tetramers, the A11 and the B57, you tested them in a secondary Dengue-2 patient. So when you tested the different Dengue-2 peptide did you also test the other serotypes?

Anuja Mathew: We have done studies, where we had all three tetramers, three variants together, and then single variants of each of the peptides. Specifically for secondary infection we would test with whatever they had currently. But for this particular donor, I know we have tested the individual variants.

Laura Rivino: Do you see a very different response?

Anuja Mathew: No, not a striking difference. You would expect that maybe you would see one, but no. In some instances, and I do not know if it is something unique about the serotype, but for Dengue-1, we see a dominant binding of Dengue-1 variant peptide tetramer. There may be something about Dengue-1 that we still have to figure out.

Laura Rivino: I want to make a comment on B57. So in ARDS, this is published very well. HLA Bs seem to be better at presenting, but I think they are just better at inducing T-cell responses in general.

Anuja Mathew: I think that is true. We have had that experience, too. I think all of the B57 donors we tested are responders. But in general, the frequencies are not as high as we expected to see given how conserved the B57 epitope is. You know, frequencies are similar to what we see in A11+ donors. It is fairly low.

Laura Rivino: And have you tested other HLAs, apart from the A11-NS3 peptide?

Anuja Mathew: We have tested A2, A11, B7, B57.

Laura Rivino: And you find A11 is partcularly low?

Anuja Mathew: We detect low responses to many HLA restricted epitopes. Every now and then, you'll get one or two donors that have a high frequency of TET+ T cells. We have mutiple time points during acute infection, and that is the strength of our studies. It is easy to miss peak frequencies when you use only one or two time points. We have about five time points in each individual during acute infection. That is our strength of our studies, I think.

George Gao: Regarding your NS1 peptide which might be the B57. If they are binding to the KIR, is that peptide detected?

Anuja Mathew: We really hope, it is. We show binding of NK cells to B57 NS1 tetramers. We show binding of the B57 NS1 tetramers with KIR3DL1 transfectants. But the question really is: can it alter NK cell functional responses? We have been struggling to find proof for that. This is a challenge in the field

including those working more actively on HIV viral peptides.

Subhash Vasudevan: What does it mean for severe dengue? Because you are saying that the NK cell response is during the defervesence period and in secondary dengue at least that is the period where you can get severe disease. So how does it fit the observed T-cell response in that period?

Anuja Mathew: What is new about this study is the unexpected NK cell regulation for a CD8+ T cell peptide. We were looking at frequencies of B57 NS1 CD8 T cells to determine whether frequencies of these T cells correlate with severe disease. Here we find an interaction with an inhibitory receptor on NK cells, which is something to consider if you do not see what you expect.

Subhash Vasudevan: So are the cytokines downstream of any importance?

Anuja Mathew: That is what we hope. Our expectation is that during the viremic phase this peptide is able to inibit NK cells. When the viremia ends, there is no longer presentation of the NS1 peptide, and that leads to NK-cell inhibition being released. So now suddenly the NK cells get activated and release the cytokines which may contribute to the cytokine storm seen during end of viremia and the critical phase of illness. That is our thinking, but that is still to proven.

References

1. Aranda-Romo S, Garcia-Sepulveda CA, Comas-Garcia A, Lovato-Salas F, Salgado-Bustamante M, Gomez-Gomez A, Noyola DE (2012) Killer-cell immunoglobulin-like receptors (KIR) in severe a (H1N1) 2009 influenza infections. Immunogenetics 64:653–662
2. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285:727–729
3. Beltrame LM, Sell AM, Moliterno RA, Clementino SL, Cardozo DM, Dalalio MM, Fonzar UJ, Visentainer JE (2013) Influence of KIR genes and their HLA ligands in susceptibility to dengue in a population from southern Brazil. Tissue Antigens 82:397–404
4. Beltran D, Lopez-Verges S (2014) NK cells during dengue disease and their recognition of dengue virus-infected cells. Front Immunol 5:1–6
5. Bettencourt A, Silva AM, Carvalho C, Leal B, Santos E, Costa PP, Silva BM (2014) The role of KIR2DS1 in multiple sclerosis: KIR in Portuguese MS patients. J Neuroimmunol 269(1–2):52–55
6. Biron CA, Byron KS, Sullivan JL (1989) Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med 320:1731–1735
7. Blish CA (2016) Natural killer cell diversity in viral infection: why and how much? Pathog Immun 1:165–192
8. Braud VM, Allan DS, O'callaghan CA, Soderstrom K, D'andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH, Lanier LL, Mcmichael AJ (1998) HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 391:795–799
9. Brown MH, Boles K, Van Der Merwe PA, Kumar V, Mathew PA, Barclay AN (1998) 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. J Exp Med 188:2083–2090
10. Brown D, Trowsdale J, Allen R (2004) The Lilr family: modulators of innate and adaptive immune pathways in health and disease. Tissue Antigens 64:215–225
11. Cantoni C, Bottino C, Vitale M, Pessino A, Augugliaro R, Malaspina A, Parolini S, Moretta L, Moretta A, Biassoni R (1999) NKP44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. J Exp Med 189:787–796
12. Cassidy SA, Cheent KS, Khakoo SI (2014) Effects of peptide on NK cell-mediated Mhc I recognition. Front Immunol 5:133
13. Cassidy S, Mukherjee S, Myint TM, Mbiribindi B, North H, Traherne J, Mulder A, Claas FH, Purbhoo MA, Das J, Khakoo SI (2015) Peptide selectivity discriminates NK cells from KIR2DL2- and KIR2DL3-positive individuals. Eur J Immunol 45:492–500
14. Chan KR, Ong EZ, Tan HC, Zhang SL, Zhang Q, Tang KF, Kaliaperumal N, Lim AP, Hibberd ML, Chan SH, Connolly JE, Krishnan MN, Lok SM, Hanson BJ, Lin CN, Ooi EE (2014) Leukocyte immunoglobulin-like receptor B1 is critical for antibody-dependent dengue. Proc Natl Acad Sci U S A 111(7):2722
15. Chau TN, Quyen NT, Thuy TT, Tuan NM, Hoang DM, Dung NT, Lien Le B, Quy NT, Hieu NT, Hieu LT, Hien TT, Hung NT, Farrar J, Simmons CP (2008) Dengue in vietnamese infants--results of infection-enhancement assays correlate with age-related disease epidemiology, and cellular immune responses correlate with disease severity. J Infect Dis 198:516–524
16. Chu H, George SL, Stinchcomb DT, Osorio JE, Partidos CD (2015) CD8+ T-cell responses in Flavivirus-Naive individuals following immunization with a live-attenuated tetravalent dengue vaccine candidate. J Infect Dis 212:1618–1628
17. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL (1993) HLA-C is the inhibitory ligand that determines dominant resistance to Lysis by NK1-

and NK2-specific natural killer cells. Proc Natl Acad Sci U S A 90:12000–12004
18. Cook KD, Waggoner SN, Whitmire JK (2014) NK cells and their ability to modulate T cells during virus infections. Crit Rev Immunol 34:359–388
19. Das J, Khakoo SI (2015) NK cells: tuned by peptide? Immunol Rev 267:214–227
20. Deaglio S, Zubiaur M, Gregorini A, Bottarel F, Ausiello CM, Dianzani U, Sancho J, Malavasi F (2002) Human CD38 and CD16 are functionally dependent and physically associated in natural killer cells. Blood 99:2490–2498
21. Della Chiesa M, Marcenaro E, Sivori S, Carlomagno S, Pesce S, Moretta A (2014) Human NK cell response to pathogens. Semin Immunol 26:152–160
22. Fadda L, Korner C, Kumar S, Van Teijlingen NH, Piechocka-Trocha A, Carrington M, Altfeld M (2012) Hla-CW*0102-restricted HIV-1 P24 epitope variants can modulate the binding of the inhibitory KIR2DL2 receptor and primary NK cell function. PLoS Pathog 8:e1002805
23. Faure M, Long EO (2002) KIR2DL4 (CD158D), an NK cell-activating receptor with inhibitory potential. J Immunol 168:6208–6214
24. Garcia G, Del Puerto F, Perez AB, Sierra B, Aguirre E, Kikuchi M, Sanchez L, Hirayama K, Guzman MG (2011) Association of MICA and MICB alleles with symptomatic dengue infection. Hum Immunol 72:904–907
25. Graef T, Moesta AK, Norman PJ, Abi-Rached L, Vago L, Older Aguilar AM, Gleimer M, Hammond JA, Guethlein LA, Bushnell DA, Robinson PJ, Parham P (2009) KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A*11 while diminishing avidity for HLA-C. J Exp Med 206:2557–2572
26. Green S, Pichyangkul S, Vaughn DW, Kalayanarooj S, Nimmannitya S, Nisalak A, Kurane I, Rothman AL, Ennis FA (1999) Early CDd69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. J Infect Dis 180:1429–1435
27. Guy B, Nougarede N, Begue S, Sanchez V, Souag N, Carre M, Chambonneau L, Morrisson DN, Shaw D, Qiao M, Dumas R, Lang J, Forrat R (2008) Cell-mediated immunity induced by chimeric tetravalent dengue vaccine in naive or flavivirus-primed subjects. Vaccine 26:5712–5721
28. Hansasuta P, Dong T, Thananchai H, Weekes M, Willberg C, Aldemir H, Rowland-Jones S, Braud VM (2004) Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. Eur J Immunol 34:1673–1679
29. Harenberg A, Begue S, Mamessier A, Gimenez-Fourage S, Ching Seah C, Wei Liang A, Li Ng J, Yun Toh X, Archuleta S, Wilder-Smith A, Shek LP, Wartel-Tram A, Bouckenooghe A, Lang J, Crevat D, Caillet C, Guy B (2013) Persistence of Th1/Tc1 responses one year after tetravalent dengue vaccination in adults and adolescents in Singapore. Hum Vaccin Immunother 9:2317
30. Hershkovitz O, Rosental B, Rosenberg LA, Navarro-Sanchez ME, Jivov S, Zilka A, Gershoni-Yahalom O, Brient-Litzler E, Bedouelle H, Ho JW, Campbell KS, Rager-Zisman B, Despres P, Porgador A (2009) NKp44 receptor mediates interaction of the envelope glycoproteins from the West Nile and dengue viruses with NK cells. J Immunol 183:2610–2621
31. Homchampa P, Sarasombath S, Suvatte V, Vongskul M (1988) Natural killer cells in dengue hemorrhagic fever/dengue shock syndrome. Asian Pac J Allergy Immunol 6:95–102
32. Ito M, Okuno T, Fujii T, Mutoh K, Oguro K, Shiraishi H, Shirasaka Y, Mikawa H (1990) ACTH therapy in infantile spasms: relationship between dose of ACTH and initial effect or long-term prognosis. Pediatr Neurol 6(4):240
33. Jamil KM, Khakoo SI (2011) KIR/HLA interactions and pathogen immunity. J Biomed Biotechnol 2011:298348
34. Jiang Y, Chen O, Cui C, Zhao B, Han X, Zhang Z, Liu J, Xu J, Hu Q, Liao C, Shang H (2013) KIR3DS1/L1 and HLA-BW4-80I are associated with HIV disease progression among HIV typical Progressors and long-term Nonprogressors. BMC Infect Dis 13:405
35. Jost S, Altfeld M (2013) Control of human viral infections by natural killer cells. Annu Rev Immunol 31:163–194
36. Kaiser BK, Barahmand-Pour F, Paulsene W, Medley S, Geraghty DE, Strong RK (2005) Interactions between NKG2x immunoreceptors and HLA-E ligands display overlapping affinities and thermodynamics. J Immunol 174:2878–2884
37. Khor CC, Chau TN, Pang J, Davila S, Long HT, Ong RT, Dunstan SJ, Wills B, Farrar J, Van Tram T, Gan TT, Binh NT, Tri Le T, Lien Le B, Tuan NM, Tham NT, Lanh MN, Nguyet NM, Hieu NT, Van NVCN, Thuy TT, Tan DE, Sakuntabhai A, Teo YY, Hibberd ML, Simmons CP (2011) Genome-wide association study identifies susceptibility loci for dengue shock syndrome at MICB and PLCE1. Nat Genet 43:1139–1141
38. Kurane I, Hebblewaite D, Brandt WE, Ennis FA (1984) Lysis of dengue virus-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. J Virol 52:223–230
39. Kusnierczyk P (2013) Killer cell immunoglobulin-like receptor gene associations with autoimmune and allergic diseases, recurrent spontaneous abortion, and neoplasms. Front Immunol 4:8
40. Lanier LL, Ruitenberg JJ, Phillips JH (1988) Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. J Immunol 141:3478–3485
41. Laoprasopwattana K, Libraty DH, Endy TP, Nisalak A, Chunsuttiwat S, Vaughn DW, Ennis FA, Rothman AL, Green S (2007) Antibody dependent cellular cytotoxicity in pre-secondary dengue virus serotype 3 (DV3) but not in DV2 infection plasma samples inversely correlated with viremia levels. J Infect Dis 195:1108–1116

42. Latchman Y, Mckay PF, Reiser H (1998) Identification of the 2B4 molecule as a counter-receptor for CD48. J Immunol 161:5809–5812
43. Libraty DH, Zhang L, Obcena A, Brion JD, Capeding RZ (2014) Circulating levels of soluble MICB in infants with symptomatic primary dengue virus infections. PLoS One 9:e98509
44. Lim DS, Yawata N, Selva KJ, Li N, Tsai CY, Yeong LH, Liong KH, Ooi EE, Chong MK, Ng ML, Leo YS, Yawata M, Wong SB (2014) The combination of Type I IFN, TNF-alpha, and cell surface receptor engagement with dendritic cells enables NK cells to overcome immune evasion by dengue virus. J Immunol 193:5065–5075
45. Lindow JC, Borochoff-Porte N, Durbin AP, Whitehead SS, Fimlaid KA, Bunn JY, Kirkpatrick BD (2012) Primary vaccination with low dose live dengue 1 virus generates a proinflammatory, multifunctional T cell response in humans. PLoS Negl Trop Dis 6:e1742
46. Ljutic B, Carlyle JR, Filipp D, Nakagawa R, Julius M, Zuniga-Pflucker JC (2005) Functional requirements for signaling through the stimulatory and inhibitory mouse NKR-P1 (CD161) NK cell receptors. J Immunol 174:4789–4796
47. Lunemann S, Martrus G, Holzemer A, Chapel A, Ziegler M, Korner C, Garcia Beltran W, Carrington M, Wedemeyer H, Altfeld M (2016) Sequence variations in HCV core-derived epitopes Alter binding of KIR2DL3 to HLA-C *03:04 and modulate NK cell function. J Hepatol 65:252–258
48. Maenaka K, Juji T, Stuart DI, Jones EY (1999) Crystal structure of the human P58 killer cell inhibitory receptor (KIR2DL3) specific for HLA-CW3-related MHC Class I. Structure 7:391–398
49. Mallone R, Funaro A, Zubiaur M, Baj G, Ausiello CM, Tacchetti C, Sancho J, Grossi C, Malavasi F (2001) Signaling through CD38 induces NK cell activation. Int Immunol 13:397–409
50. Manser AR, Weinhold S, Uhrberg M (2015) Human KIR repertoires: shaped by genetic diversity and evolution. Immunol Rev 267:178–196
51. Mathew A, Townsley E, Ennis FA (2014) Elucidating the role of T cells in protection against and pathogenesis of dengue virus infections. Future Microbiol 9:411–425
52. Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R, Bottino C, Moretta L (1995) Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J Exp Med 182:875–884
53. Ozturk OG, Sahin G, Karacor ED, Kucukgoz U (2012) Evaluation of KIR genes in recurrent miscarriage. J Assist Reprod Genet 29:933–938
54. Parolini S, Bottino C, Falco M, Augugliaro R, Giliani S, Franceschini R, Ochs HD, Wolf H, Bonnefoy JY, Biassoni R, Moretta L, Notarangelo LD, Moretta A (2000) X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. J Exp Med 192:337–346
55. Pende D, Parolini S, Pessino A, Sivori S, Augugliaro R, Morelli L, Marcenaro E, Accame L, Malaspina A, Biassoni R, Bottino C, Moretta L, Moretta A (1999) Identification and molecular characterization of NKP30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med 190:1505–1516
56. Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, Biassoni R, Moretta A (1998) Molecular cloning of NKP46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. J Exp Med 188:953–960
57. Petitdemange C, Wauquier N, Jacquet JM, Theodorou I, Leroy E, Vieillard V (2014a) Association of HLA Class-I and inhibitory KIR genotypes in gabonese patients infected by chikungunya or dengue Type-2 viruses. PLoS One 9:e108798
58. Petitdemange C, Wauquier N, Rey J, Hervier B, Leroy E, Vieillard V (2014b) Control of acute dengue virus infection by natural killer cells. Front Immunol 5:1–5
59. Petitdemange C, Wauquier N, Devilliers H, Yssel H, Mombo I, Caron M, Nkoghe D, Debre P, Leroy E, Vieillard V (2016) Longitudinal analysis of natural killer cells in dengue virus-infected patients in comparison to chikungunya and chikungunya/dengue virus-infected patients. PLoS Negl Trop Dis 10:e0004499
60. Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J (2009) CD56bright natural killer (NK) cells: an important NK cell subset. Immunology 126:458–465
61. Pozo D, Vales-Gomez M, Mavaddat N, Williamson SC, Chisholm SE, Reyburn H (2006) CD161 (human NKR-P1A) signaling in NK cells involves the activation of acid sphingomyelinase. J Immunol 176:2397–2406
62. Rajagopalan S, Long EO (2012) KIR2DL4 (CD158D): an activation receptor for HLA-G. Front Immunol 3:258
63. Ramirez-De Los Santos S, Sanchez-Hernandez PE, Munoz-Valle JF, Palafox-Sanchez CA, Rosales-Rivera LY, Garcia-Iglesias T, Daneri-Navarro A, Ramirez-Duenas MG (2012) Associations of killer cell immunoglobulin- like receptor genes with rheumatoid arthritis. Dis Markers 33:201–206
64. Raulet DH (2004) Interplay of natural killer cells and their receptors with the adaptive immune response. Nat Immunol 5:996–1002
65. Rothman AL (2011) Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat Rev Immunol 11:532–543
66. Rothman AL, Currier JR, Friberg HL, Mathew A (2015) Analysis of cell-mediated immune responses in support of dengue vaccine development efforts. Vaccine 33:7083–7090
67. Sconocchia G, Titus JA, Mazzoni A, Visintin A, Pericle F, Hicks SW, Malavasi F, Segal DM (1999)

CD38 triggers cytotoxic responses in activated human natural killer cells. Blood 94:3864–3871
68. Seymour LA, Nourse JP, Crooks P, Wockner L, Bird R, Tran H, Gandhi MK (2014) The presence of KIR2DS5 confers protection against adult immune thrombocytopenia. Tissue Antigens 83:154–160
69. Shwetank, Date OS, Carbone E, Manjunath R (2014) Inhibition of ERK and proliferation in NK cell lines by soluble HLA-E released from Japanese encephalitis virus infected cells. Immunol Lett 162:94–100
70. Thomas R, Matthias T, Witte T (2010) Leukocyte immunoglobulin-like receptors as new players in autoimmunity. Clin Rev Allergy Immunol 38:159–162
71. Townsley E, Woda M, Thomas SJ, Kalayanarooj S, Gibbons RV, Nisalak A, Srikiatkhachorn A, Green S, Stephens HA, Rothman AL, Mathew A (2014) Distinct activation phenotype of a highly conserved novel HLA-B57-restricted epitope during dengue virus infection. Immunology 141:27–38
72. Townsley E, O'connor G, Cosgrove C, Woda M, Co M, Thomas SJ, Kalayanarooj S, Yoon IK, Nisalak A, Srikiatkhachorn A, Green S, Stephens HA, Gostick E, Price DA, Carrington M, Alter G, Mcvicar DW, Rothman AL, Mathew A (2016) Interaction of a dengue virus NS1-derived peptide with the inhibitory receptor KIR3DL1 on natural killer cells. Clin Exp Immunol 183:419–430
73. Vivian JP, Duncan RC, Berry R, O'connor GM, Reid HH, Beddoe T, Gras S, Saunders PM, Olshina MA, Widjaja JM, Harpur CM, Lin J, Maloveste SM, Price DA, Lafont BA, Mcvicar DW, Clements CS, Brooks AG, Rossjohn J (2011) Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B. Nature 479:401–405
74. Waggoner SN, Cornberg M, Selin LK, Welsh RM (2012) Natural killer cells act as rheostats modulating antiviral T cells. Nature 481:394–398
75. Weiskopf D, Angelo MA, Bangs DJ, Sidney J, Paul S, Peters B, De Silva AD, Lindow JC, Diehl SA, Whitehead S, Durbin A, Kirkpatrick B, Sette A (2015) The human CD8+ T cell responses induced by a live attenuated tetravalent dengue vaccine are directed against highly conserved epitopes. J Virol 89:120–128
76. Welsh RM, Waggoner SN (2013) NK cells controlling virus-specific T cells: rheostats for acute vs. persistent infections. Virology 435:37–45
77. Whitehorn J, Chau TN, Nguyet NM, Kien DT, Quyen NT, Trung DT, Pang J, Wills B, Van Vinh Chau N, Farrar J, Hibberd ML, Khor CC, Simmons CP (2013) Genetic variants of MICB and PLCE1 and associations with non-severe dengue. PLoS One 8:e59067
78. Wong-Baeza I, Ridley A, Shaw J, Hatano H, Rysnik O, Mchugh K, Piper C, Brackenbridge S, Fernandes R, Chan A, Bowness P, Kollnberger S (2013) KIR3DL2 binds to HLA-B27 dimers and free H chains more strongly than other HLA class I and promotes the expansion of T cells in ankylosing spondylitis. J Immunol 190:3216–3224
79. Yossef R, Rosental B, Appel MY, Hershkovitz O, Porgador A (2012) Upregulation of MHC class I expression following dengue virus infection: the mechanism at the promoter level. Expert Rev Anti-Infect Ther 10:285–287

19 Structural Insights into the Broad-Spectrum Antiviral Target Endoplasmic Reticulum Alpha-Glucosidase II

Alessandro T. Caputo, Dominic S. Alonzi, John L. Kiappes, Weston B. Struwe, Alice Cross, Souradeep Basu, Benoit Darlot, Pietro Roversi, and Nicole Zitzmann

Abstract

Targeting the host-cell endoplasmic reticulum quality control (ERQC) pathway is an effective broad-spectrum antiviral strategy. The two ER resident α-glucosidases whose sequential action permits entry in this pathway are the targets of glucomimetic inhibitors. Knowledge of the molecular details of the ER α-glucosidase II (α-Glu II) structure was limited. We determined crystal structures of a trypsinolytic fragment of murine α-Glu II, alone and in complex with key catalytic cycle ligands, and four different broad-spectrum antiviral iminosugar inhibitors, two of which are currently in clinical trials against dengue fever. The structures highlight novel portions of the enzyme outside its catalytic pocket which contribute to its activity and substrate specificity. These crystal structures and hydrogen-deuterium exchange mass spectrometry of the murine ER alpha glucosidase II heterodimer uncover the quaternary arrangement of the enzyme's α- and β-subunits, and suggest a conformational rearrangement of ER α-Glu II upon association of the enzyme with client glycoproteins.

Keywords

Endplasmic reticulum quality control · Broad spectrum antiviral · Glucomimetic inhibitors · Hydrogen-deuterium exchange mass spectrometry · Antiviral iminosugar

A. T. Caputo · D. S. Alonzi · J. L. Kiappes
W. B. Struwe · A. Cross · S. Basu · P. Roversi
N. Zitzmann (✉)
Department of Biochemistry, Oxford Glycobiology Institute, University of Oxford, Oxford, UK
e-mail: nicole.zitzmann@bioch.ox.ac.uk

B. Darlot
Department of Biochemistry, Oxford Glycobiology Institute, University of Oxford, Oxford, UK

Ecole Nationale Supérieure de Chimie de Montpellier, Montpellier Cedex 5, France

19.1 Introduction

Endoplasmic reticulum (ER) α-glucosidase II (α-Glu II) is the central glycosyl hydrolase of the eukaryotic ER glycoprotein folding quality control (ERQC) machinery [8]. This enzyme removes a glucose residue from $Glc_2Man_9GlcNAc_2$ *N*-linked glycans attached to a nascent glycoprotein, after α-Glu I removes the first terminal glucose of the tri-glucosylated

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_19

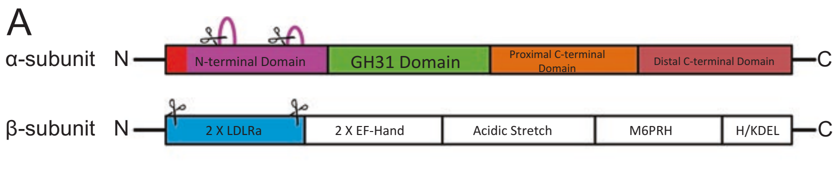
glycan. The resulting $Glc_1Man_9GlcNAc_2$ glycan enables glycoprotein binding to the ER lectins calnexin and calreticulin and the associated chaperones and refolding isomerases [6]. After a second α-Glu II-mediated glucose cleavage, the glycoprotein is left with a $Man_9GlcNAc_2$ glycan, and loses binding affinity for the ER lectins and refolding machinery: if folded correctly, the α-Glu II substrate glycoprotein is then free to proceed towards the Golgi apparatus and secretion. Thus, α-Glu II effectively acts as the eukaryotic *N*-linked glycoprotein secretion usher, admitting folding client glycoproteins into ERQC and releasing them from it [6, 13]. Importantly, most enveloped viruses also utilise α-Glu II-mediated ERQC for the correct folding of their surface glycoproteins [13]. Inhibition of α-Glu II causes viral glycoproteins to misfold and reduces virion secretion and/or infectivity [16]. The centrality of α-Glu II to viral glycoprotein folding and secretion makes it an appealing target with clinical relevance for broad-spectrum antiviral therapy. Iminosugars, a class of glycomimetics, are antiviral α-Glu II inhibitors that are well tolerated in mammals [7], with two clinical trials of α-glucosidase iminosugar inhibitors as antivirals currently in progress against dengue virus (see NCT01619969 and NCT02061358 at www.clinicaltrials.gov).

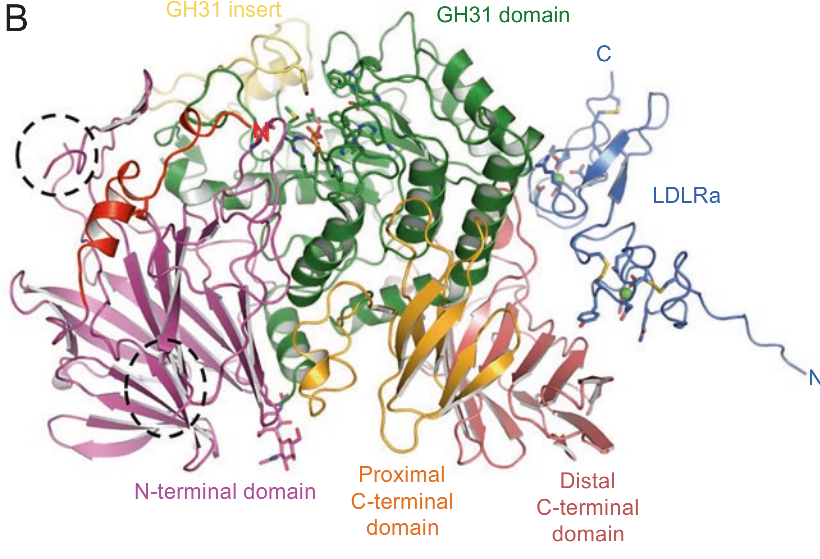
α-Glu II is a heterodimer composed of a catalytic α-subunit (104–116 kDa) and a β accessory subunit (58–80 kDa) [6, 23], and is a challenging target for biochemical and structural studies (Fig. 19.1a). The β-subunit carries a C-terminal ER-retention signal which mediates ER retention of the α-Glu II heterodimer [1], and a mannose 6-phosphate receptor homology (M6PRH) domain that binds mannose, and is thought to recruit the substrate *N*-glycan to the α-subunit active site [11] *via* interaction with a terminal α(1,2)-linked mannose [22]. In the absence of the β-subunit, α-Glu II activity is reduced *in vivo* [1] and *in vitro* [10] to about 5–10% of wild-type (wt) levels and the recombinant α-subunit is unstable unless associated with the β-subunit [13]. However, the α/β interface is not definitively known [1], nor has the role of the β-subunit been fully characterised. The catalytic α-subunit is a member of the GH31 family of glycosyl hydrolases, which includes a number of intestinal α-glucosidases. Both glycosidic bonds specifically cleaved by α-Glu II have α(1,3) linkages and therefore differ from the α(1,4) maltose-like bonds in the polysaccharides digested by intestinal GH31 α-glucosidases. Yet, cross-reactivity of α-Glu II inhibitors to intestinal α-glucosidases of the same fold gives rise to unwanted side effects in the clinic [9] and highlights the need for more selective α-Glu II inhibitors [4].

Towards this goal, and in order to acquire insight into the molecular determinants of α-Glu II activity and substrate specificity, we set out to functionally and structurally characterise a mammalian α-Glu II enzyme. We describe here production of recombinant murine heterodimeric α-GluI II (*Mm* α-Glu II), and its characterisation by enzyme kinetics, spectroscopy, single crystal X-ray crystallography, and hydrogen deuterium exchange mass spectrometry (HDX). Four crystal structures of the 107 kDa trypsinised fragment of murine α-Glu II (*Mm* α-Glu II_{Tryps}), alone and in complex with ligands, comprise an almost intact α-subunit and the associated N-terminal domain of the β-subunit, reveal the details of α/β association and rationalise the enzyme substrate specificity and the molecular basis of ligand binding [2].

19.2 Architecture of *Mm* α-Glu II_{Tryps}

We cloned, expressed and purified wt recombinant *Mm* α-Glu II and treated it with trypsin to generate the stable proteolytic fragment *Mm* α-Glu II_{Tryps} [24]. Size-exclusion chromatography multi-angle laser light scattering (SEC-MALLS) confirmed folded molecules of 166 kDa and 107 kDa, respectively for *Mm* α-Glu II and *Mm* α-Glu II_{Tryps}, assembling as α/β heterodimers in solution. Electrospray ionisation mass spectrometry (ESI-MS) confirmed the boundaries of the





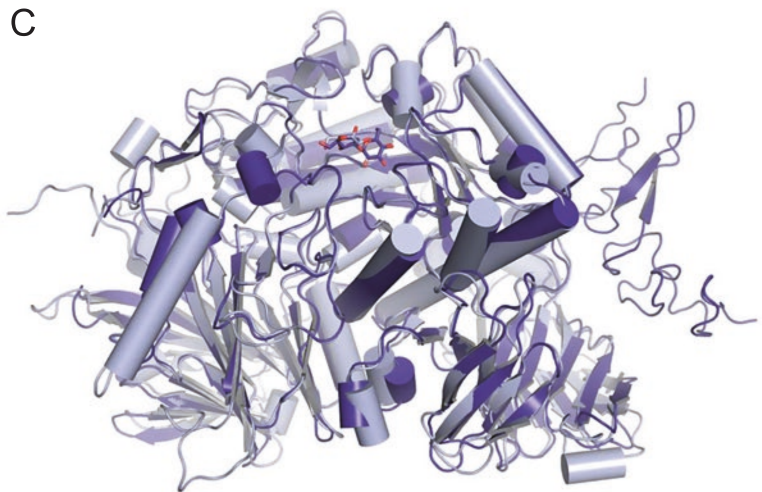


Fig. 19.1 Architecture of the *Mm* α-Glu II_{Tryps} crystal structure. (**a**) Schematic of the domain arrangement of both subunits of α-Glu II. The two loops removed by trypsin in the α-subunit are represented by cartoon scissors and similarly the portion of the β-subunit remaining are in blue between the cartoon scissors. (**b**) Three-dimensional structure of *Mm* α-Glu II_{Tryps} (PDB ID: 5F0E) in cartoon representation coloured according to (**a**) and labelled by domain. Overlay of disaccharide bound *Mm* α-Glu II_{Tryps} (in dark purple, PDB ID 5HJR) with the *Chaetomium thermophilum* α-subunit crystal structure (in light purple, PDB ID 5DKZ) both containing the bound carbohydrates sitting in their active sites (Figure adapted from [2])

resulting polypeptides of the *Mm* α-Glu II_{Tryps} species.

Mm α-Glu II_{Tryps} yielded diffracting crystals that enabled structure solution by molecular replacement at a resolution of 1.74 Å (Fig. 19.1b). The crystal structure of *Mm* α-Glu II_{Tryps} also reveals two tandem low-density-lipoprotein receptor class A (LDLRa) subdomains, corresponding to the N-terminal portion of the β-subunit (in blue in Fig. 19.1b). This is the only portion of the β-subunit to survive trypsinolysis, because of its close association with the α-subunit. Each LDLRa module contains an octahedrally coordinated calcium ion, explaining the known *S.pombe* β-subunit mutants E73A and E114A, inactive against Glc_1Man_9 [21], which correspond to *Mm* α-Glu II β-subunit E64 and E105: both residues are part of a calcium ion coordination sphere in one of the β-subunit LDLRa subdomains. The recent structure of the α-subunit from *Chaetomium thermophilum (Ct)*, a thermophilic fungus, shows a similar domain architecture and agreement with our murine structures (Fig. 19.1c) [15]. The two α-subunits have an RMSD of 0.68 Å and the bound disaccharide poses match well. A second structure with a bound glucose-α(1,3)-mannose indicates how substrate discrimination may occur in the active site.

The interface between the α- and β-subunits in the crystals spans 700 $Å^2$ of solvent accessible area, and is organised around salt bridges between aspartic acid residues coordinating the two calcium ions in the β-subunit (D53, D54, D57, D94 and D98) and four arginines and one lysine in the α-subunit (R837, R839, R840, R951 and K952 in *Mm* α-Glu II), see Fig. 19.2a. The acidic residues in the β-subunit and the basic residues in the α-subunit at this interface are conserved across sequences of eukaryotic α-Glu II enzyme subunits (Fig. 19.2b, c). As expected on the basis of the crystal structure, the *Mm* α-Glu II α-subunit single mutant R840E loses binding capacity for the β-subunit *in vitro:* the β-subunit no longer copurifies with the 6xHis-tagged α-subunit R840E mutant by immobilised metal affinity chromatography (Fig. 19.2d). The observations form this set of crystal structures has been recently confirmed with a similar crystal structure of the *Chaetomium thermophilum* α-subunit with a tandem LDLRa construct [14].

The α/β interface was further studied by hydrogen deuterium exchange mass spectrometry experiments (HDX) conducted on the full-length *Mm* α-Glu II heterodimer. Following pepsinolysis a total of 158 peptides were detected (101 α-subunit and 57 β-subunit) and support the observed α/β interface. Peptides including residues α 837-840 and β 94-98 were not detected, however good coverage was measured for residues α 951-952 and β 50-61, which were protected from deuterium uptake for up to 60 min (Fig. 19.3a, b), consistent with the α/β interface in the crystals. Interestingly, the helices α 427-441 and α 470-482, which contact each other on the surface of the α-subunit, also seem to be protected from H/D exchange (Fig. 19.3c).

19.3 Snapshots of the Catalytic Cycle of ER α-Glu II

For the first time, all key stages of the catalytic cycle of a single member of the GH31 family have been trapped in crystal structures by soaking of ligands (Fig. 19.4). All ligands displace most of the water molecules in the −1 catalytic pocket of the *apo* enzyme (Fig. 19.4a). α-Glycosidases can catalyse transglucosylation reactions at high substrate concentration [19], and *Mm* α-Glu II proves no exception: soaking the *Mm* α-Glu II_{Tryps} crystals with the 1,2-unsaturated glucose analogue D-glucal we obtained a complex with a bound α(1,3)-linked pseudo-disaccharide, illustrating the binding mode of substrate α(1,3) disaccharides in the enzyme's catalytic pocket: in particular, the hydroxyl moieties at C4 and C6 on the ring at the +1 site form H-bonds to the conserved R624 and D305, respectively (Fig. 19.4b). The complex obtained by soaking the crystals with 5-fluoro-α-D-glucopyranosyl fluoride traps the reaction intermediate after the first nucleophilic attack (Fig. 19.4c), confirming that D564 is the nucleophile. The complex with D-glucose shows the

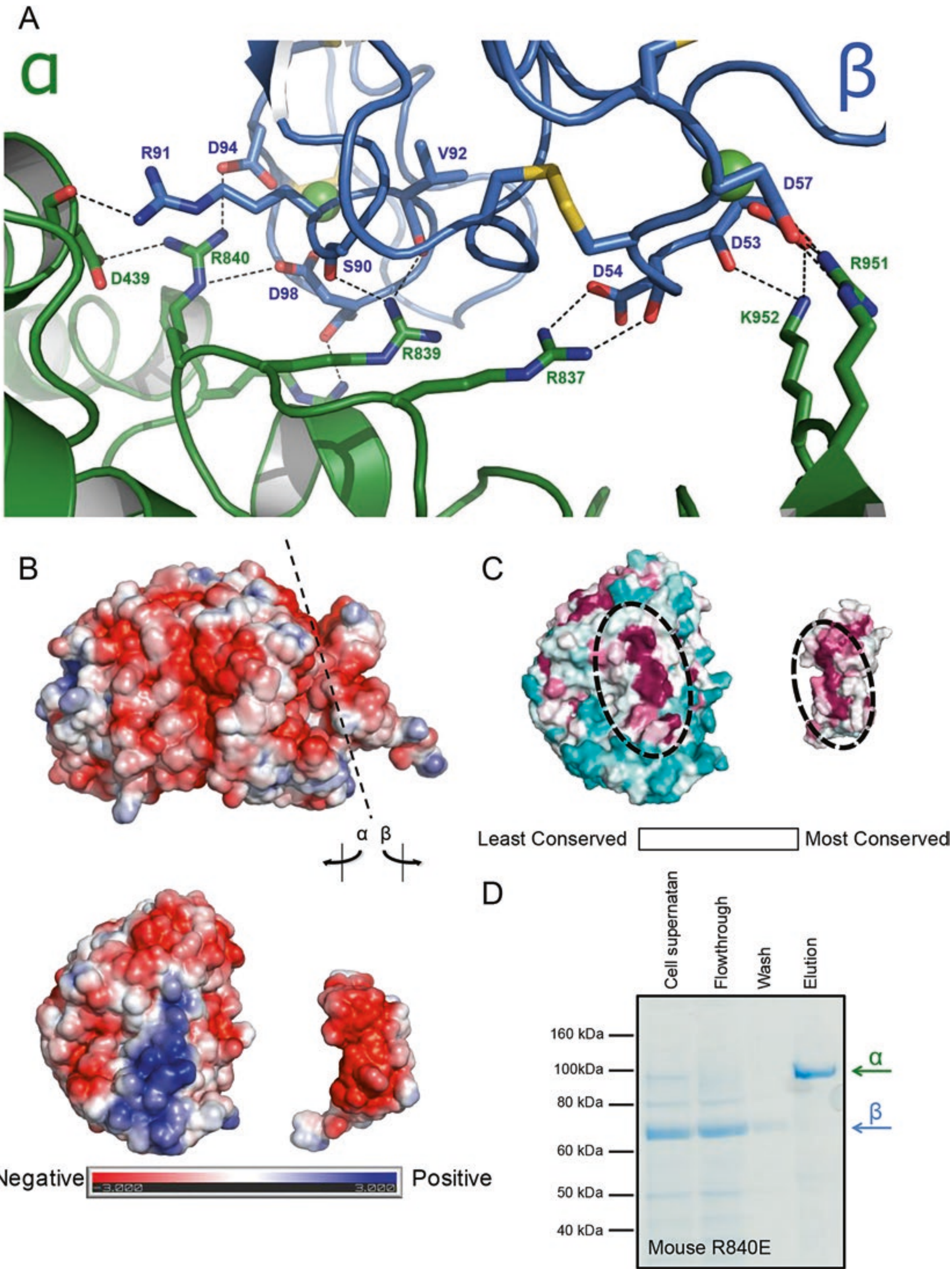
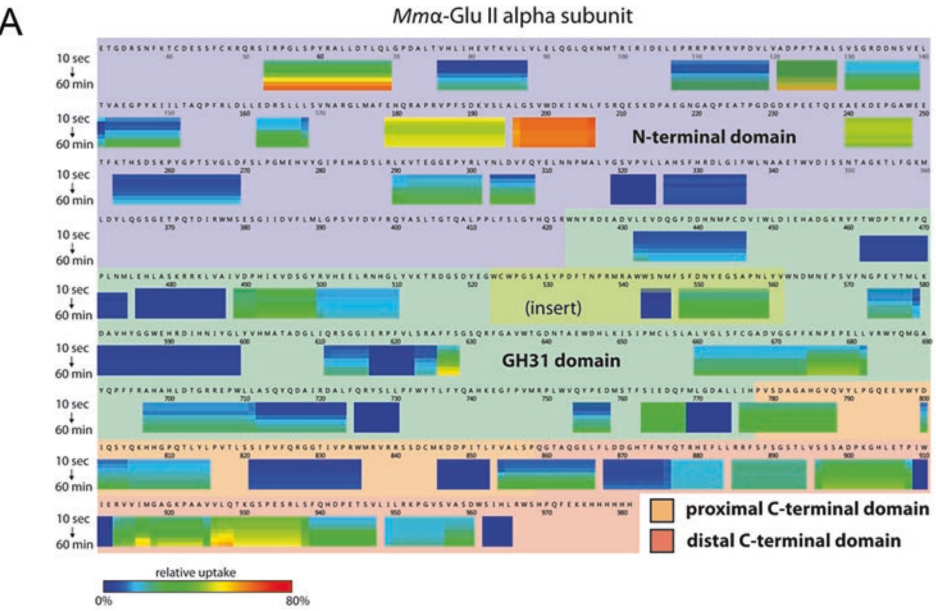
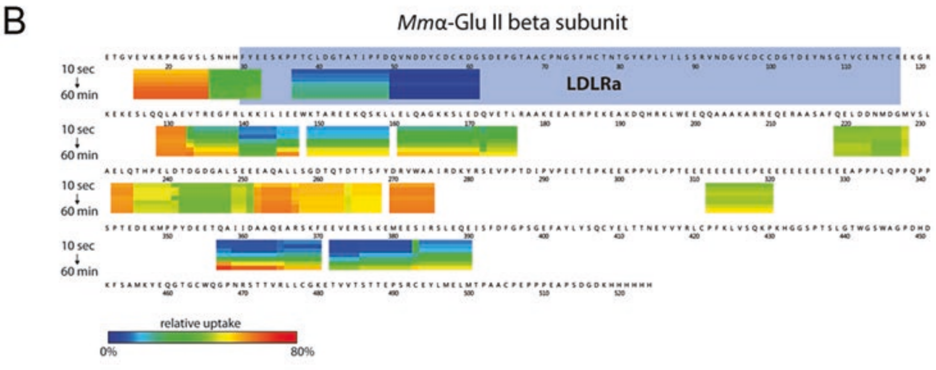


Fig. 19.2 The interface between the α- and β-subunits is comprised of a highly conserved, charged interface. (**a**) Molecular detail of the interaction between the two subunits (α-subunit in green and β-subunit in blue) highlighting a number of salt bridges and hydrogen bonds that form the interface. (**b**) Electrostatic potential on the surface of the dimer and the individualised subunits calculated by Adaptive Poisson-Boltzmann Solver plugin of PyMOL. (**c**) Sequence conservation over 30 sequences of both subunits painted onto the surface of the subunits produced with the Consurf server. (**d**) Abrogation of the dimerisation interaction *in vitro* is possible by mutation of one of the key arginines (R840) to a glutamate when co-expressed with the β-subunit. Purification using the hexahistidine tag on the α-subunit causes the β-subunit to flow through affinity resin (Figure adapted from [2])





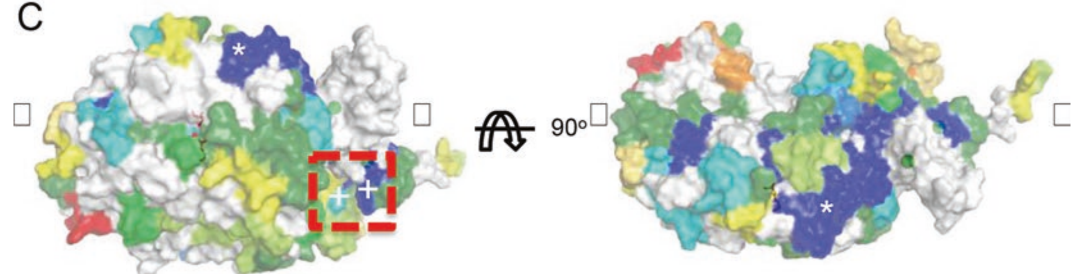


Fig. 19.3 Characterisation of the α-Glu II interface by hydrogen-deuterium exchange mass spectrometry. Pepsinolysis of a time course of deuterated full-length *Mm* α-Glu II over 1 h. (**a**) and (**b**) Heat maps for the identified peptides with the shaded domains present in the *Mm* α-Glu II_{Tryps} crystal structure. (**c**) Surface representation of *Mm* α-Glu II_{Tryps} coloured by rate of hydrogen-deuterium exchange obtained from the wt *Mm* α-Glu II. The α/β interface residues α -951-952 and β -50-61 are boxed by a dashed red line and marked by white "+" signs. The helices α 427-441 and α 470-482, protected by the β-subunit in the context of the heterodimer are marked by a white asterisk (Figure taken from [2])

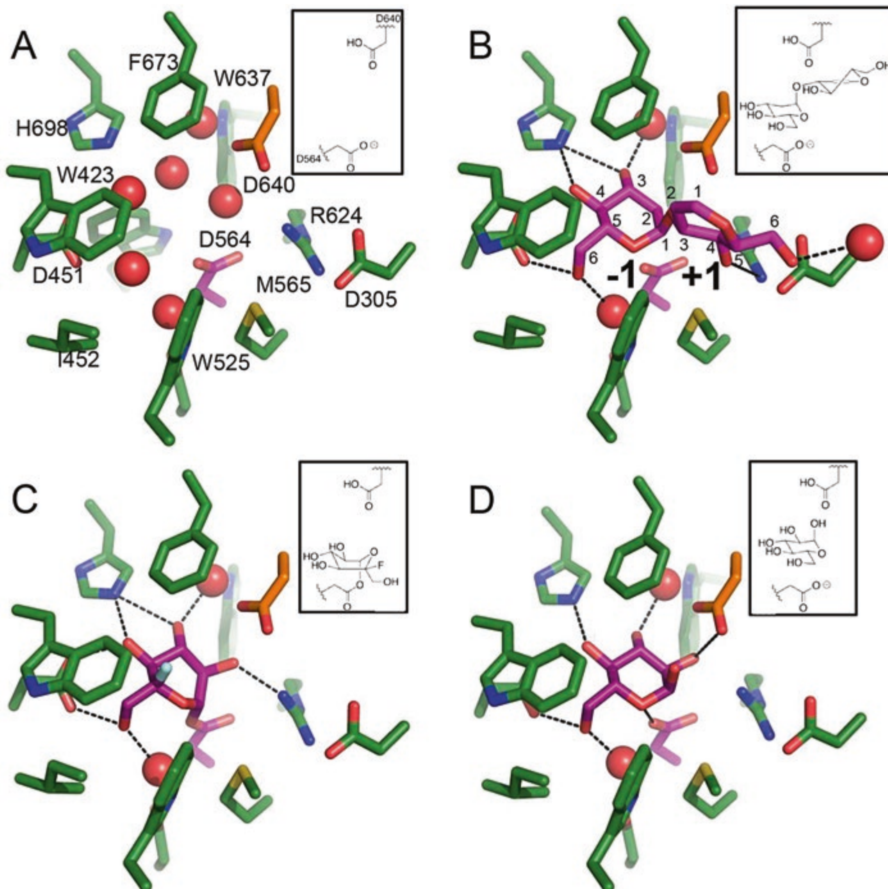


Fig. 19.4 Details of the *Mm* α-Glu II active site at key stages during its catalytic cycle (with corresponding chemical schemes in the corners). (**a**) *Apo* enzyme, the pocket is filled with ordered water molecules (red spheres). (**b**) Complex with the D-glucal transglucosylation product, an analogue of a α(1,3) disaccharide substrate. The −1 and +1 sites are marked. (**c**) Covalent complex after nucleophilic attack from D564 to 1, forming a reaction intermediate analogue. (**d**) Complex with D-glucose, one of the products of the enzyme's reactions. The C atoms of the catalytic nucleophile D564 and the ligands are coloured purple. The C atoms of the catalytic acid/base D640 are coloured orange (Figure taken from [2])

product bound to the −1 pocket of the enzyme after glycosidic bond hydrolysis (Fig. 19.4d).

19.4 Novel Features of ER α-Glu II

The structure of the *Mm* α-Glu II α-subunit reveals two novel structural features corresponding to stretches of amino acid sequence that are both unique to eukaryotic α-Glu II enzymes as well as conserved across them (Fig. 19.5). Firstly, the N-terminus of the α-subunit contains a helical turn (residues 41–43) connected to a short alpha helix (residues 45–52) by a disulfide bond (between C41 and C47). This conserved N-terminal portion reaches the rim of the catalytic pocket (in red in Fig. 19.5). Secondly, all eukaryotic α-Glu II sequences possess a two-residue insertion, which is absent in other GH31 family members, in an N-terminal domain loop that flanks the active site. This loop corresponds to residues 306–313 (and the insertion residues are F307 and Q308) in *Mm* α-Glu II (Fig. 19.5b). We overlayed our structure of the *Mm* α-Glu II α-subunit onto the structure (PDB ID 2QMJ) of the N-terminal domain of human maltase-

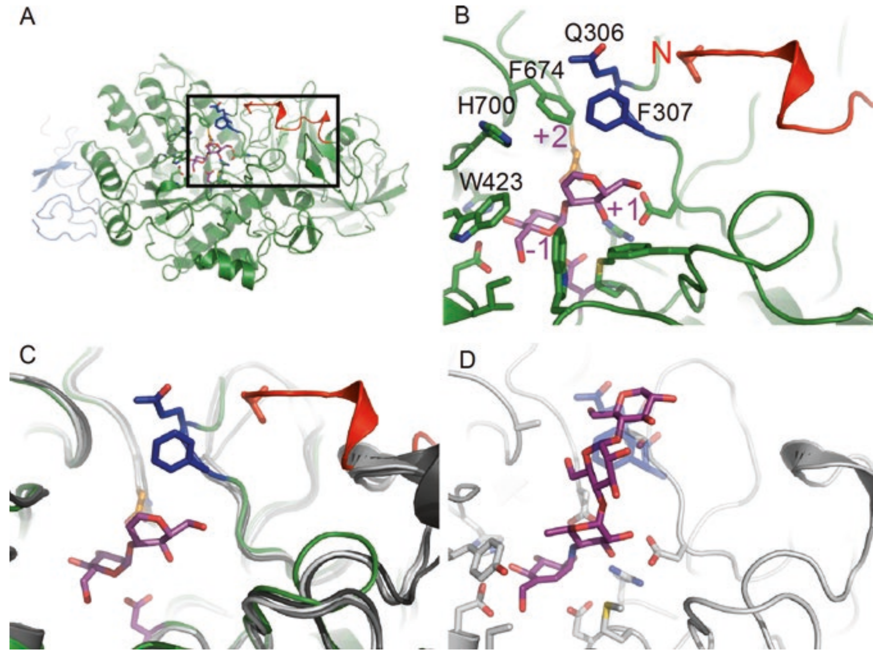


Fig. 19.5 The N-terminus extension and exclusion loops are novel α-Glu II-specific determinants of enzyme activity. Structural alignment of *Mm* α-Glu II_{Tryps} (green) with its bound disaccharide analogue (purple) against the maltase domain of *H. sapiens* maltase-glucoamylase (grey, PDB ID 2QMJ). (**a**) Red: highlight of the position of the N-terminus in relation to the exclusion loop only present in *Mm* α-Glu II_{Tryps}. (**b**) Detail of acarbose (grey) binding in 2QMJ, again overlayed with the *Mm* α-Glu II_{Tryps} and bound disaccharide analogue (purple). The mode of binding for acarbose observed in *H. sapiens* maltase-glucoamylase is not possible in *Mm* α-Glu II_{Tryps}, due to steric hindrance from the exclusion loop residues F307 and Q308 (Figure adapted from [2])

glucoamylase (Fig. 19.5c) in complex with acarbose, a non-cleavable α(1,4) tetrasaccharide mimic and a well-known inhibitor of intestinal α-glucosidases. We observed that acarbose cannot fit into the substrate binding pocket of the *Mm* α-Glu II α-subunit because of steric hindrance by the insertion loop residues F307 and Q308 (Fig. 19.5d). As it is this loop which prevents acarbose from binding and inhibiting α-Glu II, we name this part of the α-Glu II enzyme the *exclusion loop*.

