Targeting chikungunya virus replication : insights into chikungunya virus replication and the antiviral activity of suramin in vitro

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Chapter 1

General introduction, aim and chapter outline

Introduction and aim

Viruses are submicroscopic entities that can infect organisms from all kingdoms of life. They are obligatory parasites and do not grow or multiply by division, but instead are assembled from components produced by their host, which also supplies them with the energy and protein synthesis required for virus replication. There are numerous viruses that can cause serious disease in humans, sometimes leading to problematic recovery or even death. They can be spread by contact with an infected individual via bodily fluids, rely on airborne transmission, or the bite of a virus-containing vector (mosquitos, ticks, etc.). The latter viruses are classified as arboviruses (arthropod-borne viruses). In this era of increased travel, urbanization and climate change, viruses have the potential to cause larger and faster spreading outbreaks. The recent chikungunya, Ebola, yellow fever, and Zika virus epidemics are only a few examples of this trend. Unfortunately, for many infections no specific vaccine or antiviral treatment is available.

Chikungunya virus (CHIKV) belongs to the arbovirus group and is a member of the *Togaviridae* family. CHIKV is transmitted to susceptible hosts through the bite of an infected mosquito. Although this virus was discovered in 1952 in what is currently Tanzania [1], research has intensified in the past two decades due to the large epidemics (and painful burden) that the virus has caused outside of Africa [2]. This led to increased interest in this neglected tropical virus, which is classified as a biosafety level 3 pathogen, meaning that special containment facilities are required to work with it.
This thesis aims to provide a deeper understanding of CHIKV replication in cell culture and to uncover particular characteristics that can facilitate the development of targeted inhibitors. It includes studies on the inhibition of CHIKV replication by the registered antiparasitic drug suramin. Suramin was found to also inhibit the replication of Zika virus, an arbovirus that emerged and caused a massive epidemic while the research on CHIKV described in this thesis was ongoing. More in-depth studies into suramin's mode of action were performed and the possibilities to repurpose this antiparasitic drug as a broad-spectrum antiviral compound are discussed.

CHIKV and the *Togaviridae* family

CHIKV is a member of the *Togaviridae* family, which based on the Baltimore classification of viruses belongs to group IV, the positive-sense and single-stranded RNA (+ssRNA) viruses [3].

The *Togaviridae* family (toga=cloak in Greek) was established in 1974 [4], and to date it contains 32 members that are subdivided into two genera: *Rubivirus*, with rubella virus as its only member, and *Alphavirus* containing the other 31 species, listed at [5, 6]. Representative species and isolates are depicted in the phylogenetic tree presented in Fig. 1.

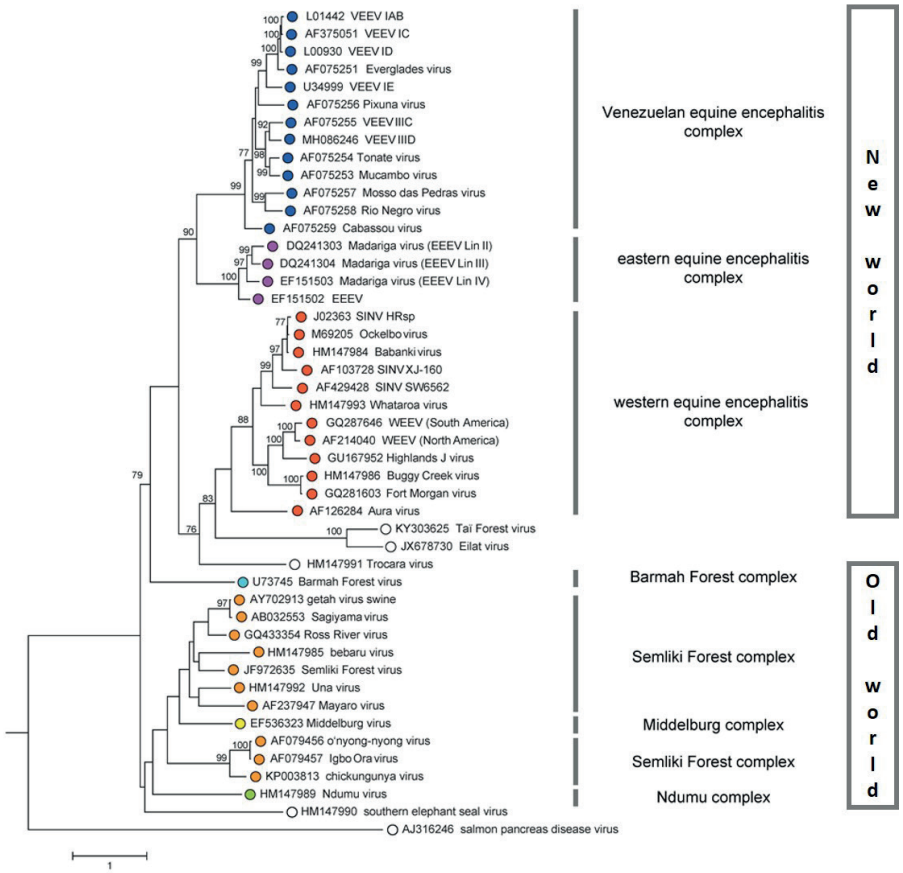

Figure 1. Phylogenetic tree with representative species and isolates from the *Alphavirus* genus. The tree was generated based on a conserved region of 2184 nt encoding part of the envelope proteins; the alphaviruses are further grouped depending on the antigenic complex they belong to, as well as their geographic distribution (adapted from [9]).

Togavirus nomenclature is based predominantly on their geographical origin, or, alternatively, refers to a feature of the disease they cause (*e.g.*, rubella virus – rubis = red in latin, causes a red rash; Venezuelan equine encephalitis virus or VEEV requires no further explanation). Sindbis virus (named after an Egyptian village) and Semliki Forest virus (originating from Uganda) are two of the most-studied alphaviruses, because their low

pathogenicity in humans allows them to be studied at lower containment facilities. Studies on SINV and SFV have led to many advancements in the understanding of the replication cycle of alphaviruses and viruses in general [7].

Togaviruses differ from one another from an antigenic point of view. They can, however, be grouped into seven antigenic complexes (established based on cross-neutralization assays), containing members that are more closely related to each other [8].

Host range and transmission

The host range of togaviruses is quite broad, as they are able to replicate in both arthropod vectors (mosquitoes, ticks, lice, and cliff swallow bugs) as well as vertebrate host such as humans, non-human primates, equines, seals, fish, birds, rodents, and swine [8, 10]. In the case of CHIKV, there is a sylvatic cycle with the virus circulating between mosquitoes (*Aedes* sp.) and non-human primates, with occasional spillover transmission into humans occurring in urbanized areas. Transmission is horizontal, predominantly via the bite of an infected vector, with the exception of Rubella virus that has airborne transmission and infects only humans [11], and Eilat virus, which only replicates in insect cells and is thought to be vertically transmitted in mosquitoes [12].

Geographical distribution of alphaviruses and epidemiology of CHIKV

The *Togaviridae* family distribution relies on favorable ecological conditions and preferred vectors as well as host availability. Rubella virus can be found worldwide, but is more prevalent in underdeveloped countries where vaccination is scarce infection is characterized by mild fever and rash, and is considered a measles-like disease that can have severe consequences for fetal development during pregnancy when maternal immunity is lacking [13]. The alphaviruses are thought to have a marine origin based on phylogenetic analysis of members infecting fish and marine mammals, and likely spread from the southern ocean across the rest of the world [14]. They are further separated into new-world and old-world alphaviruses (Fig. 1). New-world alphaviruses are mainly present in the Americas, and are known to cause encephalitis (in equines and humans) and arthritic/rheumatic syndromes. The Old-world alphaviruses, including CHIKV, are found in Africa, Asia, and Australia, and in humans predominantly cause arthritic/rheumatic syndromes [10].

