Cracking the Code of Chikungunya Virus: Inhibitors as Tools to Explore Alphavirus Biology

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DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

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CRACKING THE CODE OF CHIKUNGUNYA VIRUS:
INHIBITORS AS TOOLS TO EXPLORE ALPHAVIRUS BIOLOGY

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Biological and Environmental Sciences, for public examination in the auditorium 1041 of Biocenter 2, Viikinkaari 5, Helsinki on 2nd December, 2016 at 12 o’clock noon

HELSINKI 2016
The Road goes ever on and on
Down from the door where it began.
Now far ahead the road has gone,
And I must follow, if I can,
Pursuing it with weary feet,
Until it joins some larger way,
Where many paths and errands meet.
And whither then? I cannot say.

J.R.R. Tolkien, The Lord of the Rings
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I Discovery of berberine, abamectin and ivermectin as antivirals against chikungunya and other alphaviruses.


I The antiviral alkaloid berberine reduces chikungunya virus-induced mitogen-activated protein kinase (MAPK) signaling.

Varghese FS, Thaa, B, Naqiah SA, Simarmata D, Rausalu K, Nyman TA, Merits A, McInerney GM, Ng LFP, Ahola T.

III Obatoclax inhibits alphavirus fusion by neutralizing the acidic environment in endocytic compartments.

Varghese FS, Rausalu K, Hakanen M, Kümmerer BM, Susi P, Merits A, Ahola T.
Manuscript.

The author’s contribution:

I FSV designed all experiments together with PK and TA. FSV performed all the experiments excluding the screening, primary validation of the hits and the experiments with yellow fever virus. FSV analyzed the data and made the final figures. FSV wrote the paper with amendments by TA.

II FSV designed all the experiments with advice from TA, excluding the in vivo experiments in the mouse model. FSV performed all the experiments, except antiviral dose response assays in HEK-293T and CRL-2522 cells, antiviral activity against different CHIKV strains, Northern blot, signaling Western blots for virus-induced MAPK activation and the mouse experiments. FSV analyzed the data together with BT and made the final figures, excluding the one for in vivo experiments. FSV wrote the paper with suggestions and contributions by the other authors.

III FSV designed all the experiments with advice from TA, excluding the experiments with picornaviruses. FSV performed all the experiments, except Northern blot, sequencing of resistant mutants and experiments with viruses from other families. FSV analyzed the data and made the final figures. FSV wrote the paper with suggestions and contributions by the other authors.
Chikungunya virus (CHIKV) is an arbovirus spread by the *Aedes* sp. of mosquitoes. Chikungunya fever results in a sudden onset of a febrile disease with headache, nausea and maculopapular rash. Additionally, a large proportion of the affected individuals experience persistent arthralgia months after all other signs of the disease have vanished. Originally discovered in Tanzania in 1952, it re-emerged with a massive outbreak in several islands of the Indian Ocean in 2004 and spilled over onto the Indian sub-continent and South-east Asia. Later CHIKV invaded Southern Europe and since the last two years has ventured into the western hemisphere, causing more than 1 million suspected infections in the Caribbean islands, Central and Latin America. The explosive nature of these outbreaks has led to a tremendous strain on the public health system of many of the affected countries already burdened with the endemically circulating Dengue virus.

So far, no licensed vaccines or antivirals exist to counter this virus. Besides, it is paramount to have an in-depth understanding of the replication mechanisms of this re-emerging pathogen in order to come up with novel and effective therapeutic measures. A previously characterized CHIKV replicon cell line was used to conduct a high-throughput screen of ~3000 bioactive compounds, which are in clinical use or in clinical trials against other diseases. This led to the discovery of abamectin, ivermectin and berberine as novel antivirals effective at low micromolar concentrations and having broad-spectrum anti-alphaviral activity.

Deciphering the mode of action of berberine led to the discovery that CHIKV infection robustly activates the three main branches of the mitogen-activated protein kinase (MAPK) signaling – extracellular signal regulated kinase (ERK), p38 MAPK and c-Jun NH2-terminal kinase (JNK). Berberine was shown to reduce this virus-induced MAPK activation and also suppressed virus-independent ERK activation. These pathways were shown to be important for CHIKV replication, as specific inhibitors of the ERK and JNK pathways significantly reduced the viral progeny release. Most importantly, berberine reduced CHIKV-induced inflammatory disease in a mouse model and is one of the few compounds reported to show in vivo efficacy.

Exploring the antiviral mechanism of obatoclax, an anticancer compound previously reported to be active against different viruses, including influenza A virus and Sindbis virus, revealed the compound to be active against other alphaviruses, including SFV and CHIKV. Further characterization showed that obatoclax inhibits viral fusion by rapidly neutralizing the acidic environment of endolysosomal organelles. Additionally, characterization of escape mutants showed that a single mutation in the SFV E1 fusion protein was sufficient to confer partial resistance against obatoclax.

This study has unearthed effective candidate antivirals against alphaviruses, which have served as useful tools to help us gain further insight into alphavirus biology, when characterizing their modes of action.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AdoMet</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ATF-2</td>
<td>Activating transcription factor 2</td>
</tr>
<tr>
<td>AUD</td>
<td>Alphavirus Unique Domain</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 homologous antagonist killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
</tr>
<tr>
<td>BIM</td>
<td>Bcl-2-like protein 11</td>
</tr>
<tr>
<td>BST2</td>
<td>Bone marrow stromal antigen 2</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CHIKV</td>
<td>Chikungunya virus</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>CSE</td>
<td>Conserved sequence elements</td>
</tr>
<tr>
<td>CV</td>
<td>Coxsackievirus</td>
</tr>
<tr>
<td>DALY</td>
<td>Disability Adjusted Life Year</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin</td>
</tr>
<tr>
<td>DENV</td>
<td>Dengue virus</td>
</tr>
<tr>
<td>DMXAA</td>
<td>5,6-dimethylxanthenone-4-acetic acid</td>
</tr>
<tr>
<td>EBOV</td>
<td>Ebola virus</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>ECSA</td>
<td>East/Center/South African</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>G3BP</td>
<td>Ras-GAP SH3 domain-binding protein</td>
</tr>
<tr>
<td>GADD153</td>
<td>Growth arrest and DNA-damage-inducible protein 153</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GTase</td>
<td>Guanylyltransferase</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Anti-Retroviral Therapy</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HCMV</td>
<td>Human cytomegalovirus</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HGPRT</td>
<td>Hypoxanthine guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methyl-glutaryl-coenzymeA</td>
</tr>
<tr>
<td>HOS</td>
<td>Human osteosarcoma</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
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<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>Huh</td>
<td>Human hepatoma</td>
</tr>
<tr>
<td>HVD</td>
<td>Hypervariable Domain</td>
</tr>
<tr>
<td>IAV</td>
<td>Influenza A virus</td>
</tr>
<tr>
<td>ICRES</td>
<td>Integrated chikungunya research</td>
</tr>
<tr>
<td>IFIT1</td>
<td>Interferon-induced protein with tetratricopeptide repeats 1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH$_2$-terminal kinase</td>
</tr>
<tr>
<td>L-SIGN</td>
<td>Liver/lymph node-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>MAPK kinase kinase</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid leukemia cell differentiation protein 1</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MTase</td>
<td>Methyltransferase</td>
</tr>
<tr>
<td>MSK</td>
<td>Mitogen and stress-activated protein kinase</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Nonnucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>ONNV</td>
<td>O’nyong nyong virus</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>RRV</td>
<td>Ross river virus</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>Severe Acute Respiratory Syndrome-Coronavirus</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki forest virus</td>
</tr>
<tr>
<td>SINV</td>
<td>Sindbis virus</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VEEV</td>
<td>Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella zoster virus</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>YFV</td>
<td>Yellow fever virus</td>
</tr>
<tr>
<td>ZBD</td>
<td>Zinc-binding domain</td>
</tr>
<tr>
<td>ZIKV</td>
<td>Zika virus</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Viruses that are spread to a mammalian host through a hematophagous (blood-feeding) arthropod vector are called arboviruses (ARthropod-BOrne viruses). Mainly spread by mosquitoes, ticks, midges and sandflies (common arbovirus vectors are shown in Fig. 1), these viruses are usually maintained in nature through transmission cycles between the arthropod vector and a vertebrate animal reservoir. Humans are primarily accidental dead-end hosts in the majority of the transmitted arboviruses, but viruses like dengue and chikungunya are also spread through a man-mosquito-man urban transmission cycle (2, 3).

Since the advent of the exploration age and the slave trade, vector mosquitoes were disseminated to different corners of the world. Sporadic outbreaks of dengue and yellow fever were on the rise in tropical areas of Africa and the Americas. It was only in the beginning of the 20th century that the *Aedes aegypti* mosquito was implicated as the vector for Yellow fever. This was due to the ingenuity of Carlos Finlay, who proposed the theory and Major Walter Reed from the United States (U.S.) Army who confirmed the theory with well-designed experiments, albeit with voluntary human test subjects (4, 5).

We have come a long way since then and the U.S. Centers for Disease Control and Prevention (CDC) now lists over 600 identified arboviruses, out of which more than 150 are human pathogens. Arbovirus infections when symptomatic, usually cause a flu-like illness with fever, headache and nausea. With some viruses, this can progress to a severely debilitating rash and arthralgia (chikungunya virus (CHIKV) and Ross river virus (RRV)), neurological symptoms like myelitis or encephalitis (Venezuelan equine encephalitis virus (VEEV), tick-borne encephalitis virus, West Nile virus (WNV)) and hemorrhagic fever (dengue virus, Crimean Congo hemorrhagic fever virus, Kyasanur forest disease virus), where thrombocytopenia may result in constant bleeding into the tissues, shock and elevated levels of liver enzymes (2). Recently, this list of symptoms has been expanded to include Guillain-Barré syndrome and virus-induced microencephaly in the unborn fetuses and newborns of infected pregnant women caused by Zika virus (ZIKV) (6).

Except one DNA virus causing African swine fever, all other arboviruses belong to different RNA virus families viz. *Flaviviridae*, *Togaviridae*, *Bunyaviridae* and *Reoviridae*, which have a highly mutable genome and utilize diverse replication strategies, allowing these viruses to circulate and effectively multiply in various vertebrate and invertebrate hosts. Arbovirus epidemiology is in a state of constant flux due to several environmental factors. An ever burgeoning world population, urbanization, globalization, expanding air travel and climate change have ensured that vector mosquitoes continue to expand their habitats, thereby introducing viruses like dengue, chikungunya and Zika into new geographical areas (7).

DENV, a flavivirus primarily spread by *Aedes aegypti* mosquitoes has by far the highest disease burden among the arboviruses. It comprises four circulating serotypes, and is endemic throughout the world in the tropical belt. Yearly, there are approximately 50-100 million infections of dengue fever, with 10,000 – 15000 deaths due to severe complications caused by dengue hemorrhagic fever and
dengue shock syndrome (2, 3, 7). Recently, another flavivirus spread by the same vector mosquitoes - ZIKV, has also emerged as a dangerous arboviral threat, presenting new clinical manifestations and also the ability to be sexually transmitted (6). CHIKV, an alphavirus that causes severe and persistent arthralgia was recently introduced to the New World and has wreaked havoc in Central and South America by causing more than 1.5 million infections since the beginning of 2014 (8). These are just few of the examples of the major arboviral diseases plaguing the global population. Several others arboviruses like West Nile virus, Japanese encephalitis virus (JEV), Crimean-Congo hemorrhagic fever virus and Rift valley fever virus can cause sporadic, life threatening outbreaks, calling for improved surveillance, vector control and development of vaccines and antiviral therapeutics.

So far, vaccines exist only for yellow fever, Japanese encephalitis and tick-borne encephalitis. Recently, Sanofi Pasteur launched Dengvaxia®, a tetravalent vaccine against all four circulating serotypes of dengue that has shown good efficacy in clinical trials (9). The vaccine has been approved in five countries, with the World Health Organization recently recommending that other countries affected with dengue should consider employing the vaccine in areas with high endemicity (http://outbreaknewstoday.com/costa-rica-approves-dengue_vaccine-dengvaxia-5th-country-to-approve-67888/). Other than these, no antiviral therapies exist for any arbovirus and current treatment only involves symptomatic management.

Figure 1. Arbovirus vectors. (A) Aedes aegypti mosquito which spreads dengue, yellow fever, Zika and chikungunya viruses (photo credit: James Gathany, CDC). (B) Aedes albopictus mosquito which is competent to spread the same viruses as Aedes aegypti (photo credit: Susan Ellis, Bugwood.org). (C) Culex pipiens mosquito which spreads West Nile, Japanese encephalitis and Usutu viruses (photo credit: EU 7TH Framework Vectorie Consortium). (D) Ixodes scapularis tick which transmits tick-borne encephalitis virus (photo credit: M. Plonsky). (E) Phlebotomus papatasii sandfly which transmits several different viral serotypes that cause phlebotomus fever (photo credit: Frank Collis, CDC). (F) Culicoides imicola midge which spreads bluetongue virus to sheep and cattle (photo credit: French Agricultural Research Centre for International Development).
2. LITERATURE REVIEW

2.1 Alphaviruses

The Alphavirus genus is contained in the Togaviridae (toga is the Latin word for ‘cloak’ or ‘shroud’ and refers to the viral envelope) family (10). Alphaviruses (so called because they were the first viruses to be classified under Group A arboviruses) are positive-sense single-stranded RNA viruses that are all arthropod-borne with the exception of salmon pancreatic disease virus and Southern elephant seal virus. The genus comprises 29 different virus species grouped into 10 antigenic complexes. They are also classified as Old World and New World alphaviruses, depending on the geographical location where they were discovered and where they are currently distributed (11, 12).

Old World alphaviruses which include Semliki forest virus (SFV), Sindbis virus (SINV), O’nyong nyong virus (ONNV), CHIKV and RRV cause a febrile illness associated with a maculopapular rash, myalgia and arthralgia, which can often be painful and chronic and lead to considerable economic loss and suffering. New World alphaviruses on the other hand cause encephalitic disease associated with different neurological complications like ataxia, mental depression and aseptic meningitis, with VEEV, Eastern and Western equine encephalitis viruses being some of the examples (11, 12). In particular, VEEV is important for biothreat preparedness due to its ability to be weaponized and spread in aerosol form (13). Alphaviruses have been used as tools to study enveloped viruses and additionally as vectors for gene therapy (14).