19.5 The Binding Mode of the Broad-Spectrum Iminosugar Antiviral Compounds

We went on to determine the crystal structures of *Mm* α-Glu II_{Tryps} in complex with four antiviral iminosugars: castanospermine, 1-deoxynojirimycin (DNJ), and its alkylated derivatives *N*-butyl-1-deoxynojirimycin (*N*B-DNJ, *aka* miglustat) and *N*-9′-methoxynonyl-1-deoxynojirimycin (M*O*N-DNJ, *aka* UV-4) (Fig. 19.6a–d) [3]. The iminosugars all occupy the −1 subsite and their hydroxyl moieties interact with the enzyme similarly to what is observed for glucose, 5F-glucose and D-glucal disaccharide. DNJ orients itself with the endocyclic nitrogen atom in close proximity to the catalytic D564, compatible with the idea that the molecule acts as a transition state mimic of the first step of glycosidic bond hydrolysis (Fig. 19.6a) [5]. The castanospermine hydrophobic five-membered ring fits in a pocket formed by the conserved W423, I448 and W525 (Fig. 19.6b). The alkyl tail of *N*B-DNJ displaces the side chain of W525, disordering it (Fig. 19.6c). In the crystal structure of the N-terminal domain of human maltase-glucoamylase in complex with the iminosugar miglitol (PDB ID 3L4W) [18], a similar movement of the side chain of W406 (equivalent to *Mm* α-Glu II W525)

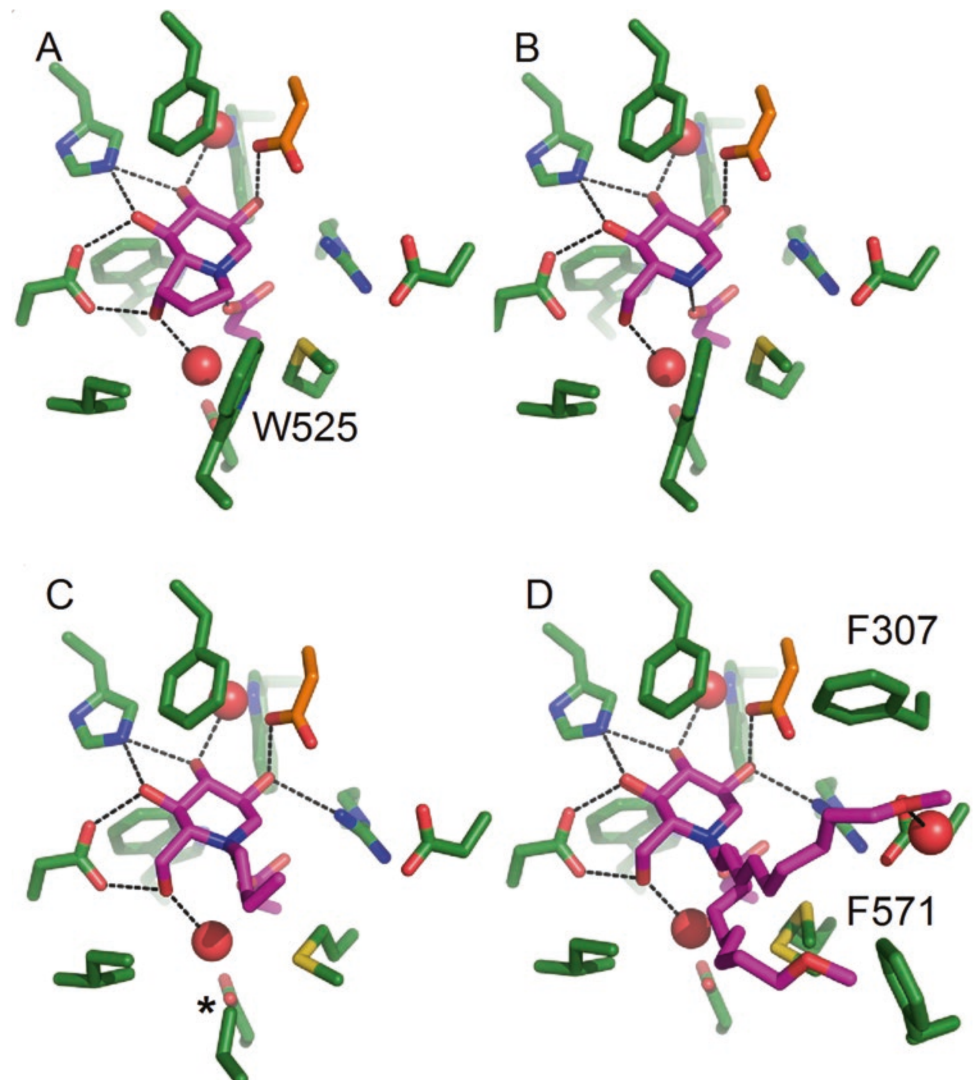


Fig. 19.6 Crystal structures of *Mm* α-Glu II_{Tryps} in complex with iminosugar inhibitors. Active site of complexes with (**a**) castanospermine, (**b**) DNJ, (**c**) *N*B-DNJ, and (**d**) M*O*N-DNJ. Upon alkylation of the endocyclic nitrogen, the W525 side chain in the *N*B-DNJ complex is disordered (asterisk in **c**), while the longer chain of M*O*N-DNJ causes complete disordering of the α 525-527 hairpin loop. The side chain of M*O*N-DNJ adopts two main conformations, which interact with F307 and F571. (Figure taken from [2])

was caused by the *N*-hydroxyethyl group of the ligand. The alkyl chain of *N*B-DNJ is longer than that of miglitol and stretches towards the side chains of the conserved residues F307 and F571, suggesting that *N*-alkylated iminosugar derivatives act by blocking access to the +1 subsite as well as occupying the −1 subsite with the iminosugar ring. This is confirmed by the structure with M*O*N-DNJ (Fig. 19.4d), which has a longer alkyl chain, and is the most potent of the *N*-alkylated iminosugars tested (M*O*N-DNJ has an IC_{50} of about 1.8 ± 0.3 μM [22] *vs.* 5.2 ± 1.0 μM for *N*B-DNJ and 11.4 ± 4.3 μM for DNJ [17]). M*O*N-DNJ displaces the whole loop α 523-528, which changes conformation, and whose terminal hairpin α 525-527 is disordered in the crystal after soaking. M*O*N-DNJ's alkyl chain is in two main conformations (of refined occupancies 0.61 and 0.39), the major conformer docking

against the exclusion loop F307 and the minor one against the hydrophobic side chain of F571 (Fig. 19.6d).

Taken together, these crystal structures show that the DNJ moiety and castanospermine bind in a manner similar to that observed for the iminosugar miglitol in the active site of the intestinal maltase-glucoamylase and sucrase-isomaltase. Further away from the catalytic pocket, an insertion in a loop at the edge of the +1 subsite of α-GluII explains the lack of α-GluII inhibition by the non-cleavable α(1,4) tetrasaccharide mimic acarbose, a well-known inhibitor of intestinal α-glucosidases. M*O*N-DNJ so far is the best known inhibitor of α-GluII *in vivo* [12], active against dengue and influenza [20, 25] and currently in clinical trials for dengue fever. The alkyl-chains of *N*B-DNJ and M*O*N-DNJ displace and induce disorder in a surface loop. Both the disordering of portions of the protein and the multiple conformations of the alkyl chain make favorable entropic contributions to the free energy of binding. Based on these observations, the conserved ring of hydrophobic residues between the α-Glu II +1 and +2 subsites could be a good target for inhibitors with the potential to achieve increased potency and selectivity for α-Glu II.

19.6 Conclusions

In our recent work we have identified α-Glu II in particular as a suitable target for the development of a broad-spectrum antiviral agent. Inhibition of α-Glu II was necessary and sufficient to inhibit *e.g.* dengue virus *in vitro* and in dengue disease mouse models [12]. The crystal structures reported here suggest that the pharmacological search for α-Glu II inhibitors selective over GH31 intestinal glucosidases should be extended to a much broader drug epitope space, aiming at regions of the enzyme that this work has uncovered as specific to α-Glu II and not shared by other GH31-family α-glucosidases, such as the exclusion loop, the α-subunit N-terminus and the α/β interface. These structural features may constitute druggable targets, and *Mm* α-GluII$_{Tryps}$ crystals may assist in drug candidate discovery.

Discussion of Chapter 19 in *Dengue and Zika: Control and Antiviral Treatment Strategies*

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Nicole Zitzmann.

Aravinda de Silva: I guess this is more of a basic question about how the quality control system works. So you said that you have the sugar with one glucose and then, when it is strained, it is released and calnexin binds. Or am not i getting it?".

Nicole Zitzmann: Slightly. It is not possible [to happen at the same time; ie for calnexin and gluII to bind to the monoglucosylated substrate]. The ER alpha-glucosidase II has two substrates, the diglucosylated and the monoglucosylated [N-linked glycan]. And then [the question is] does calnexin get there first [i.e. to the monoglucosylated glycan], and calnexin is obviously also going on-off, on- off. And so eventually, you can't have alpha-glucosidase cleaving the last glucose off with calnexin still bound to it, it's physically impossible. So the glucosidase II will have to come in there at some point when calnexin is not bound.

Aravinda de Silva: So the question is: How does the quality control work? Is it the glucosidase that actually does that?

Nicole Zitzmann: No. The real player here is UGGT, the UDP-glucose-glucosyl transferase, the one on the other end of the calnexin cycle. That is the real quality controller that scans the protein that is trying to fold and sees wether it is folded or not. Nobody knows exactly how that works, so we wanted the structure. Now that we have the structure we can address some of these questions.

Aravinda de Silva: So it mysteriously scans proteins in the act of folding...

Nicole Zitzmann: Well, it possibly samples for disulfide bridges and you know that the textbooks always say that exposed hydrophobic patches can't be there. It has a lot of thioredoxin-like domains doing all sorts of jobs.

Aravinda de Silva: Do you know the fate of the viral proteins? What happens to them?

Nicole Zitzmann: That entirely depends on the virus and that's sadly, although it is broad spectrum, or at least in vitro it is antiviral against all of these viruses, why it totally depends on the virus. Say, in HIV, you'll get a slight misfolding of the gp120 and the virus can still get secreted, but you have a slight misfolding in the V1 and V2 loop. The virus can still bind to the next cell and it can bind to CD4, but it cannot make the conformational change to bind to the coreceptors. So in HIV you get virus out, so if you just measure the RNA, you wouldn't know any better, but you have a totally non-infectious virus. Other viruses see gross misfolding. For dengue, I think Joanna [Miller] will show some data. It looks like we actually retain them. And if you retain things inside the cell, they eventually will be degraded by the ERAD pathway. And for most other viruses, you'll get grossly misfolded [viral envelope glyco] proteins inside the cell and they get degraded inside the cell.

George Gao: Are there any evidences for quality control factors or putative factors such as your enzyme being upregulated in the ER during virus infection like HIV or Ebola?

Nicole Zitzmann: The enzymes of the calnexin cycle? Yes they are. So when you have a viral infection, there are thousands of viral proteins trying to fold in addition to the normal host proteins that are trying to fold. So yes, the cell senses this extra burden and triggers the unfolded protein response, the UPR response. This can lead to the upregulation of the main players I mentioned.

George Gao: So what happens for a chronic infection like HIV? Is it also upregulated?

Nicole Zitzmann: It depends on wether you have active viral replication in the cell. So if you have active viral replication it will be upregulated. But HIV is often latent.

Laura Rivino: So you are inhibiting an enzyme that is a host enzyme. And so what is the effect on the host?

Nicole Zitzmann: It is actually not very dramatic. We have a large therapeutic window with alpha glucosidases and we hit the viruses much earlier, before we hit the host proteins. The reason for this is so far only speculative, but we have a paper hopefully coming out soon where we have proven that for HIV. Most viruses need to oligomerize their envelope glycoproteins. For instance, gp120 in HIV is a trimer. So even if you only slightly misfold one of them, the whole trimer cannot work anymore and for HIV, we know that actually for viral fusion, you'll need several, about seven, of these to come together. So you can actually have a massive amplification effect with only a little bit of misfolding and that is why we hit the viruses before we hit the host. Most host enzymes can also use endomonosidases in the Golgi to get around these problems but viruses can't. So you could speculate if it has something to do with the fact that viral proteins have not necessarily, at least initially, co-evolved with our cells. They really need a lot of help to fold their proteins in the ER.

Siew Pheng Lim: So you mentioned the alpha-glucosidase inhibitors have been repeatedly looked at for HIV, HBV, HCV etc. What are your thoughts in terms of what makes a better inhibitor and which angle are you are aiming at?

Nicole Zitzmann: So personally, my group is after an allosteric inhibitor at the moment for this enzyme, but there are a lot of approaches in this field and we are not the only players in this. There is a lot of medicinal chemistry going on and having determined the structure you know there are some very obvious things that one would be able to do.

References

1. Arendt CW, Ostergaard HL (1997) Identification of the CD45-associated 116-kDa and 80-kDa proteins as the alpha- and beta-subunits of alpha-glucosidase II. J Biol Chem 272:13117–13125
2. Caputo AT, Alonzi DS, Marti L, Reca I-B, Kiappes JL, Struwe WB, Cross A, Basu S, Lowe ED, Darlot B et al (2016) Structures of mammalian ER α-glucosidase II capture the binding modes of broad-spectrum iminosugar antivirals. Proc Natl Acad Sci 113:E4630–E4638
3. Chang J, Block TM, Guo J-T (2013) Antiviral therapies targeting host ER alpha-glucosidases: current status and future directions. Antivir Res 99:251–260
4. Dalziel M, Crispin M, Scanlan CN, Zitzmann N, Dwek RA (2014) Emerging principles for the therapeutic exploitation of glycosylation. Science 343:1235681–1235681
5. Gloster TM, Vocadlo DJ (2012) Developing inhibitors of glycan processing enzymes as tools for enabling glycobiology. Nat Chem Biol. 8:683-94
6. Hammond C, Braakman I, Helenius A (1994) Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc Natl Acad Sci U S A 91:913–917
7. Jeyakumar M, Dwek RA, Butters TD, Platt FM (2005) Storage solutions: treating lysosomal disorders of the brain. Nat Rev Neurosci 6:713–725
8. Khoury GA, Baliban RC, Floudas CA (2011) Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. Sci Rep 1
9. Lyseng-Williamson KA (2014) Mitglustat: a review of its use in Niemann-Pick disease type C. Drugs. 74:61-74
10. Olson LJ, Orsi R, Alculumbre SG, Peterson FC, Stigliano ID, Parodi AJ, D'Alessio C, Dahms NM (2013) Structure of the lectin mannose 6-phosphate receptor homology (MRH) domain of glucosidase II, an enzyme that regulates glycoprotein folding quality control in the endoplasmic reticulum. J Biol Chem 288:16460–16475
11. Olson LJ, Orsi R, Peterson FC, Parodi AJ, Kim J-JP, D'Alessio C, Dahms NM (2015) Crystal structure and functional analyses of the lectin domain of glucosidase II: insights into Oligomannose recognition. Biochemistry 54:4097–4111
12. Perry ST, Buck MD, Plummer EM, Penmasta RA, Batra H, Stavale EJ, Warfield KL, Dwek RA, Butters TD, Alonzi DS et al (2013) An iminosugar with potent inhibition of dengue virus infection in vivo. Antivir Res 98:35–43
13. Pieren M, Galli C, Denzel A, Molinari M (2005) The use of calnexin and calreticulin by cellular and viral glycoproteins. J Biol Chem 280:28265–28271
14. Satoh T, Toshimori T, Noda M, Uchiyama S, Kato K (2016a) Interaction mode between catalytic and regulatory subunits in glucosidase II involved in ER glycoprotein quality control. Protein Sci:1–27
15. Satoh T, Toshimori T, Yan G, Yamaguchi T, Kato K (2016b) Structural basis for two-step glucose trimming by glucosidase II involved in ER glycoprotein quality control. Sci Rep 6:20575
16. Sayce AC, Miller JL, Zitzmann N (2010) Targeting a host process as an antiviral approach against dengue virus. Trends Microbiol 18:323–330
17. Sayce AC, Alonzi DS, Killingbeck SS, Tyrrell BE, Hill ML, Caputo AT, Iwaki R, Kinami K, Ide D, Kiappes JL et al (2016) Iminosugars inhibit Dengue virus production via inhibition of ER alpha-glucosidases-not glycolipid processing enzymes. PLoS Negl Trop Dis 10:e0004524
18. Sim L, Jayakanthan K, Mohan S, Nasi R, Johnston BD, Pinto BM, Rose DR (2010) New glucosidase inhibitors from an ayurvedic herbal treatment for type 2 diabetes: structures and inhibition of human intestinal maltase-glucoamylase with compounds from Salacia reticulata. Biochemistry 49:443–451
19. Sinnott ML (1990) Catalytic mechanism of enzymic glycosyl transfer. Chem Rev 90:1171–1202
20. Stavale EJ, Vu H, Sampath A, Ramstedt U, Warfield KL (2015) In vivo therapeutic protection against influenza A (H1N1) oseltamivir-sensitive and resistant viruses by the iminosugar UV-4. PLoS One.
21. Stigliano ID, Alculumbre SG, Labriola CA, Parodi AJ, D'Alessio C (2011) Glucosidase II and N-glycan mannose content regulate the half-lives of monoglucosylated species in vivo. Mol Biol Cell 22:1810–1823
22. Totani K, Ihara Y, Matsuo I, Ito Y (2006) Substrate specificity analysis of endoplasmic reticulum glucosidase II using synthetic high mannose-type glycans. J Biol Chem 281:31502–31508
23. Trombetta ES, Simons JF, Helenius A (1996) Endoplasmic reticulum glucosidase II is composed of a catalytic subunit, conserved from yeast to mammals, and a tightly bound noncatalytic HDEL-containing subunit. J Biol Chem 271:27509–27516
24. Trombetta ES, Fleming KG, Helenius A (2001) Quaternary and domain structure of glycoprotein processing glucosidase II. Biochemistry 40:10717–10722
25. Warfield KL, Plummer E, Alonzi DS, Wolfe GW, Sampath A, Nguyen T, Butters TD, Enterlein SG, Stavale EJ, Shresta S, Ramstedt U (2015) A Novel Iminosugar UV-12 with Activity against the Diverse Viruses Influenza and Dengue (Novel Iminosugar Antiviral for Influenza and Dengue). Viruses 7:2404-27
26. Warfield KL, Plummer EM, Sayce AC, Alonzi DS, Tang W, Tyrrell BE, Hill ML, Caputo AT, Killingbeck SS, Beatty PR et al (2016) Inhibition of endoplasmic reticulum glucosidases is required for in vitro and in vivo dengue antiviral activity by the iminosugar UV-4. Antivir Res 129:93–98

20 Mechanisms of Antiviral Activity of Iminosugars Against Dengue Virus

Joanna L. Miller, Beatrice E. Tyrrell, and Nicole Zitzmann

Abstract

The antiviral mechanism of action of iminosugars against many enveloped viruses, including dengue virus (DENV), HIV, influenza and hepatitis C virus, is believed to be mediated by inducing misfolding of viral *N*-linked glycoproteins through inhibition of host endoplasmic reticulum-resident α-glucosidase enzymes. This leads to reduced secretion and/or infectivity of virions and hence lower viral titres, both *in vitro* and *in vivo*. Free oligosaccharide analysis from iminosugar-treated cells shows that antiviral activity correlates with production of mono- and tri-glucosylated sugars, indicative of inhibition of ER α-glucosidases. We demonstrate that glucose-mimicking iminosugars inhibit isolated glycoprotein and glycolipid processing enzymes and that this inhibition also occurs in primary cells treated with these drugs. Galactose-mimicking iminosugars that have been tested do not inhibit glycoprotein processing but do inhibit glycolipid processing, and are not antiviral against DENV. By comparison, the antiviral activity of glucose-mimetic iminosugars that inhibit endoplasmic reticulum-resident α-glucosidases, but not glycolipid processing, demonstrates that inhibition of α-glucosidases is responsible for iminosugar antiviral activity against DENV. This monograph will review the investigations of many researchers into the mechanisms of action of iminosugars and the contribution of our current understanding of these mechanisms for optimising clinical delivery of iminosugars. The effects of iminosugars on enzymes other than glucosidases, the induction of ER stress and viral receptors will be also put into context. Data suggest that inhibition of α-glucosidases results in inhibited release of virus and is the primary antiviral mechanism of action of iminosugars against DENV.

Keywords

N-linked glycoproteins · ER α-glucosidases · Glucose-mimicking iminosugars · Galactose-mimicking iminosugars · ER-associated degradation · Dengue virus

20.1 Introduction

Transmitted by female *Aedes* mosquitoes, dengue virus (DENV) infects almost 400 million people each year [5], and is a growing global

J. L. Miller (✉) · B. E. Tyrrell · N. Zitzmann
Department of Biochemistry, Antiviral Drug Discovery Group, Oxford Glycobiology Institute, University of Oxford, Oxford, UK
e-mail: joanna.miller@bioch.ox.ac.uk

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_20

health problem. Despite the licensure of the first dengue vaccine, Dengvaxia® (CYD-TDV) in 2015, the development and clinical testing of novel antiviral therapies against dengue virus remains imperative. Dengvaxia® displays differential efficacy against the four dengue serotypes and it is not licenced for people in non- or low-endemicity areas (e.g. travellers and military personnel), due to reduced efficacy in seronegative recipients and an increased risk of antibody-dependent enchantment (ADE). A licenced antiviral therapy to treat dengue disease is thus still of vital importance for reducing morbidity and mortality.

20.1.1 *N*-Linked Glycoprotein Production and Processing

Inhibition of biosynthesis of *N*-linked oligosaccharides, involving both glycosylation and glycoprotein processing, has been targeted as an antiviral approach for a number of decades. *N*-linked glycans are added to proteins at specific amino acid sequences, initially by addition of $Glc_3Man_9GlcNAc_2$, where Glc is glucose, Man is mannose and GlcNAc is *N*-acetyl glucosamine (Fig. 20.1). Once this precursor oligosaccharide is transferred to a glycoprotein, the carbohydrate chain is subjected to a variety of processing reactions, including both removal

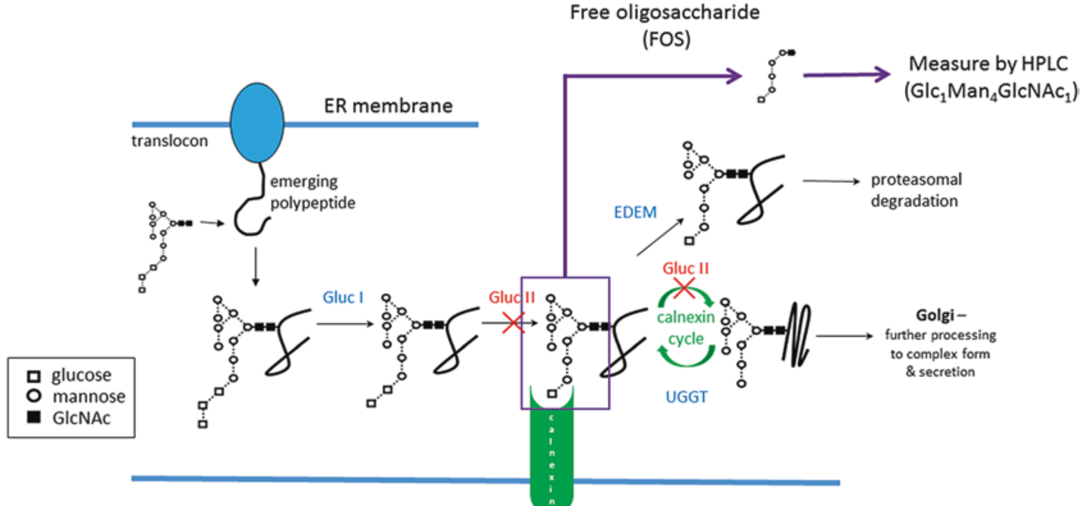


Fig. 20.1 Trimming of *N*-linked glycans in the ER and production of FOS. Following translocation of a newly transcribed peptide into the ER, a preassembled precursor oligosaccharide, consisting of 3 glucose (Glc), 9 mannose (Man) and 2 N-acetylglucosamine (GlcNAc) residues ($Glc_3Man_9GLcNAc_2$) is transferred to the nascent polypeptide. Stepwise removal of first the outer α1,2-linked glucose residue by α-glucosidase I (GluI), then the next two α1,3-linked glucose residues by α-glucosidase II (GluII) occurs. The lectin chaperones calnexin and calreticulin can bind to the monoglucosylated oligosaccharide intermediate ($Glc_1Man_9GLcNAc_2$) to slow glycoprotein progression through the ER and allow for correct folding. UDP-glucose glycoprotein:glycoslytransferase (UGGT) detects misfolded proteins and reglucosylates the oligosaccharides to allow for repeated interaction with calnexin and other folding chaperones and enzymes (calnexin cycle). Correctly folded proteins can bud from the ER for transport to the Golgi and further processing, or alternatively undergo additional processing by ER degradation-enhancing α-mannosidase I–like protein (EDEM) and other ER mannosidases to be targeted for degradation. Free oligosaccharides (FOS) are generated during protein *N*-glycosylation in mammalian cells and specific species can be detected by HPLC in cell lysates. For example, inhibition of GluII results in a monoglucosylated glycoprotein. After trimming of the glycan precursor by mannosidases and recognition of the glycoprotein as terminally misfolded, a $Glc_1Man_4GlcNAc_1$ FOS species is cleaved from the peptide during ER-associated degradation (ERAD). In the case of inhibition of GluI, (not shown) the $Glc_3Man_5GlcNAc_1$ FOS species can be detected (Adapted from [67])

and addition of sugar residues in the endoplasmic reticulum (ER) and Golgi to produce the typical high-mannose, hybrid, and complex types of oligosaccharides. Glycans play a specific role in glycoprotein folding through the calnexin/calreticulin cycle. α-glucosidase I (GluI) and α-glucosidase II (GluII) sequentially trim the terminal glucoses from the precursor oligosaccharide, resulting in the monoglucosylated $Glc_1Man_9GlcNAc_2$ which is bound by calnexin [81]. Through interactions with various protein disulphide isomerases and other chaperones, the glycoprotein has the chance to fold correctly while being held in the ER, before progressing to the Golgi. The glycoprotein eventually plays a role in the viral life cycle or is incorporated into a new virion. These host cell glycosylation processes are required by viruses expressing glycoproteins as no virus has been identified to encode the enzymes required for biosynthesis of *N*-linked oligosaccharides. DENV possesses four *N*-linked glycoproteins: envelope, premembrane, non-structural protein 1 (NS1), and non-structural protein 4B.

20.1.2 Iminosugars

Iminosugars are so named due to their resemblance to monosaccharides, differing where the ring oxygen has been replaced with a nitrogen atom. Replacement of the oxygen reduces iminosugar susceptibility to cleavage by glucosidases and allows derivatization at the nitrogen atom. Several iminosugars are inhibitors of cellular glycosidase enzymes in the *N*-linked glycan processing pathway. In 1982 deoxynojirimycin (DNJ) was first shown to inhibit the formation of complex glycans in *Saccharomyces cerevisiae* [66] by inhibiting the action of the ER GluI and GluII, the earliest enzymes in the *N*-glycan processing pathway (Fig. 20.1). Subsequently, their antiviral potential was realised when castanospermine and *N*-alkyl derivatives of DNJ were shown to inhibit HIV [28, 84].

20.1.3 Iminosugars as Anti-Dengue Compounds

ER chaperones such as BiP and heat shock protein 90 (HSP90) can assist protein folding in a carbohydrate-independent manner, unlike calnexin/calreticulin. DENV E protein binds to BiP in infected cells and knockdown of BiP, calnexin or calreticulin resulted in reduced production of infectious virus, indicating that these chaperones all play a role in folding and assembly of dengue proteins [39]. Interestingly, both HSP90 [61] and BiP [34] have been proposed to act as attachment or entry receptors for DENV in certain cells. Despite this chaperone redundancy, ER α-glucosidase inhibitors were first shown to have antiviral effects on DENV by Courageot et al. [17] via a mechanism that did not affect viral protein synthesis, but appeared to reduce prME heterodimer formation and stability. They concluded that the formation of properly folded DENV envelope complexes requires a lectin chaperone pathway.

Since then many studies have shown that iminosugars are antiviral against DENV [67]. Here we summarise the published antiviral data of iminosugars against DENV *in vitro* (Table 20.1) and *in vivo* (Table 20.2), to highlight the progress in this field. Many different DNJ-derivative and bicyclic iminosugars have now demonstrated antiviral effects against DENV. The number of studies since we last reviewed the field in 2010 has more than tripled, with particular progress in *in vivo* studies, an important step for progression to clinical trials.

20.1.4 Clinical Trials of Iminosugars Against Dengue

Two lead therapeutic candidate iminosugars, methoxy-*N*-nonyl deoxynojirimycin (M*O*N-DNJ, UV-4B) and celgosivir (the prodrug of castanospermine) have progressed to Phase I and Ib clinical trials, respectively. Both appear very safe with no serious adverse events reported.

Table 20.1 Antiviral efficacy of six-membered ring iminosugars against DENV in *in vitro* experiments

Iminosugar	DENV serotype (strain)	Cell type (MOI)	Effect on			Reference
			Viral glycoproteins	Viral replication	Infectious virus secretion	
DNJ-derivative iminosugars						
DNJ	1 (FGA/89)	Neuro 2a (400)	prME dimer formation impaired	ND	Reduced to 20% control at 500 μM	[17]
	2 (16681)	MDMΦ (1)	ND	ND	EC_{50} 308 μM	[68]
*N*B-DNJ	2 (16681)	MDMΦ (1)	ND	ND	IC_{50} 6 ± 7.31 μM; IC_{90} 62.1 ± 60.7 μM	[45]
	2 (NGC)	Vero	ND	ND	IC_{50} 162 μM	[51]
	2 (16681)	MDMΦ (1)	ND	Secreted DENV reduced in 1:1 ratio with infectious DENV	EC_{50} 10.6 μM	[68]
*N*N-DNJ	2 (PL046)	BHK-21 (0.1)	Dose-dependent reduced intracellular E and NS1, and reduced secretion	Reduced RNA replication (16-fold at 100 μM)	Reduced to plaque assay limit of detection with 5 μM; only significantly antiviral when drug added post-infection	[94]
	2	BHK-21 (0.05)	ND	ND	EC_{50} 1.1 μM; EC_{90} 3.3 μM	[13]
			ND	ND	IC_{50} 1 μM	[29]
	2 (16681)	MDMΦ (1)	ND	ND	IC_{50} 0.91 ± 0.40 μM; IC_{90} 8.02 ± 4.14 μM	[45]
	2 (NGC)	Vero	ND	ND	IC_{50} 9 μM	[14]
	2 (16681)	MDMΦ (1)	ND	ND	EC_{50} 1.25 μM	[68]
N-7-oxadecyl-DNJ	2 (NGC)	Vero	ND	ND	IC_{50} 41 μM	[51]
M*O*N-DNJ (UV-4)	1 (779,172)	Vero (0.01)	ND	ND	IC_{50} 5.15 ± 3.85 μM	[89]
	1 (SH 29177)	Vero (0.01)	ND	ND	IC_{50} 2.10 ± 2.50 μM	[89]
	1 (PRS 41393)	Vero (0.01)	ND	ND	IC_{50} 37.69 ± 10.95 μM	[89]
	2 (16681)	MDMΦ (1)	ND	ND	IC_{50} 3.09 ± 3.93 μM; IC_{90} 7.74 ± 3.63 μM	[45]
			ND	1:1 ratio in reduction of secreted total and infectious virus		[89]
	2 (NGC)	Vero	ND	ND	IC_{50} 17 μM	[51]
		Vero (0.01)	ND	ND	IC_{50} 6.49 ± 1.65 μM	[89]
	2 (SL 5–17-04)	Vero (0.01)	ND	ND	IC_{50} 22.34 ± 16.36 μM	[89]
	2 (UIS 1288)	Vero (0.01)	ND	ND	IC_{50} 18.69 ± 7.21 μM	[89]
	3 (SL 5–29-04)	Vero (0.01)	ND	ND	IC_{50} 3.64 ± 1.39 μM	[89]

(continued)

Table 20.1 (continued)

Iminosugar	DENV serotype (strain)	Cell type (MOI)	Effect on Viral glycoproteins	Viral replication	Infectious virus secretion	Reference
	3 (UIS 776)	Vero (0.01)	ND	ND	IC_{50} 6.56 ± 2.80 μM	[89]
	3 (H87)	Vero (0.01)	ND	ND	IC_{50} 86.49 ± 1.58 μM	[89]
	4 (779,157)	Vero (0.01)	ND	ND	IC_{50} 18.18 ± 24.44 μM	[89]
	4 (C258/97)	Vero (0.01)	ND	ND	IC_{50} 8.95 ± 1.25 μM	[89]
	4 (H241)	Vero (0.01)	ND	ND	IC_{50} 2.78 ± 1.42 μM	[89]
*N*AP-DNJ	2 (16681)	MDMΦ (1)	ND	ND	IC_{50} 0.04 ± 0.01 μM; IC_{90} 0.28 ± 0.14 μM	[45]
	2 (NGC)	Vero	ND	ND	IC_{50} 2 μM	[14]
2THO-DNJ (UV-12)	2 (NGC)	Vero (0.01)	ND	ND	IC_{50} 21.71 μM	[87]
CM-9-78	2 (TSV01)	A549 (0.3)	ND	EC_{50} 1.5 μM	ND	[14]
	2	BHK-21 (0.05)	ND	ND	EC_{50} 6.75 μM; EC_{90} 13 μM	[13]
CM-10-18	2 (TSV01)	A549 (0.3)	ND	EC_{50} 1.1 μM	ND	[14]
	2 (NGC)	BHK-21 (0.01)	ND	ND	EC_{50} 4.5 ± 2.0 μM; EC_{90} 47.2 ± 27.6 μM	[16]
CM-10-18 plus ribavirin	2 (TSV01)	A549	ND	Synergistic antiviral effect	ND	[14]
IVHR11029	2 (NGC)	BHK-21 (0.01)	ND	ND	EC_{50} 0.75 ± 0.06 μM; EC_{90} 6.3 ± 3.5 μM	[16]
IVHR17028	2 (NGC)	BHK-21 (0.01)	ND	ND	EC_{50} 0.3 ± 0.03 μM; EC_{90} 1.7 ± 0.8 μM	[16]
IVHR19029	2 (NGC)	BHK-21 (0.01)	ND	ND	EC_{50} 1.25 ± 1.1 μM; EC_{90} 22.5 ± 10.6 μM	[16]
OSL95-ii	2	BHK-21 (0.05)	ND	ND	EC_{50} 4 μM; EC_{90} 8.7 μM	[13]
	2	BHK-21 (0.05)	ND	ND	IC_{50} 2 μM	[29]
PBDNJ0801	2	BHK-21 (0.05)	ND	ND	EC_{50} 0.1 μM; EC_{90} 0.2 μM	[13]
PBDNJ0803	2	BHK-21 (0.05)	ND	ND	EC_{50} 0.1 μM; EC_{90} 0.6 μM	[13]
PBDNJ0804	2	BHK-21 (0.05)	ND	ND	EC_{50} 0.075 μM; EC_{90} 0.6 μM	[13]
N-butyl-cyclohexyl-DNJ	2	BHK-21 (0.05)	ND	ND	IC_{50} 3 μM	[29]
N-propyl-cyclohexyl-DNJ	2	BHK-21 (0.05)	ND	ND	IC_{50} 1.5 μM	[29]
Bicyclic iminosugars						
CAST	1 (Brazil)	BHK-21 (0.01)	ND	ND	IC_{90} < 50 μM	[92]

(continued)

Table 20.1 (continued)

Iminosugar	DENV serotype (strain)	Cell type (MOI)	Effect on Viral glycoproteins	Effect on Viral replication	Effect on Infectious virus secretion	Reference
	1 (FGA/89)	Neuro 2a (400)	E protein misfolded; prME dimer formation impaired	ND	Reduced to 5% control at 500 μM	[17]
	2	BHK-21 (0.05)	ND	ND	IC_{50} 6 μM	[29]
	2 (16681)	BHK-21 (0.1–10)	prM glycosylation affected	Replicon expression reduced by <40%	IC_{50} 1 μM; $IC_{90} < 50$ μM	[92]
		Huh-7 (0.1–10)	ND	ND	IC_{50} 85.7 μM	[92]
		MDMΦ (1)	ND	ND	EC_{50} 36.4 μM	[68]
	2 (N1042)	BHK-21	ND	ND	$IC_{90} < 50$ μM	[92]
	3 (Sri Lanka)	BHK-21	ND	ND	$IC_{90} < 50$ μM	[92]
	4 (Tahiti)	BHK-21	ND	ND	$IC_{90} < 50$ μM	[92]
Celgosivir	1 (2402)	BHK-21 (0.3)	ND	EC_{50} 0.65 ± 0.16 μM	ND	[58]
			ND	ND	EC_{50} 0.105 ± 0.059 μM	[91]
		BHK-21 (0.01)	ND	ND	EC_{50} 0.066 ± 0.019 μM	[91]
		Huh-7 (0.3)	ND	ND	EC_{50} 17.430 ± 4.921 μM	[91]
		Huh-7 (0.01)	ND	ND	EC_{50} 5.961 ± 1.258 μM	[91]
		Vero (0.3)	ND	ND	EC_{50} 51.035 ± 14.47 μM	[91]
		Vero (0.01)	ND	ND	EC_{50} 13.805 ± 1.902 μM	[91]
		THP-1 (2)	ND	ND	EC_{50} 3.236 μM	[91]
	2 (3295)	BHK-21 (0.3)	E transport to Golgi blocked. NS1 in cells reduced (immuno-fluorescence) and colocalises with ER not Golgi.	EC_{50} 0.22 ± 0.01 μM	ND	[58]
			ND	ND	EC_{50} 0.061 ± 0.003 μM	[91]
		Huh-7 (0.3)	ND	ND	EC_{50} 0.824 ± 0.109 μM	[91]
		Vero (0.3)	ND	ND	EC_{50} 2.434 ± 0.773 μM	[91]
		THP-1 (50)	ND	ND	EC_{50} 0.756 μM	[91]

(continued)

Table 20.1 (continued)

Iminosugar	DENV serotype (strain)	Cell type (MOI)	Effect on Viral glycoproteins	Viral replication	Infectious virus secretion	Reference
	2 (S221)	BHK-21 (0.3)	ND	ND	EC_{50} 0.119 ± 0.000 μM	[91]
		Huh-7 (0.3)	ND	ND	EC_{50} 5.093 ± 1.036 μM	[91]
		Vero (0.3)	ND	ND	EC_{50} 8.336 ± 0.773 μM	[91]
		THP-1 (2)	ND	ND	EC_{50} 2.135 μM	[91]
	16 DENV-1 and −2 isolates from CELADEN trial	Huh-7 (various)	ND	ND	Only one strain less sensitive to 3 μM celgosivir than DENV-1 (2402)	[91]
	2 (16681)	MDMΦ (1)	ND	Secreted DENV reduced in 1:1 ratio with infectious DENV	EC_{50} 5.17 μM	[68]
	3 (863)	BHK-21 (0.3)	ND	EC_{50} 0.68 ± 0.02 μM	ND	[58]
	4 (2270)	BHK-21 (0.3)	ND	EC_{50} 0.31 ± 0.12 μM	ND	[58]
DGJ-derivative iminosugars						
*N*B-DGJ	2 (16681)	MDMΦ (1)	ND	ND	No inhibition	[68]
*N*N-DGJ	2 (16681)	MDMΦ (1)	ND	ND	No inhibition	[68]
M*O*N-6d-DGJ	2 (16681)	MDMΦ (1)	ND	ND	No inhibition	[89]

Abbreviations: *DNJ deoxynojirimycin, DGJ deoxygalactonojirimycin MON-DNJ methoxy-nonyl-DNJ, MON-6d-DGJ methoxy-nonyl-6-deoxy-DGJ, NAP-DNJ* N-(6'-4"- azido-2"-nitrophenylamino) hexyl-1-DNJ, *2THO-DNJ* N-8'-(2"-tetrahydrofuranyl)-octyl-DNJ, *CAST* castanospermine, *NB-* N-butyl-, *NN-* N-nonyl-

Celgosivir (BuCAST) is the butylated prodrug cleaved in cells to produce castanospermine, a bicyclic iminosugar. It has submicromolar activity against DENV *in vitro* and in an *in vivo* mouse model [58, 90]. These results, combined with encouraging pre-clinical pharmacology results and human safety data obtained from clinical trials of celgosivir against HIV and hepatitis C virus (HCV) [20, 35], where it had modest antiviral effects, supported a Phase 1b randomised, double-blind, placebo-controlled clinical trial in 50 adult dengue patients (CELADEN, NCT01619969). This trial recruited patients with a fever (≥38 °C) for less than 48 h and dosed celgosivir at an initial 400 mg loading dose, followed by 200 mg every 12 h for a total of nine doses. While this study failed to show a decrease in fever duration or viral load [40, 76], the authors have subsequently investigated optimisation of dosing to give higher minimum concentrations [91] and will perform a Phase IIa clinical trial with four-times daily treatment (NCT02569827). In the first trial, a more rapid clearance of NS1 antigen was observed in patients treated with celgosivir compared to the placebo: an effect that was more prominent in patients with secondary dengue infection. They also highlighted the possibility of a therapeutic difference between patients with primary or secondary infections, indicating that future trials should be powered to investigate this.

Table 20.2 Iminosugar antiviral efficacy against DENV in *in vivo* experiments

Iminosugar	DENV infection	Animal model	Outcome	Reference
*N*B-DNJ	10^5 p.f.u. i/v DENV-2 (D2S10) with ADE (4G2 anti-E antibody)	AG129 mice; n = 5–18/group	PBS or PBS-containing PERLs: death at day 4–5 p.i. Non-significant reduction in liver and spleen viral titres.	[45]
			0.088 mg/kg/day: no effect on survival. Non-significant reduction in liver and spleen virus titres.	
			250 mg/kg/day: 20% survival	
			1000 mg/kg/day: 90% survival. Significant viral load reduction in liver, small intestine, serum and spleen at day 3.5 p.i.	
			0.094 mg/kg/day encapsulated in PERLs: 20% survival, with encapsulation providing >1900-reduction in dose able to increase survival. Non-significant reduction in liver and spleen viral titres.	
	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 10 (drug), 5 (ribavirin)	Water or ribavirin 100 mg/kg day: euthanised day 4–6 p.i., median survival 4 days	[51]
			100 mg/kg BID orally for 7 days: no significant difference	
*N*N-DNJ	2×10^6 p.f.u. i/p DENV-2 (TSV01)	7–9-week old AG129 mice; n = 8/group	75 mg/kg orally BID for 3 days: 93% reduced viraemia, 68% reduced splenomegaly; significantly reduced pro-inflammatory cytokines and chemokines (TNF-α, IL-6, IL-12, IFN-γ, MCP-1)	[71]
	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 13 (drug), 5 (ribavirin)	Water or ribavirin 100 mg/kg day: euthanised day 4–6 p.i., median survival 4 days	[51]
			100 mg/kg BID orally for 7 days: median survival 8 days, gradual decrease in mean group weight	
N-7-oxadecyl-DNJ	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 5 (drug or ribavirin)	Water or ribavirin 100 mg/kg day: euthanised day 4–6 p.i., median survival 4 days	[51]
			100 mg/kg BID orally for 7 days: no significant difference	

M*O*N-DNJ (UV-4)	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 18 (drug), 5 (ribavirin)	Water or ribavirin 100 mg/kg daily: euthanised day 4–6 p.i., median survival 4 days	[51]
			100 mg/kg BID orally for 7 days: median survival 7.5 days, mean weight significantly higher than control group throughout	
	2×10^4 p.f.u. i/v DENV-2 (S221) with ADE (2H2 anti-prM antibody)	AG129 mice; n = 33 (water), 29 (drug)	Water: 10% survival at day 9 p.i., median survival 4 days	
			100 mg/kg TID orally for 7 days: 89% survival at day 9 p.i. with no symptoms. Serum viral RNA and titres reduced 4-fold at 48 h p.i. equivalent at 72 h p.i., and 100-fold lower at 96 h p.i. Viral RNA levels reduced 100–1000-fold in liver, small intestine and kidney. Lower but significant reduction in viral titre in liver and kidney. No effect on DENV-specific IgM or IgG.	
	2×10^4 p.f.u. i/v DENV-2 (S221) with ADE (2H2 anti-prM antibody)	AG129 mice; n = 11 (water), 10/drug group	Water: 0% survival at day 5 p.i.	
			2.5 mg/kg TID orally for 7 days: 30% survival at day 9 p.i.	
			5 mg/kg TID orally for 7 days: 50% survival at day 9 p.i.	
			10 mg/kg TID orally for 7 days: 90% survival at day 9 p.i.	
			100 mg/kg TID orally for 7 days: 100% survival at day 9 p.i.	
	2×10^4 p.f.u. i/v DENV-2 (S221) with ADE (2H2 anti-prM antibody)	AG129 mice; n = 10 (water), 8/drug group	Water: 0% survival at day 9 p.i.	
			Drug dosing 100 mg/kg TID orally for 7 days.	
			From time of infection: 90% survival at day 12 p.i.	
			Beginning 24 h p.i.: 100% survival at day 12 p.i.	
			Beginning 48 h p.i.: 40% survival at day 12 p.i., median survival 11 days	
			Beginning 72 h p.i.: 0% survival at day 10 p.i., no significant difference from control	
	1×10^{10} GEs in 1st passage, 1×10^8 GEs for 2nd-4th passage	$STAT1^{-/-}/2^{-/-}$ 129/Sv mice	100 mg/kg orally TID for 72 h beginning -1 h from infection. 19 nonsynonymous mutations identified in glycoproteins after four serial passages in mice, none of which provided evidence of a true escape mutant.	[52]

(continued)

Table 20.2 (continued)

Iminosugar	DENV infection	Animal model	Outcome	Reference
	10^9 GEs DENV-2 (S221) with ADE (2H2 anti-prM antibody)	AG129 mice; n = 10/group	Vehicle: 10–20% survival, significantly worse clinical scores and weight loss than drug-treated.	[89]
			10 mg/kg TID orally for 7 days: starting -1 h relative to infection, 60% survival; starting 24 h p.i., 56% survival; starting 48 h p.i., 36% survival (not significant).	
			20 mg/kg TID orally for 7 days: starting -1 h relative to infection, 85% survival; starting 24 h p.i., 100% survival; starting 48 h p.i., 70% survival.	
			40 mg/kg TID orally for 7 days: starting -1 h relative to infection, 100% survival; starting 24 h p.i., 100% survival; starting 48 h p.i., 90% survival	
			100 mg/kg TID orally for 7 days: starting -1 h relative to infection, 90% survival; starting 24 h p.i., 90% survival; starting 48 h p.i., 100% survival.	
			100 mg/kg *MON*-6d-DGJ TID orally for 7 days: No protection	
*N*AP-DNJ	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 10 (drug), 5 (ribavirin)	Water or ribavirin 100 mg/kg daily: euthanised day 4–6 p.i., MSD 4 days	[51]
			100 mg/kg BID orally for 7 days: no significant difference from water.	
2THO-DNJ (UV-12)	1×10^4 p.f.u. i/v DENV-2 (S221) with ADE (2H2 anti-prM antibody)	5–6 week old AG129 mice	Vehicle: 0% survival, MSD 5 days	[87]
			20 mg/kg TID for 7 days, starting 1 h pre-infection: 100% survival to day 9 p.i.	
			100 mg/kg TID for 7 days, starting 1 h pre-infection: 100% survival to day 9 p.i. Viral loads reduced in kidney (12.9-fold at 72 h p.i., 5.23-fold at 96 h p.i.), small intestine (6.1-fold at 72 h p.i.), but not in serum or liver at 72 or 96 h p.i. Spleen viral load increased 5-fold at 72 h p.i. but no difference at 96 h p.i.	
CAST	10^5 p.f.u. i/c DENV-2 (mouse-adapted NGC)	4-week old A/J mice; n = 30–45/ group	Vehicle: 0% survival	[92]
			10 mg/kg (10 days i/p): 20% survival	
			50 mg/kg (10 days i/p): 90% survival	
			250 mg/kg (10 days i/p): 85% survival	
	2×10^5 p.f.u. i/p DENV-2 (S221) with ADE (4G2 anti-E antibody)	AG129 mice; n = 8 (vehicle), 10 (drug)	Vehicle: 0% survival at day 5 p.i.	[90]
			50 mg/kg BID for 5 days: 60% survival at day 10 p.i.	

