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Targeting chikungunya virus replication

Insights into chikungunya virus replication and the antiviral activity of suramin *in vitro*

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PhD thesis, Leiden University, 2019.

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Targeting chikungunya virus replication

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"I know, I know the sun is hot
Mosquitos come suck your blood
Leave you there all alone
Just skin and bone
…
Swallow and chew
Eat you alive
All of us food
That hasn't died"

(lyrics from the "Mosquito Song", by Queens of the Stone Age)

Contents

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.

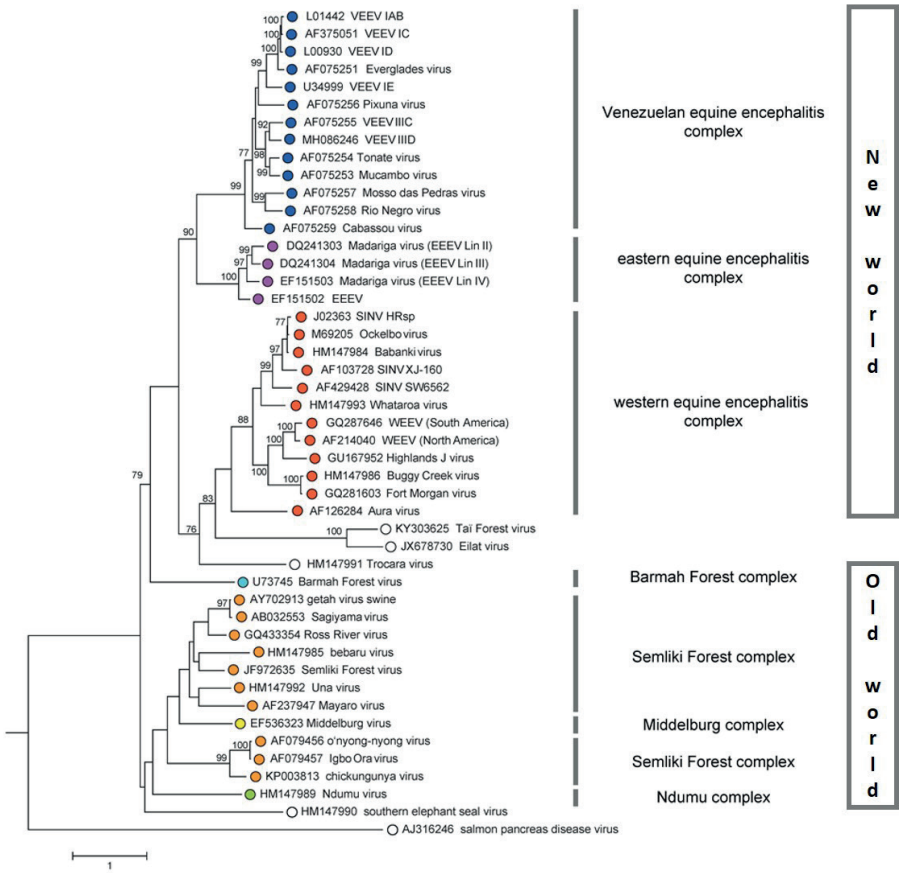

Figure 1. Phylogenetic tree with representative species and isolates from the *Alphavirus* genus. The tree was generated based on a conserved region of 2184 nt encoding part of the envelope proteins; the alphaviruses are further grouped depending on the antigenic complex they belong to, as well as their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

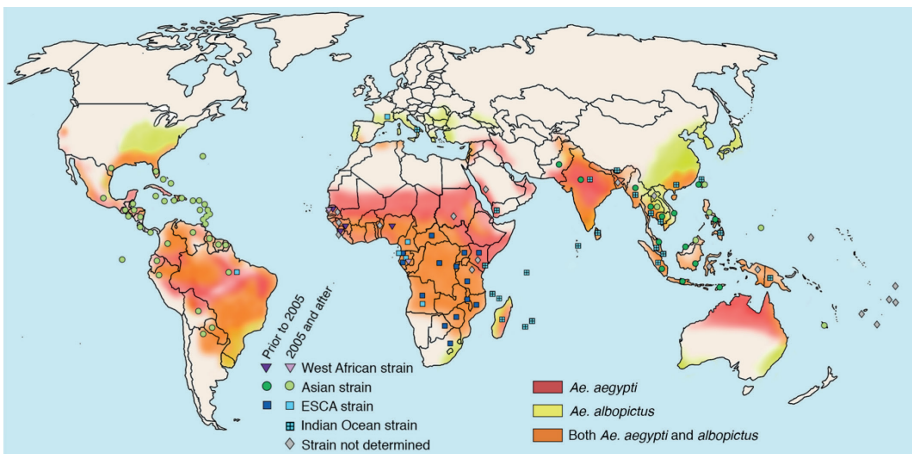

Figure 2. Global distribution of mosquito vectors and areas where CHIKV is endemic (with permission from [23]).

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).

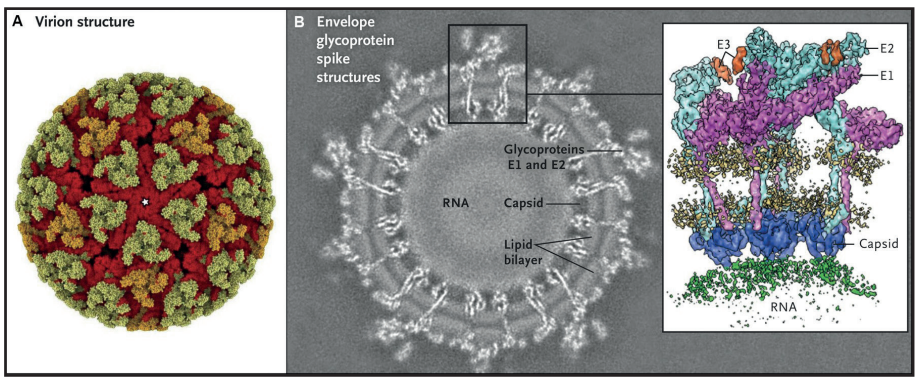

Figure 3. Morphology of a CHIKV particle with ultrastructure details. **A.** Structure of the virion. **B.** View through a section of the virus based on a cryolectron microscopic reconstruction of CHIKV and VEEV particles. The insert shows the structure of an immature envelope spike [30], [31], while 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.

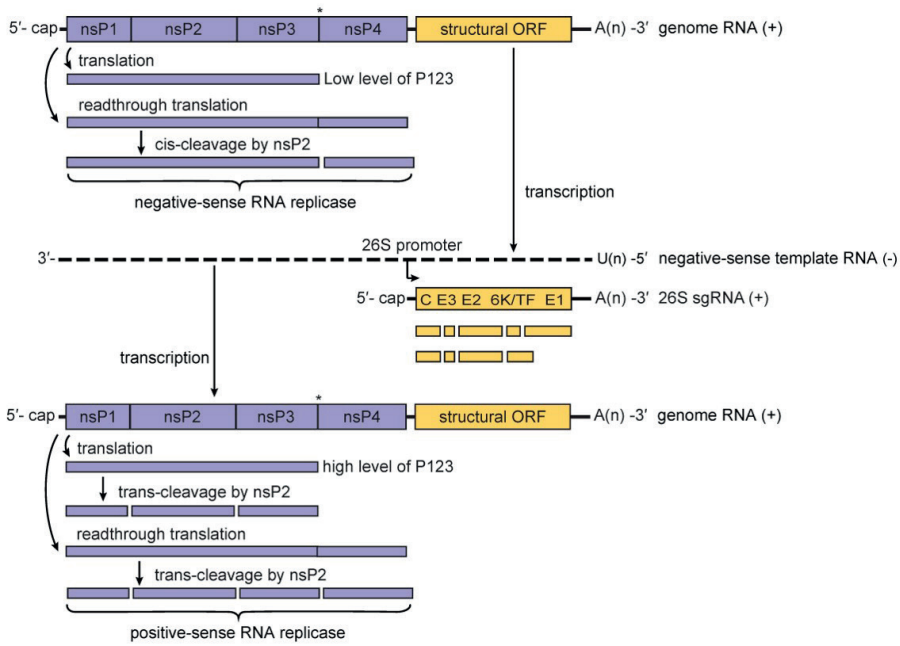

Figure 4. Schematic representation of the alphavirus genome, its translation products, polyprotein processing, and the types of RNA produced during replication/transcription. Upon genome translation the polyproteins nsP123 and nsP1234 are produced, the latter after read-through of the opal stop codon 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].

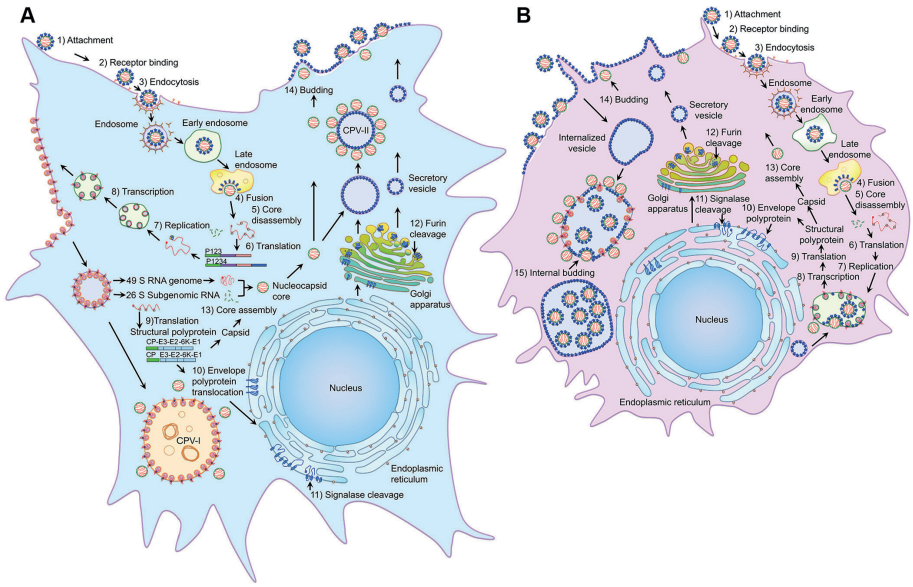

Figure 5. Schematic representation of the alphavirus replication cycle in mammalian (A) and insect (B) cells. (A) Upon attachment and receptor binding (steps 1 and 2), alphaviruses are internalized via endocytosis (step 3). At low pH the virus fuses (step 4) with the late endosome membrane and the nucleocapsid 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.

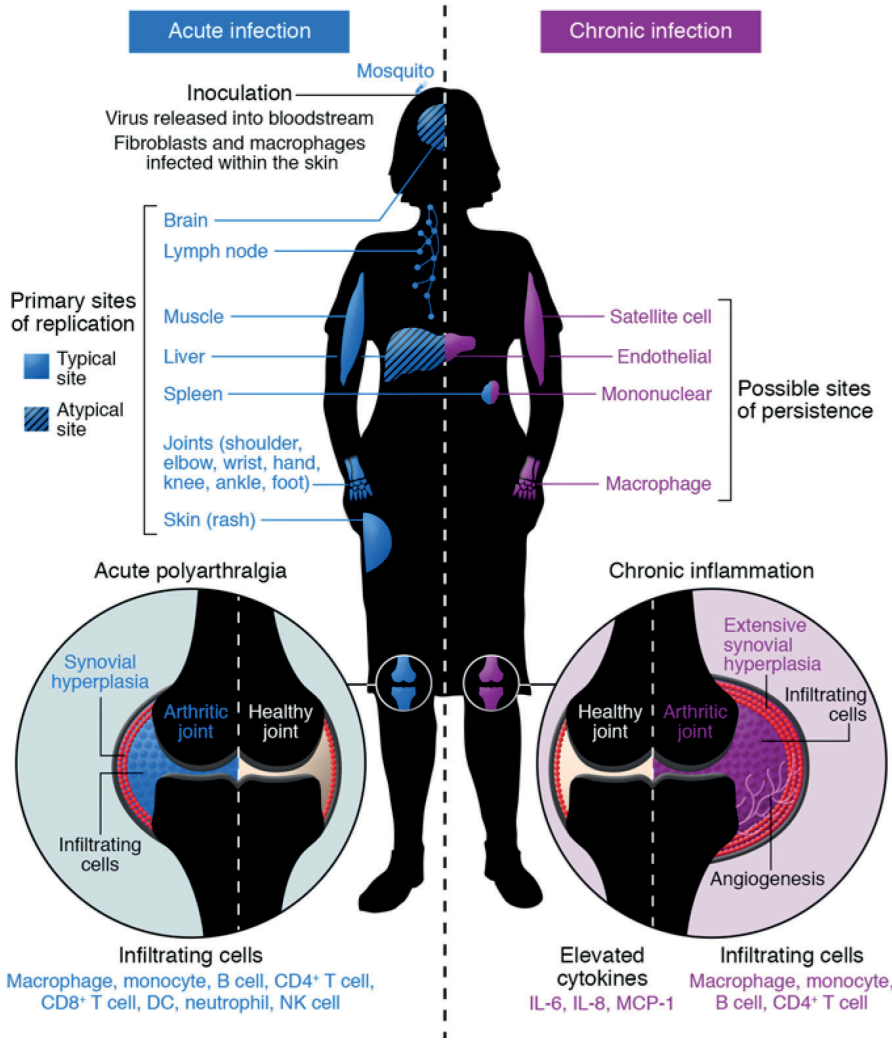

Figure 6. Representation of acute and chronic chikungunya fever symptoms with underlying events (with permission from [23]).

