2014

Chikungunya virus: emerging targets and new opportunities for medicinal chemistry

Adel Rashad
University of Wollongong, ar917@uowmail.edu.au

Suresh Mahalingam
Griffith University

Paul Keller
University of Wollongong, keller@uow.edu.au

Publication Details

Rashad, A. A., Mahalingam, S. & Keller, P. A. (2014). Chikungunya virus: emerging targets and new opportunities for medicinal chemistry. Journal of Medicinal Chemistry, 57 (4), 1147-1166.

Chikungunya virus: emerging targets and new opportunities for medicinal chemistry

Abstract
Chikungunya virus is an emerging arbovirus that is widespread in tropical regions and is spreading quickly to temperate climates with recent epidemics in Africa and Asia and documented outbreaks in Europe and the Americas. It is having an increasingly major impact on humankind, with potentially life-threatening and debilitating arthritis. There is no treatment available, and only in the past 24 months have lead compounds for development as potential therapeutics been reported. This Perspective discusses the chikungunya virus as a significant, new emerging topic for medicinal chemistry, highlighting the key viral target proteins and their molecular functions that can be used in drug design, as well as the most important ongoing developments for anti-chikungunya virus research. It represents a complete picture of the current medicinal chemistry of chikungunya, supporting the development of chemotherapeutics through drug discovery and design targeting this virus.

Keywords
CMMB

Publication Details
Rashad, A. A., Mahalingam, S. & Keller, P. A. (2014). Chikungunya virus: emerging targets and new opportunities for medicinal chemistry. Journal of Medicinal Chemistry, 57 (4), 1147-1166.

Chikungunya Virus: Emerging Targets and New Opportunities for Medicinal Chemistry

Adel A. Rashad[1], Suresh Mahalingam[2] and Paul A. Keller[1]*

[1]Centre for Medicinal Chemistry, School of Chemistry, University of Wollongong, Wollongong, Australia, 2522

[2]Emerging Viruses and Inflammation Research Group, Institute for Glycomics, Griffith University, Gold Coast, QLD, Australia, 4222

[*]*Correspondence to:*

Paul A. Keller, keller@uow.edu.au

Tele: +61 2 4221 4692

Fax: +61 4221 4287

Abstract: Chikungunya virus is an emerging arbovirus that is widespread in tropical regions and is spreading quickly to temperate climates with recent epidemics in Africa and Asia, and documented outbreaks in Europe and the Americas. It is having an increasingly major impact on humankind, with potentially life-threatening and debilitating arthritis. There is no treatment available, and only in the last 24 months have lead compounds for development as potential therapeutics been reported. This perspective discusses the chikungunya virus as a significant, new emerging topic for medicinal chemistry, highlighting the key viral target proteins and their molecular functions that can be used in drug design, as well as the most important ongoing

developments for anti-chikungunya virus research. It represents a complete picture of the current medicinal chemistry of chikungunya, supporting the development of chemotherapeutics through drug discovery and design targeting this virus.

Key words: alphaviruses; arboviruses; chikungunya virus (CHIKV); chikungunya fever (CHIKF); non-structural proteins; envelope proteins; drug discovery

I. INTRODUCTION

I.1. Classification, History and Clinical Features. Chikungunya virus (CHIKV) is an emerging arthrogenic arbovirus that belongs to the *alphavirus* genus, family *Togaviridae*. It has been responsible for major outbreaks of devastating human arthritis disease during the past five years.[1] Chikungunya fever caused by the virus was first described in 1952,[2] after an outbreak on the Makonde Plateau (named after an ethnic group from East Africa), along the border between Tanganyika and Mozambique. During this period, a high proportion of residents of all ages were affected by a distinctive disease with a sharp onset of crippling joint pains, severe fever, and a conspicuous rash.[2] The elders of the Makonde tribes could not remember any previous, similar epidemics with these symptoms, suggesting that this was a new illness. The word ‘‘Chikungunya’’ translates to ‘‘that which bends up’’ relating to the stooped posture developed as a result of rheumatologic inflammation.[3] Subsequently, only minor outbreaks occurred periodically in Africa, however major epidemics were reported in the 1960s and 70s in India and Southeast Asia.[4] After the 1973 outbreak in India, only sporadic activities were detected for the next 30 years, with no major recurrence until a large outbreak in Kenya in 2004.[5] This initiated a spreading epidemic that reached numerous islands of the Indian Ocean, India, and parts of Southeast Asia, and was further detected in 18 countries throughout Asia, Europe, and North

America via imported infectious carriers. Over the course of five years, an estimated more than 2 million cases occurred, with outbreaks in several countries where the virus had not been previously documented.[6] The first CHIKV hit in Europe occurred in Italy in 2007.[7,8]

Nearly 40 countries have detected chikungunya virus infected cases thus far (Figure 1). The US National Institute of Allergy and Infectious Diseases (NIAID) in 2008 listed CHIKV as a category C priority pathogen: this category includes pathogens that could be engineered for mass dissemination in the future, or due to their high morbidity and mortality rates and those with major health impacts.[9, 10] Recent epidemics were reported in India (1.4 to 6.5 million cases in 2006-2007), and 3,000 - 42,000 cases were detected in 2009 in Malaysia and Thailand.[11,12] The CHIKV mortality rate has been estimated to be 1:1000 and most of the deaths occur in neonates, adults with underlying conditions and the elderly.[10]

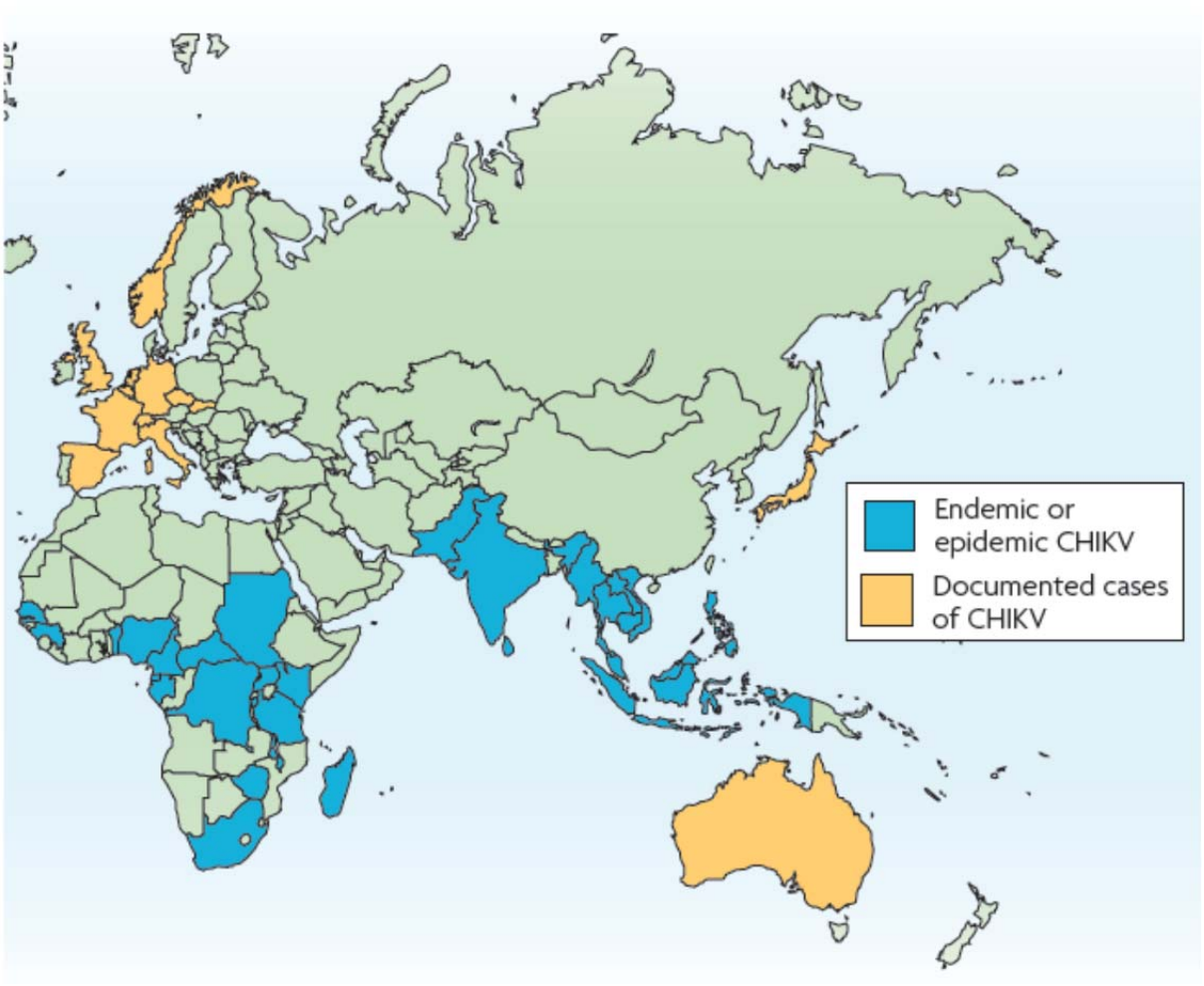


Figure 1. Worldwide distribution of the Chikungunya virus.[10] Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, (Vol 8), copyright (2010).

CHIKV can be transmitted through an urban cycle, man to mosquito to man, or a sylvatic cycle, animal to mosquito to man.[13] The virus is transmitted to humans by mosquitoes of the *Aedes* genus (*Aedes furcifer* in Africa and *Aedes aegypti* in Asia), similar to the dengue fever causing virus. However, the *Ae. albopictus* mosquito was extensively implicated in CHIKV transmission during the 2005-2006 outbreak on Reunion island.[10]

The switch of the CHIKV vector from *Ae. aegypti* to *Ae. albopictus* arose from an insufficient number of *Ae. aegypti* for its transmission.[14] A mutation in the E1 envelope protein, A226V, increased the CHIKV fitness in *Ae. albopictus* and improved the transmissibility of the

virus through *Ae. albopictus* to vertebrate species.[15] *Ae. albopictus* has spread to Madagascar, the Indian Ocean nations, Africa, Southern Europe and the USA.[16, 17] Virus transmission has been also reported as a result of maternal-foetal transmission in recent epidemics.[18]

Chikungunya fever *(CHIKF)*: The symptoms of CHIKF infection generally start 4–7 days after the mosquito bite. Infection presents in two phases, the first being acute, while the second stage is persistent (chronic), causing disabling polyarthritis.[19] Acute infection lasts 1–10 days and is characterized by a painful polyarthralgia, high fever, asthenia (weakness), headache, vomiting, rash, and myalgia (muscle pain). Rash is the least reliable symptom, presenting in as few as 19% of patients. When a rash is present, it is typically maculopapular in nature, but recent studies have also noted vesiculobullous lesions with desquamation.[20] The persistent chronic CHIKF phase is characterized by polyarthralgia (aches in the joints, joint pains) that can last from weeks to years beyond the acute stage.[21] Eighteen months after disease onset, 40% of patients are found to still have anti-CHIKV immunoglobulin M (IgM).[22]

CHIKV attacks fibroblasts, explaining the involvement of muscles, joints, and skin connective tissues. The high number of nerve endings within the joints and muscle tissues explains the pain associated with CHIKF. Neurological manifestations have also been described during the most recent epidemics in India, including disorders such as encephalitis, peripheral neuropathy, myelopathy, myeloneuropathy and myopathy.[23] Moreover, some cases with multi-organ failure have also been noted.[24] Eye infection (Chikungunya neuroretinitis) has also been reported where patients suffered from a sudden, painless diminution of vision in both eyes.[25,26] CHIKV can also infect the cornea and be transmitted via the ocular route.[27]

I.2. Virology of the CHIKV. The CHIKV genome (Figure 2) is a positive sense, single stranded RNA genome of about 11.8 Kb in size. It consists of two open reading frames (ORFs),[28] one in the 5` end encoding the non-structural protein precursors:

- nsP1: involved in viral mRNA capping via its guanine-7-methyltransferase and guanylyltransferase enzymatic activities,
- nsP2: acts as protease and helicase,
- nsP3: part of the replicase unit and an accessory protein involved in RNA synthesis,
- nsP4: RNA-dependent-RNA polymerase,

The nsP123 precursor and nsP4 function as part of a complex for viral negative-strand RNA synthesis. The 3` end ORF encodes the structural proteins, the capsid (C), envelope glycoproteins E1 and E2 and two small cleavage products (E3, 6K). The untranslated junction region (J) (Figure 2) contains its internal promoter, a conserved sequence of 21 nucleotides, for transcription of the sub-genomic mRNA in other *alphaviruses* (sindbis virus, for more details see ref 29).[29]

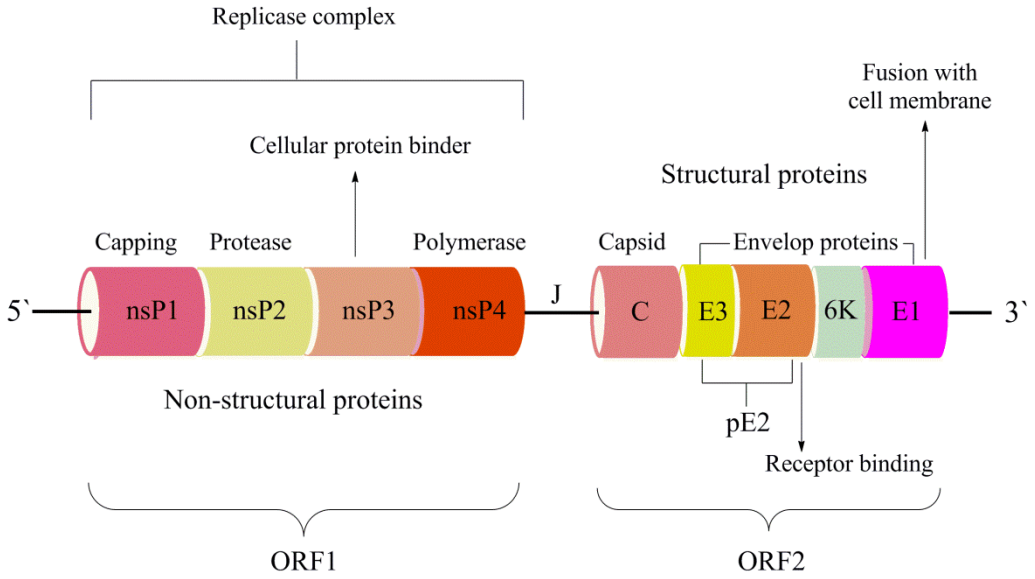


Figure 2. Schematic representation for The CHIKV genome showing the RNA sequence ORFs.

The CHIKV surface consists of 80 trimeric spikes composed of heterodimers of the envelope glycoproteins (E1 and E2) in the lipid bilayer. Similar to other members of the *alphaviruses*, the

CHIKV starts its life cycle (Figure 3) by entering the target cells by pH dependent endocytosis in clathrin coated vesicles *via* receptor mediated interaction,[30] but the exact mechanism by which it does so remains unclear. CHIKV has been shown to replicate in a large number of cell types including epithelial, endothelial and fibroblast cells as well as monocyte derived macrophages.[30] A recent study identified prohibitin (PHB) as a microglial cell expressed CHIKV binding protein.[31] PHB is an evolutionarily conserved and ubiquitous protein that consists of two highly homologous proteins of different molecular weights. PHB1 has a mass of approximately 30 kDa while PHB2 is approximately 37 kDa. The two proteins oligomerize, and hetero-oligomerization is essential for protein stability.[32] PHB has been shown to be present in multiple cell compartments including the mitochondria, cytoplasm and nucleus in addition to its expression on the cell surface.[33] PHB1 was confirmed as a CHIKV E2 binding protein, but not PHB2.[31] PHB1 was found to be involved in the internalization process either on its own or as part of a complex, further suggesting that a PHB-virus interaction may be mediated by the specific PHB molecule that interacts with the virus. Experimental down-regulation of PHB1 significantly reduced the level of infection in tested cell lines. However, the authors believe that this mechanism might be only one pathway by which CHIKV can enter the susceptible cells.[31]

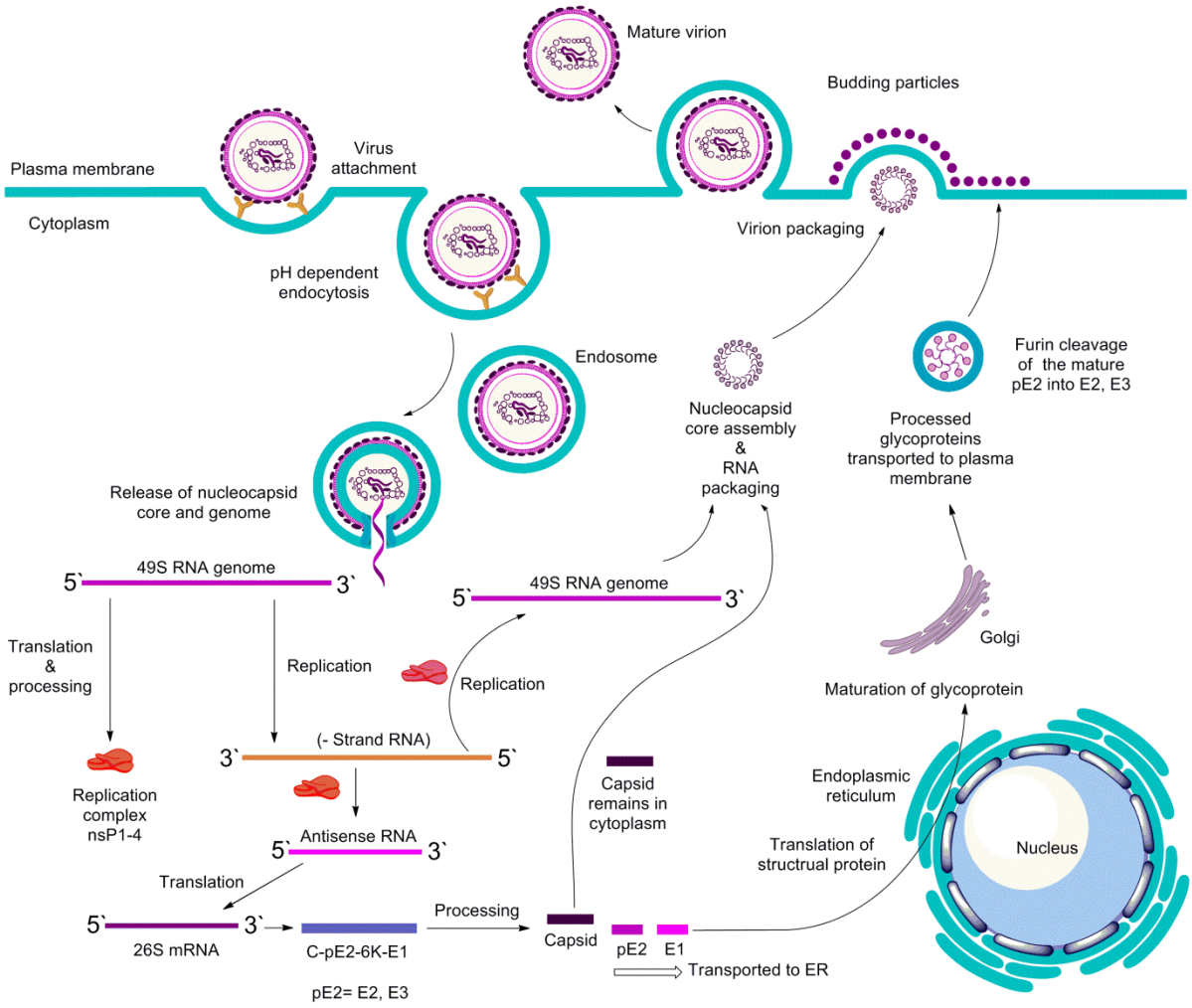


Figure 3. Schematic representation of the Chikungunya virus life cycle.