Celgosivir	2 × 10^6 p.f.u. i/p DENV-2 (TSV01)	7–9-week old AG129 mice; n = 8/group	7.5 mg/kg orally BID for 3 days: 62% reduced viraemia	[71]
			75 mg/kg orally BID for 3 days: 88% reduced viraemia	
			1 day delay then 75 mg/kg orally BID for 2 days: 55% reduced viraemia	
	2 × 10^5 p.f.u. i/p DENV-2 (S221)	AG129 mice; n = 8/group	Vehicle: 75% survival at day 10 p.i.	[58]
			50 mg/kg i/p BID for 5 days: 100% survival at day 10 p.i.	
	2 × 10^5 p.f.u. i/p DENV-2 (S221) with ADE (4G2 anti-E antibody)	AG129 mice; n = 8/group	Vehicle: 0% survival at day 5 p.i.	[58]
			50 mg/kg i/p BID for 5 days: 100% survival at day 10 p.i., reduced to 50% survival if administered from day 2 p.i.	
	2 × 10^5 p.f.u. i/p DENV-2 (S221) with ADE (4G2 anti-E antibody)	AG129 mice; n = 7 (50 mg/kg) or 8/group	Vehicle: 0% survival at day 5 p.i.	[90]
			10 mg/kg BID for 5 days: 13% survival at day 10 p.i.	
			25 mg/kg BID for 5 days: 63% survival at day 10 p.i., reduced viraemia at day 3 p.i.	
			50 mg/kg BID for 5 days: 100% survival at day 10 p.i., reduced viraemia at day 3 p.i.	
			100 mg/kg daily for 5 days: 0% survival at day 6 p.i., no viraemia reduction	
	10^5 p.f.u. i/v DENV-2 (D2S10) with ADE (4G2 anti-E antibody)	AG129 mice	33.3 mg/kg every 8 h until sacrifice at 80 h p.i. Viral RNA load significantly reduced, trend towards reduced circulating infectious virus and viral RNA in kidney, parenteral lymph nodes, liver and small intestine. Enhanced viral RNA levels in spleen.	[68]
	7 × 10^7 p.f.u. i/v DENV-1 (2402) with ADE (4G2 antibody)	AG129 mice; n = 5–6/group	Vehicle: 0% survival at day 5 p.i.	[91]
			10 mg/kg orally BID: 0% survival at day 6 p.i., 1.8-fold viraemia reduction at day 3 p.i.	
			50 mg/kg orally BID: 100% survival at day 10 p.i., viraemia reduced 4.3-fold. No additional reduction in viraemia if treatment started at peak viraemia.	
	1 × 10^8 p.f.u. i/v DENV-2 (3295) with ADE (4G2 antibody)	AG129 mice; n = 5–6/group	Vehicle: 0% survival at day 5 p.i.	[91]
			10 mg/kg orally BID: 100% survival at day 10 p.i., 3.7-fold viraemia reduction at day 3 p.i.	
			50 mg/kg orally BID: 100% survival at day 10 p.i., viraemia reduced 16.5-fold. No additional reduction in viraemia if treatment started at peak viraemia.	
	2 × 10^4 p.f.u. i/v DENV-2 (S221) with ADE (4G2 antibody)	AG129 mice; n = 5–6/group	Vehicle: 0% survival at day 5 p.i.	[91]
			10 mg/kg orally BID: 0% survival at day 6 p.i., 1.4-fold viraemia reduction at day 3 p.i.	
			50 mg/kg orally BID: 100% survival at day 10 p.i., viraemia reduced 2.4-fold	

(continued)

Table 20.2 (continued)

Iminosugar	DENV infection	Animal model	Outcome	Reference
	2×10^7 p.f.u. i/v DENV-2 DENV-2 (#013)	AG129 mice; n = 6/group	50 mg/kg orally BID: from infection, viraemia on day 3 p.i. reduced 6.8-fold; from 3 days p.i., VLR from day 3 to day 6 not significantly different from control	[91]
	1×10^7 p.f.u. i/v DENV-2 (#031)	AG129 mice; n = 6/group	50 mg/kg orally BID: from infection, viraemia on day 3 p.i. reduced 7.8-fold; from 3 days p.i., VLR from day 3 to day 6 not significantly different from control	[91]
	2×10^7 p.f.u. i/v DENV-2 (#036)	AG129 mice; n = 6/group	50 mg/kg orally BID: from infection, viraemia on day 3 p.i. reduced 12.5-fold; from 3 days p.i., VLR from day 3 to day 6 not significantly different from control	[91]
CM-9-78	5×10^6 p.f.u. i/p DENV-2 (TSV01)	7–8 week old AG129 mice; n = 6/group	75 mg/kg orally BID for 3 days: 2.3-fold viraemia reduction at 3 days p.i.	[14]
			25 and 10 mg/kg orally BID for 3 days: no significant effects on viraemia	
CM-10-18	5×10^6 p.f.u. i/p ENV-2 (TSV01)	7–8 week old AG129 mice; n = 6/group	75 mg/kg orally BID for 3 days: 1.8-fold viraemia reduction at 3 days p.i.	[14]
	2×10^7 p.f.u. i/v DENV-2 (mouse-adapted D2S10)	AG129 mice; n = 5/group	PBS: euthanised day 6 p.i.	[15]
			40 mg/kg/day ribavirin: euthanised day 5 p.i.	
			75 mg/kg or 150 mg/kg orally BID for 3 days: 100% survival to day 15	
	10^7 p.f.u. i/p DENV-2 (D2Y98P-rc)	AG129 mice; n = 5/group	PBS: MSD 9 ± 2.2	[15]
			25 mg/kg BID NITD008: 100% survival at day 24 p.i.	
			3 mg/kg orally BID for 3 days: MSD 12 ± 2.0	
			10 mg/kg orally BID for 3 days: MSD 14 ± 1.1	
			25 mg/kg orally BID for 3 days: MSD 17 ± 2.3	
			75 mg/kg orally BID for 3 days: 40% survival at day 24 p.i.	
CM-10-18 plus ribavirin	5×10^6 p.f.u. i/p DENV-2 (TSV01)	7–8 week old AG129 mice; n = 6/group	CM-10-18 75 mg/kg orally BID for 3 days p.i.: 1.9-fold viraemia reduction at day 3 p.i.	[14]
			Ribavirin 40 mg/kg daily for 3 days p.i.: no effect on viraemia at day 3 p.i.	
			Combination: 4.7-fold viraemia reduction at day 3 p.i.	

Abbreviations: *CAST* castanospermine, *DNJ* deoxynojirimycin, *NAP-DNJ* N-(6′-4″- azido-2″-nitrophenylamino) hexyl-1-DNJ, *NB-* N-butyl-, *NN-* N-nonyl-, *BID* bis in die (twice daily), *i/p* intraperitoneal, *i/v* intravenous, *MSD* mean survival days, *PERL* polyunsaturated endoplasmic reticulum-targeting liposome, *PBS* phosphate buffered saline, *p.f.u.* plaque forming units, *p.i.* post-infection, *TID* ter in die (three times daily), *VLR* virological log reduction

M*O*N-DNJ was developed to be a more potent yet similarly non-toxic derivative of *N*-butyl-DNJ (*N*B-DNJ) through alkyl chain elongation and oxygenation [44, 45] and has demonstrated more potent *in vivo* antiviral effects than *N*B-DNJ against dengue virus. M*O*N-DNJ has antiviral activity against a range of viruses *in vitro,* and *in vivo* efficacy in animal models against dengue [51, 89] and influenza virus [74, 88]. A Phase I single-ascending dose clinical trial of M*O*N-DNJ in humans (NCT02061358) has recently been completed, in which 64 volunteers received a single oral dose ranging from 3–1000 mg, with no serious adverse events reported. Even the highest dose of 1000 mg was overall well tolerated. Multiple-ascending dose studies are currently underway in preparation for efficacy testing against DENV in humans.

20.1.5 Iminosugars Are Broad Spectrum Antivirals

Several members of the class of small molecules known as iminosugars have broad-spectrum antiviral activity *in vitro* against both DNA and RNA viruses and against viruses that bud from either the ER or the plasma membrane (Table 20.3). Furthermore, iminosugars have demonstrated promising *in vivo* results against influenza, Ebola, Marburg, dengue and woodchuck hepatitis (a model for hepatitis B) [8] viruses (Table 20.3). With respect to understanding mechanism of action, it is informative to ask what susceptible viruses have in common, and of equal interest to define what determines lack of susceptibility to iminosugars. Theoretically any virus that depends non-redundantly upon the calnexin/calreticulin pathway for glycoprotein folding would be sensitive to glucosidase inhibitors. This requires at least one *N*-linked glycan on a (viral, but in some cases host [98]) glycoprotein essential for viral infectivity. Interestingly, a single glycan can be sufficient to confer susceptibility to glucosidase inhibition, as is demonstrated in the case of the glycosylation sequon in the pre-S2 domain of M protein of hepatitis B [43]. However, currently it is not possible to predict which if any *N*-glycan may be utilized to engage with the calnexin cycle, and which proteins may depend on it for proper folding. The degree of *N*-glycosylation and number of disulphide bonds, the complexity of folding required for oligomerisation and co-translational cleavage events, amongst other factors, have been proposed to contribute to sensitivity to iminosugars. Ongoing and future studies will continue to elucidate the relationship between glucosidase inhibition and antiviral action.

The formative paper by Hammond, Braakman and Helenius [30] over 20 years ago on the role the calnexin cycle, and specifically the monoglucosylated glycan, played in correct glycoprotein folding was critical in the development of our perception of how iminosugars are antiviral. While research in the last decade has significantly progressed our understanding, the mechanism is not fully elucidated. Iminosugars are known to inhibit α-glucosidases, enzymes that trim terminal glucose residues from nascent glycoproteins in the ER, controlling interaction with the calnexin cycle and hence proper glycoprotein folding and transport. Evidence suggests that by preventing the appropriate folding of viral glycoproteins, iminosugars prevent the formation of infectious viral particles. How has our understanding of the mechanism/s of antiviral action of iminosugars progressed in the last decade? We shall put new findings into context within the field.

20.2 Investigations into Mechanism of Action of Iminosugars against DENV

20.2.1 Reduced Virus Secretion

Experiments published in 2015 have clarified that treatment of DENV-infected cells with a range of iminosugars results in reduced secretion of DENV, rather than a reduction in virion infectivity [68, 89]. Iminosugars have demonstrated antiviral activity against all four serotypes of DENV [92] (Table 20.1) with IC_{50} values falling within a tenfold range across the four serotypes [51].

Table 20.3 Iminosugar antiviral efficacy against viruses relevant for human health

Virus (*N*-linked glycoproteins, where known)	Efficacious iminosugars *in vitro*	Efficacious iminosugars *in vivo*	References
Flaviviridae			
Dengue (E, prM, NS1, NS4b)	See Tables 20.1 and 20.2		
Japanese encephalitis (E, prM, NS1)	*N*N-DNJ	*N*N-DNJ	[94]
West Nile (E, prM, NS1)	*N*N-DNJ, SP169, SP173 OSL-1, OSL-3, OSL95-II, CAST, PBDNJ0801, PBDNJ0803, PBDNJ0804	ND	[13, 29, 92]
Kunjin (E)	*N*N-DNJ	ND	[41]
Hepatitis C (E1, E2, NS4B)	DNJ, *N*B-DNJ, *N*N-DNJ, *N*N-DGJ, OSL-95II, CM-10-18, CM-9-78, PBDNJ0802, PBDNJ0803, PBDNJ0804	ND	[56, 75]
Yellow fever (prM, E, NS1)	CAST	ND	[92]
Bunyaviridae			
Rift valley fever (Gn, Gc, LGp)	*N*B-DNJ, *N*N-DNJ, *N*-7-oxadecyl-DNJ, M*O*N-DNJ, *N*AP-DNJ, IHVR11029, IHVR17028, IHVR19029	ND	[16, 57]
Filoviridae			
Ebola (GP, sGP)	ND	IHVR11029, IHVR17028, IHVR19029, *N*B-DNJ, M*O*N-DNJ	[16, 46]
Marburg (GP)	ND	IHVR11029, IHVR17028, IHVR19029	[16]
Togaviridae			
Sindbis (E1, PE2)	DNJ, *N*M-DNJ, DMJ, CAST	ND	[42, 70]
Semliki forest (E1, E2, E3)	*N*M-DNJ	ND	[36]
Chikungunya (E1, E3E2)	*N*B-DNJ, *N*N-DNJ, *N*-7-oxadecyl-DNJ, M*O*N-DNJ, *N*AP-DNJ	ND	[57]
Orthomyxoviridae			
Influenza A (HA, NA)	DNJ, *N*B-DNJ, *N*N-DNJ, M*O*N-DNJ, 2THO-DNJ, *N*N-DGJ, CAST, celgosivir, HNJ, DMJ, *N*-benzyl-1,5-dideoxy-1,5-imino-D-glucitol, *N*,2-*O*-dibenzyl-1,5-dideoxy-1,5-imino-D-glucitol, *N*-benzyl-1,5-dideoxy-1,5-imino-D-mannitol, *N*-benzyl-1,5-dideoxy-1,5-imino-4,6-O-isopropylidene-D-mannitol, 3-episiastatin B	M*O*N-DNJ, 2THO-DNJ, celgosivir, HNJ	[11, 31, 32, 48, 65, 74, 83, 88, 87, 96, 97]

(continued)

Table 20.3 (continued)

Virus (*N*-linked glycoproteins, where known)	Efficacious iminosugars *in vitro*	Efficacious iminosugars *in vivo*	References
Influenza B (HA, NA)	M*O*N-DNJ	M*O*N-DNJ	[88]
Paramyxoviridae			
Measles (F, H)	*N*AP-DNJ, CAST, DMJ	ND	[9, 57]
Newcastle disease (F, HANA)	DNJ, CAST	ND	[82]
Herpesviridae			
Herpes simplex type 1 (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL)	Celgosivir	Celgosivir	[10]
Herpes simplex type 2 (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL)	*N*M-DNJ, *N*B-DNJ, *N*H-DNJ, CAST, celgosivir	ND	[33, 1]
Cytomegalavirus (gN)	DNJ, *N*B-DNJ, CAST, fagomine	ND	[27, 33, 78]
Retroviridae			
Human immunodeficiency 1 (gp160 → gp120)	DNJ, DNJ derivatives 1–8 & 10–11 [[73]], *N*M-DNJ, *N*E-DNJ, *N*B-DNJ, CAST, celgosivir, MDL 43305, MDL 28653, MDL 29435, MDL 29204, MDL 44370, MDL 29270, DMJ, *N*M-DMJ, L-fuconic-1,5-lactam, *N*-methyl-FT, *N*-acetyl-FT, *N*-(5-carboxy methyl-1-pentyl)-FT, DMDP	ND	[4, 18, 25, 28, 38, 47, 49, 53, 59, 60, 72, 73, 77, 79, 84, 86]
Human immunodeficiency 2 (gp160 → gp120)	*N*B-DNJ	ND	[53]
Hepadnaviridae			
Hepatitis B (S, M, L)	*N*B-DNJ	ND	[7]
Rhabdoviridae			
Vesicular stomatitis (G)	DNJ, CAST, Miglitol	ND	[6, 69]
Coronaviridae			
Severe acute respiratory syndrome (M, S)	Compound 7 & 15	ND	[95]

Iminosugars with six-membered rings have broad-spectrum antiviral activity against viruses infecting humans. Investigations using only native virions are included (pseudotyped viruses and replicon systems are not included). Abbreviations: *DMDP* 2R,5R-dihydroxymethyl-3R,4R-dihydroxypyrrolidin, *FT* 1,5-dideoxy-1,5-imino-L-fucitol, *Miglitol* (2R,3R,4R,5S)-1-(2-hydroxyethyl)-2-(hydroxymethyl) piperidine-3,4,5-triol, *DNJ* deoxynojirimycin, *DGJ* deoxygalactonojirimycin, *HNJ* homonojirimycin, *DMJ* deoxymannojirimycin, *MON-DNJ* methoxy-nonyl-DNJ, *NAP-DNJ* N-(6'-4"- azido-2"-nitrophenylamino) hexyl-1-DNJ, *2THO-DNJ* N-8'-(2"-tetrahydrofuranyl)-octyl-DNJ, *CAST* castanospermine, *NM-* N-methyl-, *NB-* N-butyl-, *NN-* N-nonyl-, *NH-* N-hexyl, *NE-* N-ethyl-, *NN-* N-nonyl.

When supernatant from iminosugar-treated DENV-infected primary human macrophages is assayed for both infectious virus (by plaque assay) and total virus production (by qRT-PCR for DENV RNA), the levels of virus decrease concomitantly. This is consistent with ER α-glucosidase inhibition resulting in sufficient misfolding of viral glycoproteins such that they are targeted for degradation and there is reduced secretion of virus. However, reduced secretion is not the outcome of iminosugar treatment of all viruses. In the case of HIV, iminosugars affect infectivity to a greater degree than secretion: iminosugar treatment alters the glycosylation of gp120 resulting in only modest reductions in virus released from infected cells, but strongly impaired viral entry at a stage post CD4-binding [22, 23]. It is not possible to predict whether iminosugar treatment will result in reduced virion infectivity alone, or also in reduced secretion, and as such this has to be determined on a virus-by-virus basis [38]. The differential effects of iminosugars on

secretion and infectivity seen with different viruses may also be compound or cell type specific, as in baby hamster kidney (BHK)-21 cells *N*-nonyl-DNJ (*N*N-DNJ) reduced DENV RNA replication in addition to effects on the DENV glycoproteins [94]. This is unlike observations for other, and related viruses, such as bovine viral diarrhoea virus (BVDV) [21] and HCV [75] where an absence of direct effect on RNA replication was shown. Additional research monitoring effects of iminosugars on DENV replication in relevant primary cells would be valuable to clarify the relative contributions of inhibition of viral RNA replication and glycoprotein folding on the overall antiviral effect observed.

20.2.2 Inhibition of ER α-Glucosidases

Findings from the only two documented living individuals with a genetic deficiency in GluI provide support for the treatment of viral infections with α-glucosidase inhibitors as the two children seem to be resistant to infection with enveloped viruses [64]. These children have no history of viral disease and despite substantial hypogammaglobulinemia, did not produce immune responses to live viral vaccines while still producing a normal response to protein, polysaccharide and conjugated protein-polysaccharide immunogens. Cells cultured from these children (shown to express GluII) were equally susceptible to infection with HIV in comparison to control cells; however virions produced were less infectious than those produced when the GluI gene was re-complemented back in. Displaying a similarly antiviral phenotype, but manifesting at the initial stage of cell infection, monocyte-derived macrophage cultures from each patient were either only very weakly or not productively infected with influenza virus (and less infectious virus was produced from these cells). These phenotypes are consistent with the hypothesis that inhibition of GluI is sufficient for antiviral activity.

There is convincing evidence to show that inhibition of α-glucosidases correlates with antiviral activity against DENV, much of which depends on quantification of mono- and tri-glucosylated free oligosaccharide (FOS) species. Generation of these specific FOS as the end products of protein misfolding gives a measure of both accessibility of iminosugars to the ER combined with their ER α-glucosidase inhibition activity. As a consequence of inhibition of ER GluII, a monoglucosylated glycoprotein is produced (Fig. 20.1). After trimming of the glycan precursor by mannosidases and recognition of the glycoprotein as terminally misfolded, $Glc_1Man_4GlcNAc_1$ and $Glc_1Man_6GlcNAc_1$ FOS species are cleaved from the peptide during ER-associated degradation (ERAD). In the case of inhibition of ER GluI, a similar process produces a $Glc_3Man_5GlcNAc_1$ species. Thus, the presence of each species of FOS can be correlated with successful inhibition of the respective cellular α-glucosidase. Addition of 100 μM *N*B-DNJ, *N*N-DNJ, M*O*N-DNJ or celgosivir to macrophages led to the generation of both mono- and tri-glucosylated FOS species, demonstrating inhibition of both α-glucosidases [68, 89]. When the degree of inhibition of just GluII (ie. generation of $Glc_1Man_4GlcNAc_1$ FOS) is plotted against the antiviral activity for a range of iminosugar concentrations a clear correlation is observed between these two parameters (Fig. 20.2), consistent with GluII inhibition being sufficient to achieve an antiviral effect.

20.2.3 Inhibition of Glycoprotein Folding

Interaction of dengue E with ER chaperones facilitates DENV production [39]. Ideally, to confirm that blocking viral glycoprotein entry to the ER calnexin quality control cycle results in the formation of misfolded viral glycoproteins with subsequent antiviral effect, a demonstration that iminosugar treatment of DENV-infected cells results in misfolded DENV glycoprotein(s) would be important. The three studies that monitored the effects of iminosugars on DENV glycoprotein folding and secretion [17, 58, 94] are documented in Table 20.1. Castanospermine treatment of DENV-infected cells reduced levels of immunoprecipitated E to 15–30% compared to that from untreated cells, when a conformation-sensitive monoclonal antibody was used, and the

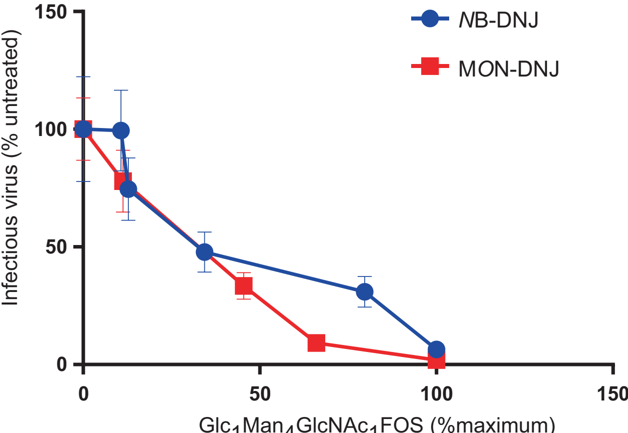


Fig. 20.2 Correlation between the antiviral activity of iminosugars and inhibition of GluII. Infectious titre (% untreated) of DENV is plotted against $Glc_1Man_4GlcNAc_1$ FOS (% maximum) (as a measure of inhibition of α-GluII) for titrations of *N*B-DNJ and M*O*N-DNJ. The viral titres and FOS are means +/− SD of samples generated in at least duplicate from at least 2 donors, at equal concentrations of iminosugar, originally published in [68, 89]

amount of E co-precipitated with prM was about 25% of that from untreated cells, demonstrating that the iminosugar decreased formation of the prME heterodimer, and that inhibition of glucosidases affects the correct folding of DENV E in this system [17]. Such a study monitoring the effects of iminosugars on folding of DENV glycoproteins could be expanded utilizing larger panels of monoclonal antibodies which recognise both conformation-dependent and -independent epitopes to all four DENV glycoproteins. This has been performed for the heavily studied HIV gp120, for which many more validated reagents are available. Studies on the effects of *N*B-DNJ on gp120 folding demonstrated that interaction with the calnexin/calreticulin pathway was critical for correct folding of the V2 loop of HIV gp120, as blocked entry into this folding pathway resulted in conformation defects in this region [23]. A recent study took this observation a step further, assessing regional folding using monoclonal antibodies against conformational epitopes in combination with mathematical modelling, which showed that misfolding of only a portion of the gp120 was sufficient to produce an amplified effect on infectivity (S.G. Spiro, 2016, unpublished results). Additional studies that compare both the level of expression of DENV glycoproteins and their state of folding in the presence and absence of iminosugars, will clarify whether iminosugars can induce misfolding and degradation of DENV glycoproteins.

20.2.4 Consequences of α-Glucosidase Inhibition for Glycans

There are at least two outcomes of α-glucosidase inhibition. By preventing the production of the monoglucosylated glycan it blocks glycoprotein binding to calnexin/calreticulin as has been described above and in Wu et al. [94]. Secondly, the retained terminal glucoses block access of α-mannosidase enzymes to the D1 arm of the glycan, theoretically preventing the formation of complex glycans. The hypothesis that glucosidase inhibitors may be antiviral due to the absence of complex sugars was the basis of the original studies on iminosugars and viruses in the 1980s. In cells with an active Golgi endomannosidase, this can be partially salvaged; however, the activity of endomannosidase is highly cell line dependent [21, 50, 75]. For example, BHK-21 cells and the human hepatoma cell line HepG2 express high levels of endomannosidase [37]

while Chinese hamster ovary (CHO) and Madin-Darby canine kidney (MDCK) cells have no detectable endomannosidase activity [63]. Production of DENV prM and NS1 with hyper-glycosylated glycans has been demonstrated by mobility shift of the viral glycoprotein in electrophoresis [58, 92]. This has been confirmed by analysing glycan structures attached to specific glycoproteins for SARS coronavirus [62] and influenza virus [32] though not yet for DENV glycoproteins. Human cells have active endo-mannosidases so it is important that *in vitro* experiments into the effects of glucosidase inhibition use cell lines that express endomannosidase for relevance. It will be interesting to ascertain whether DENV glycoproteins produced in the presence of iminosugars bear triglucosylated glycans.

20.2.5 Off-Target Effects

Being glucose mimetics, it is not surprising that iminosugars inhibit more than just ER α-glucosidases. Iminosugars also target intestinal digestive enzymes including sucrases and isomaltases, and this interaction is consistent with the mild/moderate, reversible, gastrointestinal symptoms (including flatulence and diarrhoea) seen in some participants in celgosivir and *N*B-DNJ clinical trials. Combinations of low sucrose/starch, high glucose diets and anti-diarrhoea agents can control these symptoms. Of interest, iminosugar inhibition of intestinal α-glucosidases can be used for benefit in patients with non-insulin dependent diabetes. *N*-2′-hydroxyethyl-DNJ is marketed as Miglitol®, and through preventing digestion of carbohydrates lowers the degree of postprandial hyperglycemia to establish greater glycemic control in diabetes mellitus type 2.

Long alkyl chain iminosugars such as *N*N-DNJ and the galactose mimetic *N*N- deoxygalactonojirimycin (*N*N-DGJ) mediate an antiviral effect on BVDV and HCV via inhibiting viral p7 ion channel activity, in a manner independent of inhibiting glucose-recognising host cell enzymes [21, 50, 75]. For the same antiviral mechanism of action to be evoked for DENV, the existence of a dengue ion channel needs to be postulated, as well as its inhibition by long alkyl chain iminosugars. Although some controversy exists in the literature [55, 93], studies by Wong et al. [93] suggesting that neither DENV1 nor DENV2 prM or M proteins show pH-activated ion channel activity when expressed on the surface of *Xenopus* oocytes, in combination with the observation that *N*N-DGJ is not antiviral against DENV *in vitro* at up to 100 μM [68], would indicate that this is an unlikely mechanism of action in the case of DENV.

*N*B-DNJ, as well as other DNJ- and DGJ-derivative iminosugars with longer alkyl tails, also inhibits glucosyl-ceramide synthase (GCS), a glucosyltransferase: an effect that is not dependent on mimicking glucose stereochemistry, but on mimicking the other substrate of GCS, ceramide. Through a comparison of antiviral activity with inhibition of glycolipid processing using glucose and galactose analogues of iminosugars, we have recently shown that antiviral activity of iminosugars against DENV is a function of inhibition of glycoprotein processing rather than due to any effects on GCS [68, 89].

20.2.6 Induction of ER Stress

Blocking productive folding of viral glycoproteins can alter retention times in the ER lumen followed by accumulation and/or increased ERAD. Accumulation of misfolded DENV glycoproteins induces the unfolded protein response (UPR) as the cell attempts to redress the imbalance in homeostasis. While DENV infection alone induces and regulates UPR pathways in human monocytic cells [85], stimulating BiP and XBP-1 mRNA transcription, addition of celgosivir appeared to reduce downstream effector mechanisms of UPR pathways, as demonstrated by reduced phosphorylation of EIF2α [58]. Celgosivir treatment, alone or in the presence of DENV, upregulated transcription of EDEM-1, an ER chaperone that promotes degradation of unfolded proteins, clearing the ER to reduce stress. Taken together, Vasudevan and colleagues suggest that DENV infection in the presence of celgosivir is

characterised by reduced ER stress and enhanced survival. Modulation of the UPR induced in response to increased viral protein levels in the ER has been proposed as a therapeutic target [19, 26].

20.2.7 Iminosugar Effects on Viral Receptors

Modulation of receptors important in the DENV lifecycle and pathogenesis is an additional potential pathway by which iminosugars may exert their antiviral effect. Many host proteins vital for DENV attachment, uptake, signalling and the immune response are themselves *N*-linked glycoproteins and thus perturbations in their expression and function could have implications for virus growth. As with the effects of iminosugars on secretion of different viruses, their effects on host glycoproteins are predicted to be protein specific. Treatment with IHVR-17028, a DNJ-derivative, altered the *N*-linked glycan structure of angiotensin I-converting enzyme 2 (ACE2) in a manner that did not affect its expression or binding to SARS-CoV spike glycoprotein but disrupted its ability to participate in virus envelope-triggered membrane fusion [98]. Very few host glycoproteins have been examined specifically for effects of iminosugars on expression and function.

20.3 Iminosugars As Pharmaceuticals

A number of challenges lie ahead to optimise clinical delivery of iminosugars for pharmaceutical use. One of the specific difficulties of using iminosugars to treat dengue disease, which is more broadly applicable to its use against any acute viral infection, is the short window available for treatment. By the time a dengue patient presents to the healthcare system, they typically may have had a fever for 2–4 days, at which stage there is only 24–48 h before viral load drops as the immune system controls viral replication. The task for an antiviral to reduce viral load in such a window will require a safe, fast acting, highly potent drug. In considering whether an iminosugar could be administered to people living in an endemic setting who present with fever, independent of the differential diagnosis, a dengue therapeutic would need to have an excellent safety profile. Phase I single-ascending dose trial results recently released for MON-DNJ are promising in this respect (NCT02061358), however recruitment for the clinical trial testing the safety and pharmacokinetics of MON-DNJ administered as multiple ascending doses (NCT02696291) was terminated for business reasons in March 2018. All these challenges will be relevant for the use of iminosugars therapeutically against a number of acute viruses, while treating chronic viral disease will present different challenges.

The rapid clearance of iminosugars *in vivo* makes reaching sufficient concentrations to mediate antiviral effects a specific challenge for these compounds. Following oral administration celgosivir had a plasma half-life of 2.5 h in patients [76], which is similar to 5.14 h in mice given a single dose of M*O*N-DNJ orally at 200 mg/kg [51]. In addition, iminosugars are generally excreted rapidly in the urine [2]. The clinical trial testing *N*B-DNJ against HIV concluded that sufficient plasma concentrations could not be achieved to obtain a convincing antiviral effect [24, 80]. In efforts to maximise the mean trough concentrations, and increase the chance of success in testing celgosivir efficacy against DENV, coordinators of the next celgosivir trial performed pharmacokinetic modelling and propose increasing the number of doses per day [76]. An alternate approach, previously shown to enhance antiviral activity of iminosugars against HIV >100,000-fold *in vitro* [53, 54], is encapsulation of the compounds in liposomes, a system used clinically to mediate intracellular delivery of anti-cancer and anti-fungal treatments. When tested *in vivo* against DENV in an ADE mouse model, liposome-mediated delivery of *N*B-DNJ, in comparison with free *N*B-DNJ, resulted in a 3-$\log_{10}$ reduction in the dose of drug required to enhance animal survival [45]. Although a promising approach, the specific formulation of liposomes tested in this study was costly and not sufficiently stable for liposome-mediated delivery to be investigated further. The availability of iminosugars with generally low toxicity makes the

optimisation of pharmacokinetics and dosing regimens currently a more promising approach to optimising clinical iminosugar delivery.

20.3.1 Selectivity

Because ER glucosidases control glycan processing of both viral and host cellular glycoproteins, it would not necessarily be predicted that inhibition of ER glucosidases selectively suppresses viral replication, and yet, in animals at least, a therapeutic window clearly exists where iminosugars are antiviral and well tolerated, at least for acute treatment. A number of possible explanations exist for this dichotomy but further experiments will need to be performed to determine their relative contributions. When viruses infect cells, their proteins are the predominant proteins being synthesised and hence may be more susceptible to inhibition of ER glucosidases. In addition, the DENV virion is comprised of a closely packed, repetitive and coordinated interaction of E and prM proteins which may increase virion susceptibility to any perturbation. Interestingly, in the case of HIV, very little protein misfolding is required to affect virion infectivity (S.G. Spiro, 2016, unpublished results). When the proportions of misfolded gp120 in the presence of *N*B-DNJ were modelled, HIV infectivity was shown to be highly sensitive to the misfolding of only a small proportion of total gp120, suggesting an amplification effect that may contribute to the selectivity of iminosugars against viruses over the host.

20.4 Conclusions

Against the background of historical findings, we highlight advances made in the last decade in understanding the mechanisms of antiviral activity of iminosugars against DENV. The generally accepted antiviral mechanism of ER glucosidase inhibitors, that inhibition of GluI and/or GluII prevents the removal of the terminal glucose moieties on *N*-linked glycans and results in misfolding and retention of glycoproteins in the ER and ultimate degradation via ERAD is supported by a number of pieces of evidence. These include the observations that iminosugars induce the electrophoretic mobility shift of viral glycoproteins, as well as structural changes of *N*-linked glycans and measurement of FOS consistent with GluI and GluII inhibition correlating with antiviral effect. Substrate flux along the *N*-linked glycosylation pathway makes the correlation between key enzymes and an antiviral effect complex [3]. Though both GluI and GluII are targets of iminosugars, the slower removal of the third glucose residue by GluII, even though iminosugars bind approximately tenfold more avidly to GluI [2], is more sensitive to inhibition by iminosugars. As a result of inhibiting these enzymes, viral envelope glycoproteins cannot interact with ER chaperones such as calnexin and calreticulin, preventing correct glycoprotein folding, oligomerization and assembly of infectious virions.

Uniting the conclusions from these multiple studies also allows us to highlight areas where the mechanism of action of iminosugars against DENV could be understood in greater molecular detail. The use of panels of anti-DENV glycoprotein monoclonal antibodies with known specificity binding to DENV glycoproteins produced in the presence of iminosugars has the potential to enable mapping of regional iminosugar-induced misfolding, which may inform our understanding of, for example, E dimerization. The degree or location of misfolding may be protein dependent, potentially even down to strain-dependent differences. Use of iminosugars with galactose stereochemistry (DGJ compounds) has allowed the conclusion that the antiviral effect of piperidine iminosugars (monocylic iminosugars with an iminopyranose structure) against DENV observed in macrophages is not mediated by effects on enzymes of the glycolipid pathway. Development of selective ER α-glucosidase inhibitors would allow both confirmation that ER α-glucosidase inhibition (and not other enzymes) is responsible for the antiviral effect of iminosugars and avoidance of gastrointestinal side effects due to inhibition of intestinal glucosidases. Recently published structures of GluII [12], alone and in complex with M*O*N-DNJ and castanospermine

(see Chap. 19), provide the opportunity for rational drug design and greater understanding of the biochemical detail underlying the inhibition of this host enzyme.

Acknowledgements JLM is supported by Oxford Glycobiology Institute Endowment, BET is supported by The Wellcome Trust [105402/Z/14/Z] and NZ is a Fellow of Merton College, Oxford.

References

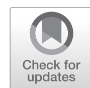
1. Ahmed SP, Nash RJ, Bridges CG, Taylor DL, Kang MS, Porter EA, Tyms AS (1995) Antiviral activity and metabolism of the castanospermine derivative MDL 28, 574, in cells infected with herpes simplex virus type 2. Biochem Biophys Res Commun 208:267–273
2. Alonzi DS, Neville DC, Lachmann RH, Dwek RA, Butters TD (2008) Glucosylated free oligosaccharides are biomarkers of endoplasmic- reticulum alpha-glucosidase inhibition. Biochem J 409:571–580
3. Alonzi DS, Scott KA, Dwek RA, Zitzmann N (2017) Iminosugar antivirals: the therapeutic sweet spot. Biochem Soc Trans 45:571–582
4. Asano N, Kizu H, Oseki K, Tomioka E, Matsui K, Okamoto M, Baba M (1995) N-alkylated nitrogen-in-the-ring sugars: conformational basis of inhibition of glycosidases and HIV-1 replication. J Med Chem 38:2349–2356
5. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI (2013) The global distribution and burden of dengue. Nature 496:504–507
6. Blatt LM, Tan HP, Seiwert S (2011) Use of alpha-glucosidase inhibitors to treat alphavirus infections. US7638488B2.
7. Block TM, Lu X, Platt FM, Foster GR, Gerlich WH, Blumberg BS, Dwek RA (1994) Secretion of human hepatitis B virus is inhibited by the imino sugar N-butyldeoxynojirimycin. Proc Natl Acad Sci U S A 91:2235–2239
8. Block TM, Lu X, Mehta AS, Blumberg BS, Tennant B, Ebling M, Korba B, Lansky DM, Jacob GS, Dwek RA (1998) Treatment of chronic hepadnavirus infection in a woodchuck animal model with an inhibitor of protein folding and trafficking. Nat Med 4:610–614
9. Bolt G, Pedersen IR, Blixenkrone-Moller M (1999) Processing of N-linked oligosaccharides on the measles virus glycoproteins: importance for antigenicity and for production of infectious virus particles. Virus Res 61:43–51
10. Bridges CG, Ahmed SP, Kang MS, Nash RJ, Porter EA, Tyms AS (1995) The effect of oral treatment with 6-O-butanoyl castanospermine (MDL 28,574) in the murine zosteriform model of HSV-1 infection. Glycobiology 5:249–253
11. Burke B, Matlin K, Bause E, Legler G, Peyrieras N, Ploegh H (1984) Inhibition of N-linked oligosaccharide trimming does not interfere with surface expression of certain integral membrane proteins. EMBO J 3:551–556
12. Caputo AT, Alonzi DS, Marti L, Reca IB, Kiappes JL, Struwe WB, Cross A, Basu S, Lowe ED, Darlot B, Santino A, Roversi P, Zitzmann N (2016) Structures of mammalian ER alpha-glucosidase II capture the binding modes of broad-spectrum iminosugar antivirals. Proc Natl Acad Sci U S A 113:E4630–E4638
13. Chang J, Wang L, Ma D, Qu X, Guo H, Xu X, Mason PM, Bourne N, Moriarty R, Gu B, Guo JT, Block TM (2009) Novel imino sugar derivatives demonstrate potent antiviral activity against flaviviruses. Antimicrob Agents Chemother 53:1501–1508
14. Chang J, Schul W, Butters TD, Yip A, Liu B, Goh A, Lakshminarayana SB, Alonzi D, Reinkensmeier G, Pan X, Qu X, Weidner JM, Wang L, Yu W, Borune N, Kinch MA, Rayahin JE, Moriarty R, Xu X, Shi PY, Guo JT, Block TM (2011a) Combination of alpha-glucosidase inhibitor and ribavirin for the treatment of dengue virus infection in vitro and in vivo. Antivir Res 89:26–34
15. Chang J, Schul W, Yip A, Xu X, Guo JT, Block TM (2011b) Competitive inhibitor of cellular alpha-glucosidases protects mice from lethal dengue virus infection. Antivir Res 92:369–371
16. Chang J, Warren TK, Zhao X, Gill T, Guo F, Wang L, Comunale MA, Du Y, Alonzi DS, Yu W, Ye H, Liu F, Guo JT, Mehta A, Cuconati A, Butters TD, Bavari S, Xu X, Block TM (2013) Small molecule inhibitors of ER alpha-glucosidases are active against multiple hemorrhagic fever viruses. Antivir Res 98:432–440
17. Courageot MP, Frenkiel MP, Dos Santos CD, Deubel V, Despres P (2000) Alpha-glucosidase inhibitors reduce dengue virus production by affecting the initial steps of virion morphogenesis in the endoplasmic reticulum. J Virol 74:564–572
18. Dedera D, Vander Heyden N, Ratner L (1990) Attenuation of HIV-1 infectivity by an inhibitor of oligosaccharide processing. AIDS Res Hum Retrovir 6:785–794
19. Diwaker D, Mishra KP, Ganju L (2015) Effect of modulation of unfolded protein response pathway on dengue virus infection. Acta Biochim Biophys Sin Shanghai 47:960–968
20. Durantel D (2009) Celgosivir, an alpha-glucosidase I inhibitor for the potential treatment of HCV infection. Curr Opin Investig Drugs 10:860–870
21. Durantel D, Branza-Nichita N, Carrouee-Durantel S, Butters TD, Dwek RA, Zitzmann N (2001) Study of the mechanism of antiviral action of iminosugar derivatives against bovine viral diarrhea virus. J Virol 75:8987–8998
22. Fischer PB, Collin M, Karlsson GB, James W, Butters TD, Davis SJ, Gordon S, Dwek RA, Platt FM (1995) The alpha-glucosidase inhibitor

N-butyldeoxynojirimycin inhibits human immunodeficiency virus entry at the level of post-CD4 binding. J Virol 69:5791–5797
23. Fischer PB, Karlsson GB, Butters TD, Dwek RA, Platt FM (1996) N-butyldeoxynojirimycin-mediated inhibition of human immunodeficiency virus entry correlates with changes in antibody recognition of the V1/V2 region of gp120. J Virol 70:7143–7152
24. Fischl, M. A., Resnick, L., Coombs, R., Kremer, A. B., Pottage, J. C., Jr., Fass, R. J., Fife, K. H., Powderly, W. G., Collier, A. C., Aspinall, R. L. & Et al. (1994). The safety and efficacy of combination N-butyl-deoxynojirimycin (SC-48334) and zidovudine in patients with HIV-1 infection and 200-500 CD4 cells/mm3. J Acquir Immune Defic Syndr*,* 7**,** 139–147
25. Fleet GW, Karpas A, Dwek RA, Fellows LE, Tyms AS, Petursson S, Namgoong SK, Ramsden NG, Smith PW, Son JC, Et A (1988) Inhibition of HIV replication by amino-sugar derivatives. FEBS Lett 237:128–132
26. Fraser JE, Wang C, Chan KW, Vasudevan SG, Jans DA (2016) Novel dengue virus inhibitor 4-HPR activates ATF4 independent of protein kinase R-like Endoplasmic Reticulum Kinase and elevates levels of eIF2alpha phosphorylation in virus infected cells. Antivir Res 130:1–6
27. Gretch DR, Gehrz RC, Stinski MF (1988) Characterization of a human cytomegalovirus glycoprotein complex (gcI). J Gen Virol 69(Pt 6):1205–1215
28. Gruters RA, Neefjes JJ, Tersmette M, De Goede RE, Tulp A, Huisman HG, Miedema F, Ploegh HL (1987) Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. Nature 330:74–77
29. Gu B, Mason P, Wang L, Norton P, Bourne N, Moriarty R, Mehta A, Despande M, Shah R, Block T (2007) Antiviral profiles of novel iminocyclitol compounds against bovine viral diarrhea virus, West Nile virus, dengue virus and hepatitis B virus. Antivir Chem Chemother 18:49–59
30. Hammond C, Braakman I, Helenius A (1994) Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc Natl Acad Sci U S A 91:913–917
31. Huang R, Dietsch E, Lockhoff O, Schuller M, Reutter W (1991) Antiviral activity of some natural and synthetic sugar analogues. FEBS Lett 291:199–202
32. Hussain S, Miller JL, Harvey DJ, Gu Y, Rosenthal PB, Zitzmann N, Mccauley JW (2015) Strain-specific antiviral activity of iminosugars against human influenza A viruses. J Antimicrob Chemother 70:136–152
33. Jacob GS, Tyms AS, Rademacher TW, Dwek RA (1990) Method of treating herpesviruses. US 07/288,528
34. Jindadamrongwech S, Thepparit C, Smith DR (2004) Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch Virol 149:915–927
35. Kaita K, Yoshida E, Kunimoto D, Anderson F, Sherman M, Marotta P, Scully L, Peltekian KE, Worobetz L, Pankovich J, Petersen A (2007) Phase II proof of concept study of celgosivir in combination with pegIFNa-2b and ribavirin in chronic hepatitis c genotype-1 non responder patients. Digestive Diseases Week, Washington, DC
36. Kaluza G, Repges S, Mcdowell W (1990) The significance of carbohydrate trimming for the antigenicity of the Semliki Forest virus glycoprotein E2. Virology 176:369–378
37. Karaivanova VK, Luan P, Spiro RG (1998) Processing of viral envelope glycoprotein by the endomannosidase pathway: evaluation of host cell specificity. Glycobiology 8:725–730
38. Karpas A, Fleet GW, Dwek RA, Petursson S, Namgoong SK, Ramsden NG, Jacob GS, Rademacher TW (1988) Aminosugar derivatives as potential antihuman immunodeficiency virus agents. Proc Natl Acad Sci U S A 85:9229–9233
39. Limjindaporn T, Wongwiwat W, Noisakran S, Srisawat C, Netsawang J, Puttikhunt C, Kasinrerk W, Avirutnan P, Thiemmeca S, Sriburi R, Sittisombut N, Malasit P, Yenchitsomanus PT (2009) Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production. Biochem Biophys Res Commun 379:196–200
40. Low JG, Sung C, Wijaya L, Wei Y, Rathore AP, Watanabe S, Tan BH, Toh L, Chua LT, Hou Y, Chow A, Howe S, Chan WK, Tan KH, Chung JS, Cherng BP, Lye DC, Tambayah PA, Ng LC, Connolly J, Hibberd ML, Leo YS, Cheung YB, Ooi EE, Vasudevan SG (2014) Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): a phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial. Lancet Infect Dis 14:706–715
41. Mackenzie JM, Westaway EG (2001) Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. J Virol 75:10787–10799
42. Mcdowell W, Romero PA, Datema R, Schwarz RT (1987) Glucose trimming and mannose trimming affect different phases of the maturation of Sindbis virus in infected BHK cells. Virology 161:37–44
43. Mehta A, Lu X, Block TM, Blumberg BS, Dwek RA (1997) Hepatitis B virus (HBV) envelope glycoproteins vary drastically in their sensitivity to glycan processing: evidence that alteration of a single N-linked glycosylation site can regulate HBV secretion. Proc Natl Acad Sci U S A 94:1822–1827
44. Mehta A, Ouzounov S, Jordan R, Simsek E, Lu X, Moriarty RM, Jacob G, Dwek RA, Block TM (2002) Imino sugars that are less toxic but more potent as antivirals, in vitro, compared with N-n-nonyl DNJ. Antivir Chem Chemother 13:299–304
45. Miller JL, Lachica R, Sayce AC, Williams JP, Bapat M, Dwek R, Beatty PR, Harris E, Zitzmann N (2012) Liposome-mediated delivery of iminosugars enhances efficacy against dengue virus in vivo. Antimicrob Agents Chemother 56:6379–6386