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.

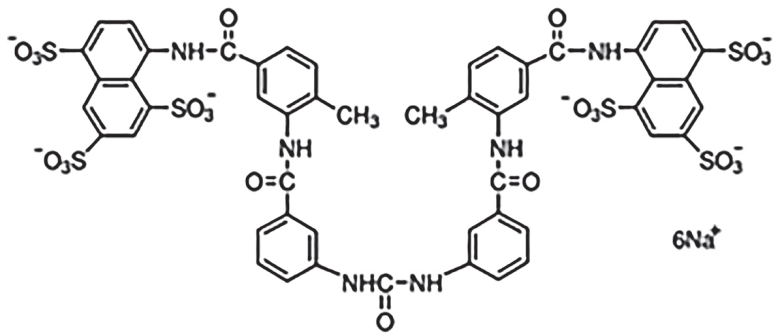

Figure 7. The chemical structure of suramin hexasodium. The full IUPAC nomenclature is available at PubChem (https://pubchem.ncbi.nlm.nih.gov/).

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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Chapter 2

An *in vitro* assay to study chikungunya virus RNA synthesis and the mode of action of inhibitors

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Summary

Chikungunya virus (CHIKV) is a re-emerging mosquito-borne alphavirus that causes severe persistent arthralgia. To better understand the molecular details of CHIKV RNA synthesis and the mode of action of inhibitors, we have developed an *in vitro* assay to study CHIKV replication/transcription complexes isolated from infected cells. In this assay ^{32}P-CTP was incorporated into CHIKV genome and subgenomic (sg) RNA, as well as into a ~7.5 kb positive-stranded RNA, termed RNA II. We mapped RNA II, which was also found in CHIKV-infected cells, to the 5'-end of the genome up to the start of the sgRNA promoter region. Most of the RNA-synthesizing activity, negative-stranded RNA and a relatively large proportion of nsP1 and nsP4 were recovered from a crude membrane fraction obtained by pelleting at 15,000 x g. Positive-stranded RNA was mainly found in the cytosolic S15 fraction, suggesting it was released from the membrane-associated RTCs. The newly synthesized RNA was relatively stable and remained protected from cellular nucleases, possibly by encapsidation. A set of compounds that inhibit CHIKV replication in cell culture was tested in the *in vitro* RTC assay. In contrast to 3'dNTPs, which act as chain terminators and were potent inhibitors of RTC activity, ribavirin triphosphate and 6-aza-UTP did not affect the RNA-synthesizing activity *in vitro*. In conclusion, this *in vitro* assay for CHIKV RNA synthesis is a useful tool for mechanistic studies on the CHIKV RTC and mode of action studies on compounds with anti-CHIKV activity.

Introduction

Chikungunya virus (CHIKV) is an alphavirus that is mainly transmitted by *Aedes* mosquitoes and in general causes a severe arthralgia that may persist for months. The virus re-emerged in 2005 in an epidemic form and has since affected millions of people mainly in Asia [1]. Hundreds of infected travelers have arrived in the USA [2] and Europe, which even led to local transmission in Italy in 2007 and France in 2010 [3, 4]. A massive, CHIKV outbreak that started in October 2013 on the Caribbean island of Saint Martin marked [5] the arrival of CHIKV in the Americas [6, 7] and up to July 2014 over 100,000 cases were already reported [8]. These outbreaks illustrate the increasing disease burden of CHIKV, for which there is still no registered vaccine or specific antiviral therapy.

The CHIKV replication cycle involves the synthesis of negative-stranded RNA (-RNA) early in infection, which serves as a template for the subsequent production of the genome and a subgenomic (sg) mRNA. Genome and sgRNA are capped and polyadenylated and serve to express polyproteins comprising the nonstructural proteins (nsPs) 1-4 and structural proteins C, E3, E2, 6K and E1, respectively. CHIKV RNA synthesis presumably takes place on the cytoplasmic side of the plasma membrane and/or modified endosomal membranes, as described for other alphaviruses [5, 9-11].

In vitro activity studies on replication complexes from a variety of positive-stranded RNA (+RNA) viruses, like poliovirus [12] hepatitis C virus [13-16], West Nile virus [17, 18], Dengue virus [19], and SARS-coronavirus [20], have contributed to our understanding of +RNA virus replication. For the alphaviruses Semliki forest virus (SFV) and Sindbis virus (SINV) such *in vitro* assays have been developed as well [21].

We have now established an *in vitro* assay to characterize the activity and composition of CHIKV replication/transcription complexes (RTCs) isolated from infected cells. Besides developing a useful tool to screen for inhibitors of CHIKV RNA synthesis, our study provided more insight into the molecular details of CHIKV RNA synthesis and the mode of action of several inhibitors. Our *in vitro* studies also revealed the synthesis of a previously unrecognized 7.5 kb CHIKV RNA (RNA II) that is collinear with the 5' end of the genome up to the start of the sgRNA promoter region. This RNA species was also found in CHIKV-infected cells and resembles the RNA II that has been observed in SINV-infected cells [22].

Results

Isolation of active RTCs from CHIKV-infected cells

We set out to isolate CHIKV RTCs with the highest possible RNA-synthesizing activity from infected Vero E6 cells. We therefore first analyzed CHIKV RNA synthesis *in vivo* by metabolic labeling with ^{3}H-uridine at various time points post infection. Quantification of ^{3}H-labeled CHIKV RNA following denaturing agarose gel electrophoresis and fluorography (Fig. 1) showed that the rate of RNA synthesis increased up to 8 h p.i., after which a modest

decline was observed. Based on these results, we begun the isolation procedure of CHIKV RTCs at 6 h p.i. to ensure that a good amount of activity would remain after the relatively lengthy procedure (1-2 h).

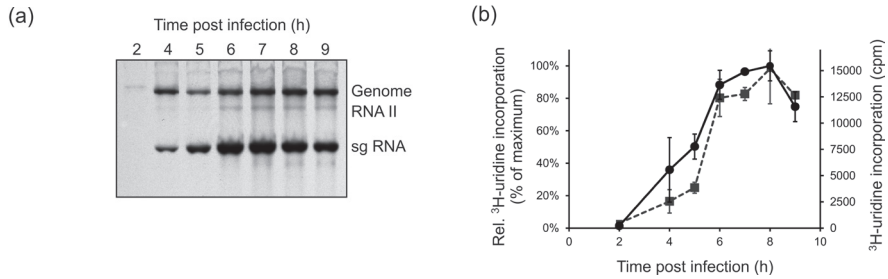

Figure 1. Kinetics of CHIKV RNA synthesis in Vero E6 cells. (a) CHIKV-infected cells were pulse labeled for 1 h with ^{3}H-uridine starting 30 min before the time points indicated above the gel, followed by isolation of RNA, denaturing agarose gel electrophoresis and visualization by fluorography. The positions of genomic and sgRNA (determined by hybridization of CHIKV RNA with a specific probe) and an additional CHIKV-specific ~7.5 kb product (RNA II) are indicated. (b) The rate of CHIKV RNA synthesis (replication and transcription combined) at each time point post infection was quantified by densitometry of the bands shown in panel a (circles) or by direct liquid scintillation counting of isolated RNA (squares). Ribosomal 18S RNA levels were used to correct for variations in isolation. The graph shows ^{3}H-uridine incorporation (relative to the highest observed value) and represents the average ±SD of two experiments.

Approximately 1 x 10^{8} CHIKV-infected Vero E6 cells were harvested by trypsinization at 6 h p.i., followed by homogenization in hypotonic buffer. Nuclei and debris were pelleted and the resulting post-nuclear supernatant (PNS) was fractionated by centrifugation yielding a 15,000 x g pellet (P15) and supernatant (S15; Fig. 2a). The PNS, P15 and S15 fractions were assayed for CHIKV RNA-synthesizing activity by measuring the incorporation of ^{32}P-CTP using the protocol described in the Materials section.

Reactions performed with PNS prepared from CHIKV-infected cells yielded two major ^{32}P-labeled reaction products with sizes corresponding the CHIKV genome and sgRNA (Fig. 2b). RNA II, a minor product running between genome and sgRNA will be discussed below. No radiolabeled RNA was detected when the assay was performed using PNS prepared from mock-infected cells (Fig. 2b). Approximately 58% of the RTC activity present in the PNS could be recovered in the P15 fraction, while 6% remained in the S15 fraction. Combining the P15 and S15 fractions yielded an activity that was comparable to the sum of their individual activities, suggesting that the RTCs in the P15 fraction did not require (host) factors from the S15 fraction for their activity. The ~35% overall activity loss compared to the PNS, was likely due to damage caused by RTC pelleting and resuspending.

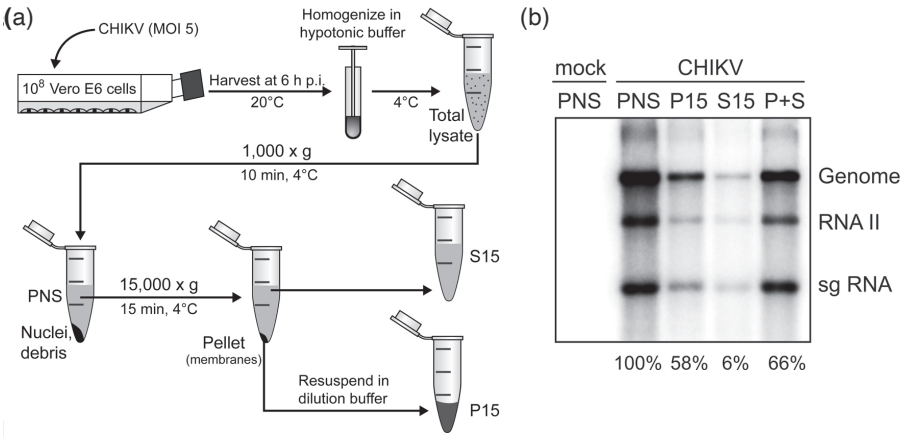

Figure 2. Isolation of active CHIKV RTCs from infected Vero E6 cells. (a) Overview of the isolation procedure. (b) *In vitro* incorporation of ^{32}P-CTP into RNA in PNS prepared from mock-infected cells, or PNS, P15 and S15 fractions obtained from CHIKV-infected cells. RNA was extracted, separated in denaturing agarose gels and visualized using a phosphor imager. The amount of ^{32}P-CTP incorporated into CHIKV genome and sgRNA is indicated under each lane, expressed as % of the radioactivity incorporated by the PNS fraction.

CHIKV RTCs synthesize RNA II in addition to genome and sgRNA

During the analysis of *in vitro* synthesized RNA we noticed that besides products with the size of genome and sgRNA, a ~7.5-kb product was synthesized, which we termed RNA II (Fig. 2b and 3a). Approximately 4% of the incorporated ^{32}P-CTP was present in RNA II, which was only observed under reaction conditions that allowed sgRNA synthesis. RNA II was not an artifact of the *in vitro* system as it was also detected in RNA isolated from CHIKV-infected cells metabolically labeled with ^{3}H-uridine (see Fig. 1a and 3a). At the peak of RNA synthesis *in vivo*, about 2% of the ^{3}H-label was found in RNA II, while 27% and 71% of the radioactivity was found in genome and sgRNA, respectively. Based on its size and the correlation between RNA II and sgRNA synthesis, we hypothesized it represents the 5'-proximal ~7.5 kb of the genome, up to the sgRNA promoter region (starting at nt 7480). We have termed this molecule RNA II, since this name was used for a similar RNA product described for SINV [22]. To map the 3' end of CHIKV RNA II, RNA from CHIKV-infected cells was hybridized with probes that bind to +RNA between nucleotides 4572-4601 (IA1), or immediately upstream (IA2) or ~100 nucleotides downstream (IA3) of the subgenomic promoter region (Fig. 3c). As anticipated, probes IA1 and IA2 hybridized to the genome and RNA II, while probe IA3 recognized sgRNA but not RNA II (Fig. 3b and c). This result confirmed our hypothesis that RNA II corresponds to the 5'-proximal 7.5 kb of the CHIKV genome up to subgenomic promoter region.

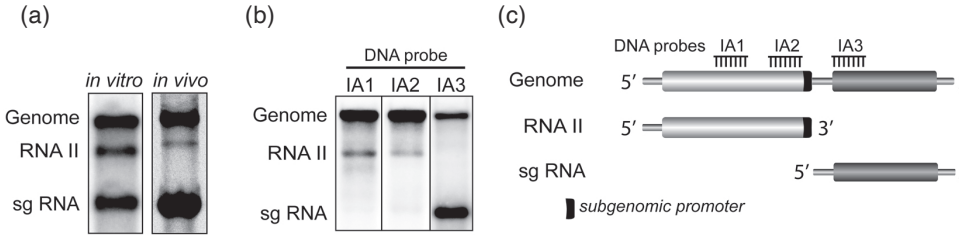

Figure 3. Analysis of the origin of CHIKV RNA II. (a) CHIKV RNA was metabolically labeled with ^{3}H-uridine *in vivo* or with ^{32}P-CTP *in vitro*. The positions of the genome, sgRNA and the minor ~7.5 kb CHIKV-specific product RNA II are indicated. (b) Hybridization analysis of CHIKV RNA using probes that specifically bind the regions of CHIKV+RNA indicated at (c). (c) Schematic representation of the 3 +RNAs synthesized by CHIKV and the locations of the probes IA1, IA2 and IA3 that were used to map the origin of RNA II.