After entering the cell, the endosome acidic environment triggers conformational changes with the viral envelope proteins (E1 and E2 complex) reorganizing, leading to dissociation of the E2-E1 heterodimers, and formation of E1 homotrimers. E1 trimerizes and inserts into the target membrane with the hydrophobic fusion peptide (fusion loop) and refolds to form a hairpin-like structure. Exposure of the E1 fusion peptide mediates virus host cell membrane fusion, releasing the nucleocapsid into the cytoplasm.[34,35] This process depends on low pH and cholesterol, which is also required for budding during *alphavirus* infection.[36,37]

Two non-structural proteins precursors are translated from the viral mRNA, and are then cleaved generating nsP1, 2, 3 and 4. During translation, nsP123 binds to free nsP4 and with some cell proteins, forming the replication complex,[38,39] which synthesizes a full-length negative-strand RNA intermediate required for replication. When the nsP123 concentration increases, it is cleaved into nsP1, nsP2, nsP3 and nsP4 which forms, along with host cell proteins, the positive strand replicase, producing the 26S sub-genomic positive strand RNAs and genomic (49S) RNAs.[39] Promoters present in the negative strand initiate the transcription of 26S sub-genomic positive stranded RNA which encodes the structural proteins precursors. The latter is cleaved by a serine protease to yield the capsid (C) which remains in the cytoplasm (Figure 3), pE2, 6K and E1.[40] The C protein might be responsible for such autoproteolytic activity as it has a number of conserved sequences which have similar activity, and are common in other *alphaviruses*.[41]

pE2 and E1 are translated in the endoplasmic reticulum (Figure 3) and processed in the Golgi, and are then moved to the plasma membrane, where pE2 is cleaved by furin-like protease activity in the host cell into E2 and E3.[42] The assembly of virions begins in the cytoplasm of the cell, where the formation of the nucleocapsid with 120 dimers of the C protein starts to occur.[43] The assembled particle buds at the cell membrane as spherical particles of 65 to 70 nm in diameter, composed of genomic RNA molecules and the capsid proteins and enveloped in a host-derived lipid membrane.

I.3. The Development of CHIKV Vaccine. There is an urgent need to control the spreading of CHIKV, however, there is little understanding of the interaction between the chronic CHIKV infection and the immune system in defending the body against any subsequent reinfection.[10] The immune responses are strongly accepted to induce autoimmunity, by cross reactivity between viral and host antigens. The B cells and T cells might respond to CHIKV and this may contribute to the long-term joint disease experienced by many convalescent patients.[44]

Thus far, there is no licensed CHIKV vaccine. Some vaccine preparations that involved either formalin inactivation or Tween-ether extracts of virus[45] showed high immune responses without any adverse effects. In 2000, the US Army carried out a Phase II clinical trial examining a live attenuated CHIKV vaccine.[46,47,48] The vaccine was formulated from a human MRC-5 cell line as a lyophilized supernatant. In this study, subjects that received the vaccine developed neutralizing antibodies, and fewer subjects showed mild to moderate joint pain.[48]

In 2009, one study reported a vero cell adapted formalin inactivated prototype vaccine with alhydrogel as adjuvant that was prepared using an Indian CHIKV strain implicated in the 2006 epidemic. The humoral immune response was characterized by high titer antibodies that have been confirmed through microcytotoxicity assays and *in vivo* neutralization tests. Therefore, this could be a promising, safe and effective vaccine eliciting a long lasting protective immune response.[49]

A live CHIKV vaccine was developed in 2011 that elicits a protective immune response with no detectable disease in mice. It is also unable to infect mosquito vectors, which is an important safety feature for a live virus vaccine that could be used in non endemic areas to immunize travellers or laboratory personnel. However, this vaccine candidate is still under evaluation in nonhuman primates.[50] Other promising CHIKV vaccine candidates that depend on virus-like particles are in early stages of preclinical development.[51,52] A successful virus-like particle vaccine based on viral structural proteins was tested on nonhuman primates and was found to produce neutralizing antibodies that protect against viremia after high-dose challenge. When these antibodies were transferred into an immunodeficient host (a mice), the host was protected indicating a passive immunity.[53]

With the ongoing vaccine development research against the CHIKV, the world remains under the threat of rapidly spreading CHIKV infections, and this emphasizes the importance of

developing chemotherapeutics targeting the virus for controlling already existing infections in devastated areas.

II. EMERGING NOVEL CHIKV TARGETS

As previously mentioned, the CHIKV genome is formed of 2 ORFs, one from the 5` end coding for nsP1, nsP2, nsP3 and nsP4. The 3` end ORF encodes the the capsid (C), envelope proteins E1, E2, E3 and 6k (Figure 2). These proteins, which mediate essential steps in the lifecycle (Figure 3) of the virus,[10] could be possible targets for drug design.

II.1. Non-Structural Proteins

II.1.1. Non-structural protein 1. Like in other *alphaviruses*, CHIKV nsP1 is a palmitoylated 535 amino acid protein. The N-terminal region is a methyltransferase and guanylyltransferase involved in capping and methylation of the newly formed viral genomic and subgenomic RNAs.[54] In early 2013, it was shown that CHIKV nsP1 acts as antagonist for the bone marrow stromal antigen 2 (BST-2).[55] BST-2 is one of the host cell defence mechanisms, and is induced by interferon (INFα). BST-2 expression results in retaining viruses at the surface of the infected cells.[56] BST-2 was found to co-localize with CHIKV E1 and nsP1, but only nsP1 is able to down-regulate BST-2 expression, thereby inhibiting virus tethering on the cell surface.[55] This activity of the CHIKV nsP1 is similar to that of the HIV-1 Vpu protein[56] in that both repress BST-2. This discovery will help in developing BST-2 mediated therapeutics targeting the nsP1.

II.1.2. Non-structural protein 2. The non-structural protein 2 (nsP2) of *alphaviruses* is a multifunctional protein.[57,58,59,60] The proteolytic domain has been allocated to its C-terminal section which forms a papain like cysteine protease (also known as thiol protease).[57,61] The nsP2 proteolytic activity is critical for virus replication and is responsible for cleavage of the non-structural polyprotein complex.[62,63]

The proteolytic activity of the CHIKV nsP2 has been demonstrated,[64] and the enzymatic activities within the N-terminus have been recently investigated. It was found to have RNA triphosphatase activity that performs the first of the viral RNA capping reactions. It was also found to have a nucleotide triphosphatase (NTPase) activity, fueling the RNA helicase activity performed by the C-terminal domain.[65] CHIKV-nsP2 also has 5`-triphosphatase (RTPase) activity that removes the γ-phosphate from the 5` end of RNA. Both NTPase and RTPase activities are completely dependent on Mg^{2+} ions.[65]

Both N and C domains are composed of α-helices and β-strands (Figure 4). The N terminus is dominated by α-helices, whereas the C-terminal domain contains helices and strands. The central β-sheets are flanked by α-helices. The crystal structure of CHIKV nsP2 protease has been solved, and is composed of 324 residues. Being a cysteine protease, the catalytic mechanism involves a nucleophilic cysteine thiol in a catalytic dyad.[66] Analysis of the CHIKV nsP2 crystal structure shows 6 cysteine residues, three in the N-terminus (Cys1013, Cys1057 and Cys1121) and three in the C-terminus (Cys1233, Cys1274 and Cys1290) as shown in Figure 4. Since the proteolytic activity is isolated in the C-terminus,[64] one of the three cysteine residues in the C-domain might contribute as the catalytic thiol.

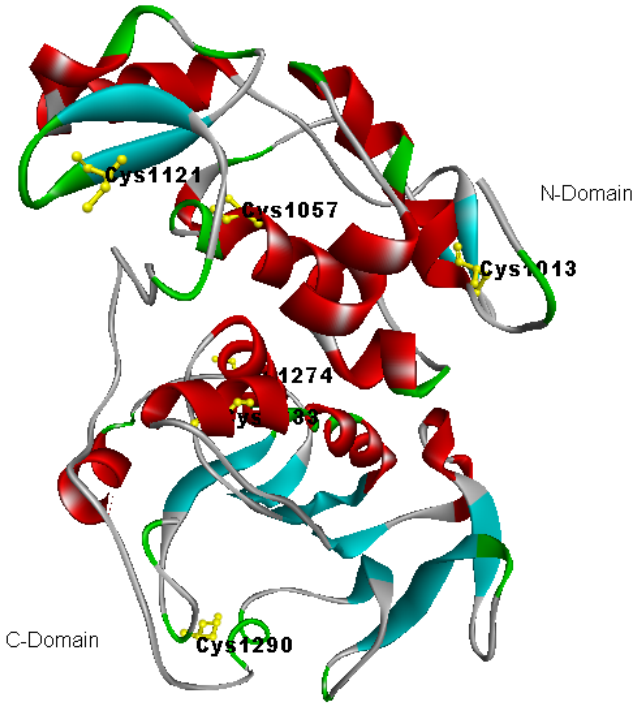


Figure 4. CHIKV nsP2 crystal structure showing the N- and C-terminal domains, cysteine residues shown in yellow balls and stick form (pdb code: 3TRK[67], no citation was found for the crystal structure).

The first step in the mechanism of cysteine proteases catalysis is usually the deprotonation of a thiol group within the enzyme active site by an adjacent amino acid containing a basic side chain, often a histidine residue.[68] Among the three cysteine residues in the C-terminus (Figure 5), the Cys1274 residue is less likely to be involved in the catalytic mechanism as only one His residue (His1314) is nearby, whereas for the other cysteine residues, four His residues, His1222, His1228, His1229 and His1236 could be associated in the deprotonation mechanism (Figure 5).

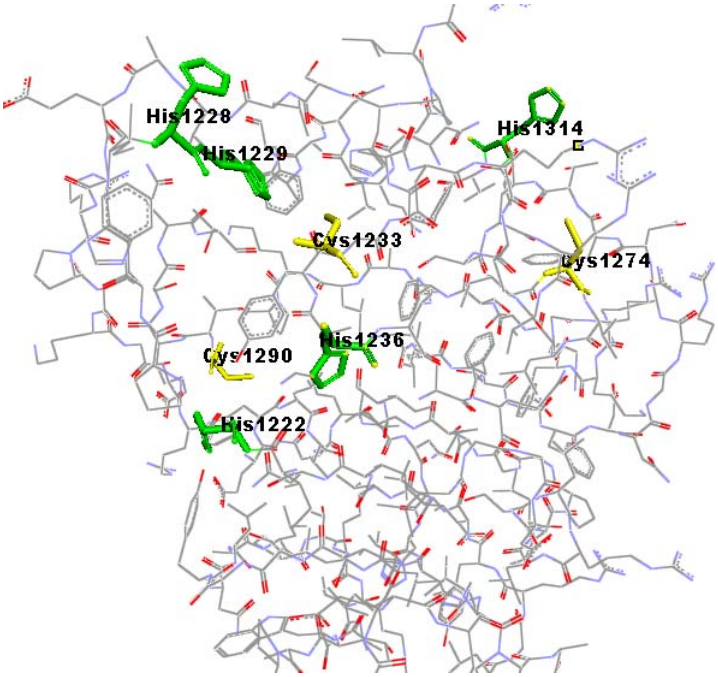


Figure 5. Schematic representation of the CHIKV nsP2 C-Domain showing the positions of the cysteine residues (yellow) and histidine residues (green), generated from the crystal structure pdb file code: 3TRK.

In 2012, Singh Kh *et al*. reported the development of a homology model of the CHIKV nsP2 protein based on the crystal structure of the nsP2 protein of Venezuelan equine encephalitis virus (VEEV),[69] in order to locate the active site of the protease. The critical residues in nsP2 were identified by docking three different peptides to identify the residues responsible for non-structural protein cleavage of the nsP1-2, nsP2-3 and nsP3-4 peptides. These three peptide sequences represent the substrates for the nsP2 proteolytic processing with a remarkable preference of nsP3-4>nsP1-2>nsP2-3.[70]

The active site was investigated and was found to lie in the C-terminal domain[69] (Figure 5). The key residues Gln1039, Lys1045, Glu1157, Gly1176, His1222, Lys1239, Ser1293, Glu1296 and Met1297 were found to interact with the non-structural protein sequence complex

to be cleaved, and were considered an individual functional unit. Only two residues are located in the N-domain, Gln1039 and Lys1045, with all the other residues located in the C-domain. Analogous work by Bassetto *et al*.[71] reported the development of a homology model for the nsP2 protease active site within the C-domain.

The predicted active site by Singh Kh *et al*.[69] and Bassetto *et al*.[71] matches with the above mentioned explanation of the positioning of the active site within the C-domain. In particular, they found the His1222 residue to be lying within the predicted active site pocket. Analysis of the enzyme surface shows that the predicted active site is located in a major surface groove as shown in Figure 6, with the major cavity on the enzyme surface more likely to accommodate the substrate polyprotein sequence to be cleaved.

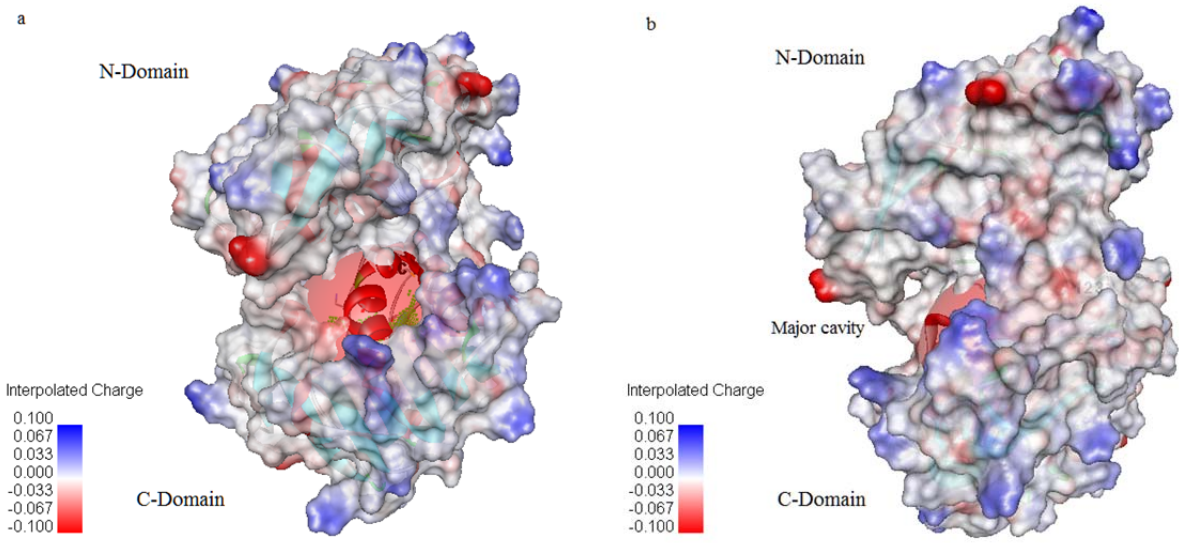


Figure 6. Electrostatic potential surface of the CHIKV nsP2 and its active site pocket within the C-domain, a) The active enzyme surface front view showing the active site as a transparent red sphere surrounding the active site with the Cys1233 and His1222 residues lying within the sphere. b) Side view of the enzyme (rotated to the left by 90º about the vertical axis from the

view in a) showing the major accessible cavity to the active site with a part of the sphere protruding toward the outer surface, generated from the pdb file 3TRK.

This major enzyme groove (Figure 6b) may act as the enzyme mouth holding the protein to be processed. Therefore, targeting the residues identified above,[69] as well as the residues within the active site, would be applicable strategy to inhibit the enzyme function and consequently inhibiting the virus replication.