2.2 Alphavirus life cycle

The alphavirus particle is 70 nm in diameter and is made up of a nucleocapsid core surrounded by a lipid bilayer derived from the host cell membrane. The viral envelope glycoproteins E1 and E2 are embedded in this lipid layer as 80 trimeric spikes, with each constituent monomer made up of E1-E2 heterodimers (240 copies of each protein) arranged in a T = 4 icosahedral lattice. The viral core is made up of 240 copies of the capsid protein and this one-to-one relationship between the capsid and envelope glycoproteins has been shown to be essential for virus assembly. Inside the viral core is packaged the ~11.5kb long viral genome, which is a single-stranded positive sense RNA (11, 12).

2.2.1 Receptor attachment and entry

Alphaviruses have a broad host range and cellular tropism, owing to their ability of adapt to multiple host requirements, most probably by being able to bind to several different types of cellular receptors. Virus attachment is brought about by the E2 glycoprotein. Numerous receptor molecules have been implicated – class I major histocompatibility protein, heparan sulfate, DC-SIGN, L-SIGN, α1β1 integrin,
glycosaminoglycans, prohibitin etc., and are usually cell-type and species-specific. It has been noticed that infection can proceed even in the absence of some of these cellular receptors and it is therefore thought that they only aid in the attachment process and are not an absolute requirement [reviewed in (15, 16)]. Additionally, various residues in the E2 glycoprotein have also been implicated in cell tropism, penetration, immunogenicity and pathogenicity of different alphaviruses (17-20). Following attachment, internalization is mediated by clathrin-coated endocytosis which delivers the viral particle into early endosomes. VEEV, though, has been reported to be internalized into late endosomes (21). The mildly acidic pH of the early endosome triggers drastic conformational changes in the envelope glycoproteins. Initially, E1-E2 heterodimers dissociate, and in this process which is regulated by a conserved histidine E2-H170, one of the E2 domains moves away to expose the fusion loop of the E1 membrane fusion protein (22). E1 then extends and inserts the fusion loop into the target membrane in a cholesterol and pH dependent process [reviewed in (15, 16)]. It was shown that a specific mutation in E1-226 from alanine to valine made some CHIKV strains have an increased cholesterol and lower pH requirement for fusion (23, 24). Further reduction in the pH then leads to a complete dissociation of E1 and E2 and leads to E1 trimerization – initially a core trimer between Domains I and II of E1 and later an E1 homotrimer when Domain III folds back towards the core trimer [reviewed in (15, 16)]. Another conserved histidine (H3 in E1) in SFV is essential for the trimerization process (25). The opposing membranes are pulled together as a result and the outer membrane leaflets merge to form a hemifusion state. Eventually, the formation of a fusion pore and its expansion allows the nucleocapsid to enter the cytoplasm [reviewed in (15, 16)].

2.2.2 Genome replication and functions of non-structural proteins

Once the capsid enters the cytoplasm, it is disassembled in association with ribosomes and translation of genomic RNA proceeds concomitantly (26). The alphavirus genome is a single-stranded RNA (~11.5 – 12kb in length) with plus polarity and has a 5’ cap structure and a 3’ polyadenylic acid [poly (A)] tail. The RNA is divided into 2 open-reading frames (ORFs) – the initial two-thirds coding for the viral non-structural proteins (nsPs) and the latter one-third coding for the structural proteins, with two untranslated regions (UTRs) on either side of the genome.

Conserved sequence elements

The alphavirus genome has certain cis-acting elements that are essential for the overall replication process. Some of these elements are conserved between the alphaviruses and are called Conserved Sequence Elements (CSEs).

CSE1

It spans the first 44nt of the 5’ UTR and forms a stem loop structure that helps initiate minus strand synthesis. On the other hand, this region also serves as the promoter for plus strand synthesis using the minus strand as a template (27). Additionally, this stem-loop structure was found to prevent the recognition of the alphaviral 5’ cap structure by cellular IFIT1 (Interferon-induced protein with
tetrameric peptide repeats), thereby blocking activation of the innate immune system leading to enhanced virus replication (28).

**CSE2**

This region is 51 nt long and is situated in the initial part of the nsP1 coding sequence. This element forms two stem-loop structures (29) that are needed for both minus and plus strand RNA synthesis (27). CSE2 was more critical for replication in insect cells as compared to vertebrate cells (30). Recently, using the SFV trans-replication system it was shown that CSE2 can compensate to some extent for the absence of CSE1, inducing some level of minus strand synthesis, although the precise mechanism remains to be elucidated (31).

**CSE3**

This element is the minimal promoter (24 nt) for the initiation of subgenomic RNA synthesis. The full length subgenomic promoter overlaps with the sequence coding for nsP4 and continues into the junction region (short sequence between the two open reading frames coding for the non-structural and structural proteins) for some alphaviruses (32). In alphavirus vector based gene expression systems, a duplicated subgenomic promoter is often used to efficiently express the desired gene to high levels (33).

**CSE4**

CSE4 is a short stretch of nucleotides (19-24 nt long) immediately before the poly-A tail in the 3' UTR (34, 35). It forms the core promoter that is needed for initiation of minus strand synthesis. Mutations in CSE4 were found to affect minus strand synthesis. A minimum length of at least 11-12 poly A residues were needed for minus strand synthesis (36).

From the genome, a long polyprotein precursor P1234 is synthesized, which then gets cleaved *in cis* by the autoproteolytic activity of nsP2 into P123 and nsP4 [the viral RNA-dependent RNA polymerase (RdRp)] (37, 38). This process differs between alphaviruses, where many of them have an opal stop codon at the end of nsP3, which is read through to form P1234, with preferential production of P123 (39). On the other hand, in most isolates of SFV, this opal codon is replaced by an arginine residue, resulting in higher production of full length P1234 (40). P123 and nsP4 form the early polymerase complex, which is then presumably targeted to the plasma membrane to form membrane invaginations called spherules which is the active viral replication complex. The recruitment of the viral replicase and genome to the plasma membrane to form spherules is still an enigmatic process that probably needs the concerted effort of hitherto unknown host factors and specific conformational changes and stoichiometric arrangement of the nsPs. The cleavage of nsP4 and the membrane-binding property of nsP1 are absolutely essential for the formation of replication complexes (41, 42). Recently, using a SFV trans-replication system (43) it was shown that the size of these replication complex spherules is dependent on the length of the template RNA that is being replicated (44). In SFV, these replication complexes are completely internalized in a PI3K (phosphatidylinositol-3-kinase)-Akt, actin and microtubule-dependent process into cytopathic vacuoles (45). On the other hand, in CHIKV (46) as well as SINV (47), the spherules are predominantly present on the plasma membrane.
Figure 2. Alphavirus RNA genome organization and polyprotein processing. The genome is capped and polyadenylated and functions like cellular mRNA, which is directly translated. The replicase ORF (Open Reading Frame) codes for the non-structural (ns) polyprotein precursor, whereas the structural protein precursor is translated from the 2nd ORF located on the subgenomic RNA, which is transcribed from the minus strand. Both precursors are cleaved by viral and cellular proteases into the individual proteins. Nature of the cleavages are indicated by triangles of different colors. C – capsid; E – envelope.

P123 and nsP4 initiate minus strand synthesis from a promoter on the 3’ UTR using the genomic RNA as a template (48). Further proteolytic processing then cleaves nsP1, which results in nsP1, P23 and nsP4 and brings about a switch from minus strand to plus strand synthesis. Additionally, the subgenomic RNA (2nd ORF) is transcribed from an internal promoter on the minus strand. P23 species is short-lived and is cleaved in trans to form the final late polymerase complex comprising the individual nsPs, which continue synthesis of the viral genomic RNA and preferentially the subgenomic RNA in larger amounts [reviewed in (49, 50)] (Fig. 2).

Before describing the subsequent processes of the alphavirus replication cycle, a brief overview of the individual non-structural proteins is given to describe their functions in the replication process.

nsP1
nsP1 is the viral capping enzyme and possesses methyltransferase (MTase) and guanylyltransferase (GTase) activities at its N-terminal end. The alphavirus RNA capping reaction proceeds in a different manner as compared to the capping of cellular RNAs. GTP and S-adenosyl methionine (AdoMet) are first bound by nsP1.
nsP1 then catalyzes a reaction where bound GTP is first hydrolyzed to GMP and then methylated at nitrogen 7 using AdoMet as a donor, leading to the formation of a covalently bound m\textsuperscript{7}GMP-nsP1 complex. The m\textsuperscript{7}GMP is then transferred to the 5’ end of the newly synthesized RNA genome via a 5’-5’-linkage (\textsuperscript{51}, \textsuperscript{52}). Cellular capping reactions on the other hand first transfer GMP to the nascent RNA, followed by subsequent methylation (\textsuperscript{53}). Conserved residues H38 and D64 have been shown to be responsible for m\textsuperscript{7}GMP and AdoMet binding respectively and bioinformatics analyzes also revealed homologous MTase and GTase domains in other plus-strand RNA viruses of the \textit{Nodaviridae} family (\textsuperscript{52}, \textsuperscript{54}). Interestingly, the presence of uncapped viral genomes was recently observed during SINV and RRV infection, which was shown to be responsible for induction of Type I interferon (IFN) response (\textsuperscript{55}).

nsP1 is thought to be responsible for targeting the replication complex to the membrane due to the presence of an amphipathic membrane-binding peptide in the central region of nsP1. This binding is strengthened by the palmitoylation of three cysteine residues at the C-terminus (\textsuperscript{56}, \textsuperscript{57}). Membrane association was reported to be critical for SFV replication and the W259 residue in nsP1 was shown to be crucial for this property (\textsuperscript{42}). The enzymatic activity of purified SFV nsP1 requires membrane association (\textsuperscript{41}), but this was not the case with SINV nsP1 (\textsuperscript{58}). The membrane association property of nsP1 induces filopodia-like membrane and cytoskeletal rearrangements in infected cells (\textsuperscript{59}), which probably have a role in cell-to-cell virus transmission (\textsuperscript{60}). Recently, Jones and co-workers showed that CHIKV nsP1 counteracted the tethering restriction effect of a cellular interferon-stimulated gene BST2 (bone marrow stromal antigen 2) (\textsuperscript{61}). Additionally, second site suppressor mutations have been observed in nsP1 for nsP4 mutants defective in minus strand synthesis, suggesting a physical interaction between them within the replication complex [reviewed in (\textsuperscript{50})].

\textbf{nsP2}

nsP2 has multiple functions in the infectious life cycle of alphaviruses. The N-terminal end contains a helicase domain, which also possesses NTPase (nucleotide triphosphatase) and RTPase (RNA triphosphatase) activities (\textsuperscript{62}-\textsuperscript{64}). Helicase activity unwinds dsRNA during replication and requires NTPase activity (\textsuperscript{63}, \textsuperscript{65}, \textsuperscript{66}). Mutation of a critical residue K192 completely abolished all three enzyme activities (\textsuperscript{63}, \textsuperscript{66}). K192 was shown to be absolutely essential for both RNA synthesis and replication complex formation (\textsuperscript{67}). RTPase activity aids in the RNA capping reaction by cleaving the terminal phosphate bond at the 5’ end of the nascent genomic RNA, before transfer of m\textsuperscript{7}GMP cap (\textsuperscript{64}). CHIKV nsP2 was shown to possess RNA helicase unwinding activity in the 5’-3’ direction as well as RNA reannealing activity (\textsuperscript{65}). Das and colleagues also showed that the truncated N-terminal domain of purified CHIKV nsP2 alone was not sufficient for these RNA modulating effects, but needed to interact with the C-terminal part of the protein (\textsuperscript{65}). The helicase activity of a G641D CHIKV nsP2 mutant in combination with a C483Y mutation in nsP4 was found to provide greater fidelity to the viral polymerase and resistance to the misincorporation of nucleotide analogues, suggesting an intimate interaction between the viral helicase and polymerase to regulate replication fidelity (\textsuperscript{68}).
The other important function of nsP2 is its protease activity, which resides at the C-terminal end of the protein. The alphavirus nsP2 protease is a papain-like cysteine protease, with conserved cysteine and histidine residues forming the catalytic diad. This function is critical for virus replication and mutation of the catalytic cysteine completely abolishes polyprotein processing (69). Cleavage of P1234 at the 3/4 and 1/2 sites occur in cis (38, 70), whereas cleavage of 2/3 site must take place in trans (38). As mentioned earlier, the proteolytic cleavage of the viral non-structural polyprotein precursor by nsP2 is a highly orchestrated event, which regulates the temporal dynamics of the switch from minus strand to plus strand synthesis (70). The processing of the final 2/3 site in SFV requires the N-terminal part of nsP2 as well as the N-terminal part of the nsP3 (macrodomain), ~165 amino acids downstream of the 2/3 cleavage site (71). The crystal structures of VEEV and CHIKV nsP2 protease domains are one of the few alphavirus protein structures that have been resolved, which has now made it an attractive target for virtual screening of alphavirus protease inhibitors (72-74).

The nsP2 of Old World alphaviruses has a putative nuclear localization signal (NLS) in the C-terminal region and is known to be shuttled to the nucleus during virus infection (75). The nuclear translocation of nsP2 was shown to be responsible for shut off of host cell transcription (76) and translation (77) as well as evasion of type I IFN innate immune response (78, 79). nsP2 brought about the degradation of the catalytic subunit of cellular RNA polymerase II (76), and also inhibited cellular JAK/STAT signaling to inhibit the Type I interferon response (80). P726G mutation in SINV (81, 82) and P718G mutations in SFV (83) and CHIKV (84) were shown to either abolish or reduce virus infection induced cytotoxicity. The P718G mutation in a CHIKV replicon coupled with the selection of a 5 amino acid insertion in nsP2 resulted in the generation of a stable CHIKV replicon cell line (85), which has now been used for antiviral studies [I and (86, 87)].

nsP3

nsP3 remains the most enigmatic of the alphaviral non-structural proteins. Although nsP3 is undoubtedly indispensable for virus replication, no enzymatic activity that has a role in the viral life cycle has been assigned to it yet. The protein is divided into three-distinct domains. The N-terminal macro domain, whose crystal structure has been determined for some alphaviruses (88, 89) has binding activity to ADP ribose moieties and RNA. It also possesses a weak ADP-ribose-1'-phosphate-phosphatase activity, although the RNA binding property is thought to be its main function (88, 90, 91). This is the only domain which has some level of structural conservation and homology with similar domains from other viruses (92). Recently, it was shown that viral macrodomains from three different virus families (Coronaviridae, Togaviridae and Hepeviridae) could hydrolyse mono-ADP-ribose from cellular proteins, and this property was hypothesized to be an important pathogenicity determinant (93). The C-terminal region of macrodomain is also needed for proper presentation and cleavage of the 2/3 site during polyprotein processing (71). Mutations in the macro domain of SINV were shown to reduce neuropathogenicity (94).