Optimization of reaction conditions for in vitro CHIKV RNA synthesis

A number of assay parameters were tested to determine the optimal conditions for CHIKV RNA synthesis *in vitro*. Activity remained relatively constant over a range of temperatures from 20 to 37°C (Fig. 4a), probably reflecting that CHIKV replicates at low temperatures in mosquito vectors and at higher temperatures in vertebrate hosts. Synthesis of sgRNA decreased at higher temperatures, while the ^{32}P-incorporation into genomic RNA increased. In further experiments, 30°C was used as the standard assay temperature, as this allowed the analysis of both genome replication and sgRNA synthesis. Magnesium was required for RTC activity and a concentration of 3 mM was optimal (Fig. 4b). An ATP-regenerating system was essential as no radiolabeled products were observed when creatine phosphate and creatine phosphokinase were omitted (data not shown). The incorporation of ^{32}P-CTP into CHIKV RNA was readily detectable after a 5-min incubation at 30°C, and the signal rapidly increased up to 90 min, after which it hardly increased further (Fig. 4c and e). Addition of pyrophosphatase did not increase the yield suggesting that the decreasing activity was not due to inhibition by the pyrophosphate that is released during NTP incorporation (data not shown). The addition of fresh NTPs, creatine phosphate and creatine phosphokinase after a 90-min reaction, followed by an additional 60-min incubation led to an almost 3-fold increase in sgRNA labeling compared to a reaction that was incubated for an additional 60 min without replenishing components (Fig. 4d). The incorporation of radioactivity into genomic RNA was only 1.2-fold higher. This suggests that the reaction rate dropped after 90 min due to exhaustion of one or more reaction components, like NTPs, and not due to RTC instability. Indeed, RTCs retained most of their activity when they were first kept at 30°C for 1 h before a reaction was started (data not shown). To assess the stability of the *in vitro*-synthesized RNAs, a reaction was terminated after 90 min by adding the obligate chain terminator 3'dUTP, after which RNA samples were taken every 20 min during a 60-min chase period. In untreated control samples the amount of radioactive CHIKV RNA increased ~1.2 fold over the chase period (Fig. 4f, squares). The level of radioactive CHIKV RNA in samples in which RNA synthesis was blocked slowly decreased (half-life >60 min)

over the chase period (Fig. 4f, circles). The half-life of a (naked) control RNA was less than 5 min (Fig. 4f, triangles), suggesting that the newly synthesized CHIKV RNA was somehow protected from cellular nucleases.

While optimizing the NTP concentration, we discovered that even in a reaction to which no CTP, GTP and UTP was added, a substantial amount of mainly genomic RNA was synthesized (Fig. 3g). This was likely due to the pool of endogenous NTPs in PNS [23]. Addition of 10 μM of NTPs to the reaction hardly had an effect, probably because the endogenous NTP concentration was already at least 10-fold higher. Addition of 1 mM of ATP increased the reaction rate and resulted especially in increased sgRNA transcription and generation of RNA II. Reactions performed with the P15 fraction (not expected to contain endogenous NTPs) were strongly dependent on the supplied NTPs, as no ^{32}P-CTP incorporation was observed in the absence of NTPs (Fig. 3g). A final concentration of 10 μM CTP, UTP and GTP and 1 mM of ATP was optimal. Higher NTP concentrations did not substantially increase the activity (not shown).

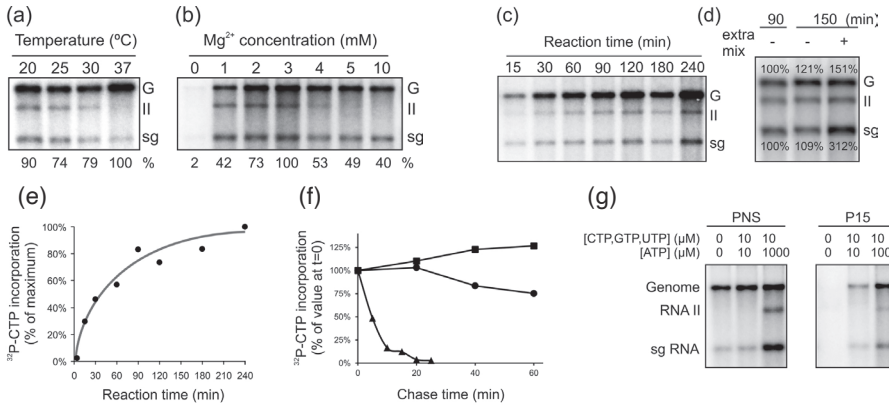

Figure 4. Optimization of reaction conditions for the *in vitro* RNA-synthesizing activity of CHIKV RTCs. The temperature (a), Mg^{2+} concentration (b) or incubation time (c) were varied and ^{32}P-CTP-labeled RNA products were analyzed (see Fig. 1). The percentages depicted under the lanes indicate ^{32}P incorporation normalized to the highest observed activity in a given series (100%), after correction for variations in RNA recovery based on quantification of 18S rRNA (not shown). (d) After a 90-min standard reaction (left lane) fresh NTPs, creatine phosphate and creatine phosphokinase (+) or an equal volume of dilution buffer (-) were added and the reaction was continued for an additional hour. (e) Kinetics of the incorporation of ^{32}P-CTP into CHIKV genomic and sgRNA. (e) Stability of newly synthesized CHIKV RNA in PNS. The incorporation of ^{32}P-CTP into CHIKV RNA was allowed to proceed for 90 min, after which it was blocked by the addition of 0.1 mM 3'dUTP, followed by the quantification of the remaining radioactive CHIKV RNA at the indicated time points of the chase (circles). A reaction that was chased in the absence of 3'dUTP was included as a control (squares). The triangles show the decay of a *in vitro* transcribed control RNA that was incubated with PNS. (f) Assays performed with either PNS or the P15 fraction in the presence of various concentrations of (added) ATP and the other NTPs.

CHIKV RTCs incorporate ^{32}P-CTP into single-stranded +RNA

We next assessed whether *in vitro* synthesized radioactive CHIKV RNAs resulted from genuine RdRp-mediated incorporation of ^{32}P-label or whether they were merely the result of end-labeling of existing RNA molecules by a viral or host cell activity. This was done by hybridizing a small DNA probe at a specific position in the viral RNA, ~2.2 kb from the 3' end of both genome and sgRNA, and subsequently cleaving the DNA:RNA duplex using RNase H (Fig. 5a). The fact that, after this targeted cleavage, the ~9.6-kb 5'-terminal genome fragment was radioactively labeled confirmed that ^{32}P-CTP had been incorporated at other positions than merely the 3' end of the CHIKV RNAs (Fig. 5b). A radioactive fragment corresponding to the predicted ~2.1 kb 5' fragment of the sgRNA was also observed, although migrating very close to the ~2.2 kb 3'-terminal fragment. Treatment of ^{32}P-labeled reaction products with single strand-specific RNAse A/T1 resulted in the degradation of more than 90% of the *in vitro* synthesized RNA, along with the 18S RNA that was used as an internal control (Fig. 5c). Treatment with double-stranded RNA-specific RNase III led to a 10-20% decrease of radioactive CHIKV RNA, suggesting that radioactivity had mainly accumulated in single-stranded RNA.

To determine the polarity of the newly synthesized RNA, radioactive reaction products were hybridized to a membrane containing specific capture probes for positive- and negative-stranded CHIKV RNA and a non-specific control RNA. Figure 5d shows that mainly +RNA was synthesized *in vitro*.

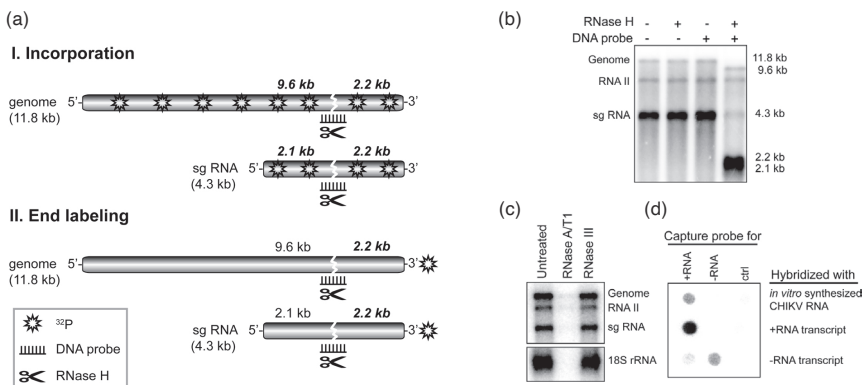

Figure 5. Characterization of *in vitro* synthesized CHIKV RNAs. (a) Schematic representation of the two possible mechanisms that could lead to generation of ^{32}P-labeled CHIKV RNAs and the targeted RNA cleavage assay with DNA probe and RNase H that was performed to distinguish between the two. Genuine RdRp activity would incorporate ^{32}P-CTP throughout newly synthesized RNA and would result in 4 radioactive fragments (sizes in bold italic) after cleavage. If RNAs would only be labeled at their 3' end, due to terminal transferase activity, only the 2.2 kb 3'-terminal fragment of genome and sgRNA would be radioactively labeled. (b) Result of the targeted cleavage assay using reaction products from an *in vitro* RTC assay. RNA II was not cleaved since it was not recognized by the DNA probe. (c) Treatment of *in vitro* synthesized CHIKV RNA with single strand-specific RNase A/T1 or the double-stranded RNA-specific RNase III, followed by denaturing agarose gel electrophoresis and direct detection of ^{32}P-labeled products by phosphor imaging. Ribosomal 18S RNA, was included as a control. (d) Binding of radioactive *in vitro* synthesized CHIKV RNA to a membrane with capture probes specific for CHIKV RNA of positive or negative polarity or a non-specific control probe.

Distribution of CHIKV RNA and proteins between the P15 and S15 fraction

To obtain more insight into the composition of the P15 fraction that contains most of the RTC activity (Fig. 2b), the distribution of CHIKV RNA, viral proteins and several cellular marker proteins between P15 and S15 was studied.

Approximately 90% of -RNA was found in the P15 fraction, where it likely serves as template for +RNA synthesis (Fig. 6a). In contrast, the P15 fraction contained about 6 times less +RNA than the cytoplasmic S15 fraction (Fig. 6a).

The P15 fraction contained 20% or 16% of the total amount of nsP2 and nsP3, respectively. In contrast, 30% of nsP4 and 50% of the nsP1 were found in P15 (Fig. 6b). The E2 envelope protein was enriched in the membrane fraction (P15), which also contained about 15% of the capsid protein (Fig 6b). The absence of the nuclear marker fibrillarin indicated that the P15 and S15 fraction (and PNS) were not notably contaminated with nuclear material. The P15 fraction did not contain detectable amounts of the cytosolic marker cyclophilin A or actin, while it contained most of the endoplasmic reticulum marker cyclophilin B and the bulk of the plasma membrane marker Na^+/K^+ ATPase (Fig. 6b, right panel). The early endosome marker, Rab5, was predominantly found in the S15 fraction, whereas Rab7, a late endosome marker, was present both in P15 and S15. These results indicated that P15 was a rather crude fraction that contained membranes of various cellular origins.

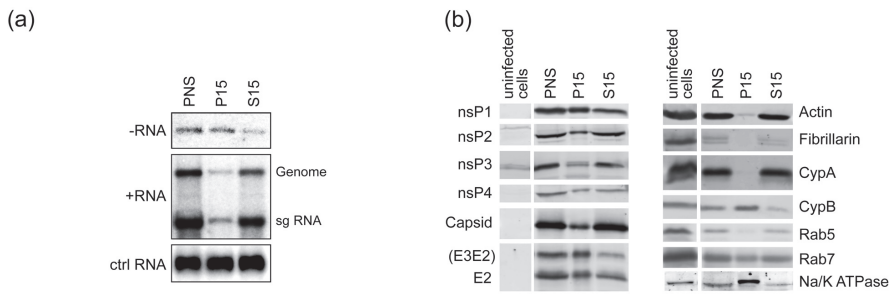

Figure 6. Distribution of CHIKV RNA and viral and cellular marker proteins between the P15 and S15 fractions. (a). Total RNA was isolated from PNS, P15 and S15 fractions (volumes reflecting equal cell numbers) obtained from CHIKV-infected Vero E6 cells. After denaturing agarose gel electrophoresis, -RNA and +RNA were specifically detected by hybridization with ^{32}P-labeled DNA probes. Samples were spiked with a control RNA to correct for variations in the recovery and/or loading of RNA. (b) Western blot analysis of samples reflecting equal cell numbers of PNS, P15, and S15 of CHIKV-infected cells or a whole cell lysate of uninfected cells using antibodies specific for the protein indicated next to each panel.