46. Miller JL, Spiro SG, Dowall SD, Taylor I, Rule A, Alonzi DS, Sayce AC, Wright E, Bentley EM, Thom R, Hall G, Dwek RA, Hewson R, Zitzmann N (2016) Minimal in vivo efficacy of iminosugars in a lethal Ebola virus guinea pig model. PLoS One 11(11):e0167018
47. Montefiori, D. C., Robinson, W. E., Jr. & Mitchell, W. M. (1989). Antibody-independent, complement-mediated enhancement of HIV-1 infection by mannosidase I and II inhibitors. Antivir Res, 11, 137–146
48. Nishimura Y, Umezawa Y, Kondo S, Takeuchi T, Mori K, Kijima-Suda I, Tomita K, Sugawara K, Nakamura K (1993) Synthesis of 3-episiastatin B analogues having anti-influenza virus activity. J Antibiot (Tokyo) 46:1883–1889
49. Pal R, Kalyanaraman VS, Hoke GM, Sarngadharan MG (1989) Processing and secretion of envelope glycoproteins of human immunodeficiency virus type 1 in the presence of trimming glucosidase inhibitor deoxynojirimycin. Intervirology 30:27–35
50. Pavlovic D, Neville DC, Argaud O, Blumberg B, Dwek RA, Fischer WB, Zitzmann N (2003) The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. Proc Natl Acad Sci U S A 100:6104–6108
51. Perry ST, Buck MD, Plummer EM, Penmasta RA, Batra H, Stavale EJ, Warfield KL, Dwek RA, Butters TD, Alonzi DS, Lada SM, King K, Klose B, Ramstedt U, Shresta S (2013) An iminosugar with potent inhibition of dengue virus infection in vivo. Antivir Res 98:35–43
52. Plummer E, Buck MD, Sanchez M, Greenbaum JA, Turner J, Grewal R, Klose B, Sampath A, Warfield KL, Peters B, Ramstedt U, Shresta S (2015) Dengue virus evolution under a host-targeted antiviral. J Virol 89:5592–5601
53. Pollock S, Dwek RA, Burton DR, Zitzmann N (2008) N-Butyldeoxynojirimycin is a broadly effective anti-HIV therapy significantly enhanced by targeted liposome delivery. AIDS 22:1961–1969
54. Pollock S, Antrobus R, Newton L, Kampa B, Rossa J, Latham S, Nichita NB, Dwek RA, Zitzmann N (2010) Uptake and trafficking of liposomes to the endoplasmic reticulum. FASEB J 24:1866–1878
55. Premkumar A, Horan CR, Gage PW (2005) Dengue virus M protein C-terminal peptide (DVM-C) forms ion channels. J Membr Biol 204:33–38
56. Qu X, Pan X, Weidner J, Yu W, Alonzi D, Xu X, Butters T, Block T, Guo JT, Chang J (2011) Inhibitors of endoplasmic reticulum alpha-glucosidases potently suppress hepatitis C virus virion assembly and release. Antimicrob Agents Chemother 55:1036–1044
57. Ramstedt U, Klose B, Zitzmann Z, Dwek RA, Butters TD (2013) Iminosugars and methods of treating bunyaviral and togaviral disease. US 12/813,882
58. Rathore AP, Paradkar PN, Watanabe S, Tan KH, Sung C, Connolly JE, Low J, Ooi EE, Vasudevan SG (2011) Celgosivir treatment misfolds dengue virus NS1 protein, induces cellular pro-survival genes and protects against lethal challenge mouse model. Antivir Res 92:453–460
59. Ratner L, Vander Heyden N (1993) Mechanism of action of N-butyl deoxynojirimycin in inhibiting HIV-1 infection and activity in combination with nucleoside analogs. AIDS Res Hum Retrovir 9:291–297
60. Ratner L, Vander Heyden N, Dedera D (1991) Inhibition of HIV and SIV infectivity by blockade of alpha-glucosidase activity. Virology 181:180–192
61. Reyes-Del Valle J, Chavez-Salinas S, Medina F, Del Angel RM (2005) Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol 79:4557–4567
62. Ritchie G, Harvey DJ, Feldmann F, Stroeher U, Feldmann H, Royle L, Dwek RA, Rudd PM (2010) Identification of N-linked carbohydrates from severe acute respiratory syndrome (SARS) spike glycoprotein. Virology 399:257–269
63. Roth J, Ziak M, Zuber C (2003) The role of glucosidase II and endomannosidase in glucose trimming of asparagine-linked oligosaccharides. Biochimie 85:287–294
64. Sadat MA, Moir S, Chun TW, Lusso P, Kaplan G, Wolfe L, Memoli MJ, He M, Vega H, Kim LJ, Huang Y, Hussein N, Nievas E, Mitchell R, Garofalo M, Louie A, Ireland DC, Grunes C, Cimbro R, Patel V, Holzapfel G, Salahuddin D, Bristol T, Adams D, Marciano BE, Hegde M, Li Y, Calvo KR, Stoddard J, Justement JS, Jacques J, Long Priel DA, Murray D, Sun P, Kuhns DB, Boerkoel CF, Chiorini JA, Di Pasquale G, Verthelyi D, Rosenzweig SD (2014) Glycosylation, hypogammaglobulinemia, and resistance to viral infections. N Engl J Med 370:1615–1625
65. Saito T, Yamaguchi I (2000) Effect of glycosylation and glucose trimming inhibitors on the influenza a virus glycoproteins. J Vet Med Sci 62:575–581
66. Saunier B, Kilker RD Jr, Tkacz JS, Quaroni A, Herscovics A (1982) Inhibition of N-linked complex oligosaccharide formation by 1-deoxynojirimycin, an inhibitor of processing glucosidases. J Biol Chem 257:14155–14161
67. Sayce AC, Miller JL, Zitzmann N (2010) Targeting a host process as an antiviral approach against dengue virus. Trends Microbiol 18:323–330
68. Sayce AC, Alonzi DS, Killingbeck SS, Tyrrell BE, Hill ML, Caputo AT, Iwaki R, Kinami K, Ide D, Kiappes JL, Beatty PR, Kato A, Harris E, Dwek RA, Miller JL, Zitzmann N (2016) Iminosugars inhibit dengue virus production via inhibition of ER alpha-glucosidases-not glycolipid processing enzymes. PLoS Negl Trop Dis 10:e0004524
69. Schlesinger S, Malfer C, Schlesinger MJ (1984) The formation of vesicular stomatitis virus (San Juan strain) becomes temperature-sensitive when glucose residues are retained on the oligosaccharides of the glycoprotein. J Biol Chem 259:7597–7601
70. Schlesinger S, Koyama AH, Malfer C, Gee SL, Schlesinger MJ (1985) The effects of inhibitors of

glucosidase I on the formation of Sindbis virus. Virus Res 2:139–149
71. Schul W, Liu W, Xu HY, Flamand M, Vasudevan SG (2007) A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J Infect Dis 195:665–674
72. Shimizu H, Tsuchie H, Honma H, Yoshida K, Tsuruoka T, Ushijima H, Kitamura T (1990a) Effect of N-(3-phenyl-2-propenyl)-1-deoxynojirimycin on the lectin binding to HIV-1 glycoproteins. Jpn J Med Sci Biol 43:75–87
73. Shimizu H, Tsuchie H, Yoshida K, Morikawa S, Tsuruoka T, Yamamoto H, Ushijima H, Kitamura T (1990b) Inhibitory effect of novel 1-deoxynojirimycin derivatives on HIV-1 replication. AIDS 4:975–979
74. Stavale EJ, Vu H, Sampath A, Ramstedt U, Warfield KL (2015) In vivo therapeutic protection against influenza a (H1N1) oseltamivir-sensitive and resistant viruses by the iminosugar UV-4. PLoS One 10:e0121662
75. Steinmann E, Whitfield T, Kallis S, Dwek RA, Zitzmann N, Pietschmann T, Bartenschlager R (2007) Antiviral effects of amantadine and iminosugar derivatives against hepatitis C virus. Hepatology 46:330–338
76. Sung C, Wei Y, Watanabe S, Lee HS, Khoo YM, Fan L, Rathore AP, Chan KW, Choy MM, Kamaraj US, Sessions OM, Aw P, De Sessions PF, Lee B, Connolly JE, Hibberd ML, Vijaykrishna D, Wijaya L, Ooi EE, Low JG, Vasudevan SG (2016) Extended evaluation of virological, immunological and pharmacokinetic endpoints of CELADEN: a randomized, placebo-controlled trial of celgosivir in dengue fever patients. PLoS Negl Trop Dis 10:e0004851
77. Sunkara PS, Taylor DL, Kang MS, Bowlin TL, Liu PS, Tyms AS, Sjoerdsma A (1989) Anti-HIV activity of castanospermine analogues. Lancet 1:1206
78. Taylor DL, Fellows LE, Farrar GH, Nash RJ, Taylor-Robinson D, Mobberley MA, Ryder TA, Jeffries DJ, Tyms AS (1988) Loss of cytomegalovirus infectivity after treatment with castanospermine or related plant alkaloids correlates with aberrant glycoprotein synthesis. Antivir Res 10:11–26
79. Taylor DL, Sunkara PS, Liu PS, Kang MS, Bowlin TL, Tyms AS (1991) 6-0-butanoylcastanospermine (MDL 28,574) inhibits glycoprotein processing and the growth of HIVs. AIDS 5:693–698
80. Tierney M, Pottage J, Kessler H, Fischl M, Richman D, Merigan T, Powderly W, Smith S, Karim A, Sherman J, Et A (1995) The tolerability and pharmacokinetics of N-butyl-deoxynojirimycin in patients with advanced HIV disease (ACTG 100) The AIDS Clinical Trials Group (ACTG) of the National Institute of Allergy and Infectious Diseases. J Acquir Immune Defic Syndr Hum Retrovirol 10:549–553
81. Trombetta ES, Helenius A (1998) Lectins as chaperones in glycoprotein folding. Curr Opin Struct Biol 8:587–592
82. Tsujii E, Muroi M, Shiragami N, Takatsuki A (1996) Nectrisine is a potent inhibitor of alpha-glucosidases, demonstrating activities similarly at enzyme and cellular levels. Biochem Biophys Res Commun 220:459–466
83. Tyms AS (2003) Use of certain castanospermine esters in the treatment of influenza virus infections. WO2003006017A3.
84. Tyms AS, Berrie EM, Ryder TA, Nash RJ, Hegarty MP, Taylor DL, Mobberley MA, Davis JM, Bell EA, Jeffries DJ, Et A (1987) Castanospermine and other plant alkaloid inhibitors of glucosidase activity block the growth of HIV. Lancet 2:1025–1026
85. Umareddy I, Pluquet O, Wang QY, Vasudevan SG, Chevet E, Gu F (2007) Dengue virus serotype infection specifies the activation of the unfolded protein response. Virol J 4:91
86. Walker BD, Kowalski M, Goh WC, Kozarsky K, Krieger M, Rosen C, Rohrschneider L, Haseltine WA, Sodroski J (1987) Inhibition of human immunodeficiency virus syncytium formation and virus replication by castanospermine. Proc Natl Acad Sci U S A 84:8120–8124
87. Warfield KL, Plummer E, Alonzi DS, Wolfe GW, Sampath A, Nguyen T, Butters TD, Enterlein SG, Stavale EJ, Shresta S, Ramstedt U (2015) A novel iminosugar UV-12 with activity against the diverse viruses influenza and dengue (novel iminosugar antiviral for influenza and dengue). Virus 7:2404–2427
88. Warfield KL, Barnard DL, Enterlein SG, Smee DF, Khaliq M, Sampath A, Callahan MV, Ramstedt U, Day CW (2016a) The iminosugar UV-4 is a broad inhibitor of influenza A and B viruses ex vivo and in mice. Virus 8:71
89. Warfield KL, Plummer EM, Sayce AC, Alonzi DS, Tang W, Tyrrell BE, Hill ML, Caputo AT, Killingbeck SS, Beatty PR, Harris E, Iwaki R, Kinami K, Ide D, Kiappes JL, Kato A, Buck MD, King K, Eddy W, Khaliq M, Sampath A, Treston AM, Dwek RA, Enterlein SG, Miller JL, Zitzmann N, Ramstedt U, Shresta S (2016b) Inhibition of endoplasmic reticulum glucosidases is required for in vitro and in vivo dengue antiviral activity by the iminosugar UV-4. Antivir Res 129:93–98
90. Watanabe S, Rathore AP, Sung C, Lu F, Khoo YM, Connolly J, Low J, Ooi EE, Lee HS, Vasudevan SG (2012) Dose- and schedule-dependent protective efficacy of celgosivir in a lethal mouse model for dengue virus infection informs dosing regimen for a proof of concept clinical trial. Antivir Res 96:32–35
91. Watanabe S, Chan KW, Dow G, Ooi EE, Low JG, Vasudevan SG (2016) Optimizing celgosivir therapy in mouse models of dengue virus infection of serotypes 1 and 2: the search for a window for potential therapeutic efficacy. Antivir Res 127:10–19

92. Whitby K, Pierson TC, Geiss B, Lane K, Engle M, Zhou Y, Doms RW, Diamond MS (2005) Castanospermine, a potent inhibitor of dengue virus infection in vitro and in vivo. J Virol 79:8698–8706
93. Wong SS, Chebib M, Haqshenas G, Loveland B, Gowans EJ (2011) Dengue virus PrM/M proteins fail to show pH-dependent ion channel activity in Xenopus oocytes. Virology 412:83–90
94. Wu SF, Lee CJ, Liao CL, Dwek RA, Zitzmann N, Lin YL (2002) Antiviral effects of an iminosugar derivative on flavivirus infections. J Virol 76:3596–3604
95. Wu CY, Jan JT, Ma SH, Kuo CJ, Juan HF, Cheng YS, Hsu HH, Huang HC, Wu D, Brik A, Liang FS, Liu RS, Fang JM, Chen ST, Liang PH, Wong CH (2004) Small molecules targeting severe acute respiratory syndrome human coronavirus. Proc Natl Acad Sci U S A 101:10012–10017
96. Zhang G, Zhang B, Zhang X, Bing F (2013a) Homonojirimycin, an alkaloid from dayflower inhibits the growth of influenza a virus in vitro. Acta Virol 57:85–86
97. Zhang GB, Tian LQ, Li YM, Liao YF, Li J, Bing FH (2013b) Protective effect of homonojirimycin from Commelina communis (dayflower) on influenza virus infection in mice. Phytomedicine 20:964–968
98. Zhao X, Guo F, Comunale MA, Mehta A, Sehgal M, Jain P, Cuconati A, Lin H, Block TM, Chang J, Guo JT (2015) Inhibition of endoplasmic reticulum-resident glucosidases impairs severe acute respiratory syndrome coronavirus and human coronavirus NL63 spike protein-mediated entry by altering the glycan processing of angiotensin I-converting enzyme 2. Antimicrob Agents Chemother 59:206–216



21 Countering Zika Virus: The USAMRIID Response

Robert G. Lowen, Thomas M. Bocan, Christopher D. Kane, Lisa H. Cazares, Krishna P. Kota, Jason T. Ladner, Farooq Nasar, Louise Pitt, Darci R. Smith, Veronica Soloveva, Mei G. Sun, Xiankun Zeng, and Sina Bavari

Abstract

The United States Army Medical Research Institute of Infectious Diseases (USAMRIID) possesses an array of expertise in diverse capabilities for the characterization of emerging infectious diseases from the pathogen itself to human or animal infection models. The recent Zika virus (ZIKV) outbreak was a challenge and an opportunity to put these capabilities to work as a cohesive unit to quickly respond to a rapidly developing threat. Next-generation sequencing was used to characterize virus stocks and to understand the introduction and spread of ZIKV in the United States. High Content Imaging was used to establish a High Content Screening process to evaluate antiviral therapies. Functional genomics was used to identify critical host factors for ZIKV infection. An animal model using the temporal blockade of IFN-I in immunocompetent laboratory mice was investigated in conjunction with Positron Emission Tomography to study ZIKV. Correlative light and electron microscopy was used to examine ZIKV interaction with host cells in culture and infected animals. A quantitative mass spectrometry approach was used to examine the protein and metabolite type or concentration changes that occur during ZIKV infection in blood, cells, and tissues. Multiplex fluorescence in situ hybridization was used to confirm ZIKV replication in mouse and NHP tissues. The integrated rapid response approach developed at USAMRIID presented in this review was successfully applied and provides a new template pathway to follow if a new biological threat emerges. This streamlined approach will increase the likelihood that novel medical countermeasures could be rapidly developed, evaluated, and translated into the clinic.

Keywords

Animal models of infection · Next-generation sequencing · High content imaging · PET imaging · Rapid medical countermeasure to emerging viruses

R. G. Lowen (✉) · T. M. Bocan · C. D. Kane
L. H. Cazares · K. P. Kota · F. Nasar · L. Pitt
D. R. Smith · V. Soloveva · M. G. Sun
X. Zeng · S. Bavari
U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD, USA
e-mail: robert.g.lowen.mil@mail.mil

J. T. Ladner
The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, USA

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_21

21.1 Introduction

The United States Army Medical Research Institute of Infectious Diseases (USAMRIID) is a research organization located on at Fort Detrick, Maryland. USAMRIID is a subordinate laboratory of the US Army Medical Research and Materiel Command. Broadly stated, our mission is to provide leading edge medical capabilities to deter and defend against current and emerging biological threat agents. While our core mission is to protect the warfighter from biological threats, we also investigate disease outbreaks and threats to public health.

Emerging and reemerging infectious diseases pose a continuing threat to public health. In fact, infectious diseases are responsible for nearly 20% of global mortality [28]. Our increasingly globalized world has the ability to accelerate both the emergence and spread of diseases. For example, Zika virus (ZIKV) has emerged explosively since 2007 to cause frequent epidemics, recently with millions of human infections in the Americas. ZIKV is expected to continue to spread and numerous locally acquired mosquito-borne cases have recently been reported in the United States (Florida and Texas).

While our modernized world continues to facilitate the emergence and spread of infectious diseases, it also serves to advance the technology used to study them. New biomedical research technologies are available for genomic sequencing, drug discovery, and experimental pathology studies to help scientists better respond to these threats. In addition to the technological advances, a concerted response effort is needed to accelerate infectious disease research and the development of medical countermeasures.

Here we will highlight how USAMRIID pooled its resources and technologies to address important knowledge gaps during the ZIKV outbreak (Fig. 21.1). Researchers openly communicated to avoid duplicative work and test materials were shared between laboratories to get the maximum amount of data from every experiment. This has helped our organization to further develop our toolkit to more rapidly respond to emerging disease threats.

21.2 Next-Generation Sequencing for Tracking Pathogens

Genome characterization via next-generation sequencing (NGS) has quickly become a standard and integral part of the public-health response to emerging infectious diseases. In response to the ZIKV disease outbreak in the Americas, the Center for Genome Sciences (CGS) at USAMRIID used NGS approaches to a) rapidly characterize virus stocks that were later used for *in vitro* and *in vivo* experiments (see below) and b) assemble complete ZIKV genomes from clinical samples.

Using an approach that combines Rapid Amplification of cDNA Ends (RACE) with the SISPA random amplification protocol and NGS [26], USAMRIID-CGS was able to rapidly assemble complete genomes, including the 5′ and 3′ termini, of seven ZIKV isolates [22]. Not only were these sequences used to verify the integrity of ZIKV stocks prior to further characterization, but they also served as templates for the development of ZIKV reverse genetics systems within USAMRIID and at several other institutions around the world [2, 12, 48]. Additionally, USAMRIID-CGS utilized a probe-based targeted enrichment approach to sequence and assemble ZIKV genomes directly from clinical samples. These sequences were used to understand the introduction and spread of ZIKV in the United States [15].

21.3 *In Vitro* Cell-Based Infection for Antiviral Drug and Genetic Screening

(a) HCI Infection Assay & Antiviral Drug Screening

High Content Imaging (HCI) is a popular and basic tool of early drug discovery in multiple disease research areas. HCl is most applicable in areas of infectious diseases. The USAMRIID Molecular and Translational Sciences (MTS) division developed a HCI assay as a high throughput process and is actively using it to characterize

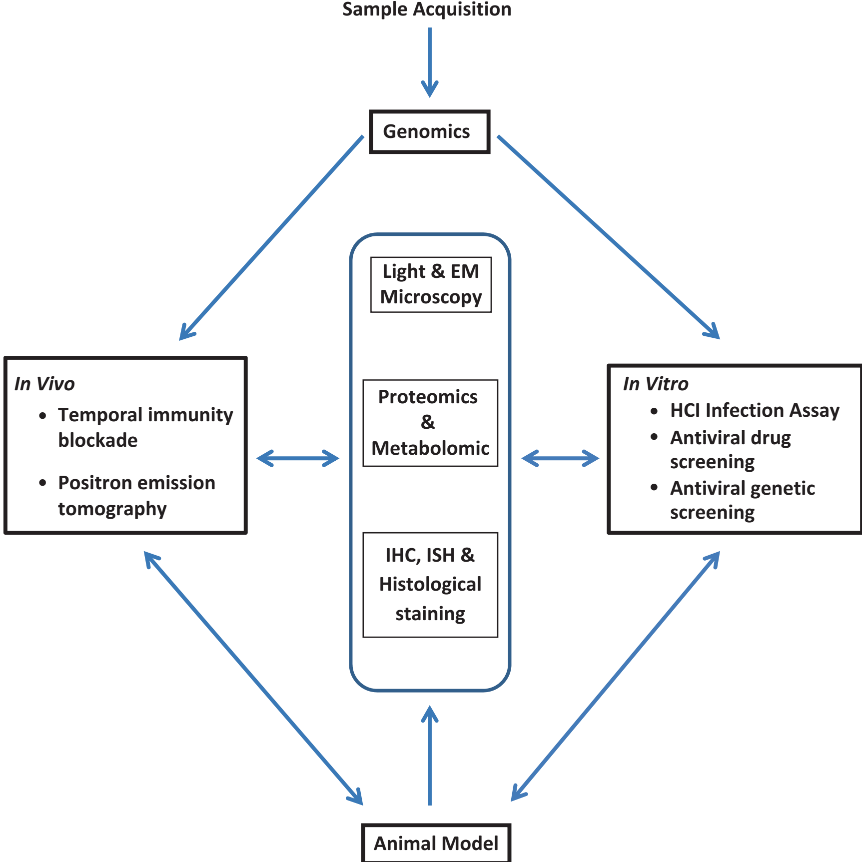


Fig. 21.1 USAMRIID integrated rapid response system

host-viral interactions for pathogens at all biosafety levels. High content screening (HCS) was established to quantify ZIKV infection in any cell-culture model as measured by signal intensity of the staining with a pan-flavivirus envelope protein-specific antibody. The HCS process for the ZIKV infection assay is shown in Fig. 21.2.

The flexibility of the HCS platform enables testing using traditional immortalized cell lines and primary cells. Figure 21.3 shows examples of images obtained from ZIKV infections conducted in 384- or 96-well plates using an African ZIKV strain (DAK ArD 41,525) in immortalized grivet (*Chlorocebus aethiops*) Vero E6, primary human astrocyte, and primary human foreskin fibroblast (HFF-1) cells.

The ZIKV HCI assay is a quantitative immunoassay with a distinct endpoint. The dose response curves for control inhibition measured using the HCl assay are similar to those of more traditional ZIKV infection testing methods, such as plaque assays or real-time PCR (Fig. 21.4). However, the HCI platform offers significantly higher throughput, precision, and cost-effectiveness for *in vitro* testing of potential ZIKV inhibitors compared to the other two methods. This advantage makes the HCl assay attractive as a high content screening tool. Its flexibility enabled the detailed and reproducible characterization of the potency and selectivity of subsets of chemical molecules and biological reagents. The results were complemented by the ability of HCS to simultaneously evaluate the potential cytotoxic effects of chemical molecules simultaneously and the efficacy of the antiviral activity of tested compounds in the same wells of the same experiment (Fig. 21.5).

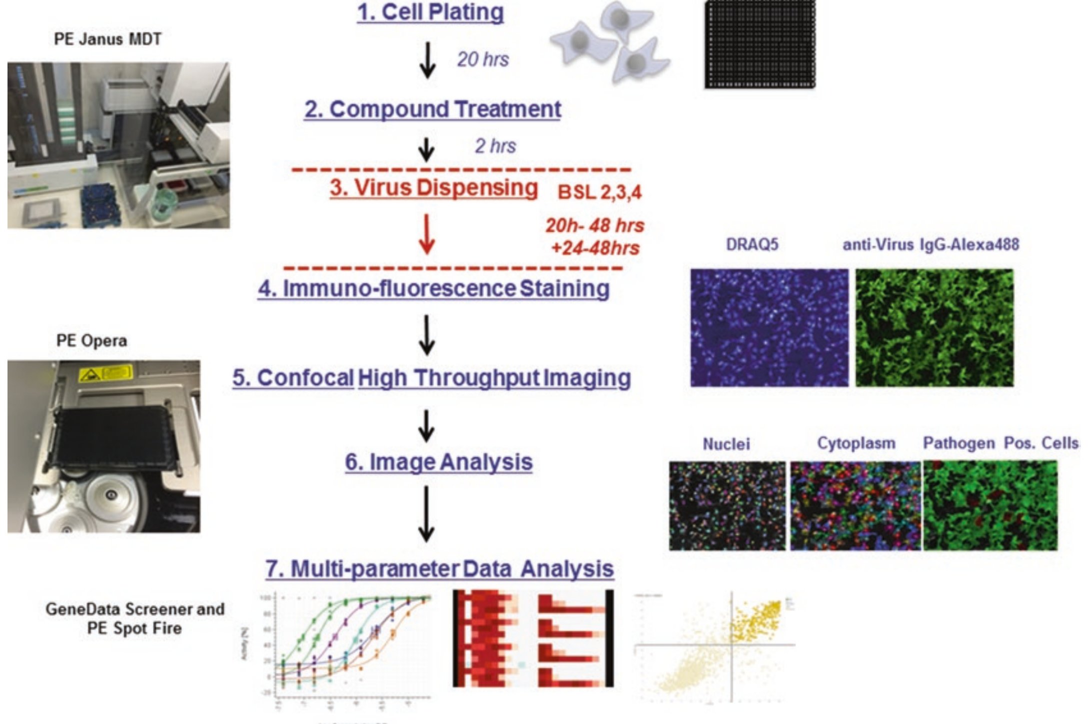


Fig. 21.2 The High Content Screening assay design. The primary antibody specific for Flavi-virus common epitope of the envelop glycoprotein followed by the secondary detection antibody IgG conjugated with Dylight488 allows detection and localization of any strain of Zika virus after it infects cells. The Opera PE confocal plate reader takes high quality, high resolution images of cells infected or not infected with virus in the 96, 384 or 1536 well plates. The image analysis software quantifies the signal of different fluorophores by segregating nuclei measured at 405 nm emission wavelength for Hoechst 33342 nuclei dye and cellular cytoplasm at 650 nm for CellMask Deep Red dye. The virus-specific antigen can be quantified by measuring fluorescence emission at a 488 nm wavelength for Dylight488. The ratio of virus-positive cells to total number of analyzed cells is used to determine the percentage of infection for each well on the assay plates. Data normalization uses GeneData analytical software for evaluation of tested compounds on viral infection as a percentage of inhibition of infection in comparison to control notinfected or infected but not treated wells. GeneData software helps analyses of dose–responses and applying the best curve- fitting strategy applying multi-parameter non-linear regression curve by applying the Levenberg–Marquardt algorithm

This HCI platform enables us to conduct combinatory studies for modulation of the known antiviral effects specifically crucial in the area of antiviral therapies (Fig. 21.6). In addition to the evaluation of these therapies, ZIKV HCI enables fast and precise detection of neutralization properties of anti-ZIKV antibodies.

(b) Antiviral Genetic Screening

Functional genomics, including loss-of-function and gain-of-function screens, have produced a wealth of data and discovered numerous host factors and cellular process that play a vital role in virus infections. Four types of genetic screens are used: random retroviral insertional mutagenesis using haploid cells (haploid cell screening), cDNA overexpression, RNA interference (RNAi), and more recently CRIPSR/Cas9. Among these screens, the latter two are at the forefront. RNAi involves targeted mRNA degradation by small double-stranded (19–21 nt-long) RNA, via complementary base pairing of one of the strands of the dsRNA to a target mRNA. Arrayed siRNA libraries targeting individual genes have been instrumental in the discovery of hundreds of host factors that play a critical role in infections caused by important

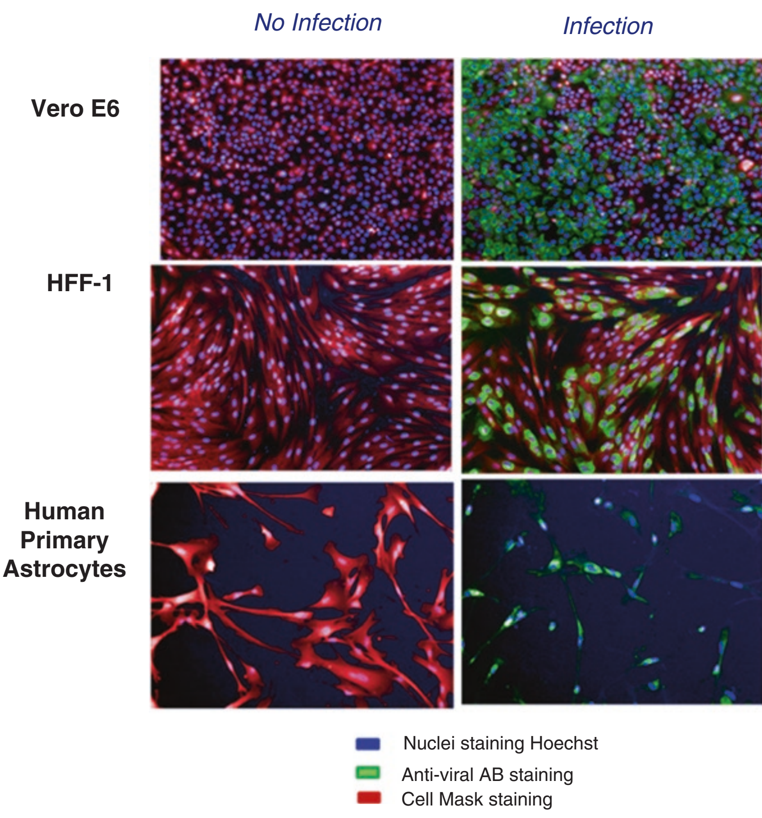


Fig. 21.3 An example of Zika infection in different cell types from the high content anti-viral screen. Zika (DAK ArD 41525) infection was conducted in 384-or 96-well plates using in Vero E6, human primary Astrocyte, and Human Foreskin Fibroblast (HFF-1) primary cells

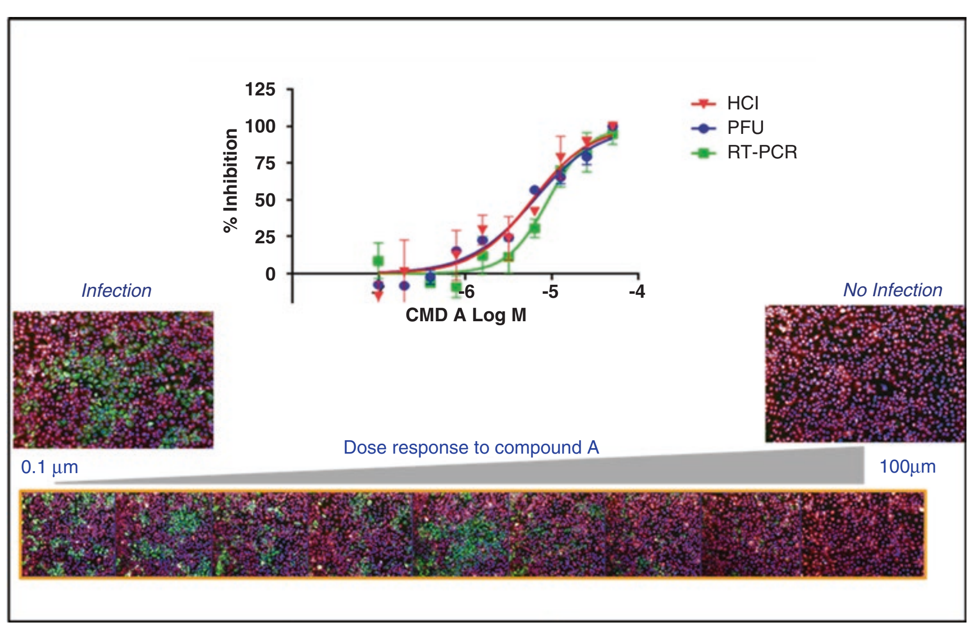


Fig. 21.4 Dose response curves for Reference inhibitor A across HCI, plaque forming, and RT-PCR assay formats. The resulted potency values are EC50 = 5.85 ± 2.65uM in HCI assay; EC50 = 6.12 ± 2.65uM in log PFU values and EC50 = 8.97 ± 3.25uM in RT-PCR assay

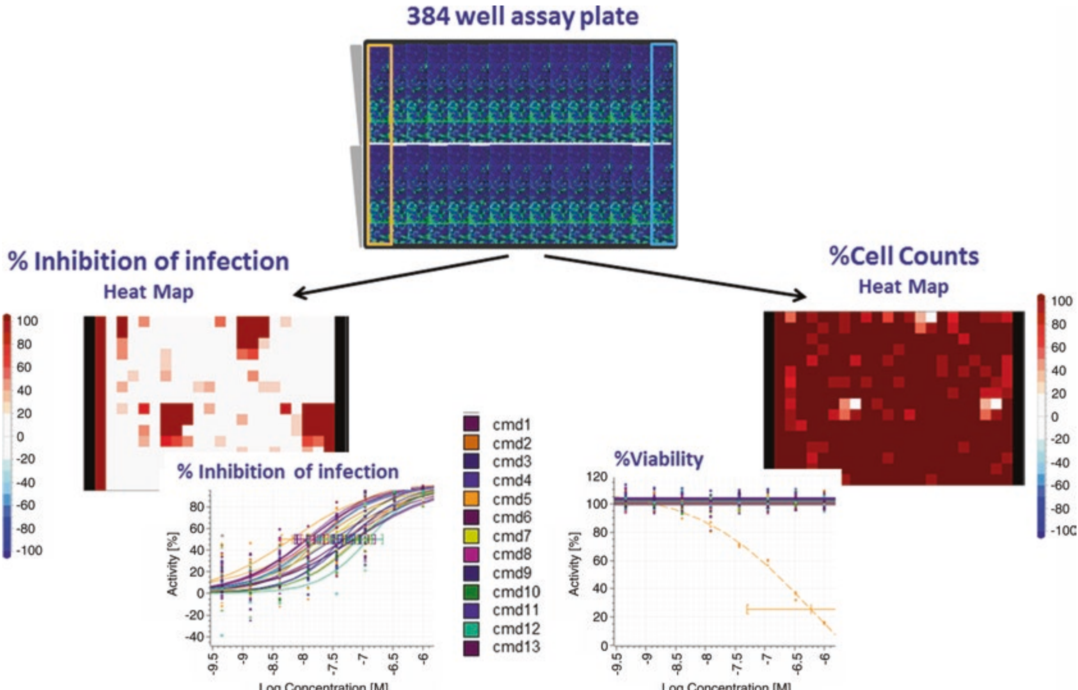


Fig. 21.5 Assessment of efficacy and selectivity for the anti-Zika compounds simultaneously on the same assay plate. The schematic image of 384 well plate represents the combined staining of cells in wells for 8-point dose responses. The heat maps are generated by GeneData software using the meta-data from a single 384 well plate done for 13 compounds tested in 8-point dose responses that are shown as fitted curves aside of each heat map. Only two parameters, the % inhibition of infection and % cell count, are exported for the data set. The clear cytotoxicity can be observed for cmd 5 by the down-word trend of the % Viability curve

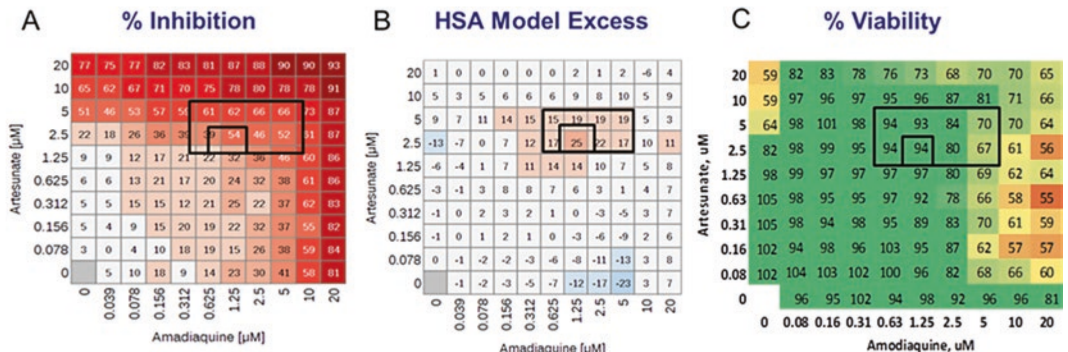


Fig. 21.6 Evaluation of the combination of two drugs in Zika HCI assay. (**a**) Heat map of % inhibition obtained from the test of the combination of two drugs artesunate and amodiaquine as potential anti-Zika therapy. (**b**) The HAS model is showing the zone in the doses of two drugs that show additive effect if compare to each drug alone. (**c**) The heat map for the % Viability results. The combination of two drugs shows the additional advantage in the increased viability of cells at the selected doses

human disease-causing viruses (reviewed in [40]). In the arrayed platform, siRNA transfections are performed in a high-throughput manner in a 96- or 384-well plate format, and require the optimization of siRNA transfection and infection conditions.

At USAMRIID, image-based high-throughput conditions have been optimized for many viruses, including ZIKV, in 384-well format [34, 39]. Virus infections were scored by enumerating the percentage of viral antigen-expressing cells after collecting images of immunofluorescent virus-infected cells. We also optimized high-throughput siRNA transfection conditions in the 96-well plate format to screen several small focused siRNA libraries targeting kinases, the ubiquitin pathway, or endosome or viral trafficking factors thought to be used or bunyaviruses [35, 42, 43]. Currently we have optimized the same protocols for ZIKV infection in HCT116 cells in 384-well plates (data not shown) and are poised to screen siRNA libraries at the genomic scale.

One of the shortcomings of siRNA screens is the high rate of siRNA off-target effects that lead to false positive hits or ineffective siRNAs that gives rise to false negative hits. To overcome this shortcoming, siRNA libraries are used that are established by two to three different vendors. This approach improves the cumulative comprehensive sequence coverage and reduces the number of false negative hits. For example, siRNA libraries constructed by Dharmacon and Ambion are largely orthologous with very small overlap (<5%) due to their proprietary siRNA design algorithms. Hits from all performed screens are then statistically evaluated using the established RNAi gene enrichment ranking (RIGER) tool to assign a *p* value, which predicts the likelihood of an identified gene playing a vital role in virus infection. These approaches successfully helped identify over 150 high-confidence candidate host factors for DENV infection (e.g., host factors regulating endocytosis, transmembrane processing, including the endoplasmic reticulum membrane complex (EMC) [47]). Similar approaches will be used to identify critical host factors for ZIKV infection.

21.4 Animal Models and Tracking Infection by Imaging

(a) Temporal Immunity Blockade

Animal and in vitro models have helped provide a better understanding of the pathogenic mechanisms induced by ZIKV infection. Laboratory mice deficient in type I or type II interferon responses were found to be highly susceptible to ZIKV infection and these mice developed severe neurological disease [1, 10, 25, 45, 57]. In an attempt to produce infection models that do not rely on knockout laboratory mice, several groups explored the temporal blockade of IFN-I in immunocompetent laboratory mice using polyclonal and monoclonal antibodies targeting either IFN-Is directly or the IFN-I receptor. A murine non-cell depleting monoclonal antibody (MAb) that efficiently targets the IFNAR-1 subunit of the laboratory mouse IFN-α/β receptor (MAb-5A3) was developed. This antibody prevents type I IFN-induced intracellular signaling in vitro and inhibits antiviral, antimicrobial, and antitumor responses in laboratory mice [49]. C57BL/6 mice treated with MAb-5A3 are now being used to study ZIKV pathogenesis [51]. We observed 40% lethality in antibody-treated laboratory mice exposed to ZIKV subcutaneously whereas all laboratory mice exposed intraperitoneally died. Laboratory mice infected by either exposure routes experienced weight loss, viremia, hind-limb paralysis, and severe neuropathology (Fig. 21.7). The most significant histopathological changes occurred in the CNS, where neuronal death, astrogliosis, microgliosis, scattered necrotic cellular debris, and inflammatory cell infiltrates were indicative of acute to subacute encephalitis/encephalomyelitis [51]. The laboratory mouse model of ZIKV encephalitis will be valuable for evaluating medical countermeasures and characterizing pathogenesis in a more relevant model because the interferon blockade occurs only at the time of infection.

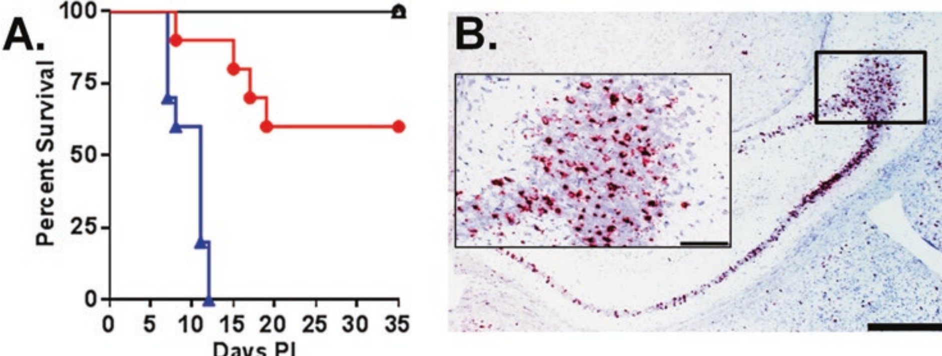


Fig. 21.7 Wild-type Mice Treated with an IFNAR1-Blocking MAb are Susceptible to ZIKV and Significant Pathology Occurs in the CNS. Five week old wild-type mice were treated with an IFNAR1-blocking MAb or PBS by IP injection and then exposed to 6 log10 of ZIKV strain DAK AR D 41525 SC or IP and monitored for (**a**) survival. (**b**) ISH findings in the hippocampus of mice succumbing to ZIKV infection demonstrating massive ZIKV infection of the brain; scale bar represents 500 µm (inset picture scale bar represents 100 µm)

(b) Positron Emission Tomography

Medical/molecular imaging has been used to characterize disease progression and evaluate drugs against neurological, cardiovascular, inflammatory and cancer research. However, using medical/molecular imaging techniques in infectious disease research is still limited [5]. Establishing individual stages of an infection helps to define the appropriate pharmacologic regimen to treat the infection. For instance, a well-characterized disease course will inform the decision on whether to use a specific antiviral or an anti-inflammatory agent and to select cohorts of animals based on the same stage of the disease to study disease mechanisms and reduce variability upon pharmacologic treatment. Current approaches to the evaluation of infectious diseases in the presence of drug therapies involve reduced lethality or extended time to death as the efficacy endpoint, plasma viremia as an index of infection, and serial necropsies to establish the consequences of infection.

Medical/molecular imaging approaches for the assessment of tissue metabolism and inflammation exist (Fig. 21.8) and can be applied to the characterization of infectious diseases. The metabolic tracer [18F]-FDG has routinely been used to assess tissue metabolism. Hypometabolism is believed to be associated with reduced neuronal activity and neuronal loss [19, 31]. In cases of ZIKV infection, one could use [18F]-FDG to demonstrate overall changes in brain metabolism and depending on the animal model being used, one could be able to map regional differences in brain metabolic activity over the course of infection and potentially before development of overt clinical signs. Inflammation and positron emission tomography (PET) tracers for the assessment of macrophage accumulation and microglia/astrocyte activation in the brain have focused on monitoring the 18 kDa translocator protein (TSPO), which is upregulated in activated microglia and inflammatory cells relative to normal cells. The TSPO radiotracer [18F]-DPA714 has been used in animal models of epilepsy, stroke [16], quinolinic acid-induced striatal inflammation [24], inflammatory bowel disease [3], and rheumatoid arthritis [41] to assess the degree of inflammation and specificity of binding of the tracer to inflammatory cells.

Thus, medical/molecular imaging has the potential, when applied preclinically, to inform the development of medical countermeasures against biological threat agents by providing data on 1) presence, biodistribution, and time course of infection in the presence or absence of a therapeutic; 2) binding of the therapeutic to the target; and 3) expression of a pharmacologic effect either related to drug mechanism, efficacy, or safety. Preclinical medical/molecular imaging could potentially be a real-time, dynamic tool to

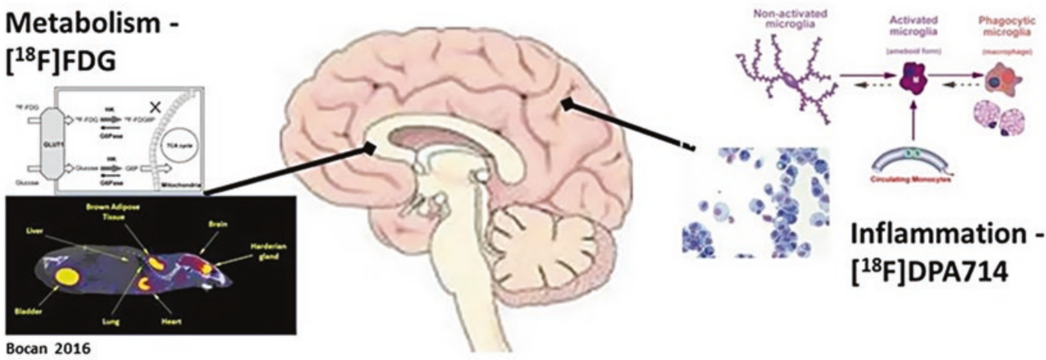


Fig. 21.8 Schematic on the use of in vivo imaging to assess neurological changes associated with infections

characterize a pathogen and animal model, and for developing medical countermeasures under the US FDA Animal Rule.

21.5 Light and Electron Microscopy in Infectious Diseases

Microscopy is an important diagnostic and research tool in infectious disease [18, 44, 53]. The electron microscope (EM) was first used to observe viral particles in 1937 [21], and today is still considered an essential tool to study virus particles with a resolution below 5 nanometers. Negative staining is a typical and routine EM technique to characterize virus particle structures and is therefore useful to study the ultrastructure of ZIKV particles and nano-particles [6, 11, 27, 33]. Currently, with cryo-EM methods, atomic-level imaging can be achieved revealing virus ultrastructural details [20, 50]. EM is also applied extensively to detect ZIKV particles in cell culture and infected animals [9, 32]. For instance, EM has been used to study ZIKV interaction with and replication in host cells (Fig. 21.9). Focused ion beam electron microscopy (FIB-SEM) was used to generate 3D-volume data to study whole-cell ZIKV particle distribution at different conditions. By combining EM ultrastructural data and FIB-SEM 3D volume data, a ZIKV trafficking model for the entire virus replication cycle could be established (data in submission).

Nearly all EM studies have some degree of correlation to light microscopy (LM). Various approaches for the correlation of microscopy techniques have existed since the early 60's [9, 36]. For many decades, correlative light and

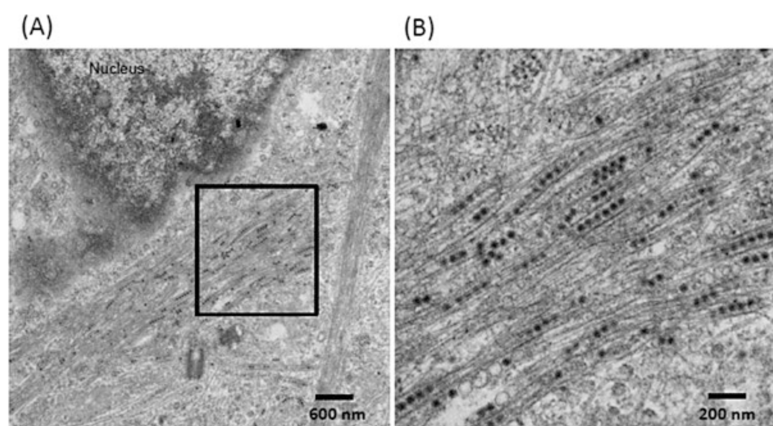


Fig. 21.9 TEM of Zika virus replication within fibroblast cells. (**a**) Low magnification image of zika virus aligning well with actin filaments. (**b**) High magnification image of the square zoom-in area of (**a**)

electron microscopy (CLEM) studies used an approach that requires samples to be prepared in different ways: formalin fixation and paraffin embedding for LM versus aldehyde fixation, osmium tetroxide staining, and epoxy resin embedding for EM [37]. The sample was then investigated using both types of microscopy but correlation to the cellular level remained impossible. Recently, several techniques and tools have been developed that improve the accuracy of CLEM correlations by enabling imaging of cellular events with both modalities. These improvements allow for correlation at the single cell level and the inclusion of time-resolved images to enhancing the study of virus replication and production [17, 23, 30, 52]. This improved CLEM method is now being applied to study ZIKV infection in cell culture as well as in animal tissues.

21.6 Proteomics and Metabolomics

The examination of protein and metabolite concentration changes that occur during ZIKV infection in blood, cells, and tissues *in vitro* or *in vivo* will provide insight into pathogenesis. Potentially, the identification of host proteins and metabolites involved in the ZIKV infection process may lead to the discovery of druggable targets for medical countermeasure development. Mass spectrometry-based proteomics and metabolomics techniques are therefore now applied to characterize ZIKV-induced host responses.

A quantitative mass spectrometry approach is now being taken for the examination of host response proteins in body fluids (plasma, CSF, urine) collected during the course of ZIKV infection. Samples are processed using filter-assisted sample processing (FASP), which captures all proteins from a body fluid sample onto a membrane on which they are then digested by trypsin. The resulting peptides are then labelled with tandem mass tags (TMT) for relative quantitation of protein expression differences between samples [54]. The use of 6-or 10-plex TMT tags makes it possible to simultaneously measure protein expression in 6–10 consecutive post-exposure time points from an individual host animal in a single high-resolution LC-MS/MS run. The data are then analyzed for protein abundance changes over the course of infection using pre-exposure sample data as the baseline, thus providing a relative quantitation of the temporal host proteomic response.

An established proteomic profile is then compared to the profile observed in vaccinated animals before and after exposure to ZIKV to provide a detailed characterization of the proteomic host response. Since most of the host response proteins previously associated with ZIKV infection in NHPs have human homologues, it should be straightforward to bridge studies using human samples from individuals vaccinated against ZIKV with candidate vaccines. An identical strategy is used to study the temporal proteomic alterations that occur in mosquito- and mammalian-derived cell lines *in vitro* during ZIKV infection. Unlike infection in mammalian cell lines, ZIKV infection in mosquito-derived cells often results in high viral titers with little to no observable CPE [8]. Therefore, the examination of temporal proteomic profiles during ZIKV infection may aid in the identification of mammalian cellular targets that can limit or reduce ZIKV virulence.

The visualization of proteins, peptides, lipids, and other metabolites directly in tissues is a rapidly growing area in clinical chemistry. Mass spectrometry imaging (MSI) techniques are at the forefront to achieve such visualization [7]. MSI is a label-free technique that allows the direct collection of mass spectra from tissue sections and adds another dimension of information to proteomic and metabolite profiling by providing *in-situ* snapshots of the spatial distribution of biologically relevant proteins and metabolites in intact tissue samples. Whereas homogenization-based sample preparation and extraction methods result in a loss of spatial information, MSI provides the *in situ* distribution profiles of metabolites within tissues. Recent improvements in matrix assisted laser desorption ionization (MALDI) imaging mass spectrometry tissue imaging (IMS) instrumentation platforms related to higher mass accuracy (MALDI-FTICR-MS)

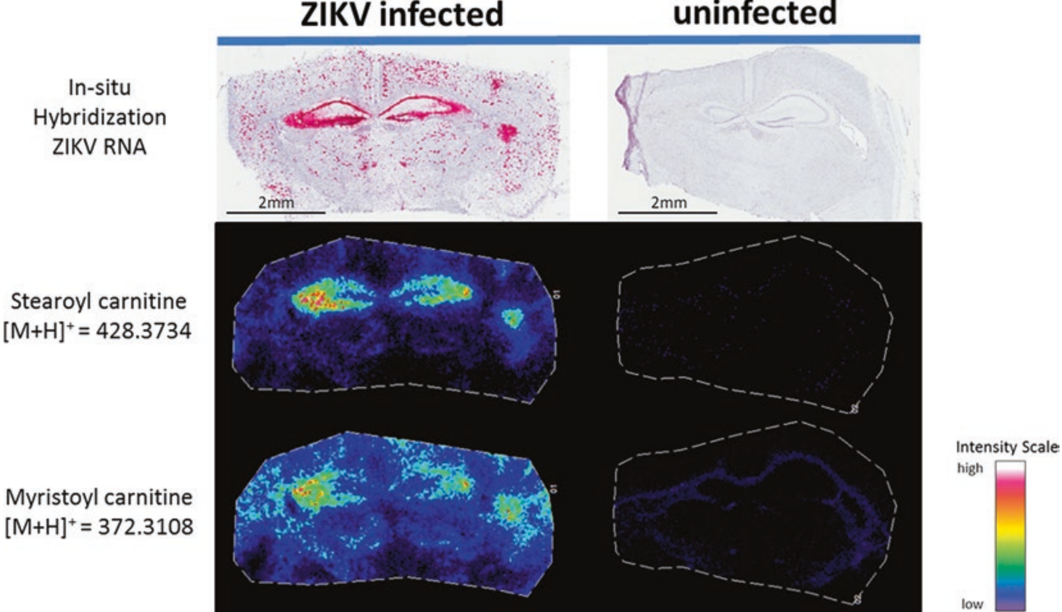


Fig. 21.10 MALDI-FTICR Mass Spectrometry Imaging (MSI) to detect metabolite changes in mouse brain tissue during ZIKV infection. (Top panel) Mid-brain tissue from a mouse model of ZIKV infection and uninfected control was sectioned and prepared for ISH for the detection of ZIKV RNA. Serial sections were prepared for MSI by coating the tissue with 2,5 dihydroxybenzoic acid (DHB) and reading in a SolariX XR 7 T FTICR using a spatial resolution of 50 microns for each laser pulse. (Bottom panels) Spectra were collected across the tissue sections and imaged using FlexImaging 4.1 (Bruker Daltonics) to visualize metabolite abundances (intensity scale provided). Masses reported for each carnitine identified are within a 1 ppm mass error

and spatial resolution improvement (specialized lasers and optics) have facilitated the detection of protein and metabolite molecules directly from the pathogen-host interface of infected tissues, thus enabling the characterization of metabolic networks that are typical for pathogenesis [13, 29]. Using a relevant mouse-model of ZIKV infection developed at USAMRIID [51] MALDI-FTICR MSI methods were established to detect lipid and small-molecule metabolites directly from ZIKV-infected mouse brain tissue sections. MSI canvassing of ZIKV infected brain tissue provides the spatial localization and abundance of dysregulated metabolites to be pinpointed to specific brain regions. In a recent study, MALDI-FTICR MSI was performed on ZIKV infected mouse mid-brain sections, demonstrating a dramatic increase in abundance of long-chain acylcarnitines in comparison to uninfected control samples. These acylcarntines were often detected in the same brain regions where ZIKV RNA was detected by ISH (see Fig. 21.10). Acylcarnitines are important lipid biomarkers that possess bioactive and inflammatory properties. A comprehensive comparison of the MSI data with PET imaging data, FISH and IHC will provide insight based on the appearance of metabolites and proteins in the context of inflammation and dissemination of ZIKV in the CNS.