The central domain is a zinc-binding domain [ZBD; also called the Alphavirus Unique Domain (AUD)]. The structural determination of the precleavage form of
P23 revealed the presence of this domain that can bind to zinc ions. Additionally, it was shown to be important for virus infectivity and thought to be important for RNA binding and aiding the macro domain during this process (89). Domain swapping of elements downstream of the ZBD between CHIKV and SFV caused defects in the resultant chimeric viruses, which were rescued to some extent by compensatory mutations in the ZBD, suggesting that the ZBD interacts with the C-terminal Hypervariable Domain (HVD) (46).

The HVD which is a highly disordered region has a variable length of 150-250 amino acids between the different alphaviruses. It has recently emerged as a key player in virus-host interactions. A series of conserved proline residues in this region was found to bind to Src-3 homology domain amphiphysin proteins, which influence membrane curvature, possibly shaping the positive curvature in the neck region of the replication complex spherule. Mutation of a critical arginine residue in the SH3 domain binding motif reduced SFV viral RNA synthesis in cell culture and pathogenesis in mice (95).

nsP3 is the only alphavirus phosphoprotein and is highly phosphorylated on specific serine/threonine residues (96, 97). Recently, it was conclusively proven that this phosphorylated region regulated the internalization of the replication complex in SFV through activation of the PI3K-Akt-MTOR pathway. CHIKV, on the other hand has fewer phosphorylated residues in this region, corresponding to replication complexes localizing to the plasma membrane and reduced activation of the PI3K-Akt pathway. Moreover, swapping the entire HVD between these viruses led to a switch in phenotypes – both in terms of internalization of spherules and the activation of the PI3K-Akt pathway (46).

nsP3 that is not localized in the replication complexes is observed in the cytoplasm as punctate foci (98). In recent years, researchers from different groups have elucidated the role of the Old World alphavirus nsP3 in sequestering cellular Ras-GAP SH3 domain binding (G3BP) proteins (or the mosquito homolog rasputin) to counteract stress granule-mediated antiviral defense (99). On the other hand, G3BP was hypothesized to play a proviral role early during CHIKV infection by helping the switch between host cell translation and genome amplification (100). The HVD has two FGDF motifs that bind to G3BP proteins. Recently, the crystal structure of G3BP in complex with the corresponding binding peptide was determined. Schulte et al showed that binding of G3BP at both FGDF motifs are necessary for efficient SFV and CHIKV replication. G3BP molecules were predicted to tether and stabilize replication complex spherules to the membrane (101). Kim and co-workers recently showed that similar motifs in the New World VEEV bind to proteins of the Fragile X syndrome family, which are different stress-granule related proteins similar to G3BP. They also showed that these stress-granule proteins play a key role in viral replication complex assembly (102). As more research is being done on nsP3-mediated virus host interactions, a complete picture of the nsP3 interactome will gradually emerge.

nsP4
nsP4 possesses the conserved GDD active site which forms the catalytic core of viral RNA-dependent RNA polymerases (RdRp) (103). The N-terminal ~100 amino acid residues are unique to alphaviruses, while the remaining ~500 residues make
up the viral RdRp with its characteristic palm and thumb domains as predicted by sequence alignment (104, 105). nsP4 is synthesized at a significantly lower level than the other nsPs due to the presence of the opal codon between nsP3 and nsP4. The read through efficiency at this codon is only ~20% (106). The nsP4 needed for viral RNA synthesis is presumably shielded in the replication complexes. Excess nsP4 in the cytoplasm is targeted for proteasomal degradation by the N-end rule due to the presence of an aromatic destabilizing Tyr (tyrosine) residue at the N-terminal end of nsP4 (107). The aromaticity of this N-terminal residue along with the R183 residue were shown to be critical for minus strand synthesis and were compensated by a suppressor mutation in nsP1 (108-110). Purification of nsP4 has proven to be quite challenging due to its poor solubility. nsP4 with a truncated N-terminal domain exhibits terminal adenylyltransferase activity, independent of the other viral nsPs (105). Full length recombinant nsP4 demonstrated minus strand RNA synthesis activity in the presence of P123 (104).

2.2.3 Packaging

The structural ORF is also synthesized as a long polyprotein, and initially, the N-terminal capsid protein gets cleaved off due to its serine protease autoproteolytic activity (Fig. 2). There is a translational enhancer present at the 5′ end of the sequence coding for the capsid protein in SFV and SINV to ensure efficient translation of the structural proteins in the infected cell. This enhancer is not a common feature in all the alphaviruses and is missing in VEEV and CHIKV (111). Viral genomic RNA is preferentially packaged into capsids, due to a packaging signal in the non-structural protein coding region. For SINV and the New World encephalitic viruses, this signal is located in nsP1, whereas it was found to be in nsP2 for SFV and other viruses belonging to the SFV clade, including CHIKV (112).

2.2.4 Egress

The viral glycoprotein heterodimers of pE2 (precursor of E2 containing E3-E2) and E1 are transported through the ER and Golgi, where they undergo conformational changes and post-translational modifications like glycosylation and palmitoylation. The presence of E3 is thought to prevent premature dissociation of E1-E2 in the low pH environment of the ER lumen (113). In the Golgi compartment, furin cleavage separates E3 from E1-E2, and the E1-E2 heterodimer is then transported to the plasma membrane. This furin cleavage of E3 is essential for subsequent fusion and virus entry into newly infected cells (114, 115). E1-E2 heterodimers self-assemble into trimeric spikes on the plasma membrane. The assembled and filled nucleocapsids are thought to diffuse to the plasma membrane, where one-to-one interaction between a hydrophobic pocket on the capsid protein and E2 tail drives the budding process (116, 117) to generate progeny virions [reviewed in (14)].

2.3 Epidemiology of chikungunya virus infections

The rapid spread of chikungunya fever across the globe has recently put the spotlight onto the alphaviruses. Although other alphaviruses like VEEV and SINV
are important human pathogens, neither of them have garnered so much research and media attention. In fact, with the re-emergence of CHIKV, alphavirologists have been able to use their existing knowledge and expertise to help dissect the finer details of this geographically expanding disease.

Chikungunya fever leads to a febrile illness that is characterized by nausea, headache, acute musculo-skeletal pain and a maculopapular rash. Long term persistent myalgia and arthralgia can occur in a majority of the affected individuals. In rare situations, neurological and ocular defects have been reported (8). It is speculated that CHIKV has been in circulation for centuries and was often misdiagnosed as dengue fever due to the similarity in clinical symptoms. In fact, there have been reports of a ‘knuckle/joint fever’ in Indonesia as far back as in 1779, which could very well have been an outbreak of CHIKV infection (118). Chikungunya has been maintained in an enzootic or sylvatic cycle involving non-human primate hosts/reservoirs and arboreal vector mosquitoes of the Aedes species. Eventually, CHIKV was introduced into the urban epidemic cycle involving a man-mosquito-man transmission pattern, being primarily circulated by the tropical urban vector Aedes aegypti (119).

CHIKV was first isolated in Tanzania in 1952 and the name of the disease comes from the Makonde language roughly translated as ‘that which bends up the joints’ (120, 121). Shortly thereafter, CHIKV was detected in several places of East Africa and these strains were classified into the ECSA (East/Central/South African) lineage. Later in the 20th century, several smaller episodes of CHIKV infection in Senegal and related areas were observed. Strains obtained from these outbreaks formed the West African lineage. At the same time, sporadic CHIKV cases were being detected in different places in Asia. In the period of 1958 – 1973, CHIKV was detected in Thailand, Cambodia and India in several different outbreaks, sometimes co-circulating with dengue fever. The viruses from these outbreaks collectively formed a separate Asian epidemic lineage. The Asian lineage disappeared from India after 1973, but maintained its presence in Southeast Asia through small scale epidemics [reviewed in (122)].

For the next 30 odd years, CHIKV faded out of memory until 2004 when a new epidemic strain emerged from the ECSA lineage. Initially circulating in coastal Kenya, in 2005 it spread to several islands in the Indian ocean like Mauritius, Comoros, Mayotte and most notably, the French territory of La Réunion, where around one third of the island’s population (~300,000) was infected (123). Genetic analysis of this emergent strain now classified as the Indian ocean lineage, along with vector competence and vector transmission studies revealed the presence of a dominant A226V mutation in the E1 fusion protein (124), which made this strain increasingly dependent on cholesterol for membrane fusion and better adapted to the less common, secondary vector - Aedes albopictus (24). This epidemic then spilled over onto the Indian subcontinent, where in a span of less than 2 years, more than 1.5 million infections occurred (125). At the same time, the virus also spread to Southeast Asia where millions of individuals in several different countries were affected. Moreover, strains from the Asian lineage also saw a resurgence with active circulation in Southeast Asia and further spread to Oceania [reviewed in (122)].
In subsequent years, the CHIKV epidemic raged on at lower intensities in these tropical endemic countries. During this period, there were several reports of imported CHIKV infections as a consequence of western tourists visiting areas with active CHIKV circulation. Some of these importations led to local CHIKV epidemics in Europe – first in Italy in 2007 (126) and later in southern France in 2010 (127), where the temperate vector *Aedes albopictus* had already been established.

![Geographical distribution of chikungunya virus and its vectors](image)

**Fig. 3 Geographical distribution of chikungunya virus and its vectors.** The map indicates the important outbreaks since the virus was first isolated in 1952, with different symbols denoting the three genotypes. Color coded areas depict the spread of the two vectors – *Aedes aegypti* and *Aedes albopictus*. (Photo credit: adapted by J. Hirshfeld, Geoatlas/Graphi-Ogre).

These events highlighted the possibility of CHIKV spreading to new geographical locations that were not restricted to tropical areas, as well as the western hemisphere, where these vector mosquitoes are already endemic.

This prediction came true not soon after when CHIKV cases were reported in some Caribbean islands in late 2013. Surprisingly, the etiologic strain was from the Asian lineage, probably imported from Southeast Asia or Oceania (128, 129). Thereafter, it rapidly spread across Central and South America as well as northward into Mexico and the U.S.A., where local transmission resulted in 11 cases in the southern state of Florida (130). Additionally, later in 2014, a strain from the ECSA lineage was introduced into Brazil and currently both these strains are circulating in the Americas and threaten to become endemic in urban settings as well as being established in the enzootic cycle like yellow fever virus (YFV) (131). So far, more than 1 million cases have been reported in the western hemisphere, with many more probably being misdiagnosed as dengue fever in regions which do not have access to diagnostic infrastructure (8).

Disability Adjusted Life Years (DALYs) is often used as an indicator of the epidemiological burden of a disease. It measures the number of ‘healthy’ years lost due to ill health, disability or early death. For the Indian outbreak of 2006-2007,
the DALY was estimated to be ~26,000 (132); whereas for the current epidemic in Central and South America the modest lower estimate DALY is > 150,000 (133). This fact highlights the explosive nature of this disease and its spread to millions of immunologically naïve individuals, leading to widespread economic losses.

Urgent intervention measures are needed to tackle this pathogen. There are several different vaccine strategies that have been employed and they seem to offer protection in mouse models, non-human primates and have progressed to Phase I clinical trials with promising results. Nevertheless, it might take quite a while before a vaccine to be used in endemic countries is licensed. Vaccine manufacturing scale-up, technical and regulatory details, feasibility for medium and large-scale companies to invest in these ventures due to the target population largely restricted to low and middle-income countries are all issues that might affect the timeline [reviewed in (134, 135)]. In such a scenario, therapeutic measures might be a likely recourse. Ever since the re-emergence of CHIKV in 2004, several groups all over the world have been in the pursuit of screening and discovering potential antiviral compounds that inhibit CHIKV. So far, no approved antivirals exist and treatment of patients is limited to symptomatic management (136).

2.4 Antivirals and antiviral validation

Antivirals are chemical agents that interfere with a productive viral infection without causing adverse toxicity to the host cell (137). The first ever antiviral to be licensed was the thymidine analogue, idoxuridine against the herpesviruses which interferes with thymidine phosphorylase and the viral DNA polymerase. Its synthesis and activity was first described in 1959 (138) and it was formally approved in 1963. Since then, over 90 antiviral drugs have been approved for human use against a wide variety of pathogenic viruses e.g., different members of the *Herpesviridae* family, human immunodeficiency virus (HIV), influenza virus, hepatitis B virus (HBV), hepatitis C virus (HCV), respiratory syncytial virus (RSV) and human papilloma virus [reviewed in (139)]. Nevertheless, as compared to the rapid advancement of antibiotics from discovery to the clinic, antiviral discovery and development has been slow and fraught with many obstacles. Viruses are obligate intracellular parasites that hijack different cellular processes to facilitate their own replication and as a result, very few inhibitors achieve selectivity against a virus-specific process without leading to detrimental effects in the host cell. Secondly, incomplete inhibition or lack of adherence to prescribed drug regimen can lead to the emergence of resistant populations. Finally, with many emerging and exotic viruses [Middle Eastern Respiratory Syndrome coronavirus, Ebola virus (EBOV)], the systematic validation of antiviral drugs requires specialized handling facilities and equipment, including high containment laboratories (biosafety level 3 and 4), which are difficult and expensive to maintain. Some viruses are difficult to propagate in cell culture (HBV), whereas some others do not have appropriate animal models for pre-clinical trials (measles virus) [reviewed in (137)]. Therefore, as new pathogenic viruses continue to emerge and other prevalent ones develop resistance to existing therapeutic measures, the search for potent and effective antivirals must continue.
2.4.1 Classes of antivirals

Antivirals can be broadly classified as direct-acting (targeting a viral component) and host-directed (targeting one or more host factors required for the viral infectious cycle) antivirals (137). Dr. Erik de Clercq from the Rega Institute of Medical Research (Leuven, Belgium), who has been a pioneer in antiviral drug development for more than 50 years, has recently published a comprehensive review of approved antiviral drugs (139), where he has classified antiviral drugs into 13 different classes based on their functional chemical groups and mode of action which are as follows:

5-substituted 2’-deoxyuridine analogues
These were the first antiviral drugs to be approved and include idoxuridine, trifluuridine and brivudine. They act as thymidine analogues and target the viral DNA polymerase of HSV-1 (140). Brivudine is also active against Varicella zoster virus (VZV) DNA polymerase (141).