The in vitro RTC assay as a tool for mode of action studies on inhibitors of CHIKV replication

To determine whether our *in vitro* assay for CHIKV RNA synthesis is a suitable tool for mode of action studies on inhibitors of CHIKV replication, we have studied the effect of a variety of compounds. We first tested a set of compounds that inhibit CHIKV replication in cell culture [24], but were not expected to directly inhibit RNA synthesis. These compounds either affect another step in the CHIKV replication cycle (chloroquine, 3-deaza-adenosine), act as antimetabolite (mycophenolic acid, ribavirin, 6-aza-uridine) and/or need to be first

converted by cellular enzymes to their active form (ribavirin, 6-aza-uridine). Indeed, none of these compounds had a measurable inhibitory effect in the *in vitro* assay for CHIKV RNA synthesis (Fig. 7a).

A set of nucleoside analogs that act as obligate chain terminators strongly inhibited the synthesis of CHIKV RNA in the *in vitro* assay in a dose-dependent manner (Fig. 7b). The weaker inhibitory effect of 3'dATP compared to that of the other 3'dNTPs was likely due to an excess of ATP in the reaction (1 mM supplied and also produced by the ATP-regenerating system). In line with this notion, the inhibitory effect of 3'dUTP, which was already very strong at a concentration of 10 μM (Fig 7b, lane 7) could be reversed by adding an excess of UTP. For example a 20-fold molar excess of UTP over 50 μM 3'dUTP restored the RNA-synthesizing activity to a level comparable to that observed in the presence of 1 μM of the inhibitor (Fig. 7b, compare lanes 6 and 11). Finally, we analyzed whether 500 μM of ribavirin triphosphate or 100 μM 6-aza-UTP had an effect on the kinetics of CHIKV RNA synthesis *in vitro*. Figure 7c shows that the kinetics of RNA synthesis in the presence of these compounds was indistinguishable from that of the control reaction, suggesting these compounds do not (directly) affect the reaction rate.

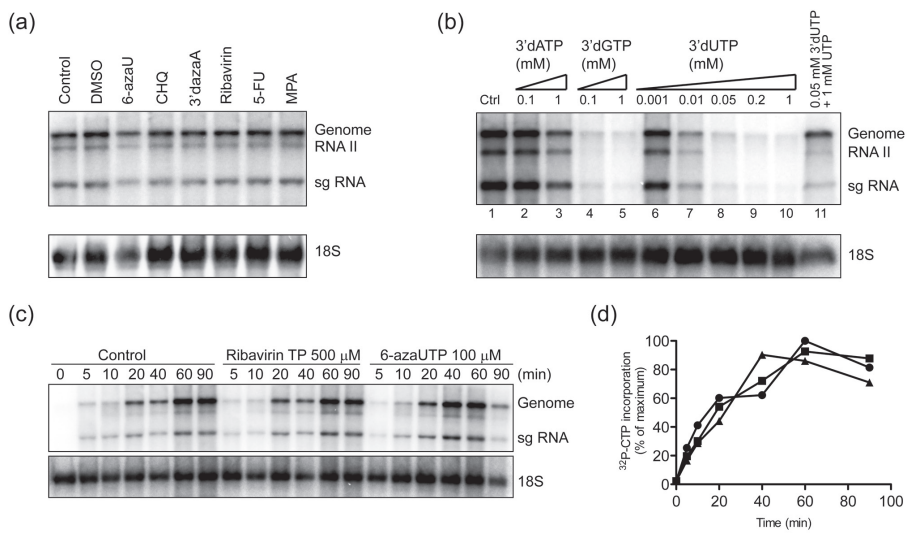

Figure 7. Effect of various compounds on the *in vitro* RNA synthesizing activity of CHIKV RTCs. (a) Standard assays were performed in the presence of 0.5% DMSO, 50 μM 6-azauridine, 100 μM chloroquine, 60 μM 3'-deaza-adenosine, 150 μM ribavirin, 500 μM fluorouracil, or 6 μM mycophenolic acid. (b) Standard reactions performed in the presence of the obligate chain terminators 3'dATP, 3'dGTP and 3'dUTP at the concentrations indicated above the lanes. The rightmost lane contains RNA synthesized in the presence of 50 μM 3'dUTP and 1 mM UTP. (c) Reaction kinetics of *in vitro* RNA synthesis in the presence of ribavirin triphosphate or 6-azaUTP or in the absence of inhibitor (control) analyzed over a time course of 5-90 min. (d) Quantified *in vitro* ^{32}P-CTP incorporation into CHIKV RNA in the presence of ribavirin triphosphate (squares) or 6-aza-UTP (triangles) represented as % of the maximum incorporation in the control reaction (circles).

Discussion

We have successfully developed an *in vitro* system to study CHIKV RNA synthesis, in which ^{32}P-CTP is incorporated into CHIKV genome and sgRNA. Mainly single-stranded +RNA accumulated, which was not unexpected considering lysates were prepared at 6 h p.i., when -RNA synthesis has ceased and RTCs are exclusively involved in +RNA synthesis [24].

A crude membrane fraction (P15) had an approximately 10-fold higher RNA synthesizing activity than the cytosolic S15 fraction. It remains to be determined whether the low activity in S15 represents a pool of structurally and functionally distinct RTCs or whether the activity is merely due to RTCs that were released (dissociated) from the pool of P15-associated complexes. We are currently developing more elaborate subcellular fractionation procedures to study these possibilities in detail. Our fractionation data showed that the pelleted CHIKV RTCs were not dependent on cytosolic host factors for their activity, in contrast to what was found for nidoviruses [20, 25]. This is in line with previous studies on SINV and SFV [21].

The P15 fraction contained most of the -RNA, the template for +RNA synthesis. The bulk of the +RNA was found in the S15 fraction, suggesting it was released from the membrane-associated RTCs. The *in vitro* synthesized CHIKV RNA was relatively stable in the lysate and appeared to be somehow protected from cellular nucleases. This might be due to its structure, membrane association, presence within polysomes, or encapsidation soon after its synthesis (capsid protein was also detected in P15). The S15 fraction contained about 80% of nsP2 and nsP3, proteins that besides their role in the RTC are known to have several other functions, like inducing shut-off of host transcription and translation and interacting with a variety of host proteins. Compared to nsP2 and nsP3, the RdRp nsP4 and especially nsP1 were enriched in the P15 fraction. The latter protein is presumably involved in the anchoring of RTCs to membranes.

We discovered that a previously unrecognized CHIKV RNA, which we termed RNA II, was produced besides genome and sgRNA. RNA II was synthesized both *in vitro* and *in vivo*, and we demonstrated that it represents the first 7.5 kb of the genome.

Earlier metabolic labeling studies already suggested the presence of similar molecules in SINV- and SFV-infected cells [26, 27] and Wielgosz *et al.* [22] have identified and mapped RNA II in SINV-infected cells [22]. RNA II was then believed to be unique for SINV, but we have now also identified it in CHIKV-infected cells. RNA II probably results from premature termination (near the sgRNA promoter) of RNAs initiated on the 3' end of a -RNA template that is also involved in transcription. This is supported by the observation that RNA II was only found under conditions that favored sgRNA synthesis. We are currently investigating whether RNA II is capped and polyadenylated and whether it is merely a by-product of genome replication or has a function in the infected cell.

We have tested several compounds in the *in vitro* assay for CHIKV RNA synthesis to demonstrate it is a useful addition to the toolbox for mode of action studies. As expected chloroquine showed no effect, because *in vivo* it blocks CHIKV entry. Ribavirin and 6-azauridine had no effect as their conversion to an active (triphosphorylated) form by cellular enzymes probably does not occur (to a sufficient level) *in vitro*. These compounds may also act as antimetabolites that *in vivo* affect the cellular NTP pool, like 5-fluorouracil and mycophenolic acid, and hence have no effect in the *in vitro* assay, as NTPs are supplied in the reaction buffer. In cell culture 3-deaza-adenosine inhibits CHIKV replication and it was hypothesized that this might be due to its effect on the capping of CHIKV RNA [24]. In line with this, we found that 3-deaza-adenosine had no direct effect on RNA synthesis *in vitro*. The obligate chain terminators 3'dATP, 3'dGTP and 3'dUTP inhibited CHIKV RNA synthesis *in vitro* at low micromolar concentrations. Their inhibitory effect could be reversed by addition of NTPs, which stresses the importance of not supplying a large excess of NTPs in *in vitro* assays. Otherwise the inhibitory effect of (novel) compounds might be underestimated or missed completely. Using the newly developed assay, we found that ribavirin triphosphate and 6-aza-UTP had no measurable effect on the kinetics of CHIKV RNA synthesis. This might indicate that either these compounds were not incorporated by the RTC or they were incorporated without affecting the reaction rate. In addition, these compounds could also indirectly affect RNA synthesis *in vivo* through their effect on cellular NTP pools. Obviously, this would have no effect in the *in vitro* system as NTPs are supplied. This is further supported by the fact that the IMPDH inhibitor mycophenolic acid had no effect *in vitro*, while it inhibited CHIKV in cell culture. Since ribavirin and 6-azauridine increase the viral mutation frequency [28, 29], at least part of their mode of action can probably be attributed to their incorporation into viral RNA.

The in vitro assay described here may be used to enhance our understanding of the molecular details of CHIKV RNA synthesis, to screen for inhibitors, and to study the mode of action of antiviral compounds originating from other (cell-based) screens.

Methods

Cells, virus, antisera and compounds

Vero E6 cells were infected with CHIKV strain LS3 at an MOI of 5 as described [24]. Antisera against CHIKV nsP1, nsP2, nsP3, nsP4, and capsid protein were a generous gift from prof. Andres Merits (Tartu, Estonia). The E2 antiserum [30] was obtained from dr. Gorben Pijlman (Wageningen, The Netherlands). Antisera against cellular markers were purchased from Sigma (actin), Santa Cruz (fibrillarin, cyclophilins A&B), and Cell Signalling (Rab5, Rab7 and Na/K ATPase). Ribavirin triphosphate, 3'dATP, 3'dGTP and 3'dUTP were from TriLink BioTechnologies and 6-aza-UTP from Jena Bioscience. Stock solutions of ribavirin, 6-azauridine, 5-fluorouracil, 3'-deaza-adenosine, chloroquine and mycophenolic acid were prepared as described [24].

RNA isolation, denaturing agarose gel electrophoresis, in gel hybridization and detection of ^{32}P- and ^{3}H-labeled RNA

RNA isolation [25] and denaturing formaldehyde agarose gel electrophoresis were performed as described [24]. For detection of ^{32}P-labeled RNA Phosphor Imager screens were directly exposed to dried gels, followed by scanning with a Typhoon 9410 imager (GE Healthcare). CHIKV -RNA and +RNA were specifically detected by in-gel hybridization with ^{32}P-labeled probe Hyb2 and Hyb4 [24]. Probes IA1 (5'-CAATATCGCAGTCTA-TGGAGATGTGCTCAT-3'), IA2 (5'-GTTATGACG-GGTCCTCTGAGCTTCTCGA-3') and IA3 (5'- CCTCCTATT-GTAAAAAGTTT-GGGTTGGG-ATG-3') are complementary to nt 4572-4601, 7452-7479, and 7575-7605 of the genome, resp. The 18S ribosomal RNA, detected with probe 5'-ATGCCCCCGGCCGTCCCTCT-3', was used as isolation efficiency and loading control for RNA isolated from cells or PNS. P15 and S15 fractions were spiked with an *in vitro* transcribed RNA containing the 3' end of the CHIKV genome that was detected with probe Hyb4, to correct for variations in isolation and/or loading.

Metabolic labeling of CHIKV-infected cells

Metabolic labeling of CHIKV-infected cells with 40 µCi ^{3}H-uridine was done basically as described [24] except that we used Vero E6 cells seeded in 12-well clusters at a density of $2x10^5$ cells/well and infected at an MOI of 5.

Isolation of RTCs from CHIKV-infected cells

Approximately 1 x 10^8 CHIKV-infected VeroE6 cells were harvested by trypsinization at 6 h p.i. Cells were resuspended in 4 ml of hypotonic buffer containing 20 mM HEPES pH 7.5, 10 mM KCl, 1 mM DTT, 0.2 U/µl RiboLock (Thermo Scientific), and 2 µg/ml ActD. After incubation at 4°C for 15 min, the cells were disrupted using a Dounce homogenizer. To increase the osmotic value sucrose was added to a final concentration of 250 mM. HEPES and DTT were also added to a final concentration of 35 mM and 2.5 mM, respectively. The lysate was cleared by centrifugation at 1,000 x g for 10 min to remove unlysed cells, nuclei and cellular debris, yielding a post-nuclear supernatant (PNS). Part of the PNS was further separated by centrifugation at 15,000 x g for 15 min into a heavy membrane pellet fraction (P15) and a supernatant fraction S15. The P15 pellet was resuspended in dilution buffer (35 mM HEPES pH 7.5, 250 mM Sucrose, 2.7 mM DTT, 7 mM KCl, 2 µg/ml ActD, and 0.2 U/µl RiboLock) using 1/5 of the volume of the PNS from which the pellet originated (now S15). This 5x concentrated P15 fraction and all other fractions were aliquoted and stored at -80°C.