Moreover, the *alphaviruses* nsP2 proteins have been described as virulence factors responsible for the transcriptional and translational shutoff in infected host cells and the inhibition of interferon (IFN)-mediated antiviral responses contributing to the controlling of translational machinery by viral factors.[72,73] This controlling comes through interactions with cellular RNA binding proteins, including heterogeneous nuclear ribonucleoproteins (hnRNPs), ribosomal protein S6 (RpS6), and cellular filament components. Recently reported was the believed interaction of 22 cellular components with nsP2 or nsP4, contributing to the CHIKV replication, mainly heterogeneous nuclear ribonucleoprotein K (hnRNP-K) and ubiquilin 4 (UBQLN4). It was also noted that the interaction of nsP2 with the tetratricopeptide repeat protein 7B (TTC7B) plays a significant role in the cellular machinery control induced by the CHIKV infection.[74]

II.1.3. Non-structural protein 3. The function of *alphaviruses* nsP3 remains unknown, although mutations can affect different steps of the viral replication machinery.[75] It is constructed of two domains, the first being a unique macro domain in the conserved N-terminal region. The C-terminal region is less conserved and is phosphorylated in approximately 16 positions on serines and threonines.[76,77] The function of phosphorylation is not understood, but it was found that deletion of these phosphorylated residues decreases the level of RNA synthesis.[78] Interestingly, viral pathogenicity of Semliki Forest virus (SFV), another *alphavirus*, is decreased

in absence of that phosphorylation on the nsP3, and the absence of the C terminus alters SFV neurovirulence.[79]

The N-terminus of nsP3 contains a macro domain (known also as the X domain), which binds to ADP-ribose derivatives and RNA, and is able to hydrolyse ADP-ribose-1``-phosphate,[80,81] a side product of cellular pre-tRNA splicing. Therefore, it is believed to control the metabolism of ADP-ribose 1``- phosphate and/or other ADP-ribose derivatives which have regulatory functions in the cell. The ADP ribose–binding site within the nsP3 macro domain is solvent-exposed and points away from the other domains in the nsP23 polyprotein. Based on sequence conservation in *alphaviruses*, it has been shown that residues just after the nsP3 macro domain play a role in positioning of the nsP23 complex cleavage site.[82] It can be inferred from the crystal structure of the nsP23 precursor protein of the closely related *alphaviruses*, SINV, that the nsP2 is connected to the nsP3 through the macro domain of the nsP3.[83] The nsP23 cleavage site is located in a narrow cleft formed between nsP2 and nsP3 that is inaccessible for proteolysis, and all the nsP2 non-cytopathic mutants lie at the interface between nsP2 and nsP3.[83] The inaccessibility of the nsP23 cleavage site indicates that access is tightly regulated. It is believed that the activator segment is located in the amino-terminus of the nsP2 which becomes exposed after cleavage from the nsP12 precursor poly protein.[84]

In 2010, the crystal structure of the nsP3 macro domain for the CHIKV was solved[80] (Figure 7). It consists of 672 residues and contains six-stranded β sheets with three α helices. The intermolecular interactions between the residues in the binding pocket of the enzyme and the ADP-ribose,[85] as analysed from the crystal structure, are shown in Figure 8.

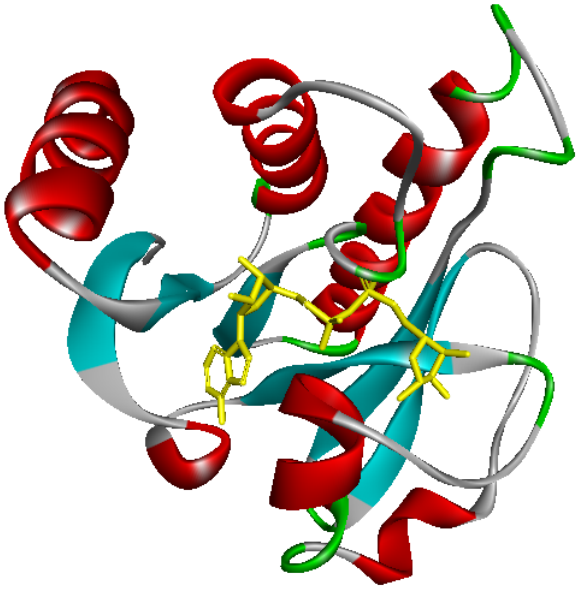


Figure 7. Crystal structure of the CHIKV macro domain with the bound ADP-ribose (yellow colour), generated from the pdb file code: 3GPO.

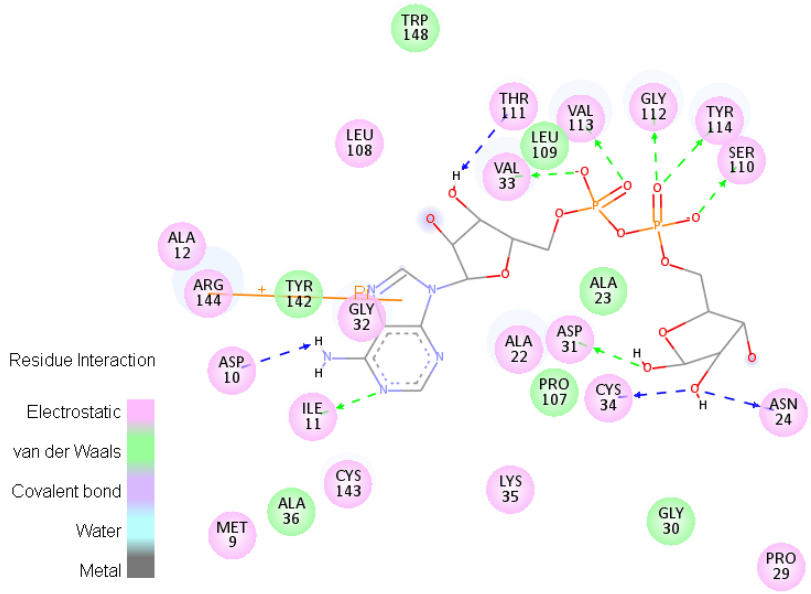


Figure 8. 2D representation of the interaction of ADP-ribose inside the nsP3 macro domain binding pocket showing the other residues inside the active site (Generated from the pdb file: 3GPO).

As shown in Figure 8, the key binding residues are: Arg144, Asp10, Ile11 Thr111, Gly112, Ser110, Tyr114, Val113, Asn24, Asp31 and Val33. The binding complex is formed of 10 H-bonds and one π-cation interaction.[85] The PO_4^{2-} moiety showed the strongest interactions with these residues in the enzyme pocket. Also, the ribose (with Thr111) and the diphosphate (with Val 33, Ser110, Gly112, Val113, Tyr114) units were found to play major roles in the CHIKV nsP3 ADP-ribose complex.[85]

Understanding this binding interaction of the ADP-ribose to the macro domain of the CHIKV could therefore be a useful element to further assist in drug design and development of inhibitors for this virus. Bound inhibitors to the ADP-ribose binding pocket will alter the function of the nsP3 either cleaved or in a polyprotein complex, and consequently will alter the function of the viral replication machinery.

Recently, more insights on the molecular function of the nsP3 revealed interesting findings, it was reported that the nsP3 of CHKV, as with other *alphaviruses*, use a conserved proline-rich motif to interact with the Src-homology-3 (SH3) domain of amphiphysin-1 and amphiphysin-2 proteins of the host cell, two related member proteins of the BAR (Bin-Amphiphysin-Rvsp) protein superfamily implicated in several cellular functions.[86]

More recently,[87] the nsP3 has been shown to be the inhibitor of stress granule assembly by recruiting G3BP into cytoplasmic foci. The conserved nsP3 SH3 domain-binding motif (the proline-rich motif) is essential for both nsP3-G3BP interactions and viral RNA replication. G3BP (Ras GTPase-activating protein-binding protein) is an enzyme in human cells and a member of the heterogeneous nuclear RNA-binding proteins.[88] This protein plays a major role during infection and in the assembly of stress granules. Stress granules are membranous cytoplasmic focal structures (foci) that immediately aggregate in response to cellular stress. This

last action leads to impaired translation of most mRNAs.[89] These stress granules may have antiviral activity that is inhibited by CHIKV replication by the nsP3 SH3 domain-binding motif.[87]

II.1.4. Non-structural protein 4. The non-structural protein 4 was identified as the RNA-dependent-RNA polymerase,[29,90] and was recently found to suppress the host cell unfold protein response (UPR), also named as the endoplasmic reticulum (ER) stress response.[91] The UPR is a mechanism that maintains the cellular protein homeostasis and prevents over-loading of unfolded protein in the lumen of the ER during normal and diseased cellular conditions. The UPR is multi-stepped and involves contributing proteins, including the PKR-like ER kinases (PERK).[92] During the UPR, PERK is activated by self-dimerization and phosphorylation, which then phosphorylates the serine 51 position of the eukaryotic translation initiation factor 2, alpha subunit (eIF2α), an essential factor for protein synthesis. Phosphorylated eIF2α inhibits the general protein synthesis, and consequently, will inhibit the pathogen protein replication. The CHIKV nsP4 was found experimentally to significantly reduce the phosphorylation (serine 51) of eIF2α, and thus ensuring the translation of the viral protein.[91] This discovery can be exploited as possible target for anti-CHIKV intervention. The crystal structure of the CHIKV nsP4 is not yet available for the structure based inhibitors discovery, and more efforts should be devoted to decipher the mechanism of action for that protein as well as experimental tools to evaluate possible potential protein inhibitors.

II.2. Structural Proteins

The invasion of susceptible cells by the CHIKV is performed by two viral glycoproteins, E1 and E2. Both carry the basic antigenic determinants and form the icosahedral shell of the virion particle. E2 and E3 are produced from furin cleavage of the p62 (also known as PE2, see Figure

2) precursor. E2 is responsible for receptor binding whereas E1 mediates the membrane fusion.[10] E3 contains the 64-amino-terminal residues of p62.[93]

E1 and p62 peptide are type I membrane proteins and are derived from a structural polyprotein precursor. They are translated in the infected cell endoplasmic reticulum, into a p62‑E1 heterodimer and processed by the Golgi (Figure 3). E3 protects the E2-E1 heterodimer from premature fusion with cellular membranes.[94] The heterodimers trimerize forming the viral spikes. Cleavage of p62 into E3 and E2 during transport to the cell surface prepares the spikes for the fusogenic activation to enter the cell. At the plasma membrane, the formed virions bud through interactions between E2 and genome-containing viral nucleocapsids in the cytoplasm.[95]

In a recent study, the roles of four amino acid residues (G91, V178, A226, and H230) in the CHIKV E1 protein were linked to the E1 and cell fusion process.[96] The study revealed that the highly conserved amino acid residues, G91 and H230, were important for membrane fusion functionality. The glycine residue (G91) is critical for the fusion process whereas any mutation or substitution in this residue lead to complete loss of E1 fusion ability. The E1 histidine 230 is located outside of the fusion sequence, but still critical for the fusion. Other structural proteins also affect the E1 fusogenic capacity, *e.g.* the E2 protein facilitates both E1 folding and regulates E1 fusogenic properties in a pH and cholesterol dependent process.[96] As an *alphavirus* family member, the hydrophobic fusion peptide of the CHIKV was found to be a trimer of hairpins composed of β-sheets in the post fusion state (type II fusion proteins).[97,98] Figure 9 shows the crystal structure of the CHIKV fusion peptide,[99] consisting of 18 amino acid residues, which are residues 84−101 in the full-length E1 glycoprotein.[95]

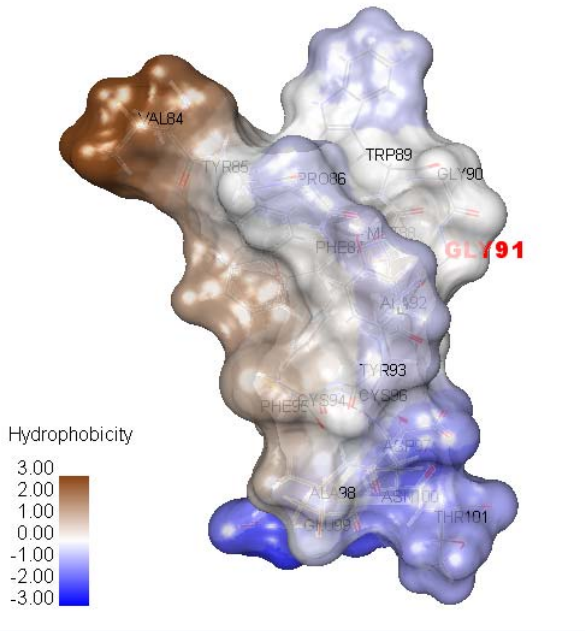


Figure 9. Hydrophobic surface view of the CHIKV fusion peptide showing the residue G91 in red label located at the back side (generated from the crystal structure of the fusion peptide, pdb file code: 2RSW[99]).

The crystal structures of the CHIKV p62-E1 (immature) and the E3-E2-E1 (mature) glycoprotein complexes are shown in Figures 10-12. E1 is folded into three domains I, II and III (Figure 11) that are rich in β-sheets. E2 is an immunoglobulin β protein, with three domains (A, B and C). Domain A is at the centre and domain B is at the membrane upper end whereas domain C is towards the viral membrane. The latter binds to domain II of E1 by hydrogen bonding due to the hydrophilic contact region between them (Figure 10). The long β – ribbon of E2 makes most of the connection with E3 (Figure 12). Furin loop (Figure 10) is the E2E3 junction in the immature complex, this junction contains a functional proprotein convertase motif which is cleaved by the cellular proteases, furin-like proprotein convertases, during the maturation (Figure 3) of the glycoproteins.[42] Variations within this junction site among the

different CHIKV isolates greatly affect the cleavage susceptibility by furin proteases. The amino acid His60 (residue 56 in the crystal structure pdb file: 3N40) is the critical residue that determines the spectrum of furin and furin-like convertases that process E2E3 glycoprotein complexes.[100] It can be inferred from the comparison between the immature and the mature glycoprotein complexes structures,[95] that the short peptide sequence (Pro59, His60, Arg63, Glu64, Ser65, Thr66, Lys67 and Asp68) is cleaved form the immature complex after furin cleavage.

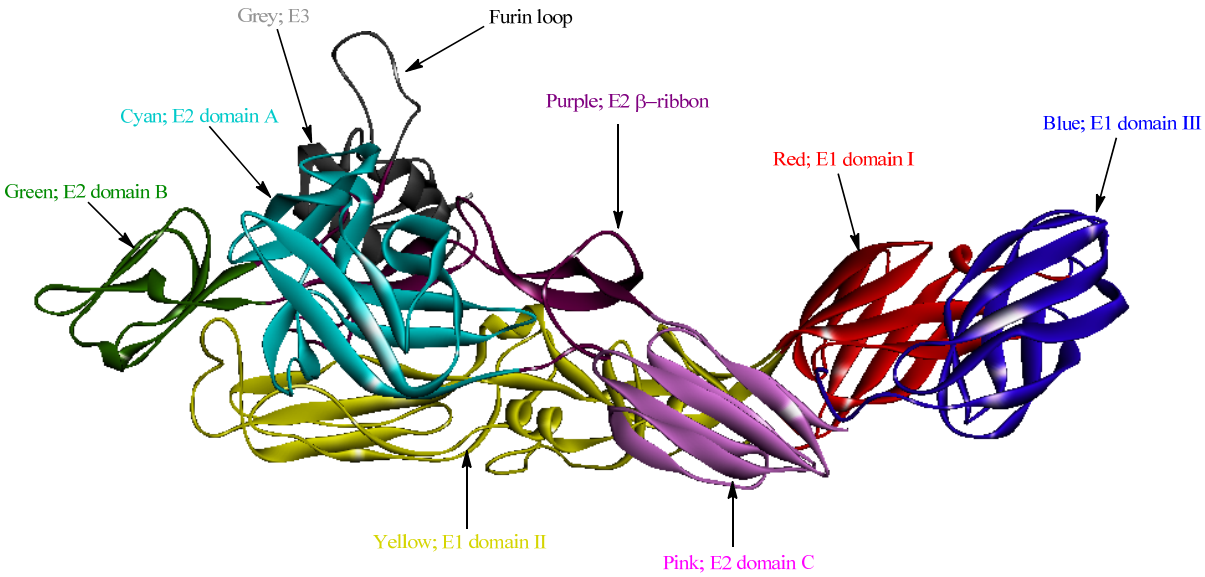


Figure 10. Crystal structure of the immature envelope glycoprotein complex of Chikungunya virus, E1 is formed from domains I, II and III. E2 contains domains A, B and C. Structure generated from the pdb file code: 3N40[95]. E3 stabilizes the E2 β–ribbon connector[94] being associated with domain A of E2 and domain II of E1, allowing domain B to protect the fusion loop.

The U shaped fusion loop of E1 is inserted in a cavity that lies between the E2 domains A and B, being stabilized by hydrogen bonds (Figure 13) with E2 histidine side chains.[95] At

neutral pH, E3 maintains the relative orientation of E2 domains B and A creating a cavity space that accommodates the E1 fusion loop. This orientation by E3 protects the virus from premature fusion with other cellular membranes.[95,101] The fusogenic activity of the E1 fusion peptide is therefore highly dependent on pH change. The histidine residues of E2 act as the pH sensor for the activation of the fusion protein at lower pH[95] due to the increased probability of histidines to become positively charged at lower pH values (acidic endosome), based on the fact that the imidazole ring of the histidine residue is the only amino acid side chain whose apparent dissociation constant from protons (pKa) falls within the physiological range.

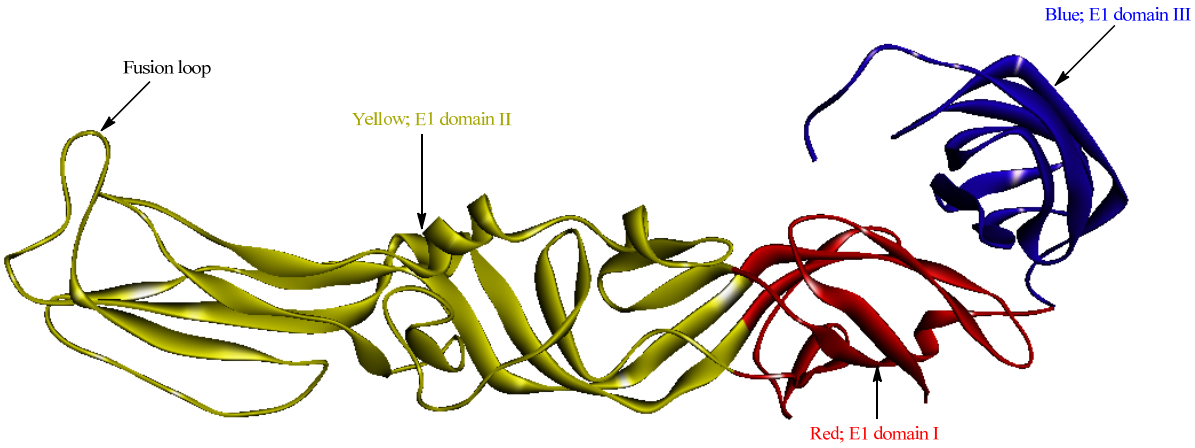


Figure 11. Crystal structure of E1 glycoprotein formed of 393 residues (residue 402-residue 794 within the whole complex structure), separated from the complex for visualization, generated from the pdb file code: 3N40[95].