Nucleoside analogues
Vidarabine, entecavir and telbivudine are the approved antiviral nucleoside analogues. Vidarabine triphosphate acts against the DNA polymerase of HSV and VZV by competing against dATP (142), whereas both entecavir and telbivudine are used especially against HBV infection, where they act by inhibiting the HBV DNA polymerase (139).

Pyrophosphate analogues
Foscarnet is the only approved drug in this class. Its uniqueness lies in the fact that unlike nucleoside analogues, it does not need to be phosphorylated before it binds to the target viral DNA polymerase (143). Foscarnet has broad spectrum antiviral activity against viral DNA polymerases of different DNA viruses like HSV1 and 2, human cytomegalovirus (HCMV), VZV, Epstein-Barr virus (EBV) and HBV; but it does not show any activity against the RdRps of RNA viruses. Additionally, it is also used against HIV (144).

Nucleoside reverse transcriptase inhibitors (NRTIs)
There are several approved drugs belonging to this class – zidovudine, didanosine, zalcitabine, stavudine, lamivudine and abacavir. They compete with cellular nucleotides to act as chain terminators for viral DNA synthesis. Most of them have been approved and used against HIV, except lamivudine which is also used against HBV [reviewed in (139)].

Nonnucleoside reverse transcriptase inhibitors (NNRTIs)
Five approved anti-HIV drugs fall in this category – nevirapine, delavirdine, efavirenz, etravirine and rilpivirine. These compounds are non-competitive allosteric inhibitors of the HIV reverse transcriptase and do not need to be metabolically processed (145). They are often used as first-line treatment in the clinical setting (146).

Protease inhibitors
In this particular class, there are 12 HIV aspartic protease inhibitors viz. saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, atazanavir, fosamprenavir, lopinavir, tipranavir, darunavir. Most of them are peptidomimetic compounds that bind to the active site of the HIV protease and interfere with the processing of its proteins. They form an integral part of the HAART (Highly Active Anti-Retroviral Therapy) regimen for patients infected with HIV-1 and HIV-2. Recently, directly-acting antivirals have been approved against HCV which include NS3/4A serine protease inhibitors: asunaprevir, boceprevir, paritaprevir, simprevir, telaprevir, vaniprevir and grazoprevir. In some countries like Japan, they have been approved in combination with other drugs and PEGylated interferon to treat infections with HCV genotype 1. Other HCV protease inhibitor candidates are in clinical development [reviewed in (139)].

**Integrase inhibitors**
These are compounds that block the strand-transfer reaction catalyzed by HIV integrase to integrate its proviral DNA into the host chromosome (147). Three integrase inhibitors - raltegravir, elvitegravir and dolutegravir have been approved for clinical use. These compounds are also part of the HAART therapy in combination with different NRTIs and protease inhibitors (139). They generally have a better safety profile and a higher genetic barrier to the development of resistance (148).

**Entry inhibitors**
Within this class of antivirals, there are several different types of drugs. Enfuvirtide (also known as T-20) is a polypeptide drug that binds to a heptad repeat in the HIV envelope protein GP41 and prevents the fusion of the viral and cellular membranes (149). Maraviroc is a chemokine receptor antagonist that prevents the binding of HIV with one of its essential co-receptors CCR5 on the surface of CD4+ T cells and macrophages (150). RSV-IGIV and palivizumab are immunoglobulins that were approved for prophylactic use against RSV. RSV-IGIV may prevent binding of F & G glycoproteins of RSV to host cells, whereas palivizumab binds to a specific conserved residue on the RSV fusion protein and offered neutralization as well as inhibition of fusion (151). VZIG and VariZIG are immunoglobulins against VZV used prophylactically as well as for post-exposure prophylaxis to reduce clinical illness in immunocompromised patients (152). Docosanol is a saturated primary alcohol that is used in topical creams against HSV and supposedly prevents virus entry by blocking attachment of HSV envelope proteins to cell membrane receptors (153, 154).

**Acyclic guanosine analogues**
Acyclovir, ganciclovir, penciclovir and their phosphorylated prodrugs are used to treat both HSV and VZV. Once they are in their triphosphorylated forms, they compete with the cellular dGTP substrate of viral DNA polymerase to inhibit DNA synthesis [reviewed in (139)].

**Acyclic nucleoside phosphonate analogues (ANPs)**
Cidofovir, adefovir dipoxil, tenofovir disoproxil fumarate (TDF) are compounds belong to this class of drugs. Cidofovir is primarily used to treat HCMV, adefovir and TDF are used in combination therapy to treat HIV and HBV. ANPs are effective chain terminators of DNA synthesis, because of the presence of their phosphonate
linkage, which cannot be cleaved by cellular hydrolase esterases [reviewed in (155)].

**HCV NS5A inhibitors**
Direct-acting antivirals against HCV are recent entrants in the market. So far, four of them have been approved – daclatasvir, ledipasvir, ombitasvir and elbasvir. These compounds bind specifically to HCV NS5A and were shown to block replication by disrupting the formation of the membranous web that is found in HCV replication complexes (156).

**HCV NS5B polymerase inhibitors**
Two drugs, sofosbuvir and dasabuvir have been approved. These compounds target the allosteric site of the HCV NS5B polymerase. They are used in combination with other drugs for effectively treating HCV [reviewed in (139)].

**Influenza virus inhibitors**
Amantadine and rimantadine are both influenza virus M2 ion channel protein blockers which prevent proton translocation into the endosomes, thereby preventing the uncoating of the virus particle (157). Zanamivir, the relatively more successful oseltamivir and other derivatives like peramivir and laninamivir octanoate are the results of a rational computer-based design against IAV neuraminidase (158). These compounds prevent viral release from infected cells by binding to neuraminidase and preventing the cleavage of sialic acid residues on the viral hemagglutinin protein (159). Ribavirin is a broad-spectrum antiviral drug that is approved for treatment of IAV infections, but also works against many other RNA viruses like HCV, RSV, DENV etc. (160). This compound blocks the de novo synthesis of GTP by inhibiting the inosine-5′ monophosphate (IMP) dehydrogenase enzyme (161), thereby inhibiting the activity of the IAV RNA polymerase (162). Another compound favipiravir which has been approved to treat IAV in Japan gets converted intracellularly to its active triphosphate metabolite (163) and shows broad-spectrum activity against many positive and negative-sense RNA viruses (164).

**Antiviral agents not directed against viral proteins**
Interferon (pegylated interferon alpha 2b) therapy, which triggers innate immune antiviral defenses has been approved for treating HBV and HCV infections in combination with ribavirin, although its use is not widespread in clinical practice [reviewed in (139)]. Imiquimod is a cytokine stimulant and it is approved as a topical cream for treating HPV-induced genital warts (165). Sinecatechins is a plant-derived immunomodulatory product used as a topical ointment for treating HPV-induced warts (166). Podofilox is an antimitotic compound approved as a topical solution or gel to treat external warts. It works by disrupting mitotic spindle formation during the metaphase stage (167). Fomiviren is an antisense oligonucleotide targeting the expression of an immediate early gene during HCMV infection (168). It was approved as an intravitreal injection into human eyes to treat HCMV retinitis in AIDS (Acquired Immunodeficiency Syndrome) patients [reviewed in (139)].
This brief overview of approved antiviral drugs highlights the fact that antiviral
drug research has primarily focused on diseases that are prevalent in the western
world and quite often diseases endemic in the tropical areas and in third-world
developing countries have been neglected.

2.4.2 Antiviral drug discovery

The process of a systematic antiviral drug discovery program entails the following
steps:

1. Target identification
This process involves finding a potential drug target in the viral life cycle, which
can either be a virus-encoded protein or host factors that are needed for efficient
virus replication.

2. Target validation
Targets that are indispensable for virus replication need to be identified and
validated, wherein targeting them leads to reduced virus replication in cell culture
or confers a therapeutic effect in an animal model.

3. Lead identification
Following validation of a target, assays need to be developed and optimized,
wherein the biological function mediated by the target can be accurately assessed.
This is usually followed by a screening process where compounds depicting dose-
dependent activity and selectivity against the target, within predefined cytotoxicity
thresholds are selected for further optimization.

4. Lead optimization
In this process, the lead compound or molecule undergoes further refinement,
possibly in terms of structural modifications to enhance its potency and selectivity
towards the target.

Once the candidate drug clears this initial phase of testing and optimization, it
needs to undergo rigorous pre-clinical evaluation in animal models to ascertain its
effectiveness and also to obtain pharmacokinetic and toxicity profiles. The next
step is clinical trials in human volunteer test subjects that are classified into four
phases, with approval granted if the drug clears the first three phases [reviewed in
(137, 169)].

2.1.1 Antiviral screening

Screening for inhibitors is an integral process of the drug discovery program,
which contributes significantly to the generation of new lead candidates. This
becomes especially important when the pathogen in question is an exotic or re-
emerging virus, where both the life cycle and the processes involved, or the
structures of the different virus-encoded proteins are unknown. In such cases,
infection assays are often used to screen for inhibitors interfering with different
stages of the viral life cycle. There are different readouts that are employed in
these infection assays, such as CPE (cytopathic effect) reduction (170), viral
protein expression determined by immunofluorescence (171), high throughput dot blot assays (172), FRET-based viral protease activity readout (173) etc. Often, a reporter gene cloned into the viral genome is used as a readout of a productive infection and its reduction as a measure of antiviral activity of the compounds being screened (174). In case of viruses which have a higher biosafety requirement, often non-pathogenic, but related surrogate viruses (175), pseudotyped viruses (non-pathogenic viruses expressing only the envelope proteins of the virus under study) (176, 177), viral replicons (178) or cell lines stabling expressing replicons are used (85, 179). Indirect approaches using cell-cell fusion assays and measuring resultant syncytia formation in infected cells (180) or measuring cellular electrical impedance (181) are innovative methods to screen for viral fusion inhibitors. Another approach is to use recombinant purified active viral enzymes in cell-free enzyme substrate assays (182, 183). High-resolution crystal structures of viral enzymes offer an additional advantage for the screening process. Computational methods for structure or ligand-based virtual screening of millions of compounds helps to narrow the list of potential inhibitors to those that presumably bind with highest affinity (184). This approach is often constrained by predefined thresholds based on the programs and software being used for this purpose (137). The hits generated from such in silico approaches are then validated either with in vitro enzyme substrate assays or isothermal titration calorimetry binding affinity assays and infection assays using live virus (185).

So far, we have concentrated on screening for inhibitors that interfere with viral processes. In a complementary approach, host factors that are critical or essential for the viral life cycle are also screened. Since the discovery of RNAi (RNA interference) a decade ago, knockout of specific cellular processes via siRNA to identify crucial proteins or pathways in the viral life cycle has been a widespread approach (186). These screens are often unbiased genome-wide screens (187) or involve selectively knocking out only specific types of proteins like kinases (188). In recent years, with the advent of the CRISPR-Cas9 gene editing tool, it has been possible to achieve high efficiency in knockdown, few off target effects and less signal/noise ratio, thereby increasing the effectiveness of such screens (189). Yet another exciting recent development is the use of haploid human cells for screening host factors (190), where knockout of a particular gene results in complete loss of function, due to only one copy of the particular gene being present (191). Some studies have even combined the use of these powerful tools to reveal novel host factors regulating viral infection (192). Some of these host factors can be targets for other diseases like diabetes and cancer and there is a good chance that FDA (Food and Drug Administration)-approved drugs that target these factors are available. These existing medications can then be repurposed for antiviral use (193). Inversely, using libraries of FDA-approved drugs in viral screening setups could also yield similar results.
2.4.3 Antivirals against chikungunya virus

Potential drug targets in the alphavirus life cycle are represented in Fig. 4 and select inhibitors against these targets are listed in Table 1. There are several other inhibitors with unknown targets that have been reported to possess antiviral activity against CHIKV. The reader is referred to comprehensive reviews on antivirals against CHIKV by Abdelnabi et al (2015) and Kaur and Chu (2013) for further details.