The most prominent alphavirus members, which have raised awareness due to their pathology and high mortality rate, are the encephalitic viruses: Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus or VEEV [15]. The arthritogenic viruses like CHIKV, Ross River virus (RRV), Barmah Forest virus (BFV), o'nyong-nyong virus (ONNV), Mayaro virus (MAV), SINV and SFV have caused occasional epidemics leading to a much larger number of infections [16]. Among the arthritogenic alphaviruses, CHIKV has caused the largest epidemics so far,

exhibiting highly effective spread within immunologically naïve populations. The first time that CHIKV attracted widespread attention was in 2004-2008, when the epidemics that originated from Africa reached the island of La Reunion, after which CHIKV further spread into India and other countries surrounding the Indian Ocean, causing at least 6 million cases [17, 18]. This was followed by the 2013-2014 'New-world epidemic' with an estimated number of 2.9 million human infections [19]. As far as we know, CHIKV is also the only Old-world alphavirus that has crossed into the New world on two different occasions: in 2013 via the Caribbean island of Saint Martin [20] and in 2014 in Brazil [21].

Based on phylogenetic analysis, CHIKV is considered to have originated in Africa, about 500 years ago [22] and it can now be classified into three major genotypes (or lineages): West African (WA), East, central and South African (ECSA; including the Indian Ocean lineage (IOL) as a subgenotype) and Asian (including the Asian/American subgenotype) [23]. Aedes aegypti was considered to be the main vector for transmission, but in the early 2000s the IOL evolved from the ECSA genotype after acquiring a mutation (A226V) in the E1 envelope protein that allowed CHIKV to replicate in, and hence be transmitted by, *Aedes albopictus*, the Asian tiger mosquito, an avid daytime biter [24]. There are differences in virulence between CHIKV genotypes, with the Asian/American type being the less virulent as compared to the Asian, ECSA and WA viruses [25]. The difference in virulence of the Asian/American genotype is presumably caused by its shorter 3' UTR – as explained later in this chapter. The distribution of CHIKV genotypes is depicted in Fig. 2, alongside the territories of their main vectors, *Aedes aegypti* and *albopictus*.

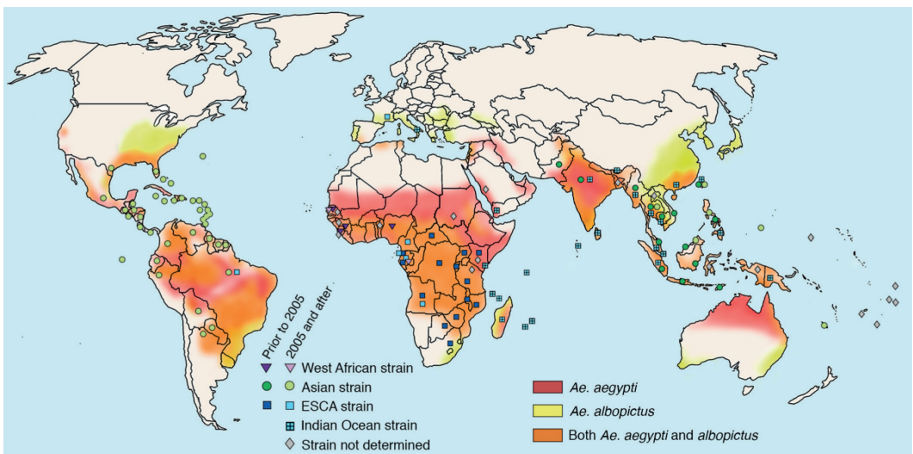

Figure 2. Global distribution of mosquito vectors and areas where CHIKV is endemic (with permission from [23]).

Morphology and replication cycle of alphaviruses and CHIKV in particular

Virion morphology and characteristics

A typical alphavirus particle is icosahedral (T=4 symmetry) and has a diameter of approximately 70 nm (Fig. 3A). The outer layer consists of a lipid bilayer originating from the host cell, in which the envelope glycoproteins E1 and E2 are embedded as groups of three heterodimers, forming projections or spikes that give the particle its coarse surface (Fig. 3B). Below the envelope, the nucleocapsid core has a regular, icosahedral structure, and contains 240 copies of the capsid protein, which interacts with the cytoplasmic tails of the envelope proteins while packaging the genome. In the virion the stoichiometric ratio between the major structural proteins capsid: E1: E2 is presumed to be 1:1:1 [8].

Regarding their physical properties, it is known that alphaviruses have a buoyant density of 1.20 g/cm^3 in sucrose gradients and a sedimentation coefficient of ~280S, corresponding to a molecular mass of 5.2×10^7 Daltons [26, 27]. The infectivity of the particle is lost upon treatment with denaturing agents (formaldehyde, 70% ethanol) or detergents, acids, and lipid solvents [28]. Moreover, exposure to heat, UV light, or radiation greatly decreases infectivity ([29] and WHO Technical Report, Series No. 924, 2004, Annex 4).

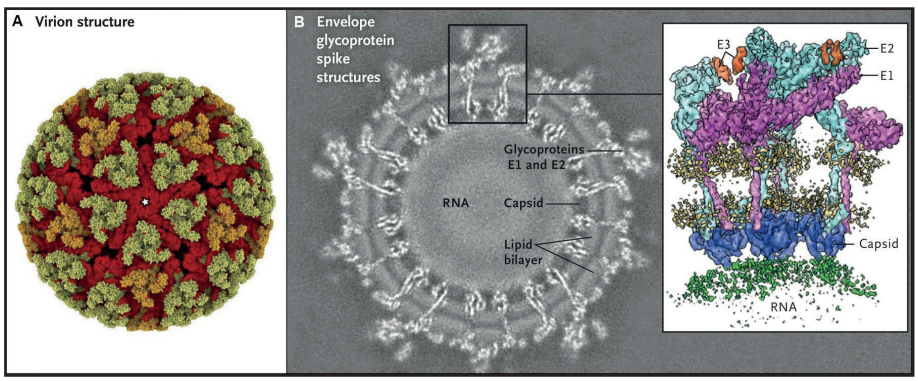

Figure 3. Morphology of a CHIKV particle with ultrastructure details. **A.** Structure of the virion. **B.** View through a section of the virus based on a cryolectron microscopic reconstruction of CHIKV and VEEV particles. The insert shows the structure of an immature envelope spike [30], [31], while the lipid bilayer and the nucleocapsid are shown at atomic resolution (adapted from [2]).

Besides the basic structural components described above, some alphaviruses can contain other components, as is the case for Aura virus that can have a subgenomic RNA packaged alongside the genome [32].

Other alphavirus preparations have been found to contain additional virus proteins, such as the envelope protein E3 (as a sign that the particles contain immature spikes). The 6K or transframe protein TF [33] can also be present in sub-stoichiometric amounts, as determined

by mass spectrometry. However, further studies, e.g. using cryolectron microscopy, are needed to understand their arrangement (perhaps as oligomeric channels) within the virion [8]. Surprisingly, the non-structural protein 2 (nsP2) has been postulated to be important for virus encapsidation in the case of VEEV [34], and very recently has been shown to actually be incorporated into the virion, though not as a structural component [36]. Besides these additional virus proteins, virions can also contain small amounts of host proteins, such as ribosomal subunits [35] or phospholipid scramblase [36]. Particles generated in mosquito cells have a homogeneous density, while those obtained from vertebrate cells include two populations: light and heavy particles [35]. Furthermore, particles derived from mammalian cells contain considerably more cholesterol in their envelope. In general the mass of an alphavirus particle consists of 6% RNA, 62% proteins, 26% lipids, and 6% glycans, with the abundance of the latter two components depending on the cell type from which the particles were generated [27].