21.7 New Developments in Immunological Staining of Cells and Tissues

Immunohistochemistry (IHC), *in situ* hybridization (ISH), and histological staining have been widely used to detect infectious pathogens and evaluate pathologic changes in infected animals and human. ISH is used to detect nucleic acids of infectious agents within conserved tissue architectures especially when pathogen-specific

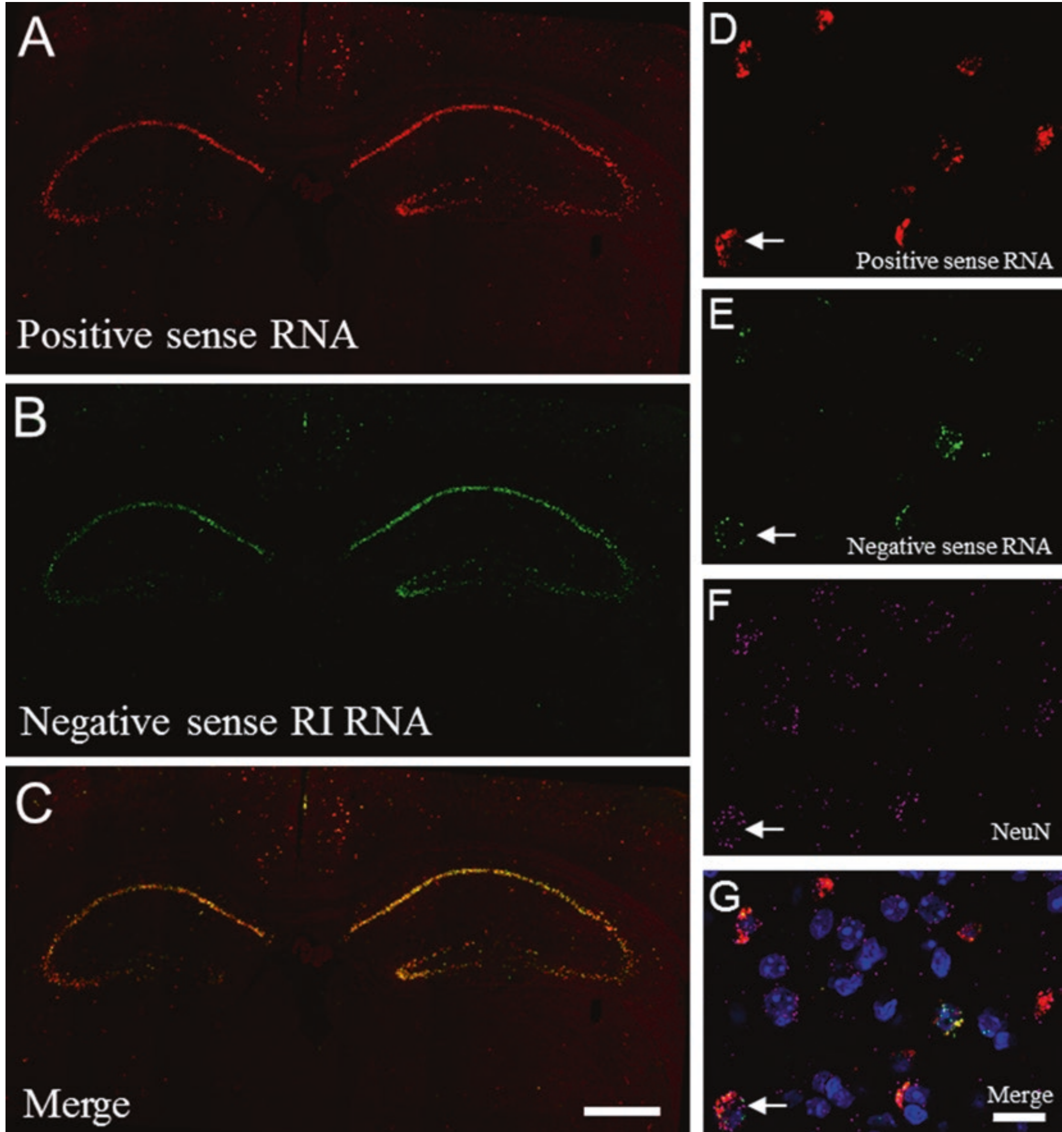


Fig. 21.11 Detection of Zika virus replication in tissue using Multiplex fluorescence in situ hybridization. (**a**–**c**) Negative sense RI (**b**, green) RNA were highly detected in hippocampus of ZIKV infected mouse brain in addition to positive sense ZIKV RNA (**a**, red), which indicates active ZIKV replication. (**d**–**g**) Detection of ZIKV replication (**a**, positive sense RNA; **b**, negative sense RNA) in a NeuN-labeled neuron (**f**, magenta; arrow) of ZIKV infected mouse brain. Blue, nuclear stain by DAPI. Scale bar, 500 um in A–C and 20 um in **d**–**g**

antibodies have not been available. A newly developed ISH technique, RNAscope, has extremely high sensitivity and specificity in detection of RNA in tissues or cultured cells due to its unique double Z probe design and branched DNA amplification technique [56]. RNAscope ISH has been extensively used to detect ZIKV RNA in human and animal (murine and NHP)

tissues [4, 14, 38, 46, 51, 55]. ZIKV has a positive sense single-stranded RNA genome consisting of one single open reading frame approximately 11 kb in length. ZIKV genome replication involves synthesis of a negative-sense RNA replication intermediate (RI), which in turn serves as a template to generate progeny (positive-sense RNA) genomes. Detection of ZIKV negative sense RI is considered evidence of viral replication. Multiplex fluorescence *in situ* hybridization (mFISH) was developed to detect both ZIKV positive-sense and negative-sense RI RNA in formalin-fixed, paraffin-embedded (FFPE) tissue using RNAscope ISH techniques. mFISH has been successfully used to confirm ZIKV replication in ZIKV-infected mouse and NHP FFPE tissues. As shown in Fig. 21.11a–c, negative sense RI (green) RNAs were highly detected in hippocampi of ZIKV-infected laboratory mouse brains in addition to positive-sense ZIKV RNAs (red). Furthermore, ZIKV replication could be detected in a cell type-specific manner by using three probes: one probe targeting cell-type specific markers and the other two probes targeting ZIKV positive- and negative-sense RNAs, respectively (Fig. 21.11d–g). By measuring the ratio between the fluorescence intensity of ZIKV negative-sense RI and positive-sense RNA, it is easy to identify ZIKV target cells with the most active viral replication.

21.8 Summary

USAMRIID possesses an array of expertise in diverse capabilities for the characterization of emerging infectious diseases from the pathogen itself to human or animal infection models. The recent ZIKV outbreak was a challenge and an opportunity to work as a cohesive unit to quickly respond to a rapidly developing threat. The integrated rapid response approach developed at USAMRIID (Fig. 21.1) was successful and should be taken again if a new biological threat emerges. This approach will increase the likelihood that novel medical countermeasures could be rapidly developed, evaluated, and translated into the clinic.

21.9 Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

Acknowledgments Funding for ZIKV research was provided by DARPA. The authors would like to thank Dr. Michael Wiley, Ms. Karla Prieto, Mr. Daniel Reyes, Ms. Elyse Nagle, Dr. Mariano Sanchez-Lockhart and Dr. Gustavo Palacios for their contributions toward the genomic characterization. The authors would like to acknowledge Mr. Kyle Kuszpit and Robert Stafford for their assistance in performing the imaging studies and Drs. Xiang Zhang, Falguni Basuli, and Rolf Swenson from the Imaging Probe Development Center at NIH for providing the PET radiotracers. The authors would also like to thank Mr. Brandon Kline and Ms. Kathy Kuehl for EM support and Ms. Tara Kenny, Ms. Xiaoli Chi, Mr. Rouzbeh Zamani, Mr. Glenn Gomba, Mr. Collin Dube for their support of HCI screening work.

Discussion of Chapter 21 at Dengue and Zika: Control and Antiviral Treatment Strategies

This discussion was held at the 2nd Advanced Study Week on Emerging Viral Diseases at Praia do Tofo, Mozambique.
Transcribed by Hilgenfeld R and Vasudevan SG (Eds); approved by Dr. Robert Lowen.

Anuja Mathew: I just had a quick question: in the antibody suppression model, is the antibody that you are using, against type-1 interferon or is it something else?

Robert Lowen: I believe, yes, it is against type-1 interferon.

Anuja Mathew: Thank you!

References

1. Aliota MT et al (2016) Characterization of Lethal Zika virus infection in AG129 mice. PLoS Negl Trop Dis 10(4):e0004682
2. Atieh T, Baronti C, de Lamballerie X, Nougairede A (2016) Simple reverse genetics systems for Asian and African Zika viruses. Sci Rep 6:39384
3. Bernards N, Pottier G, Theze B, Dolle F, Boisgard R (2015) In vivo evaluation of inflammatory bowel disease with the aid of muPET and the translocator protein 18 kDa radioligand [18F]DPA-714. Mol Imaging Biol 17(1):67–75
4. Bhatnagar J, Rabeneck DB, Martines RB, Reagan-Steiner S, Ermias Y, Estetter LB, Suzuki T, Ritter J, Keating MK, Hale G, Gary J, Muehlenbachs A, Lambert A, Lanciotti R, Oduyebo T, Meaney-Delman D, Bolanos F, Saad EA, Shieh WJ, Zaki SR (2017) Zika virus RNA replication and persistence in brain and placental tissue. Emerg Infect Dis 23(3):405–414
5. Bocan TM, Panchal RG, Bavari S (2015) Applications of in vivo imaging in the evaluation of the pathophysiology of viral and bacterial infections and in development of countermeasures to BSL3/4 pathogens. Mol Imaging Biol 17(1):4–17
6. Boigard H, Alimova A, Martin GR, Katz A, Gottlieb P, Galarza JM (2017) Zika virus-like particle (VLP) based vaccine. PLoS Negl Trop Dis 11(5):e0005608
7. Cazares LH, Troyer DA, Wang B, Drake RR, Semmes OJ (2011) MALDI tissue imaging: from biomarker discovery to clinical applications. Anal Bioanal Chem 401(1):17–27
8. Chan JF, Yip CC, Tsang JO, Tee KM, Cai JP, Chik KK, Zhu Z, Chan CC, Choi GK, Sridhar S, Zhang AJ, Lu G, Chiu K, Lo AC, Tsao SW, Kok KH, Jin DY, Chan KH, Yuen KY (2016) Differential cell line susceptibility to the emerging Zika virus: implications for disease pathogenesis, non-vector-borne human transmission and animal reservoirs. Emerg Microbes Infect 5:e93
9. Cortese M, Goellner S, Acosta EG, Neufeldt CJ, Oleksiuk O, Lampe M, Haselmann U, Funaya C, Schieber N, Ronchi P, Schorb M, Pruunsild P, Schwab Y, Chatel-Chaix L, Ruggieri A, Bartenschlager R (2017) Ultrastructural characterization of Zika virus replication factories. Cell Rep 18(9):2113–2123
10. Dowall SD, Graham VA, Rayner E, Atkinson B, Hall G, Watson RJ, Bosworth A, Bonney LC, Kitchen S, Hewson R (2016) A susceptible mouse model for Zika virus infection. PLoS Negl Trop Dis 10(5):e0004658
11. Dowd KA, Ko SY, Morabito KM, Yang ES, Pelc RS, DeMaso CR, Castilho LR, Abbink P, Boyd M, Nityanandam R, Gordon DN, Gallagher JR, Chen X, Todd JP, Tsybovsky Y, Harris A, Huang YS, Higgs S, Vanlandingham DL, Andersen H, Lewis MG, De La Barrera R, Eckels KH, Jarman RG, Nason MC, Barouch DH, Roederer M, Kong WP, Mascola JR, Pierson TC, Graham BS (2016) Rapid development of a DNA vaccine for Zika virus. Science 354(6309):237–240
12. Ellison DW, Ladner JT, Buathong R, Alera MT, Wiley MR, Hermann L, Rutvisuttinunt W, Klungthong C, Chinnawirotpisan P, Manasatienkij W, Melendrez MC, Maljkovic Berry I, Thaisomboonsuk B, Ong-Ajchaowlerd P, Kaneechit W, Velasco JM, Tac-An IA, Villa D, Lago CB, Roque Jr VG, Plipat T, Nisalak A, Srikiatkhachorn A, Fernandez S, Yoon IK, Haddow AD, Palacios GF, Jarman RG, Macareo LR (2016). Complete genome sequences of Zika virus strains isolated from the blood of patients in Thailand in 2014 and the Philippines in 2012. Genome Announc 4(3)
13. Fujimura Y, Miura D (2014) MALDI mass spectrometry imaging for visualizing in situ metabolism of endogenous metabolites and dietary phytochemicals. Meta 4(2):319–346
14. Govero J, Esakky P, Scheaffer SM, Fernandez E, Drury A, Platt DJ, Gorman MJ, Richner JM, Caine EA, Salazar V, Moley KH, Diamond MS (2016) Zika virus infection damages the testes in mice. Nature 540(7633):438–442
15. Grubaugh ND, Ladner JT, Kraemer MUG, Dudas G, Tan AL, Gangavarapu K, Wiley MR, White S, Theze J, Magnani DM, Andersen KG (2017) Genomic epidemiology reveals multiple introductions of Zika virus into the United States. Nature 54(7658):401–405
16. Harhausen D, Sudmann V, Khojasteh U, Muller J, Zille M, Graham K, Thiele A, Dyrks T, Dirnagl U, Wunder A (2013) Specific imaging of inflammation with the 18 kDa translocator protein ligand DPA-714 in animal models of epilepsy and stroke. PLoS One 8(8):e69529
17. Hellstrom K, Vihinen H, Kallio K, Jokitalo E, Ahola T (2015) Correlative light and electron microscopy enables viral replication studies at the ultrastructural level. Methods 90:49–56
18. Herderschee J, Fenwick C, Pantaleo G, Roger T, Calandra T (2015) Emerging single-cell technologies in immunology. J Leukoc Biol 98(1):23–32
19. Herholz K (2003) PET studies in dementia. Ann Nucl Med 17(2):79–89
20. Kostyuchenko VA, Lim EX, Zhang S, Fibriansah G, Ng TS, Ooi JS, Shi J, Lok SM (2016) Structure of the thermally stable Zika virus. Nature 533(7603):425–428
21. Kruger DH, Schneck P, Gelderblom HR (2000) Helmut Ruska and the visualisation of viruses. Lancet 355(9216):1713–1717
22. Ladner JT, Wiley MR, Prieto K, Yasuda CY, Nagle E, Kasper MR, Reyes D, Vasilakis N, Heang V, Weaver SC, Haddow A, Tesh RB, Sovann L, Palacios G (2016) Complete genome sequences of five Zika virus isolates. Genome Announc 4(3)
23. Larson DR, Johnson MC, Webb WW, Vogt VM (2005) Visualization of retrovirus budding with correlated light and electron microscopy. Proc Natl Acad Sci U S A 102(43):15453–15458
24. Lavisse S, Inoue K, Jan C, Peyronneau MA, Petit F, Goutal S, Dauguet J, Guillermier M, Dolle F,

Rbah-Vidal L, Van Camp N, Aron-Badin R, Remy P, Hantraye P (2015) [18F]DPA-714 PET imaging of translocator protein TSPO (18 kDa) in the normal and excitotoxically-lesioned nonhuman primate brain. Eur J Nucl Med Mol Imaging 42(3):478–494
25. Lazear HM, Govero J, Smith AM, Platt DJ, Fernandez E, Miner JJ, Diamond MS (2016) A Mouse Model of Zika Virus Pathogenesis. Cell Host Microbe 19(5):720–730
26. Leguia M, Loyola S, Rios J, Juarez D, Guevara C, Silva M, Prieto K, Wiley M, Kasper MR, Palacios G, Bausch DG (2015) Full genomic characterization of a Saffold virus isolated in Peru. Pathogens 4(4):816–825
27. Li S, Shi Y, Zheng K, Dai J, Li X, Yuan S, Chen L, Huang J (2017) Morphologic and molecular characterization of a strain of Zika virus imported into Guangdong, China. PLoS One 12(1):e0169256
28. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Almazroa M, Memish ZA (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380(9859):2095–2128
29. Ly A, Buck A, Balluff B, Sun N, Gorzolka K, Feuchtinger A, Janssen KP, Kuppen PJ, van de Velde CJ, Weirich G, Erlmeier F, Langer R, Aubele M, Zitzelsberger H, McDonnell L, Aichler M, Walch A (2016) High-mass-resolution MALDI mass spectrometry imaging of metabolites from formalin-fixed paraffin-embedded tissue. Nat Protoc 11(8):1428–1443
30. Martinez MG, Snapp EL, Perumal GS, Macaluso FP, Kielian M (2014) Imaging the alphavirus exit pathway. J Virol 88(12):6922–6933
31. Mielke R, Kessler J, Szelies B, Herholz K, Wienhard K, Heiss WD (1998) Normal and pathological aging–findings of positron-emission-tomography. J Neural Transm (Vienna) 105(8–9):821–837
32. Miner JJ, Cao B, Govero J, Smith AM, Fernandez E, Cabrera OH, Garber C, Noll M, Klein RS, Noguchi KK, Mysorekar IU, Diamond MS (2016) Zika virus infection during pregnancy in mice causes placental damage and fetal demise. Cell 165(5):1081–1091
33. Morgan C, Godman GC, Breitenfeld PM, Rose HM (1960) A correlative study by electron and light microscopy of the development of type 5 adenovirus. I. Electron microscopy. J Exp Med 112:373–382
34. Mudhasani R, Kota KP, Retterer C, Tran JP, Tritsch SR, Zamani R, Whitehouse CA, Bavari S (2015) High-content image-based screening of a signal transduction pathway inhibitor small-molecule library against highly pathogenic RNA viruses. J Biomol Screen 20(1):141–152
35. Mudhasani R, Tran JP, Retterer C, Kota KP, Whitehouse CA, Bavari S (2016) Protein Kinase R degradation is essential for fift valley fever virus infection and is regulated by SKP1-CUL1-F-box (SCF)FBXW11-NSs E3 ligase. PLoS Pathog 12(2):e1005437
36. Nazerian K, Purchase HG (1970) Combined fluorescent-antibody and electron microscopy study of Marek's disease virus-infected cell culture. J Virol 5(1):79–90
37. Noda T, Kolesnikova L, Becker S, Kawaoka Y (2011) The importance of the NP: VP35 ratio in Ebola virus nucleocapsid formation. J Infect Dis 204(Suppl 3):S878–S883
38. Osuna CE, Lim SY, Deleage C, Griffin BD, Stein D, Schroeder LT, Omange R, Best K, Luo M, Hraber PT, Andersen-Elyard H, Ojeda EF, Huang S, Vanlandingham DL, Higgs S, Perelson AS, Estes JD, Safronetz D, Lewis MG, Whitney JB (2016) Zika viral dynamics and shedding in rhesus and cynomolgus macaques. Nat Med 22(12):1448–1455
39. Panchal RG, Kota KP, Spurgers KB, Ruthel G, Tran JP, Boltz RC, Bavari S (2010) Development of high-content imaging assays for lethal viral pathogens. J Biomol Screen 15(7):755–765
40. Perreira JM, Meraner P, Brass AL (2016) Functional genomic strategies for elucidating human-virus interactions: will CRISPR knockout RNAi and haploid cells? Adv Virus Res 94:1–51
41. Pottier G, Bernards N, Dolle F, Boisgard R (2014) [(1)(8)F]DPA-714 as a biomarker for positron emission tomography imaging of rheumatoid arthritis in an animal model. Arthritis Res Ther 16(2):R69
42. Radoshitzky SR, Pegoraro G, Chi XO, Dong L, Chiang CY, Jozwick L, Clester JC, Cooper CL, Courier D, Langan DP, Underwood K, Kuehl KA, Sun MG, Cai Y, Yu SQ, Burk R, Zamani R, Kota K, Kuhn JH, Bavari S (2016) siRNA screen identifies trafficking host factors that modulate alphavirus infection. PLoS Pathog 12(3):e1005466
43. Reid SP, Tritsch SR, Kota K, Chiang CY, Dong L, Kenny T, Brueggemann EE, Ward MD, Cazares LH, Bavari S (2015) Sphingosine kinase 2 is a chikungunya virus host factor co-localized with the viral replication complex. Emerg Microbes Infect 4(10):e61
44. Rella CE, Ruel N, Eugenin EA (2014) Development of imaging techniques to study the pathogenesis of biosafety level 2/3 infectious agents. Pathog Dis 72(3):167–173
45. Rossi SL, Tesh RB, Azar SR, Muruato AE, Hanley KA, Auguste AJ, Langsjoen RM, Paessler S, Vasilakis N, Weaver SC (2016) Characterization of a novel murine model to study Zika virus. Am J Trop Med Hyg 94(6):1362–1369
46. Sapparapu G, Fernandez E, Kose N, Bin C, Fox JM, Bombardi RG, Zhao H, Nelson CA, Bryan AL, Barnes T, Davidson E, Mysorekar IU, Fremont DH, Doranz BJ, Diamond MS, Crowe JE (2016) Neutralizing human antibodies prevent Zika virus replication and fetal disease in mice. Nature 540(7633):443–447
47. Savidis G, McDougall WM, Meraner P, Perreira JM, Portmann JM, Trincucci G, John SP, Aker AM, Renzette N, Robbins DR, Guo Z, Green S, Kowalik TF, Brass AL (2016) Identification of Zika virus and

Dengue virus dependency factors using functional genomics. Cell Rep 16(1):232–246
48. Shan C, Xie X, Muruato AE, Rossi SL, Roundy CM, Azar SR, Yang Y, Tesh RB, Bourne N, Barrett AD, Vasilakis N, Weaver SC, Shi PY (2016) An infectious cDNA clone of Zika virus to study viral virulence, mosquito transmission, and antiviral inhibitors. Cell Host Microbe 19(6):891–900
49. Sheehan KC, Lai KS, Dunn GP, Bruce AT, Diamond MS, Heutel JD, Dungo-Arthur C, Carrero JA, White JM, Hertzog PJ, Schreiber RD (2006) Blocking monoclonal antibodies specific for mouse IFN-alpha/beta receptor subunit 1 (IFNAR-1) from mice immunized by in vivo hydrodynamic transfection. J Interf Cytokine Res 26(11):804–819
50. Sirohi D, Chen Z, Sun L, Klose T, Pierson TC, Rossmann MG, Kuhn RJ (2016) The 3.8 A resolution cryo-EM structure of Zika virus. Science 352(6284):467–470
51. Smith DR, Hollidge B, Daye S, Zeng X, Blancett C, Kuszpit K, Bocan T, Koehler JW, Coyne S, Minogue T, Kenny T, Chi X, Yim S, Miller L, Schmaljohn C, Bavari S, Golden JW (2017) Neuropathogenesis of Zika virus in a highly susceptible immunocompetent mouse model after antibody blockade of type I interferon. PLoS Negl Trop Dis 11(1):e0005296
52. Spuul P, Balistreri G, Hellstrom K, Golubtsov AV, Jokitalo E, Ahola T (2011) Assembly of alphavirus replication complexes from RNA and protein components in a novel trans-replication system in mammalian cells. J Virol 85(10):4739–4751
53. Sridhar S, K. K. To, Chan JF, Lau SK, Woo PC, Yuen KY (2015) A systematic approach to novel virus discovery in emerging infectious disease outbreaks. J Mol Diagn 17(3):230–241
54. Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C (2003) Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 75(8):1895–1904
55. van der Eijk AA, van Genderen PJ, Verdijk RM, Reusken CB, Mogling R, van Kampen JJ, Widagdo W, Aron GI, GeurtsvanKessel CH, Pas SD, Raj VS, Haagmans BL, Koopmans MP (2016) Miscarriage associated with Zika virus infection. N Engl J Med 375(10):1002–1004
56. Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ, Luo Y (2012) RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn 14(1):22–29
57. Zmurko J, Marques RE, Schols D, Verbeken E, Kaptein SJ, Neyts J (2016) The viral polymerase inhibitor 7-Deaza-2′-C-Methyladenosine is a potent inhibitor of in vitro Zika virus replication and delays disease progression in a Robust Mouse infection model. PLoS Negl Trop Dis 10(5):e0004695

22 Dengue Antiviral Development: A Continuing Journey

Jenny G. Low, Rene Gatsinga,
Subhash G. Vasudevan, and Aruna Sampath

Abstract

Dengue fever is a leading cause of illness and mortality in the tropics and subtropics. There are no therapeutics currently available and a recently approved vaccine is not very efficacious demanding an urgent need to develop an effective antiviral. The path to successful dengue drug development depends on availability of relevant preclinical testing models and better understanding of dengue pathogenesis. In recent years, efforts to develop dengue therapeutics have focused on both repurposing approved drugs as well as discovery of new chemical entities that act via virus or host targeted mechanisms. Here, we discuss the various innovative approaches, their outcome, and the lessons gleaned from the development efforts.

Keywords

Drug repurposing · Alpha glucosidase inhibitors · Host targets · FDG imaging · Dengue biomarker

22.1 Introduction

Dengue fever is the most prevalent mosquito-borne viral disease globally. It is endemic in more than 100 countries, and causes an estimated 400 million infections and 25,000 deaths every year [5]. As these numbers have been rising steadily over the past decades, developing efficacious antiviral agents and vaccine is imperative to control the disease burden. Current treatment guidelines rely entirely on supportive care and aggressive monitoring [23]. Recently, a tetravalent vaccine was licensed in several dengue-endemic countries, which is a considerable breakthrough [63]. Its efficacy, however, varies widely hence there remains a strong need to develop effective therapeutic modalities for dengue virus (DENV) infections [27].

Since the early 2000s, several compounds have been tested in early proof-of-concept trials but, none was able to demonstrate clinical efficacy. In this chapter, we discuss the progress that has been made and the main challenges faced in dengue therapeutics research and attempt to draw a parallel to the development of other antivirals like oseltamivir.

J. G. Low
Department of Infectious Diseases, Singapore General Hospital, Singapore, Singapore

Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore, Singapore

R. Gatsinga · S. G. Vasudevan
Emerging Infectious Diseases Program, Duke-NUS Medical School Singapore, Singapore, Singapore

A. Sampath (✉)
Emergent BioSolutions Inc., Gaithersburg, MD, USA
e-mail: sampatha1@ebsi.com

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_22

22.2 Candidate Drugs

Consideration of the nature of the disease and the impacted demographics is essential for drug development – and defining a Target Product Profile (TPP) is a key step [30]. Dengue fever (DF) results in an acute self-limiting disease, with a small proportion of patients, mostly children, developing constellations of life threatening complications referred to as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DF mostly affects low-income, tropical countries. Consequently, the ideal drug should have an excellent safety profile not to surpass the low risk posed by uncomplicated DF. In addition, it should be an oral drug, require minimum frequency dosing with total course of therapy not to exceed 5 days duration given the acute nature of the disease, and produce measurable clinical benefits. It should be easily manufactured and distributed at affordable cost, and be able to withstand temperatures and high humidity prevalent in tropical regions. These general TPP goals should be kept in mind but they should not stand in the way of new innovations such as prophylactic drugs or biologics in the form of potent therapeutic antibodies that target the viral envelope protein to block its infectivity.

22.2.1 Repurposed Agents

Starting from scratch, drug development is a long and expensive process, and for dengue the experiences of Novartis pharmaceutical company which formed the first major industrial effort to develop a drug that could be used as defined in the TPP has been reviewed [34]. The lack of any compounds from these ventures and the urgent need for affordable anti-dengue drugs has resulted in several attempts at repurposing drugs that were developed for other indications. The main advantage of this approach is that it has a relatively short development path as the initial safety data in animals and humans are already established. In fact, all seven compounds evaluated in dengue clinical trials to date were repurposed. These agents were either already approved for different indications or were discontinued for further development due to reasons other than acute safety and tolerability. There have been 5 completed and published and 2 ongoing randomized clinical trials for anti-dengue agents since the early 2000s.

22.2.1.1 Chloroquine

Tested by the Oxford University Clinical Research Unit in Vietnam (OUCRU), chloroquine is a 4-amino-quinoline derivative with lysosomotropic and weak base properties (Trial Identifier: ISRCTN38002730). It has evidence of in vitro and in vivo (aotus monkey) anti-viral activity that is thought to be mediated by disrupting endosomal fusion and viral maturation [21]. This oral drug is cheap and widely available, with a strong safety profile. The clinical trial for this drug monitored time to resolution of viremia and NS1 antigenemia as primary measures of drug efficacy. There was no observed difference in efficacy between the drug and placebo, but chloroquine was associated with a higher incidence of adverse events [61].

22.2.1.2 Prednisolone

Prednisolone is a corticosteroid with potent anti-inflammatory activity. Some non-randomized trials have reported possible benefits of corticosteroids as a rescue treatment for severe DF, but this remains controversial [47]. It is, however, universally accepted that inflammatory factors play a crucial role in the pathogenesis of DSS. On this ground, the OUCRU in Vietnam conducted a Randomized Controlled Trial (RCT) to assess the safety of corticosteroids and outcome in viremia but was not powered to measure its therapeutic efficacy, although clinical outcomes such as DSS, ICU admission, and bleeding were measured (Trial Identifier: ISRCTN39575233). The safety profiles were similar in both groups but no clinical benefit was detected [57].

22.2.1.3 Balapiravir

A prodrug of nucleoside analogue 4′-azidocytidine, balapiravir was originally developed as a therapeutic agent against hepatitis C virus (HCV), another flavivirus. Balapiravir development against HCV was halted after evidence of excessive toxicity upon prolonged exposure. Due to the known similarities between DENV and HCV RNA-dependent RNA polymerases, this drug was evaluated as an anti-dengue agent by OUCRU in Vietnam (Trial identifier: NCT01096576). The phase II study monitored time to fever clearance, viremia and NS1 antigenemia to measure the drug efficacy. The results showed no significant difference between control and treated arms [14, 41].

22.2.1.4 Ribavirin

Ribavirin is a drug commonly used to treat HCV and also sometimes used as a broad-spectrum antiviral for RNA viruses. It has RNA-dependent RNA polymerase inhibitory activity, but its antiflaviviral activity is believed to be mediated by intracellular GTP depletion. Ribavirin is used in conjunction with interferon-α against HCV as they show synergistic effect. Ribavirin was evaluated against DENV infection in combination with traditional Chinese medicine by the Guangzhou 8th People's Hospital, but the results have not been made publicly available yet (Trial identifier: NCT01973855).

22.2.1.5 Celgosivir

Celgosivir is another drug that was originally developed for HCV. It is an inhibitor of α-glucosidase, a host enzyme necessary for glycosylation of viral coat proteins that aids in proper protein folding. It was tested in a RCT as an anti-dengue agent by Duke-National University of Singapore (Duke-NUS)/Singapore General Hospital (SGH) in Singapore using fever and viremia reduction as measures of drug efficacy. No statistically significant difference was seen between control and treatment groups, but celgosivir was found to have a good safety profile in dengue patients [36]. Extended pharmacokinetics studies and alternative regimen testing in mouse models of dengue infection predicted that increased exposure may impact efficacy [55, 70]. To test this possibility a small pharmaceutical company 60° Pharma has partnered with Singapore General Hospital to test an altered regimen that will result in an almost 300% increase in exposure with only a modest increase in dose. The clinical trial is scheduled to start recruiting in 2017 (Trial Identifier: NCT02569827).

22.2.1.6 Lovastatin

Lovastatin is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, widely used for its lipid lowering properties. Among their other properties, statin drugs improve endothelial function and stabilize lipid membranes, two mechanisms known to be disrupted in DF pathogenesis. On this ground, a clinical trial was conducted in Vietnam by OUCRU to assess the safety of lovastatin as an anti-DENV agent. The safety outcome was measured clinically by trending liver and muscle injury serum markers, two commonly encountered side effects of lovastatin. Lovastatin was reported to be a safe and tolerable treatment in dengue patients, but there was no evidence of beneficial effect in terms of clinical progress or viremia reduction [71].

22.2.1.7 Ivermectin

This anti-parasitic agent commonly used to treat nematode infections, scabies, and lice has been shown to have anti-viral activities by inhibiting host nuclear import receptors importin-α and importin-β [64]. These receptors are necessary for DENV nonstructural protein 5 (NS5) migration to the nucleus for efficient replication. Nevertheless the precise mechanism by which ivermectin inhibits dengue is still unclear because the nuclear localization of NS5 is not required for viral RNA replication [59]. On the positive side, the drug has been identified as a potent inhibitor

of the related flavivirus, zika virus in recent screens with FDA approved drugs [2, 74]. A clinical trial as a treatment for dengue fever with ivermectin is being carried out by the Mahidol University in collaboration with the Ministry of Health of Thailand (clinical identifier: NCT02045069). Preliminary results suggest reduction in serum NS1 antigenemia and body temperature despite the lack of detectable difference in viremia levels [1].

22.2.1.8 Ketotifen

Ketotifen is a non-competitive anti-histamine and a mast cell stabilizer generally used to treat atopic conditions like asthma and allergic rhinitis. It is being evaluated as an anti-dengue agent based on preclinical data suggesting that mast cell degranulation is an important mediator of DF pathogenesis [16, 22]. A clinical trial (NCT026773840) is currently being conducted by Duke-NUS, SGH, and the National University Hospital in Singapore to assess its clinical safety and efficacy.

22.3 DENV Specific Drug Development

Typically, new drug discovery involves identifying a target; developing an assay to assess activity against the target; high-throughput screening of potential candidate compounds; and chemically modifying the successful candidates to optimize activity, pharmacokinetics and toxicology in in vitro and in animal models of infection. Once a lead candidate is identified, manufacturing methods are adapted to produce the drug candidate in sufficient quantities to be tested in clinical trials [54].

Usually, the target is either a viral or host protein required for entry, proteolytic processing of the newly translated polyprotein, RNA replication, viral genome packaging and virion release from infected cells. DENV proteome comprises of 3 structural proteins: Capsid (C), premembrane (prM), and envelope (E) proteins; and 7 nonstructural (NS) proteins involved in protein processing and viral replication: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The replication apparatus, especially NS3 and NS5 have been the most intensively investigated targets in dengue drug development [37]. Commonly studied host targets include α-glucosidase, inosine monophosphate dehydrogenase, and dihydroorotate dehydrogenase among others [7].

These endeavors require large amounts of investments and carry high financial risks. Like many other neglected tropical diseases, dengue has been understudied consequently. To date, no candidate drug developed specifically for dengue has had positive results in a phase II human trial. This section highlights the considerable milestones achieved in this field and the promising projects underway.

NITD-008, a nucleoside inhibitor that targets viral RNA-dependent RNA polymerase (RdRp) is a potent antiviral developed by the Novartis Institute of Tropical Diseases in Singapore. It has remarkable nanomolar efficacy against all four DENV serotypes and other Flaviviruses in vitro and *in vivo* [76]. Toxicology studies conducted in animals revealed significant renal toxicity hence further development of this candidate was halted. Nonetheless, NITD-008 is still considered the standard of preclinical efficacy in dengue drug development and widely used to benchmark new drug development.

Recently, Emergent BioSolutions Inc. developed UV-4B, an antiviral agent that shows in vitro and in vivo activity against DENV. The proposed mechanism of action is through the inhibition of the enzymatic activities of host endoplasmic reticulum α-glucosidases. Viruses require these cellular enzymes for proper processing of their proteins. Since this is a host targeted mechanism of action, it is anticipated that development of viral resistance to UV-4B is less likely to occur than with directly acting antiviral agents [44, 66, 67]. This hypothesis was tested in vitro where DENV-infected cells treated with 38 cycles of UV-4B showed no drug-induced resistance. In vivo,

UV-4B efficacy was maintained through 5 DENV passages in a mouse model [45].

An investigational new drug application has been opened for UV-4B based on preclinical safety and efficacy data. A phase 1 clinical trial completed in 2016 documented good tolerability and no serious adverse events after administration of single doses of UV-4B ranging between 3 and 1000 mg (NCT02061358). The pharmacokinetics data showed low inter-individual variability and linearity over a broad dose range. Another phase 1 clinical study has been initiated to determine the safety and pharmacokinetics of UV-4B administered orally as multiple ascending doses to healthy volunteers (NCT02696291). UV-4B is also being evaluated in preclinical studies for the treatment of influenza as an additional indication [66, 67].

Siga Technologies reported the development of the inhibitor ST-148, which acts by inducing structural rigidity. In a nonlethal model of DENV infection in AG129 mice, ST-148 reduced viremia and viral load and lowered cytokine levels in the plasma [6].

In recent years several groups have reported the discovery and development of NS4B inhibitors. NITD reported the activity of spiropyrazolopyridone compound NITD-618. Although, the compound showed reduced virema against DENV-2 in AG129 model of infection they were unable to identify a candidate that could show pan serotypic activity. In another screening effort, van Cleef et al. reported a new inhibitor, SDM25N, from screening the NIH Clinical Collection (NCC); a library of drug-like small molecules a stably replicating DENV serotype 2 (DENV2) subgenomic replicon. SDM25N, which restricts genomic RNA replication by – directly or indirectly – targeting the viral NS4B protein [62, 65, 72]. Janssen is also currently developing a NS4B inhibitor [43].

22.3.1 Recent Structural Discoveries

Recent advances in immunological, molecular, and structural virology have offered new ways to develop drugs more efficiently. Nuclear magnetic resonance spectrometry, X-ray crystallography, and cryo-electron microscopy have provided detailed structural data of DENV proteins. This information can be combined with molecular tools such as in silico approaches and infectious clone technology to discover new drug targets against DENV and potentially other flaviviridae [6, 33, 35, 73]. The viral processes that can be targeted include entry/fusion (E protein), translation/polyprotein processing of nonstructural (NS) proteins (NS3, NS2B-NS3 complex), replication (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), and viral packaging (Capsid) [54]. Being essential to replication and infectivity and highly conserved among flaviviruses, NS3 and NS5 have been the most popular targets of DENV antiviral development.

Most notably, this structural information has been used in studies of human antibodies isolated from convalescent patients and yielded greater understanding of the epitopes that need to be targeted for effective virus neutralization [48]. Serotype-specific and cross-reactive neutralizing monoclonal antibodies are both being explored [60]. Ab513, a monoclonal antibody that binds domain III of the E protein of all 4 DENV serotypes was engineered by Visterra (Cambridge, MA). This agent has shown promising preclinical results with the ability to lower viremia and control DHS/DSS features in humanized mouse models without enhancing infection despite the presence of cross-reactive antibodies. It is poised to enter clinical trial by early 2018.

Compound and structure	Mechanism of action	Study site	Preclinical results	Clinical results
1.	Lysosomal fusion inhibitor	OUCRU, Ho Chi Minh City, Vietnam	Cell-based assay (U937): EC_{50}: 50 μM Aotus monkey: significant reduction in viremia [21]	No change in viremia or NS1 antigenemia
2. Prednisolone	Anti-inflammatory activity	OUCRU, Ho Chi Minh City, Vietnam	NA	No change in hematological virological or clinical endpoints
3. Balapiravir	Polymerase inhibitor	OUCRU, Ho Chi Minh City, Vietnam	Cell based (Huh-7) EC_{50};1.9–11 μM [41]	No change in virological and immunological endpoints [40]
4. Ribavirin	Nucleoside analogue	Guangzhou 8th People's Hospital	Cell-based (LLC-MK2): IC_{50}: 50.9 μM [56]	Pending
5. Lovastatin	Improving endothelial function and stabilizing lipid membranes	OUCRU, Ho Chi Minh City, Vietnam	AG129 mouse model: Increased survival at dose of 200 mg/kg/day [71]	No evidence of beneficial effect on clinical progress or DENV viremia [71]
6. Ivermectin	Helicase inhibitor	Mahidol University/Siriraj Hospital, Thailand	Enzyme assay: IC_{50}: 0.5 μM [1]	NS1 antigenemia and fever reduction (preliminary)
7. Celgosivir	Alpha glucosidase inhibitor	SGH/Duke-NUS, Singapore	Cell-based assay: EC_{50}: 0.2 μM AG129 mouse model: 100% survival at dose of 50 mg/kg BD for 5 days [68–70]	No statistically significant reduction of viral load or fever [36]
8. Ketotifen	Mast cell stabilizer	SGH/Duke-NUS, Singapore	Mouse model: significant reduction of severe dengue features [51]	Pending
9. UV4B	Alpha glucosidase inhibitor	Emergent BioSolutions, Maryland USA	AG129 mouse model: 100-fold viremia reduction at 100 mg/kg PO dosing Minimum effective dose: 10 mg/kg PO [44, 66, 67]	Phase 1a Single Ascending Dose Study in healthy volunteers showed UV-4B is well tolerated up to 1000 mg single dose
10. NITD008	Nucleoside analogue-polymerase inhibitor	NITD, Singapore	Cell based assay (PBMC) EC_{50}: 0.16–0.85 μM AG129 mouse model: 100% survival at dose >10 mg/kg PO [76]	Excessive nephrotoxicity, development halted

22.4 The Clinical – Preclinical Gap

As discussed above, many compounds have shown good efficacy in preclinical studies. However, none was proven to be superior to placebo in clinical setting. The disparity between preclinical and clinical results is an inherent entity of therapeutics research. Common reasons include the nonspecific and wide spectrum of disease manifestations, the limitations of existing model organisms, and the lack of a biological marker that correlates with clinical results. A factor that makes this even more pertinent for dengue therapeutics, specifically, is the limited financial resources available for research. The recent clinical and preclinical progress achieved gives an insightful perspective on what should be future research directions.

22.4.1 Clinical Manifestation

Dengue fever has extremely nonspecific manifestations making it difficult to study in clinical settings. Contrary to preclinical setting where the time of infection is controlled, patients are recruited into clinical trials when they experience symptoms and present to their healthcare provider. Fever is virtually the only symptom clinicians base they suspicion on when screening for dengue. As with most other febrile illnesses, dengue patients do not present until later in the course of disease. Unless there is an ongoing outbreak, dengue might not be the first impression of the healthcare provider. This presents an additional challenge to clinical trial design and recruitment as administering treatment later in the course of illness when the viremia level has already past the peak can dilute any potential benefit of the therapeutic agent under investigation.

Moreover, the vagueness of early symptoms is a source of inaccuracy based on patient-reported onset of illness/fever. This is a known unreliable measure, but there is usually no better alternative.

22.4.2 Representative Model Organism

DENV is not known to be naturally pathogenic in any species other than humans. This makes it very challenging to obtain representative animal models for research. Efforts have been made to identify the specific characteristics that make us vulnerable to DENV. Multiple animals and DENV strains have been genetically engineered to replicate the identified characteristics, but, to this day, none of the models are able to accurately mimic a human DENV infection.

22.4.3 Nonhuman Primates

Nonhuman primates develop viremia and neutralizing antibody response to DENV infection but do not develop symptoms of DF [75]. They have been used to study antibody-dependent enhancement of infection (ADE) and to test candidate vaccines and antivirals efficacy by assessing viremia levels [12, 13, 24, 25]. As we discuss below, viremia is a suboptimal measure of treatment response. Some nonhuman primate models that express features of human DF have been identified; but their use is hindered by the prohibitive cost and accessibility.

22.4.4 Mouse Models

DENV does not replicate well in rodent cells. To overcome this, extensive work has been done to develop DENV mouse models. The first successful suckling mouse, was developed by serial passage DENV in mice brain, increasing the virus adaptability to mice cells [50]. Intracranial inoculation resulted in DENV encephalitis and paralysis. This model has been used by to evaluate antivirals and vaccines but the altered tropism of the virus makes the relevance to humans questionable [9].

Inoculation with high DENV titers in immunocompetent mice can induce disease comparable to human DF. Different groups have reported hepatic T cell invasion, localized hemorrhage,

thrombocytopenia and detectable viral load in serum, spleen, liver, and brain, vascular leakage; all of which are characteristic of human DF/DHS/DSS [9, 10, 12, 13, 52]. This model is useful to gain insight on the pathogenesis of DF but the high viral titer required to induce symptoms is not representative of the infection process in humans; making it inadequate for antiviral development studies.

Humanized mice developed by transplanting human hematopoietic stem cells into severe combined immunodeficient mice have been shown to be susceptible to low passage DENV. They exhibit DF-like feature such as fever, rash and thrombocytopenia, but not DHS/DSS [4]. Kuruvilla and colleagues reported that humanized mice can develop fever and viremia for up to 21 days and produce human anti-DENV Ig-M and Ig-G capable of neutralizing DENV. There was, however, no significant cellular immune response induced [32]. Several groups have tried to replicate a human like T-cell response in humanized mice, but there still has not been any model capable of mimicking severe dengue symptoms, or human-like immune response; limiting their use in therapeutics research [9].

Immunocompromised mice have been the most widely used model thus far. By knocking out key genes involved in immune response, infectible mice models have been engineered. The most established in therapeutics research is the type I and II interferon receptors deficient AG129. This model is susceptible to both mouse-adapted and clinical DENV infection. Concurrently, efforts were also being put into identifying a DENV strain that could successfully infect mice. D2S10 was developed by alternate passaging of PL046, a clinical isolate, between AG129 mice and C3/C6 mosquito cells. This DENV strain was able to cause lethal infection in mice without neurological deficits and; most importantly, D2S10 caused vascular leakage making it very pertinent to study human DF. Of note, celgosivir and lovastatin were proven to be efficacious in AG129 mice, with increased survival and decreased viremia. In clinical trials, however, no significant effect was observed [36, 68, 69, 71].

22.4.5 Need for Human Infection Model

Although there has been reports of certain DENV strains causing some features of DHS and DSS in certain mouse models, the pathogenesis remains substantially different from that in humans [58]. The ideal way around this obstacle is the use of a human infection model (HIM). HIM was an important contributor to the development of oseltamivir. The illness caused by influenza virus infection in healthy young adults is short lived and of mild severity; the use of a HIM was hence accessible to the developers of oseltamivir [28]. That enabled them to control the timing between infection and initiation of therapy, one of the most critical challenges in anti-dengue clinical trials [37]. Perhaps more importantly, it also allowed them to quickly down-select candidate compounds [19]. This was crucial to a remarkably fast drug development; oseltamivir was approved for treatment of influenza only 7 years after the search for an orally available neuraminidase inhibitor was initiated [54].

HIM has been used to study several aspects of dengue before. For instance, Startler and colleagues studied the process of DENV induced plasma leakage in healthy individuals [53]. In 2011, Gunther et al. used HIM to study the immune response to DENV in previously vaccinated people [26]. More recently, Kirkpatrick and colleagues proposed HIM to validate vaccines before moving onto large scale clinical trials [31]. Even though HIM would revolutionize dengue research, there are still strong reservations about its use. DF is typically not associated with mortality in healthy adults, but has a potential for high morbidity requiring hospitalization [19]. Another limitation of HIM is the fact that there is no possibility to access tissue samples for pathological analysis, limiting HIM studies to a descriptive nature [9].

22.5 Objectively Measurable Endpoints

22.5.1 Viremia

Since the earliest dengue studies, viremia as a biomarker for dengue disease has been a universally accepted paradigm [49, 54]. This concept was popularized by the availability of sensitive methods to detect and quantify viral particles in clinical samples. In fact, all trials for candidate antiviral drugs and vaccines have reduction of viremia as one of their primary clinical endpoints, if not the only endpoint. However, it has become evident that measured viremia does not always correlate with clinical outcome. For instance, during secondary infections, measured viremia is often markedly lower than primary despite the higher likelihood of developing severe disease. One of the possible explanations for this phenomenon is intrinsic to the viremia assay methodology itself.

22.5.1.1 qPCR

In the 7 clinical trials mentioned above, viremia was measured using quantitative polymerase chain reaction assay (qPCR). This method detects and quantifies viral RNA in serum, but it does not reflect the amount infective viruses present. RNA copies can exceed infectious viral units by 2–5 logs. This effect has been described for other viral infections as well and was attributed to a probable large quantity of cells infected by defective proviruses, masking the absolute viremia [20].

22.5.1.2 Plaque Assay

A more representative assay would be direct culturing of the clinical samples. However, this is extremely challenging because clinical isolates have widely varying abilities to grow in vitro. Unpassaged viruses, for instance, are known to be less able to infect consistently in vitro despite their potential infectivity in vivo. This makes culture and plaque assays inherently inaccurate. Mosquito inoculation can be used to account for unpassaged DENV, but this technique requires an insectary and highly skilled personnel making it inaccessible to most diagnostic virology laboratories [15].

22.5.2 NS1 Antigenemia

Serum NS1 levels rise early in the infection course making it a good diagnostic tool. However, NS1 antigenemia varies considerably by DENV serotypes and primary versus secondary infection [18, 68, 69]. There is also evidence that it may be involved in DF pathogenesis [3, 39]. Structural and in vivo mice studies suggest that, if combined with a host dependent biomarker, NS1 could be a reliable biomarker and clinical endpoint measure for therapeutic trials [40]. Further work is needed to confirm this.

22.5.3 Host Biomarkers: Cytokine, Endothelial Activation Markers, Cells, Biochemical Markers

It is believed that patients with DHS/DSS experience a “cytokine storm,” causing their clinical symptoms. Trending these cytokines may, consequently, serve as a good prognostic biomarker. Identified markers that correlate with disease progress include interleukin-10, complements C3a and C5a, and macrophage migration inhibitory factor [11, 29, 38].

The correlation between blood cellular components and dengue severity has been extensively studied, platelet and red blood cell counts in particular. Thrombocytopenia is an established hallmark of severe dengue disease [17, 23]. A study conducted in Thailand was able to predict more than 97% subsequent severe disease development using an algorithm based on white blood cell counts, percent monocytes, platelet count, and hematocrit information obtained in the first 72 h of disease [46]. A similar study conducted in

Vietnam proposed a prognostic scoring system based on the platelet count, history of vomiting, blood aspartate aminotransferase level, and NS1 rapid test status [42]. This scoring system had a sensitivity of 87% and specificity of 88%; with a negative predictive value of 99% amongst their study participants.

Biochemical markers like liver enzymes, nitric oxide, and lipids are also known to be deranged in severe disease, and have been studied as biomarkers and potential clinical endpoints. Several endothelial activation markers, including angiopoietin, von Willebrand factor, and VEGF, have also been associated with disease severity [29].

All these host biomarkers are used at varying degrees in dengue research. They all have a common short coming, however; poor specificity. There are many factors that can be responsible for host biomarkers changes and over-relying on them can result in false interpretations [29]. Nonetheless, cautiously combining them with DENV biomarkers greatly improves their prognostic power, making a case for their use in therapeutics trials [37].

22.5.4 18F-Fluorodeoxyglucose Imaging

In addition to developing optimal infection models, identifying new measurable endpoints that are consistent and comparable between animal models and humans would greatly help narrow the aforementioned preclinical-clinical gap. A team of researchers in Singapore is working on identifying new and more representative DF biomarkers by building upon the known pathogenesis. DENV particles preferentially replicate and accumulate in tissues that are not readily accessible. The ability to monitor tissues specifically instead of relying on systemic circulating markers offers a novel way to make early and robust inferences about the course of illness. 18F-Fluorodeoxyglucose uptake monitoring in dengue-infected mice has identified specific inflammation patterns in the intestines that strongly correlate with progression of disease and treatment response [8]. A human proof-of-concept trial is currently recruiting dengue infected subjects in Singapore to validate these findings in human.

22.6 Conclusion

Dengue fever imposes a significant global burden and low-income tropical countries are more heavily impacted. Efforts to develop vaccines and treatment modalities have been very slow until the 2000s. For the most part, this was due to the lack of available resources allocated to the cause. Over the past two decades, more governmental, academic, and private agencies have devoted funds to dengue research; which resulted in considerable advances, most notably a tetravalent vaccine licensed in most endemic countries. Nonetheless, the road to relieving the dengue burden is still long. The available vaccine's efficacy is not ideal, making it imperative to have effective therapeutic options. Dengue antiviral drug development endeavors with both repurposed and de novo agents have not yet yielded effective products in clinical trials but have bolstered the understanding of dengue pathogenesis and laid up the ground for further projects. Recent technological advances and innovative approaches may be offering faster, targeted, and more cost-efficient models to develop and assess new drugs.

Acknowledgements UV-4B development was supported by a contract award from the National Institute of Allergy and Infectious Diseases, HHSN272201100030C. The National Medical Research Council of Singapore is gratefully acknowledged for the support of dengue translational research grants (NMRC/CTGCoD/0001/2015 (JGL) and NMRC/CBRG/0103/2016 (SGV)).