Figure 4. CHIKV life cycle and potential drug targets. Schematic representation of the major steps/druggable targets in the CHIKV infectious cycle. 1 − receptor-binding and clathrin coated endocytosis, 2 − membrane fusion, 3 − capsid disassembly, 4 − polyprotein translation, 5 − non-structural protein enzymatic activity, 6 − RNA replication, 7 − subgenomic RNA translation, 8 − capsid protein cleavage, 9 − genome packaging, 10 − signalase cleavage in the ER, 11 − furin cleavage of the E3 protein and 12 − budding. [Adapted from (136)].
<table>
<thead>
<tr>
<th>No.</th>
<th>Target</th>
<th>Inhibitor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Receptor-binding and clathrin-coated endocytosis</td>
<td>Arbidol</td>
<td>(197, 198)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compounds with a 10H-phenothiazine core</td>
<td>(85)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flavaglines</td>
<td>(199)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epigallocatechin gallate (green tea component)</td>
<td>(200)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suramin</td>
<td>(201, 202)</td>
</tr>
<tr>
<td>2</td>
<td>Membrane fusion</td>
<td>Chloroquine</td>
<td>(203, 204)</td>
</tr>
<tr>
<td>3</td>
<td>Capsid disassembly</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Translation of viral genome</td>
<td>Phosphorodiamidate morpholino oligomers</td>
<td>(205, 206)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siRNAs against nsP3 and E1</td>
<td>(207)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>siRNAs against nsP1 and E2</td>
<td>(208)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>shRNAs against nsP1 and E1</td>
<td>(209)</td>
</tr>
<tr>
<td>5</td>
<td>Enzymatic activity</td>
<td>[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones</td>
<td>(210)</td>
</tr>
<tr>
<td></td>
<td>a) nsP1 capping activity</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>b) nsP2 helicase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>c) nsP2 protease</td>
<td>ID1452-2</td>
<td>(211)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compound 1</td>
<td>(73)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arylalkylidene derivative of 1,3-thiazolidin-4-one</td>
<td>(212)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCI_217697, NCI_61610, NCI_37553 and NCI_293778</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compounds 3a, 4b and 5d</td>
<td>(213)</td>
</tr>
<tr>
<td></td>
<td>d) nsP3 macrodomain</td>
<td>NCI_61610, NCI_25457, NCI_345647-a, NCI_670283, NCI_127133</td>
<td>(214)</td>
</tr>
<tr>
<td>Step</td>
<td>Process Description</td>
<td>Inhibitors</td>
<td>References</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>6</td>
<td>Viral genome replication</td>
<td>Ribavirin</td>
<td>(215, 216)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mycophenolic acid</td>
<td>(217)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-azauridine</td>
<td>(215)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Favipiravir (T-705)</td>
<td>(196)</td>
</tr>
<tr>
<td>7</td>
<td>Translation of subgenomic RNA</td>
<td>Hammerhead ribozymes</td>
<td>(218)</td>
</tr>
<tr>
<td>8</td>
<td>Capsid protein serine protease activity</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Viral genome packaging</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Signalase cleavage in the ER</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Furin cleavage of E3 protein</td>
<td>Decanoyl-RVKR-chloromethylketone</td>
<td>(219)</td>
</tr>
<tr>
<td>12</td>
<td>Budding</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Targeting host factors</td>
<td>Tigliane diterpenoids prostratin and 12-O-tetradecanoylphorbol 13-acetate</td>
<td>(220)</td>
</tr>
<tr>
<td></td>
<td>a) Protein kinase C activators</td>
<td>Tigliane diterpenoids prostratin and 12-O-tetradecanoylphorbol 13-acetate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b) Kinase inhibitors</td>
<td>Bryostatin analogues</td>
<td>(221)</td>
</tr>
<tr>
<td></td>
<td>c) HSP90 inhibitors</td>
<td>HS-10 and SNX-2112</td>
<td>(223)</td>
</tr>
<tr>
<td></td>
<td>d) Fatty acid synthesis</td>
<td>5-tetradecyloxy-2-furoic acid and orlistat</td>
<td>(224)</td>
</tr>
<tr>
<td></td>
<td>e) Calmodulin signaling</td>
<td>TAE684, pimozide and perphenazine</td>
<td>(224)</td>
</tr>
<tr>
<td></td>
<td>f) Sodium-potassium ATPase</td>
<td>Digoxin and ouabain</td>
<td>(225)</td>
</tr>
<tr>
<td></td>
<td>g) Polyamine biosynthesis</td>
<td>Difluoromethylornithine and diethylnorspermine</td>
<td>(226)</td>
</tr>
<tr>
<td>14</td>
<td>Modulating host immune response</td>
<td>IFN-α</td>
<td>(215)</td>
</tr>
</tbody>
</table>
There are few inhibitors which have been validated in a mouse model of CHIKV infection, which deserve more attention:

**Favipiravir**

Favipiravir (also known as T-705 or Avigan) is a pyrazinecarboxamide derivative that has been licensed in Japan for treating IAV infections. It was also shown to possess broad-spectrum antiviral activity against several different RNA viruses from various families (194). Favipiravir is a prodrug that gets converted to its active metabolite - ribofuranosyl-5'-triphosphate by the cellular HGPRT (hypoxanthine guanine phosphoribosyltransferase) enzyme and competitively inhibits the activity of the viral RdRp in a GTP-dependent manner (163). Two possible modes of action have been proposed, although they remain to be conclusively proven: a) it works as a chain terminator and prevents elongation of the nascent RNA molecule, b) it induces lethal RNA transversion mutations that eventually results in error catastrophe and a non-viable virus (195). However, unlike ribavirin (another broad-spectrum antiviral), favipiravir is not active against DNA viruses (194). Delang and co-workers described the antiviral effect of favipiravir against CHIKV. Favipiravir offered protection in a lethal mouse model of CHIKV-induced neurological disease, where disease severity was reduced and mortality was reduced by more than 50%. Furthermore, mutation K291R (conserved residue in the polymerase of all plus-strand RNA viruses) in the viral RdRp was shown to confer low level phenotypic resistance against favipiravir (196).

**Bindarit**

Bindarit is a small-molecule indazolic compound possessing anti-inflammatory properties and predominantly targeting the expression of CCL2 [also known as monocyte chemoattractant protein (MCP) 1] and other proinflammatory cytokines like CCL8, CCL7 and IL (interleukin)-12 (231). Bindarit was also reported to be active against other disorders like breast cancer (232), adjuvant-induced arthritis (233), lupus nephritis (234) etc. Chen et al had reported earlier that alphavirus infection could trigger loss in bone volume in the tibial epiphysis in an immunocompetent C57BL/6 mouse model and induce the production of proinflammatory cytokines in infected bone osteoblasts (235). Bindarit was shown to prevent the bone damage mediated by CCL2 induced osteoclastogenesis and...
bone resorption (229). This study also highlighted the potential of similar anti-inflammatory drugs used against rheumatoid arthritis being repurposed against CHIKV-induced chronic arthralgia.

**HSP-90 inhibitors**

Heat shock proteins (HSP) are chaperone proteins that aid in protein folding and regulate multiple functions in the cellular environment (236). HSP-90 was shown to interact with CHIKV nsP3 and nsP4 and siRNA knockdown of HSP90 reduced CHIKV replication. Two synthetic inhibitors of HSP90, HS-10 and SNX-2112 reduced viral RNA and protein expression. The same study also showed that these compounds reduced viral load in infected SvA129 mice (deficient in Type II IFN) and that treated mice did not show any CHIKV-induced inflammation or swelling (223).

**RNA interference strategies**

Different groups have tried the RNA interference approach to inhibit CHIKV replication in vitro and in vivo. siRNAs targeting the CHIKV nsp1 and E2 regions or a combination of both led to a significant reduction in CHIKV RNA expression in vitro. More importantly, siRNA treatment at 72 h p.i. significantly reduced viral loads in serum and muscle tissues of Swiss albino and C57BL/6 mice (208). In a similar approach, plasmid based shRNAs (short hairpin RNAs) against the viral nsp1 and E1 genes resulted in significant reduction in virus particle production in HeLa cells. Pretreatment of suckling C57BL/6 mice with 60 μg of shRNA E1 resulted in complete survival of the infected mice even after 10 days p.i (209). The same group used yet another approach to target the viral RNA, using phosphorodiamidate morpholino oligomers which are single-stranded oligonucleotides with six-membered morpholine rings and nucleotide bases linked by a phosphorodiamidate bond. These antisense nucleotides can bind to their complementary RNA sequence with high affinity and thereby create a physical steric hindrance for ribosome assembly on the target RNA molecule and prevent subsequent RNA translation. Pretreatment of HeLa cells with these antisense oligomers significantly decreased viral titer and protein expression. Moreover, 6-day old BALB/c neonate mice were completely protected on treatment with 15 μg/g of the phosphorodiamidate oligomer treatment given twice before infection and two more doses post-infection (205).

**STING agonist**

Recently, a novel approach of triggering the innate immune system against CHIKV infection has been successful. In a high throughput screen for molecules having the ability to activate the IRF (Interferon Regulatory Factor) 3/IFN-dependent innate immune responses, the compound termed as G-10 was identified which was able to dose-dependently reduce replication of CHIKV, VEEV and SINV in human cells. Using gene editing tools, the compound was determined to be a STING (Stimulator of IFN genes) agonist. Another unrelated small molecule STING activator called DMXAA (5,6-dimethylxanthenone-4-acetic acid) that works only in mice was found to reduce CHIKV viral load in 5-7 week old C57BL/6] mice when administered 3 h prior to infection and 6 h p.i. However, the reduction in viremia was not significant when the compound was given 24 h p.i. (230).
Animal models are required to investigate the pathophysiology of any disease. With regard to viral infections, having an animal model that replicates the disease symptoms and progression in humans is extremely important in order to evaluate the efficacy of vaccines as well as antiviral therapies. As early as in 1956, Ross discovered that intracerebral inoculation of neonate Swiss albino mice (< 12 days old) with sera from acute-phase CHIKV patients led to 100% mortality (121). It was only after the large-scale epidemics in 2005-2006 that there was renewed interest in CHIKV and the need to develop appropriate animal models. Adult immunocompetent mice are not susceptible to CHIKV-induced disease due to protection conferred by a robust IFN response whereas neonate mice show lethality following intradermal inoculation (237, 238). Couderc et al developed IFN-α/β receptor knockout (IFN-α/βR –/–) adult mice which were permissive to CHIKV infection and developed severe disease and mortality 3 d p.i. Interestingly, a partial abrogation of the IFN-α/β receptors (IFN-α/βR +/–) did not result in any disease signs, but viral presence was detected in the muscles and joints (238). At the same time, Ziegler and co-workers used sub-cutaneous inoculation of CHIKV in two strains (ICR and CD-1) of outbred newborn and neonate mice and observed arthritogenic disease manifestations of acute CHIKV disease including difficulty in walking, imbalance and severely reduced hind limb movement, but reduced the mortality that was observed earlier in mice from this age group (239). 2-3 day old mice when inoculated with CHIKV sub-cutaneously developed hind limb paralysis and diseased tissues were used for proteomic analysis to screen for host factors that may contribute to myositis (240).

Although these infection models provided valuable information on different aspects of CHIKV tissue tropism and the control exerted by the innate immune response in limiting in vivo disease, they could not recapitulate the muscle and joint pathology normally associated with CHIKV disease in humans [reviewed in (241)]. Secondly, the use of immune deficient or immunologically immature mice might not accurately portray the immunopathogenesis of CHIKV. Besides, in humans CHIKV infection rarely results in fatal disease, but instead depicts symptoms associated with chronic myalgia and polyarthralgia in distal joints. As a result, several groups began to develop mouse models which sought to mimic the arthralgic and myalgic complications associated with CHIKV disease – with juvenile mice (14 – 21 day old) (242) and also with adult immunologically mature mice (6 week old) (243). Instead of the more traditional routes of inoculation in mice, the site of infection was moved to the ventral side of the hind limb at the footpad, towards the ankle. As a result, this model was able to mirror the self-limiting arthritis, tenosynovitis and myositis that is associated with CHIKV disease in human patients (242, 243). Acute disease phase symptoms included peak foot swelling 6-8 days p.i., viremia was observed for 4-5 days and viral presence in the foot peaked 24 h p.i., with infectious viruses being detected in the feet up to 9 days p.i (243). Histological analyzes of feet from infected mice showed tell-tale signs of severe damage, with huge amount of mononuclear cell infiltrates around the synovial membranes, swelling, inflammatory cell infiltration near the tendons and extensive necrosis of the muscle fibers [reviewed in (241)]. Several different mutant models derived from the footpad model have been used in studies to
delineate the role of the innate immune response in CHIKV pathogenesis. Despite these advancements, there is still no mouse model that can replicate the chronic arthralgia that is observed in 30% of infected humans [reviewed in (244)]. Adult Cynomolgus macaques is a non-human primate model for CHIKV disease that can successfully recapitulate the chronic manifestations of CHIKV fever. However, they are not feasible to work with due to ethical issues and high maintenance costs (245). Humanized mouse models which have gained popularity with other diseases might be one way to go forward (244).

2.5 MAP kinase signaling in the context of viral infections

The mitogen-activated protein kinases (MAPKs) together constitute a repertoire of signal transducing Ser/Thr kinases in cells that relay a cascade of signals, triggered by external stimuli. The flow of information is usually mediated by multiple phosphorylation steps, beginning at the plasma membrane and proceeding to one or more cytoplasmic or nuclear substrates. There are three main branches of MAPK pathways which are widely studied: extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun NH2-terminal kinase (JNK) pathways. Each of these pathways is composed of a three-step signaling network of conserved serially acting kinases termed as MAPKKK (MAPK kinase kinase), MAPKK (MAPK kinase) and the final effector MAPKs, which through their phosphorylation of multiple substrates govern functions related to gene expression, cell growth, proliferation, mitosis, metabolism, migration, survival and apoptosis (pictorial representation in Fig. 5) [for more details, please refer to (246-248)].

2.5.1 ERK

The ERK module of the MAP kinases has a predominant role in cell survival and proliferation. It is mostly activated by different types of growth factors like the platelet-derived growth factor, epidermal growth factor, and nerve growth factor as well as in response to insulin. The activation starts by ligand binding at cell surface receptor tyrosine kinases which leads to a sequence of events that activate different Raf protein isoforms, which are the primary MAPKKKs in this module. Activated Ras then binds and phosphorylates MEK1/2 (the MAPKK in this pathway) which in turn phosphorylates the effector MAPKs, ERK1/2. Activation of this module leads to a significant amount of nuclear localization of ERK. Some of the important nuclear substrates of ERK are c-Fos and Elk-1, which have roles to play in cell cycle progression and survival. MEK1/2 inhibitors like PD98059 and U0126 which preferentially interact with the unphosphorylated forms of the kinase have been used to delineate the function of this pathway in different biological phenomena [reviewed in (246, 249)].

2.5.2 p38 MAPK

p38 MAPK has a major role in inflammatory immune responses and apoptosis. Four different isoforms (p38α, p38β, p38γ and p38δ) have been discovered. This pathway is strongly activated by a variety of different stress stimuli like UV irradiation, oxidative stress, hypoxia, ischemia; and inflammatory cytokines like
TNF (Tumor necrosis factor)-α and IL-1. Cytokine stimuli promote TRAF (TNF-receptor Associated Factor) protein recruitment to the intracellular domains of their cognate receptors. This leads to the activation of the initial MAPKKKs in this cascade, which include MEKK1-3, MLK2/3, ASK1, Tpl2 etc. In addition, this activation can also be triggered by certain GPCRs (G-protein Coupled Receptors), as well as Rho family GTPases like Rac and Cdc42. MKK3/6 are the MAPKKs in this cascade that form functional complexes with the different p38 isoforms, leading to their activation. p38 MAPKs in turn phosphorylate different cytoplasmic (MNK1/2, Bax, Tau etc.) and nuclear substrates (ATF1/2/6, p53, MSK1/2, GADD153 etc.) that are responsible for various proinflammatory and apoptotic responses. SB203580 and SB202190 are compounds that target the activity of p38α and p38β isoforms by competitively inhibiting ATP binding and have been used in numerous studies to shed light on the role of the p38 MAPKs in different processes [reviewed in (246, 250)].