Alphavirus genome organization and protein function

In general, the alphavirus genome is a ≈11.8-kilobase, single-stranded RNA equipped with a 5'-terminal m7G cap and a 3'-terminal poly-A tail (Fig. 3), functional features that help it mimic a host cell/eukaryotic mRNA in order to facilitate its direct translation by the host cell ribosomes. The genome contains two open reading frames, ORF1 and ORF2, encoding the non-structural (nsPs) or the structural proteins, respectively.

Alphavirus genomes have a type-0 cap that consists of a 7meGpppA and in the cytoplasm of the infected cell its formation is mediated by the activities of the non-structural proteins (nsP1 and nsP2, as explained below in more detail). It differs from the cellular type-1 cap structure, 7meGppp2meG, as it lacks the second methyl group. Interestingly, it should be noted that for the particles produced early during the infection of mammalian cells, not all genomes are capped, leading to and impairment in their infectivity because of this; it also implies that alphaviruses, for some reason, rely on a mix t of infectious and non-infectious particles for infectivity [37]. The poly(A) tail is ~70 nt long and requires a minimum length of 11-12 nt in order to support the interaction with the host's poly (A) binding protein (PABP), which in turn interacts with initiation factors (eIFα, eIF4E, etc.) bound to the 5' cap in order to circularize the RNA for efficient translation [38].

Besides the ORFs, the genome also contains untranslated regions or UTRs (some authors use the term NTR, for non-translated region) that show a high variability in length and sequence within the alphavirus genus, but also between strains of the same species. UTRs are located at either end of the genome and are important for the regulation of viral gene expression. Conserved sequence elements CSE1-4 play important roles in the alphavirus replication cycle and the nsP1- or nsP2-encoding region of the genome contains a sequence that is crucial for RNA encapsidation (table 1). These RNA sequence elements interact with the nsPs, capsid or host proteins and are crucial for key processes in the virus replication cycle.

Table 1. Conserved sequence elements identified within the genome of alphaviruses, adapted from [8].

Element	Location	Functions
CSE1	Within UTR1	Promoter for genomic RNA synthesis; recognized on the complementary -RNA by RTCs consisting of partly or fully processed nsPs
CSE2	Beginning of ORF1	Important for –RNA synthesis
CSE3	Between ORF1 and ORF2	Promoter for subgenomic RNA synthesis; recognized on the complementary -RNA by RTCs containing fully processed nsPs
CSE4	Within UTR2	Promoter for -RNA synthesis, perhaps in conjunction with CSE2, recognized RTCs composed of nsP123 and nsP4
Virion packaging sequences	Within nsP1 or nsP2	Allow specific packaging of the genomic RNA in the presence of a the more abundant sgRNA

The 3' UTR (or UTR2) of CHIKV is about 498-732 nt long and is located upstream of the poly(A) tail. Its sequence contains a series of direct-repeat elements (DRs 1-3, also known as RSEs, repeated sequence elements) and stem-loop structures, as well as a conserved sequence element, CSE4, immediately upstream of the poly A tail [39]. Although there is great sequence variation within UTR2 of the different CHIKV genotypes, these conserved elements are required for genome replication and stability, and efficient transmission. Therefore, the genetic changes that have occurred in the UTR2 sequence of the CHIKV Asian/American sub-genotype (namely a gain in length) are responsible for the impaired replication in the vector and consequently these viruses are less virulent compared to the other genotypes [40].
The 5' UTR (or UTR1) is about 76-77 nt long in the case of CHIKV, and contains CSE1 that acts as a promoter for +RNA synthesis from its complement that forms the 3' sequence of the negative strand. This element, as well as several stable secondary structures in the region, are involved in the interactions with host factors, leading to initiation of translation. This region also contributes to evasion of the innate immune response by antagonizing IFIT1, an IFN-stimulated gene encoding a protein that limits translation of non-self RNA [41].

The proteins involved in RNA replication and transcription are produced/translated from ORF1 as a polyprotein consisting of the four non-structural proteins (nsP1-4). In many alphaviruses an opal stop codon is present towards the end of the coding sequence of nsP3,

in order to downregulate the production of the viral RNA-dependent RNA polymerase, nsP4. Ribosomes can read through this relatively weak stop codon in ~10% of the cases, leading to the production of the nsP1234 and nsP123 polyproteins in a ratio of ~1:10 [42]. For SFV, ONNV, and CHIKV, it has been shown that not all isolates have the opal stop codon and that it is sometimes replaced by an arginine codon. This is attributed to evolutionary pressures allowing for the existence of both variants and suggesting that both are necessary for virus fitness in the vertebrate and invertebrate host [43-45]. The proteolytic processing of the non-structural polyproteins is carried out by nsP2, the largest of the proteins, with multiple functions that are listed in table 2. The N-terminal Cys protease activity of nsP2 is responsible for the following cleavages: rapid *cis*-cleavage of the site RAGG/Y between nsP3/4, *trans*-cleavage of the sites RAGA/G and RAGC/A between nsP1/2 and nsP2/3 respectively. The latter requires the accumulation of nsP123 [43], thereby controlling the switch from negative strand synthesis done by RTCs composed of nsP123 and nsP4 to synthesis of +RNA by complexes composed of fully cleaved nsPs.

The structural proteins are not produced by direct translation of the genome, but are derived from a polyprotein expressed from the single sgRNA. Also in this situation two polyproteins can be translated: capsid-E3-E2-6K-E1 and, following a frameshift within the 6K protein-coding region, the minor product capsid-E3-E2-TF is produced. The processing of these polyproteins starts with the release of the capsid protein, which catalyzes its own liberation, and next the other sites are gradually cleaved by host proteases (signal peptidase, furin) while trafficking through the secretory pathway.

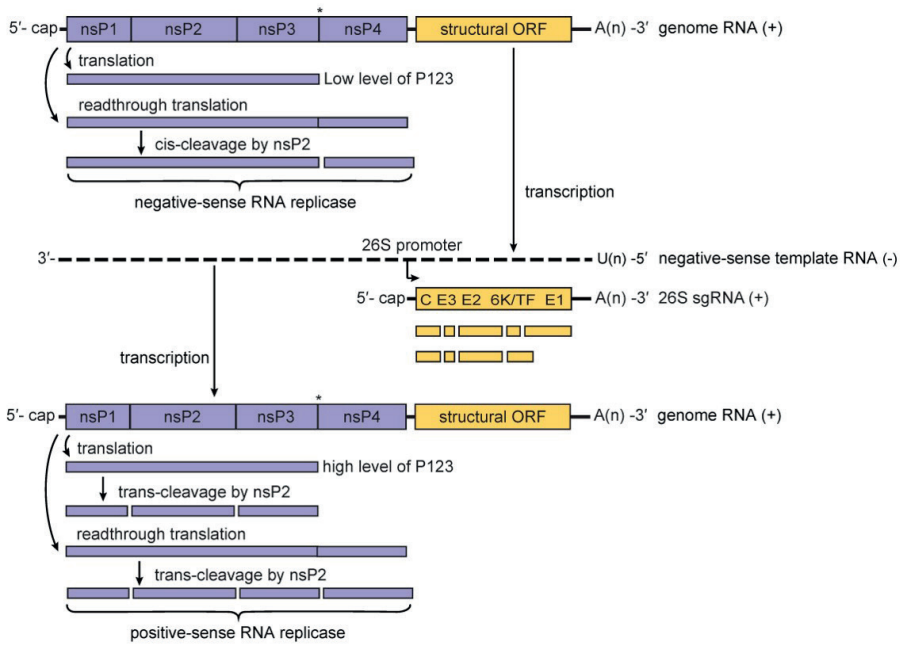

Figure 4. Schematic representation of the alphavirus genome, its translation products, polyprotein processing, and the types of RNA produced during replication/transcription. Upon genome translation the polyproteins nsP123 and nsP1234 are produced, the latter after read-through of the opal stop codon between nsp3 and nsP4. The cis-cleavage of nsP1234 produces the replicase complex (nsP123+nsP4) that synthesizes only the negative sense RNA (upper part of the figure). As the level of nsP123 increases, trans-cleavage occurs generating a short-lived nsP1+nsP23+nsP4 complex and the final nsP1+nsP2+nsP3+nsP4 complex, which is involved in synthesizing only positive sense RNA (g and sgRNA) – lower part of the figure. The structural proteins are translated from the sgRNA, also as two polyproteins due to a frameshift within the 6K protein-coding region: capsid-E3-E2-6K-E1 and capsid-E3-E2-TF. Capsid catalyzes its release from the polyprotein and the remaining polyprotein is processed by host proteases into individual proteins while trafficking along the secretory pathway. (Adapted from https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/*togaviridae*)

As outlined above, the alphavirus genome is relatively small, encoding only 10 proteins - four non-structural and six structural components (Fig. 4); therefore, like the majority of viruses, alphaviruses strongly rely on host factors, metabolic pathways and cellular building blocks for their replication. The proteins encoded by CHIKV, their size, and functions are summarized in table 2 and are further detailed in the description of the replicative cycle.