In vitro RNA synthesis assay

These assays were performed inside the BSL-3 facility, since infectious CHIKV remained present in the lysates and fractions used. Standard 30-µl reactions contained either 25 µl PNS, or 25 µl S15, or 5 µl P15 (5x concentrated) mixed with 20 µl dilution buffer, 30 mM

HEPES (pH 7.5), 220 mM sucrose, 7 mM KCl, 2.5 mM DTT, 3 mM magnesium acetate, 2 µg/ml ActD, 0.2 U/µl of RiboLock, 20 mM creatine phosphate, 10 U/ml creatine phosphokinase, 10 µCi (0.12 µM) of [α-^{32}P]-CTP (Perkin Elmer), 1 mM ATP, and either 0.01 or 0.2 mM of GTP, UTP, and CTP. Unless otherwise stated, reactions were performed for 60 min at 30°C and terminated by the addition of 60 µl of 5% lithium dodecyl sulfate, 0.1 M Tris-HCl pH 8.0, 0.5 M LiCl, 10 mM EDTA, 5 mM DTT, and 0.1 mg/ml proteinase K. After a 15-min incubation at 42 °C, unincorporated label was removed using RNase-free Micro Bio-spin 30 columns (Bio-Rad), and total RNA was isolated and analyzed as described above.

Detection of ^{32}P-labeled reaction products with capture probes

In vitro transcribed RNAs (1 µg) representing the first 1348 nucleotides of the CHIKV negative strand, the 1100 3'-terminal nt of the CHIKV genome, or nt 1-2042 of the unrelated equine arteritis virus (negative control), were immobilized to Hybond N+ membrane (GE Healthcare) as described [20]. The membrane with the immobilized probes was incubated with half of the ^{32}P-labeled RNA isolated from a 30-µl *in vitro* reaction and washing and detection of bound material were done as described [20]. ^{32}P-labeled *in vitro* transcript that contained 5' and 3' UTR of CHIKV or a transcript with the complementary sequence were used as positive controls and to assess the specificity of the immobilized probes.

RNase treatments

RNA isolated from *in vitro* RTC reactions was treated with a RNase A/T1 mixture under high salt conditions to degrade single-stranded RNA, or with RNase III to degrade double-stranded RNA as described [25].

Targeted RNA cleavage assay

For targeted cleavage, RNA isolated from *in vitro* reactions was heated to 96°C for 4 min, followed by hybridization overnight at 45°C in 20 µl of 40 mM PIPES pH 6.5, 0.4 M NaCl, 1mM EDTA, and 80% deionized formamide with DNA probe AS25 (5'-GATAACTGCGGCCAATACTTAT-3') that is complementary to nt 9548 – 9569 of the CHIKV genome. RNase H (7.5 U; Fermentas) in a volume of 150 µl 10 mM PIPES pH 6.5, 0.1 M NaCl, 0.25 mM EDTA, and 10 mM DTT was added to the hybridized RNA. After an incubation for 60 min at 37°C, 225 µl of RNase inactivation solution (Ambion RPA kit) was added, followed by incubation for 30 min at -20°C. This treatment results in the targeted cleavage of a 2,242 nucleotide fragment of the 3' end of genomic and sgRNA, The digested RNA was precipitated in the presence of GlycoBlue (Ambion) at 4°C by centrifugation for 30 min at 15,000 x g. After resuspension in 5 µl of 1 mM sodium citrate, RNA was analyzed by denaturing agarose gel electrophoresis.

SDS-PAGE and western blot analysis

SDS-PAGE and Western blotting using a Trans-Blot Turbo instrument and fluorescent detection of antibodies with a Typhoon 9410 scanner were done as described [24].

Acknowledgements

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Chapter 3

Suramin inhibits chikungunya virus replication through multiple mechanisms

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Abstract

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that causes severe and often persistent arthritis. In recent years, millions of people have been infected with this virus for which registered antivirals are still lacking. Using our recently established *in vitro* assay, we discovered that the approved anti-parasitic drug suramin inhibits CHIKV RNA synthesis (IC_{50} of ~5 μM). The compound inhibited replication of various CHIKV isolates in cell culture with an EC_{50} of ~80 μM (CC_{50} >5 mM) and was also active against Sindbis virus and Semliki Forest virus. *In vitro* studies hinted that suramin interferes with (re)initiation of RNA synthesis, whereas time-of-addition studies suggested it to also interfere with a post-attachment early step in infection, possibly entry. CHIKV (nsP4) mutants resistant against favipiravir or ribavirin, which target the viral RNA polymerase, did not exhibit cross-resistance to suramin, suggesting a different mode of action. The assessment of the activity of a variety of suramin-related compounds in cell culture and the *in vitro* assay for RNA synthesis provided more insight into the moieties required for antiviral activity. The antiviral effect of suramin-containing liposomes was also analyzed. Its approved status makes it worthwhile to explore the use of suramin to prevent and/or treat CHIKV infections.

1. Introduction

Chikungunya virus (CHIKV) is a mosquito-borne arthritogenic alphavirus that has infected millions of people since its re-emergence in 2005. In November 2013, CHIKV emerged in the Caribbean [1, 2], starting an outbreak that has thus far resulted in over 1.2 million cases in the Americas (http://www.paho.org/hq/index.php?Itemid=40931).

CHIKV replication occurs in the cytoplasm on modified endosomal membranes and is driven by replication and transcription complexes (RTCs) that contain CHIKV nonstructural proteins (nsP) nsP1-4, of which nsP4 is the RNA-dependent RNA polymerase (RdRp). Early in infection negative-stranded RNA (RNA) complementary to the viral genome is synthesized, which serves as template for the production of genomic and subgenomic RNA (sgRNA). The genome serves as mRNA for the production of nsPs and the sgRNA is translated into the structural proteins that are required for the biogenesis of new virions.

Despite intensified research efforts over the past years and the identification of a variety of compounds with anti-CHIKV activity in preclinical studies [3], there are still no registered drugs on the market for treating CHIKV infections. Suramin is a symmetrical sulfonated naphthylurea compound that was approved for the treatment of parasitic infections in 1921, but its anti-cancer and antiviral potential were discovered only 60 years later (reviewed in [4-6]). It was shown that suramin had anti-reverse transcriptase activity against tumor-inducing viruses [7] and it was actually the first documented HIV reverse transcriptase inhibitor that was tested in human patients [8], but the compound's side effects outweighed the clinical benefit due to the required long term treatment [9]. A later study revealed that suramin's anti-HIV activity *in vivo* was actually due to its inhibitory effect on the interaction between the viral gp120 and the CD4 receptor [10]. Suramin has also been shown to block

the binding or early steps of infection of several DNA and RNA viruses, like herpes simplex virus type-1 [11], cytomegalovirus [12], human hepatitis B virus [13], hepatitis delta virus [14], hepatitis C virus [15], dengue virus [16], several bunyaviruses [17-20], norovirus-like particles [21] and enterovirus 71 [22], for which the antiviral activity of suramin was also confirmed in an animal model [23]. In recent *in vitro* studies suramin was identified as a hepatis C virus and dengue virus helicase inhibitor [24, 25] and also as a norovirus RdRp inhibitor by virtual screening and biochemical assays with purified enzymes [26, 27]. In the present study we assessed the effect of suramin on CHIKV RNA synthesis using our recently established *in vitro* assay that relies on RTCs isolated from infected cells [28]. We found that suramin inhibits both CHIKV RNA synthesis *in vitro* as well as an early step in CHIKV infection of cultured cells. In addition to describing the inhibition of CHIKV replication through two independent mechanisms, we provide more insight into the moieties required for suramin's antiviral activity.

2. Material and Methods

2.1. Cell lines, viruses and virus titration.

Vero E6 and BHK-21 cell culture and infectious clone-derived CHIKV LS3 and strain ITA07-RA1 have been described previously [29]. CHIKV STM35 is an infectious clone-derived virus based on the sequence of a clinical isolate from the island of St. Martin (manuscript in preparation). CHIKV M5 is a reverse-engineered LS3-derived (nsP4) mutant virus that is resistant to favipiravir [30] and CHIKV C483Y is identical to LS3 except for a C483Y mutation in nsP4 that renders it resistant to ribavirin [31]. Sindbis virus (SINV) strain HR and Semliki Forest virus (SFV) strain SFV4 were used. Virus titers were determined by plaque assay on Vero E6 cells as described [29]. All experiments with CHIKV were performed in a Leiden University Medical Center biosafety level 3 facility.

2.2. Compounds

Suramin was from Santa Cruz and Sigma and 3'dUTP from TriLink. Suramin-related compounds were synthesized at the National Tsing Hua University in Taiwan and their synthesis and spectroscopic data will be reported separately (manuscript in preparation). All compounds were dissolved in water. Suramin-containing liposomes were prepared as previously described [32].

2.3. Cytopathic effect (CPE) protection assay

CPE protection assays with Vero E6 cells and the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation kit (Promega) were performed as described [29].

2.4. In vitro RNA synthesis assay

In vitro assays for viral RNA synthesis, based on the incorporation of ^{32}P-CTP into viral RNA, were performed as described [28] using RTCs isolated from VeroE6 cells infected with CHIKV LS3, SINV or SFV4 or BHK-21 cells transfected with CHIKV replicon RNA (see 2.6).

2.5. CHIKV protein and RNA analysis

RNA isolation from infected cells, denaturing agarose gel electrophoresis, detection of ^{32}P-RNA or viral RNA by hybridization with (strand-) specific probes have been described previously [28, 33]. CHIKV genome copy numbers were determined by internally-controlled TaqMan multiplex RT-qPCR as described [34]. Detection of CHIKV proteins by SDS-PAGE and Western blotting was done using procedures and antisera that were described previously [29, 34].

2.6. Transfection of cells with CHIKV replicon RNA

Freshly trypsinized BHK-21 cells were transfected by electroporation using 4 x 10^6 cells in 0.4 mL PBS and 4 μg of *in vitro* transcribed CHIKV replicon RNA [35] per 4 mm cuvette (Bio-Rad). After two pulses with an Eurogentec Easyjet Plus instrument set at 850 V and 25 μF, cells were transferred to T-75 flasks with pre-warmed medium, followed by a 10-h incubation at 37 °C.

2.7 Statistical analysis

Graph-Pad Prism 5.01 was used for EC_{50}, IC_{50} and CC_{50} determination by non-linear regression and for statistical analysis performed with one-way ANOVA with Dunnett's (figure 2C) or Bonferroni's (figure 6B) multiple comparison test.

3. Results and discussion

3.1. Suramin inhibits RNA synthesis of CHIKV and other alphaviruses in vitro

As suramin was previously shown to inhibit the *in vitro* activity of a number of viral polymerases, including that of noroviruses [26, 27], we set out to study its effect on CHIKV RNA synthesis using our recently established *in vitro* assay that is based on the RNA-synthesizing activity of RTCs isolated from CHIKV-infected cells. This assay measures the incorporation of [α]^{32}P-CTP into viral RNA, which was severely impaired by suramin in a dose-dependent manner, with an IC_{50} of approximately 5 μM (Fig. 1A, supplemental figure S1A). Suramin also inhibited the *in vitro* activity of RTCs derived from SINV- (Fig. 1B) or SFV-infected cells (Fig. 1C), suggesting that it is a broad-spectrum inhibitor of alphavirus RNA synthesis. A small fraction of the RNA-synthesizing activity appeared refractory to the inhibitory effect of suramin, as some residual incorporation of ^{32}P-CTP remained even in the presence of 500 μM of the compound.

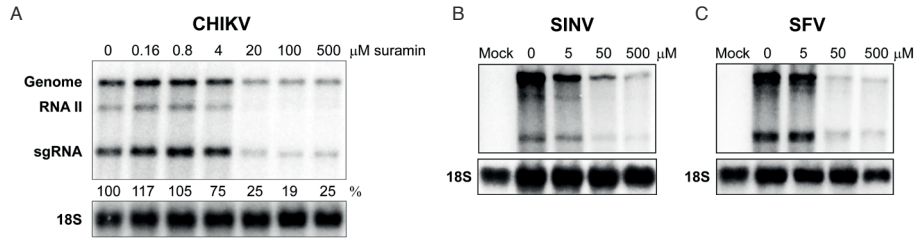


Figure 1. Effect of suramin on alphavirus RNA synthesis *in vitro*.
In vitro RNA synthesis assays with RTCs isolated from Vero E6 cells infected with CHIKV (A), SINV (B) or SFV (C) were performed in the presence of the suramin concentrations indicated above the lanes. RNA was extracted and the ^{32}P-labeled reaction products were analyzed by denaturing agarose gel electrophoresis and phosphor-imaging. A lysate from mock-infected cells was used as a negative control and 18S ribosomal RNA, detected by hybridization, was used as loading control.

3.2. Suramin inhibits the replication of CHIKV and other alphaviruses in cell culture

To determine the antiviral efficacy of suramin in cell culture, Vero E6 cells were infected with different CHIKV strains and treated with serial dilutions of the compound in a CPE protection assay. Viability assays on uninfected cells were performed in parallel to determine the CC_{50}. The EC_{50} values for infectious clone-derived CHIKV LS3, a natural isolate from Italy (ITA07-RA1) and a Caribbean CHIKV strain (STM35) were 75-80 μM (Table 1, supplemental figure S2). The EC_{50} values are ~15 times higher than the IC_{50} values, maybe due to inefficient cellular uptake or poor availability of the compound. Suramin also inhibited the replication of SINV and SFV in cell culture (Table 1). The CC_{50} of suramin was higher than 5 mM, but the compound had a cytotoxic effect at high concentrations, as viability dropped to 65% at 5 mM, the highest concentration of suramin tested. This results in a selectivity index (SI) of >60 for CHIKV and SFV. For comparison, with the same CHIKV strain we found SI values of >32 for ribavirin >7 for T-705. In a plaque reduction assay, in which suramin was only present for 1 h during infection, the concentration that reduced the number of CHIKV plaques by 50% was determined to be 80 μM (data not shown).