Figure 5. Three-tiered MAPK signaling cascade. Schematic diagram showing the three main groups of the MAPK signal transduction system – ERK, p38 and JNK. Some of the main kinases at each stage are shown, along with the inhibitors commonly used to study their effects.
in different biological scenarios. [Reprinted from (1) with permission from the American Society for Microbiology].

2.5.3 JNK

There are three isoforms of JNK [also called stress-activated protein kinase (SAPK)], which similar to the p38 MAPKs are strongly activated by external stress signals like DNA damaging agents, ionizing radiation, heat shock, cytokines, UV irradiation, DNA and protein synthesis inhibitors etc. After being activated by upstream MAPKKKs common to the p38 MAPK pathway, MKK4 and MKK7 (MAPKKs of this module) co-operatively phosphorylate and activate the JNKs. Similar to ERK and p38 MAPK, JNK also depicts a predominant, but not exclusive nuclear localization subsequent to activation. JNK also has multiple downstream substrates that participate in cellular functions related to cell proliferation (especially in hematopoietic cells) and in the intrinsic apoptotic response to cellular stress. One of the well-characterized substrates of JNK is the transcription factor c-Jun, which together with c-Fos (activated by ERK) form functionally active AP (Activator protein)-1 complexes that modulate gene expression related to cell cycle progression by interacting with their AP-1-binding sites. SP600125 and AS601245 are two reversible ATP-competitive inhibitors that inhibit the activity of the JNKs [reviewed in (246, 251)].

2.5.4 Role of MAPK signaling in viral life cycles

The functions carried out by MAPK signaling are quite complex in nature and are often context and cell-type specific. Different viruses either stimulate or suppress MAPK signaling in order to favor their replication. We will briefly take a look at the role of MAPK signaling in the life cycles of some important RNA viruses.

HIV

HIV is the causative agent of AIDS and has a complex replication strategy involving reverse transcribing its RNA genome into DNA and integrating it into the host genome (252). Additionally, HIV has several regulatory proteins like Tat, Rev, Nef, Vif, Vpu and Vpr, which have diverse roles in the viral life cycle, which influence its ability to sustain a productive infection and cause disease. The production of pro-inflammatory cytokines like IL-10 during HIV infection is induced by the Tat protein and depends on ERK and p38 MAPK, through phosphorylation of the CREB-1 transcription factor (253). In striatal neuronal cells, Tat protein induced cell death by JNK-dependent caspase-3 activation (254). HIV Vpr protein is primarily responsible for regulating the import of the HIV-1 pre-integration complex into the nucleus (255), but can also induce cell cycle arrest and apoptosis by downregulating ERK (256). HIV-1 Nef protein mediates bystander killing of both CD4+ and CD8+ T-cells through upregulation of the Fas ligand, a process that requires p38 and Nef-induced AP-1 activation. Additionally, specific inhibitors of the p38 MAPK pathway led to reduced bystander damage of CD8+ cells in vitro (257).
**IAV**

IAV is a negative-sense RNA virus that causes respiratory infections, resulting in mild to severe flu-like symptoms leading to thousands of deaths and millions of hospitalizations every year (258). MAPK signaling and in particular, the ERK signaling pathway has a proviral role in the IAV life cycle in infected host cells (259). Virus replication was not only inhibited by specific inhibitors and dominant negative mutant kinases of this pathway, but also enhanced by active kinase mutants, both in vitro as well as in vivo in mice (259-261). The nuclear export of viral RNP (ribonucleoprotein) complexes is impeded when this pathway is blocked (259). Expression of the viral HA protein and its arrangement as lipid raft domains requires ERK activation and coincides well with RNP export, suggesting that ERK activation might be crucial for IAV budding (262). p38 MAPK pathway in IAV infection is responsible for the induction of the inflammatory response, leading to production of cytokines like RANTES and IL-8 (263). As opposed to the ERK pathway, the JNK pathway is crucial for the innate antiviral response and regulating the expression of IFN-β and defects in this pathway led to increased virus production. Additionally, IAV triggered AP-1 expression also takes place through JNK phosphorylation of its transcription factors c-Jun and ATF-2. IAV-induced JNK activation correlated with the accumulation of viral RNA in infected cells (264).

**SARS-CoV (Severe Acute Respiratory Syndrome-Coronavirus)**

SARS-CoV is a single-stranded positive-sense RNA virus and has a large genome of ~30kb (265). This virus which spread globally in 2003, resulting in a worldwide pandemic, causes severe respiratory illness and pneumonia (266) with a ~9.6% case fatality rate in infected patients (http://www.who.int/csr/sars/en/). SARS-CoV infection results in ERK1/2 phosphorylation, which is a thought to promote a prosurvival signal to the cells (267). At the same time, p38 MAPK activation in SARS-CoV infected cells promotes CPE and cell death (268). There is conflicting information about the precise role of p38 MAPK activation in the context of SARS-CoV infection. While apoptosis is normally observed with this pathway in virus-infected cells, several downstream pro-survival targets of p38 MAPK like CREB (cyclic AMP response element binding protein) and HSP27 were also reported to be activated (268). JNK activation occurs late in SARS-CoV infected cells and is thought to be involved in persistent infection of the cells (268). Overall, SARS-CoV infection seems to activate both prosurvival and apoptotic cell signaling MAPK pathways, but more research needs to be done to characterize the biological significance of these activations.

In addition to the above-mentioned examples, several RNA viruses like HCV (269), DENV (270, 271), WNV (272), EBOV (273) and RSV (274) as well as pathogenic DNA viruses like Epstein-Barr virus (275, 276), HBV (269), human papilloma virus (277) and herpesviruses (278) activate different branches of the MAPK signaling. This activation can either serve to prolong the life of the infected cells in order to maximize the production of progeny virions or to trigger apoptosis later in the infectious cycle. Despite its seemingly important role across viruses of different families, MAPK signaling in the context of alphavirus infection is a relatively underexplored area and deserves more attention.
3. AIMS OF THE STUDY

At the beginning of this study, CHIKV had spread to Europe and therefore, urgent intervention measures were needed to counter this re-emerging alphavirus.

This study aimed to miniaturize a previously developed CHIKV replicon cell line (85) to a 384-well plate format for high-throughput screening. Once the assay was set up, the aim was to screen drugs previously approved for other indications or those in clinical trials for antiviral activity against the replication stage of the CHIKV infectious cycle (I).

The next step was to characterize the mechanism of a relatively non-toxic inhibitor obtained in the screen – berberine (BBC) and to assess its effectiveness in vivo in a CHIKV mouse model (II).

An anti-cancer molecule, obatoclax (OLX) was shown to be antiviral against IAV and also against SINV (279). In the final part of the study, the objective was to determine the effectiveness of obatoclax against CHIKV and to characterize its mode of action (III).
4. MATERIALS AND METHODS

Most of the methods used in this study are described in the original publications as indicated in Table 2.

Table 2. Materials and methods used in this thesis

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5. RESULTS AND DISCUSSION

5.1 CHIKV replicon screening platform and its optimization for high throughput screening (I)

CHIKV re-emerged between the years 2004 – 2006 on a massive scale and spread throughout tropical Africa and Asia (124, 125). Later, the outbreaks in Italy and France in 2007 and 2010 respectively (126, 127), garnered a lot of attention worldwide, leading to an overwhelming response from many research groups and the organization of focused research consortia. As part of one such group, the ICRES (Integrated Chikungunya Research) European Union-funded consortium, the SFV group set out to develop screening tools to discover bioactive compounds with antiviral activity against CHIKV and identify potential antiviral drug candidates. In countries where CHIKV is not endemic, the virus needs to be handled under high containment BSL (Biosafety Level)-3 conditions. These facilities are not only tedious to work in, but also expensive to maintain.

As an alternative to using live CHIKV infections, Pohjala and co-workers established a BHK-derived cell line stably expressing CHIKV replicon (85). Plus strand RNA virus replicons are based on a bicistronic RNA molecule, where one cistron encodes for a reporter and a selectable marker and the other cistron encodes for the viral non-structural proteins. Here *Renilla reniformis* luciferase gene was cloned as a fusion protein with nsP3 and served as a readout for viral protein expression levels and indirectly for replication. Puromycin acetyltransferase for antibiotic selection and EGFP (Enhanced Green Fluorescent Protein) as a visual marker were included under the subgenomic promoter of the replicon. Additionally, a Pro718Gly mutation and a 5 amino-acid insertion in nsP2, obtained during the selection process, was necessary to confer a non-cytotoxic phenotype and ensure survival of the cells expressing the replicon. This tool was used in a 96-well plate format to screen a small set (~350) of natural compounds, resulting in a few compounds effective at low micromolar concentrations. These compounds were tested for their antiviral activity against SFV (the prototype relatively non-pathogenic alphavirus) and also against infectious CHIKV (85). Replicon systems have been useful in circumventing the problem faced with handling or culturing wild type infectious viruses. With HCV, it was a challenge for many years to obtain a suitable system that would allow screening for antiviral drugs, until the development of the replicon system which allowed replication of the non-structural proteins to very high levels in Huh-7 cells (280, 281). The replicon system has since been used for antiviral screening against different plus-stranded RNA viruses (178, 282, 283). Replicon systems although amenable for high throughput screening have obvious limitations as well. They restrict the screen to compounds that target only the replication cycle and miss out any potential hits which could affect entry, maturation and exit processes of the viral life cycle. Screening facilities within high containment laboratories (284) or the use of an attenuated vaccine strain (225) has allowed for assessing the entire viral life cycle for possible targets. High content image analysis has been useful in this context where either reporter viruses containing fluorescent tags (224) or analysis
by subsequent immunofluorescence assays (285) have been used to screen for antiviral drugs or host factors critical for virus replication.

The CHIKV replicon screening platform was optimized and miniaturized for a 384-well plate format at the high-throughput screening facility at the Institute for Molecular Medicine Finland (FIMM). The assay was carried out in an automated manner using a Beckman Coulter Integrated Robotic System and compounds were added with precision to each target well at 10 μM concentration using an acoustic dispensing system. Assay conditions like cell number, assay medium and volume of detection reagents to be used were optimized. We obtained a Z’ score of 0.7 for the assay which is higher than the threshold of 0.5, indicating that the assay was suitable. The Z’ score is a statistical measure of the suitability of a high-throughput assay, to assess if differences seen in treated samples are of significant value as compared to the positive and negative controls (286). Also, the coefficients of variation were for the positive control (6-azauridine) and negative control (0.1% DMSO) were 11% and 7% respectively, suggesting that these compounds served as appropriate controls for the assay (I).

5.2 Identification of novel antiviral drugs with broad-spectrum anti-alpha viral activity at low micromolar concentrations (I, III)

The high throughput assay screened ~3000 bioactive compounds from three different libraries – the NIH Clinical Collection, ENZO FDA-approved drug library and the MicroSource Spectrum library. These were compounds either in clinical use or clinical trials against other indications, with the intent of repurposing drugs with known targets/effects as antivirals against CHIKV. Screening libraries containing previously characterized or approved compounds has a distinct advantage in terms of knowing the targets and pharmacokinetic properties of these compounds in animal models. This approach of attempting to repurpose compounds for antiviral use has been used by several different groups conducting high throughput screens for inhibitors (225, 287). Using stringent selection criteria and further primary validation, the list of candidate drugs was narrowed down to 27 compounds (Table S1, Figs. S1A, S1B; I). Additionally, all compounds showed < 75% toxicity in the replicon-containing cells over a relatively long period of 48 h. This ensured that compounds which were purchased for resupply analysis had considerable selectivity in their antiviral activity. One of the compounds, amphotericin B turned out to be a false positive, inhibiting the reporter luciferase enzyme itself (Figs. 1D & S2; I). This calls for caution while interpreting reporter expression data in high-throughput screening.

Secondary validation with infectious CHIKV-Rluc virus trimmed the list further to seven compounds with strong antiviral activity, without adverse cytotoxic effects. Next, these compounds were validated in Huh 7.5 cells, which confirmed their effectiveness in a human cell line. All the compounds showed similar or lower EC₅₀ values in Huh 7.5 cells as compared to BHK-21 cells (Table 1; I). Cerivastatin was the only exception. This compound which targets the HMG-CoA (3-hydroxy-3-methyl-glutaryl-coenzymeA) reductase enzyme and reduces cholesterol
biosynthesis did not inhibit CHIKV replication in Huh 7.5 cells, probably due to increased cholesterol levels in the used hepatoma cells (288). Out of these eight compounds, only abamectin (EC50_BHK = 1.5 μM), ivermectin (EC50_BHK = 0.6 μM) and berberine (EC50_BHK = 1.8 μM) reduced the production of infectious viruses, although some other inhibitors like bromocriptine showed marginal reduction in viral RNA synthesis and both bromocriptine and cerivastatin showed a weak reduction in viral protein expression (Fig 2; I). The higher sensitivity of the luciferase assay can amplify small changes in protein expression. Most likely, these changes were not enough to result in a significant reduction in progeny virion production. Fenretinide, one of the hits in our screen, proved to be more effective against YFV, a flavivirus. This observation was later confirmed by two groups that reported fenretinide to inhibit the replication of DENV and other flaviviruses and also offer protection against DENV in vivo (289, 290). It was proposed that fenretinide interfered with the recognition of DENV NS5A by cellular nuclear import proteins and modulated the unfolded protein response (UPR) to impede virus replication (290).

We noticed a discrepancy in the inhibition profile for abamectin, ivermectin and berberine. Both abamectin and ivermectin completely abolished viral protein expression and RNA synthesis levels and also significantly reduced viral titers. On the other hand, berberine which did not have a strong effect on viral RNA synthesis and protein expression, reduced viral titers significantly, prompting us to investigate this further in a time-of-addition experiment (Fig. 2; I). The results of this experiment correlated with our previous observations and showed that berberine indeed had an additional late effect on the maturation or exit phase of the viral life cycle (Fig. 3; I). At first glance, it seems contradictory that a compound obtained through screening only for inhibitors that target the viral life cycle has a predominant effect of virus particle production. Nevertheless, berberine seemed to have a dual mode of action, where viral protein production and RNA synthesis was also reduced to some extent and could be why it was picked up in our initial screen (Figs. 1 & 2; I). However, at this stage, we had strong suspicions and indications from recent studies that berberine most likely targets host factors essential for a productive viral infection, which reflected in the reduction of different viral components. This was addressed later in our follow-up study (II).