The alphavirus replication cycle: general and CHIKV-specific features

The CHIKV replicative cycle, as is the case for all alphaviruses, exhibits some host cell-specific differences in invertebrate cell and vertebrate cells [46]. Alphaviruses cause cytolytic infections in mammalian cells while establishing a noncytopathic, persistent infection in mosquito cells. The replication cycle in invertebrate cells is therefore different than in the vertebrate ones [36].

Regardless of the host cell, several key steps can be distinguished in the replicative cycle of alphaviruses: entry, genome translation, RNA replication and transcription, and biogenesis of new particles. These steps are represented in Fig. 5, together with the multitude of stages that occur in vertebrate and invertebrate host cells.

Virus entry and its stages

Entry, the process by which a virion delivers its genome to the viral replication site, in this case the cytoplasm, can be divided in several stages: virus attachment, uptake via receptor-mediated endocytosis (RME), and membrane fusion in order to release the nucleocapsid (numbered 1 to 5 in Fig. 5). This topic is covered in much detail in [47].

The E2 protein likely plays a role in the broad host range of alphaviruses, as it is involved in receptor interaction, and could have multiple binding sites for various cellular receptors. Alphavirus E2 proteins likely interact with receptors that are ubiquitously expressed and highly conserved among the various host species [8].

Attachment is thought to require co-factors as GAGs (such as heparan sulfate) or adhesion molecules (such as DC-SIGN, L-SING, C-type lectins, NRAMP, NRAMP2) that are very abundant on the surface of vertebrate cells [8]. This can facilitate the subsequent interaction with a specific receptor, such as matrix remodeling-associated protein 8 (Mxra8) [48] or the epidermal growth factor receptor pathway substrate (EPS15) [49], in the case of CHIKV. The choice of co-factor and/or receptor is thought to be cell type-dependent as well as CHIKV strain-specific.

Once bound to a receptor, protein rearrangements can occur while the virus is taken up by receptor-mediated endocytosis (RME) in early endosomes that will mature by fusing with lysosomes. Endosomal acidification will trigger conformational changes that destabilize the spike heterotrimers leading to the exposure of the E1 fusion loop. This loop will be inserted in the target membrane in a cholesterol-dependent fashion, followed by trimerization of E1 protein and subsequent fusion with the (late) endosomal membrane. Surprisingly, alphavirus infection could be achieved in the absence of clathrin-mediated endocytosis as has been shown for SINV [50], and the fusion events can also take place directly at the plasma membrane of the host cell.

Following fusion with the endosomal or the plasma membranes, the nucleocapsid is released in the cytoplasm where it undergoes disassembly in order to proceed to genome translation. It is unknown how this happens, but it might be triggered by the ribosomes, more specifically ribosomal RNA that could interact with the capsid protein, destabilizing the core [8]. This hypothesis is plausible, since it has been shown for SINV that the presence of host-derived ribosomal subunits within the virion can speed up the start of the translation [35].

Protein	Size (amino acids)	Main enzymatic activities and other functions
nsP1	535	Methyltransferase and guanylyl transferase activity for capping of viral RNA; Membrane anchor for the replicase complex
nsP2	798	N-terminal NTPase, helicase, and RNA triphosphatase activities; involved in processing of nonstructural polyproteins (either in cis or trans) with its C-terminal cysteine protease activity
nsP3	523 or 529(*)	Phosphoprotein that contains macro domain and SH3-binding regions and an opal stop codon; it interacts with host factors and is involved in negative-strand RNA synthesis;
nsP123	1856 or 1863(*)	Polyprotein that together with nsP4 is involved in negative-strand RNA synthesis (replication)
nsP4	611	RNA-dependent RNA polymerase (RdRp) with terminal transferase activity; it is unstable and produced in lower amounts, as compared to the other nsPs
capsid	261	Structural protein that together with the RNA form the nucleocapsid core; its carboxyl domain has autocatalytic serine protease activity
pE2	487	Precursor polyprotein containing E3 and E2; processed by host furin-like protease in the trans-Golgi vesicles; incorporated into virions at a low level
E3	64	N-terminal domain is uncleaved leader peptide for E2; glycosylated; may help shield fusion peptide in E1 during egress
E2	423	Component of the envelope projection mediating binding to receptors and attachment factors on the host cell membrane; glycosylated and palmitoylated; main target of neutralizing antibodies
6K	61	Leader peptide for E1; functions as an ion channel and may enhance particle release
TF	76	Transframe protein alternatively produced by ribosomal frameshifting; has the N-terminus and the ion channel function as 6K; may enhance particle release
E1	439	Type II fusion protein and component of the envelope surface projections; mediates fusion of viral envelope with cellular membranes via fusion peptide at acidic pH

Table 2. Summary of the CHIKV proteins, polyproteins and intermediates, with their individual functions (adapted from [7, 8, 23].
(*) The longer variant is generated in the cases when the opal stop codon is read-through

RNA replication and its stages

The next 6-9 stages of the virus replication cycle as depicted in Fig. 5 are the steps required for translation, replication and transcription of viral RNA. Translation of genomic RNA (gRNA) leads to the production of the non-structural proteins 1-4, and is initiated by recruiting the host translation machinery. It is hypothesized that alphavirus genomes are translated in the same fashion as cellular mRNAs (a process assisted by the eukaryotic initiation factors, eIFs), since later in infection their translation is downregulated [51, 52]. At first, the polyproteins nsP123 and nsP1234 are produced (in different amounts, as explained earlier), which will be gradually processed by the Cys-protease activity of nsP2. Out of these four proteins, only nsP1 is believed to interact with membranes, either through palmitoylated cysteine residues (C417-419) or by using an amphipathic α-helix formed by amino acid residues 245-264 [8]. By interacting with the plasma membrane these polyproteins are involved in the formation spherules, which are the sites of viral RNA replication and transcription [53]. Because nsP3 has been shown to interact with ampiphysin-1 and -2, proteins involved in membrane curvature, it might have an important role in the generation of these structures [54]. In the spherule environment, the RNA is protected from degradation by host RNases, miRNA targeting or/and detection by the innate immune system. The nsP123 and nsP4 complex is thought to form the bottleneck of the spherule and is responsible, as previously mentioned, for initiating replication by interacting with CSE4 and CSE2, leading to the production of the negative-strand RNA. This will be the template from which more genomes, as well as the sgRNA will be transcribed. The synthesis of the aforementioned three types of viral RNA, requires the functions of all four nsPs as listed in table 2.