References

1. Avirutnam P (2016) Ivermectin: a promising anti-dengue replication treatment. Eur Congress Clin Microbiol Infect Dis. (Abstract)
2. Barrows NJ, Campos RK, Powell ST, Prasanth KR, Schott-Lerner G, Soto-Acosta R, Galarza-Muñoz G, McGrath EL, Urrabaz-Garza R, Gao J, Wu P, Menon R, Saade G, Fernandez-Salas I, Rossi SL, Vasilakis N, Routh A, Bradrick SS, Garcia-Blanco MA (2016) A screen of FDA-approved drugs for inhibitors of Zika virus infection. Cell Host Microbe 20(2):259–270. https://doi.org/10.1016/j.chom.2016.07.004
3. Beatty PR, Puerta-Guardo H, Killingbeck SS, Glasner DR, Hopkins K, Harris E (2015) Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. Sci Transl Med 7(304):304ra141–304ra141. https://doi.org/10.1126/scitranslmed.aaa3787
4. Bente DA, Melkus MW, Garcia JV, Rico-Hesse R (2005) Dengue Fever in Humanized NOD/SCID mice. J Virol 79(21):13797–13799. https://doi.org/10.1128/JVI.79.21.13797-13799.2005
5. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI (2013) The global distribution and burden of dengue. Nature 496(7446):504–507. https://doi.org/10.1038/nature12060
6. Byrd CM, Dai D, Grosenbach DW, Berhanu A, Jones KF, Cardwell KB, Schneider C, Wineinger KA, Page JM, Harver C, Stavale E, Tyavanagimatt S, Stone MA, Bartenschlager R, Scaturro P, Hruby DE, Jordan R (2013) A novel inhibitor of dengue virus replication that targets the capsid protein. Antimicrob Agents Chemother 57(1):15–25. https://doi.org/10.1128/AAC.01429-12
7. Caputo AT, Alonzi DS, Marti L, Reca I-B, Kiappes JL, Struwe WB, Cross A, Basu S, Lowe ED, Darlot B, Santino A, Roversi P, Zitzmann N (2016) Structures of mammalian ER α-glucosidase II capture the binding modes of broad-spectrum iminosugar antivirals. Proc Natl Acad Sci U S A 113(32):E4630–E4638. https://doi.org/10.1073/pnas.1604463113
8. Chacko AM, Watanabe S, Herr KJ, Kalimuddin S, Tham JY, Ong J, Reolo M, Serrano RMF, Cheung YB, Low JGH, Vasudevan SG (2017) 18F-FDG as an inflammation biomarker for imaging dengue virus infection and treatment response. JCI Insight 2(9). https://doi.org/10.1172/jci.insight.93474
9. Chan KWK, Watanabe S, Kavishna R, Alonso S, Vasudevan SG (2015) Animal models for studying dengue pathogenesis and therapy. Antivir Res 123:5–14. https://doi.org/10.1016/j.antiviral.2015.08.013
10. Chen HC, Lai SY, Sung JM, Lee SH, Lin YC, Wang WK, Chen YC, Kao CL, King CC, Wu-Hsieh BA (2004) Lymphocyte activation and hepatic cellular infiltration in immunocompetent mice infected by dengue virus. J Med Virol 73(3):419–431. https://doi.org/10.1002/jmv.20108
11. Chen L-C, Lei H-Y, Liu C-C, Shiesh S-C, Chen S-H, Liu H-S, Lin Y-S, Wang S-T, Shyu H-W, Yeh T-M (2006) Correlation of serum levels of macrophage migration inhibitory factor with disease severity and clinical outcome in dengue patients. Am J Trop Med Hyg 74(1):142–147
12. Chen HC, Hofman FM, Kung JT, Lin YD, Wu-Hsieh BA (2007a) Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage. J Virol 81(11):5518–5526. https://doi.org/10.1128/JVI.02575-06
13. Chen L, Ewing D, Subramanian H, Block K, Rayner J, Alterson KD, Sedegah M, Hayes C, Porter K, Raviprakash K (2007b) A heterologous DNA prime-Venezuelan equine encephalitis virus replicon particle boost dengue vaccine regimen affords complete protection from virus challenge in cynomolgus macaques. J Virol 81(21):11634–11639. https://doi.org/10.1128/JVI.00996-07
14. Chen YL, Abdul Ghafar N, Karuna R, Fu Y, Lim SP, Schul W, Gu F, Herve M, Yokohama F, Wang G, Cerny D, Fink K, Blasco F, Shi PY (2014) Activation of peripheral blood mononuclear cells by dengue virus infection depotentiates balapiravir. J Virol 88(3):1740–1747. https://doi.org/10.1128/JVI.02841-13, https://www-ncbi-nlm-nih-gov.libproxy1.nus.edu.sg/pubmed/24257621
15. Choy MM, Ellis BR, Ellis EM, Gubler DJ (2013) Short report: comparison of the mosquito inoculation technique and quantitative real time polymerase chain reaction to measure dengue virus concentration. Am J Trop Med Hyg 89(5):1001–1005. https://doi.org/10.4269/ajtmh.13-0100
16. Dawicki W, Marshall JS (2007) New and emerging roles for mast cells in host defence. Curr Opin Immunol 19(1):31–38. https://doi.org/10.1016/j.coi.2006.11.006
17. Dengue: guidelines for diagnosis, treatment, prevention, and control. (2009). World Health Organization, Geneva
18. Duyen HTL, Ngoc TV, Ha DT, Hang VTT, Kieu NTT, Young PR, Farrar JJ, Simmons CP, Wolbers M, Wills BA (2011) Kinetics of plasma viremia and soluble nonstructural protein 1 concentrations in dengue: differential effects according to serotype and immune status. Journal of Infectious Diseases 203(9):1292–1300. https://doi.org/10.1093/infdis/jir014
19. Endy TP (2014) Dengue human infection model performance parameters. J Infect Dis 209(SUPPL. 2). https://doi.org/10.1093/infdis/jiu112
20. Eriksson S, Graf EH, Dahl V, Strain MC, Yukl SA, Lysenko ES, Bosch RJ, Lai J, Chioma S, Emad F, Abdel-Mohsen M, Hoh R, Hecht F, Hunt P, Somsouk M, Wong J, Johnston R, Siliciano RF, Richman DD, O'Doherty U, Palmer S, Deeks SG, Siliciano JD (2013) Comparative analysis of mea-

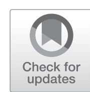
sures of viral reservoirs in HIV-1 eradication studies. PLoS Pathog 9(2). https://doi.org/10.1371/journal.ppat.1003174

21. Farias KJS, Machado PRL, Muniz JAPC, Imbeloni AA, da Fonseca BAL (2015) Antiviral activity of chloroquine against dengue virus type 2 replication in Aotus monkeys. Viral Immunol 28(3):161–169. https://doi.org/10.1089/vim.2014.0090
22. Furuta T, Murao LA, Lan NTP, Huy NT, Huong VTQ, Thuy TT, Tham VD, Nga CTP, Ha TTN, Ohmoto Y, Kikuchi M, Morita K, Yasunami M, Hirayama K, Watanabe N (2012) Association of mast cell-derived VEGF and proteases in dengue shock syndrome. PLoS Negl Trop Dis 6(2). https://doi.org/10.1371/journal.pntd.0001505
23. Global strategy for dengue prevention and control 2012–2020. (2012). World Health Organization, Geneva.
24. Goncalvez AP, Engle RE, St. Claire M, Purcell RH, Lai CJ (2007) Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. Proc Natl Acad Sci 104(22):9422–9427. https://doi.org/10.1073/pnas.0703498104
25. Guirakhoo F, Pugachev K, Arroyo J, Miller C, Zhang ZX, Weltzin R, Georgakopoulos K, Catalan J, Ocran S, Draper K, Monath TP (2002) Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever–dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. Virology 298(1):146–159. https://doi.org/10.1006/viro.2002.1462
26. Gunther VJ, Putnak R, Eckels KH, Mammen MP, Scherer JM, Lyons A, Sztein MB, Sun W (2011) A human challenge model for dengue infection reveals a possible protective role for sustained interferon gamma levels during the acute phase of illness. Vaccine 29(22):3895–3904. https://doi.org/10.1016/j.vaccine.2011.03.038
27. Halstead SB, Russell PK (2016) Protective and immunological behavior of chimeric yellow fever dengue vaccine. Vaccine 34(14):1643–1647. https://doi.org/10.1016/j.vaccine.2016.02.004
28. Hayden FG, Atmar RL, Schilling M, Johnson C, Poretz D, Paar D, Huson L, Ward P, Mills RG, the Oseltamivir Study, G (1999) Use of the selective oral neuraminidase inhibitor oseltamivir to prevent influenza. N Engl J Med 341(18):1336–1343
29. John DV, Lin Y-S, Perng GC (2015) Biomarkers of severe dengue disease – a review. J Biomed Sci 22(1):83. https://doi.org/10.1186/s12929-015-0191-6
30. Keller TH, Chen YL, Knox JE, Lim SP, Ma NL, Patel SJ, Sampath A, Wang QY, Yin Z, Vasudevan SG (2006) Finding new medicines for flaviviral targets. In: Novartis Foundation Symposium, vol 277. John Wiley, Chichester, pp 102–114. 1999
31. Kirkpatrick BD, Whitehead SS, Pierce KK, Tibery CM, Grier PL, Hynes NA, Larsson CJ, Sabundayo BP, Talaat KR, Janiak A, Carmolli MP, Luke CJ, Diehl SA, Durbin AP (2016) The live attenuated dengue vaccine TV003 elicits complete protection against dengue in a human challenge model. Sci Transl Med 8(330):330ra336–330ra336. https://doi.org/10.1126/scitranslmed.aaf1517
32. Kuruvilla JG, Troyer RM, Devi S, Akkina R (2007) Dengue virus infection and immune response in humanized RAG2(-/-)gamma(c)(-/-) (RAG-hu) mice. Virology 369(1):143–152. https://doi.org/10.1016/j.virol.2007.06.005
33. Lescar J, Luo D, Xu T, Sampath A, Lim SP, Canard B, Vasudevan SG (2008) Towards the design of antiviral inhibitors against flaviviruses: the case for the multifunctional NS3 protein from Dengue virus as a target. Antivir Res 80(2):94–101. https://doi.org/10.1016/j.antiviral.2008.07.001
34. Lim SP, Wang Q-Y, Noble CG, Chen Y-L, Dong H, Zou B, Yokokawa F, Nilar S, Smith P, Beer D, Lescar J, Shi P-Y (2013) Ten years of dengue drug discovery: progress and prospects. Antivir Res 100(2):500–519. https://doi.org/10.1016/j.antiviral.2013.09.013
35. Lim SP, Noble CG, Shi PY (2015) The dengue virus NS5 protein as a target for drug discovery. Antivir Res 119:57–67. https://doi.org/10.1016/j.antiviral.2015.04.010
36. Low JG, Sung C, Wijaya L, Wei Y, Rathore APS, Watanabe S, Tan BH, Toh L, Chua LT, Hou Y a, Chow A, Howe S, Chan WK, Tan KH, Chung JS, Cherng BP, Lye DC, Tambayah PA, Ng LC, Connolly J, Hibberd ML, Leo YS, Cheung YB, Ooi EE, Vasudevan SG (2014) Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): a phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial. Lancet Infect Dis 14(8):706–715. https://doi.org/10.1016/s1473-3099(14)70730-3
37. Low JG, Ooi EE, Vasudevan SG (2017) Current status of dengue therapeutics research and development. J Infect Dis 215(suppl_2):S96–S102. https://doi.org/10.1093/infdis/jiw423
38. Malavige GN, Gomes L, Alles L, Chang T, Salimi M, Fernando S, Nanayakkara KDL, Jayaratne SD, Ogg GS (2013) Serum IL-10 as a marker of severe dengue infection. BMC Infect Dis 13(1):341–341. https://doi.org/10.1186/1471-2334-13-341
39. Modhiran N, Watterson D, Muller DA, Panetta AK, Sester DP, Liu L, Hume DA, Stacey KJ, Young PR (2015) Dengue virus NS1 protein activates cells via Toll-like receptor 4 and disrupts endothelial cell monolayer integrity. Sci Transl Med 7(304):304ra142. https://doi.org/10.1126/scitranslmed.aaa3863
40. Muller DA, Young PR (2013) The flavivirus NS1 protein: molecular and structural biology, immunology, role inpathogenesis and application asadiagnostic

biomarker. Antivir Res 98(2):192–208. https://doi.org/10.1016/j.antiviral.2013.03.008

41. Nguyen NM, Tran CN, Phung LK, Duong KT, Huynh Hle A, Farrar J, Nguyen QT, Tran HT, Nguyen CV, Merson L, Hoang LT, Hibberd ML, Aw PP, Wilm A, Nagarajan N, Nguyen DT, Pham MP, Nguyen TT, Javanbakht H, Klumpp K, Hammond J, Petric R, Wolbers M, Nguyen CT, Simmons CP (2013) A randomized, double-blind placebo controlled trial of balapiravir, a polymerase inhibitor, in adult dengue patients. J Infect Dis 207(9):1442–1450. https://doi.org/10.1093/infdis/jis470
42. Nguyen MT, Ho TN, Nguyen VVC, Nguyen TH, Ha MT, Ta VT, Nguyen LDH, Phan L, Han KQ, Duong THK, Tran NBC, Bridget W, Wolbers M, Simmons CP (2017) An evidence-based algorithm for early prognosis of severe dengue in the outpatient setting. Clin Infect Dis 64(5):656–663. https://doi.org/10.1093/cid/ciw863
43. Partnership with the Wellcome Trust to address unmet medical needs. Retrieved from http://www.janssen.com/partnerships/dengue
44. Perry ST, Buck MD, Plummer EM, Penmasta RA, Batra H, Stavale EJ, Warfield KL, Dwek RA, Butters TD, Alonzi DS, Lada SM, King K, Klose B, Ramstedt U, Shresta S (2013) An iminosugar with potent inhibition of dengue virus infection in vivo. Antivir Res 98(1):35–43. https://doi.org/10.1016/j.antiviral.2013.01.004
45. Plummer E, Buck MD, Sanchez M, Greenbaum JA, Turner J, Grewal R, Klose B, Sampath A, Warfield KL, Peters B, Ramstedt U, Shresta S (2015) Dengue virus evolution under a host-targeted antiviral. J Virol 89(10):5592–5601. https://doi.org/10.1128/JVI.00028-15
46. Potts JA, Gibbons RV, Rothman AL, Srikiatkhachorn A, Thomas SJ, Supradish P o, Lemon SC, Libraty DH, Green S, Kalayanarooj S (2010) Prediction of dengue disease severity among pediatric Thai patients using early clinical laboratory indicators. PLoS Negl Trop Dis 4(8):2–8. https://doi.org/10.1371/journal.pntd.0000769
47. Rajapakse S, Rodrigo C, Maduranga S, Rajapakse AC (2014) Corticosteroids in the treatment of dengue shock syndrome. Infect Drug Resist 7:137–143. https://doi.org/10.2147/IDR.S55380
48. Robinson LN, Tharakaraman K, Rowley KJ, Costa VV, Chan KR, Wong YH, Ong LC, Tan HC, Koch T, Cain D, Kirloskar R, Viswanathan K, Liew CW, Tissire H, Ramakrishnan B, Myette JR, Babcock GJ, Sasisekharan V, Alonso S, Chen J, Lescar J, Shriver Z, Ooi EE, Sasisekharan R (2015) Structure-guided design of an anti-dengue antibody directed to a non-immunodominant epitope. Cell 162(3):493–504. https://doi.org/10.1016/j.cell.2015.06.057
49. Sabin AB (1952) Research on dengue during World War II. Am J Trop Med Hyg 1(1):30–50. https://doi.org/10.1086/383418
50. Sabin AB, Schlesinger RW (1945) Production of immunity to dengue with virus modified by propagation in mice. Science 101(2634):640–642. https://doi.org/10.1126/science.101.2634.640
51. St John AL (2013) Influence of mast cells on dengue protective immunity and immune pathology. PLoS Pathog 9(12):e1003783. https://doi.org/10.1371/journal.ppat.1003783
52. St John AL, Rathore APS, Raghavan B, Ng ML, Abraham SN (2013) Contributions of mast cells and vasoactive products, leukotrienes and chymase, to dengue virus-induced vascular leakage. eLife 2013(2). https://doi.org/10.7554/eLife.00481
53. Statler J, Mammen M, Lyons A, Sun W (2008) Sonographic findings of healthy volunteers infected with dengue virus. J Clin Ultrasound 36(7):413–417. https://doi.org/10.1002/jcu.20476
54. Sung C, Kumar GBS, Vasudevan SG (2014) Dengue Drug Development. In Dengue and Dengue Hemorrhagic Fever (2nd Edn) Gubler DJ, Ooi EE, Vasudevan S, Farrar J (Editors) Chapter 16 p293–321. Oxfordshire, CABI
55. Sung C, Wei Y, Watanabe S, Lee HS, Khoo YM, Fan L, Rathore AP, Chan KW, Choy MM, Kamaraj US, Sessions OM, Aw P, de Sessions PF, Lee B, Connolly JE, Hibberd ML, Vijaykrishna D, Wijaya L, Ooi EE, Low JG, Vasudevan SG (2016) Extended evaluation of virological, immunological and pharmacokinetic endpoints of CELADEN: a randomized, placebo-controlled trial of celgosivir in dengue fever patients. PLoS Negl Trop Dis 10(8):e0004851. https://doi.org/10.1371/journal.pntd.0004851
56. Takhampunya R, Ubol S, Houng H-S, Cameron CE, Padmanabhan R (2006) Inhibition of dengue virus replication by mycophenolic acid and ribavirin. J Gen Virol 87(7):1947–1952. https://doi.org/10.1099/vir.0.81655-0
57. Tam DT, Ngoc TV, Tien NT, Kieu NT, Thuy TT, Thanh LT, Tam CT, Truong NT, Dung NT, Qui PT, Hien TT, Farrar JJ, Simmons CP, Wolbers M, Wills BA (2012) Effects of short-course oral corticosteroid therapy in early dengue infection in Vietnamese patients: a randomized, placebo-controlled trial. Clin Infect Dis 55(9):1216–1224. https://doi.org/10.1093/cid/cis655
58. Tan GK, Ng JKW, Trasti SL, Schul W, Yip G, Alonso S (2010) A non mouse-adapted dengue virus strain as a new model of severe dengue infection in AG129 mice. PLoS Negl Trop Dis 4(4). https://doi.org/10.1371/journal.pntd.0000672
59. Tay MYF, Fraser JE, Chan WKK, Moreland NJ, Rathore AP, Wang C, Vasudevan SG, Jans DA (2013) Nuclear localization of dengue virus (DENV) 1–4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin. Antivir Res 99(3):301–306. https://doi.org/10.1016/j.antiviral.2013.06.002
60. Teoh EP, Kukkaro P, Teo EW, Lim AP (2012) The structural basis for serotype-specific neutralization of dengue virus by a human antibody. Sci Transl 4(139):139ra183

61. Tricou V, Minh NN, Van TP, Lee SJ, Farrar J, Wills B, Tran HT, Simmons CP (2010) A randomized controlled trial of chloroquine for the treatment of dengue in Vietnamese adults. PLoS Negl Trop Dis 4(8):e785. https://doi.org/10.1371/journal.pntd.0000785
62. van Cleef KWR, Overheul GJ, Thomassen MC, Kaptein SJF, Davidson AD, Jacobs M, Neyts J, van Kuppeveld FJM, van Rij RP (2013) Identification of a new dengue virus inhibitor that targets the viral NS4B protein and restricts genomic RNA replication. Antivir Res 99(2):165–171. https://doi.org/10.1016/j.antiviral.2013.05.011
63. Vannice KS, Durbin A, Hombach J (2016) Status of vaccine research and development of vaccines for dengue. Vaccine 34(26):2934–2938. https://doi.org/10.1016/j.vaccine.2015.12.073
64. Wagstaff KM, Sivakumaran H, Heaton SM, Harrich D, Jans D a (2012) Ivermectin is a specific inhibitor of importin α/β-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. Biochem J 443(3):851–856. https://doi.org/10.1042/BJ20120150
65. Wang QY, Dong H, Zou B, Karuna R, Wan KF, Zou J, Susila A, Yip A, Shan C, Yeo KL, Xu H, Ding M, Chan WL, Gu F, Seah PG, Liu W, Lakshminarayana SB, Kang C, Lescar J, Blasco F, Smith PW, Shi PY (2015) Discovery of Dengue virus NS4B inhibitors. J Virol 89(16):8233–8244. https://doi.org/10.1128/JVI.00855-15
66. Warfield KL, Barnard DL, Enterlein SG, Smee DF, Khaliq M, Sampath A, Callahan MV, Ramstedt U, Day CW (2016a) The iminosugar UV-4 is a broad inhibitor of influenza A and B viruses ex vivo and in mice. Viruses 8(3). https://doi.org/10.3390/v8030071
67. Warfield KL, Plummer EM, Sayce AC, Alonzi DS, Tang W, Tyrrell BE, Hill ML, Caputo AT, Killingbeck SS, Beatty PR, Harris E, Iwaki R, Kinami K, Ide D, Kiappes JL, Kato A, Buck MD, King K, Eddy W, Khaliq M, Sampath A, Treston AM, Dwek RA, Enterlein SG, Miller JL, Zitzmann N, Ramstedt U, Shresta S (2016b) Inhibition of endoplasmic reticulum glucosidases is required for in vitro and in vivo dengue antiviral activity by the iminosugar UV-4. Antivir Res 129:93–98. https://doi.org/10.1016/j.antiviral.2016.03.001
68. Watanabe S, Rathore APS, Sung C, Lu F, Khoo YM, Connolly J, Low J, Ooi EE, Lee HS, Vasudevan SG (2012a) Dose- and schedule-dependent protective efficacy of celgosivir in a lethal mouse model for dengue virus infection informs dosing regimen for a proof of concept clinical trial. Antivir Res 96(1):32–35. https://doi.org/10.1016/j.antiviral.2012.07.008
69. Watanabe S, Tan KH, Rathore APS, Rozen-Gagnon K, Shuai W, Ruedl C, Vasudevan SG (2012b) The magnitude of dengue virus NS1 protein secretion is strain dependent and does not correlate with severe pathologies in the mouse infection model. J Virol 86(10):5508–5514. https://doi.org/10.1128/JVI.07081-11
70. Watanabe S, Chan KWK, Dow G, Ooi EE, Low JG, Vasudevan SG (2016) Optimizing celgosivir therapy in mouse models of dengue virus infection of serotypes 1 and 2: the search for a window for potential therapeutic efficacy. Antivir Res 127:10–19. https://doi.org/10.1016/j.antiviral.2015.12.008
71. Whitehorn J, Nguyen CVV, Khanh LP, Kien DTH, Quyen NTH, Tran NTT, Hang NT, Truong NT, Hue Tai LT, Cam Huong NT, Nhon VT, Van Tram T, Farrar J, Wolbers M, Simmons CP, Wills B (2016) Lovastatin for the treatment of adult patients with dengue: a randomized, double-blind, placebo-controlled trial. Clin Infect Dis 62(4):468–476. https://doi.org/10.1093/cid/civ949
72. Xie X, Wang Q-Y, Xu HY, Qing M, Kramer L, Yuan Z, Shi P-Y (2011) Inhibition of dengue virus by targeting viral NS4B protein. J Virol 85(21):11183–11195. https://doi.org/10.1128/JVI.05468-11
73. Xie X, Zou J, Wang QY, Shi PY (2015) Targeting dengue virus NS4B protein for drug discovery. Antiviral Res 118:39–45. https://doi.org/10.1016/j.antiviral.2015.03.007
74. Xu M, Lee EM, Wen Z, Cheng Y, Huang W-K, Qian X, Tcw J, Kouznetsova J, Ogden SC, Hammack C, Jacob F, Nguyen HN, Itkin M, Hanna C, Shinn P, Allen C, Michael SG, Simeonov A, Huang W, Christian KM, Goate A, Brennand KJ, Huang R, Xia M, Ming G-l, Zheng W, Song H, Tang H (2016) Identification of small-molecule inhibitors of Zika virus infection and induced neural cell death via a drug repurposing screen. Nat Med 22(10):1101–1107. https://doi.org/10.1038/nm.4184
75. Yauch LE, Shresta S (2008) Mouse models of dengue virus infection and disease. Antiviral Res 80(2):87–93. https://doi.org/10.1016/j.antiviral.2008.06.010
76. Yin Z, Chen YL, Schul W, Wang QY, Gu F, Duraiswamy J, Kondreddi RR, Niyomrattanakit P, Lakshminarayana SB, Goh A, Xu HY, Liu W, Liu B, Lim JY, Ng CY, Qing M, Lim CC, Yip A, Wang G, Chan WL, Tan HP, Lin K, Zhang B, Zou G, Bernard KA, Garrett C, Beltz K, Dong M, Weaver M, He H, Pichota A, Dartois V, Keller TH, Shi PY (2009) An adenosine nucleoside inhibitor of dengue virus. Proc Natl Acad Sci U S A 106(48):20435–20439. https://doi.org/10.1073/pnas.0907010106



23 An Industry Perspective on Dengue Drug Discovery and Development

Ilane Hernandez-Morales and Marnix Van Loock

Abstract

Dengue is the most important mosquito-borne viral disease in the world, representing a major unmet medical need and a growing public health concern. The disease imposes a heavy burden to the affected individuals, to the health care systems, and to the economies of endemic countries. Vector control is the most widespread tool to curb dengue epidemics, but has been insufficient. Therefore, additional means such as vaccines and antivirals are required to aid in a coordinated response. The discovery and development of small molecule dengue virus inhibitors as a tool to prevent and/or treat dengue disease faces major hurdles in combining pan-serotypic efficacy, safety, and optimal drug-like properties. Moreover, the financial return of dengue drug projects may not compensate for the initial investment in research and development. This review article addresses the efforts undertaken to face the dengue epidemics, focusing on antiviral drug development. The dengue drug research and development process is described in detail and a dengue antiviral target product profile is proposed. The article discusses collaborations between the different players in the research field: government and government-sponsored organizations, pharmaceutical and biotechnology companies, academia, and non-profit and philanthropic organizations. Public-private partnerships are proposed as a model to boost dengue research and development towards an approved antiviral drug in the near future.

Keywords

Dengue antiviral drug development · Global funding for dengue R&D · Public-private partnerships

I. Hernandez-Morales
Janssen Pharmaceutica NV, Janssen Pharmaceutical Companies of Johnson & Johnson, Beerse, Belgium

Laboratory of Virology and Chemotherapy, Rega Institute for Medical Research, University of Leuven (KU Leuven), Leuven, Belgium

M. Van Loock (✉)
Janssen Research & Development, Janssen Pharmaceutica NV, Janssen Pharmaceutical Companies of Johnson & Johnson, Beerse, Belgium

Abbreviations

DENV	dengue virus
DVI	Dengue Vaccine Initiative
PPP	private public partnership

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_23

R&D Research and Development
SAR Structure Relationship Activity
WHO World Health Organization

23.1 Dengue: From a Neglected Tropical Disease to a Global Health Concern

Dengue disease is caused by dengue virus (DENV), which belongs to the *Flavivirus* genus in the family of the *Flaviviridae*. The virus is transmitted by mosquitoes of the genus *Aedes*, with *Aedes aegypti* being the primary and *Aedes albopictus* the secondary vector [14]. In 2009, the World Health Organization (WHO) ranked dengue as the most important mosquito-borne viral disease in the world [104]. In the last 50 years, the incidence of DENV increased 30-fold [103]; not only the number of cases and outbreaks increased, but the virus distribution expanded to new regions making dengue disease a major public health concern.

DENVs encompass four antigenically and phylogenetically distinct genotypes, DENV-1 to DENV-4 [12]. The first reports on DENV occurred in French Polynesia and Japan for DENV-1 (1943), Papua New Guinea and Indonesia for DENV-2 (1944), and the Philippines and Thailand for DENV-3 and DENV-4 (1953). Currently, the four serotypes have a global distribution, co-circulate in the dengue-endemic tropic and sub-tropic regions of the world and each can induce the full spectrum of disease [61].

Four billion people, living in more than 128 countries, are at risk of becoming infected with DENV [8, 104]. Globally, there are approximately 390 million DENV infections each year (95% confidence interval [CI]: 284–528 million) [8]. The majority of these DENV infections are asymptomatic (~75%), yet the exact incidence of symptomatic dengue is uncertain due to underreporting and lack of specific diagnostics in resource-poor settings [104]. Bhatt *et al*. estimated a total of 96 million of dengue clinical cases in 2010 (95% CI: 67–136 million) [8]. More recently, based on an analysis of data from 141 endemic countries, Shepard et al. [87] estimated that 58.4 million symptomatic cases occurred in 2013 (95% CI: 23·6 –121·9 million) [87]. Both estimates illustrate the high incidence of dengue disease and reflect the need to improve epidemiological surveillance systems. During 2015, the WHO member regions of the Americas, South-East Asia, and Western Pacific reported 3.2 million dengue disease cases, which is 1 million more than reported in 2010 [105]. The exponential growth in the number of dengue disease cases reflects the global spread of the mosquito vectors to new regions, driven by climate change, human population growth, rapid urbanization, and increased international trade and travel [39]. For instance, during the last decade, autochthonous transmission of DENV occurred for the first time in France and Croatia (2010) [37, 90], and on the island of Madeira (2012) [56]. In addition, dengue disease has re-emerged after many years of absence in regions like Japan and Florida (USA) [79, 91].

The first clinical symptoms of a DENV infection, usually an abrupt onset of high fever, appear between 3 and 8 days after a DENV-positive mosquito bite. Most patients develop dengue fever (DF), an acute, self-limiting, debilitating, febrile illness that resolves within 4–7 days due to a robust innate and adaptive immune response [101]. However, a small number of the DENV infections (<1%) results in life-threatening dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), characterized by vascular leakage [59]. These severe dengue disease cases are accountable for approximately 500,000 hospitalisations every year [89]. The case fatality rate for DF cases is <1% [103], but for severe dengue disease (DHF/DSS) it can reach up to 5% [69]. Children are more susceptible to severe dengue disease and in some Asian countries (i.e., Indonesia, Myanmar, Thailand, and Timor-Leste) it represents the leading cause of death among patients <15 years of age [103, 105]. The risk of developing DHF/DSS increases upon a secondary DENV infection with a serotype different from the primary infection, due to a process called antibody-dependent enhancement (ADE) (reviewed in [62]). Therefore, it is critical that

vaccines or antivirals are equipotent against all four serotypes of DENV.

Dengue disease outbreaks represent a heavy burden not only to the affected individuals, but also on the health care systems and economies of endemic countries. The years lost due to disability caused by dengue disease account for 1.9 million disability-adjusted life-years (DALYs) in 2015 [17]. Based on morbidity, Dengue is the tenth most important neglected tropical disease, above African trypanosomiasis and Chagas disease [17, 43]. In addition, Dengue is the tenth cause of mortality from the neglected tropical diseases, above helminth infections [17]. In terms of expenses due to hospitalization and maintenance treatment, Shepard et al. [87] estimated that in 2013, when approximately 58.4 million symptomatic cases occurred, the global cost raised to US$ 8.9 billion (95% CI: US$ 3.7 billion – US$ 19.7 billion). The estimated costs related to specific disease outcomes were: US$ 4093 million for hospitalized nonfatal cases, US$ 2987 million for ambulatory cases, US$ 1055 million for fatal cases, and US$ 752 million nonmedical cases. In total, the cost for dengue disease is higher than the cost for other infectious diseases such as Chagas (US$ 7 billion), cholera (US$ 3 billion), and rotavirus gastroenteritis (US$ 2 billion) [87]. Several studies estimated the cost of dengue illness per region or country and accounted for ambulatory cases, hospitalized cases, and deaths. “For instance” in the Americas, the annual average cost estimated due to dengue illness was US$ 2.1 billion, from 2000 to 2007 (ranging from US$ 1 to 4 billion with substantial year-to-year variation), for which 73.7% of the cost corresponded to ambulatory cases, 23.6% to hospitalized cases, and 2.7% to deaths [84]. Estimates for South East Asia account for an annual average economic burden of US$ 950 million (95% CI: US$ 610 million – US$ 1384 million) between 2001 and 2010 [86]. However, India alone reported that the cost of dengue disease raised up to US$ 1.11 billion from 2006 to 2012 [85]. For the Western Pacific region, studies in the Philippines estimate an annual average of US$ 345 million from 2008 to 2012 [29], an annual average of US$ 41,5 million in Singapore [13], and US$ 3–14 million in Cambodia during 2007 and 2008 (the highest cost was recorded during a large epidemic in 2007) [6]. The majority of dengue outbreaks affect low- or middle-income countries. In addition, the costs related to vector control increase the total economic burden [5]. Reducing the number of dengue disease cases and potentially halting dengue outbreaks would reduce significantly the associated health, social, and economic burden.

Both the expansion of dengue epidemics and the impact of the disease triggered a response of the international community, coordinated by the WHO, promoting the development, establishment, and improvement of effective tools to face the epidemics. Several approaches were part of this initiative including vaccine development, vector control, and funding for research and development (R&D). In 2002, the World Health Assembly resolution WHA55.17 urged the WHO and the member states to increase the response to DENV. Later on, the 2005 World Health Assembly resolution WHA58.3 included dengue as ‘a disease that would constitute a public health emergency of international concern’ [75]. The WHO recommended vector control and vaccine development to diminish DENV epidemics. Mosquito-control measures are still the primary method for the prevention and control of DENV infections, but have not yet proven to be effective in preventing and controlling dengue disease outbreaks [11]. In December 2015, the first vaccine against DENV was licensed. CYD-TDV (Dengvaxia®) is a tetravalent live-attenuated vaccine developed by Sanofi Pasteur. However, the immediate widespread use of the vaccine is not foreseen according to the WHO working group for immunization, as a number of factors need further consideration [106].

The increased awareness of dengue epidemics has been accompanied by a steady investment in R&D in the past decade (Fig. 23.1). During 2016 the total spending in DENV R&D reached US$ 113 million (excluding funding for dengue vaccine R&D). The dengue investment accounted for 4% of the global funding for neglected tropical diseases in 2016 [17]. Nearly half of the funding has been allocated to basic research since 2007, followed by drug development, and vector

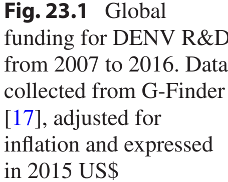
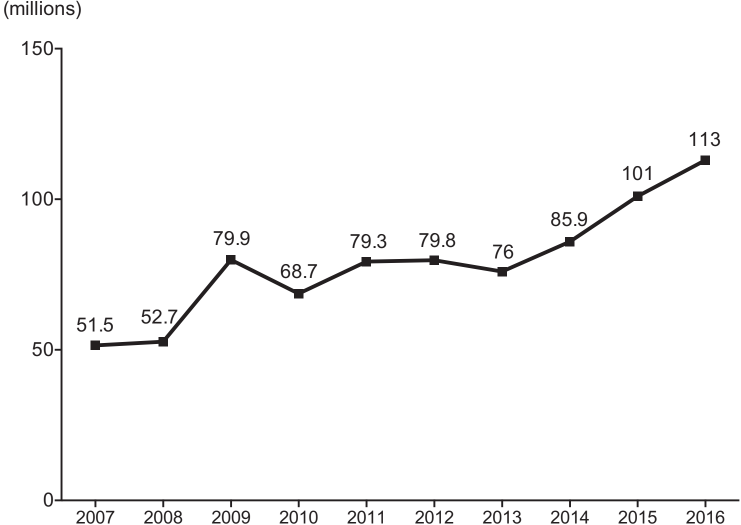


Fig. 23.1 Global funding for DENV R&D from 2007 to 2016. Data collected from G-Finder [17], adjusted for inflation and expressed in 2015 US$



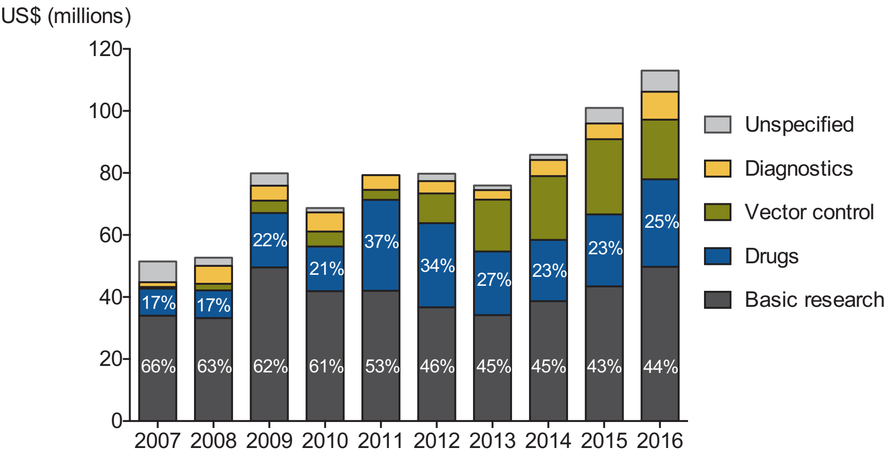


Fig. 23.2 DENV funding for R&D by product type from 2007 to 2016
Data collected from G-Finder [17], adjusted for inflation and expressed in 2016 US$. Percentages are indicated from the total investment for each year

control (Fig. 23.2) [17]. The funding for basic research has been decreasing, whereas the funding for drug research and development has been variable but with an overall decreasing trend in the last years.

Overall, DENV is recognized as a global public health concern and despite the increased awareness and investments during the past decade, dengue epidemics are increasing and the disease is expanding to new geographical regions [42]. Consequently, an integrated approach of vaccines, small molecule antivirals, vector control, and diagnostics is required to prevent, cure, and even eradicate dengue.

23.2 Dengue Vaccines and Antivirals

Until now, vector control is the most widespread tool for restraining dengue epidemics. However, vector control campaigns are not sufficient to completely curb outbreaks of the disease [11]. Therefore, other means such as vaccines and antivirals are needed to aid in a coordinated response.

During the last two decades, vaccine development received the majority of dengue R&D funding (71% in 2012) [63]. This effort resulted in the first marketed dengue vaccine, Dengvaxia®, developed by Sanofi-Pasteur, while several other vaccine candidates are in clinical development [27, 71, 81]. The FDA approved Dengvaxia® in December 2015 and since then, 11 countries licensed the vaccine. Currently, the Strategic Advisory Group of Experts (SAGE) recommended countries to consider introduction of Dengvaxia® only in geographic settings (national or subnational) with high endemicity, as indicated by seroprevalence of approximately 70% or greater in the age group targeted for vaccination [76, 106].

Dengue drug R&D progressed during the last decade, where several antiviral targets showed potential for antiviral intervention [52, 55, 77]. Although there are no approved small molecule inhibitors yet, the landscape of drugs at various stages of development is growing [55]. Currently, there are five small molecules – all targeting host proteins – in clinical development for the treatment of dengue disease: modipafant, ketotifen, ivermectin, celgosivir, and UV4. Modipafant and ketotifen are disease-modifying agents whose therapeutic target is preventing or diminishing severe dengue disease [20, 21, 88]. Ivermectin is an antiparasitic drug with demonstrated antiviral activity against DENV [18]. Celgosivir and UV4 are α-glucosidase inhibitors that target host-mediated post-translational modifications needed for viral assembly or secretion [16, 19, 20, 57, 73, 95]. The outcome of the ongoing clinical trials might bring us closer to a solution for the unmet medical need, or will at least define the direction of dengue drug R&D in terms of future clinical trials to assess the antiviral molecules currently in pre-clinical development [47, 98].

The pre-clinical pipeline of dengue small molecule inhibitors is diverse and includes both viral targets (reviewed in Lim et al. [55]) and host targets (reviewed in Krishnan and Garcia-Blanco [52]). DENV proteins for which small molecule inhibitors have been identified include: the capsid, the envelope, the NS2B-NS3 protease, the NS3 helicase, the NS5 methyl-transferase, the NS5 RNA-dependent RNA polymerase (reviewed in Lim et al. [55]), and NS4B (reviewed in Xie et al. [110] and Zmurko et al. [112]). In addition, several host proteins or enzymes participating in different cellular processes have demonstrated target potential for antiviral intervention (reviewed in Krishnan and Garcia-Blanco [52]). The further development of the pre-clinical candidates faces important hurdles such as potential lack of *in vivo* antiviral activity or toxicity in pre-clinical animal models [55]. However, screening campaigns and medicinal chemistry efforts are ongoing, and promising compounds targeting the capsid, NS4B, and polymerase are likely to enter clinical development in the coming years [66].

During recent years, prophylaxis with a small molecule has gained increased attention as a complementary approach to vaccines [100]. A prophylactic dengue antiviral would be beneficial for travellers to dengue-endemic regions (e.g., aid workers, tourists, business and military personnel, and those visiting friends and family) as well as for vulnerable populations living in dengue-endemic areas. The concept relies on the premise that the compound would be present in the body prior to the peak viral load. By inhibiting viral replication, the small molecule DENV inhibitor would inhibit completely or at least blunt viral load, and therefore dengue-associated morbidity and mortality could potentially be

reduced or even prevented [100]. An ideal dengue antiviral molecule should encompass efficacy, safety, and availability in resource-poor settings and should show prophylactic and therapeutic value (Box 23.1).

Box 23.1: Profile of an Ideal Dengue Antiviral Product

Description	Small-molecule inhibitor of DENV.
Efficacy	Potent, tetravalent antiviral activity.
Indications	Prophylactic and/or treatment indication for residents or travelers to endemic areas.
Safety and tolerability	Safe for adults and children, well-tolerated without significant side effects. Suitable to combine with supportive care drugs.
Dose	Oral, once daily dosing.
Formulation	Long-term stability for stockpiling and distribution.
Payer value	Reduced health care costs, hospitalization frequency and duration.

Adapted from Whitehorn et al. [100]

Boosting DENV inhibitor discovery will position more drug candidates into clinical development, hence increasing the probabilities to obtain approved dengue antiviral drugs [55, 67]. It is the aim of the following two sections to give an overview of the dengue R&D process and the importance of public-private partnerships (PPPs) to increase scientific innovation and decrease attrition rates during compound development.

23.3 The Roadmap of Dengue Drug Discovery and Development

The R&D roadmap towards new antiviral drugs follows a long and expensive process (Fig. 23.3), which, in the case of dengue, might result in antivirals for prophylactic and/or therapeutic intervention. In general, this process consist of three major steps: (i) basic research to increase the insight into the DENV biology and the pathogenesis of the disease, resulting in the potential identification of novel druggable targets, (ii) drug discovery to find or design new molecules that could lead to drug candidates, and (iii) drug development during which these drug candidates undergo rigorous testing to assess the safety and clinical proof of concept regarding efficacy [9, 44]. In this review, we will focus on drug discovery as an approach to increase the number of drug candidates entering clinical development.

Basic research aims at increasing the understanding of DENV biology regarding the replication cycle, pathogen-host interactions, and pathogenesis, and may be part of early discovery programs within pharmaceutical companies or academic institutions. This information is important to identify novel antiviral targets and to develop assays for target validation, drug screening, and mechanism of action studies. Druggable antiviral targets are viral or host components that can be modulated by a small molecule, which consequently interferes with viral replication [65, 70]. Upon the identification of a potential druggable target, thorough validation occurs in cell-based assays, enzymatic assays, structure-based *in silico* assays, and potentially *in vivo* assays [9, 44, 65]. During this phase of dengue drug discovery, academic research may contribute to the identification and validation of druggable targets as well as the development of appropriate *in vitro* assays and *in vivo* models. Likewise, academic teams may assemble viral panels of relevant clinical isolates that are to be used during the hit-to-lead and lead-optimization phase. Currently, validated viral targets include the non-structural (NS) proteins NS2B-NS3 protease, NS3 helicase, NS5 methyltransferase, NS5 RNA-dependent RNA polymerase [7, 55], and NS4B [110, 112], and the structural envelope and capsid proteins [55]. As mentioned above, also host components have been validated as druggable targets interfering with dengue replication [16, 18–21, 57, 73, 88, 95].

During the early discovery process, compound libraries are screened to identify hit compounds exhibiting antiviral activity. This process occurs

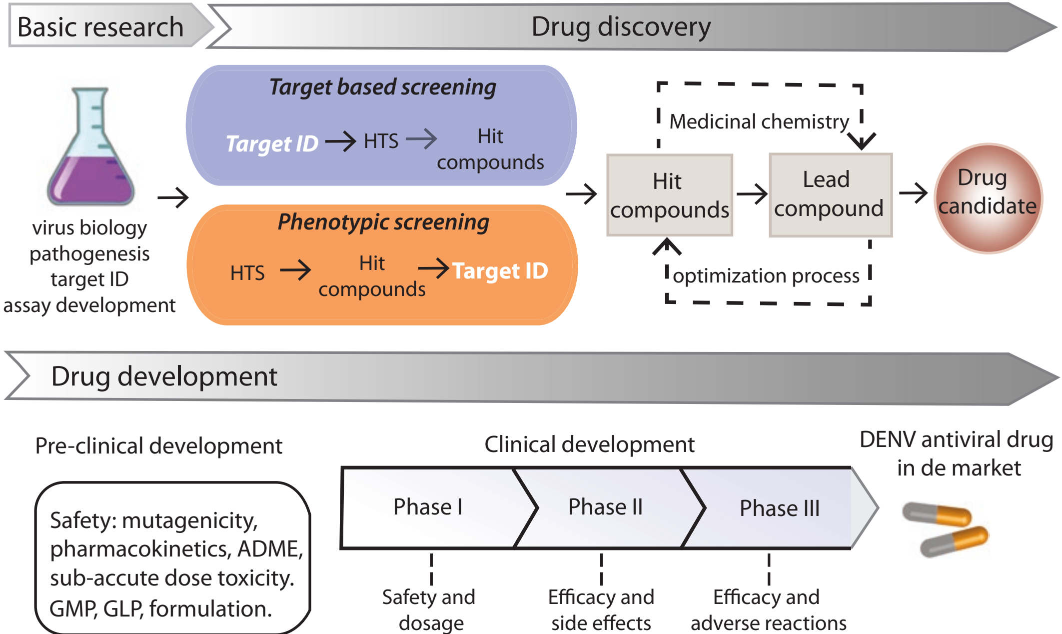


Fig. 23.3 The roadmap of dengue drug discovery and development
The dengue drug discovery and development roadmap encompasses three major steps. First, basic research increases the knowledge of the pathogen and the associated disease, enabling the identification of novel antiviral targets and the development of *in vitro* assays for drug discovery. Second, in an early discovery phase, screening of compound libraries takes place using either target-based or phenotypic screening, to identify initial, antivirally active hits. Next, the hit-to-lead phase includes optimization of hit compounds via structure-activity relationship (SAR) using an iterative process between medicinal chemistry and antiviral testing to identify a drug candidate. Prior to entering the clinical development, the drug candidate is extensively tested at the pre-clinical stage to address compound safety. In parallel, capacity is built to produce the compound at large scale, with optimal production standards. The clinical development phase constitutes the most time consuming and expensive part of the process where the drug candidate undergoes three phases of clinical testing. Finally, after approval by regulatory agencies, the process results in a drug available for the patients in need
HTS: high-throughput screening; target ID: target identification; ADME: absorption, distribution, metabolism, and excretion; GMP: good manufacturing practices; GLP: good laboratory practices

mainly via two approaches: (i) target-based screening and (ii) phenotypic screening.

In general, target-based drug discovery starts from a potential druggable target in a biochemical assay in which chemical libraries, computer or laboratory based, are evaluated to select hit compounds (Fig. 23.3) [9]. In this approach a target is any protein, e.g., enzymes or receptors, whose activity can be measured quantitatively [9, 44]. For DENV, druggable enzymatic targets include the NS2B-NS3 protease, the NS3 helicase, the NS5 methyl-transferase, and the NS5 RNA-dependent RNA polymerase [7, 55]. Recombinantly expressed enzymes can be used for such screenings. As such, a target-based screening requires a specific *in vitro* assay that enables medium- to high-throughput screening [45]. In addition, screening for molecules active against a known target enables computational structure-based design and *in silico* screening [30, 45]. Another advantage is that structure-activity relationship (SAR) optimization can be directly performed using the *in vitro* assay used for screening. Finally, a known target facilitates the deconvolution of a compound's mechanism of action. A downside from this approach is that a biochemical assay, using recombinant proteins, does not take into account aspects such as the fact that compounds need to enter the host cell at sufficiently high concentrations and should, at the same time, not be

toxic to the host cell. As a consequence, many compounds identified in target-based screens do not elicit activity when their effect is explored in cellular assays. In addition, target-based screening often reduces the chemical diversity of the compound series, compromising the discovery process success [45, 111].

In the phenotypic screening approach, chemical compounds are evaluated, without prior knowledge of a specific target, for their antiviral activity in cell-based assays during high-throughput screening campaigns (Fig. 23.3) [44, 65]; such approaches were facilitated by the development of dengue reporter replicons and reporter viruses [34, 113]. The cell-based assays are mostly viral-replication-based and provide a holistic approach to identify novel compounds and targets [65]. For instance, the DENV NS4B protein is a target identified from phenotypic screening [110, 112]. Phenotypic screenings constitute a more physiologically relevant environment where the compound interacts with cellular membranes and cellular components to exert its function. Therefore, these assays produce important information on the *in vitro* absorption, solubility, and toxicity of the compounds, as compared to enzymatic assays. However, screening exercises for DENV typically utilize Vero (green African monkey kidney cells), A549 (human lung carcinoma cells), BHK (baby hamster kidney cells), and Huh7 (human hepatoma cells) cells, which are not the primary cell target for DENV infection. Primary human cell cultures of mononuclear cells (monocytes, dendritic cells, and macrophages) represent a more relevant *in vitro* model of disease [78, 82], the throughput in assay systems based on these cells is too low for screening purposes. Nevertheless, during the development of a certain class of DENV inhibitors, the activity of molecules should be assessed in these more relevant cultures as well. Downsides of phenotypic screening may be the cost, and the need to develop more specific assays during the compound profiling stage to identify the target, to elucidate the mechanism of action, and to optimize the SAR [111].

Regardless of the screening approach, compound libraries represent the starting point and the composition of the library is a critical factor to select hit compounds during the screening process. Compound libraries incorporate so-called "drug-like molecules" that are low molecular weight molecules (<500 Dalton) that (typically) fulfill the five principles of Lipinski [44, 54]. Libraries are either commercially available or, often, pharmaceutical companies have built internal libraries [9]. These libraries can also exist virtually, where chemical entities are designed and screened *in silico* [30].

Hit compounds must show antiviral activity (single-digit micromolar activity or less), low cellular toxicity, and belong to different chemical clusters to allow further SAR improvement [44].

The hit compounds then advance into the hit-to-lead optimization phase, where they undergo structural modifications to create analogues that are subsequently tested for improved antiviral activity in the absence of cytotoxicity (Fig. 23.3). This is an iterative process to achieve a suitable SAR. When the structure of the target and if possible the structure of the target in complex with the inhibitor are available, computational medicinal chemistry may aid the optimization of hit compounds.

Finally, from thousands or even millions of initial chemical compounds one or a few lead compounds are selected as drug candidates that will advance into the development process. The lead compound(s) must show: efficacy *in vitro* and *in vivo*, high antiviral potency (ideally <1 μM activity in cellular assays), no apparent toxicity, high selectivity for the target, desirable physicochemical properties that support good absorption, distribution, metabolism, and excretion (ADME), and suitable pharmacokinetics (PK) and pharmacodynamics (PD) [41, 48]. Specifically for dengue, the lead compound should also show pan-serotypic coverage against a panel of clinical isolates.

The lead compound advances to pre-clinical development where further evaluations will generate detailed information about toxicity, dosing, mutagenicity, ADME profile, PK, and PD [68].

Next, approval for registration of a drug candidate to further clinical trials in humans is required. In order to register an investigational new drug

(IND), the candidate must fulfil the following requirements: acceptable stability and formulation, optimal ADME profile, no systemic and organ toxicity evaluated in at least two different animal species, no observed adverse effects (within the therapeutic dose), initial data on lack of cardiotoxicity and genotoxicity, proven mechanism of action, and initial plan for conducting clinical trials [33, 41].

The approved drug candidate then undergoes clinical testing in human subjects, which encompasses three phases: (i) evaluation of safety and dosage in healthy individuals, (ii) evaluation of efficacy and potential side effects in infected patients, and (iii) large-scale evaluation of efficacy and potential adverse reactions in infected patients [33]. The latter clinical phases are the most time consuming and expensive of the entire development process [72].