Table 1. Antiviral activity of suramin against various alphaviruses in cell culture. EC_{50} values were determined in CPE reduction assays and the average and standard deviation of 2 independent experiments, performed in quadruplicate are listed. (* in an independent experiment with higher concentrations suramin was found to be toxic, but the CC_{50} was still above 5 mM)

Virus	**EC_{50} (μM)**	**CC_{50} (mM)**
CHIKV LS3	79 ± 11.6	> 1*
CHIKV ITA07-RA1	76 ± 7	> 1*
CHIKV STM35	79 ± 12.9	> 1*
SINV	141 ± 18.3	> 0.4*
SFV	40 ± 10	> 0.4*

3.3. Suramin reduces CHIKV RNA and protein levels and infectious progeny titers

A dose-response experiment was performed to analyze the antiviral effect of suramin in a single replication cycle. CHIKV-infected Vero E6 cells were grown in the presence of various suramin concentrations (up to 500 μM) from -6 to 12 h p.i., when they were lysed and analyzed. Suramin reduced the accumulation of nsP1 and capsid protein in a dose-dependent manner, to hardly detectable levels in cells treated with 500 μM suramin (Fig. 2A). The accumulation of CHIKV RNA and positive-stranded RNA (+RNA) was also severely impaired at concentrations of 125 μM suramin or higher (Fig. 2B). The production of infectious CHIKV was strongly inhibited, leading to a 4-log reduction when 500 μM of the compound was present (Fig. 2C). The observed reduction of RNA levels (and consequently +RNA, nonstructural and structural proteins and infectious virus) in this single-cycle analysis suggests that suramin affects an early step in the CHIKV replication cycle.

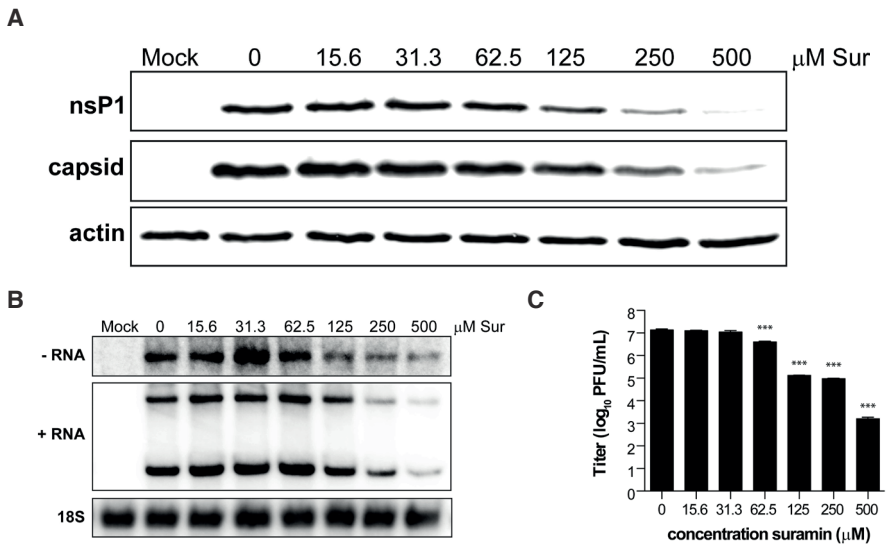


Figure 2. Effect of suramin on CHIKV replication (A) Western blot analysis of nsP1 and capsid protein levels in CHIKV-infected Vero E6 cells (MOI 1) that were treated with suramin at the concentrations indicated above the lanes and analyzed at 12 h p.i. Actin was used as loading control. (B) CHIKV RNA and +RNA were detected in total RNA samples from CHIKV-infected cells treated with suramin at the concentrations indicated above the lanes and analyzed at 12 h p.i. by hybridization with specific probes. 18S ribosomal RNA, detected with a probe was used as loading control. (C) Infectious CHIKV titers at 20 h p.i. in the culture medium of cells treated with various concentrations of suramin were determined by plaque assay. The bars represent the average (± stdev) of two independent experiments with plaque assays performed in duplicate; ***$p < 0.005$.

3.4. Suramin also inhibits an early step of the CHIKV replication cycle

To determine which step of CHIKV replication is inhibited, we performed a time-of-addition experiment in which cells were treated with 500 μM suramin. Suramin was added at 30 or 10 minutes prior to infection or at 0, 5, 10, 20 or 30 minutes after infection, and remained present up to 60 min p.i., when the inoculum was removed, cells were washed 5 times with warm PBS and incubated in medium without suramin (Fig. 3A). In addition,

cells were infected in the absence of suramin, and then treated with 500 μM suramin from 1 - 7 h p.i. (Fig. 3A, sample 8). At 7 h p.i. cells were lysed and CHIKV replication was assessed by analyzing CHIKV –RNA levels (Fig. 3A). When suramin was added very early, not later than 20 minutes p.i., it strongly reduced CHIKV replication, as indicated by the ~85 -75% reduction of RNA levels compared to those in untreated infected cells. Addition of suramin later than 30 min p.i., and even treatment from 1 - 7 h p.i., was much less effective, leading to a reduction in RNA levels of only ~20% (Fig. 3A, samples 7 & 8). These results suggest that -besides its effect on RNA synthesis- suramin also inhibits an early step of the CHIKV replicative cycle, possibly attachment or entry.

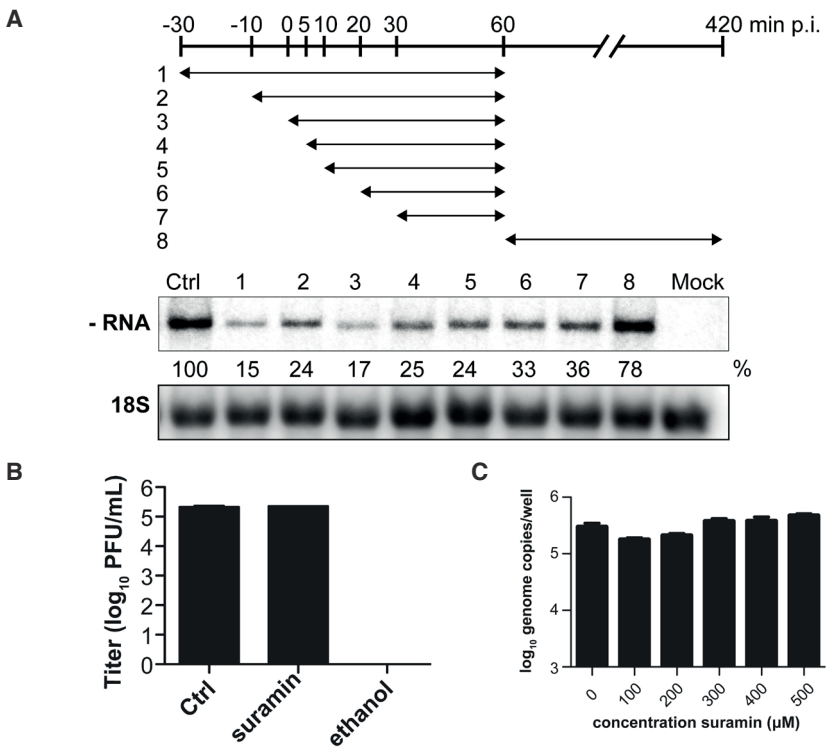


Figure 3. Effect of suramin on early steps of the CHIKV replication cycle (A) Vero E6 cells were infected with CHIKV (MOI 5) and were left untreated (Ctrl) or were treated with 500 μM suramin during the intervals schematically indicated for each sample. At 60 min p.i., cells were washed extensively and incubated in medium without suramin (sample 1-7) or with 500 μM suramin (sample 8) for an additional 6 h. At 7 h p.i. CHIKV –RNA levels were determined by hybridization with a specific probe and the normalized quantities (% of untreated control) corrected for variations in loading, based on the 18S rRNA signal, are indicated under the lanes. (B) 10^5 PFU of CHIKV were incubated for 30 min in medium without (Ctrl) or with 500 μM suramin or with 70% ethanol, followed by determination of the infectious virus titer by plaque assay. (C) CHIKV (MOI 5) was allowed to bind for 30 min at 4 °C to confluent monolayers of Vero E6 cells in 12-well clusters in the presence of various high concentrations of suramin. After extensive washing with ice-cold PBS, the amount of bound CHIKV was determined by measuring the number of genome copies per well using an internally controlled multiplex RT-qPCR.

To test whether suramin has a negative (virucidal) effect on the infectivity of virions, 10^5 PFU of CHIKV were incubated with 500 μM suramin or 70% ethanol (positive control for virucidal activity) for 30 min and the remaining infectivity was analyzed by plaque assay. Compared to the untreated control (Ctrl), suramin treatment did not reduce the infectious titer, while ethanol completely abolished infectivity (Fig. 3B). This demonstrated that suramin is not virucidal and has no irreversible negative effects on the virus. To assess whether suramin blocks attachment of CHIKV to cells, Vero E6 cells were incubated with CHIKV (MOI 5) at 4°C (to block entry by endocytosis) for 30 min in medium with various concentrations of suramin, after which the cells were washed 5 times with ice-cold PBS. The amount of cell-associated virus was quantified by RT-qPCR analysis of total RNA collected immediately after the last washing step (Fig. 3C). Suramin treatment did not affect the amount of bound virus and therefore does not appear to interfere with CHIKV attachment, but likely interferes with a later, post-attachment step such as entry, fusion of the viral envelope with the endosomal membrane and/or the release of nucleocapsids into the cytoplasm.

3.5. Suramin also inhibits CHIKV RNA synthesis in cell culture

To assess whether suramin also inhibits CHIKV RNA synthesis in infected cell culture, we analyzed the kinetics of the accumulation of CHIKV genomic RNA following treatment with various high doses of suramin added 1 h after infection. Figure 4 shows that post-infection treatment changed the kinetics of RNA synthesis in cell culture, leading to ~4-fold reduction in the number of CHIKV genome copies per cell at 7 h p.i.

To validate the effect of suramin on RNA synthesis in cell culture, independent of its effect on entry, we electroporated BHK-21 cells with CHIKV replicon RNA and treated these cells with different concentrations of suramin. Suramin inhibited RNA synthesis and expression of the eGFP reporter gene, which is dependent on transcription of the sgRNA (data not shown), suggesting that besides its effect on entry, suramin also inhibits RNA synthesis in cell culture. However, the latter effect appeared to be much weaker compared to the impact on the early step (entry), which might be explained by poor uptake or intracellular availability of suramin.

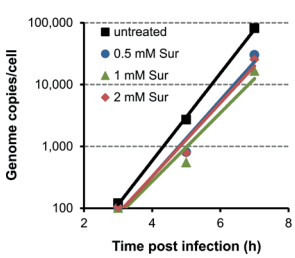


Figure 4. Effect of suramin on the kinetics of CHIKV RNA accumulation in cell culture.
Vero E6 cells were infected with CHIKV (MOI 3) and at 1 h p.i. the inoculum was removed, cells were washed extensively with warm PBS, followed by incubation in medium with 0, 0.5, 1 or 2 mM suramin. Intracellular RNA was isolated at 3, 5 and 7 h p.i. and the CHIKV genome copy numbers per cell were determined by RT-qPCR.

3.6. Mutations that confer resistance to favipiravir or ribavirin do not provide cross-resistance to suramin.

We determined the suramin sensitivity of nsP4 mutant CHIKV M5, which is resistant to favipiravir [30], and of CHIKV C483Y, which has a C483Y mutation in nsP4 rendering it resistant to ribavirin [31] (supplemental figure S3A). CHIKV M5 and C483Y exhibited an ~2-fold resistance to favipiravir (supplemental figure S3B) and ribavirin (supplemental figure S3C), respectively. In a CPE protection assay with suramin and using BHK-21 cells (to allow parallel experiments with ribavirin, which does not work in Vero E6 cells) EC_{50} values of 27 and 48 μM were found for CHIKV M5 and CHIKV C483Y, respectively, compared to 41 μM for the parental CHIKV LS3. In a CPE protection assay with Vero E6 cells EC_{50} values of 72 and 61 μM were found for CHIKV M5 and CHIKV C483Y, respectively, suggesting that these mutants are equally or even somewhat more sensitive to suramin than the parental CHIKV LS3 (EC_{50} 79 μM). The effect of suramin in the CPE protection assay is likely mainly due to its inhibition of the early step of CHIKV replication. Therefore, we also analyzed more specifically the effect of suramin on the kinetics of CHIKV RNA accumulation for wt CHIKV LS3 and the favipiravir- and ribavirin-resistant mutants (Fig. 5). As for the wt virus, the RNA synthesis of both mutants was inhibited by suramin . The lack of cross-resistance suggests that suramin acts on RNA synthesis (RdRp) through a different mechanism.