Besides their direct involvement in RNA replication and transcription, the nsPs have additional activities within the infected cell that are beneficial for the viral replication cycle. The interplay between host factors and CHIKV nsPs was extensively discussed by Wong and Chu [55] and in particular for nsP2 and nsP3 a variety of interactions with the host have been described. Despite lacking a known nuclear localization signal, nsP2 can translocate to the nucleus where it mediates the degradation of Rpb1 and many other subunits of the RNApolymerase II complex [56, 57], thus leading to a transcriptional host shut-off that could contribute to evasion of innate immune responses [58]. NsP3 was shown to interact with G3BP1 and G3BP2, components of stress granules, which are believed to be involved in the inhibition of translation. nsP3 sequesters G3BPs, which blocks the formation of true stress granules during infection, while early in infection this interaction appears to facilitate the switch from translation to viral RNA replication [59, 60]. Activation of the PI3K-Akt-mTOR pathway by nsP3, which is dependent on its C-terminal part (last 50 residues), causes spherules to pinch off into early endosomes and by fusion with lysosomes they mature in the cytoplasm into type I cytopathic vacuoles [61].

Virion assembly and egress

Stages 9-14 as depicted in Fig. 5 concern the events that lead to the assembly and release of new particles.

The structural proteins are produced upon translation of the sgRNA at a later stage in infection, when cellular and genomic RNA translation are downregulated [52]. Therefore, it is possible that the sgRNA is translated through a different mechanism, perhaps relying on the interaction with host proteins such as the heterogeneous nuclear ribonucleoproteins (hnRNPs) [62]. During translation, the capsid protein liberates itself from the polyprotein, after which the rest of the polyprotein is partially translocated across the ER membrane, a process that is assisted by the transmembrane domains of the E proteins and dependent on an N-terminal signal sequence located in E3. Following its membrane association, the polyprotein undergoes cleavage of the 6K or TF protein by signal-peptidases (step 11) and, as it travels across the secretory pathway towards the plasma membrane, it also undergoes posttranslational modifications (N-glycosylation and palmitoylation), as well as cleavage of the E3-E2 proteins by furin (step 12).

In parallel, newly produced gRNA is packaged by capsid subunits in the cytoplasm (step 13). Another type of cytopathic vacuoles are formed, CPV type II, which originate from the trans-Golgi network. CPV II can interact with nucleocapsids, and contain E1 and E2 glycoproteins arranged as helical tube-like structures resembling the trimeric organization from the envelope of the mature virions. These vacuoles are located closer to the plasma membrane, which is the site of virus budding, suggesting that they might be involved in the transport of structural components to the budding site [63].

The nucleocapsids reach the plasma membrane and interact with the cytoplasmic sides of the envelope proteins that will trigger changes in the curvature of the membrane leading to the budding of newly formed particles.

The steps of virus replication in invertebrate cells are quite similar to those in vertebrate cells, except that replication complexes are associated with endosomes in so called “virus factories”, which are also the site of protein synthesis and assembly of new particles. The newly assembled virions are released into the extracellular space by fusion of the endosome with the plasma membrane [46].

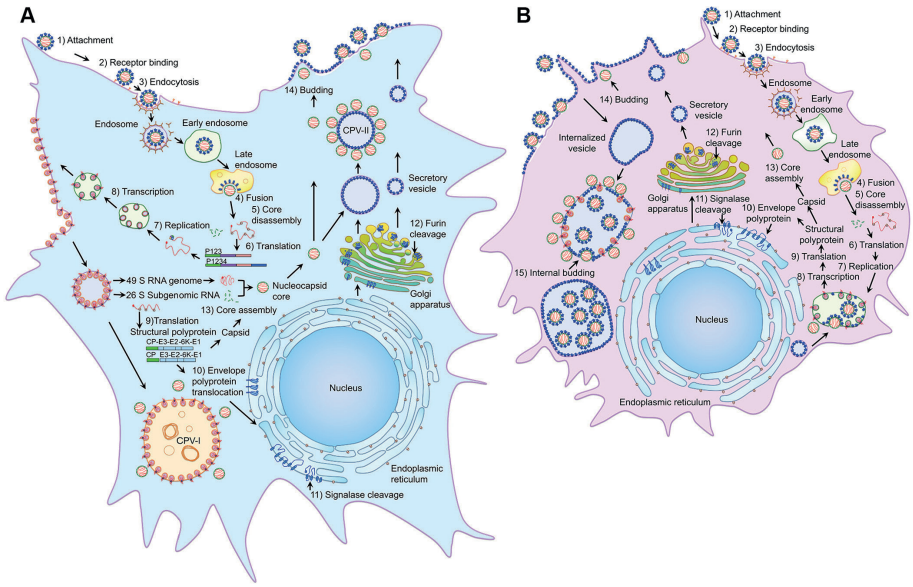

Figure 5. Schematic representation of the alphavirus replication cycle in mammalian (A) and insect (B) cells. (A) Upon attachment and receptor binding (steps 1 and 2), alphaviruses are internalized via endocytosis (step 3). At low pH the virus fuses (step 4) with the late endosome membrane and the nucleocapsid is released in the cytosol. After disassembly (step 5), the non-structural proteins (nsPs) are expressed from the genomic RNA (Step 6). Together with host proteins, the nsPs form RTCs (step 7) that will replicate and transcribe the viral RNA (step 8) producing the –RNA that serves as a template from which genomic and subgenomic RNA are transcribed. The RTCs perform their activity in spherules located at the plasma membrane or on modified endosomes termed cytopathic vacuoles type I (CPV I). The structural proteins are expressed from the sgRNA (step 9) and after capsid self-cleavage, the rest of the polyprotein is translocated to the ER (step 10) where it undergoes processing by signalase (Step 11) and several post-translational modifications (glycosylation) when the proteins reach the Golgi complex. Here the host protease furin cleaves the E3 from E2 (step 12) and the E1-E2 pre-formed heterodimers are trafficked towards the plasma membrane or to CPV-II. The capsid protein will interact with genomic RNA and form nucleocapsids (step 13) that will reach CPV-II or directly the plasma membrane in order to bud off into new virus particles (step 14). (B) In the mosquito cell, replication progresses in a similar manner, but with some differences. These concern the site of replication, which takes place on internal large vesicles that also contain nucleocapsids and internally budded virus particles (step 15). The newly formed virus particles accumulate in large vesicles that are released from the plasma membrane (with permission from [46]).

Not all released particles will be infectious. On the one hand, this can be due to immature surface projections from which E3 is not removed or due to the lack of proper post-translational modifications. On the other hand, it is possible that new virions with properly processed and arranged proteins are formed, which carry a defective genome that is incapable of starting a new round of replication when it is released into the cytoplasm of a new host cell. This is a consequence of the high error rate of the RdRp, which leads to the accumulation of mutations that can be detrimental (e.g. introducing stop codons, changing the reading frame, or point mutations that affect structure or function of proteins or regulatory RNA elements). However, the relatively high error rate of the RdRp also

provides an advantage, as it allows the generation of a large genetic diversity – quasispecies, which enhances the capability of the virus to rapidly adapt to changing selective pressures, e.g. host immune responses or replication in a novel host cell [64].

Clinical signs and pathogenesis of CHIKV infection

In humans CHIKV causes a non-lethal but debilitating, untreatable, and usually self-limiting disease characterized by extreme joint pains that can persist for months.

Clinical symptoms

Upon the bite of an infected mosquito, around 85 % of infected people develop chikunguya fever (CHIKF) symptoms, while 15% or less have asymptomatic seroconversion [2].

The onset of symptoms is rapid, after a mean incubation period of 3 days (range 2 to 6 days), and is characterized by high fever (39-40°C), intense asthenia, myalgia, rash, and headache. The polyarthralgia is the specific symptom to which CHIKV owes its name, as the word chikungunya describes a person bent over by the debilitating joint pain, in Kimakonde, the language of the Makonde tribe that lives in the border region of Mozambique and Tanzania. Polyarthralgia is symmetrical and localized in distant extremities (arms and legs). Acute arthritis may also occur in the interphalangeal joints, wrists, and ankles. This acute phase lasts around 7 days and is dominated by joint pain or/and arthritis. Some unusual symptoms were also noted, such as lymphadenopathy, digestive disorders, and pruritus. Complications of this disease were described for the extreme age groups (neonates and elderly) as well as for those with underlying comorbidities. They are rare, and include myocarditis, ocular disease, hepatitis, acute renal failure, and neurological disorders (meningoencephalitis, Guillane-Barre syndrome). In many patients arthritis can persist for months to years, suggesting the ability of CHIKV to induce immune pathogenic mechanisms specific for chronic disease, by potentially persisting in the joint tissues and replicating at a low level, leading to continuous stimulation of the immune system.