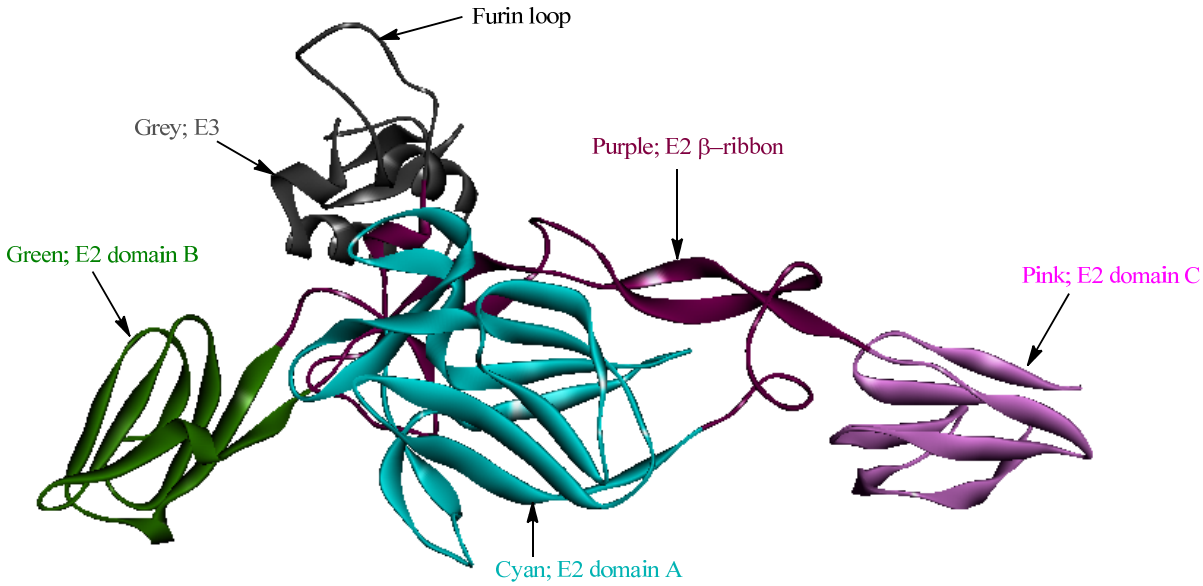


Figure 12. Crystal structure of E2, E3 glycoprotein complex formed of 401 residues (residue 1-residue 401 within the whole structural protein complex), separated from the complex for visualization. Generated from the pdb file code: 3N40[95].

Some important locations (transitional epitopes) were identified in both E1 (domain III) and E2 (domain B) of *alphaviruses*: these locations become accessible upon exposure to heat or low pH[102,103] as well as upon contact of the virions with the susceptible cells. This contact leads to conformational changes related to cell binding,[104] with domain B moving out in relation to domain A, thus opening the cavity. The fusion loop now becomes free to release,[95] without a full dissociation of the E2-E1 heterodimer. Other residues in domain B of E2 are believed to be associated with cell recognition assuming that a number of sites on the virus surface can interact with different cell surface receptors, and may be involved in the attachment and entry of the virus.[94]

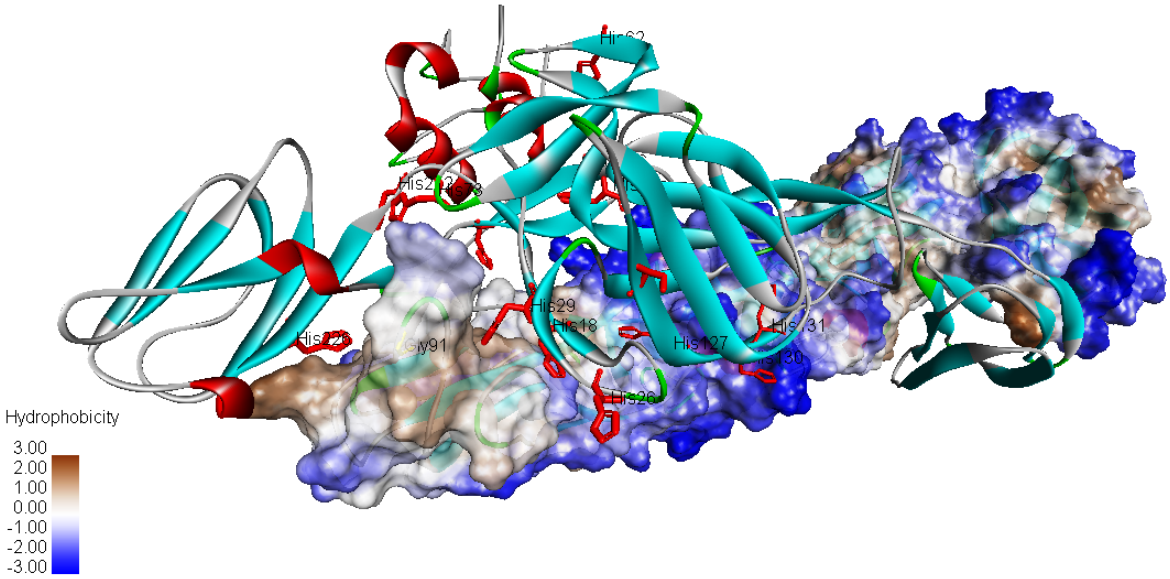


Figure 13. Crystal structure of the CHIKV mature envelope glycoprotein complex. E1 is viewed as hydrophobic surface with the critical amino acid residue Gly91[99] of the fusion loop labelled in black. E2 and E3 are viewed as a solid ribbon, and histidine residues on E2 are viewed as stick structures in red surrounding the fusion loop and acting as the pH sensors.[95] Generated from the pdb file code: 3N42[95].

Recently, the possible druggable pockets within the CHIKV envelope proteins have been determined (Figure 14), with two found to be critical to the protein functions, mainly the fusion process.[105] The green pocket (Figure 14) represents a surface cavity that lies between the E1 domain II and E2 β–ribbon that connects E2 domain A to E2 domain C. It extends downwards as a channel between E1 domain II and E2 domain A. The blue pocket (Figure 14) is a narrow channel extending just behind the fusion loop and is surrounded by both E2 domains A and B. These pockets make contact with residues from E1 and E2 and therefore, ligands for these pockets can affect the relative movement of E1 and E2 domains in the pre- and post-fusion states. Furthermore, the blue pocket (Figure 14) makes contact with the fusion loop residues,

and therefore, designing antagonists for this pocket with would be applicable strategy to block the fusion function.[105]

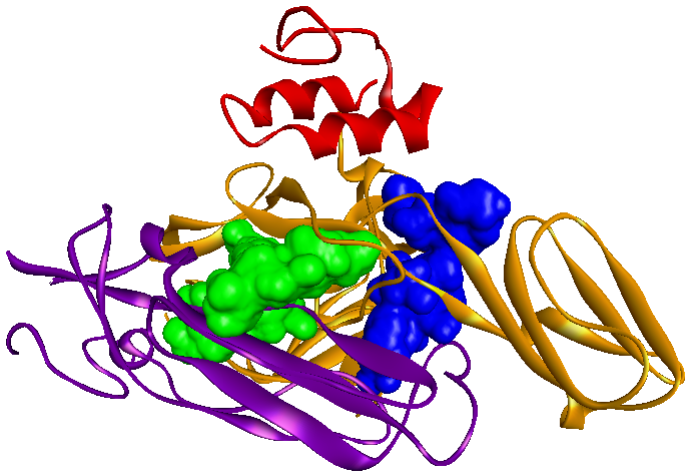


Figure 14. Possible druggable cavities within the CHIKV envelope proteins illustrated as blue and green space-filling pockets. E1 is coloured in violet, E2 is coloured in orange and E3 is shown in red. For simplicity, the figure illustrates only a component of the glycoprotein mature envelope complex. Generated from the pdb file code: 3N42[95].

II.3. Highlights for the CHIKV target enzymes

With the recent growing knowledge and available structural information about the CHIKV genome, drug design of specific inhibitors targeting individual viral proteins has become more viable. The most promising targets from a chemical and biological standpoint would be the viral protease (nsP2) and the viral envelope proteins. The first protein has a complete crystal structure for the N and C domains, with the critical residues for proteolytic activity already investigated. This nsP2 protein also functions through the N domain, allowing the possibility of medicinal chemistry intervention at both domains on this protein. The challenge is the current lack of individual enzyme-based assays to investigate the inhibitory effects of the designed protein

antagonists, rather than the whole cell assay protocols. The second promising protein target is the viral envelope proteins where specific residues have been identified to be essential for the viral fusion process, *e.g.* the Gly91 and His230 residues. Designing specific inhibitors targeting the viral fusion process would be valuable for the inhibition of *alphaviruses* in general. The viral nsP3 also represents a possible drug design target, with the structure of the conserved macro domain is already known. However, this target requires further investigation, *e.g.* the solving of the complete protein structure.

III. DEVELOPMENT OF CHEMOTHERAPEUTICS AGAINST CHIKV: NEW MEDICINAL CHEMISTRY LEADS

There is currently no recognised single antiviral treatment for chikungunya. During the recent outbreaks that occurred in the Indian Ocean nations, only treatments for symptoms were available, based on non-steroidal anti-inflammatory, non-salicylate analgesics and fluids. Mild physical exercise is believed to decrease the joint stiffness, but heavy exercise may increase the rheumatic pain. During chronic CHIKV infection, corticosteroids may be used to help decrease the inflammation.[106] The status of drug discovery for the CHIKV is still in the very early stages with no drugs currently in clinical trials. The first mouse model to study the pathophysiology of the resulting disease was developed in 2008,[107] after which several animal models were developed to aid the understanding of the drug-disease interactions that would facilitate the development of effective therapy.[108]

III.1. Protease inhibitors. Targeting the CHIKV nsP2 protease activity within the C-domain, would have an inhibitory effect on the viral replication. Using the developed homology model for the nsP2, Singh Kh and co-workers screened a library of compounds *in silico* and identified four compounds (**1-4**, Figure 15) as potential inhibitors of the nsP2 protease.[69] Ideally, binding

to this active site will block protein function stopping the replication cycle. However, the antiviral activity of these hits has yet to been reported.

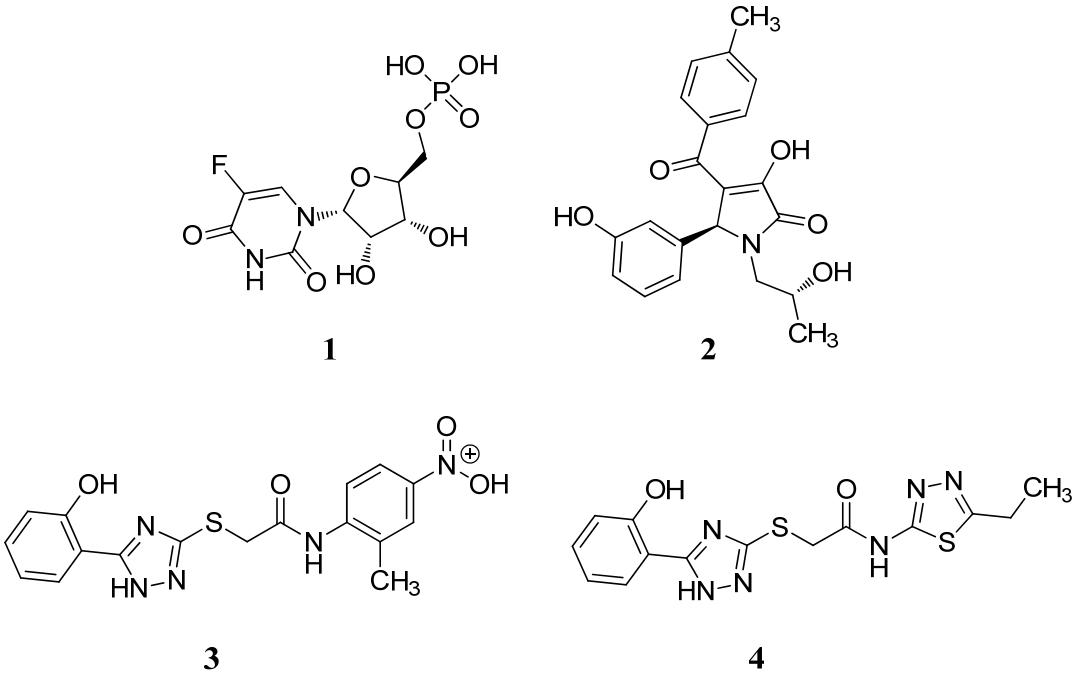


Figure 15. Structures of the four *in silico* predicted CHIKV nsP2 inhibitors.

In a similar work, Bassetto *et al.*[71] reported the identification of *in silico* CHIKV nsP2 inhibitors through a virtual screening of a large compounds library using the developed homology model for the CHIKV nsP2. One of the hits, compound (**5**, Figure 16) was predicted to bind to central portion of the nsP2 protease active site, with its hydrazone group placed in the region defined by the catalytic dyad. The *in vitro* activity of this hit compound was assessed and it was found to inhibit the virus at EC_{50} value of 5 μM with a selectivity index (SI) value of 14, through the inhibition of the virus-induced cytopathic effect.[71] The central cyclopropyl and the hydrazone moieties were found, through a structure activity relationship study, to be important for the anti-chikungunya activity. A series of derivatives were also designed based on these identified pharmacophores of **(5)**, whereas the cyclopropyl group was replaced with a *trans*-

ethenylic moiety (**6**, Figure 16), maintaining length and geometry of the original linker. The antiviral activity was slightly improved with **(6)** displaying an EC_{50} value of 3.2 μM and a selectivity index (SI) of 32. The binding modes of both compounds (**5**, **6**) inside the nsP2 pocket were similar.[71]

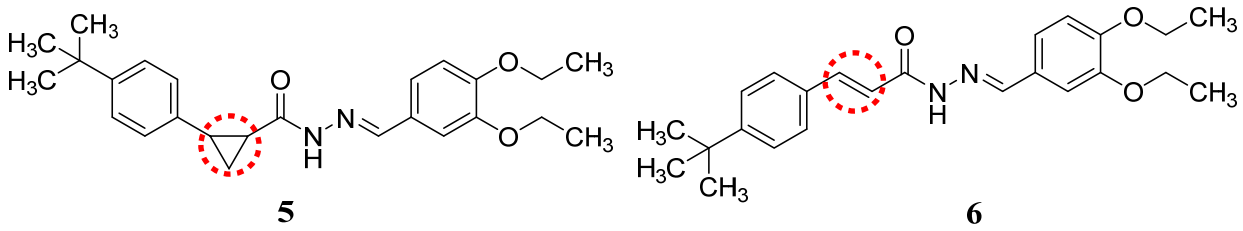
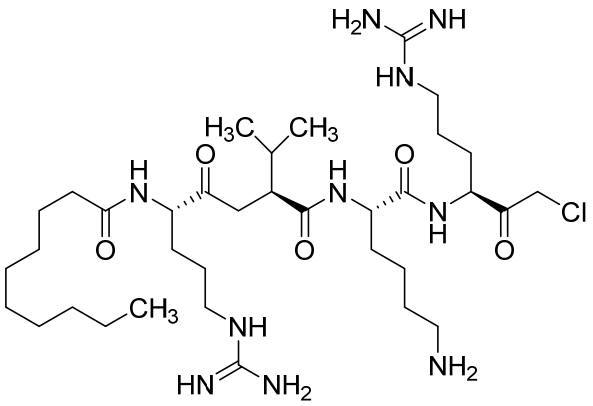


Figure 16. Chemical structures of the CHIKV inhibitors identified from *in silico* screening, with subsequent testing confirming substantial activities. Red circles indicate the only difference in the structures responsible for the activity change.

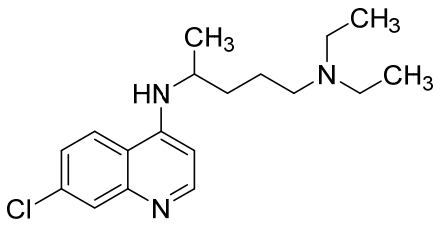
III.2. Furin inhibitors. Infection by *alphaviruses* can be inhibited *in vitro* by blocking the intracellular furin-mediated cleavage of viral envelope glycoproteins: the E2E3 or p62 precursors. This blocking was demonstrated by showing the inhibitory effect of an irreversible furin-inhibiting peptide, decanoyl-RVKR-chloromethyl ketone (dec-RVKR-cmk, **7**, Figure 17) on *in vitro* CHIKV infection.[100] This peptide significantly reduced the processing of E3E2 CHIKV glycoproteins in infected myoblast cultures and led to the formation of immature viral particles and impaired viral spreading among cells, but not the replication in cells already infected.[100] Therefore, the chemical structure of the furin-inhibiting peptide **(7)** could be a starting point for generating novel generations of active peptidomimetics using the ligand-based drug design techniques, targeting the intracellular furin cleavage step.



7

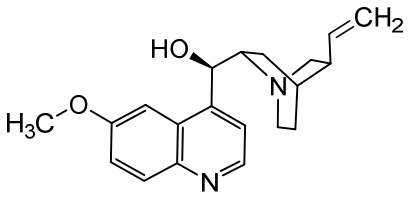
Figure 17. Structures of the CHIKV furin-inhibiting peptide **(7)**.