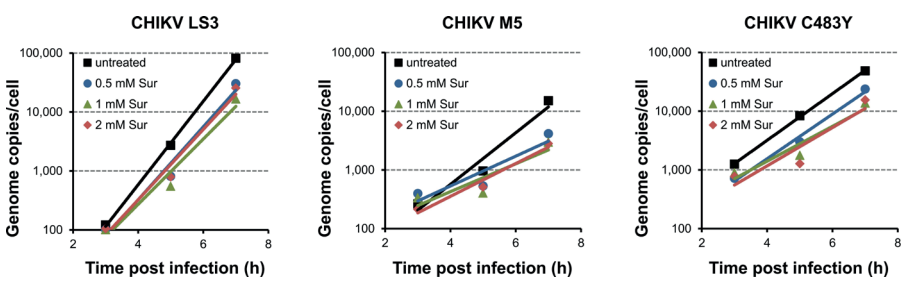


Figure 5. Effect of suramin on the kinetics of CHIKV RNA accumulation of wt CHIKV and two mutants that are resistant to ribavirin and favipiravir. Vero E6 cells were infected with CHIKV LS3, CHIKV M5 or CHIKV C483Y at an MOI of 3 and at 1 h p.i. the inoculum was removed, cells were extensively washed with warm PBS, followed by incubation in medium with 0, 0.5, 1 or 2 mM suramin. Intracellular RNA was isolated at 3, 5 and 7 h p.i. and the CHIKV genome copy numbers per cell were determined by RT-qPCR.

3.7. Suramin appears to inhibit (re)initiation of CHIKV RNA synthesis

To gain more insight into the mechanism by which suramin inhibits CHIKV RNA synthesis, *in vitro* assays with RTCs isolated from CHIKV replicon-transfected cells were employed. Also in this biosafe system, suramin inhibited RNA synthesis, with an IC_{50} of 6.7 μM (Fig. 6A, supplemental figure 1B). The inhibitory effect of nucleoside analogs can be reversed by

adding an excess of NTPs [28], as can be seen for 3'dUTP in Fig. 6B. The inhibitory effect of suramin could not be reversed by an excess of NTPs, suggesting the compound does not compete with NTPs.

As can be seen in figures 1 and 6B, even at very high suramin doses some incorporation of ^{32}P-CTP into viral RNA remained. We hypothesized that this might be caused by complexes already involved in RNA synthesis (interacting with the template) that are insensitive to suramin, which would then mainly inhibit (re)initiating RTCs. To test our hypothesis, we allowed reactions to proceed for 15 min in the absence of ^{32}P-CTP (so products will not be detected), and in the presence or absence of 500 μM of suramin or the nucleoside analog 3'dUTP as a control. After 15 min, ^{32}P-CTP was added and the reactions were allowed to proceed for 60 min (Fig. 6C, condition 1). Under this condition suramin completely blocked the synthesis of radiolabeled RNA, suggesting it was able to inhibit (re)initiating RdRps during the first 15 min of the reaction, during which the "suramin-resistant RTCs" finished their products that are non-radioactive (and therefore are not detected). Merely preincubating RTCs with suramin for 15 min before starting the *in vitro* reaction did not abolish the residual activity (Fig. 6C, condition 2), demonstrating that the effect (condition 1) is not due to just giving the compound time to access the RTC.

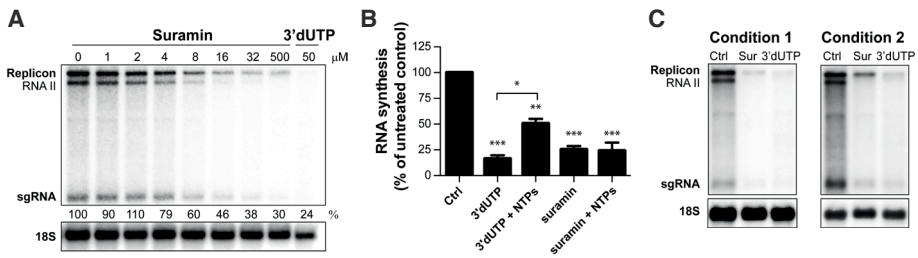


Figure 6. Analysis of the mechanism of inhibition of CHIKV RNA synthesis *in vitro*.
(A) Inhibition of the *in vitro* RNA-synthesizing activity of RTCs isolated from CHIKV replicon-transfected cells by suramin. The nucleoside analog 3'dUTP was used as a control. (B) The inhibitory effect of 50 μM 3'dUTP or 32 μM suramin in a standard *in vitro* reaction and in a reaction supplemented with 200 μM NTPs. Reaction products were quantified and normalized to untreated control reactions (100%). (C) RNA synthesizing activity in a 60-min reaction that followed a 15-min pretreatment with 32 μM suramin or 50 μM 3'dUTP under conditions that sustain (condition 1) or do not sustain (condition 2) RNA synthesis. ^{32}P-CTP was absent during the first 15 min, but was present during the following 60 min. For details see section 3.7. The bars represent the average (± stdev) of two independent experiments; ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$.

3.8. Effect of suramin containing liposomes on CHIKV replication

Due to its charged groups suramin poorly crosses the cell membrane. In an attempt to improve suramin delivery into the cell we tested various cationic liposome formulations containing the negatively charged suramin for their efficacy to inhibit CHIKV replication in CPE protection assays (Table 2, supplemental figure S4). This approach is expected to decrease drug-related toxicity, enhance cellular uptake, and might lead to higher

accumulation in macrophage-rich (CHIKV-infected) tissues when used *in vivo*. Control liposomes without suramin exhibited a relatively high cytotoxicity, while suramin-containing liposomes were less cytotoxic, with CC_{50} values of 50-100 μM. The enhanced toxicity of the control liposomes was previously reported for similar cationic liposomes extensively investigated and employed as non-viral gene delivery systems. It is well known that cationic lipids (i.e. DDAB and DDAC included in the liposomes) are "membrane active" molecules, which interfere with membrane function and affect the integrity of the cell or subcellular compartments, leading to toxicity. The formation of ionic complexes between the positively charged lipids and the negatively charged suramin leads to a reduction of the toxicity of the cationic liposomes. Formulation #PC3-Cl1-sur.2 inhibited CHIKV replication with an EC_{50} of ~62 μM, which is slightly better than suramin dissolved in water (79 μM). The #PC3-Cl1-sur.2 formulation is an interesting starting point for further optimization to improve the efficacy of suramin. The liposome-mediated direct delivery into cells might also help in studying specifically the effect of suramin on viral RNA synthesis, separate from the compound's effect on the early stage of infection.

Table 2. Antiviral and cytotoxic effects of suramin-containing and empty control liposomes, determined by CPE protection assay with Vero E6 cells.

Formulation identification name	PC content (mM)	DDAC content (mM)	DDAB content (mM)	Suramin (mM)	EC_{50} (μM)	CC_{50} (μM)
#PC3-Cl1-sur0	3.0	1.0	-	-	ND	4
#PC3-Br1-sur0	3.0	-	1.0	-	ND	7
#PC3-Cl1-sur.2	3.0	1.0	-	0.2	62	~100
#PC3-Br1-sur.2	3.0	-	1.0	0.2	~ 100	~ 100
#PC9-Cl1-sur0	9.0	1.0	-	-	ND	7
#PC9-Br1-sur0	9.0	-	1.0	-	ND	35
#PC9-Cl1-sur.2	9.0	1.0	-	0.2	ND	~ 50
#PC9-Br1-sur.2	9.0	-	1.0	0.2	ND	~ 50

ND: EC_{50} not determined due to low CC_{50}. PC: phosphatidylcholine, DDAC: distearyldimethylammonium chloride, DDAB: dimethyl-dioctadecylammonium bromide.

3.9. Essential moieties in the structure of suramin

Suramin is a symmetric molecule (Fig. 7A; 1a in Table 3) with in the center a urea (NH–CO–NH) functional group as the "neck". Suramin also contains two benzene rings with amide linkers on each side as the "arms" and possesses two naphthalene rings as the

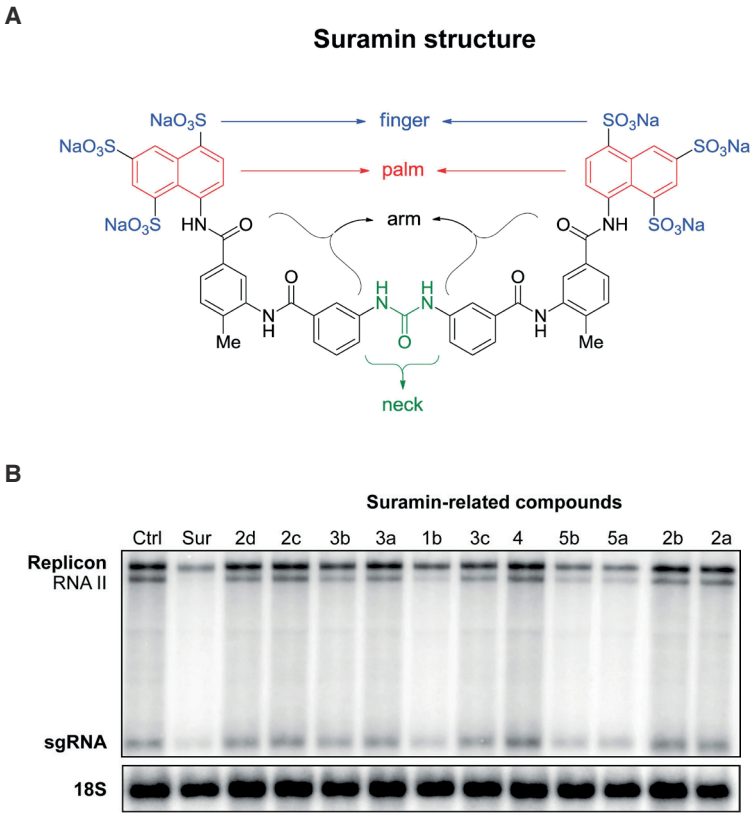
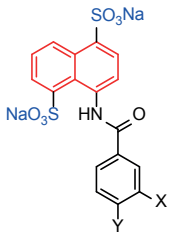
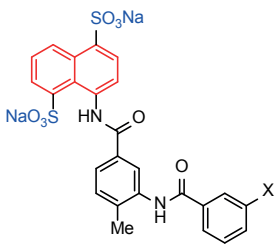
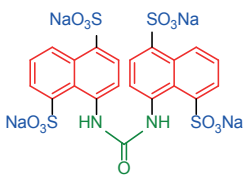
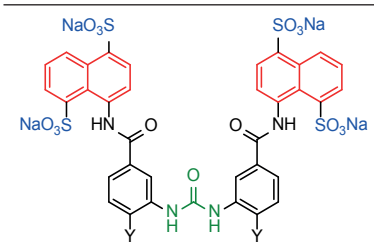
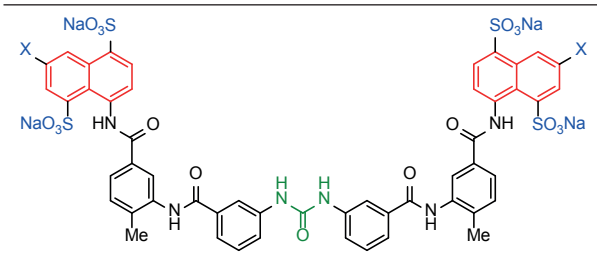


Figure 7. Effect of suramin-related molecules on CHIKV RNA synthesis.
(A) Structure of suramin. (B) Effect of the suramin-related compounds indicated above the lanes (structures are depicted in table 3) on CHIKV RNA synthesis *in vitro*. See legend of Fig. 1 for details.

"palms," and six sulfonate groups as the "fingers". Table 3 lists its structure (1a) and those of ten related compounds (1–5), which have fewer sulfonate fingers, shorter arms, only one side, or no neck in comparison with suramin. These compounds were tested for their ability to inhibit CHIKV RNA synthesis *in vitro* (Fig. 7B; Table 3) and CHIKV replication in cell culture in a CPE protection assay. Examination of the biological activities of compounds 1–5 shown in Table 3 indicates that CHIKV RNA synthesis was inhibited by compounds 1a, 1b, 5a and 5b. Unsymmetrical compounds 2a–d and 3a–c, which had only one arm, were

inactive regardless of its length. Suramin (1a) with six sulfonate groups exhibited greater anti-CHIKV activity (EC_{50} 80 μM) than tetrasulfonate 1b (EC_{50} 200 μM) in cell culture. This preliminary structure-activity analysis gives a first indication of the moieties that are important for suramin's activity and suggest that the two antiviral activities can be separated and optimized independently. The synthesis of additional related compounds is currently in progress and further analysis of structures and biological activities is required to design a rational route for optimization of suramin-like molecules into effective antiviral drugs.