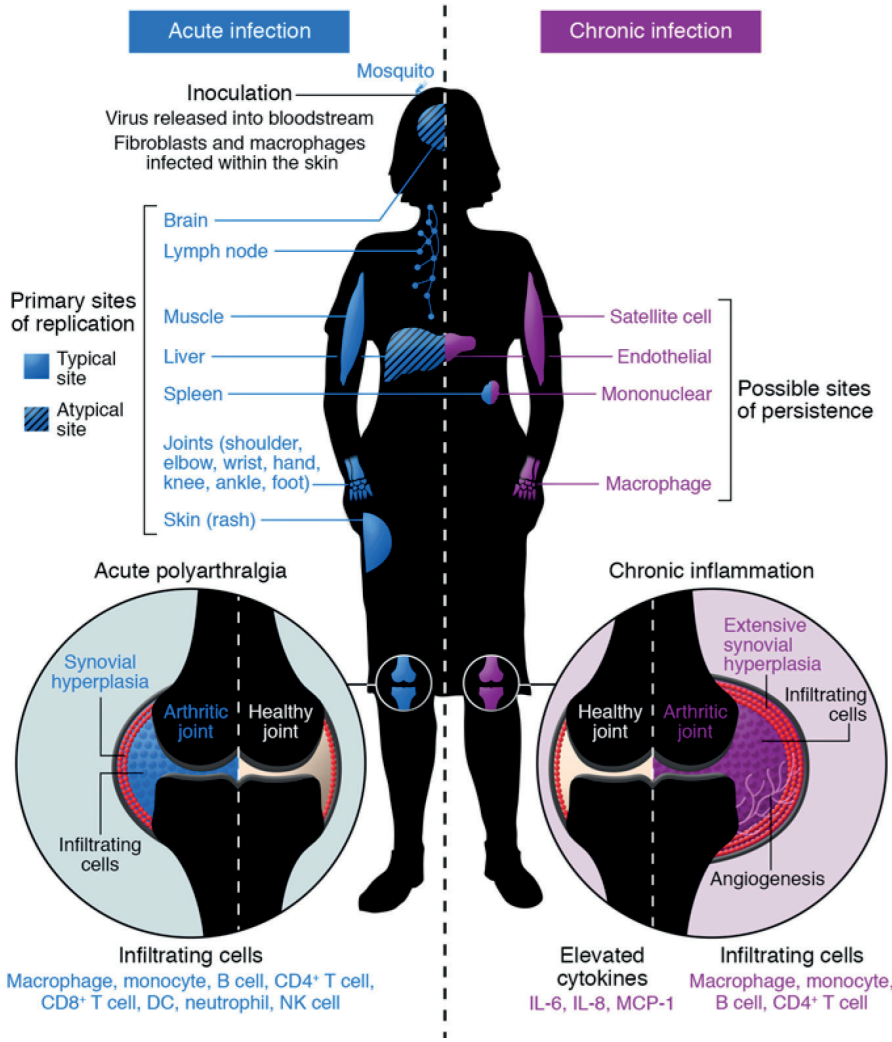

Figure 6. Representation of acute and chronic chikungunya fever symptoms with underlying events (with permission from [23]).

Mechanism of dissemination and pathogenesis

An important role in virus dissemination can be attributed to the mosquito saliva that has been shown to enhance the infectivity of arboviruses [65]. After reaching the skin of its host, CHIKV infects and replicates in fibroblasts as well as macrophages, then spreads via the lymphatic system to specific sites: lymph nodes, spleen, muscle, skin, peripheral joints, tendons, but also to aspecific sites as brain and liver (see Fig. 6, left side).

Viral replication can lead to high levels of viremia, $>10^9$ particles/mL in the blood, which is correlated with the acute onset of the disease. This will initiate the immune response - elevation of pro-inflammatory cytokines, chemokines, growth factors, and type I IFNs. It also leads to activation and proliferation of T lymphocytes (CD8- and CD4-positive), which

contribute to virus clearance. IgM antibody levels are detected within 5-6 days (overlapping with the acute phase of the disease) and IgG antibodies are detected 7-10 days after the onset of chikungunya fever, and their presence aids with the infection clearing.
The chronic phase (Fig. 6, right side), follows a partial clearance of the infection (no CHIKV titers detected), but perhaps antigens still remain in many tissues because of persistence in infected cells: endothelial cells, macrophages, mononuclear cells, satellite cells in the muscle [23]. In some individuals this can last for years, leading to long-term impairment of the quality of life [66]. In rare cases, some patients can suffer from bone erosions due to the CHIKV-induced arthritis [67].

Much of the above information has been acquired by long-term monitoring of patients recovering from CHIKV infection, but also by using animal models – a wide range of mouse and non-human primate models are available to study the progression of CHIKV infection [68]. These approaches have offered a more in-depth view of the molecular mechanisms involved in CHIKV pathogenesis, persistence, and (difficulties in) clearance.

Chikungunya fever diagnosis
Accurate chikungunya fever diagnosis has been an issue for decades, because it has been mistaken for malaria or dengue fever, the major infections in tropical areas. It can also be misdiagnosed as rheumatoid arthritis, since CHIKV-induced joint pain can mimic this autoimmune disease, though the onset of arthritis is usually abrupt in CHIKF [69]. Another contributing factor is the absence of fast and cost-effective diagnostic methods, and unlike flaviviruses (Zika virus), CHIKV can be detected reliably only in blood, and not saliva or urine [70].

At the moment, the World Health Organization recommends several methods for diagnosis. For samples collected soon after the onset of symptoms virus-detection methods like RT-PCR (that can also allow proper genotyping of the virus) can be used as well as serological methods. At later stages, the detection of infection can be done only by serological tests to determine the presence of IgM and IgG anti-CHIKV antibodies [71]. Virus isolation can also be performed from blood shortly after the symptoms have appeared and viral RNA extracted from blood should directly be used for genotyping, preferably by NGS, to avoid the accumulation of adaptive mutations due to repeated passaging in cell culture. Passaging of the isolated virus on mammalian cells or in various animal models, as was conventionally done, could lead to accumulation of adaptive mutations that would hamper proper characterization of the original clinal isolate.
Recently a new and promising method for rapid diagnosis of CHIKV infection, even in DENV co-infected samples, has been proposed, which is based on CHIKV antigen detection by immune-chromatography [72].

Preventive and antiviral strategies against CHIKV infection

Preventive strategies/prophylaxis

In the case of untreatable (infectious) diseases, prevention is the best strategy for controlling the spread of the infection. At the same time, substantial focus should also be on developing proper treatments in order to reduce the symptoms and cure those already infected. Prevention can be achieved by vector control, and by vaccinating the population in endemic areas.

Since CHIKV is an arbovirus, vector control is an important strategy for containing an epidemic. Wearing long clothes and using insect repellent sprays are recommended measures to be taken in areas with endemic CHIKV (or other arboviruses and parasites). Mosquito nets and insecticide-impregnated curtains should be used to limit vector entry into homes and prevent nighttime bites. Additionally, insecticides can be sprayed or added to treat the water from containers (in order to kill the immature larvae or adult mosquitoes) during outbreaks. The use of insecticides can be detrimental for public health, as well as the environment (affecting multiple ecosystems), and it can ultimately become inefficient due to insecticide resistance. A more recently developed approach for vector control is the production of genetically engineered (GE) male mosquitoes that upon release will produce offspring unable to grow in the absence of tetracycline [73]. Another promising strategy could be the use of *Wolbachia*-infected mosquitos, which appears to diminish vector competence for CHIKV (as well as dengue virus). More research should be dedicated to these approaches in order to establish whether the use of GE insects poses a risk to the environment [74].