In conclusion, the CHIKV replicon cell line represents a feasible platform to rapidly screen thousands of different compounds that target alphavirus replication. Nevertheless, there are also limitations with this system. There can be false negatives that might target viral processes other than replication. Secondly, the non-toxic phenotype of the CHIKV replicon abrogates nsP2 translocation to the nucleus, which does not represent the scenario of live virus infection. Besides, the parental cell line, BHK-21 is IFN-defective and does not accurately portray the cellular environment in CHIKV infections. We tried to address some of these limitations in our subsequent study (II).

The work conducted as part of this dissertation also revealed another small-molecule compound – obatoclax possessing broad-spectrum activity against different alphaviruses. Obatoclax, an anti-cancer drug was first reported to have antiviral activity against IAV and several other viruses, including SINV (279). We report that obatoclax was highly active against both SFV (EC50 = 0.23 μM) and
CHIKV (EC_{50} = 0.04 \mu M) with remarkably high selectivity indices (Table 1; III) and also characterized its mode of action (III), which will be discussed in detail below.

Altogether, these molecules represent novel antivirals against CHIKV and other alphaviruses, which are effective at low micromolar concentrations and could serve as starting points for further drug development. Although, ivermectin (structural analogue of abamectin) was quite toxic in Huh 7.5 cells (Table 1; I), it has been in clinical use against different parasitic diseases like onchocerciasis and elephantiasis (291), suggesting that ivermectin is well tolerated in humans. Further work needs to be done to evaluate its effectiveness against alphaviruses in other cell lines and in animal models. The modes of action of berberine and obatoclax were explored further and will be described later.

5.3 CHIKV-induced MAPK signaling (II)

In our pursuit to dissect the mechanism of berberine, we serendipitously stumbled on to signaling pathways activated by CHIKV. Published literature on berberine seemed to be conflicting and contradictory and quite often cell-type or context-specific. Therefore, a human phosphokinase array was used to assess the effects of CHIKV infection and berberine treatment on the activation of a panel of 48 cellular kinases and the phosphorylation of their substrates. This array helped to depict for the first time, the robust activation of the major MAPK signaling pathways – ERK, p38 MAPK and JNK by CHIKV infection (Figs. 5A, 5B & 6E; II).

Earlier, MAPK activation by alphavirus infection was shown for SINV (292) and VEEV (293). Similar activation by CHIKV indicates the conserved nature of these pathways in the alphavirus life cycle. Contrarily, ERK signaling was found to be antiviral against SINV in Aedes aegypti Aag2 cells, and this pathway was triggered in the insect gut epithelium by the insulin present in the vertebrate blood meal (294). Arbovirus infections progress differentially in mammalian and insect cells and this may be reflected in distinct signaling patterns during virus infection. ERK signaling seems to confer a prosurvival state to infected cells in the life cycle of different viruses (259, 269, 295). ERK signaling was also shown to be crucial in IAV budding from infected cells (262). ERK exhibits a typical biphasic activation pattern during infection with several different viruses – at the time of viral binding or entry and next coinciding with virus replication or egress (296-298). It will be interesting to know if this pattern is also exhibited by CHIKV and assess the effects of inhibitors of the ERK pathway on CHIKV entry. Indeed, for VEEV, ERK activation was noticed at early time points of infection (293). In our analysis, we chose a time point based on nsP3 expression levels in order to visualize the overall effect on signaling pathways as a result of CHIKV replication (Fig. 5; II). One of the substrates of Erk is the transcription factor, c-Fos, the phosphorylation of which was reported to be upregulated by HCV (299) and coxsackievirus B3 (300). It will be interesting to analyze the effect of c-Fos activation in the context of CHIKV and/or alphavirus infection. The upstream MEK1/2 inhibitor U0126 could significantly reduce viral titers under high MOI (multiplicity of infection) conditions, even though there seemed to be only a marginal effect on viral protein
expression (Fig. 6; II). This suggests that the ERK pathway might be essential for CHIKV maturation/egress.

SINV was also shown to activate the p38 MAPK and JNK at later time points (292). These pathways are usually induced by pro-inflammatory cytokines and cellular stress and are responsible for regulating immune response and apoptotic signaling (246). However, Nakatsue and colleagues showed that SINV infection leads to the phosphorylation of HSP27, which is usually associated with cell survival (292). We did not observe HSP27 phosphorylation with CHIKV infection in the phosphokinase array (data not shown), which might suggest differences in the eventual fate of MAPK activation between SINV and CHIKV. Another probable explanation is that these differences could be cell-type specific: the SINV study was performed in Vero cells (292), whereas we used IFN-competent HOS (human osteosarcoma) cells. There are quite a few examples of viruses or viral proteins which activate (301, 302) or inhibit (303, 304) JNK and its downstream transcription factor, AP-1 which seems to have stimulus-specific cell proliferative or growth arrest functions. For some viruses like JEV, it was shown that the UPR initiated due to high level viral protein expression in the endoplasmic reticulum results in apoptosis in a p38 MAPK and JNK dependent manner (305, 306). It would be interesting to assess the effects of alphavirus-induced UPR on MAPK signaling. The importance of the JNK cascade in the CHIKV life cycle was highlighted by a 2 log reduction in viral titers in response to treatment with a specific JNK inhibitor, SP600125 (Figs 6C & 6D; II). Unlike the MEK1/2 inhibitor, treatment with SP600125 also reduced virus-mediated reporter protein expression, suggesting that the JNK pathway could be subverted by CHIKV infection for multiple purposes. Treatment with the p38 MAPK inhibitor, SB203580 seemed to activate the ERK and JNK pathways and did not show any reduction in viral titers. This was surprising, as virus-mediated reporter expression had been significantly reduced on SB203580 treatment (Figs. 6C – 6E; II). This made us conduct a comprehensive literature review, and indeed there were reports of SB203580 and one of its metabolites cross-activating the ERK and JNK pathways (307, 308). This could in turn compensate for the loss of p38 activity in SB203580 treated cells and therefore no reduction in viral titers is seen, whereas in a luciferase based system, small differences can sometimes be amplified. In the luciferase assay, there is no assembly or budding and it is likely that inhibitions at the luciferase levels (thus, on replication) were “compensated” in the infection assay (by not having impact on assembly/budding). This underscores the importance of assessing viral titers in addition to reporter protein expression data to study the effect of different inhibitors on the viral life cycle. This study emphasizes the potential of cellular MAP kinases as therapeutic targets against CHIKV and other pathogenic alphaviruses. Indeed, our observations were independently confirmed by another study screening for host factors affecting CHIKV replication, where some of the upstream kinases in the p38 MAPK and JNK modules were implicated to have proviral functions. The same study also showed that FDA-approved drugs targeting receptor tyrosine and other upstream kinases activating MAPK, like sorafenib, tivozanib and linifanib showed antiviral activity against CHIKV with good therapeutic indices (224).
5.4 Berberine as a novel antiviral modulating host signaling pathways (II)

Berberine is an isoquinoline plant-derived alkaloid that has been used in traditional Chinese and Ayurvedic medicine for thousands of years (309, 310). Additionally, this compound has been in clinical trials and in clinical use for a wide variety of disorders like diabetes, hypercholesterolemia, and different forms of cancer and has also been shown to possess anti-inflammatory and anti-microbial properties (309, 311). More importantly, there have been several reports describing its antiviral properties against viruses from different families like HCMV (312), HSV (313), HPV (314) and RSV (315), some of them implicating different MAPKs as probable targets of berberine. As described above, CHIKV infection induced the activation of the major MAPK signaling pathways. In our initial array as well as in confirmatory immunoblots performed in two different cell lines, berberine was capable of concomitantly reducing MAPK activation (Figs 5B & 6E; II). This was in line with earlier observations where berberine reduced p38 MAPK activation in RSV infection and JNK activation in HSV-1 infection.

Berberine was also capable of reducing virus-independent, serum-induced ERK activation (Fig. 7; II), demonstrating that berberine affects host-processes which in turn could inhibit virus replication and consequently dampen other interconnected pathways. We did not observe any effect of berberine treatment on anisomycin (protein synthesis inhibitor)-induced JNK activation (data not shown). Nevertheless, we did notice reduction in basal JNK signaling in unstimulated cells (Fig. 6E; II). Further work needs to be done to elucidate the precise molecular target of berberine.

Earlier, it was shown that both SFV and CHIKV differentially activate the PI3K-Akt pathway. SFV is a strong inducer of the pathway, whereas SFV-Δ50 virus (mutant virus lacking 50 phosphorylated amino acids in the HVD of nsP3) as well as CHIKV only moderately activated this pathway (46). In our study, we also observed the CHIKV-induced Akt phosphorylation and its reduction in the presence of berberine (Figs. 5B & 6E; II). Berberine was found to significantly reduce SFV titers under low MOI conditions (Fig. 4A; I), but under high MOI conditions, berberine could not reduce the robust activation of Akt by SFV [Fig. 6 (316)]. This might
correspond to the higher EC$_{50}$ value (19.5 μM) obtained for berberine with SFV-Rluc virus (data not shown), where luciferase is expressed as a fusion protein with nsP3. Contrarily, the moderate Akt phosphorylation induced by SFV-Δ50 was drastically reduced by berberine, similar to that observed for CHIKV. This suggests that the effect of berberine on alphavirus-induced Akt phosphorylation depends on the Akt activation capacity of the virus in question and does not correspond to the observed reduction in viral titers. Berberine remains an enigmatic compound with myriad effects on multiple interconnected signaling pathways and this might be a reason for its relative non-toxic nature.

In study II, we overcame some of the limitations of our previous work. Berberine was proven to be effective against CHIKV in different IFN-competent human cell lines at high selectivity indices (Fig. 1; II) and worked against a variety of circulating CHIKV strains (Fig. 2; II). Additionally, berberine was antiviral against CHIKV even under high MOI conditions, reducing viral protein expression and titer. Berberine did not have a remarkable effect on RNA synthesis at the same concentrations (Fig. 3; II), highlighting once again that berberine does not interfere directly with the viral replication process. However, the defect may occur at a post-RNA synthesis stage by probably affecting host factors needed for viral structural protein expression or maturation and egress. This was also confirmed by the observation that berberine did not affect alphavirus entry or the trans replication of separately provided template RNA by SFV and CHIKV replicase proteins (Fig. 4; II).

5.5 The in vivo efficacy of berberine (II)

No antiviral showing promise in cell culture models can progress further, without proving to be efficacious in vivo. Therefore, berberine was tested in a mouse model that mimics the arthritic and myalgic manifestations seen during CHIKV infection in humans. Four-week-old female C57BL6/J mice were infected with CHIKV at the ventral side of the right hind footpad. This model uses footpad swelling as an indicator of CHIKV-induced inflammatory disease. We sought to replicate a typical antiviral drug regimen taken after onset of acute disease, treating the infected mice 24 h post-infection. Berberine was capable of controlling viral RNA levels in serum as compared to control-treated mice. Although this reduction in RNA levels was not statistically significant, it was enough to significantly reduce disease score as compared to the control mice at all days of peak disease severity. On day 6 p.i., joint swelling was reduced to marginal levels. Additionally, a marked reduction in inflammatory cell infiltrates in the infected joint footpad was observed in berberine-treated mice (Fig. 8; II). These results highlight both the antiviral and anti-inflammatory properties of berberine.

The observed LD$_{50}$ for berberine in mice is ~57 mg/kg (317), which is considerably higher than the dose of 10 mg/kg we employed in our study. Although, we did not achieve statistically significant differences in all of the parameters measured, this could well be due to the specific dosage conditions. In several studies validating antivirals against CHIKV in the same or different mouse models, compounds were also administered prophylactically (196, 230, 318),
which may have contributed to its efficacy. In our study, berberine was administered through the intra-peritoneal route due to its reduced oral bioavailability, which may not be ideal. Several groups are working on methods to improve the bioavailability of this compound (319, 320). Besides, further optimization of this compound may lead to improved compounds with better antiviral properties and enhanced bioavailability and tolerance. Therefore, further investigation is needed with berberine in terms of dosing, treatment schedules and assessment of immune functions. Nevertheless, berberine is now added to the list of the few compounds that have demonstrated antiviral efficacy against CHIKV in a mouse model.

5.6 Obatoclax as a novel viral fusion inhibitor (III)

A derivative of bacterial prodigiosin, obatoclax was originally designed as an anti-cancer drug. Bcl-2 family proteins control cellular fate by regulating mitochondrial permeabilization and subsequent apoptosis. The pro-survival members of the Bcl-2 family like Bcl-2, Bcl-XL and Mcl-1 (myeloid leukemia cell differentiation protein-1) bind and sequester their pro-apoptotic counterparts, Bak (Bcl-2 homologous antagonist killer) and Bax (Bcl-2 associated X protein). BH3-only proteins (members of the Bcl-2 family that contain only a BH3 domain) like NOXA, Bim (Bcl-2-like protein 11) and Bad (Bcl-2-associated death promoter) dock their α-helical BH3 domain into a hydrophobic groove of the pro-survival protein, thereby disrupting their interaction with Bak/Bax [reviewed in (321)]. Obatoclax is a BH3 domain mimetic that antagonizes the anti-apoptotic protein, Mcl-1, a protein that is often over-expressed in cancer cells (322, 323). Only a few years back, it was shown to possess antiviral activity against IAV. In the same study, obatoclax was also tested against other RNA and DNA viruses with differential results. In particular, it was highly effective against SINV at low concentrations (279). This led us to assess the effectiveness of obatoclax against the other alphaviruses – SFV and CHIKV.

The low EC_{50} values (0.04 μM_{CHIKV}), coupled with high selectivity indices against these viruses (Table 1; III) highlights the potential of obatoclax for further drug development. Indeed, the plasma level concentrations in clinical trials reached a C_{max} of 0.4 μM (324, 325), indicating that obatoclax could offer a probable prophylactic and therapeutic alternative against CHIKV and other alphaviruses. Denisova and co-workers had shown that obatoclax interferes with the entry and uptake of IAV and suggested the targeting of Mcl-1 to be the mode of action. Indeed, silencing Mcl-1 reduced IAV protein expression (279). In our study, we also noticed a predominant effect of obatoclax on the early phase of alphavirus infection (Fig. 2; III). An entry assay performed with temperature-sensitive SFV-ts9 mutant clearly showed that obatoclax inhibited alphavirus entry (Fig. 3A; III). Time of addition assays measuring both viral titers as well as luciferase signals in the entry assay showed that obatoclax gradually loses its effect when added at later time points of infection (Figs. 2C, 2D & 3B; III).