The drug discovery and development process is costly, the development of one drug is estimated to be US$ 2.6 billion [3, 25]. The time from discovery to final approval of one drug may take from 8 up to 20 years with an average of 13.5 years [24, 72]. Moreover, the probability of technical and regulatory success is only 4.1% [72]. For neglected tropical diseases, the scenario is even more challenging because the financial return may not compensate for the initial investment in R&D programs [92]. However, certain mechanisms offer incentives to the industry to register new treatments for neglected tropical diseases –including dengue; for instance the FDA grants, upon approval, a priority review voucher that allows a fast-track revision for approval of a new molecular entity [32].

In addition, there are specific challenges to position a dengue antiviral drug in the market. During early drug discovery it is important to select compounds, from different chemical series, showing tetravalent serotype coverage [7, 100]. Compounds active against all four DENV genotypes are challenging to obtain, as the four serotypes have only 70% sequence homology; the challenge is comparable to developing an antiviral compound active against all six HCV genotypes [55]. Though more relevant *in vitro* cell cultures for compound screening may increase the possibility to select potent compounds with tetravalent serotype coverage [26].

Furthermore, despite the currently available knowledge of dengue disease, it is important to better understand its pathogenesis. Gaining insight in the factors which may drive the pathogenesis to severe dengue can improve predictability in clinical trials [47]. *In vivo* models, mostly mice studies, have greatly contributed to the study of dengue pathogenesis and have enabled the assessment of the potential efficacy of antiviral compounds. Yet, there is still a need for an animal model that encompasses all the aspects of the disease in humans [15].

Ultimately the therapeutic efficacy in patients must be evaluated in humans [38, 99], as failed clinical trials with, e.g., balapiravir and lovastatin, have shown that there is currently little understanding of the predictive power of preclinical models towards the human situation [47]. Improving viral kinetic modelling will increase the understanding of the therapeutic window and assess the feasibility to intervene successfully with dengue antivirals [60].

Equally important is the modelling of epidemiological data to support the understanding on how the virus circulates and persists in different populations and regions [40, 50]. In addition, a strongly reliable diagnostic platform enabling early-on detection of DENV infection is essential to control dengue outbreaks and determine the timely implementation of an antiviral intervention [104].

These challenges may be covered with dedicated PPPs that reinforce different aspects of dengue drug R&D, as exemplified by the malaria partnerships [67].

23.4 Public-Private Partnership Initiatives for Dengue: Progress, Challenges, and Opportunities

After decades of intense dengue research, there is still no DENV-specific small molecule antiviral medicine on the market. The handful of drugs that did advance to clinical development were repurposed from other medical indications, e.g.,

boceprevir, lovastatin, or prednisolone [38, 74]. Yet, these compounds failed to show efficacy in DENV-infected patients. Therefore, it is crucial to fuel the drug development pipeline by boosting the discovery of new drug candidates [2, 4, 22, 23, 68].

From a pharmaceutical industry perspective, development of dengue antivirals is hampered by two main challenges. On one hand there are the limited incentives of investing in drugs that do not offer sufficient financial returns [92]; on the other hand there are the high attrition rates, translating into low efficiency and lack of innovation [80, 83]. To address these issues, an orchestrated response from different stakeholders in the public and private sector is required. A popular model to organize such collaborative responses is a PPP.

PPPs were developed to establish collaborations between the public and private sector to implement projects or provide social services, and are wide spread across many sectors such as infrastructure, car industry, health care, etc. [4]. PPPs for health care are especially important to combat neglected tropical diseases and rare diseases, which do not offer enough economic incentives to big private investors [68, 92]. Such PPPs bring together expertise and resources of the participants at different R&D stages to move medicines from the 'bench to the bedside' [4, 36 23, 108]. Three sectors participate in health care PPPs: (i) public: government and government-sponsored organizations such as the WHO; (ii) for-profit: pharmaceutical industry, biotechnology companies, etc.; (iii) civil society: academia, non-profit and philanthropic organizations [108]. The characteristics, benefits, and risks of PPPs for health care are listed in Box 23.2.

PPPs dedicated to solve health-related issues do so in different ventures: product development, improvement of product distribution and accessibility, strengthening health care services, public health education, improvement of product quality or regulation, and coordination of multifaceted efforts [46, 107, 108]. In this review we will focus on product development PPPs, which are the majority of currently active PPPs in the healthcare field [22, 108].

Box 23.2: PPPs for Health Care

Characteristics for successful partnerships:

- Share a mission
- Establish clear and realistic objectives
- Define clear roles and responsibilities
- Allow equal power for decision making
- Promote mutual, but distinct benefits for each partner
- Shared risk associated to the project
- Accomplish timelines for deliverables.
- Promote equal participation among the partners
- Maintain an active collaboration
- Ensure transparency and accountability
- Bound by a clear legal and regulatory framework

Potential benefits:

- Introduce state of the art technology and research
- Stimulate participation of pharmaceutical industry in neglected tropical diseases
- Enhance the development of local capabilities in endemic, developing countries
- Attract investment and funding for neglected tropical diseases

Potential risks:

- Conflict of interest for intellectual property
- Exceed allocated budget
- Unforeseen situations not covered in the legal agreement

Source: Widdus [107, 108], Austin [2], Bagley [4], Croft [22], and Demotes-Mainard [23]

Product development PPPs, aiming at bringing novel solutions to patients, apply the so-called 'portfolio' approach (adopted from the industry), which relies on the development of several candidates in parallel to manage the risk of failure associated with individual products [22]. A specific example for dengue disease is the Dengue Vaccine Initiative (DVI) that has several vaccine candidates in its portfolio, and which recently resulted in the licensing of the first dengue vaccine [27, 71, 81]. The DVI works in association with governments, industries, and scientific and medical communities to consolidate concrete actions for vaccine development. For instance, it accelerates the progression of vaccine candidates, evaluates the degree of underreporting in endemic areas and, together with the WHO, develops criteria for the clinical evaluation of vaccine candidates [27].

For dengue drug development, there is no PPP as broadly organized as the DVI. In 2013, the Partnership for Dengue Control (PDC) was established to 'create synergies among the many new and innovative tools in the development pipeline to ensure the most effective control strategies' for dengue (www.controldengue.org). The PDC includes dengue antiviral drugs in its toolbox for effective control, however, its focus is on vector control, vaccine development, and epidemiological surveillance [35].

Drug development PPPs have in common that they have to move drug candidates towards the market in an efficient and timely manner. In the field of neglected tropical diseases, the Medicines for Malaria Venture (MMV) is the largest PPP dedicated to develop products, including new medicines (www.mmv.org). MMV has successfully advanced into a first of a kind, open-source drug discovery campaign where 400 compounds with reported activity against malaria were distributed free of charge to research laboratories for their evaluation. The results suggested the mechanism of action for 135 compounds and helped to prioritize the most promising drug candidates against malaria for further development. In addition, the screening campaign identified compounds active against other medical indications, e.g., infectious diseases and even cancer. As a result, more than 30 new drug follow-on programs for different indications were initiated (reviewed in [94]).

MMV has embraced an open environment of collaboration based on four pillars: open science, open source, open innovation, and open access [97].

Open science refers to making primary data publicly available, which may include DNA and protein sequences, crystallography structures, and should include cross-validated data from biological assays [94, 97]. For dengue specifically, differences in laboratory procedures and resources, such as cell types and DENV strains, may vary considerably among labs. Therefore, an open cross validation of *in vitro* and *in vivo* assays, conducted within the scope of open science, would provide reliable information for clear decision-making. Moreover, harmonization in testing protocols offers opportunities for pre-competitive collaborations. Examples of open science include the various drug-like small molecule databases, such as Drug Bank, GDB-13 [10, 109], and the TDR targets database [58].

Open innovation involves a higher degree of coordination where partners share information under confidentially agreements. This is the case for MMV that shares validated assays to new partners that want to engage in screening campaigns. Other examples include the (Eli) Lilly Phenotypic Drug Discovery Initiative (https://openinnovation.lilly.com), the GlaxoSmithKline clinical trial data platform (www.gsk-clinical-studyregister.com), the Structural Genomics Consortium (www.thesgc.org), and the Single Nucleotide Polymorphism Database (dbSNP) (http://www.ncbi.nlm.nih.gov/projects/SNP/) [97].

Open access refers to making scientific publications available to the public. In drug discovery, this involves exchanging information regarding novel targets, novel compounds, and phenotypic screenings, as such attempting to boost R&D capabilities in endemic countries. In 2015, an WHO consultation of international stakeholders signed a consensus statement to enhance and engage in a timely and transparent open access (pre) publication of research findings during pub-

lic health emergencies [96]. For instance, during the Zika outbreak, open access facilitated the rapid understanding of the virus pathogenesis and accelerated decision making [28, 51].

Open source is a model where the wider community is invited to participate, and where each partner offers its particular expertise and/or resources. This participation could include advice, work force (e.g., student internships), chemical synthesis, and screening. Such an effort needs continuing financial support, good communications for example via easy to use online interfaces, and strong leadership and guidance [97]. The Open Source Malaria (http://opensourcemalaria.org/) platform is an example of this type of open collaboration.

Existing dengue partnerships integrate some principles of these four models to promote open collaboration projects. Current dengue partnerships, not related to vaccines, and their collaborators are enlisted in Table 23.1.

Pharmaceutical companies have established several initiatives to provide medical solutions to neglected tropical diseases. The Global Public Health (GPH) initiative from Johnson & Johnson was created in late 2015 to generate healthier futures for the most vulnerable populations in the developing world (www.jnj.com/global-public-health). The growing GPH R&D portfolio is focusing on HIV/AIDS, tuberculosis, vaccines development (HIV, Ebola), and other neglected tropical diseases such as soil-transmitted helminthiasis, leprosy, chagas, malaria, and dengue. In addition, GPH is focussing on novel technology platforms, e.g., long acting. Through this global strategy, Johnson & Johnson will mobilize its global public health capabilities and resources behind a unified, multidisciplinary effort to deliver a sustainable, measurable impact. This model is based on innovation, collaboration, and local empowerment. The project includes partnerships with governments, donors, non-profits, and multilateral institutions to develop and deliver integrated, evidence-based solutions for public health.

Another initiative is the Novartis Institute of Tropical Diseases (NITD) that is dedicated to small-molecule drug discovery research for the treatment of infectious tropical diseases such as dengue, human African trypanosomiasis, and malaria (https://www.nibr.com/our-research/institutes/novartis-institute-tropical-diseases). Regarding dengue antiviral drug research NITD has worked on characterizing the NS4B target, establishing animal models, defining structure and function of viral proteins, and developing screening assays [55]. NITD funding partners are Novartis and the Singapore Economic Development board.

In 2016, the investment in DENV R&D originated from public sector (60%) from high-income countries (academia, government programs, and grants), followed by philanthropic associations (18%), mostly form the Gated Foundation, and industry (13%), e.g., multinational pharmaceutical companies [17]. In general, for drug discovery, the majority of biology-related knowledge comes from academia, whereas most of the chemistry technology and resources come from the industry. Unfortunately, the interaction between academia and the industry is precarious due to the competitive intellectual property environment, which dominates both sides [94]. Hence to ensure open-science collaborations, it is imperative to understand the benefits associated with it and to accept the intellectual property responsibilities.

The top five funders of dengue R&D in 2016 were, in order of the highest investment to the lowest: (i) the US NIH, (ii) the pharmaceutical industry, (iii) the Gates Foundation, (iv) the Welcome Trust, and (v) the Indian Council of Medical Research (ICMR) (the complete list can be retrieved in G-Finder) [17]. These organizations are actively supporting dengue projects and offer funding opportunities.

The US NIH is currently funding nearly .60 dengue research projects encompassing: community-based prevention programs, surveillance programs based on laboratory-confirmed cases, the development of better and faster diagnostic tools, antiviral therapies, and vaccine development [64].

The Gates foundation and Wellcome Trust are the major philanthropic organizations supporting R&D for neglected tropical diseases. The Gates Foundations has its major focus on vector control

Table 23.1 Dengue partnerships and initiatives

Name	Focus	Funding partners	Operating partners	Website	Status
Break Dengue	An open platform to connect different initiatives around the world that are addressing the issue of dengue so that together they can have a bigger impact.	Multi-stakeholder partnerships: Sanofi-Pasteur, Bayer Crop Sciences, Health Map, Malaria consortium, Americas Health foundation, The Global Health Network, The International Society for Neglected Diseases, John Snow Labs.		http://breakdengue.wpengine.com/break-dengue/	Ongoing
Partnership for Dengue Control	Prevention tools: vaccines, antiviral drugs, insecticides, new diagnostic, surveillance techniques, and innovative mosquito control.	Bill & Melinda Gates Foundation, Sanofi Pasteur, Takeda, Bayer, GSK, bioMérieux.	Foundation Mérieux, Dengue Vaccine Initiative (DVI), National Institutes of Health (NIH), Sabin Vaccine Institute.	http://www.controldengue.org/	Ongoing
Dengue Relief Foundation	Study and prevention of dengue in Latin America.	Non-profit public benefit corporation. Donation opportunities for general public include "donor trips" to Nicaragua.		www.denguerelief.org	Ongoing
Dengue Antigenic Cartography Consortium	Characterize antigenic variation among a large sample of DENVs.	WHO Collaborating Centre at the University of Cambridge, several international leading dengue research laboratories [49].		http://www.whocc.infectiousdisease.cam.ac.uk/antigenic-cartography/dengue	Ongoing
International Research Consortium on Dengue Risk Assessment, Management, and Surveillance (IDAMS)	Improving diagnostics and clinical management, assess risk of dengue spread.	Nineteen international research partners.	European Commission.	www.idams.eu	Completed
Dengue Research Framework for Resisting Epidemics in Europe (DENFREE)	Prevention of dengue epidemics.	Fourteen international research partners.	European Commission.	http://cordis.europa.eu/project/rcn/102500_en.html	Completed

(continued)

Table 23.1 (continued)

Name	Focus	Funding partners	Operating partners	Website	Status
Innovative Tools and Strategies for the Surveillance and Control of Dengue (DengueTools)	Monitoring dengue fever transmission.	Thirteen international partners: research and government institutions.	European Commission.	http://cordis.europa.eu/project/rcn/100455_en.html	Completed
Asia-Pacific dengue partnership [102]	Increase preparedness to face dengue outbreaks in the Asia-Pacific region. Focus on improvement of health systems, diagnostics, vector control, information sharing. Support for developing innovative tools for diagnostics, prevention and treatment.	Health care officials of some Asia-Pacific countries.	WHO and Asia Pacific countries	http://www.who.int/iris/handle/10665/204914	Completed
UNITEDengue [93]	Dengue surveillance and information sharing on Dengue control.	Public Institutions. Founding partners: Malaysia, Singapore, and Indonesia. Current members: Philippines, Thailand, Viet Nam, Sri Lanka, Brunei Darussalam, Cambodia, and Pakistan.		https://www.unitedengue.org/	Ongoing

and vaccine development (Bill-&-Melinda-Gates-Foundation 2017). The Wellcome Trust has engaged in dengue drug development and granted a Seeding Drug Discovery award to the University of Leuven (KU Leuven) in Belgium. The KU Leuven identified a series of compounds that are highly potent against DENV and entered in a collaboration with Johnson & Johnson for further drug development [53].

The EU launched the program 'Comprehensive control of Dengue fever under changing climatic conditions' in 2011. This program granted €18 million to three consortia: IDAMS, DENFREE, and DengueTools (Table 23.1). Another consortium interested in antiviral research was the European Training Network on (+) RNA Virus Replication and Antiviral Drug Development [31]. This consortium was an EU FP7 funded project that included dengue antiviral R&D, e.g., target identification, target validation, *in silico* design, and evaluation of *in vitro* antiviral activity.

Several partnerships happen at the level of academic-industry collaborations. Both parts are actively seeking collaborations and funding to carry on dengue antiviral research. We, as the industrial counterpart, encourage active collaborations across different sectors to optimize drug discovery processes and ultimately, be beneficial to the patients. Specific aspects of dengue drug discovery can be explored to maximize innovation and discovery of drug candidates (Fig. 23.4). Our recommendations include creating a repository of dengue compound libraries, strengthening *in silico* design and screening capabilities, supporting target validation and identification in dedicated academic labs, committing mechanism of action studies to academic research, delegating PK/PD and ADME evaluations to the industrial partners, and ensuring optimal communication among the participants.

23.5 Conclusions and Future Perspectives

The increasing global burden of dengue disease evidences the need to implement a comprehensive approach to control and prevent future dengue outbreaks [104]. Vector control has been, for many years, the sole measure to restrain dengue; unfortunately it has been insufficient [1]. The first approved dengue vaccine (Dengvaxia® by Sanofi-Pasteur) is now licensed in more than 11 countries but is only recommended for people from 9 to 45 years of age living in endemic areas, yet not recommended for individuals who have not been previously infected with dengue virus [76, 106]. Taking into account that over 40% of the global population is at risk of becoming infected with dengue [104], it is clear that antiviral treatments are required to prevent and cure dengue. However, there is not yet any dengue-specific drug treatment available on the market [100].

The major challenges for an antiviral dengue drug are to encompass efficacy against the four DENV serotypes and the need for an excellent safety profile [55]. It is evident that a coordinated response is required to develop potent, effective, and safe dengue antivirals. Existing dengue PPPs and initiatives could benefit from the lessons learned from malaria (see MMV) and should catalyse the development of wider collaborations for dengue drug discovery. The development of a PPP solely dedicated to dengue antiviral drug R&D has a potential to deliver results, as has been the case for the MMV initiative and the dengue vaccine initiative (DVI). Collaborations between academia and industry are limited but have been increasing over the last years. These PPPs may benefit maximally when the tasks are assigned, based on the resources and expertise of the different members; with the overall goal to optimize the dengue drug discovery process.

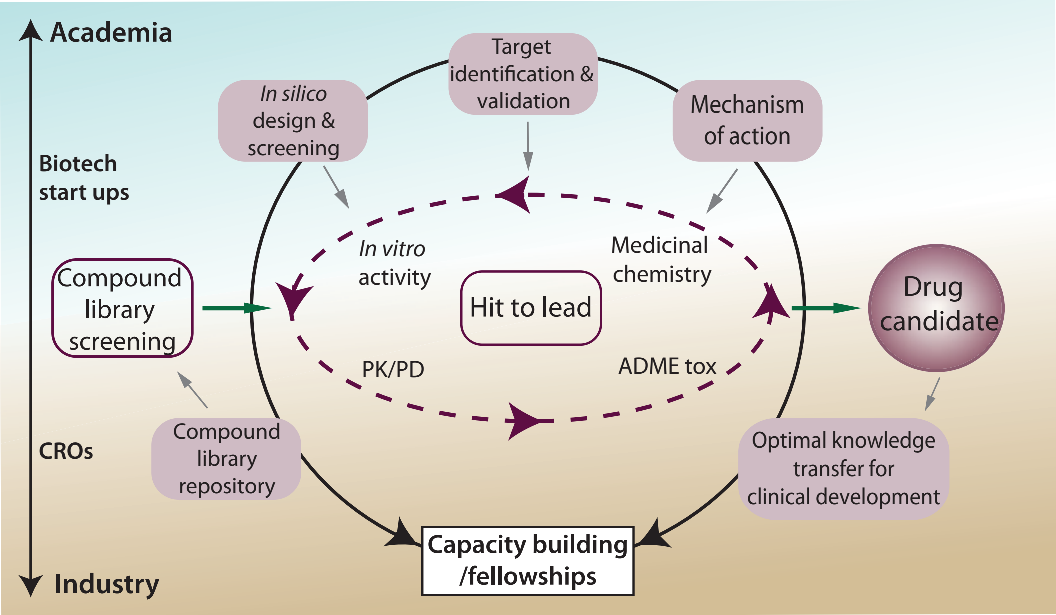


Fig. 23.4 Key aspects to increase innovation and reduce attrition rates in dengue drug discovery. Focusing on collaboration in the basic research and drug discovery phase, the highlighted "violet" boxes represent key elements for fostering collaborative projects and/or consortia. The activities located in the blue part are more feasible to be conducted in academic labs. Whereas the activities located in the brown region are more feasible for the industrial setting. All the violet-boxes represent a network in which dengue R&D could strengthen its capacity building and fellowships to develop human power to support dengue drug R&D in the near future. This scheme depicts the intricate process of discovering a drug candidate. The complete process can happen either in industry or academia. However, we propose a coordinated collaboration between both partners to optimize the usage of resources and expertise from each of them

We propose to boost such PPPs dedicated to dengue drug discovery to build a sustainable and diverse pre-clinical antiviral drugs pipeline for clinical development. Specifically, we propose a model, where different tasks can be conducted either in the industry or in an academic setting according to the skills and capabilities of each one (Fig. 23.4).

Although better vector control strategies as well as the development of improved diagnostics and vaccines will remain much needed in the battle against dengue, having a potent, tetravalent, and safe dengue antiviral drug at hand for the treatment and/or prophylaxis of DENV infections will be of utmost importance to control the impact of this ever-expanding pathogen.

References

1. Achee NL, Fred Gould TAP, Reiner RC Jr, Morrison AC, Ritchie SA, Gubler DJ, Teyssou R, Scott TW (2015) A critical assessment of vector control for Dengue prevention. PLoS Negl Trop Dis 9(5):e0003655. https://doi.org/10.1371/journal.pntd.0003655
2. Austin JE (2000) The collaboration challenge: how nonprofits and business succeed through strategic alliances. Jossey-Bass, San Francisco

3. Avorn J (2015) The $2.6 Billion Pill – methodologic and policy considerations. N Engl J Med 372(20):1877–1879. https://doi.org/10.1056/NEJMp1500848
4. Bagley CE, Tvarno CD (2013) Pharmaceutical public-private partnerships in the United States and Europe: moving from the bench to the bedside. *Lecturer and Other Affiliate Scholarship Series* 12 (Paper 12)
5. Beatty ME, Beutels P, Meltzer MI, Shepard DS, Hombach J, Hutubessy R, Dessis D et al (2011) Health economics of Dengue: a systematic literature review and expert panel's assessment. Am J Trop Med Hyg 84(3):473–488. https://doi.org/10.4269/ajtmh.2011.10-0521
6. Beauté J, Vong S (2010) Cost and disease burden of Dengue in Cambodia. BMC Public Health 10(1):521. https://doi.org/10.1186/1471-2458-10-521
7. Behnam MAM, Nitsche C, Boldescu V, Klein CD (2016) The medicinal chemistry of Dengue virus. J Med Chem 59(12):5622–5649. https://doi.org/10.1021/acs.jmedchem.5b01653
8. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM et al (2013) The global distribution and burden of dengue. Nature 496(7446):504–507. https://doi.org/10.1038/nature12060
9. Blass BE (2015) Chapter 1 – drug discovery and development: an overview of modern methods and principles. In: Blass BE (ed) Basic principles of drug discovery and development. Academic Press, Boston, pp 1–34
10. Blum LC, Reymond J-L (2009) 970 million druglike small molecules for virtual screening in the chemical universe database GDB-13. J Am Chem Soc 131(25):8732–8733. https://doi.org/10.1021/ja902302h
11. Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, Moyes CL et al (2012) Refining the global spatial limits of Dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis 6(8):e1760. https://doi.org/10.1371/journal.pntd.0001760
12. Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, Brandt WE (1989) Antigenic relationships between Flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J Gen Virol 70(1):37–43. https://doi.org/10.1099/0022-1317-70-1-37
13. Carrasco LR, Lee LK, Lee VJ, Ooi EE, Shepard DS, Thein TL, Gan V et al (2011) Economic impact of Dengue illness and the cost-effectiveness of future vaccination programs in Singapore. PLoS Negl Trop Dis 5(12):e1426. https://doi.org/10.1371/journal.pntd.0001426
14. Carrington LB, Simmons CP (2014) Human to mosquito transmission of Dengue viruses. Front Immunol 5:290. https://doi.org/10.3389/fimmu.2014.00290
15. Chan KWK, Watanabe S, Kavishna R, Alonso S, Vasudevan SG (2015) Animal models for studying dengue pathogenesis and therapy. Antiviral Res 123:5–14. https://doi.org/10.1016/j.antiviral.2015.08.013
16. Chang J, Block TM, Guo J-T (2013) Antiviral therapies targeting host ER alpha-glucosidases: current status and future directions. Antivir Res 99(3):251–260. https://doi.org/10.1016/j.antiviral.2013.06.011
17. Chapman N, Doubell A, Oversteegen L, Chowdhary V, Rugarabamu G, Zanetti R, Ong M, Borri J (2017) G-Finder 2017 report. Neglected disease research and development: Reflecting on a decade of global investment. Policy Cures. Accessed 12 March 2018. http://policycuresresearch.org/downloads/Y10_G-FINDER_full_report.pdf
18. ClinicalTrials.gov (2014) Efficacy and safety of ivermectin against dengue infection. NCT02045069. Last Modified October 29, 2015. Accessed 12 Jan. https://clinicaltrials.gov/ct2/show/NCT02045069
19. ClinicalTrials.gov (2014) Study to determine the safety, tolerability and pharmacokinetics of UV-4B solution administered orally in healthy subjects (UV). NCT02061358. Last Modified September 17, 2016. Accessed 12 Jan. https://clinicaltrials.gov/ct2/show/results/NCT02061358
20. ClinicalTrials.gov (2015) Celgosivir or modipafant as treatment for adult participants with uncomplicated Dengue fever in Singapore. NCT02569827. Last Modified April 19, 2016. Accessed 11 Jan. https://clinicaltrials.gov/ct2/show/NCT02569827
21. ClinicalTrials.gov (2015) Ketotifen as a Treatment for Vascular Leakage During Dengue Fever (KETODEN). NCT02673840. Last Modified Februry 3, 2016. Accessed 11 Jan. https://clinicaltrials.gov/ct2/show/record/NCT02673840
22. Croft SL (2005) Public-private partnership: from there to here. Trans R Soc Trop Med Hyg 99(Supplement 1):S9–S14. https://doi.org/10.1016/j.trstmh.2005.06.008
23. Demotes-Mainard J, Canet E, Segard L (2006) Publi-private partnership models in France and Europe. Therapie 61(4):325–334
24. Dickson M, Gagnon JP (2004) Key factors in the rising cost of new drug discovery and development. Nat Rev Drug Discov 3(5):417–429
25. DiMasi JA, Grabowski HG, Hansen RW (2015) The cost of drug development. N Engl J Med 372(20):1972–1972. https://doi.org/10.1056/NEJMc1504317
26. Durbin AP, Vargas MJ, Wanionek K, Hammond SN, Gordon A, Rocha C, Balmaseda A, Harris E (2008) Phenotyping of peripheral blood mononuclear cells during acute dengue illness demonstrates infection and increased activation of monocytes in severe cases compared to classic dengue fever. Virology 376(2):429–435. https://doi.org/10.1016/j.virol.2008.03.028
27. DVI (2016) Dengue vaccine initiative. Accessed 20 Oct. 2016. http://www.denguevaccines.org/vaccine-development

28. Dye C, Bartolomeos K, Moorthy V, Kieny MP (2016) Data sharing in public health emergencies: a call to researchers. Bull World Health Organ 94:158. https://doi.org/10.2471/BLT.16.170860
29. Edillo FE, Halasa YA, Largo FM, Erasmo JNV, Amoin NB, Alera MTP, Yoon I-K, Alcantara AC, Shepard DS (2015) Economic cost and burden of Dengue in the Philippines. Am J Trop Med Hyg 92(2):360–366. https://doi.org/10.4269/ajtmh.14-0139
30. Ekins S, Mestres J, Testa B (2007) In silico pharmacology for drug discovery: methods for virtual ligand screening and profiling. Br J Pharmacol 152(1):9–20. https://doi.org/10.1038/sj.bjp.0707305
31. EUVIRNA (2015) European training network on (+) RNA virus replication and antiviral drug development. Accessed 30 Nov. http://euvirna.phrmy.cf.ac.uk/?q=node/2
32. FDA (2008) Guidance for industry. Tropical disease priority review vouchers. Accessed 20 Jan. https://www.fda.gov/OHRMS/DOCKETS/98fr/FDA-2008-D-0530-gdl.pdf
33. FDA (2015) The drug development process. Last Modified 24 June 2015. Accessed 29 Nov. http://www.fda.gov/ForPatients/Approvals/Drugs/ucm405658.htm
34. Fischl W, Bartenschlager R (2013) High-throughput screening using Dengue virus reporter genomes. In: Gong EY (ed) Antiviral methods and protocols. Humana Press, New York, pp 205–219
35. Foundation-Merieux (2016) Partnership for Dengue control. Accessed 13 Dec. http://www.controldengue.org/about-us/
36. Gaspar R, Aksu B, Cuine A, Danhof M, Takac MJ-M, Linden HH, Link A et al (2012) Towards a European strategy for medicines research (2014–2020): the EUFEPS position paper on Horizon 2020. Eur J Pharm Sci 47(5):979–987. https://doi.org/10.1016/j.ejps.2012.09.020
37. Gjenero-Margan I, Aleraj B, Krajcar D, Lesnikar V, Klobučar A, Pem-Novosel I, Kurečić-Filipović S, Komparak S, Martić R, Đuričić S, Betica-Radić L, Okmadžić J, Vilibić-Čavlek T, Babić Erceg A, Turković B, Avšić-Županc T, Radić I, Ljubić M, Šarac K, Benić N, Mlinarić-Galinović G (2011) Autochthonous dengue fever in Croatia, August–September 2010. Euro Surveill 16(9):19805–19808
38. Gu F, Shi P-Y (2014) The challenges of dengue drug discovery and development. Clin Investig 4(8):683–685. https://doi.org/10.4155/cli.14.67
39. Gubler DJ (2008) Dengue/Dengue haemorrhagic fever: history and current status. In: Bock G, Goode J (eds) New treatment strategies for Dengue and other Flaviviral diseases: novartis foundation symposium 277. Wiley, Chichester, pp 3–22
40. Hales S, de Wet N, Maindonald J, Woodward A (2002) Potential effect of population and climate changes on global distribution of dengue fever: an empirical model. Lancet 360(9336):830–834. https://doi.org/10.1016/S0140-6736(02)09964-6
41. Hefti FF (2008) Requirements for a lead compound to become a clinical candidate. BMC Neurosci 9(Suppl 3):S7–S7. https://doi.org/10.1186/1471-2202-9-S3-S7
42. Horstick O, Tozan Y, Wilder-Smith A (2015) Reviewing Dengue: still a neglected tropical disease? PLoS Negl Trop Dis 9(4):e0003632. https://doi.org/10.1371/journal.pntd.0003632
43. Hotez PJ, Alvarado M, Basáñez M-G, Bolliger I, Bourne R, Boussinesq M, Brooker SJ et al (2014) The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. PLoS Negl Trop Dis 8(7):e2865. https://doi.org/10.1371/journal.pntd.0002865
44. Hughes JP, Rees S, Kalindjian SB, Philpott KL (2011) Principles of early drug discovery. Br J Pharmacol 162(6):1239–1249. https://doi.org/10.1111/j.1476-5381.2010.01127.x
45. Ian HG, Didier L, Frearson Julie A (2011) Finding new hits in neglected disease projects: target or phenotypic based screening? Curr Top Med Chem 11(10):1284–1291. https://doi.org/10.2174/156802611795429176
46. Jomo KS, Chowdhury A, Sharma K, Platz D (2016) Public-private partnerships and the 2030 Agenda for sustainable development: fit for purpose?: United Nations, Department of Economics and Social Affairs
47. Kaptein SJF, Neyts J (2016) Towards antiviral therapies for treating dengue virus infections. Curr Opin Pharmacol 30:1–7. https://doi.org/10.1016/j.coph.2016.06.002
48. Katsuno K, Burrows JN, Duncan K, van Huijsduijnen RH, Kaneko T, Kita K, Mowbray CE et al (2015) Hit and lead criteria in drug discovery for infectious diseases of the developing world. Nat Rev Drug Discov 14(11):751–758. https://doi.org/10.1038/nrd4683
49. Katzelnick LC, Fonville JM, Gromowski GD, Arriaga JB, Green A, James SL, Lau L et al (2015) Dengue viruses cluster antigenically but not as discrete serotypes. Science 349(6254):1338
50. Khormi HM, Kumar L (2011) Modeling dengue fever risk based on socioeconomic parameters, nationality and age groups: GIS and remote sensing based case study. Sci Total Environ 409(22):4713–4719. https://doi.org/10.1016/j.scitotenv.2011.08.028
51. Kieny M-P, Moorthy V, Bagozzi D (2016) Public health: use open data to curb Zika virus. Nature 533(7604):469–469. https://doi.org/10.1038/533469b
52. Krishnan MN, Garcia-Blanco MA (2014) Targeting host factors to treat West Nile and Dengue viral infections. Virus 6(2):683–708. https://doi.org/10.3390/v6020683
53. KULeuven-News (2013) Wellcome trust, KU Leuven and Janssen join forces to combat dengue fever. Accessed 20 Oct. https://www.kuleuven.be/english/news/2013/wellcome-trust-ku-leuven-and-janssen-join-forces-to-combat-dengue-fever

54. Leeson P (2012) Drug discovery: chemical beauty contest. Nature 481(7382):455–456
55. Lim SP, Wang Q-Y, Noble CG, Chen Y-L, Dong H, Zou B, Yokokawa F et al (2013) Ten years of dengue drug discovery: progress and prospects. Antivir Res 100(2):500–519. https://doi.org/10.1016/j.antiviral.2013.09.013
56. Lourenço J, Recker M (2014) The 2012 Madeira Dengue outbreak: epidemiological determinants and future epidemic potential. PLoS Negl Trop Dis 8(8):e3083. https://doi.org/10.1371/journal.pntd.0003083
57. Low JG, Sung C, Wijaya L, Wei Y, Rathore APS, Watanabe S, Tan BH et al (2014) Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): a phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial. Lancet Infect Dis 14(8):706–715. https://doi.org/10.1016/S1473-3099(14)70730-3
58. Magariños MP, Carmona SJ, Crowther GJ, Ralph SA, Roos DS, Shanmugam D, Van Voorhis WC, Agüero F (2012) TDR targets: a chemogenomics resource for neglected diseases. Nucleic Acids Res 40(Database issue):D1118–D1127. https://doi.org/10.1093/nar/gkr1053
59. Martina BEE, Koraka P, Osterhaus ADME (2009) Dengue virus pathogenesis: an integrated view. Clin Microbiol Rev 22(4):564–581. https://doi.org/10.1128/cmr.00035-09
60. Medeiros LC d C, Castilho CAR, Braga C, de Souza WV, Regis L, Monteiro AMV (2011) Modeling the dynamic transmission of Dengue fever: investigating disease persistence. PLoS Negl Trop Dis 5(1):e942. https://doi.org/10.1371/journal.pntd.0000942
61. Messina JP, Brady OJ, Scott TW, Zou C, Pigott DM, Duda KA, Bhatt S et al (2013) Global spread of dengue virus types: mapping the 70 year history. Trends Microbiol 22(3):138–146. https://doi.org/10.1016/j.tim.2013.12.011
62. Moi, ML, Takasaki T, Kurane I (2016) Human antibody response to dengue virus: implications for dengue vaccine design. Trop Med Health 44(1) https://doi.org/10.1186/s41182-016-0004-y
63. Moran M, Chapman N, Abela-Oversteegen L, Chowdhary V, Doubell A, Whittall C, Howard R et al (2015). G-FInder 2015 report. Neglected disease research and development: the Ebola effect. Policy cures. Accessed 23 Nov 2016. http://policycures.org/downloads/Y8GFINDER full report web.pdf
64. NIH (2016) National institute of allergy and infectious diseases. Dengue fever. NIH. Accessed 30 Nov. https://www.niaid.nih.gov/diseases-conditions/dengue-fever
65. Noble CG, Chen Y-L, Dong H, Feng G, Lim SP, Schul W, Wang Q-Y, Shi P-Y (2010) Strategies for development of dengue virus inhibitors. Antivir Res 85(3):450–462. https://doi.org/10.1016/j.antiviral.2009.12.011
66. Noble CG, Shi P-Y (2013) Difficulties and advancements in developing drugs for the treatment of dengue fever. Expert Opin Orphan Drugs 1(12):947–949. https://doi.org/10.1517/21678707.2013.858597
67. Nwaka S, Hudson A (2006) Innovative lead discovery strategies for tropical diseases. Nat Rev Drug Discov 5(11):941–955
68. Nwaka S, Ridley RG (2003) Virtual drug discovery and development for neglected diseases through public-private partnerships. Nat Rev Drug Discov 2(11):919–928
69. Ong A, Sandar M, Chen MI, Sin LY (2007) Fatal dengue hemorrhagic fever in adults during a dengue epidemic in Singapore. Int J Infect Dis 11(3):263–267. https://doi.org/10.1016/j.ijid.2006.02.012
70. Owens J (2007) Determining druggability. Nat Rev Drug Discov 6(3):187–187
71. Pang EL, Loh H-S (2017) Towards development of a universal dengue vaccine – how close are we? Asian Pac J Trop Med 10(3):220–228. https://doi.org/10.1016/j.apjtm.2017.03.003
72. Paul SM, Mytelka DS, Dunwiddie CT, Persinger CC, Munos BH, Lindborg SR, Schacht AL (2010) How to improve R&D productivity: the pharmaceutical industry's grand challenge. Nat Rev Drug Discov 9(3):203–214. http://www.nature.com/nrd/journal/v9/n3/suppinfo/nrd3078_S1.html
73. Perry ST, Buck MD, Plummer EM, Penmasta RA, Batra H, Stavale EJ, Warfield KL et al (2013) An iminosugar with potent inhibition of dengue virus infection in vivo. Antivir Res 98(1):35–43. https://doi.org/10.1016/j.antiviral.2013.01.004
74. PPPIRC (2016) Public-private-partnership in infrastructure resource center. Government objectives: benefits and risks of PPPs. World Bank Group, Last Modified 31 October 2016. Accessed 13 Dec. https://ppp.worldbank.org/public-private-partnership/overview/ppp-objectives
75. Runge-Ranzinger S, Kroeger A, Olliaro P, McCall PJ, Tejeda GS, Lloyd LS, Hakim L et al (2016) Dengue contingency planning: from research to policy and practice. PLoS Negl Trop Dis 10(9):e0004916. https://doi.org/10.1371/journal.pntd.0004916
76. SAGE (2016) Summary of the April 2016 meeting of the Strategic Advisory Group of Experts on Immunization (SAGE) – Dengue Vaccine. Accessed 11 Oct. http://www.who.int/immunization/sage/meetings/2016/april/SAGE_April_2016_Meeting_Web_summary.pdf?ua=1
77. Sampath A, Padmanabhan R (2009) Molecular targets for flavivirus drug discovery. Antivir Res 81(1):6–15. https://doi.org/10.1016/j.antiviral.2008.08.004
78. Souza S, Fernando H, da Silva Almeida B, Boscardin SB (2016) Early dengue virus interactions: the role of dendritic cells during infection. Virus Res 223:88–98. https://doi.org/10.1016/j.virusres.2016.07.001
79. Satoshi K, Yasuyuki K, Ling MM, Akira K, Masayuki O, Koh S, Tetsuro K et al (2015) Autochthonous

Dengue fever, Tokyo, Japan, 2014. Emer Infect Dis J 21(3):517. https://doi.org/10.3201/eid2103.141662
80. Scannell JW, Blanckley A, Boldon H, Warrington B (2012) Diagnosing the decline in pharmaceutical R&D efficiency. Nat Rev Drug Discov 11(3):191–200. http://www.nature.com/nrd/journal/v11/n3/suppinfo/nrd3681_S1.html
81. Scherwitzl I, Mongkolsapaja J, Screaton G (2017) Recent advances in human flavivirus vaccines. Curr Opin Virol 23:95–101. https://doi.org/10.1016/j.coviro.2017.04.002
82. Schmid MA, Diamond MS, Harris E (2014) Dendritic cells in Dengue virus infection: targets of virus replication and mediators of immunity. Front Immunol 5:647. https://doi.org/10.3389/fimmu.2014.00647
83. Schuhmacher A, Gassmann O, Hinder M (2016) Changing R&D models in research-based pharmaceutical companies. J Transl Med 14:105. https://doi.org/10.1186/s12967-016-0838-4
84. Shepard DS, Coudeville L, Halasa YA, Zambrano B, Dayan GH (2011) Economic impact of dengue illness in the Americas. Am J Trop Med Hyg 84(2):200–207. https://doi.org/10.4269/ajtmh.2011.10-0503
85. Shepard DS, Halasa YA, Tyagi BK, Vivek Adhish S, Nandan D, Karthiga KS, Chellaswamy V et al (2014) Economic and disease burden of Dengue illness in India. Am J Trop Med Hyg 91(6):1235–1242. https://doi.org/10.4269/ajtmh.14-0002
86. Shepard DS, Undurraga EA, Halasa YA (2013) Economic and disease burden of Dengue in Southeast Asia. PLoS Negl Trop Dis 7(2):e2055. https://doi.org/10.1371/journal.pntd.0002055
87. Shepard DS, Undurraga EA, Halasa YA, Stanaway JD (2016) The global economic burden of dengue: a systematic analysis. Lancet Infect Dis 16(8):935–941. https://doi.org/10.1016/S1473-3099(16)00146-8
88. Souza DG, Fagundes CT, Sousa LP, Amaral FA, Souza RS, Souza AL, Kroon EG et al (2009) Essential role of platelet-activating factor receptor in the pathogenesis of Dengue virus infection. Proc Natl Acad Sci 106(33):14138–14143. https://doi.org/10.1073/pnas.0906467106
89. St. John AL, Abraham SN, Gubler DJ (2013) Barriers to preclinical investigations of anti-dengue immunity and dengue pathogenesis. Nat Rev Microbiol 11(6):420–426. https://doi.org/10.1038/nrmicro3030
90. Succo T, Leparc-Goffart I, Ferré J, Roiz D, Broche B, Maquart M, Noel H, Catelinois O, Entezam F, Caire D, Jourdain F, Esteve-Moussion I, Cochet A, Paupy C, Rousseau C, Paty M, Golliot F (2015) Autochthonous dengue outbreak in Nîmes, South of France, July to September 2015. Euro Surveill 21(21):30240. https://doi.org/10.2807/1560-7917.ES.2016.21.21.30240
91. Teets FD, Ramgopal MN, Sweeney KD, Graham AS, Michael SF, Isern S (2014) Origin of the dengue virus outbreak in Martin County, Florida, USA 2013. Virol Rep 1-2:2–8. https://doi.org/10.1016/j.virep.2014.05.001
92. Trouiller P, Olliaro P, Torreele E, Orbinski J, Laing R, Ford N (2002) Drug development for neglected diseases: a deficient market and a public-health policy failure. Lancet 359(9324):2188–2194. https://doi.org/10.1016/S0140-6736(02)09096-7
93. UNITEDengue (2012) UNited in tackling epidemic Dengue. Accessed 15 Sept. https://www.unitedengue.org/
94. Van Voorhis WC, Adams JH, Adelfio R, Ahyong V, Akabas MH, Alano P, Alday A et al (2016) Open source drug discovery with the Malaria box compound collection for neglected diseases and beyond. PLoS Pathog 12(7):e1005763. https://doi.org/10.1371/journal.ppat.1005763
95. Warfield KL, Plummer EM, Sayce AC, Alonzi DS, Tang W, Tyrrell BE, Hill ML et al (2016) Inhibition of endoplasmic reticulum glucosidases is required for in vitro and in vivo dengue antiviral activity by the iminosugar UV-4. Antivir Res 129:93–98. https://doi.org/10.1016/j.antiviral.2016.03.001
96. Wellcome-Trust (2016) Sharing data during Zika nd other global health emergencies. Last Modified 10 February 2016. Accessed 14 Dec. https://blog.wellcome.ac.uk/2016/02/10/sharing-data-during-zika-and-other-global-health-emergencies/
97. Wells TNC, Willis P, Burrows JN, van Huijsduijnen RH (2016) Open data in drug discovery and development: lessons from malaria. Nat Rev Drug Discov 15(10):661–662. https://doi.org/10.1038/nrd.2016.154
98. Whitehorn J, Van Vinh Nguyen C, Khanh LP, Kien DTH, Quyen NTH, Tran NTT, Hang NT et al (2016) Lovastatin for the treatment of adult patients with Dengue: a randomized, double-blind, placebo-controlled trial. Clin Infect Dis 62(4):468–476. https://doi.org/10.1093/cid/civ949
99. Whitehorn J, Van VCN, Simmons CP (2014) Dengue human infection models supporting drug development. J Infect Dis 209(Suppl 2):S66–S70. https://doi.org/10.1093/infdis/jiu062
100. Whitehorn J, Yacoub S, Anders KL, Macareo LR, Cristina Cassetti M, Van VCN, Shi P-Y, Wills B, Simmons CP (2014) Dengue therapeutics, chemoprophylaxis, and allied tools: state of the art and future directions. PLoS Negl Trop Dis 8(8):e3025. https://doi.org/10.1371/journal.pntd.0003025
101. Whitehorn J, Simmons CP (2011) The pathogenesis of dengue. Vaccine 29(42):7221–7228. https://doi.org/10.1016/j.vaccine.2011.07.022
102. WHO (2007) Regional office for South-East Asia. Core group for Asia-Pacific dengue partnership: report of the meeting, Singapore, 8–10 February 2007. Accessed 14 Dec. http://www.who.int/iris/handle/10665/204914
103. WHO (2009) Dengue guidelines for diagnosis, treatment, prevention and control. New ed. Geneva, Switzerland World Health Organization and the Special Programme for Research and Training in Tropical Diseases. electronic

104. WHO (2012) Global strategy for dengue prevention and control, 2012–2020. [WHO report]. Accessed 30 Sept. http://apps.who.int/iris/bitstream/10665/75303/1/9789241504034_eng.pdf?ua=1
105. WHO (2014) Dengue and severe dengue. Fact sheet. Last Modified July 2016. Accessed 22 Nov. http://www.who.int/mediacentre/factsheets/fs117/en/
106. WHO (2016) Dengue vaccine: WHO position paper – July 2016. Wkly Epidemiol Rec 91(30):349–364
107. Widdus R (2001) Public-private partnerships for health: their main targets, their diversity, and their future directions. Bull World Health Org 79:713–720
108. Widdus R (2005) Public-private partnerships: an overview. Trans R Soc Trop Med Hyg 99(Supplement 1):S1–S8. https://doi.org/10.1016/j.trstmh.2005.06.005
109. Wishart DS, Knox C, An CG, Shrivastava S, Hassanali M, Stothard P, Chang Z, Woolsey J (2006) DrugBank: a comprehensive resource for in silico drug discovery and exploration. Nucleic Acids Res 34(suppl 1):D668–D672. https://doi.org/10.1093/nar/gkj067
110. Xie X, Zou J, Wang Q-Y, Shi P-Y (2015) Targeting dengue virus NS4B protein for drug discovery. Antivir Res 118(0):39–45. https://doi.org/10.1016/j.antiviral.2015.03.007
111. Zheng W, Thorne N, McKew JC (2013) Phenotypic screens as a renewed approach for drug discovery. Drug Discov Today 18(0):1067–1073. https://doi.org/10.1016/j.drudis.2013.07.001
112. Zmurko J, Neyts J, Dallmeier K (2015) Flaviviral NS4b, chameleon and jack-in-the-box roles in viral replication and pathogenesis, and a molecular target for antiviral intervention. Rev Med Virol 25(4):205–223. https://doi.org/10.1002/rmv.1835
113. Zou G, Hao Ying X, Qing M, Wang Q-Y, Shi P-Y (2011) Development and characterization of a stable luciferase dengue virus for high-throughput screening. Antivir Res 91(1):11–19. https://doi.org/10.1016/j.antiviral.2011.05.001

24 The Use of *Wolbachia* by the World Mosquito Program to Interrupt Transmission of *Aedes aegypti* Transmitted Viruses

Scott L. O'Neill

Abstract

The biological control of mosquito transmission by the use of the naturally occurring insect-specific bacterial endosymbiont Wolbachia has been successfully tested in small field trials. The approach has been translated successfully to larger field sites in Townsville, Australia and expanded to more than 10 countries through the Eliminate Dengue Program. The broader application of the program beyond limiting the transmission of dengue and including other *Aedes aegypti* borne mosquitoes has seen the program growing into a global not-for-profit initiative to be known as the World Mosquito Program.

Keywords

World Mosquito Program · Biological mosquito control · Cytoplasmic Incompatibility · wMel *Wolbachia* strain · Randomised control cluster trial

Wolbachia is a naturally occurring bacterial endosymbiont of insects that is estimated to occur in up to 40–60% of all insect species [6, 14]. It has been of interest to basic biologists for many years due to the unusual ways it manipulates host insect reproduction to ensure its efficient transmission into populations. *Wolbachia* is not infectious but instead is maternally inherited through the insect egg cytoplasm. It has evolved mechanisms to transmit itself very efficiently into host populations by either directly or indirectly favouring female insects that carry Wolbachia to leave behind more offspring than uninfected counterparts [12]. One of the best studied of these mechanisms is cytoplasmic incompatibility (CI) in which embryonic development is arrested in *Wolbachia* uninfected embryos that are fertilised by sperm that have matured in the presence of *Wolbachia* (Fig. 24.1), or in embryos fertilized by sperm matured in the presence of a different strain of *Wolbachia* than in the female egg.

The World Mosquito Program (WMP), farmerly known as the Eliminate Dengue Program is a non-profit research consortium operating in a number of countries www.worldmosquitoprogram.org (Fig. 24.2). It aims to develop Wolbachia as an intervention to control mosquito-transmitted viruses such as dengue, zika and chikungunya. The key feature of the Eliminate Dengue Program is the intentional release of *Wolbachia*-infected mosquitoes into target areas that will then transmit *Wolbachia* into wild Aedes mosquito populations [7]. CI provides the mechanism by which

S. L. O'Neill (✉)
Institute Vector Borne Disease, Monash University, Clayton, VIC, Australia
e-mail: scott.oneill@monash.edu

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_24

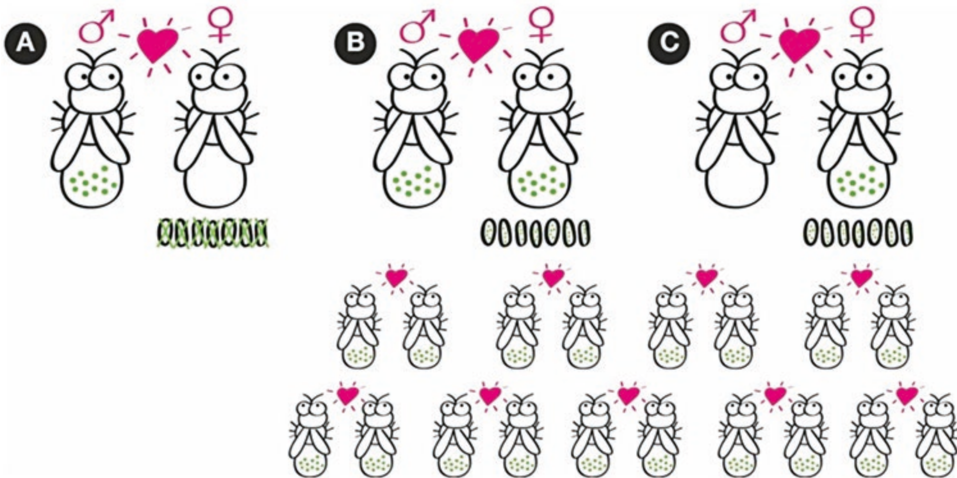


Fig. 24.1 *Wolbachia* infections induce a phenomenon known as cytoplasmic incompatibility in infected hosts that acts as a drive mechanism to push *Wolbachia* into the host populations by indirectly favouring *Wolbachia* infected females. This is done by reducing the reproductive output of *Wolbachia* uninfected females in a population which benefits the maternally transmitted *Wolbachia*

Fig. 24.2 Locations where Eliminate Dengue release activities are being undertaken as of 2016. A number of new sites will be added in 2017

Wolbachia will establish and maintain itself in wild mosquito populations over a number of mosquito generations once released, even if the *Wolbachia* strain places a mild genetic load on the mosquito it infects [8]. Given that *Wolbachia* is quite ubiquitous in the natural environment it is somewhat intuitive that environmental or human health risks associated with its introduction into urban areas should be minimal. This is supported by independent risk analysis [10].