Passive immunization could also be considered as a treatment alternative, since the humoral immune response is the one limiting the infection. Several mouse studies have shown the success of administering anti-CHIKV antibodies (either as antisera or isolated monoclonal antibodies) in protecting against the disease [23, 75]. Nevertheless, in general vaccines have been one of the most efficient ways of infectious disease prevention, and even eradication. The factors that contribute to efficiency of a vaccine efficient are still not completely understood today and the requirements for bringing a vaccine to the market, for use in humans, have become increasingly strict and time-consuming.

The most successful attempt, in the case of alphavirus vaccines, that led to a marketed product is re the TC-83 IND vaccine against the encephalitic alphavirus VEEV, which was shown to have good efficiency in equines [76]. In the case of CHIKV, multiple attempts have been made (reviewed by [77]), but no vaccine has made it to the market, e.g. due to reasons such as insufficient market interest or financing. One of the most promising attempts was done by the US Army Medical Research Institute, that have produced the live-attenuated vaccine (LAV) candidate TS1-GSD-218 (or 181/clone25) based on an Asian

CHIKV strain, AF15561. The production was stopped after the phase II clinical trial, when 8% of the human subjects developed mild arthralgia [78]. Also the dominant attenuation mutation of that strain, G82R in E2, was observed to revert to wild-type in mice and humans leading to safety concerns ([23] and references herein). However, due to the massive last two epidemics, the global market for a Chikungunya vaccine is estimated at approximately €500 million annually (estimate supported by independent market studies – www.valneva.com), and therefor new vaccine candidates are being tested in clinical trials (see table 3).

Table 3. A summary of the CHIKV vaccines currently in clinical trials.

Vaccine	Platform	Phase of clinical research	Company
VRC-CHKVLP059-00-VP	Measles vectored	Phase II	National Institute of Allergy and Infectious Diseases (NIAID)
MV-CHIK	Measles vectored	Phase II	Themis Bioscience GmbH
VLA1553	LAV	Phase I; data available early 2019	Valneva
PXVX0317 CHIKV-VLP	VLP	Phase II; with or without Alhydrogel adjuvant; study completion December 2020	PaxVax Inc.
VAL-181388	Not specified	Phase I	Moderna Therapeutics
ChAdOx1 Chik	Not specified	Phase I	University of Oxford

New promising avenues in vaccine development are also being explored, such as the production of a CHIKV/IRESv1 vaccine candidate that contains an encephalomyocarditis virus internal ribosomal entry site (IRES) instead of the subgenomic promoter. This limits the production of the structural proteins and at the same time blocks the replication in insect cells, where the IRES is not functional [25].

Antiviral treatment perspectives

It has been historically proven that vaccines are one of the most efficient ways to prevent or limit outbreaks of infectious disease. Unfortunately, there are issues with their large-scale production, especially in a short timeframe, and there are concerns related to storage/distribution in tropical areas. Another downside of vaccines is that their target must be known or related to a (pandemic) pathogen variant that is already known, otherwise a long development process will have to be taken into consideration. Vaccines can become obsolete when (immune) escape mutants of the target virus emerge.

Consequently, antiviral drugs are needed as a therapeutic alternative, offering the advantages that they are relatively easy to produce and store, compared to most vaccines. However, designing effective and specific antiviral drugs is challenging. Viruses extensively rely on their host for replication and thus it is difficult to target virus replication without affecting cellular processes/pathways.

However, viruses express proteins with unique virus-specific functions, which can be targeted by direct acting antiviral compounds (DAAs) and such compounds have been successfully developed for the treatment of viral infections caused by e.g. flu, human immunodeficiency virus (HIV), hepatitis C virus (HCV), human cytomegalovirus (hCMV), varicella zoster virus (VZV), respiratory syncytial virus (RSV), and others [79]. Alternatively, cellular factors crucial for certain steps of the viral replication cycle can be targeted with host-directed antivirals (HDAs), circumventing the potentially rapid development of antiviral resistance, which is often observed when using DAAs. In the case of CHIKV, compounds targeting host factors (HDAs) as well as viral proteins (DAAs) have been reviewed extensively [55, 80-82]. Some of the more relevant therapeutic approaches are discussed here.

Host-targeting antivirals (HDAs) against (alpha)viruses

Of the many approved drugs that are on the market for other conditions, several have been shown to also have antiviral properties, likely because their targets are important factors for the replication of certain viruses. Such compounds have been identified for several viruses, by e.g. screening of FDA-approved drug libraries.

Based on knowledge of host factors involved in replication, specific cellular functions can be targeted. For instance, inhibition of the activities of furin or heat shock protein 90 (HSP90) – involved in particle maturation and (viral) protein folding, respectively, has been investigated ([81], references herein). However, a major bottleneck for the success of this approach is the associated side-effects due a general cytotoxicity of these compounds. Side effects also can be expected when considering to modulate the activity of kinases, which are involved in regulation of a multitude of complex signaling pathways within cells. Inhibition of sphingosine kinase 2 (SK2), a host factor that co-localizes with CHIKV RTCs and is

essential during infection, has been proposed as a potential therapeutic strategy [83]. Not only inhibition of kinases should be considered, as activation of protein kinase C (PKC) by prostatin had a negative impact on CHIKV replication [84].

Another host-directed anti-CHIKV strategy concerns interference with the metabolism of polyamines by the use of difluoromethylornithine (DFMO or eflornithine, marketed as Vaniqa® for the treatment of second stage African trypanosomiasis) [85].

The immunosuppressing drug mycophenolic acid (MPA), sold as Myfortic® or CellCept® for its immunosuppressant role, was also shown to inhibit CHIKV replication [86]. This is due to its inhibitory action on the inosine monophosphate dehydrogenase (IMPDH), which leads to a strong depletion of the intracellular GTP pool, hence indirectly affecting virus replication. However, the immunosuppressive properties of this compound complicate its application in the treatment of infections.

Arguably more promising host-directed antiviral strategies would be to use drugs that upregulate host factors with antiviral activities, such as virperin, a protein that participates in the synthesis of the chain terminator ddhCTP. In this manner the toxic effects could perhaps be bypassed, since viperin is naturally produced in the human body [87].

Immune modulators that stimulate the activity of retinoic acid inducible gene I (RIG-I), a host protein that recognizes dsRNA and triggers antiviral pathways that will lead to the inhibition of virus replication, have also shown to be promising molecules [88].

Recently, the inhibition of Granzyme A, a promoter of arthritic inflammation, with Serpinb6b has been identified as a potential treatment for CHIKV infections. Granzyme A levels were elevated in CHIKV-infected non-human primates as well as a cohort of human patients and treatment with Serpinb6b reduced CHIKV-induced arthritic inflammation in a mouse model [89].

In clinical practice, several marketed therapeutics have been used with moderate success to alleviate the symptoms of patients with chronic CHIKV-induced arthritis. These include non-steroidal anti-inflammatory drugs (NSAIDs), short-term course of corticosteroids and disease modifying antirheumatic drugs (DMARDs) [90].

Direct acting antivirals (DAAs) against alphaviruses

Chloroquine, marketed as Aralen® for the treatment of malaria and additionally displaying a broad antiviral activity in cell culture, was shown to be an entry inhibitor of CHIKV as well, but in clinical trials it did not prove to be effective and even led to increased arthralgia complaints from treated patients [91]. In a non-human primate model chloroquine was even stimulating CHIKV replication while delaying cell and humoral immune responses [92].