The most remarkable observation came with the neutral red retention assay, which depicted the swift neutralization of the acidic organelles brought about by
obatoclax treatment (Fig. 4A; III). Surprisingly, the basic nature of obatoclax was sufficient to neutralize an acidic buffer of pH 5.5. This precluded reliable conclusions from the endosomal bypass assay, forcing us to modify the protocol and include a treatment schedule beginning after the induction of fusion at the plasma membrane, which was able to rescue majority of the viruses from inhibition by obatoclax (Fig. 4B; III). Obatoclax could represent a new class of lysomotrophic agents representing viral fusion inhibitors. This finding represents a new property of this compound and is independent of its antagonism of Mcl-1. As controls, we tested other compounds that inhibited the same targets. These compounds (TW-37 and ABT-199) neither affected virus entry nor neutralized the acidic environment of endolysosomal compartments (Fig. 6; III). While study III was being prepared, three different groups observed the loss of endosomal acidification induced by obatoclax, providing independent corroboration for our observations (326-328). Furthermore, we showed that other viruses dependent on low pH for fusion (WNV, YFV and the re-emerging ZIKV) were inhibited by obatoclax to a similar extent (Table 2 & Fig. S3; III). On the other hand, certain viruses from the Picornaviridae family (CV-A9, E-6 and E-7), which do not require low pH for entry or uncoating, were not affected by obatoclax treatment (Table 2 & Fig. S4; III). Obatoclax was shown to accumulate in lysosomes (326) and also to cause lysosomal clustering in some other cell types (328). Nevertheless, the exact molecular mechanism of by which obatoclax mediates endolysosomal neutralization needs further investigation. Further lead optimization to develop related molecules with lower toxicity could propel obatoclax to become an effective broad-spectrum antiviral drug. The anti-malarial drug chloroquine which operates with a similar mechanism has also shown promise with numerous viruses in vitro, but has met with very little success in vivo (329).

5.7 Isolation of drug-resistant SFV mutants (unpublished, III)

One of the classical methods to gain insight into the putative mode of action of an antiviral compound, is the isolation of drug-resistant mutants. In this process, the virus in question is usually passaged in the presence of increasing concentrations of the compound, until a resistance phenotype emerges. For the isolation of resistant mutants against the compounds discovered in this study, we decided to use SFV as a bio-safe surrogate for CHIKV, due to the cumbersome nature of the work, which would have to be done in a BSL-3 laboratory. Initially, a short passaging scheme of 8 h at MOI 0.1 was used. BHK-21 cells were infected with the wild type virus in the presence of the compound (10 μM for cerivastatin), which was present throughout the infection. Viral titers were analyzed after the first round of passaging and then adjusted for every subsequent passage to continue with approximately the same MOI of 0.1. Titers were evaluated after every fifth passage. Cerivastatin was the only compound for which this approach yielded results.

After 10 rounds of passaging, we obtained a mutant showing complete resistance against cerivastatin, growing to titers as high as the untreated wild type virus. After plaque purification of several drug-resistant clones, subsequent sequencing showed a single point mutation in the E2 envelope glycoprotein. Recombinant
virus containing this mutation was generated and analyzed once again for phenotypic resistance. Interestingly, the untreated mutant virus grew to higher titers than the untreated wild type version. Nevertheless, in cerivastatin-treated samples, the mutant grew to even higher titers than the wild type virus under the same conditions (Fig. 7). Cerivastatin inhibits cholesterol biosynthesis by targeting the HMG-CoA reductase enzyme. Cholesterol has been implicated in the SFV life cycle for both fusion (330) and exit (331). Pre-treatment of BHK-21 cells for 2 h with 10 μM cerivastatin and prolonged treatment for another 3 h, did not inhibit or enhance virus entry in the SFV-ts9-entry assay (data not shown). This may suggest that cerivastatin likely affects virus exit by downregulating cellular cholesterol levels. The specific role of this mutation in the E2 protein in viral exit and cerivastatin resistance is currently being analyzed.

We did not observe phenotypic resistance for any of the other compounds using this short passaging scheme. In order to allow sufficient time for resistant mutants to emerge, a longer time window was used, where BHK-21 cells were infected at MOI 0.01 for 16 h (schematic layout in Fig. 5A; III). This approach gave better results as we could obtain partially drug-resistant mutants against abamectin, berberine and obatoclax.

For abamectin, it took 30 rounds of passaging the wild type virus in the presence of increasing concentrations of the compound (beginning with 3 μM for 10 passages and 5 μM for passages 16 to 30) to obtain partially resistant mutants. Phenotypic analysis of plaque purified virus stocks revealed that the mutant resistant to abamectin also confers resistance to ivermectin (Fig. 8), which could be expected as these compounds are structural analogues and probably have the same modes of action. Sequencing this mutant revealed three non-synonymous mutations in viral nsP1, nsP2 and E2 proteins. The nsP2 mutation lies in the helicase domain.

Fig. 7 Cerivastatin-resistant SFV mutant. BHK-21 cells were infected with wild type SFV and the SFV mutant containing the mutation in E2 at MOI 0.1 for 8 h, in the presence or absence of 10 μM cerivastatin. Plaque assay titers from viral supernatants collected at 8 h p.i. Titrations were performed in duplicates. Representative data set from two independent experiments.
Interestingly, ivermectin was reported to target the flavivirus NS3 helicase at low micromolar concentrations (332) and was recently found to be active also against ZIKV (287). It would be interesting to assess the effect of ivermectin and abamectin on the helicase activity of alphavirus nsP2. Importantly, the isolation of mutants resistant to abamectin and ivermectin indicate that despite the high toxicity and low selectivity indices observed with these compounds, their antiviral activity is selective and can be separated from their toxicity. Ivermectin was also found to be effective against HIV, DENV (333) and VEEV (334), where the nuclear export of different viral proteins was blocked by ivermectin by disrupting their interaction with nuclear transport proteins, importins α and β. The VEEV capsid protein is known to impede nuclear trafficking and thereby affect host transcription by forming a complex with nuclear importin α/β1 and exportin 1 (335). It would be interesting to know if ivermectin-resistant VEEV mutants could emerge and in which viral proteins would these mutations map to. The capsid protein of CHIKV is not known to exhibit this phenomenon, even though a putative nuclear localization signal has been identified (336). The Old World alphaviruses, including CHIKV, mediate host shut-off through the nuclear shuttling of nsP2. Examining the nuclear localization of CHIKV or SFV nsP2 in the presence of ivermectin could shed more light on its mode of action.

Using the same passaging scheme, passaging SFV in the presence of 3 μM berberine was sufficient to obtain a partially resistant mutant. We found two non-synonymous mutations in one of the nsPs, which in combination conferred partial resistance against berberine, similar to the original resistant isolate (Fig. 9A). This result seems unexpected, when berberine has a predominant effect on the latter stage of the viral life cycle, by modulating MAPK signaling. This prompted us to analyze the ERK activation phenotype of the mutant. Interestingly, the resistant mutant showed increased ERK activation as compared to the wild type. Even though this activation is reduced by berberine, it is still as high as that observed for the untreated wild type sample (compare phospho ERK; lanes 3 and 6) [Fig. 9B; (316)].
There could be two possible explanations for this particular observation: i) the mutations in the nsP region has increased the affinity of the viral enzymes to their substrates, allowing the virus to replicate more efficiently and at levels higher than the wild type, which then accounts for the increased ERK activation or ii) excess nsPs, which are not part of the viral replication complex, transiently interact with one or more components of the ERK pathway and that the activation of ERK and/or downstream targets could serve as a trigger for viral progeny release. These mutations could conceivably increase the affinity of such an interaction, allowing for increased ERK activation and thereby, increased viral egress. Indeed, the ERK signaling pathway has been implicated in the release of IAV particles from virus-infected host cells (262). These lines of research are currently being pursued.

With obatoclax, which we discovered to be a fusion inhibitor for alphaviruses, partially resistant mutants could only be obtained after 30 rounds of passaging in the presence of 0.5 μM of the compound. Four common non-synonymous mutations were present in two different resistant viral clones – two in nsP2 (E46D and V601I) and two in the E1 fusion protein (L369I & S395R). Phenotypic analysis of recombinant viruses containing different combinations of mutations revealed that the nsP2 mutations or the E1 mutations alone could confer some amount of resistance. However, the combination of all four mutations together was most resistant, similar to the original resistant isolate. Unexpectedly though, the virus
containing only the L369I mutation also showed the same resistance profile as the combination of all four and the original resistant isolate. On the other hand, the virus containing only the S395R mutation showed only weak resistance (Fig. 5B; III). Mutations in the E1 fusion protein lend further support to our observations of obatoclax inhibiting virus fusion. Analyzing the E1 mutations in the SFV-ts9 entry assay, corroborated our results where SFV-ts9 virus containing only the L369I mutation had a seven-fold higher EC_{50} value for entry inhibition as compared to the wild type ts9 virus (Fig. 5D; III). Moreover, the ts9 viruses containing the E1 mutations seemed to have increased fusogenic potential and gave higher luciferase signals (Fig. 5C; III), suggesting that these mutations may have been selected to confer the ability to fuse better and enhance the overall infectivity of the virus. In summary, these results indicate that the L369I mutation in the SFV E1 fusion protein is sufficient to confer partial resistance to obatoclax. However, we cannot exclude that the specific passaging conditions used to isolate these mutants could have led to mutations arising in nsP2. Similar to that observed with the cerivastatin-resistant mutant, all the recombinant viruses showed increased fitness and grew to higher titers as compared to the wild type virus (Fig. 5B; III). Earlier, it was shown both for CHIKV and HCV that serial passaging of the virus in cells led to increased viral fitness and reduced sensitivity to different viral mutagens and antiviral drugs (337, 338).

It is generally considered to be quite difficult for escape mutants to arise when host processes required for different steps of the viral life cycle are targeted. However, recently there have been examples with IAV (339), CVB3 (340) and HCV (341) that escape mutants resistant to host-directed antivirals can arise, that somehow help circumvent the modulated cellular environment. Our new findings together with these previously published results suggests a paradigm shift from the long-held notion that antiviral resistance can emerge only if viral components or viral enzymatic processes are targeted. Our method of isolating drug-resistant mutants involves assessing viral titers of progeny virions that have accumulated over multiple replication cycles. Infection was performed at a low MOI of 0.01, allowing the possibility of escape mutants to arise. Other groups have used a different approach, where virus-induced CPE is observed after several days of infection in the presence of the particular antiviral compound (196, 210). The compounds presented in this thesis would not be tolerated in BHK-21 cells over such extensive periods. Nevertheless, using this approach of monitoring virus titers at intervals has enabled us to obtain fully or partially drug-resistant mutants for four different compounds. Notably, for the most effective inhibitors, extensive passaging was required, indicating a high barrier to resistance.
6. CONCLUSIONS AND FUTURE PERSPECTIVES

The present study identifies some of the most effective potential candidate antivirals against CHIKV. It also highlights the importance of the drug repositioning strategy to obtain effective host-directed antivirals. Additionally, two of the compounds presented in this thesis (berberine and obatoclax) helped us to gain more insight into different aspects of alphavirus biology.

The main highlights of this dissertation are as follows:

a) The CHIKV replicon cell line is a viable and robust platform, amenable to automation and high throughput screening of antivirals targeting the replication phase of the CHIKV life cycle. In the future, the screening can be expanded to include larger chemical collections and compounds obtained from natural sources.

b) Compounds abamectin and ivermectin were found to affect the early and replication stages of the viral life cycle. Ivermectin being in clinical use, it would be interesting to test the compound in a CHIKV mouse model. Furthermore, the resistant mutant obtained against this compound should be further characterized.

c) CHIKV infection showed a robust activation of the MAPK signaling pathways. The molecular features of the viral components needed to trigger this activation remain to be determined.

d) Berberine is an effective antiviral, which has a major effect on viral maturation/egress, through the modulation of one or more MAPK signaling networks. Most importantly, berberine is one of the few compounds to show reduction of CHIKV-induced inflammatory disease in a mouse model. Mutations that confer resistance to berberine enhanced activation of the ERK pathway. Further work needs to be done to elucidate the precise role of these mutations and the specific molecular target of berberine.

e) Obatoclax is an anti-cancer drug that was revealed to neutralize the acidic environment of endolysosomes, thereby inhibiting viral fusion. Furthermore, mutation L369I in the SFV E1 fusion protein was enough to gain partial resistance against obatoclax.
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Nijmegen, November 2016

Finny S. Varghese
CLUES

ACROSS

1. Happens when a bug gets inside of you
3. This glowing protein makes studying viruses easier
7. Energy currency in the cell
12. Used to compare and assess biological processes and experiments
13. Clothing for viral genetic material
17. Pursuit of testable knowledge through systematic study
18. Often co-circulates with chikungunya and infects millions every year
20. Novel fusion inhibitor described in this thesis
24. Degree to which a substance causes damage to an organism
27. Particle with a capsid shell and a nucleic acid core
28. The species responsible for a million deaths every year
29. The viruses in this thesis have this as a genetic material
30. This thesis involved discovering more of these
31. Country where chikungunya virus was first isolated in 1952
32. Process of narrowing down a selection

DOWN

2. Enveloped viruses enter a cell through this process
4. E3-E2 envelope proteins are cleaved by this enzyme
6. First line of information in the central dogma
7. Non-inflammatory joint pain
8. Designed to pinpoint a specific target
9. Abbreviation for a prototype virus belonging to the same family as chikungunya
11. Viruses hijack this cellular process to gain entry into the host cell
14. Thousands of these were counted during the course of this thesis
15. This technique was used the most for this thesis
16. This genus was the first group of arboviruses to be classified
17. This was the focal point of the second study in this thesis
19. Viral envelope proteins do some crazy moves when exposed to this environment
23. Final step in the viral life cycle
25. Biological catalyst
26. Investigative procedures that require plenty of optimization
28. A cellular pathway studied in this thesis


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