The key attribute of *Wolbachia* that the World Mosquito Program is basing its intervention on is its demonstrated ability to interfere with the replication of human pathogens in *Wolbachia* infected *Aedes* mosquitoes. This includes Flaviviruses like dengue, West Nile and Zika [1, 3, 5, 9, 11], Alphaviruses like chikungunya [2] as well as a range of other viruses and parasites. Analysis of dengue blocking data where mosquitoes have been fed on bloods from dengue patients indicates that the establishment of *Wolbachia* in *Aedes aegypti* populations can be predicted to reduce R0 for dengue by more than 70%, which in most epidemiological settings should completely stop local dengue transmission [4].

To implement a World Mosquito Program intervention it is necessary to release *Wolbachia* infected mosquitoes, both male and female, until the local frequency of *Wolbachia* in wild *Aedes aegypti* mosquitoes surpasses an unstable equilibrium point estimated to be less than 0.3 for the wMel strain of *Wolbachia*. Once this unstable equilibrium point is surpassed it is expected that *Wolbachia* will locally establish and if the establishment area is sufficiently large then start to slowly spread out from the release area. This theory has now been tested in five countries where establishment of wMel has been achieved according to these principles. Typically, quite small numbers of mosquitoes need to be released to surpass the unstable equilibrium point. In Northern Australia in the first release experiments undertaken 10 mosquitoes (both male and female) were released per house per week for 10 weeks and this was sufficient to achieve establishment [7]. In other countries longer release periods have been required if target mosquito populations are larger. Despite the need to release females the experience to date has been that most members of a community undergoing releases do not complain of increased biting pressure, presumably because nuisance biting by other species dominates the personal experience of residents.

Once a series of releases has been undertaken *Wolbachia* is expected to then maintain itself in the local population indefinitely under the action of CI. The deployment is predicted to be robust if it becomes successfully established initially and is demonstrated from data from our earliest release sites in Northern Australia where Wolbachia has sustained itself in local mosquito populations at frequencies above 80–90% since establishment from 10 weeks of releases in 2011 (Fig. 24.3). This is an extremely important attribute of the interventions that WMP is undertaking as costs for implementing the intervention are essentially front loaded during releases and then restricted to periodic monitoring. This avoids the need for ongoing expenditure as is the case for other interventions such as vector suppression technologies or vaccines and makes the WMP approach both sustainable and highly cost-effective.

It can be noted from Fig. 24.3 that while Wolbachia maintains itself at a very high frequency in the wild mosquito population it is rarely at complete fixation. We presume that this is a result of some leakiness in maternal transmission rates of *Wolbachia*, possibly through the action of environmental heat in some breeding sites. Of particular note though is that frequencies of Wolbachia of around 80–90% may be more optimal for disease reduction than complete fixation. At lower infection levels we can expect incompatible crosses generated from Wolbachia via the CI mechanism to put downward pressure on mosquito population sizes that should act in concert with the transmission blocking properties of Wolbachia to enhance the effects of pathogen blocking. Even at frequencies of around 80% in populations the effects of reduced vector competence should still have very large impacts on

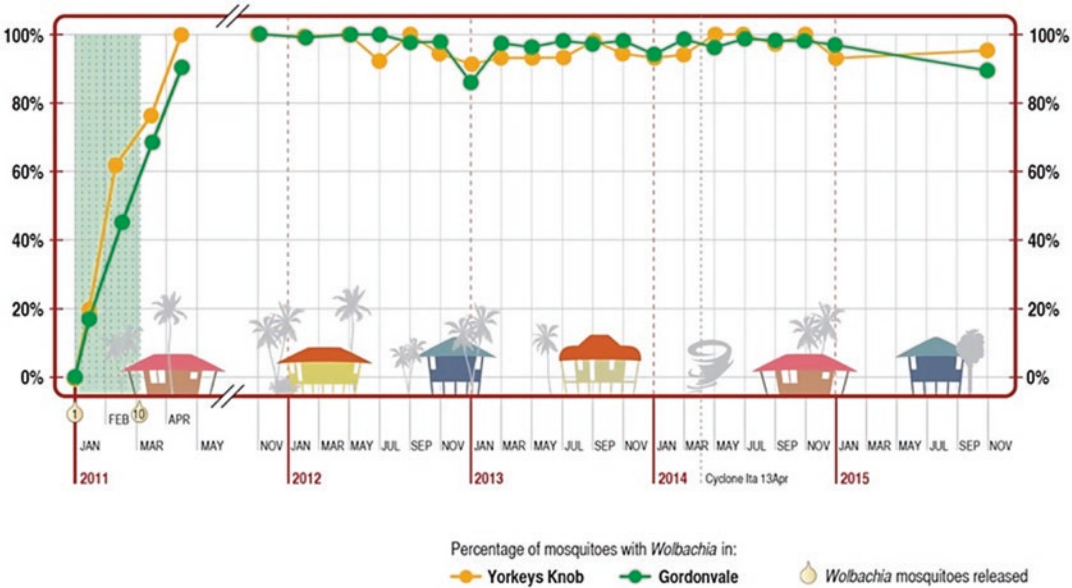


Fig. 24.3 Results of *Wolbachia* monitoring in the first two sites in Northern Australia where wMel releases were undertaken showing the frequency of Wolbachia in the sampled wild mosquito population in both sites. Wolbachia has maintained itself at high frequency since introduction in 2011

transmission, consistent with the observational data gathered so far.

Pilot releases have now been successfully undertaken in five countries including Australia, Indonesia, Vietnam, Brazil and Colombia and indicate that the wMel strain can be deployed successfully in diverse settings both ecologically and culturally. Observational data from these deployments supports large impacts on disease transmission as predicted by modelling. In all areas where *Wolbachia* has now established in these five countries we have not detected any examples of local transmission of dengue to date, defined as clustering of dengue cases in time and space, despite local transmission occurring in neighbouring areas. A key feature of all these deployments is that they have all occurred with strong community support and virtually no opposition. Similarly, there have been no adverse impacts identified in any of these deployments either human related or environmental.

In 2014 the WMP undertook its first scaled release over the entire city of Townsville using a mixture of egg and adult deployments. This scale up required a new form of community engagement requiring community consent rather than individual informed consent. The deployment in Townsville also successfully used community deployments to augment programmatic deployments. Community deployments featured the use of small mosquito release containers supplied with *Wolbachia* mosquito eggs and fish food and required only the addition of water and placement of the container in a suitable shady location for 2–3 weeks until all mosquitoes had emerged. As part of the community release program a targeted program also ran in schools where school students undertook the releases in a citizen science experiment. An area of 95 km^2 was targeted in the city of Townsville (almost the entire city) and the intervention was successfully deployed over three stages in 2.5 years providing the first indications that the method could be scaled effectively over small cities. As per earlier pilot releases there have been no examples of locally transmitted dengue cases in Townsville in any areas where *Wolbachia* has been established at the time of writing.

Within the last 2 years there has been considerable alarm in the international community of

the enormity and difficulty in controlling the South American outbreaks of Zika virus which have now spread to nearly all the countries where dengue transmission occurs. Given the similarity in the ecology of dengue and Zika we can expect ultimately that Zika transmission should co-exist with dengue transmission in the same geographies that have the main transmission vector, *Aedes aegypti*. Since Zika virus is quite closely related to dengue viruses there was an expectation that *Wolbachia* should block Zika transmission in much the same way as dengue viruses and these assumptions have since demonstrated empirically [1, 3]. Indeed the degree of blocking that has been demonstrated for Zika in the laboratory appears stronger if not similar to dengue, which bodes well for using the WMP *Wolbachia* approach to block Zika transmission in the field.

In March 2016 a special advisory group to the WHO made a public recommendation that the Wolbachia interventions being undertaken by WMP should move to pilot deployments over larger scales than previously attempted given the encouraging preliminary evidence for potential impact against Zika [13]. Based on this recommendation two large pilot deployments have commenced in Rio de Janeiro/Niteroi in Brazil and Medellin/Bello in Colombia targeting populations of around 2–2.5 M in each deployment.

At the same time a randomised controlled cluster trial is underway in the city of Yogyakarta which is expected to complete in late 2019 and another randomised trial planned to start in Vietnam by 2018. Together these approaches will provide a basket of evidence to understand the impact of the WMP intervention on arbovirus transmission. The measures include: (1) Laboratory studies showing impaired vector competence, (2) Mathematical modelling predicting large impacts on transmission, (3) Observational time series data capturing before and after impact on dengue cases measured through the existing health surveillance system, (4) Randomised cluster trials and (5) Large pilot deployments over large populations centres.

Over the next 2 years these deployments and measurements of impact will accumulate so that we will have accurate measures of effectiveness. At the same time, we will have learned how to deploy at the scale of very large cities and reduced our costs with a goal of reaching a target of US$1/person protected. If the results on these studies continue to be positive then it will be our goal to collaborate with governments in disease affected areas to make this technology and best practice methods for its deployment available to countries in need.

References

1. Aliota MT, Peinado SA, Velez ID, Osorio JE (2016) The wMel strain of *Wolbachia* reduces transmission of Zika virus by *Aedes aegypti*. Sci Rep 6:28792
2. Aliota MT, Walker EC, Uribe Yepes A, Dario Velez I, Christensen BM, Osorio JE (2016) The wMel strain of *Wolbachia* reduces transmission of chikungunya virus in *Aedes aegypti*. PLoS Negl Trop Dis e0004677:10
3. Dutra HL, Rocha MN, Dias FB, Mansur SB, Caragata EP, Moreira LA (2016) *Wolbachia* blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti* mosquitoes. Cell Host Microbe 19:771–774
4. Ferguson NM, Kien DT, Clapham H, Aguas R, Trung VT, Chau TN, Popovici J, Ryan PA, O'Neill SL, McGraw EA, Long VT, Dui Le T, Nguyen HL, Chau NV, Wills B, Simmons CP (2015) Modeling the impact on virus transmission of *Wolbachia*-mediated blocking of dengue virus infection of *Aedes aegypti*. Sci Transl Med 7:279ra37
5. Frentiu FD, Zakir T, Walker T, Popovici J, Pyke AT, Van Den Hurk A, McGraw EA, O'Neill SL (2014) Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with *Wolbachia*. PLoS Negl Trop Dis 8:e2688
6. Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008) How many species are infected with *Wolbachia*? a statistical analysis of current data. FEMS Microbiol Lett 281:215–220
7. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny N, Omodei C, Mcgraw EA, Ryan PA, Ritchie SA, Turelli M, O'Neill SL (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature 476:454–457
8. Hoffmann M, Coy MR, Kingdom Gibbard HN, Pelz-Stelinski KS (2014) *Wolbachia* infection density in populations of the Asian citrus psyllid (Hemiptera: Liviidae). Environ Entomol 43:1215–1222
9. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH,

Mcgraw EA, Van Den Hurk AF, Ryan PA, O'Neill SL (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya, and plasmodium. Cell 139:1268–1278
10. Murray JV, Jansen CC, Ede Barro P (2016) Risk associated with the release of *Wolbachia*-infected *Aedes aegypti* mosquitoes into the environment in an effort to control dengue. Front Public Health 4:43
11. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, Mcmeniman CJ, Leong YS, Dong Y, Axford J, Kriesner P, Lloyd AL, Ritchie SA, O'Neill SL, Hoffmann AA (2011) The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature 476:450–453
12. Werren JH, Baldo L, Clark ME (2008) Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol 6:741–751
13. WHO (2016) Mosquito (vector) control emergency response and preparedness for Zika virus [Online]. Available: http://www.who.int/neglected_diseases/news/mosquito_vector_control_response/en/. Accessed 15 May 2016
14. Zug R, Hammerstein P (2012) Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS One 7:e38544

Check for updates

25 Seroepidemiological Studies of Arboviruses in Africa

Eduardo Samo Gudo, S. Ali, V. S. António, I. R. Chelene, I. Chongo, M. Demanou, K. Falk, O. C. Guiliche, N. Heinrich, V. Monteiro, A. F. Muianga, J. Oludele, F. Mula, F. Mutuku, N. Amade, P. Alho, E. Betsem, Z. Chimbuinhe, A. J. Cristovam, G. Galano, A. Gessain, E. Harris, M. Heise, F. Inalda, I. Jala, E. Jaszi, C. King, U. Kitron, B. M. Kümmerer, A. D. LaBeaud, N. Lagerqvist, G. Malai, M. Mazelier, S. Mendes, D. Mukoko, B. Ndenga, R. Njouom, G. Pinto, A. Tivane, D. M. Vu, and J. Vulule

Abstract

The literature on sero-epidemiological studies of flaviviral infections in the African continent is quite scarce. Much of the viral epidemiology studies have been focussing on diseases such as HIV/AIDS because of their sheer magnitude and impact on the lives of people in the various affected countries. Increasingly disease outbreaks caused by arboviruses such as the recent cases of chikungunya virus, dengue virus and yellow fever virus have prompted renewed interest

E. S. Gudo (✉) · S. Ali · V. S. António · I. R. Chelene
I. Chongo · O. C. Guiliche · V. Monteiro
A. F. Muianga · J. Oludele · F. Mula · N. Amade
P. Alho · F. Inalda · A. Tivane · G. Pinto
National Institute of Health, Maputo, Mozambique

M. Demanou (✉) · M. Mazelier · R. Njouom
Laboratoire des arbovirus et des virus de fièvres hémorragiques, Centre Pasteur du Cameroun (CPC), Yaoundé, Cameroon

K. Falk (✉) N. Lagerqvist
The Public Health Agency of Sweden and Karolinska Institute, Solna, Sweden
e-mail: kerstin.falk@folkhalsomyndigheten.se

N. Heinrich (✉)
Infectious Diseases & Tropical Medicine, Medical Center of the University of Munich (LMU), Munich, Germany
e-mail: heinrich@lrz.uni-muenchen.de

F. Mutuku (✉)
Technical University of Mombasa, Mombasa, Kenya

E. Betsem
Faculté de médecine et des Sciences Biomédicales, Université de Yaoundé 1, Yaoundé, Cameroon

Unité d'Epidémiologie et Physiopathologie des Virus Oncogènes, Institut Pasteur, Paris, France

Z. Chimbuinhe · G. Galano
Pemba Provincial Hospital, Pemba, Mozambique

A. J. Cristovam
Direccao Provincial de Maputo, Maputo, Mozambique

A. Gessain
Unité d'Epidémiologie et Physiopathologie des Virus Oncogènes, Institut Pasteur, Paris, France

© Springer Nature Singapore Pte Ltd. 2018

R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1_25

in studying these viruses. International agencies from the US, several EU nations and China are starting to build collaborations to build capacity in many African countries together with established institutions to conduct these studies. The Tofo Advanced Study Week (TASW) was established to bring the best scientists from the world to the tiny seaside town of Praia do Tofo to rub shoulders with African virologists and discuss cutting-edge science and listen to the work of researchers in the field. In 2015 the 1st TASW focussed on Ebola virus. The collections of abstracts from participants at the 2nd TASW which focused on Dengue and Zika virus as well as presentations on other arboviruses are collated in this chapter.

Keywords

Differential diagnosis · Virology in Africa · Molecular surveillance of emerging viruses in Africa · Seroprevalence of emerging viruses in Africa · Prospective studies of emerging virus transmission in Africa

25.1 Seroepidemiology of West Nile Virus in Caia, Mozambique, 2015/2016

S. Ali · A. F. Muianga · F. Mula · I. R. Chelene · I. Chongo · V. Monteiro · O. C. Guiliche · P. Alho · G. Pinto · J. Oludele · V. S. António · K. I. Falk · Eduardo Samo Gudo

25.1.1 Introduction

West Nile virus (WNV) is a vector-borne flavivirus that is transmitted in an enzootic cycle between birds and by mosquitoes. Although the majority of the human infections are asymptomatic, WNV may also cause febrile and neuro-invasive diseases. However, approximately 80% of people who are infected will not show any symptoms. Infection with WNV is dependent on many factors including climate, mosquito habitats and immunologically-naïve bird populations. The objective of this study was to investigate seroprevalence of WNV among febrile patients in Caia, Mozambique.

E. Harris
Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, CA, USA

M. Heise
University of North Carolina School of Medicine, Chapel Hill, NC, USA

I. Jala
Laboratoire des arbovirus et des virus de fièvres hémorragiques, Centre Pasteur du Cameroun (CPC), Yaoundé, Cameroon

Tropical Disease Research Center (TDRC), Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

E. Jaszi
Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA

C. King · U. Kitron
Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, USA

B. M. Kümmerer
Institute of Virology, University of Bonn Medical Centre, Bonn, Germany

A. D. LaBeaud
Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, USA

Department of Pediatrics, Division of Infectious Diseases, Stanford University School of Medicine, California, USA

G. Malai
Polana Caniço General Hospital Mozambique, Maputo, Mozambique

S. Mendes
Nampula Central Hospital, Nampula, Mozambique

D. Mukoko
Vector Borne Disease Control Unit, Ministry of Health, Nairobi, Kenya

B. Ndenga · J. Vulule
Centre for Global Health Research, Kenya Medical Research Institute, Nairobi, Kenya

D. M. Vu
Department of Pediatrics, Division of Infectious Diseases, Stanford University School of Medicine, California, USA

25.1.2 Methods

We conduct a retrospective study among 313 paired human sera (acute- and convalescent-phase) from febrile patients collected from December 2014 to September 2016 in Caia, a rural area of Sofala Province in the Centre of Mozambique, as part of the sentinel surveillance systems for emerging infectious diseases. Samples were tested by enzyme-linked immunosorbent assay (ELISA) for IgM and IgG antibodies against WNV.

25.1.3 Results

The median age of study participants was 22.1 yers, of which 60.1% (188/313) were female A total of 149 (47.6%) of 313 patients had positive serology result for acute- or convalescent serum samples. All acute serum samples were screened by using 1-Step real-time reverse transcription PCR for WNV. Results were negative for viral RNA.

25.1.4 Discussion and Conclusion

Data from our study provide evidence that WNV occurs in Caia, Mozambique. Investigations should be conducted in order to confirm serological evidence and determine the genotype of the circulating virus. Altogether, our data suggests that establishment of animal and human surveillance systems for WNV is urgently needed in Mozambique.

25.2 Seroepidemiology of Chikungunya in Measles and Rubella Negative Samples, 2009–2014

V. S. António · N. Amade · A. F. Muianga · V. Monteiro · F. Mula · I. R. Chelene · I. Chongo · S. Ali · J. Oludele · K. Falk · Eduardo Samo Gudo

Keywords Chikungunya, Misdiagnosis

25.2.1 Introdution

Chikungunya virus (CHIKV) is a rapidly spreading arbovirus transmitted by the bites of *Aedes* mosquitoes that belongs to the genus *Alphavirus*. However, because of lack of diagnostics as well as low level of clinical awareness, most of CHIKV cases remain unsuspected and the vast majority are misdiagnosed and treated as malaria. The main symptoms of CHIKV infection are rash, arthralgia, fever, myalgia and arthritis that are similar to other viral diseases such as Measles and Rubella. The objective of this study was to investigate the frequency of CHIKV in patients with suspected infection by measles and rubella in Mozambique during 2009–2014.

25.2.2 Methodology

A total of 372 samples that were negative for measles and rubella collected between 2009–2014 in Mozambique were included in this study. All samples were screened for IgG antibodies against CHIKV using commercially available ELISA (Euroimmun).

25.2.3 Results

The mean age of participants was 7 years (IQR: 0–38 years) Of the 372 samples, 19.6% (73/372) were positive for IgG anti-CHIKV. Positive samples were reported in the following districts: Funhaloro, Mabote, Inhassoro, Xai-xai, Namuno, Mueda, Manhiça, Lugela, Pebane, Namakura, Mocuba, Alto Molócue, Ile, Namarroi, Quelimane, Morrumbala, Gorongosa Barue,

25.2.4 Conclusion

Our results suggest that CHIKV is endemic in Mozambique for several years and most of cases occurs unsuspected. Lack of diagnostics and lower level of clinical awareness are considered main challenges and barriers for diagnostics of

CHIKV. Altogether, we recommend that training of clinicians, incorporation of CHIKV diagnostics into the laboratory network, as well as establishment of surveillance of CHIKV are urgently needed in Mozambique.

25.3 Seroepidemiology of Zika Virus in Sarampo and Rubella Negative Samples

I. R. Chelene · A. F. Muianga · N. Amade · F. Mula · I. Chongo · V. S. António · S. Ali · J. Oludele · V. Monteiro · A. Tivane · Eduardo Samo Gudo

Keywords Zika, Measles, Rubella

25.3.1 Introduction

Zika virus is an arbovirus belonging to the *Flavivirus* genus of the family *Flaviridade*. Zika virus is transmitted by mosquitoes of the *Aedes* genus, especifically *Aedes aegypti* and *Aedes albopictus*. Most common symptoms of the infection are fever, rash, arthralgias and non-purulent conjunctivitis. Because symptoms of zika infection are similar to dengue and chikungunya, patients should be evaluated for these infections. Other considerations in the diagnosis include malaria and rubella, especially because of rash. In Mozambique, there are no recent data on the epidemiology of zika virus. The aim of the study was to investigate the seroepidemiology of Zika in patients with suspected infection by measles and rubella.

25.3.2 Methodology

Overall, 638 samples collected between 2009 and 2014, from throughout the country as part of the national measles and rubella surveillance, were included in this study. Negative samples for measles and rubella were tested for Zika virus using commercial ELISA reagents for Zika IgM and IgG antibodies (EUROIMMUN).

25.3.3 Results

Of the 638 enrolled samples, 4.9% (29/589) presented positive results for IgM and 1.0% (6/591) presented positive results for IgG. IgM positive samples were from Nampula (9), Niassa (6), Sofala (5), Zambézia (4), Manica (2), Gaza (2) and Maputo City (1). IgG positive samples were from Niassa (2), Nampula (1) Sofala (1), Zambézia (1) and Cabo Delgado (1).

25.3.4 Conclusion

Our data provide preliminary evidence that Zika virus maybe causing unsuspected outbreaks or disease throughout the country for many years and should be considered in differential diagnosis of sarampo and rubella, especially those without other apparent cause of rash. Further testing should be performed to confirm this preliminary data.

25.4 Antibodies Against Arboviruses in Non-febrile Patients in Northern Mozambique, Pemba, 2014

I. Chongo · K. Falk · N. Lagerqvist · I. Chelene · F. Inalda · A. Muianga · G. Pinto · S. Ali · J. Oludele · V. S. António · Z. Chimbuinhe · G. Galano · Eduardo Samo Gudo

Keywords Dengue, Chikungunya, West Nile Virus, Rift Valley Fever, Arbovirus

25.4.1 Introduction

Arboviruses are a group of rapidly spreading mosquito borne virus, of which dengue virus (DENV), chikungunya virus (CHIKV), West Nile Virus (WNV) and Rift Valley Fever (RVFV) have been reported to circulate in Sub-Saharan Africa. Those viruses are mostly transmitted by mosquitoes from *Aedes* genus. In Mozambique, arboviruses have been heavily neglected and for this reason, they are not considered in the differ-

ential diagnosis of acute non-febrile illness. Past and recent evidence indicates that Pemba city, situated in northern Mozambique represents a hotspot for arbovirus in the country. The aim of the study was to investigate the presence of antibodies against DENV, CHIKV, WNV and RVFV in non-febrile patients in Pemba, after the outbreak of dengue in 2014.

25.4.2 Methodology

Non-febrile people residing in Pemba city were enrolled in this study between March and April 2014. Blood samples were collected from 243 individuals and shipped to the Virus Isolation Laboratory at National Institute of Health in Maputo. Serum samples were tested for IgG and IgM antibodies against DENV by an ELISA (Panbio). Then they were tested for IgG antibodies against CHIKV and RVFV by immunofluorescence. IgM and IgG antibodies against WNV were tested using commercially available ELISA reagents (EUROIMMUN).

25.4.3 Results

Of the 243 non-febrile participants, 24% (58/243) were positive for dengue IgG, 21% (50/243) for dengue IgM, 49% (118/243) for CHIKV IgG, 1% (3/243) for RVF IgG, 87% (212/243) for WNV IgG and 7% (17/243) for WNV IgM.

25.4.4 Conclusion

Our data provide evidence that DENV, WNV, CHIKV and RVFV circulate in Pemba, situated in northern Mozambique, suggesting that not only dengue but also other arboviruses such as chikungunya, West Nile and Rift Valley Fever, should be considered in the differential diagnosis of acute in non-febrile patients.

25.5 Serological Evidence of *Flavivirus* Infections Among Pygmy Populations in Cameroon

I. Jala · M. Mazelier · R. Njouom · E. Betsem · A. Gessain · M. Demanou

25.5.1 Introduction

Arboviruses are the causative agents of significant morbidity and mortality among humans and domestic animals globally. Although there are several types of life cycles, many arboviruses have basically a sylvatic cycle. Pygmies are known to be the first inhabitants of the forest. In Cameroon, little is known about arboviruses prevalence among these populations [1]. Since 2004, Centre Pasteur of Cameroon (CPC) is the reference center for the yellow fever case-based surveillance. In 2013, CPC launched the first sentinel surveillance project of arboviruses [2]. All these monitoring activities however do not involve the Pygmy populations, who seldom visit hospitals and whose lifestyle increases the risk of exposure to arboviruses.

AIM: To assess the endemicity of *Flaviviruses* such as yellow fever virus (YFV), dengue virus (DENV) and West Nile virus (WNV) in the Baka Pygmy population of Cameroon.

25.5.2 Materials and Methods

A cross-sectional study was carried out in different pygmy camps in Cameroon between 2005 and 2010. Serum samples from volunteers were collected and processed. A total of 242 samples obtained from the Baka pygmies were included in this study. These sera were tested for the detection of Immunoglobulin M antibodies (IgM) to three most common *Flaviviruses* such as YFV, DENV and WNV; using in-house M Antibody Capture

Enzyme Link ImmunoSorbent Assay (MAC-ELISA) technique adapted from CDC (Center for Disease Control and Prevention, Atlanta, USA) and World Health Organization (WHO) protocols. Antigens used were either produced by the CPC arbovirus laboratory (DENV and WNV antigens) or provided by WHO (YFV antigens).

25.5.3 Results

Of the 242 serum samples tested, 9 (4%) were IgM positive for *Flavivrus* antibodies, synonym of recent or ongoing arbovirus infections. IgM ELISA was positive in 8 (3.3%) and 1 (0.4%) samples respectively for YFV and WNV. One sample was IgM positive for both YFV and DENV suggesting *Flavivirus*-induced antibody cross-reactivity.

25.5.4 Conclusion

Flaviviruses are prevalent in pygmy camps in Cameroon and remind us to pay attention to these populations when implementing public health activities. Therefore, further clinical and virological surveillance of arbovirus infections among Pygmies should be consider, and could lead to their isolation and characterization.

25.6 Chikungunya-Where Can We Find the Virus?

K. Falk

Chikungunya fever is an acute febrile illness associated with severe, often debilitating polyarthralgias. The disease is caused by Chikungunya virus (CHIKV), an arthropod-borne virus that is transmitted to humans primarily via the bite of an infected mosquito. Chikungunya virus is a member of the genus Alphavirus and the family Togaviridae.

The global expansion of CHIKV, the clinical features of the disease, laboratory diagnostics European perspective, and the spread on the African continent will be discussed.

25.7 Preliminary Results of an Investigation of Chikungunya Fever Among Acute Febrile Patients Attending 4 Medical Centers in Quelimane, Mozambique

O. C. Guiliche · A. J Cristovam · A. F. Muianga · E. Jaszi · Eduardo Samo Gudo

25.7.1 Introduction

Quelimane city in central Mozambique has a large burden of non-malaria undifferentiated illness, representing 63% of all acute febrile illnesses registered in public health facilities in 2015. In January 2016, a surveillance system for acute febrile illness was established at the Coalane Health Center in Quelimane to investigate the etiology of undifferentiated fever and a high rate of positivity was observed. Health officials suspected of a potential outbreak of CHIKV and an outbreak investigation team was deployed from the National Institute of Health to conduct a preliminary investigation of the magnitude of the disease in the city.

25.7.2 Methods

A cross-sectional investigation was conducted between May 21st and June 3rd at 4 medical centers. Acute febrile patients were consecutively enrolled at the medical centers. Patients who tested positive for malaria using routine clinic testing were included to assess the rate of co-infection. Acute samples were screened using rapid testing (SD BIOLINE immunochromatografic lateral-flow test for CHIKV (IgM) and dengue (NS1, IgM and IgG). Samples were transported to INS for future confirmatory testing. A questionnaire was

used to collect socio-demographic and clinical information from all participants.

25.7.3 Results

A total of 66 patients were enrolled at 4 health centers. Of these, 60 (90.9%) had a history of fever for less than 5 days. The median age of study participants was 28 years (IQR: 6–62 years). Headache, cold and arthralgia were the most frequent symptoms reported in 59 (89.4%), 51 (77.3%) and 48 (72.7%) patients, respectively. A total of 29 (43.9%) tested positive for IgM anti-CHIKV antibodies; 21 (31.8%) tested positive for malaria; and 3 had positive NS1 results for dengue. Twenty seven (40.9%) patients had negative results for all three diseases. Among patients suspected of CHIKV, 11 (37.9%) were co-infected with malaria, 1 (3.4%) co-infected with suspected dengue, and 2 (6.9%) co-infected with both malaria and dengue. Among patients positive for anti-CHIKV antibodies 11 (37.9%) received antibiotics.

25.7.4 Conclusion

This investigation provides serological evidence that CHIKV is a highly prevalent in Quelimane city and that co-infection with malaria is common. Confirmatory testing of samples using ELISA and PCR is planned. CHIKV as well as dengue should be considered in the differential diagnosis of fever in Mozambique, and there is a need to train health professionals in the diagnosis and management of these diseases. Interventions for *Aedes* mosquito vector control are also urgently needed. Additional research and strengthening of surveillance systems is required to better understand the burden and etiology of non-malarial febrile illnesses throughout Mozambique.

25.8 Cross-Sectional Seropepidemiology of Rift Valley Fever and Alphavirus in South-Western Tanzania, and a Prospective Fever Study

N. Heinrich

Background: the contributions of arthropod-borne disease to febrile disease etiology are unknown for most sub-saharan African settings. Rift Valley fever (RVf) and Chikungunya (CHIK) are important viral diseases with a potential for large outbreaks, but are also encountered in low intensity endemic transmission.

Methods: we conducted a seroprevalence study on 1228 patients from South-Western Tanzania, stratified by age, elevation of residence, and ownership of domestic mammals. The findings of this seroprevalence study were used to design a study on acutely febrile outpatients in two centers. The target sample size of more than 1000 patients was thought necessary to detect endemic cycling of RVf and CHIKV.

Results: we found an overall seroprevalence of 5,2% for RVf, with a local maximum of 29,3% at the shore of Lake Malawi. Seroprevalence for *Alphavirus*, with CHIKV as the most probably causative agent, was 18% overall. Distribution of *Alphavirus* seropositivity across the study area was more even than for RVf, mostly influenced by elevation of residence. Geographical distribution and associated covariates allow some conclusions on virus cycling in the area, e.g. a dependence of RVfV on seasonal flooding, and on the presence of cattle. A near-linear increase of seroprevalence with participant age suggested endemic, rather than epidemic cycling of both viruses.

Results on acutely febrile patients are currently being generated and will be presented.

Conclusion: seroprevalence findings suggest that RVf and CHIK circulate in South-Western

Tanzania, an area with no previous report of these infectious agents. These assumptions suggest that a high sample size is necessary to detect these diseases in an outpatient population.

25.9 Seroepidemiology of CHIKV in Maputo, Mozambique, March–April 2016

V. Monteiro · A. F. Muianga · F. Mula · I. R. Chelene · V. S. António · I. Chongo · G. Pinto · S. Ali · J. Oludele · A. Tivane · G. Malai · O. C. Guiliche · Eduardo Samo Gudo

Keywords Dengue, Chikungunya, Co-circulation

25.9.1 Introduction

Chikungunya virus (CHIKV) is a rapidly emerging vector borne virus transmitted by the bites of *Aedes* mosquitoes. Spread of CHIKV has been mostly attributed to global warming, rapid urbanization and globalization. However, the disease is seriously neglected in settings where Malaria is endemic. In these settings, most of chikungunya infections go undetected because of similarity of clinical presentation with malaria, lack of diagnostic capacity for CHIKV and low level of awareness. Although *Aedes* has been shown to be endemic in Mozambique, few studies have been conducted to investigate the circulation of CHIKV.

25.9.2 Methodology

A total of 82 patients with fever of unknown etiology for less than 7 days being attended at Polana Caniço Hospital, in Maputo Mozambique were enrolled and screened for CHIKV using commercially available ELISA to measure IgG antibodies against CHIKV (EUROIMMUN).

25.9.3 Results

The mean age of the enrolled participants was 33 years old (IQR: 21–45 years old) and 51.2% (42/82) were female. Of the 82 enrolled patients, 26,8% (22/82) presented positive results for malaria, 36.6% (30/82) for CHIKV IgG, and 6,1% (5/82) were dual positive for malaria and CHIKV IgG. All samples were negative for dengue IgG and NS1.

25.9.4 Conclusion

This study shows that CHIKV is prevalent in Maputo city and might be causing several unsuspected outbreaks. Altogether, we recommend that CHIKV should be considered in the differential diagnostics of febrile diseases in Maputo city and also recommend that training of clinicians accompanied by strengthening of surveillance systems are urgently needed.

25.10 Preliminary Results of Neutralizing Anti-Chikungunya Antibody in Central and Northern Mozambique, 2015–2016

A. F. Muianga · B. M. Kümmerer · V. Monteiro · F. Mula · I. R. Chelene · I. Chongo · G. Pinto · S. Ali · J. Oludele · V. Antonio · Eduardo Samo Gudo

Keywords Chikungunya, Neutralization assay

25.10.1 Introduction

Chikungunya fever (CHIKV) is an emerging arbovirus that have been overlooked and under diagnosed in most of the countries including Mozambique, despite its rapid spread worldwide.

CHIKV is transmitted by *Aedes aegytpi* and *Aedes albopictus* mosquitoes. Main signs and symptoms are arthralgia, fever, headache, diffuse back pain, myalgia, nausea and rash. In Mozambique, most of the cases of CHIKV are misdiagnosed and treated as malaria. The aim of the study is to investigate the frequency of anti-chikungunya neutralizing antibodies in provinces situated central and northern Mozambique in 2015–2016.

25.10.2 Methodology

Overall, 573 acute febrile patients with undifferentiated fever were enrolled from the outpatient appointment visit at health facilities in Pemba, Lichinga, Nampula, Quelimane, Tete and Caia cities, between 2015 and 2016. Blood sample was collected from each participant. Samples were tested on site using rapid immunochromatograhpic test for IgM anti-CHIKV (SD Bioline), ELISA for IgM and IgG anti-CHIKV (EUROIMMUN) and neutralization assay (NT) using Virus Replicon Particles (VRP).

25.10.3 Results

Of the 573 enrolled patients, 6.9% (40/573) presented positive results for both IgM and IgG anti-CHIKV from ELISA test; all positive samples (40/573) from ELISA test were tested using NT of which 62.5% (25/40) showed neutralization activity against CHIKV.

25.10.4 Conclusion

Our data provide evidence that Chikungunya circulates in central and northern Mozambique, suggesting that CHIKV represents an important yet neglected cause of acute febrile syndrome in the country. Our results also suggest that strengthening of surveillance systems for arbovirus, training of clinicians and implementation of interventions to control *Aedes* mosquitoes are urgently needed in Mozambique.

25.11 Sero-Prevalence of Dengue in Patients with Suspected Infections by Measles and Rubella in Mozambique: 2009–2014

F. Mula · A. Muianga · I. Chelene · N. Amade · V. S. António · I. R. Chongo · S. Ali · J. Oludele · A. Tivane · V. Monteiro · Eduardo Samo Gudo

Keywords Dengue, Measles, Rubella, Co-infection

25.11.1 Introduction

Dengue (DENV) is a febrile viral infection and the most important arboviral disease worldwide. This virus is transmitted by *Aedes aegypti* and *Aedes albopictus*. Despite the difference in the mode of transmission between DENV, measles and rubella, they have similar clinical presentation such as presence of fever and rash, and for this reason most of time is difficult to differentiate the diseases based on clinical presentation. However, no data exist in Mozambique on the burden of DENV among patients with suspicion of measles and rubella whose laboratory results are negative for both. The aim of the study was to determine the sero- prevalence of Dengue in patients with suspected infection by measles and rubella between 2009 and 2014, Mozambique.

25.11.2 Methodology

Samples were collected from individuals suspected of measles and rubella infection from throughout the country between 2009 and 2014. This samples were sent in a cold chain to the

Serology Laboratory at National Institute of Health (INS) in Maputo, and were all tested for both IgM anti-measles and anti- rubella using ELISA. All negative samples were further screened for DENV infection using commercially available ELISA to detect DENV NSI antigen and IgG and IgM anti-DENV antibodies (all from Panbio).

25.11.3 Results

Of 638 samples with measles and rubella negative results, 2,8% (16/566) and 29% (40/136) samples were positive for NS1 antigen and for IgM respectively, however of the 234 samples tested for IgG, all were negative.

25.11.4 Conclusion

Our data suggest that DENV is circulating in Mozambique since several decades and may be endemic in several parts of the country. In addition, DENV represents an important proportion of febrile patients with rash in Mozambique should be considered in the differential diagnosis of measles and rubella which highlight that DENV surveillance might be integrated into existing case based surveillance for measles and rubella.

25.12 Evidence of Transmission of Dengue and Chikungunya Viruses in Coastal and Western Kenya

F. Mutuku · D. M. Vu · B. Ndenga · E. Harris · M. Heise · D. Mukoko · J. Vulule · U. Kitron · C. King · A. D. LaBeaud

Dengue virus (DENV) and chikungunya virus (CHIKV) are important re-emerging mosquito-borne pathogens that have been spreading rapidly, causing endemic and epidemic disease in tropical and sub-tropical regions. For many African countries, limited resources and lack of national surveillance systems make evaluating the true burden of DENV and CHIKV disease difficult. In 2014, we initiated a prospective study to measure DENV and CHIKV prevalence and incidence in children who develop febrile illnesses, and seroprevalence among healthy children in western and coastal Kenya. Testing of serum samples for IgG and IgM to DENV and CHIKV by ELISA is ongoing, and neutralizing antibodies will be measured in a subset of samples. Preliminary results from IgG assays of samples from children residing in coastal and western Kenya confirm active transmission of both DENV and CHIKV. Specifically, among children who presented at the local health center with undetermined febrile illness from whom paired acute and 1-month convalescent serum samples were obtained. Out of 1526 paired serum samples tested, 69 (4.5%, 95% CI 3.5–5.7%) subjects were DENV IgG positive and 11 (0.7%, 95% CI 0.4–1.3%) seroconverted. The incidence of DENV infection was 452 per 10,000 cases of febrile illness in Kenyan children. Chikungunya seroincidence was estimated at 0.6% (95% CI: 0.271% – 1.475%), and chikungunya seroprevalence was significantly higher in western Kenya than on the coast. Further, IgG seroprevalence in healthy children from Ukunda was 1.7% for CHIKV (CI_{95} 0.008–0.029) and 1.4% for DENV (CI_{95} 0.006–0.26). PCR testing for DENV for the acutely ill children from western Kenya indicated a prevalence of 10.7% (44/410) in Chulaimbo health centre and 7.2%, (34/470) in Obama children hospital. These data will need to be confirmed by plaque reduction neutralization testing, as DENV and CHIKV can cross react with closely related flaviviruses and alphaviruses, respectively, on ELISA. However, our finding suggest that DENV and CHIKV transmission are presently occurring in coastal Kenya and underscore the need for surveillance of these rapidly re-emerging infections to monitor developing outbreaks and allocate limited public health resources.

25.13 Post Outbreak Surveillance of Dengue Reveals Dengue Endemicity in Northern Mozambique, 2014–2016

J. Oludele · A. F. Muianga · S. Ali · G. Pinto · V. Monteiro · O. C. Guiliche · F. Mula · I. R. Chelene · V. S. António · I. Chongo · G. Gallano · S. Mendes · A. Tivane · Eduardo Samo Gudo

Keywords Dengue, Endemicity, ELISA (IgM, IgG, NS1) and PCR.

25.13.1 Introduction

A Dengue outbreak was reported in two cities in northern Mozambique in 2014, namely, Nampula and Pemba cities. The National Institute of Health established in 2014 a surveillance system of Dengue in these cities. The objective of post outbreak surveillance of Dengue was to monitor the trend in the prevalence of Dengue and provide evidence for definition of interventions for controlling Dengue in Mozambique.

25.13.2 Method

Case based surveillance of Dengue was established in 2014 in ambulatory patients attended in Pemba Provincial Hospital and Nampula Central Hospital. From patients who met the case definition, a 5 mL whole blood sample was collected and sent to the Virus Isolation Laboratory at National Institute of Health in Maputo. All samples were tested using ELISA commercial reagents to detect IgM and IgG antibodies against Dengue as well as Dengue NS1 antigen (http://www.who.int/csr/resources/publications/dengue/034–47.pdf). PCR was performed using samples from both cities to confirm and identify dengue virus serotype (http://www.cdc.gov/dengue/clinicalLab/realTime.html).

25.13.3 Results

Between January 2015 and March 2016, a total of 235 samples were collected, of which 130 were from Pemba and 105 from Nampula. Positivity rate for IgM anti-Dengue, IgG anti-Dengue and Dengue NS1 antigen were 65 (27.6%), 27 (11.4%), and 60 (25.5%). Positivity rate for IgM anti-Dengue and Dengue NS1antigen from Pemba and Nampula samples are 25 (19.2%), 7(5.3%), 40(38%), and 53(50.4%) respectively. PCR results of a total of 38 samples of which 22 is from Pemba and 16 from Nampula indicates 11(28.9%) were positive for DENV2 as the only serotype in circulation in both cities.

25.13.4 Conclusion

Preliminary finding from surveillance of Dengue shows that the virus has become endemic in northern Mozambique with probability of further spread to other regions in Mozambique, demanding the implementation of urgent public health interventions to control the disease. We also demonstrated that the establishment of permanent surveillance of Dengue plays an important role in monitoring the burden of Dengue as well as the prevailing serotypes.

References

1. Boche R, et al. (1974) Bull Soc Path Exot 67(2):126–1240
2. Demanou M, et al. (2015) Virologica Sinica 30(6):470–473

Index

A
Alpha glucosidase inhibitors, 275, 279, 292, 296, 324, 357
Animal models of infection, 309–311
Antibody detection, 24–26
Antiviral drug discovery, 125, 142
Antiviral iminosugar, 266, 272–274
anti-Zika virus drug discovery, 141, 142
Arboviruses, 1–9, 11–17, 24, 25, 35, 184, 361–371
Arbovirus transmission, 3, 5–8, 15, 359
Arthropod-borne viruses, 1, 2, 6, 171, 366

B
Biological mosquito control, 355
Boronic-acid inhibitor, 134, 135, 138
Broad spectrum antiviral, 265–275, 289, 321

C
CD4+ T cells, 241, 242, 244–248, 257–259, 275
CD8+ T cells, 226, 241, 242, 244–248, 255–259, 261
Chikungunya, 2, 12, 14, 15, 290, 357, 363, 366, 368, 370
Climate change impacts, 8
Conditional knockout IFNAR mouse model, 227
Congenital Zika syndrome (CZS), 132–133
Crystal structure, 80, 82, 89–94, 122, 123, 134, 137–143, 149, 150, 154, 155, 158, 176, 177, 183, 188–190, 194, 196, 204, 266–268, 270, 272–274
Cytoplasmic incompatibility (CI), 355–357

D
Dengue
  animal models, 215–231
  antiviral drug development, 319–328, 337–338
  associated vascular leak, 96, 97
  biomarker, 327, 328
  diseases, 89, 94, 95, 226, 244, 252, 253, 255, 256, 258, 274, 278, 295, 327, 334, 335, 337, 341, 343, 347
  epitopes, 65–67
  NS1, 37, 39, 89, 111, 112, 256
  NS1 induced toxicity, 96
  T cells, 243–247
  vaccines, 72, 176, 216–226, 229, 230, 242, 244, 247, 252, 258–259, 278, 337–338, 343, 347
Dengue virus (DENV), 1, 11, 20, 47, 63–64, 78, 89, 109, 116, 132, 148, 166, 175, 188, 199, 215, 242, 252, 266, 277, 309, 319, 334, 359, 364
Dengue virus infectious clone, 122, 152, 204
Differential diagnosis, 20, 21, 24, 25, 33–34, 295, 364, 365, 367, 370
Drug discovery, 149, 177, 180, 183, 184, 304, 322, 333–348
Drug repurposing, 320

E
Endplasmic reticulum quality control (ERQC), 265, 266
Entry inhibitors, 107
Envelope (E) protein, 46, 63, 64, 67, 68, 70, 73, 78–79, 93, 108, 109, 112, 113, 116, 117, 133, 148, 166–168, 170, 200, 201, 220, 228, 231, 252–254, 279, 282, 320, 322, 323
Enzyme-linked immunosorbent assay (ELISA), 21–38, 151, 216–221, 228, 363–371
ER α-glucosidases, 265–275, 279, 291, 292, 294, 296
ER-associated degradation (ERAD), 278, 292, 294, 296

F
Flavivirus
  entry mechanism, 107
  reverse genetics technology, 166, 171
Fluorodeoxyglucose (FDG) imaging, 328

G
Galactose-mimicking iminosugars, 294
Global funding for dengue R&D, 335, 336
Glucomimetic inhibitors, 266, 274
Glucose-mimicking iminosugars, 294

© Springer Nature Singapore Pte Ltd. 2018
R. Hilgenfeld, S. G. Vasudevan (eds.), *Dengue and Zika: Control and Antiviral Treatment Strategies*, Advances in Experimental Medicine and Biology 1062,
https://doi.org/10.1007/978-981-10-8727-1

H
High content imaging (HCI), 304–308, 315
Host targets, 322, 337
Human antibodies, 63–73, 175, 323
Humanized mice for dengue and Zika vaccine testing, 229
Hydrogen-deuterium exchange mass spectrometry, 123, 157, 158, 268, 270
N(4-hydroxyphenyl) retinamide (4-HPR), 206–208

I
Indirect immunofluorescence test (IIFT), 21, 23–26, 29, 31–36
Inhibitor, 97, 110, 119, 134–138, 149, 175–184, 189, 201, 266, 279, 305, 321, 337
Innate immune system, 51, 118, 133, 251
Innate immunity evasion, 115–125

K
KIR peptide-MHC interactions, 256–257

M
Malaria seroepidemiology, 12
Mechanism of inhibition, 192–195
Molecular surveillance of emerging viruses in Africa, 364, 366, 368, 371

N
Natural killer (NK) cells
  activating receptors on, 251, 253–255, 258
  inhibitory receptors on, 252, 253, 255–258
Neutralizing antibodies, 12, 14, 15, 54, 64, 65, 68–72, 78–80, 98, 108, 170, 171, 216, 230, 325, 369, 370
Next-generation sequencing (NGS), 304
Niénokoué virus (NIEV), 171
N-linked glycoproteins, 266, 278–279, 290, 291, 295
Non-IFN-receptor-based immuno-compromised mouse models, 226–227
Non-infectious replicons, 166
Non-structural protein 1 (NS1)
  antigen specific test for dengue, 28
  antigen specific test for Zika, 24–26
  delipidation, 111
  dengue, 37, 39, 89, 111, 112, 256
  pathogenecity, 113
  secreted, 80, 82, 90–97, 99
NS3 antibody, 149–150
NS5 antibody, 150–151
NS3 helicase (NS3H), 122, 149, 150, 202, 337–339
NS5 RNA-dependent RNA polymerase (NS5 RdRp), 47, 48, 149, 202, 208, 337–339
Nuclear magnetic resonance (NMR) spectroscopy, 140–141, 149
Nuclear trafficking, 202–205, 209
Nucleocytoplasmic trafficking, 199–210
Nucleus, 150, 153–158, 167, 201–205, 321

O
Original antigenic sin, 241–244

P
Pathogenesis, 52, 55, 78, 80, 89, 96, 97, 99, 226, 256, 295, 309, 312, 313, 320–322, 326–328, 338, 341, 344
Peptides, 120, 122, 137, 138, 151, 152, 181–183, 209, 242, 243, 246, 247, 252, 253, 255, 256, 258, 259, 268, 270, 312
Positron emission tomography (PET) imaging, 310, 313
Protease, 63, 78, 90, 116, 131–143, 148, 166, 176, 196, 200, 337
Protein-protein interactions (PPI), 115–125, 148, 152
Public-private partnerships (PPPs), 338, 341–347

R
Randomised control cluster trial, 359
Rapid medical countermeasure to emerging viruses, 304, 305
Rational design, 195
Replication, 45–56, 71, 72, 78, 80, 90, 93, 95, 96, 99, 115–125, 133, 147–158, 166–171, 180, 181, 192, 194, 195, 200–202, 204, 206, 209, 216, 226, 227, 246, 257, 280–283, 292, 295, 296, 311, 312, 314, 315, 321–323, 337, 338, 357
Replication complex (RC), 46, 51, 53, 90, 93, 115–125, 148, 195, 200–202, 204
Resistance phenotype, 195
RNA dependent RNA polymerase (RdRp), 46, 48, 52, 118, 123, 124, 133, 148–151, 153, 155, 157, 158, 187–197, 203, 205, 321, 322

S
Secreted NS1 (sNS1), 80, 82, 90–97, 99
Selectable marker proteins, 170
Serological tests, 19–39
Seroprevalence of emerging, 362, 367, 370
Site-directed mutagenesis, 93, 118, 123, 157
Skin homing marker, 246
Small-molecular, 177–183
Structures, 45–56, 65, 77–84, 89–94, 117, 118, 120, 123, 137–142, 150, 151, 154, 155, 157, 169, 176, 177, 183, 188, 194, 196, 204, 266, 268, 272–274, 294, 296, 311, 343

T
T cells
  CD4+, 241, 242, 244–248, 257–259, 275
  CD8+, 226, 241, 242, 244–248, 255–259, 261
  dengue specific, 243–247
  response to dengue vaccine, 258–259
  tissue-resident, 246, 247
Therapeutic antibodies, 109, 230, 320

V
Virology in Africa, 361–371
Virosphere, 2

W
West Nile, 2, 21, 176, 178, 181, 182, 290, 357
wMel *Wolbachia* strain, 357
World Mosquito Program (WMP), 355–359

X
X-ray crystallography, 79, 119, 122, 123, 140, 190, 195, 266, 323

Y
Yellow fever virus NS2A protein, 168

Z
Zika non-human primate model, 220–221
Zika virus (ZIKV)
  disease, 166
  history, 132
  protease, 131–143, 149, 176, 177, 181–184
  protease dimerization, 137, 138
Zoonotic infections, 5