III.3. Chloroquine and Quinine. The *in vitro* antiviral activity of chloroquine (**8**, Figure 18) was first reported more than 35 years ago and has been successfully used as an anti-malarial drug.[109,110] With respect to the *alphaviruses*, chloroquine was found to be effective *in vitro*,[111,112,113] however, recently a mouse model revealed that chloroquine may enhance viral replication *in vivo* leading to aggravation of the disease.[114] Regarding the CHIKF, chloroquine and chloroquine phosphate have been used in the treatment of chronic chikungunya arthritis,[115] but only for the anti-inflammatory properties of the molecule (used in chronic rheumatologic diseases) rather than for any antiviral effect. Some studies suggest that chloroquine might interact with the endosome-mediated internalisation process during the infection cycle, stating that chloroquine might be classified as an entry inhibitor. Compound **8** entered phase 3 clinical trials in France as a therapy for the CHIKV in 2006, however, these studies were terminated in 2007 with no definite anti-viral results.



8

IC_{50} = 1.1 μg/ml



9

IC_{50} = 0.1 μg/ml

Figure 18. The structure and reported anti-CHIKV activities of the quinolines **8** and **9**.

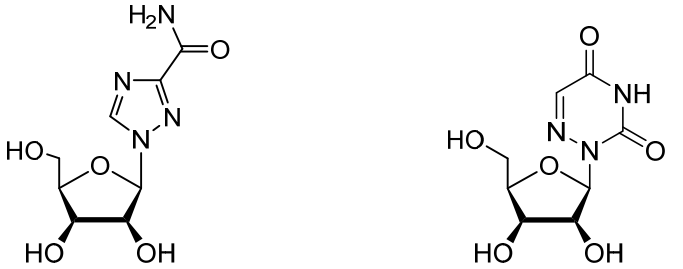
In one clinical study, the effect of **8** on CHIKF patients was investigated using another placebo treated group of patients, and at the conclusion, there was no statistical difference between the chloroquine and the placebo treated groups regarding the mean duration of febrile arthralgia or the decrease of viraemia (viral count in plasma).

Another anti-malarial drug, quinine (**9**, Figure 18), also inhibits the virus *in vitro* at a concentration less than that of chloroquine (IC_{50} = 0.1 μg/ml for quinine, 1.1 μg/ml for chloroquine). Also, quinine is suggested to affect the nsP1 as mutations in this protein occur upon growing the virus in a high concentrations of quinine.[106]

Overall, the contradicting results from different studies casts serious doubt as to the effectiveness of chloroquine as an effective chemotherapeutic against CHIKV and until resolved, it should be treated with caution as a drug lead.[106]

III.4. Ribavirin and 6-Azauridine. The antiviral ribavirin (**10**, Figure 19), is well known to inhibit *in vitro* many RNA viruses, by different mechanisms.[116] It is being used either alone for treatment of infections as Respiratory Syncytial virus, or in combination with alpha-interferon in the treatment of hepatitis C virus (HCV) infection. This combination showed a synergistic effect

in vitro in the inhibition of CHIKV.[117] A combination of alpha-interferon, at a concentration of 3.9 IU/ml, and ribavirin at a concentration of 18.75 μg/ml, inhibited CHIKV replication by 50%, whereas **10** alone without interferon, inhibited CHIKV with EC_{50} value of 83.3 μg/ml. However, there is no evidence supporting the clinical efficacy of **10** on CHIKV, and the combination with interferon should be subjected to clinical trials for the treatment of CHIKV infections.[106] Furthermore, the exact mechanism of ribavirin is still unclear as it may change from virus to another, however, it is believed that ribavirin can interact with the intracellular viral RNA production.



10 EC_{50} = 83.3 μg/ml

11 EC_{50} = 0.2 μg/ml

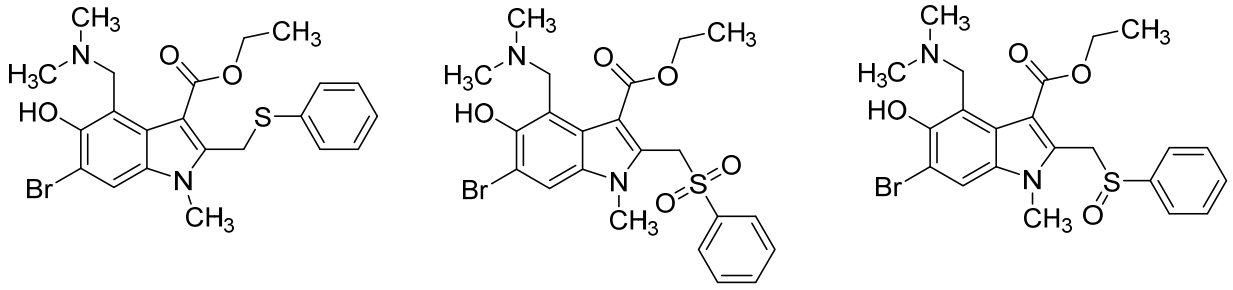
Figure 19. The structure of nucleosides **10** and **11**.

The broad-spectrum anti-metabolite, 6-azauridine (**11**, Figure 19) inhibits both DNA and RNA virus replication, and the activity might be through the inhibition of orotidine monophosphate decarboxylase, an enzyme involved in the *de novo* biosynthesis of pyrimidine, cytidine and thymidine.[118] It showed a significant inhibition of CHIKV at a low concentration (0.2 μg/ml) and was more effective against the CHIKV compared to **10**.[117] Compound **11** is not approved for human use and therefore was not included in a combination study with alpha-interferon. However, the corresponding 6-azauridine triacetate is used for treatment of different

diseases without notable adverse effects.[119] Therefore, 6-azauridine should be evaluated *in vivo* as CHIKV inhibitor.[117]

III.5. Arbidol. The antiviral drug arbidol (**12**, Figure 20) was originally developed 20 years ago at the Russian Research Chemical and Pharmaceutical Institute.[120] Since 1990, it has been used in Russia for acute respiratory infections including influenza. So far, arbidol shows a wide range of activity against many RNA, DNA, enveloped and non-enveloped viruses.[121] This broad spectrum of activity may be attributed to the different modes of actions including the inhibition of virus mediated fusion,[122] and blocking of the viral entry into the target cells through inhibition of glycoprotein conformational changes that are essential for the fusion process, as in case of influenza virus and hepatitis C virus.[123,124]

In 2011, **12** and two derived metabolites (Figure 20), **13** (HZ1) and **14 (**HZ3), were tested *in vitro* on the chikungunya virus using two cell lines, and under different conditions (pre and post-infection treatments). The only active compound was **12** with an IC_{50} value much lower than the toxic concentration (IC_{50} = 12.2 μM, $CC_{50} \geq$ 200 μg/ml).[125] Compounds **13** and **14** were assumed to be responsible for the anti-viral properties of arbidol, however in this study, they showed only weak effects on CHIKV replication.



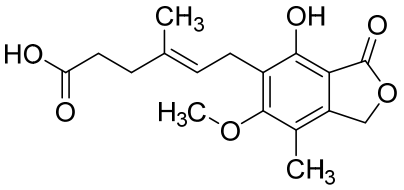
12 **13** **14**

IC_{50} = 12.2 μM IC_{50} = 56.4 μM IC_{50} = 54.5 μM

Figure 20. The structures of indole-based derivatives **12**, **13** and **14**.

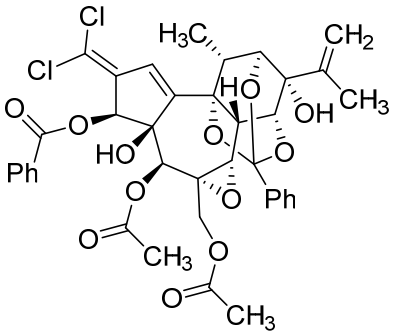
Compound **12** was found to be not viricidal, rather, it blocks the earliest stages of the viral replication, virus attachment and/or virus entry as previously reported.[121] For CHIKV, it works by targeting the cellular membrane (E2 viral envelope protein) which was confirmed by the use of an arbidol resistant CHIKV strain (mutation in the E2 protein, G407R).[125] However, all these studies were performed *in vitro* and *in vivo* studies are required to validate the activity of arbidol on CHIKV.

III.6. Mycophenolic acid (MPA). Mycophenolic acid (**15**, Figure 21) was isolated approximately one hundred years ago.[126] It acts as an inhibitor for the inosine monophosphate dehydrogenase (IMPDH), an enzyme evolved in the *de novo* biosynthesis of guanine nucleotide. It has a good anti-proliferation activity and has been established as an anticancer[127] and antiviral agent, and as an immunosuppressant.[128]



15

$IC_{50} = 0.2\ \mu M$



16

$EC_{50} = 1.5\ \mu M$

Figure 21. The structures of inhibitors of IMPDH, **15** and **16**.

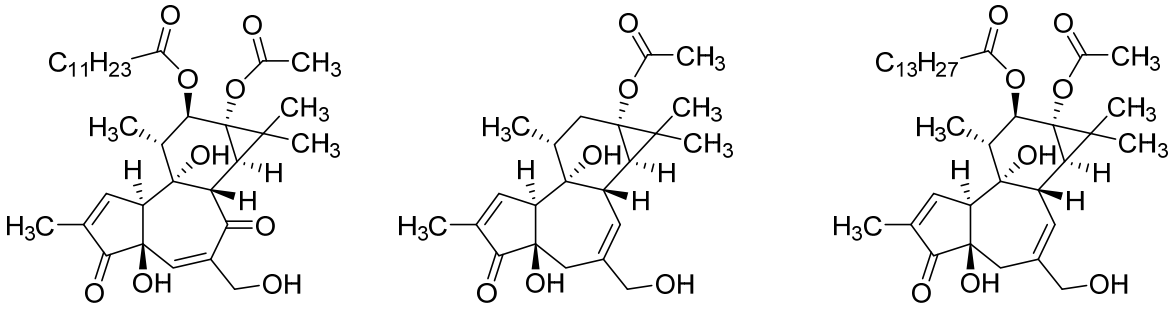
Recently, **15** was shown to inhibit the CHIKV replication by virus induced cell death. The IC_{50} value was 0.2 μM with a selectivity index of 150,[129] and it was found to induce CHIKV apoptosis. When the treated CHIKV infected cells were provided with exogenous guanosine (GMP), **15** could no more prevent the CHIKV induced cell death, indicating that it worked by inhibiting the IMPDH enzyme. It is also suggested that **15** increases the mutation rate during the

viral replication.[129] Considered as a good lead compound, the *in vivo* activity of **15** on CHIKV requires further investigation as it is known that the compound suffers from a metabolic drawback associated with rapid conjugation of the C-7 phenolic hydroxyl group with glucuronic acid.[130,131]

III.7. Trigocherrin A. Trigocherrin A (**16**, Figure 21), is a highly oxygenated and chlorinated daphnane diterpenoid orthoester, that had been isolated from the bark of *Trigonostemon cherrieri* Veillon (Euphorbiaceae), a tree collected in the sclerophyllous forest of New-Caledonia. This genus comprises about 80 species occurring in tropical Asia, from India and Sri Lanka to New Guinea.[132] These diterpenoids have been shown to have cytotoxic[133,134] and antiviral[135,136] properties. Recently, this natural product was tested against CHIKV and was found to inhibit virus-induced cell death in a virus-cell-based assay with an EC_{50} of 1.5 μM and only caused significant anti-metabolic effects at a concentration (CC_{50}) of 35 μM. The selectivity index (SI) value was 24. Different concentrations of **16** were able to protect the host cells from the virus cytopathic effect without any adverse side-effects. It was also found to be more potent than the reference compound used in this study (**8**).[137,138] The assay protocol suggests that this compound might inhibit the viral replicase functions, however, for the drug discovery process, both the *in vivo* activity, the precise mode of action as well as the total synthesis of this lead compound should be investigated.

III.8. Trigowiin A, Prostratin and 12-*O*-Tetradecanoylphorbol 13-Acetate. In late 2012, , an extract from the bark of *Trigonostemon howii* from the Euphorbiaceae species out of central Vietnam was tested against the CHIKV.[139] A new tigliane diterpenoid, trigowiin A (**17**, Figure 22) was isolated and was found to be structurally closely related to the tigliane diterpenes

(Figure 22**)**, prostratin (a promising adjuvant for anti-HIV therapy)[140] **(18)** and 12-*O*-tetradecanoylphorbol 13-acetate **(19)**.[139]



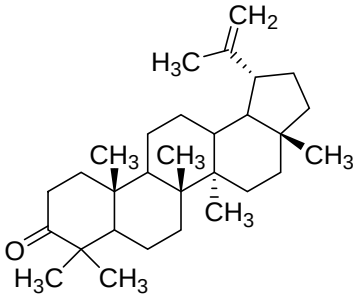
17 **18** **19**

EC_{50} = 43.5 μM EC_{50} = 2.6 μM EC_{50} = 0.0029 μM

Figure 22. Chemical structures of **17**, **18** and **19**.

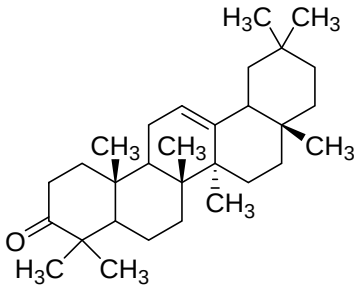
In a CHIKV assay, **17** showed weak antiviral activity, with an EC_{50} of 43.5 ± 12.8 μM whereas **19** and **18** proved to be the most potent inhibitors, with low EC_{50} values and higher selectivity indices (SI), for **19**, EC_{50} = 0.0029 ± 0.0003 μM and SI = 1965 while the EC_{50} for **18** was 2.6 ± 1.5 μM and SI = 30.3 showing that **19** was 65 times more potent than **18**.[139] At the same time, these compounds did not show activity against the Sindbis virus (SINV) nor the Semliki forest virus (SFV) which indicates an excellent selectivity for inhibition of the CHIKV. The authors[139] believe that this selectivity for **19** might be due to a specific mechanism of virus inhibition through the activation of the signal transduction enzyme protein kinase C (PKC), similar to the proposed mechanism of HIV replication inhibition.[141] However, **19** currently is one of the most potent tumor-promoting agents known to date,[142] therefore, the less potent **18** is more likely to be further investigated as a potential candidate, especially as it is shown not to be a tumor-promoting agent. The exact mode of action of these compounds is currently unknown and needs further investigation.

III.9. Lupenone and β-amyrone. In the continuous effort to identify novel inhibitors of Chikungunya from natural sources, a phytochemical study on the leaves of *Anacolosa pervilleana* (Madagascan plant) was performed in a virus-cell-based assay for CHIKV.[144] The triterpenoids isolated showed moderate anti-CHIKV activity (Figure 23) (lupenone **(20)** EC_{50} = 77 μM and β-amyrone **(21)** EC_{50} value of 86 μM).[144] Due to their weak activity, these natural products require medicinal chemistry optimization before being considered as promising lead compounds.



20

EC_{50} = 77 μM



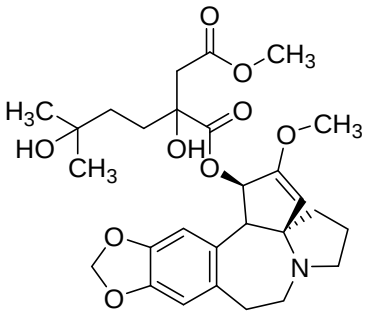
21

EC_{50} = 86 μM

Figure 23. Chemical structures of **20** and **21**.

III.10. Harringtonine. Harringtonine (**22**, Figure 24) is an alkaloid from *Cephalotaxus harringtonia* trees, which is native to Japan and it has a known antitumor activity. It is known to inhibit the first cycle of the elongation phase of eukaryotic translation.[145] It was shown recently[146] that **22** displayed potent inhibition of CHIKV infection (EC_{50} = 0.24 μM) with minimal cytotoxicity, through the inhibition of the early stages of infection after cellular endocytosis. Also, it was found to affect the CHIKV RNA production inside the infected cell, as well as viral protein expression such as the nsP3 and the E2 proteins.[146] The *in vivo* studies of **22**

are still ongoing which could make it a promising lead towards the discovery of anti-CHIKV drugs.

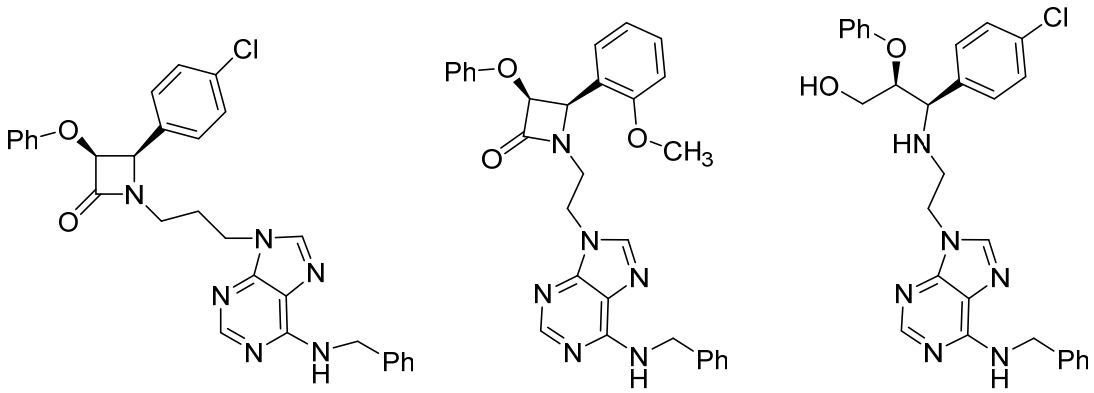


22

EC_{50} = 0.24 μM

Figure 24. Chemical structure of the alkaloid **22**.

III.11. Purine based inhibitors. In 2012, D'hooghe *et al*. reported the design and synthesis of a new series of purine-*β*-lactam hybrids and purine-aminopropanol hybrids and their evaluation as potential antiviral candidates depending on the antiviral templates purines and *β*-lactams.[147] These new scaffolds were screened against nine different viruses including the chikungunya virus. Two purine-*β*-lactam hybrids and one purine-aminopropanol hybrid (Figure 25) were found to possess promising activity and cytotoxicity profiles, the purine-*β*-lactam **(23)** with EC_{50} = 17.11 μM and SI > 5.75, the purine-*β*-lactam **(24)** with EC_{50} = 13.01 μM and SI > 4 and the purine-aminopropanol **(25)** with EC_{50} = 11.51 μM and SI > 6.[147] The mode of action has not been investigated. The synthesis of this class of compounds is already established and therefore, they represent good subject for further medicinal chemistry optimization.