CHIKV protein synthesis, and subsequently virus replication, was strongly inhibited by the use of siRNA or shRNA targeting combinations of non-structural and structural genes, and these results were successfully confirmed in animal experiments [93, 94].

Small molecules targeting the capping functions of nsP1 are a promising strategy, since alphaviruses employ unique enzymatic activities for their RNA capping and therefore this cytoplasmic process could be specifically targeted with minimal cytotoxic effects [95, 96].
Most efforts involved targeting of the Cys-protease function of nsP2, mainly through the in silico identification of inhibitors, followed by assessment of their activity using *in vitro* enzymatic assays [80]. The challenge with protease inhibitors is to obtain high selectivity, i.e. they should not inhibit host Cys-proteases.
Ribavirin is a nucleoside analogue, marketed as Rebetol® (and various other names) that has proven to effectively inhibit CHIKV replication in patients. It can act through various mechanisms, either by blocking the activity of IMPDH, an enzyme involved in GTP metabolisms (so, through an indirect effect). Additionally, it could also interfere with viral RNA capping or, by being incorporated into newly synthesized viral RNA, it can induce lethal mutagenesis ([82], references herein).
Similarly, 6-Azauridine (a uridine analogue), marketed for the treatment of psoriasis, has been shown to have a broad-spectrum antiviral activity. Its anti-CHIKV activity has been validated in multiple *in vitro* studies, and it is suspected to act indirectly by interfering with cellular UTP metabolism, or to act as DAA upon its incorporation into viral RNA leading to lethal mutagenesis ([97] and references herein). It requires further pre-clinical evaluation in an appropriate animal model, before it can be tested in the clinic.
Though many of the nucleoside analogues with anti-CHIKV activity that have been identified so far, seem to inhibit virus replication by blocking enzymes involved in the nucleoside metabolism, there are some that potentially act directly on the alphavirus RNA polymerase. β-D-N(4)-hydroxycytidine (NHC) is a potent anti-VEEV drug that causes accumulation of mutations in virus-specific RNAs, and could potentially have a broader anti-alphavirus activity [98]. Similarly, favipiravir is a compound marketed as an anti-influenza A drug, but it has been shown to have a broader antiviral activity, since it can inhibit the replication of several noroviruses, bunyaviruses, flaviviruses, and alphaviruses [99]. Favipiravir specifically targets the viral RdRps, including that of CHIKV [100]. The compound has a broad spectrum of activity in cell culture and animal models, is already approved for the treatment of influenza infections in Japan and clinical trials for other infections are ongoing. Therefore, it could be an inexpensive and fast option for the development of anti-CHIKV therapy.

Due to the lengthy process of bringing a new compound to the market, there is great interest in antiviral screening using previously approved drugs or those considered safe in clinical trials but not yet on the market. This approach is called drug repositioning or repurposing [101] and is an expanding field of research. A number of compounds that have become "block busters' are described by Naylor S. *et al* [102], and an interesting example (that the opposite route is possible as well) is Gemzar® (Eli Lilly), a nucleoside-based inhibitor that was intended as an antiviral compound but ended up on the market for the treatment of

various cancers. Suramin is one of the oldest drugs on the market, intensively studied in the past 30 years for its incredibly broad spectrum, and it is anti-CHIKV and anti-ZIKV properties are discussed in chapters 3, 4, and 5 of this thesis.

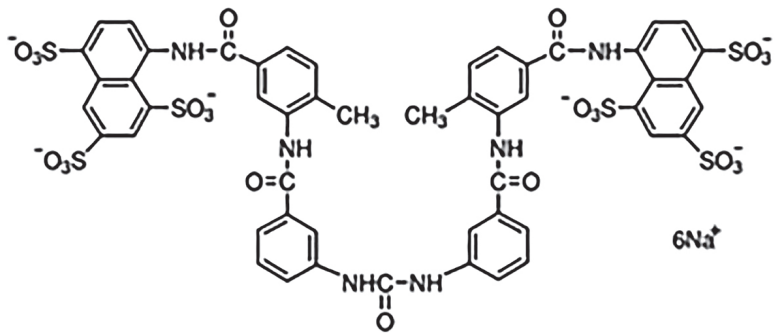

Figure 7. The chemical structure of suramin hexasodium. The full IUPAC nomenclature is available at PubChem (https://pubchem.ncbi.nlm.nih.gov/).

Suramin was synthesized in 1916 by scientists working at Bayer AG, and is still commercialized, also under the original name Germanin®. Its discovery was urged by the colonial powers being exposed to vast epidemics of sleeping sickness in Africa. The disease is transmitted through the bite of the Tsetse flies infected with *Trypanosoma* sp. Suramin, a "colorless dye" related to trypan blue, became the therapy for the first stage of the infection [103]. Soon after, it was proven to be effective also in the treatment of another parasitic infection, onchocerciasis. The mode of action of suramin against these parasites remains unclear until now. It is suspected that the compound impairs the parasites' energy metabolism, leading to their death.

The chemical properties, pharmacokinetics, toxicity and pharmacological actions of suramin have been reviewed in detail [104]. It has a high affinity for proteins, including serum proteins, which leads to a long half-life in humans. Studies on the administration of suramin at high doses and or over a long period of time, which led to accumulation in the plasma, revealed a number of side effects, including fever, skin reactions, malaise, reversible polyneuropathy, adrenal insufficiency, vomiting, blood clotting inhibition, etc. Nevertheless, due to its success in treating African trypanosomiasis, suramin is on the WHO list of essential medicines, 20th edition [105], a list summarizing the most effective and safe medicines required in a health-care system.

Additional pharmacological activities of suramin have been discovered [106, 107]. Due to its capacity to interact with growth factor receptors, suramin has anti-proliferative effects and therefore has been evaluated in cancer therapy [108]. In low doses, suramin has been shown to improve the outcome of children with autism spectrum disorder (ASD), and therefore it is also considered a promising new therapy in the field of neurological disorders [109].

Last but not least, the antiviral activity of suramin has also been explored, and the findings are summarized in the introduction of chapter 3 with all the relevant references [110]. Briefly, through *in vitro* studies it was identified as an inhibitor of viral helicases, polymerases, and reverse-transcriptases. In cell-based assays, the mechanism of action seemed to be different from the one observed *in vitro*, and surprisingly similar for a multitude of viruses - suramin blocks the early events of virus entry. Suramin was proven to be effective against enterovirus A71, the etiological agent of hand foot and mouth disease in humans. This finding was also confirmed in mouse and non-human primate animal models, which showed that suramin treatment lowered the viral burden and decreased mortality, leading to a faster recovery and better outcome [111].

Thesis outline

The main focus of this thesis is on CHIKV replication and the anti-CHIKV activity of suramin, a compound which also inhibited replication of ZIKV, another medically important arbovirus that emerged more recently.

Chapter 1 contains the general introduction that summarizes the current knowledge regarding alphavirus genome organization, protein function, replication, CHIKV pathogenesis and preventive or therapeutic strategies.

Chapter 2 presents the development of an *in vitro* replication assay (IVRA) that can be used to study CHIKV RNA synthesis, as well as to identify inhibitors of this process and perform mode of action studies on these compounds.

Chapter 3 describes the identification of suramin as a direct-acting inhibitor of CHIKV RNA synthesis *in vitro*. Cell-based assays revealed that suramin's main mode of action is dependent on the inhibition of an early step of the replicative cycle and this is also the hypothesis for its antiviral activity *in vivo*.

Chapter 4 shows the antiviral effect of suramin is indeed quite broad, as it can also inhibit ZIKV replication by interfering with virus entry and biogenesis of virions, at a later stage of the replication cycle.

Chapter 5 describes the identification of the CHIKV E2 protein as the target of suramin and mode of action studies that suggest that suramin blocks attachment to cells, and subsequent fusion of the viral particle with cellular membranes.

Chapter 6 contains the general discussion based on the key findings presented in this thesis and their implications, followed by a short conclusion.

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