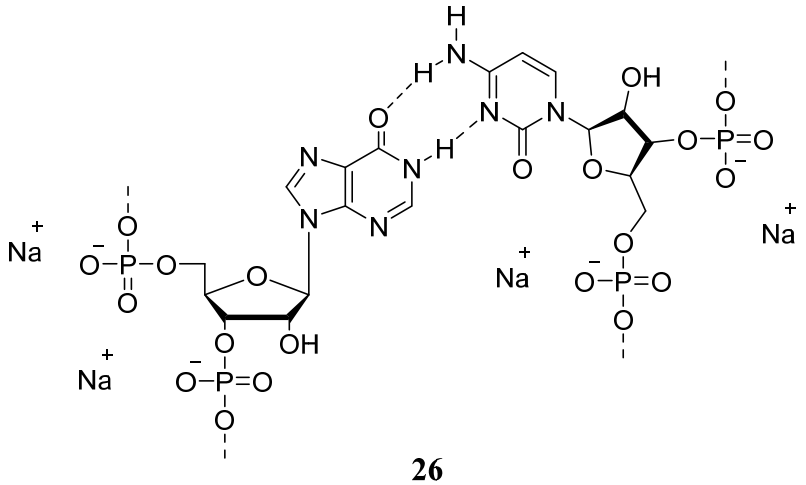


23 **24** **25**

EC_{50}= 17.11 μM EC_{50}= 13.01 μM EC_{50}= 11.51 μM

Figure 25. Chemical structures of **23**, **24** and **25**.

III.12. Polyinosinic acid. Polycytidylic acid [Poly (I:C)] (**26**, Figure 26), a synthetic double-stranded RNA (dsRNA) analogue, is an immunostimulant acting as an inducer for the most potent interferon (IFN) *via* interaction with the toll-like receptor 3 (TLR3).[148] It can induce IFN-α/β production and natural killer (NK) cells activation *in vivo* after intraperitoneal injection.[149] Activation of the TLR3 contributes to an innate immune response against many viruses.[150] In CHIKV infection, the virus was found to be sensitive to the innate immune response induced by **26**. This sensitivity was noticed as a decreased cytopathic effect and inhibition of the virus replication in the infected cell lines. This sensitivity has been explained to be a result of the overstimulation of the TTLR3 as well as the other anti-viral genes by **26**.[151]



26

Figure 26. The structure of the dsRNA analogue **26**.

III.13. Gene silencers. New trends in the CHIKV treatment trials are the use of genes silencers targeting specific viral proteins (capsid protein, E1, nsP1 and nsP3). Silencing the target viral genes will consequently lead to the shutdown of the protein expression process, and thereby stopping viral replication. After viral infection, the exogenous small interfering RNA (siRNA) induces RNA interference mechanism, resulting in the assembly of the RNA-induced silencing complex (RISC) which inhibits subsequent protein expression. Using the siRNA targeting CHIKV E1 and nsP3 effectively suppressed *in vitro* CHIKV replication.[152] Similarly, a plasmid based small hairpin RNA (shRNA) against CHIKV replication targeting the capsid, E1 and nsP1 proteins has been used. Simply, the plasmid is introduced to cell and expressed inside the nucleus, resulting in the formation of small-hairpin RNA (shRNA) which are processed by the cytoplasmic Dicer enzyme to siRNAs, leading to activation of the RNA silencing machinery. This silencing machinery recognizes and degrades the target CHIKV single strand RNA, consequently stopping viral protein expression.[153] Clinical studies should be able to prove the applicability of these trends in developing effective anti-CHIKV therapeutics.

III.14. Envelope protein antagonists. Targeting the CHIKV envelope proteins,[105] possible *in silico* antagonists have been reported based on predicted binding sites. Compounds **27-30** (Figure 27) were projected to interact with the CHIKV envelope residues critical for the fusion process. The (*S*)-stereochemistry of the OH group in **28-30** was found to be important for the *in silico* binding.[105] The antiviral activity for these compounds is yet to be evaluated.

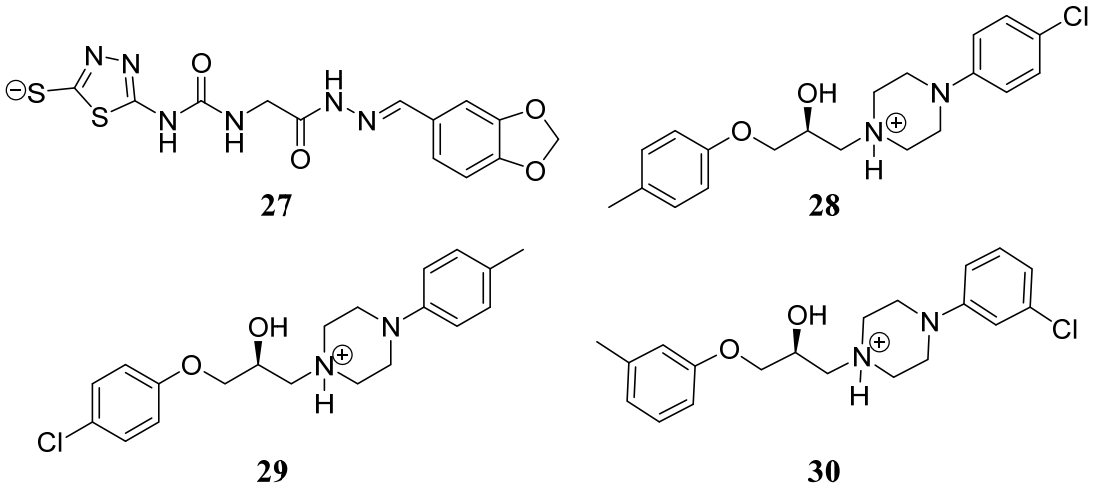


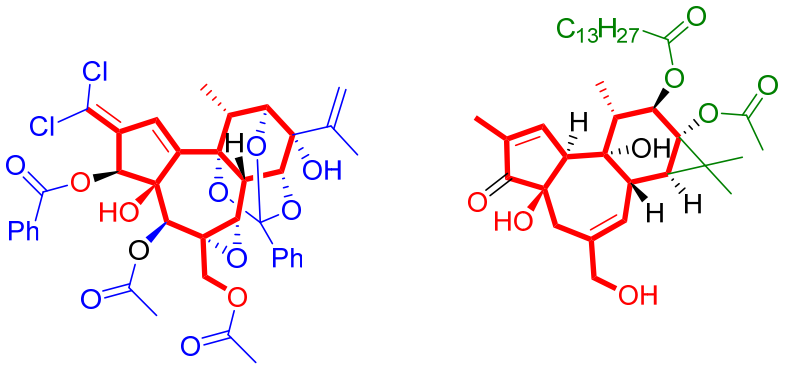
Figure 27. The identified *in silico* antagonists for the CHIKV envelope proteins.

IV. HIGHLIGHTS FOR CHIKV INHIBITORS

Several molecules have been tested against the emerging CHIKV with weak to moderate activities. Those included drugs already in the market, being used for other diseases, such as **8**, **10**, **12** and **15**. The challenge here is evolving these structures onto more novel molecules with improved potency and maintaining any drug-like qualities. The putative protease inhibitors **(5**, **6)** and the predicted fusion inhibitors (**27-30**) developed would be attractive candidates for further investigation, however, the challenge lies in being able to identify specific enzyme assay protocols to confirm the selectivity for the specific viral proteins. Protein crystallization with the inhibitor would be a useful element to validate such studies. Some of the tested compounds

represent complex natural products and have sufficient activity as antiviral agents, such as **16**, **18**, **19** and **22**. It will be a significant task to chemically access these structures and to simplify the chemical skeletons to more drug like molecules with acceptable ADME properties.

The anti-CHIKV activity of the tested molecules ranged from strong to weak inhibition depending on the type of the assay used, with the **19** being the strongest inhibitor with EC_{50} = 0.0029 µM and **20** which displayed the weakest activity with EC_{50} = 86 µM, similar activity to that of **10**, EC_{50} = 83.3 µM. It is worth noting that the active agents, **19** and the less active **16**, shared a common structural feature, the substituted benzo[*e*]azulene derived structure, (Figure 27). The structure of **19** is simpler than that of **16**, lacking the extra phenyl rings, two chlorine atoms, four oxygen bridges and the alkene side chain (Figure 27), however, **19** has a characteristic long tetradecanoic ester moiety which was responsible for the activity over **17** and **18**, two derivatives that were even less active than **16**.



16 **19**

EC_{50}= 1.5 µM EC_{50}= 0.0029 µM

Figure 27. Chemical structures of **19** and **16**. The common skeleton is highlighted in bold red colour, and groups unique to **19** are shown in green while the excess groups of **16** are shown in blue colour.

V. CONCLUDING REMARKS

Chikungunya virus (CHIKV) is an emerging arbovirus that has had devastating effects in recent years in wide ranging areas around the world. Chikungunya virus infection can develop into an arthritis disease that remains with the patient for years. The mutations in the viral envelope protein genes increased the fitness of the virus in another mosquito vector, *Ae. albopictus* which was responsible for cases reported in temperate zones. With no licensed vaccine for immunization against this virus, disease control is currently non-existent, and the defence line would be through the development of chemotherapeutics. Some promising lead compounds have been discovered recently and could be starting points towards effective treatments. The discovery of these leads was mostly based on random screening of drugs already in the market, newly discovered natural products or the antiviral evaluation of synthetic compounds. Also, in the last two years, the molecular function as well as the crystal structures of a number of critical enzymes involved in the virus life cycle have been reported, with no known specific inhibitors thus far. Some *in silico* screens against these proteins have been performed, and while compounds with antiviral activity have been identified, their mechanism has yet to be confirmed. Substantial medicinal chemistry efforts will be required to advance drug discovery and development in this area. Further, despite the availability of whole cell anti-CHIKV testing, the development of individual CHIKV enzyme assays would greatly benefit SAR research programs as well as helping to identify targets for newly discovered inhibitors. Considering the neglected status of this virus and the emerging knowledge of the biology of CHIKV, the opportunity for small molecule inhibitors to be discovered and developed remains high.

Corresponding Author

Paul A Keller

Tele: +61 2 4221 4692

keller@uow.edu.au

Biographies

Adel Rashad

Adel A. Rashad received his Bachelor of Pharmaceutical Sciences from the Faculty of Pharmacy of Zagazig University, Egypt, in 2005. He started working in medicinal chemistry research in 2006, focussing on the development of antiviral chemotherapeutics targeting HIV, HAV, HSV-1, HSV-2 and poxviruses and was awarded a Masters degree in medicinal chemistry in 2009. In addition to his research, teaching medicinal chemistry to undergraduate pharmacy students is a priority. He has just completed a PhD degree in the School of Chemistry, University of Wollongong, Australia, on the medicinal chemistry development targeting the Chikungunya virus and *T brucei*. His focus is analysis of bimolecular viral targets that can be used in drug design along with the development of the synthetic chemistry for chemotherapeutics.

Suresh Mahalingam

Professor Suresh Mahalingam PhD is currently Principal Research Leader and Professor of Virology at the Institute for Glycomics, Griffith University. He has an international reputation in the field of viral pathogenesis and has a strong research interests in the virus-host relationship and the factors that influence the outcomes of infection, with the aim of developing new interventions against disease. He extends basic medical research objectives into the clinical and applied research areas of infectious diseases and inflammation.

Paul Keller

Paul Keller completed his BSc(Hons) (1985) and PhD at the University of New South Wales, Australia, before undertaking an Alexander von Humboldt funded post-doctoral fellowship at the University of Wuerzburg, Germany, working in collaboration with Gerhard Bringmann. Since 1994, he has worked at the University of Wollongong, Australia, and is currently Associated Professor in Organic and Medicinal Chemistry. His interests lie in the drug design and development of new generation anti-infectives with a particular emphasis on anti-virals, new chemistry of indigo and chiral ligand design for sterically hindered Suzuki reactions.

Abbreviations Used:

BAR, Bin-Amphiphysin-Rvsp; CHIKF, Chikungunya fever; CHIKV, Chikungunya virus; eIF2α, Eukaryotic Translation Initiation Factor 2, Alpha Subunit; ER, Endoplasmic Reticulum; G3BP, Ras GTPase-activating protein-binding protein; GMP, Guanosine Mono Phosphate; hnRNPs, Heterogeneous Nuclear Ribonucleoproteins; IFN, Interferon; IMPDH, Inosine Monophosphate Dehydrogenase; MPA, Mycophenolic acid; ns, Non-structural; NTPase, Nucleotide Triphosphatase; ORFs , Open Reading Frames; PHB, Prohibitin; RNAi, Ribonucleic Acid Interference; RpS6, Ribosomal Protein S6; SFV, Semliki Forest Virus; SH3, Src-homology-3; SINV, Sindbis Virus; TTC7B, Tetratricopeptide Repeat Protein 7B; UBQLN4, Ubiquilin 4; UPR, Unfold Protein Response.

References

1. Her, Z.; Kam, Y. W.; Lin, R. T.; Ng, L. F. Chikungunya: a bending reality. *Microbes Infect.* **2009**, *11*, 1165-1176.
2. Robinson, M. C. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. I. Clinical features. *Trans. R. Soc. Trop. Med. Hyg.* **1955**, *49*, 28-32.

3. Lumsden, W. H. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. II. General description and epidemiology. *Trans. R. Soc. Trop. Med. Hyg.* **1955**, *49*, 33-57.
4. Nimmannitya, S.; Halstead, S. B.; Cohen, S. N.; Margiotta, M. R. Dengue and chikungunya virus infection in man in Thailand, 1962-1964. I. Observations on hospitalized patients with hemorrhagic fever. *Am. J. Trop. Med. Hyg.* **1969**, *18*, 954-971.
5. Njenga, M. K.; Nderitu, L.; Ledermann, J. P.; Ndirangu, A.; Logue, C. H.; Kelly, C. H. L.; Sang, R.; Sergon, K.; Breiman, R.; Powers, A. M. Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa. *J. Gen. Virol.* **2008**, *89*, 2754-2760.
6. Ravi, V. Re-emergence of chikungunya virus in India. *Indian J. Med. Microbio.* **2006**, *24*, 83-84.
7. Sambri, V.; Cavrini, F.; Rossini, G.; Pierro, A.; Landini, M. P. The 2007 epidemic outbreak of Chikungunya virus infection in the Romagna region of Italy: a new perspective for the possible diffusion of tropical diseases in temperate areas? *New Microbiol.* **2008**, *31*, 303-304.
8. Enserink, M. Infectious diseases - Chikungunya: No longer a Third World disease. *Science* **2007**, *318*, 1860-1861.
9. Powers, A. M.; Logue, C. H. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *J. Gen. Virol.* **2007**, *88*, 2363-2377.
10. Schwartz, O.; Albert, M. L. Biology and pathogenesis of chikungunya virus. *Nat. Rev. Microbiol.* **2010**, *8*, 491-500.
11. Outbreak news. Chikungunya, India. *Releve epidemiologique hebdomadaire / Section d'hygiene du Secretariat de la Societe des Nations = Weekly epidemiological record / Health Section of the Secretariat of the League of Nations* **2006**, *81*, 409-410.

12. Saxena, S. K.; Singh, M.; Mishra, N.; Lakshmi, V. Resurgence of chikungunya virus in India: an emerging threat. *Euro. Surveill.* **2006**, *11*, E060810 2.
13. Chhabra, M.; Mittal, V.; Bhattacharya, D.; Rana, U.; Lal, S. Chikungunya fever: a re-emerging viral infection. *Indian J. Med. Microbiol.* **2008**, *26*, 5-12.
14. Vazeille, M.; Moutailler, S.; Coudrier, D.; Rousseaux, C.; Khun, H.; Huerre, M.; Thiria, J.; Dehecq, J. S.; Fontenille, D.; Schuffenecker, I.; Despres, P.; Failloux, A. B. Two Chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, Aedes albopictus. *PLoS One* **2007**, *2*, e1168.
15. Pages, F.; Peyrefitte, C. N.; Mve, M. T.; Jarjaval, F.; Brisse, S.; Iteman, I.; Gravier, P.; Tolou, H.; Nkoghe, D.; Grandadam, M. Aedes albopictus mosquito: the main vector of the 2007 Chikungunya outbreak in Gabon. *PLoS One* **2009**, *4*, e4691.
16. Gratz, N. G. Critical review of the vector status of Aedes albopictus. *Med. Vet. Entomol.* **2004**, *18*, 215-227.
17. Smith, C. E. The history of dengue in tropical Asia and its probable relationship to the mosquito Aedes aegypti. *J. Trop. Med. Hyg.* **1956**, *59*, 243-251.
18. Gerardin, P.; Barau, G.; Michault, A.; Bintner, M.; Randrianaivo, H.; Choker, G.; Lenglet, Y.; Touret, Y.; Bouveret, A.; Grivard, P.; Le Roux, K.; Blanc, S.; Schuffenecker, I.; Couderc, T.; Arenzana-Seisdedos, F.; Lecuit, M.; Robillard, P. Y. Multidisciplinary prospective study of mother-to-child chikungunya virus infections on the island of La Reunion. *PLoS Med* **2008**, *5*, e60.
19. Ziegler, S. A.; Lu, L.; da Rosa, A. P.; Xiao, S. Y.; Tesh, R. B. An animal model for studying the pathogenesis of chikungunya virus infection. *Am. J. Trop. Med. Hyg.* **2008**, *79*, 133-139.

20. Queyriaux, B.; Simon, F.; Grandadam, M.; Michel, R.; Tolou, H.; Boutin, J. P. Clinical burden of chikungunya virus infection. *Lancet Infect. Dis.* **2008**, *8*, 2-3.
21. Santhosh, S. R.; Dash, P. K.; Parida, M. M.; Khan, M.; Tiwari, M.; Lakshmana Rao, P. V. Comparative full genome analysis revealed E1: A226V shift in 2007 Indian Chikungunya virus isolates. *Virus Res.* **2008**, *135*, 36-41.
22. Robin, S.; Ramful, D.; Le Seach, F.; Jaffar-Bandjee, M. C.; Rigou, G.; Alessandri, J. L. Neurologic manifestations of pediatric chikungunya infection. *J. Child. Neuro.* **2008**, *23*, 1028-1035.
23. Chandak, N. H.; Kashyap, R. S.; Kabra, D.; Karandikar, P.; Saha, S. S.; Morey, S. H.; Purohit, H. J.; Taori, G. M.; Daginawala, H. F. Neurological complications of Chikungunya virus infection. *Neurol. India* **2009**, *57*, 177-180.
24. Pialoux, G.; Gauzere, B. A.; Jaureguiberry, S.; Strobel, M. Chikungunya, an epidemic arbovirosis. *Lancet Infect. Dis.* **2007**, *7*, 319-327.
25. Mahesh, G.; Giridhar, A.; Shedbele, A.; Kumar, R.; Saikumar, S. J. A case of bilateral presumed chikungunya neuroretinitis. *Indian J. Ophthalmol.* **2009**, *57*, 148-150.
26. Nair, A. G.; Biswas, J.; Bhende, M. P. A case of bilateral Chikungunya neuroretinitis. *J. Ophthalmic Inflammation Infect.* **2012**, *2*, 39-40.
27. Couderc, T.; Gangneux, N.; Chretien, F.; Caro, V.; Le Luong, T.; Ducloux, B.; Tolou, H.; Lecuit, M.; Grandadam, M. Chikungunya virus infection of corneal grafts. *J. Infect. Dis.* **2012**, *206*, 851-859.
28. Singh, S. K.; Unni, S. K. Chikungunya virus: host pathogen interaction. *Rev. Med. Virol.* **2011**, *21*, 78-88.

29. Grakoui, A.; Levis, R.; Raju, R.; Huang, H. V.; Rice, C. M. A Cis-Acting Mutation in the Sindbis Virus Junction Region Which Affects Subgenomic Rna-Synthesis. *J. Virol.* **1989**, *63*, 5216-5227.

30. Sourisseau, M.; Schilte, C.; Casartelli, N.; Trouillet, C.; Guivel-Benhassine, F.; Rudnicka, D.; Sol-Foulon, N.; Le Roux, K.; Prevost, M. C.; Fsihi, H.; Frenkiel, M. P.; Blanchet, F.; Afonso, P. V.; Ceccaldi, P. E.; Ozden, S.; Gessain, A.; Schuffenecker, I.; Verhasselt, B.; Zamborlini, A.; Saib, A.; Rey, F. A.; Arenzana-Seisdedos, F.; Despres, P.; Michault, A.; Albert, M. L.; Schwartz, O. Characterization of reemerging chikungunya virus. *PLoS Pathog* **2007**, *3*, e89.

31. Wintachai, P.; Wikan, N.; Kuadkitkan, A.; Jaimipuk, T.; Ubol, S.; Pulmanausahakul, R.; Auewarakul, P.; Kasinrerk, W.; Weng, W. Y.; Panyasrivanit, M.; Paemanee, A.; Kittisenachai, S.; Roytrakul, S.; Smith, D. R. Identification of prohibitin as a Chikungunya virus receptor protein. *J. Med. Virol.* **2012**, *84*, 1757-1770.

32. Berger, K. H.; Yaffe, M. P. Prohibitin family members interact genetically with mitochondrial inheritance components in Saccharomyces cerevisiae. *Mol. Cell Biol.* **1998**, *18*, 4043-4052.

33. Kolonin, M. G.; Saha, P. K.; Chan, L.; Pasqualini, R.; Arap, W. Reversal of obesity by targeted ablation of adipose tissue. *Nature med.* **2004**, *10*, 625-632.

34. Kielian, M.; Rey, F. A. Virus membrane-fusion proteins: more than one way to make a hairpin. *Nature Rev.Microbiol.* **2006**, *4*, 67-76.

35. Marsh, M.; Helenius, A. Virus entry: open sesame. *Cell* **2006**, *124*, 729-740.

36. Chatterjee, P. K.; Vashishtha, M.; Kielian, M. Biochemical consequences of a mutation that controls the cholesterol dependence of Semliki Forest virus fusion. *J. Virol.* **2000**, *74*, 1623-1631.

37. Smit, J. M.; Bittman, R.; Wilschut, J. Low-pH-dependent fusion of Sindbis virus with receptor-free cholesterol- and sphingolipid-containing liposomes. *J. Virol.* **1999**, *73*, 8476-8484.

38. Barton, D. J.; Sawicki, S. G.; Sawicki, D. L. Solubilization and immunoprecipitation of alphavirus replication complexes. *J. Virol.* **1991**, *65*, 1496-1506.

39. Shirako, Y.; Strauss, J. H. Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. *J. Virol.***1994**, *68*, 1874-1885.

40. Schilte, C.; Couderc, T.; Chretien, F.; Sourisseau, M.; Gangneux, N.; Guivel-Benhassine, F.; Kraxner, A.; Tschopp, J.; Higgs, S.; Michault, A.; Arenzana-Seisdedos, F.; Colonna, M.; Peduto, L.; Schwartz, O.; Lecuit, M.; Albert, M. L. Type I IFN controls chikungunya virus via its action on nonhematopoietic cells. *J. Exp. Med.* **2010**, *207*, 429-442.

41. Khan, A. H.; Morita, K.; Parquet Md Mdel, C.; Hasebe, F.; Mathenge, E. G.; Igarashi, A. Complete nucleotide sequence of chikungunya virus and evidence for an internal polyadenylation site. *J. Gen. Virol.* **2002**, *83*, 3075-3084.

42. Tang, B. L. The cell biology of Chikungunya virus infection. *Cell. Microbiol.* **2012**, *14*, 1354-1363.

43. Perera, R.; Owen, K. E.; Tellinghuisen, T. L.; Gorbalenya, A. E.; Kuhn, R. J. Alphavirus nucleocapsid protein contains a putative coiled coil alpha-helix important for core assembly. *J. Virol.* **2001**, *75*, 1-10.

44. Maek-a-nantawat, A. ; Silachamroon, U. Presence of autoimmune antibody in chikungunya infection. *Case Report Med.* **2009**, *2009*, 840183, doi:10.1155/2009/840183.

45. Eckels, K. H.; Harrison, V. R.; Hetrick, F. M. Chikungunya virus vaccine prepared by Tween-ether extraction. *Appl. Microbiol.* **1970**, *19*, 321-325.

46. Levitt, N. H.; Ramsburg, H. H.; Hasty, S. E.; Repik, P. M.; Cole, F. E.; Lupton, H. W. Development of an Attenuated Strain of Chikungunya Virus for Use in Vaccine Production. *Vaccine* **1986**, *4*, 157-162.

47. McClain, D. J.; Pittman, P. R.; Ramsburg, H. H.; Nelson, G. O.; Rossi, C. A.; Mangiafico, J. A.; Schmaljohn, A. L.; Malinoski, F. J. Immunologic interference from sequential administration of live attenuated alphavirus vaccines. *J. Infect. Dis.* **1998**, *177*, 634-641.

48. Edelman, R.; Tacket, C. O.; Wasserman, S. S.; Bodison, S. A.; Perry, J. G.; Mangiafico, J. A. Phase II safety and immunogenicity study of live chikungunya virus vaccine TSI-GSD-218. *Am. J. Trop. Med. Hyg.* **2000**, *62*, 681-685.

49. Tiwari, M.; Parida, M.; Santhosh, S. R.; Khan, M.; Dash, P. K.; Rao, P. V. Assessment of immunogenic potential of Vero adapted formalin inactivated vaccine derived from novel ECSA genotype of Chikungunya virus. *Vaccine* **2009**, *27*, 2513-2522.

50. Plante, K.; Wang, E. Y.; Partidos, C. D.; Weger, J.; Gorchakov, R.; Tsetsarkin, K.; Borland, E. M.; Powers, A. M.; Seymour, R.; Stinchcomb, D. T.; Osorio, J. E.; Frolov, I.; Weaver, S. C. Novel Chikungunya Vaccine Candidate with an IRES-Based Attenuation and Host Range Alteration Mechanism. *Plos Pathogens* **2011**, *7,* e1002142 DOI: 10.1371/journal.ppat.1002142.

51. Velez, R. A.; de Matos, A. P. A.; Parreira, R.; Piedade, J.; Matos, B.; Correia, C.; Esteves, A. Expression of Chikungunya virus-like particles. *Microsc. Microanal.* **2012**, *18*, 59-60.

52. Akahata, W.; Nabel, G. J. A Specific Domain of the Chikungunya Virus E2 Protein Regulates Particle Formation in Human Cells: Implications for Alphavirus Vaccine Design. *J. .Virol.* **2012**, *86*, 8879-8883.

53. Akahata, W.; Yang, Z. Y.; Andersen, H.; Sun, S.; Holdaway, H. A.; Kong, W. P.; Lewis, M. G.; Higgs, S.; Rossmann, M. G.; Rao, S.; Nabel, G. J. A virus-like particle vaccine for

epidemic Chikungunya virus protects nonhuman primates against infection. *Nature Medicine* **2010**, *16*, 334-338.

54. Solignat, M.; Gay, B.; Higgs, S.; Briant, L.; Devaux, C. Replication cycle of chikungunya: a re-emerging arbovirus. *Virology* **2009**, *393*, 183-197.
55. Jones, P. H.; Maric, M.; Madison, M. N.; Maury, W.; Roller, R. J.; Okeoma, C. M. BST-2/tetherin-mediated restriction of chikungunya (CHIKV) VLP budding is counteracted by CHIKV non-structural protein 1 (nsP1). *Virology* **2013**, *438*, 37-49.
56. Jones, P. H.; Mehta, H. V.; Maric, M.; Roller, R. J.; Okeoma, C. M. Bone marrow stromal cell antigen 2 (BST-2) restricts mouse mammary tumor virus (MMTV) replication in vivo. *Retrovirology* **2012**, *9*, 10-22.
57. Hardy, W. R.; Strauss, J. H. Processing the Nonstructural Polyproteins of Sindbis Virus - Nonstructural Proteinase Is in the C-Terminal Half of Nsp2 and Functions Both in Cis and in Trans. *J. Virol.* **1989**, *63*, 4653-4664.
58. Merits, A.; Vasiljeva, L.; Ahola, T.; Kaariainen, L.; Auvinen, P. Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein by nsP2 protease. *J. Gen. Virol.* **2001**, *82*, 765-773.
59. Strauss, E. G.; De Groot, R. J.; Levinson, R.; Strauss, J. H. Identification of the active site residues in the nsP2 proteinase of Sindbis virus. *Virology* **1992**, *191*, 932-940.
60. Vasiljeva, L.; Valmu, L.; Kaariainen, L.; Merits, A. Site-specific protease activity of the carboxyl-terminal domain of Semliki Forest virus replicase protein nsP2. *J. Biol. Chem.* **2001**, *276*, 30786-30793.
61. Gorbalenya, A. E.; Koonin, E. V. Helicases - Amino-Acid-Sequence Comparisons and Structure-Function-Relationships. *Curr. Opin. Struct. Biol.* **1993**, *3*, 419-429.

62. Kim, K. H.; Rumenapf, T.; Strauss, E. G.; Strauss, J. H. Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. *Virology* **2004**, *323*, 153-163.

63. Vasiljeva, L.; Merits, A.; Golubtsov, A.; Sizemskaja, V.; Kaariainen, L.; Ahola, T. Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. *J. Biol. Chem.* **2003**, *278*, 41636-41645.

64. Pastorino, B. A.; Peyrefitte, C. N.; Almeras, L.; Grandadam, M.; Rolland, D.; Tolou, H. J.; Bessaud, M. Expression and biochemical characterization of nsP2 cysteine protease of Chikungunya virus. *Virus Res.* **2008**, *131*, 293-298.

65. Karpe, Y. A.; Aher, P. P.; Lole, K. S. NTPase and 5 '-RNA Triphosphatase Activities of Chikungunya Virus nsP2 Protein. *PLoS One* **2011**, *6*, e22336, DOI: 10.1371/journal.pone.0022336.

66. Domsalla, A.; Melzig, M. F. Occurrence and properties of proteases in plant latices. *Planta medica* **2008,** 74, 699-711.

67. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28*, 235-242.

68. Grudkowska, M.; Zagdanska, B. Multifunctional role of plant cysteine proteinases. *Acta Biochim. Pol.* **2004**, *51*, 609-624.

69. Singh Kh, D.; Kirubakaran, P.; Nagarajan, S.; Sakkiah, S.; Muthusamy, K.; Velmurgan, D.; Jeyakanthan, J. Homology modeling, molecular dynamics, e-pharmacophore mapping and docking study of Chikungunya virus nsP2 protease. *J. Mol. Model.* **2012**, *18*, 39-51.

70. Russo, A. T.; Malmstrom, R. D.; White, M. A.; Watowich, S. J. Structural basis for substrate specificity of alphavirus nsP2 proteases. *J. Mol. Graphics Modell.* **2010**, *29*, 46-53.

71. Bassetto, M.; De Burghgraeve, T.; Delang, L.; Massarotti, A.; Coluccia, A.; Zonta, N.; Gatti, V.; Colombano, G.; Sorba, G.; Silvestri, R.; Tron, G. C.; Neyts, J.; Leyssen, P.; Brancale, A. Computer-aided identification, design and synthesis of a novel series of compounds with selective antiviral activity against chikungunya virus. *Antiviral Res.* **2013**, *98*, 12-18.

72. Breakwell, L.; Dosenovic, P.; Karlsson Hedestam, G. B.; D'Amato, M.; Liljestrom, P.; Fazakerley, J.; McInerney, G. M. Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. *J. Virol.***2007**, *81*, 8677-8684.

73. Frolov, I.; Garmashova, N.; Atasheva, S.; Frolova, E. I. Random Insertion Mutagenesis of Sindbis Virus Nonstructural Protein 2 and Selection of Variants Incapable of Downregulating Cellular Transcription. *J. Virol.* **2009**, *83*, 9031-9044.

74. Bourai, M.; Lucas-Hourani, M.; Gad, H. H.; Drosten, C.; Jacob, Y.; Tafforeau, L.; Cassonnet, P.; Jones, L. M.; Judith, D.; Couderc, T.; Lecuit, M.; Andre, P.; Kummerer, B. M.; Lotteau, V.; Despres, P.; Tangy, F.; Vidalain, P. O. Mapping of Chikungunya virus interactions with host proteins identified nsP2 as a highly connected viral component. *J. Virol.* **2012**, *86*, 3121-3134.

75. De, I.; Fata-Hartley, C.; Sawicki, S. G.; Sawicki, D. L. Functional analysis of nsP3 phosphoprotein mutants of Sindbis virus. *J. Virol.* **2003**, *77*, 13106-13116.

76. Lastarza, M. W.; Grakoui, A.; Rice, C. M. Deletion and duplication mutations in the C-terminal nonconserved region of Sindbis virus nsP3: effects on phosphorylation and on virus replication in vertebrate and invertebrate cells. *Virology* **1994**, *202*, 224-232.

77. Li, G. P.; La Starza, M. W.; Hardy, W. R.; Strauss, J. H.; Rice, C. M. Phosphorylation of Sindbis virus nsP3 in vivo and in vitro. *Virology* **1990**, *179*, 416-427.

78. Vihinen, H.; Ahola, T.; Tuittila, M.; Merits, A.; Kaariainen, L. Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. *J. .Biol Chem.* **2001**, *276*, 5745-5752.

79. Tuittila, M. T.; Santagati, M. G.; Roytta, M.; Maatta, J. A.; Hinkkanen, A. E. Replicase complex genes of Semliki Forest virus confer lethal neurovirulence. *J. Virol.* **2000**, *74*, 4579-4589.

80. Malet, H.; Coutard, B.; Jamal, S.; Dutartre, H.; Papageorgiou, N.; Neuvonen, M.; Ahola, T.; Forrester, N.; Gould, E. A.; Lafitte, D.; Ferron, F.; Lescar, J.; Gorbalenya, A. E.; de Lamballerie, X.; Canard, B. The crystal structures of Chikungunya and Venezuelan equine encephalitis virus nsP3 macro domains define a conserved adenosine binding pocket. *J. Virol.* **2009**, *83*, 6534-6545.

81. Neuvonen, M.; Ahola, T. Differential activities of cellular and viral macro domain proteins in binding of ADP-ribose metabolites. *J. Mol. Biol.* **2009**, *385*, 212-225.

82. Lulla, A.; Lulla, V.; Merits, A. Macromolecular assembly-driven processing of the 2/3 cleavage site in the alphavirus replicase polyprotein. *J. Virol.* **2012**, *86*, 553-565.

83. Shin, G.; Yost, S. A.; Miller, M. T.; Elrod, E. J.; Grakoui, A.; Marcotrigiano, J. Structural and functional insights into alphavirus polyprotein processing and pathogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 16534-16539.

84. Vasiljeva, L.; Merits, A.; Golubtsov, A.; Sizemskaja, V.; Kaariainen, L.; Ahola, T. Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. *J. Biol. Chem.* **2003**, *278*, 41636-41645.

85. Rungrotmongkol, T.; Nunthaboot, N.; Malaisree, M.; Kaiyawet, N.; Yotmanee, P.; Meeprasert, A.; Hannongbua, S. Molecular insight into the specific binding of ADP-ribose to the nsP3 macro domains of chikungunya and venezuelan equine encephalitis viruses: Molecular dynamics simulations and free energy calculations. *J. Mol. Graphics Modell.* **2010**, *29*, 347-353.

86. Neuvonen, M.; Kazlauskas, A.; Martikainen, M.; Hinkkanen, A.; Ahola, T.; Saksela, K. SH3 domain-mediated recruitment of host cell amphiphysins by alphavirus nsP3 promotes viral RNA replication. *PLoS Pathog.* **2011**, *7*, e1002383.

87. Fros, J. J.; Domeradzka, N. E.; Baggen, J.; Geertsema, C.; Flipse, J.; Vlak, J. M.; Pijlman, G. P. Chikungunya virus nsP3 blocks stress granule assembly by recruitment of G3BP into cytoplasmic foci. *J. Virol.* **2012**, *86,* 10873-10879.

88. Parker, F.; Maurier, F.; Delumeau, I.; Duchesne, M.; Faucher, D.; Debussche, L.; Dugue, A.; Schweighoffer, F.; Tocque, B. A Ras-GTPase-activating protein SH3-domain-binding protein. *Mol. Cell. Biol.* **1996**, *16*, 2561-2569.

89. Anderson, P.; Kedersha, N. Stress granules. *Curr. Biol.* **2009**, *19*, R397-398.

90. Shirako, Y.; Strauss, E. G.; Strauss, J. H. Suppressor mutations that allow Sindbis virus RNA polymerase to function with nonaromatic amino acids at the N-terminus: Evidence for interaction between nsP1 and nsP4 in minus-strand RNA synthesis. *Virology* **2000**, *276*, 148-160.

91. Rathore, A. P.; Ng, M. L.; Vasudevan, S. G. Differential unfolded protein response during Chikungunya and Sindbis virus infection: CHIKV nsP4 suppresses eIF2alpha phosphorylation. *Virol. J.* **2013**, *10*, 36, DOI: 10.1186/1743-422X-10-36.

92. Tardif, K. D.; Waris, G.; Siddiqui, A. Hepatitis C virus, ER stress, and oxidative stress. *Trends Microbiol.* **2005**, *13*, 159-163.

93. Salminen, A.; Wahlberg, J. M.; Lobigs, M.; Liljestrom, P.; Garoff, H. Membrane fusion process of Semliki Forest virus. II: Cleavage-dependent reorganization of the spike protein complex controls virus entry. *J. Cell Biol.* **1992**, *116*, 349-357.

94. Li, L.; Jose, J.; Xiang, Y.; Kuhn, R. J.; Rossmann, M. G. Structural changes of envelope proteins during alphavirus fusion. *Nature* **2010**, *468*, 705-708.

95. Voss, J. E.; Vaney, M. C.; Duquerroy, S.; Vonrhein, C.; Girard-Blanc, C.; Crublet, E.; Thompson, A.; Bricogne, G.; Rey, F. A. Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. *Nature* **2010**, *468*, 709-712.

96. Kuo, S. C.; Chen, Y. J.; Wang, Y. M.; Tsui, P. Y.; Kuo, M. D.; Wu, T. Y.; Lo, S. J. Cell-based analysis of Chikungunya virus E1 protein in membrane fusion. *J. Biomed. Sci.* **2012**, *19*, 44, DOI: 10.1186/1423-0127-19-44.

97. Kielian, M.; Rey, F. A. Virus membrane-fusion proteins: more than one way to make a hairpin. *Nat. Rev. Microbiol.* **2006**, *4,* 67-76.

98. Weissenhorn, W.; Hinz, A.; Gaudin, Y. Virus membrane fusion. *FEBS Lett.* **2007**, 581, 2150-2155.

99. Mohanram, H.; Nip, A.; Domadia, P. N.; Bhunia, A.; Bhattacharjya, S. NMR structure, localization, and vesicle fusion of chikungunya virus fusion peptide. *Biochemistry* **2012**, *51*, 7863-7872.

100. Ozden, S.; Lucas-Hourani, M.; Ceccaldi, P. E.; Basak, A.; Valentine, M.; Benjannet, S.; Hamelin, J.; Jacob, Y.; Mamchaoui, K.; Mouly, V.; Despres, P.; Gessain, A.; Butler-Browne, G.; Chretien, M.; Tangy, F.; Vidalain, P. O.; Seidah, N. G. Inhibition of Chikungunya virus infection in cultured human muscle cells by furin inhibitors: impairment of the maturation of the E2 surface glycoprotein. *J. Biol. Chem.* **2008**, *283*, 21899-21908.

101. Lobigs, M.; Zhao, H. X.; Garoff, H. Function of Semliki Forest virus E3 peptide in virus assembly: replacement of E3 with an artificial signal peptide abolishes spike heterodimerization and surface expression of E1. *J. Virol.* **1990**, *64*, 4346-4355.

102. Meyer, W. J.; Johnston, R. E. Structural Rearrangement of Infecting Sindbis Virions at the Cell-Surface - Mapping of Newly Accessible Epitopes. *J. Virol.* **1993**, *67*, 5117-5125.

103. Meyer, W. J.; Gidwitz, S.; Ayers, V. K.; Schoepp, R. J.; Johnston, R. E. Conformational alteration of Sindbis virion glycoproteins induced by heat, reducing agents, or low pH. *J. Virol.* **1992**, *66*, 3504-3513.

104. Flynn, D. C.; Meyer, W. J.; Mackenzie, J. M., Jr.; Johnston, R. E. A conformational change in Sindbis virus glycoproteins E1 and E2 is detected at the plasma membrane as a consequence of early virus-cell interaction. *J. Virol.* **1990**, *64*, 3643-3653.

105. Rashad, A. A.; Keller, P. A. Structure Based Design towards the Identification of Novel Binding Sites and Inhibitors for the Chikungunya Virus Envelope Proteins. *J. Mol. Graphics Modell.* **2013**, *44*, 241-252.

106. de Lamballerie, X.; Ninove, L.; Charrel, R. N. Antiviral treatment of chikungunya virus infection. *Infec.t Disord. Drug Targets* **2009**, *9*, 101-104.

107. Couderc, T.; Chretien, F.; Schilte, C.; Disson, O.; Brigitte, M.; Guivel-Benhassine, F.; Touret, Y.; Barau, G.; Cayet, N.; Schuffenecker, I.; Despres, P.; Arenzana-Seisdedos, F.; Michault, A.; Albert, M. L.; Lecuit, M. A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. *PLoS Pathog.* **2008**, *4*, e29.

108. Yeo, L. S.; Chu, J. J. H. Recent developments and challenges in mouse models of Chikungunya virus infection. *Future Virol.* **2013**, *8*, 423-426.

109. Inglot, A. D. Comparison of the Antiviral Activity in vitro of some Non-steroidal Anti-inflammatory Drugs. *J. Gen. Virol.* **1969**, *4*, 203-204.

110. Shimizu, Y.; Yamamoto, S.; Homma, M.; Ishida, N. Effect of chloroquine on the growth of animal viruses. *Arch. Gesamte Virusforsch.* **1972**, *36*, 93-104.

111. Coombs, K.; Mann, E.; Edwards, J.; Brown, D. T. Effects of chloroquine and cytochalasin B on the infection of cells by Sindbis virus and vesicular stomatitis virus. *J. Virol.* **1981**, *37*, 1060-1065.

112. Helenius, A.; Marsh, M.; White, J. Inhibition of Semliki forest virus penetration by lysosomotropic weak bases. *J. Gen. Virol.* **1982**, *58*, 47-61.

113. Cassell, S.; Edwards, J.; Brown, D. T. Effects of lysosomotropic weak bases on infection of BHK-21 cells by Sindbis virus. *J. Virol.* **1984**, *52*, 857-864.

114. Maheshwari, R. K.; Srikantan, V.; Bhartiya, D. Chloroquine enhances replication of Semliki Forest virus and encephalomyocarditis virus in mice. *J. Virol.* **1991**, *65*, 992-995.

115. Brighton, S. W. Chloroquine phosphate treatment of chronic Chikungunya arthritis. An open pilot study. *S. Afr. Med. J.* **1984**, *66*, 217-218.

116. Leyssen, P.; De Clercq, E.; Neyts, J. The anti-yellow fever virus activity of ribavirin is independent of error-prone replication. *Mol. Pharmacol.* **2006**, *69*, 1461-1467.

117. Briolant, S.; Garin, D.; Scaramozzino, N.; Jouan, A.; Crance, J. M. In vitro inhibition of Chikungunya and Semliki Forest viruses replication by antiviral compounds: synergistic effect of interferon-alpha and ribavirin combination. *Antiviral Research* **2004**, *61*, 111-117.

118. Rada, B.; Dragun, M. Antiviral action and selectivity of 6-azauridine. *Ann. N. Y. Acad. Sci.* **1977**, *284*, 410-417.

119. Crutcher, W. A.; Moschella, S. L. Double-blind controlled crossover high-dose study of Azaribine in psoriasis. *Br. J. Dermatol.* **1975**, *92*, 199-205.

120. Panisheva, E. K. F., A. N.; Nikolaeva, I. S.; Galenko-Yaroshevskii, P. A.; Bartashevich, V. V.; Cherkasova, A. A.; Linchenko, S. N.; Egik'yan, A. L.; Golovanova, E. A.; Pushkina, T. V. . Synthesis and biological activity of substituted 5-hydroxy-6-bromoindoles. *Khim.-Farm. Zh.* **1988**, *22 565-569*.

121. Boriskin, Y. S.; Leneva, I. A.; Pecheur, E. I.; Polyak, S. J. Arbidol: A broad-spectrum antiviral compound that blocks viral fusion. *Curr. Med. Chem.* **2008**, *15*, 997-1005.

122. Villalain, J. Membranotropic effects of arbidol, a broad anti-viral molecule, on phospholipid model membranes. *J. Phys. Chem. B* **2010**, *114*, 8544-8554.

123. Leneva, I. A.; Russell, R. J.; Boriskin, Y. S.; Hay, A. J. Characteristics of arbidol-resistant mutants of influenza virus: Implications for the mechanism of anti-influenza action of arbidol. *Antiviral Res.* **2009**, *81*, 132-140.

124. Pecheur, E. I.; Lavillette, D.; Alcaras, F.; Molle, J.; Boriskin, Y. S.; Roberts, M.; Cosset, F. L.; Polyak, S. J. Biochemical mechanism of hepatitis C virus inhibition by the broad-spectrum antiviral arbidol. *Biochemistry* **2007**, *46*, 6050-6059.

125. Delogu, I.; Pastorino, B.; Baronti, C.; Nougairede, A.; Bonnet, E.; de Lamballerie, X. In vitro antiviral activity of arbidol against Chikungunya virus and characteristics of a selected resistant mutant. *Antiviral Res.* **2011**, *90*, 99-107.

126. Bentley, R. Mycophenolic Acid: a one hundred year odyssey from antibiotic to immunosuppressant. *Chem. Rev.* **2000**, *100*, 3801-3826.

127. Chen, L.; Pankiewicz, K. W. Recent development of IMP dehydrogenase inhibitors for the treatment of cancer. *Curr. Opin. Drug Discov. Devel.* **2007**, *10*, 403-412.

128. Ratcliffe, A. J. Inosine 5'-monophosphate dehydrogenase inhibitors for the treatment of autoimmune diseases. *Cur.r Opin. Drug Discov. Devel.* **2006**, *9*, 595-605.

129. Khan, M.; Dhanwani, R.; Patro, I. K.; Rao, P. V. L.; Parida, M. M. Cellular IMPDH enzyme activity is a potential target for the inhibition of Chikungunya virus replication and virus induced apoptosis in cultured mammalian cells. *Antiviral Res.* **2011**, *89*, 1-8.

130. Sweeney, M. J.; Hoffman, D. H.; Esterman, M. A. Metabolism and biochemistry of mycophenolic acid. *Cancer Res.* **1972**, *32*, 1803-1809.

131. Bopp, R. J.; Schirmer, R. E.; Meyers, D. B. Determination of mycophenolic acid and its glucuronide metabolite in plasma. *J. Pharm. Sci.* **1972**, *61*, 1750-1753.

132. Fan, D.; Ju, H.; Shao-Yong, Y.; Xia, C.; Da-Cai, Z.; Ye-Na, T. Trigonostemon tuberculatus (Euphorbiaceae), a peculiar new species from Yunnan Province, China. *Kew Bull.* **2010**, *65*, 111-113.

133. Lin, B. D.; Han, M. L.; Ji, Y. C.; Chen, H. D.; Yang, S. P.; Zhang, S.; Geng, M. Y.; Yue, J. M. Trigoxyphins A-G: diterpenes from Trigonostemon xyphophylloides. *J. Nat. Prod.* **2010**, *73*, 1301-1305.

134. Dong, S. H.; Zhang, C. R.; Xu, C. H.; Ding, J.; Yue, J. M. Daphnane-type diterpenoids from Trigonostemon howii. *J. Nat. Prod.* **2011**, *74*, 1255-1261.

135. Zhang, L.; Luo, R. H.; Wang, F.; Jiang, M. Y.; Dong, Z. J.; Yang, L. M.; Zheng, Y. T.; Liu, J. K. Highly functionalized daphnane diterpenoids from Trigonostemon thyrsoideum. *Org. Lett.* **2010**, *12*, 152-155.

136. Zhang, L.; Luo, R. H.; Wang, F.; Dong, Z. J.; Yang, L. M.; Zheng, Y. T.; Liu, J. K. Daphnane diterpenoids isolated from Trigonostemon thyrsoideum as HIV-1 antivirals. *Phytochemistry* **2010**, *71*, 1879-1883.

137. Allard, P. M.; Martin, M. T.; Tran Huu Dau, M. E.; Leyssen, P.; Gueritte, F.; Litaudon, M. Trigocherrin A, the First Natural Chlorinated Daphnane Diterpene Orthoester from Trigonostemon cherrieri. *Org. Let.t* **2012**, *14*, 342-345.

138. Allard, P. M.; Leyssen, P.; Martin, M. T.; Bourjot, M.; Dumontet, V.; Eydoux, C.; Guillemot, J. C.; Canard, B.; Poullain, C.; Gueritte, F.; Litaudon, M. Antiviral chlorinated daphnane diterpenoid orthoesters from the bark and wood of Trigonostemon cherrieri. *Phytochemistry* **2012**, *84*, 160-168.

139. Bourjot, M.; Delang, L.; Nguyen, V. H.; Neyts, J.; Gueritte, F.; Leyssen, P.; Litaudon, M. Prostratin and 12-O-Tetradecanoylphorbol 13-Acetate Are Potent and Selective Inhibitors of Chikungunya Virus Replication. *J. Nat. Prod.* **2012**, *75*, 2183-2187.

140. Hezareh, M. Prostratin as a new therapeutic agent targeting HIV viral reservoirs. *Drug News Perspect.* **2005**, *18*, 496-500.

141. Chowdhury, M. I. H.; Koyanagi, Y.; Kobayashi, S.; Hamamoto, Y.; Yoshiyama, H.; Yoshida, T.; Yamamoto, N. The Phorbol Ester Tpa Strongly Inhibits Hiv-1-Induced Syncytia Formation but Enhances Virus Production - Possible Involvement of Protein Kinase-C Pathway. *Virology* **1990**, *176*, 126-132.

142. Voet, D. V., J. G. In *In Biochimie; de Boeck, Ed.*, Université: Bruxelles: 2005; p 712.

143. Szallasi, Z.; Krsmanovic, L.; Blumberg, P. M. Nonpromoting 12-deoxyphorbol 13-esters inhibit phorbol 12-myristate 13-acetate induced tumor promotion in CD-1 mouse skin. *Cancer Res.* **1993**, *53*, 2507-2512.

144. Bourjot, M.; Leyssen, P.; Eydoux, C.; Guillemot, J. C.; Canard, B.; Rasoanaivo, P.; Gueritte, F.; Litaudon, M. Chemical constituents of Anacolosa pervilleana and their antiviral activities. *Fitoterapia* **2012**, *83*, 1076-1080.

145. Takeda, S.; Yajima, N.; Kitazato, K.; Unemi, N. Antitumor activities of harringtonine and homoharringtonine, cephalotaxus alkaloids which are active principles from plant by intraperitoneal and oral administration. *J. Pharmacobio-Dyn.* **1982**, *5*, 841-847.

146. Kaur, P.; Thiruchelvan, M.; Lee, R. C.; Chen, H.; Chen, K. C.; Ng, M. L.; Chu, J. J. Inhibition of chikungunya virus replication by harringtonine, a novel antiviral that suppresses viral protein expression. *Antimicrob. Agents Chemother.* **2013**, *57*, 155-167.

147. D'Hooghe, M.; Mollet, K.; De Vreese, R.; Jonckers, T. H.; Dams, G.; De Kimpe, N. Design, synthesis, and antiviral evaluation of purine-beta-lactam and purine-aminopropanol hybrids. *J. Med. Chem.* **2012**, *55*, 5637-5641.

148. Field, A. K.; Tytell, A. A.; Lampson, G. P.; Hilleman, M. R. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proc. Natl. Acad. Sci. U. S. A.* **1967**, *58*, 1004-1010.

149. Djeu, J. Y.; Heinbaugh, J. A.; Holden, H. T.; Herberman, R. B. Role of macrophages in the augementation of mouse natural killer cell activity by poly I:C and interferon. *J. Immunol.* **1979**, *122*, 182-1588.

150. Guillot, L.; Le Goffic, R.; Bloch, S.; Escriou, N.; Akira, S.; Chignard, M.; Si-Tahar, M. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J. Biol. Chem.* **2005**, *280*, 5571-5580.

151. Li, Y. G.; Siripanyaphinyo, U.; Tumkosit, U.; Noranate, N.; A, A. N.; Pan, Y.; Kameoka, M.; Kurosu, T.; Ikuta, K.; Takeda, N.; Anantapreecha, S. Poly (I:C), an agonist of toll-like receptor-3, inhibits replication of the Chikungunya virus in BEAS-2B cells. *Virology journal* **2012**, *9*, 114, DOI: 10.1186/1743-422X-9-114.

152. Dash, P. K.; Tiwari, M.; Santhosh, S. R.; Parida, M.; Rao, P. V. L. RNA interference mediated inhibition of Chikungunya virus replication in mammalian cells. *Biochem. Biophys. Res. Commun.* **2008**, *376*, 718-722.

153. Lam, S.; Chen, K. C.; Ng, M. M.; Chu, J. J. Expression of plasmid-based shRNA against the E1 and nsP1 genes effectively silenced Chikungunya virus replication. *PLoS One* **2012**, *7*, e46396.

Table of Contents Graphic