Table 3. Structures of suramin-related compounds and their effect on CHIKV replication in cell culture and RNA synthesis *in vitro*.

compound structure	label	x =	y =	finger
	**a**	NH_2	H	2
	b	NO_2	H	2
	c	NH_2	Me	2
	d	NO_2	Me	2
	**a**	NH_2	—	2
	b	NO_2	—	2
	c	NHC(=S)OEt	—	2
	—	—	—	4
	**a**	—	H	4
	b	—	Me	4
	**a** (suramin)	SO_3Na	—	6
	b	H	—	4

palm	arm	neck	Effect on RNA synthesis	EC_{50} (μM)	CC_{50} (μM)
1	short × 1	0	-	739	>800
1	short × 1	0	-	>800	>800
1	short × 1	0	-	>800	>800
1	short × 1	0	-	>800	>800
1	long × 1	0	-	420	>800
1	long × 1	0	-	>800	>800
1	long × 1	0	-	>800	>800
2	0	1	-	>800	>800
2	short × 2	1	+	403	>800
2	short × 2	1	+	>800	>800
2	long × 2	1	+	79	>800
2	long × 2	1	+	210	>800

4. Conclusion

In this study we show that the anti-parasitic drug suramin inhibits the replication of CHIKV and other alphaviruses. We discovered that while *in vitro* suramin is a potent inhibitor of RNA synthesis, in cell culture the compound mainly inhibits an earlier, post-attachment step of the CHIKV replicative cycle, likely viral entry. The inhibition of an early step in infection (receptor binding or entry or uncoating) has also been reported for a variety of other viruses [11-13, 15, 16, 18, 22]. Suramin appears to inhibit (re)initiation of CHIKV RNA synthesis, maybe by interfering with binding of the template RNA. This would be in line with earlier *in vitro* studies, reporting that suramin inhibits various RNA-binding enzymes like viral polymerases [7, 27, 36] and helicases [24, 25]. Several suramin-related compounds were analyzed, and though these compounds were not more effective, they provided insight into the (different) structural elements that are important for both inhibitory activities of suramin observed *in vitro* and in cell culture.

Clinical trials that evaluated the efficacy of suramin for treating patients chronically infected with HIV or HBV [9, 37] revealed serious side-effects during the required long term treatment. As a result and also because more promising drugs like 2',3'-dideoxy-3'-azidothymidine (AZT) became available [38], further clinical development was halted. The treatment of CHIKV infections would likely not require prolonged treatment and, therefore, we feel it is still worthwhile to obtain more insight into the modes of action of suramin and to explore its therapeutic potential.

Acknowledgements

This work was supported by the EU-FP7 EUVIRNA (#264286) and SILVER (#260644) grants, as well as by the Ministry of Science and Technology of R.O.C. (# NSC 103-2923-I-008-001 and MOST 103-2113-M-007-018-MY3) and the National Central University, Taiwan (# 103G603-14). We are grateful to the LUMC biosafety officer dr. Gijsbert van Willigen for his support in keeping our BSL-3 facility operational and to dr. Gilles Querat (Aix Marseille University) for supplying CHIKV RNA isolated during the outbreak at St. Martin.

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Suplemental Figures

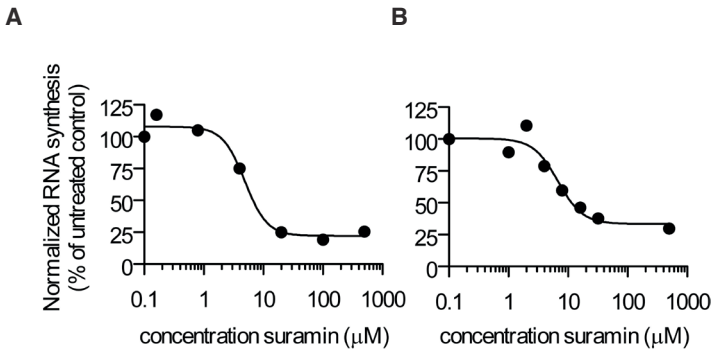

Figure S1. Quantification of the effect of suramin on alphavirus RNA synthesis *in vitro*.
In vitro RNA synthesis assays were performed with RTCs, either isolated from CHIKV-infected Vero E6 cells (A) or from BHK-21 cells transfected with a CHIKV replicon (B), in the presence of increasing concentrations of suramin. RNA synthesis, i.e. the amount of incorporated ^{32}P-CTP, was quantified by determining the volume of the bands that are visible in Fig. 1A and Fig. 6A using the Quantity One® software. The values (sum of genome and subgenomic RNA) were normalized to the untreated control sample (100%) and were corrected for variations in RNA isolation efficiency and loading using 18S ribosomal RNA signal (detected by hybridization). The corrected normalized values (amount of RNA synthesized) obtained at each suramin concentration were plotted against the log of the suramin concentration and the IC_{50} value was determined by non-linear regression using GraphPad Prism 5.

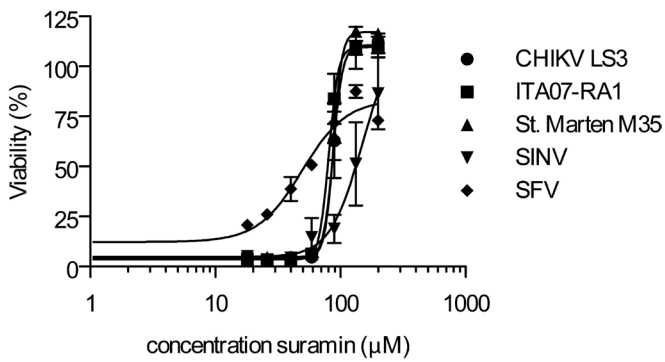

Figure S2. Antiviral activity of suramin in cell culture. Results of CPE protection assays using three different CHIKV strains, SINV and SFV. EC_{50} values for suramin (mentioned in table 1) were determined by non-linear regression using GraphPad Prism. The average and SD were calculated based on 2 independent experiments, performed in quadruplicate.

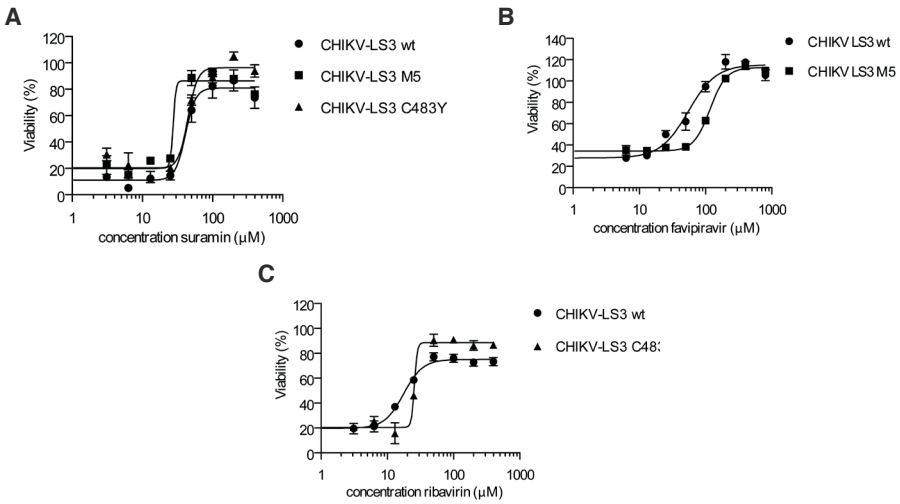

Figure S3. Effect of suramin on the replication of wt CHIKV LS3 and two mutants resistant to favipiravir and ribavirin in BHK-21 cells. CPE reduction assays were performed in BHK-21 cells, to make it possible to do parallel experiments with ribavirin, as ribavirin is converted to its active triphosphate form only very inefficiently in Vero-E6 cells. The cells were infected with CHIKV LS3 (wt), CHIKV M5 (favipiravir resistant) or CHIKV C483Y (ribavirin resistant) and treated with various concentrations of suramin (A), favipiravir (B) or ribavirin (C).

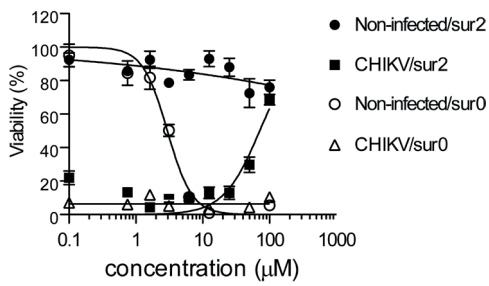

Figure S4. Effect of suramin-liposome preparation #PC3-Cl1-sur.2 and empty control liposomes on cell viability and CHIKV replication. Vero E6 cells were treated with suramin containing liposome formulation #PC3-Cl1-sur.2 (sur2) or 'empty' control liposomes #PC3-Cl1-sur.0 (sur0) and their effect on viability (uninfected cells) and CHIKV replication (by CPE reduction assay) was determined.

Chapter 4

Suramin inhibits Zika virus replication through multiple mechanisms

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Abstract

Zika virus (ZIKV) is a mosquito-borne flavivirus that mostly causes asymptomatic infections or mild disease characterized by low-grade fever, rash, conjunctivitis, and malaise. However, the recent massive ZIKV epidemics in the Americas have also linked ZIKV infection to fetal malformations like microcephaly and Guillain-Barré syndrome in adults, and have uncovered previously unrecognized routes of vertical and sexual transmission. Here we describe inhibition of ZIKV replication by suramin, originally an anti-parasitic drug, which was more recently shown to inhibit multiple viruses. In cell culture-based assays, using reduction of cytopathic effect as read-out, suramin had an EC_{50} of ~40 μM and a selectivity index of 48. In single replication cycle experiments, suramin treatment also caused a strong dose-dependent decrease in intracellular ZIKV RNA levels and a >3-log reduction in infectious progeny titers. Time-of-addition experiments revealed that suramin inhibits a very early step of the replication cycle as well as the release of infectious progeny. Only during the first two hours of infection suramin treatment strongly reduced the fraction of cells that became infected with ZIKV, suggesting the drug affects virus binding/entry. Binding experiments at 4°C using ^{35}S-labeled ZIKV demonstrated that suramin interferes with attachment to host cells. When suramin treatment was initiated post-entry, viral RNA synthesis was unaffected, while both the release of genomes and the infectivity of ZIKV were reduced. This suggests the compound also affects virion biogenesis, possibly by interfering with glycosylation and the maturation of ZIKV during its traffic through the secretory pathway. The inhibitory effect of suramin on ZIKV attachment and virion biogenesis and its broad-spectrum activity warrant further evaluation of this compound as a potential therapeutic.

Highlights

Suramin inhibits Zika virus replication in cell culture
Suramin interferes with Zika virus attachment to host cells
Suramin also affects release of infectious Zika virus

1. Introduction

Zika virus (ZIKV) is a flavivirus that was first isolated in 1947 in Uganda [1] and is primarily transmitted by Aedes mosquitos. Up to 2007, it received little attention, as only sporadic outbreaks were reported and these were not linked to serious disease. ZIKV has gained attention after the 2007 outbreak on Yap island and the 2013 epidemic in French Polynesia. Research efforts and public concern were further sparked by the massive epidemic in Brazil and many other South- and Central-American countries, which uncovered a link between ZIKV infection and serious foetal neurodevelopmental defects and neurological complications in adults.

About 80% of ZIKV infections are asymptomatic. In combination with ZIKV persistence in semen and the potential for sexual transmission, this poses a risk to women that are pregnant or trying to conceive. Symptomatic ZIKV infections are commonly characterized by low-grade fever, skin rash, conjunctivitis, and general malaise. However, the enormous scale of the recent epidemics has also revealed uncommon but more serious consequences, like the Guillan-Barré syndrome and foetal neurodevelopmental defects, like microcephaly. These could be due to ZIKV's ability to infect and persist in several immune-privileged tissues, like the central nervous system, placenta and testis [2].

ZIKV has a 10.7 kb ssRNA genome of positive polarity, which is 5' capped and lacks a poly(A) tail. The replicative cycle begins with binding to receptor and co-receptors, like AXL and TIM1 [3, 4], followed by entry through receptor-mediated endocytosis, uncoating and translation of the genome into a single polyprotein. The latter is processed by cellular and viral (NS3) proteases to yield the structural proteins C, prM and E and 7 nonstructural proteins, which are responsible for RNA replication in association with modified endoplasmic reticulum (ER) membranes and interactions with the host, including counteracting innate immune responses. Newly synthesized genomes, together with C, prM and E, are assembled into immature virions that bud into the ER lumen. These pass through the secretory pathway during which prM cleavage by the host protease furin leads to maturation, before particles are released into the extracellular space.

There are no registered vaccines against ZIKV and efforts to identify inhibitors of ZIKV replication have been initiated only recently, for example in the form of two large-scale compound screens that yielded surprisingly few common hits [5-9]. Considering the generally low success rate in antiviral drug development, it is crucial to identify a large number of ZIKV inhibitors in cell culture and elucidate their mode of action, in order to have sufficient lead compounds with the potential to advance further towards clinical development.

Suramin is an approved anti-parasitic drug that also blocks the replication of a variety of viruses (see [10] and references therein), including arboviruses like chikungunya virus (CHIKV) [10-12] and dengue virus [13]. Animal experiments have indicated that suramin can be used for preventing and treating enterovirus-71 and CHIKV infections [14, 15].

In this study we show that suramin effectively blocks ZIKV replication by interfering with attachment, while the compound also affects a later step, as it reduces the release of infectious progeny. Our results warrant further evaluation of suramin as an anti-ZIKV compound. Its broad spectrum of activity, limited toxicity and approved status offer interesting perspectives for repurposing this anti-parasitic drug as an antiviral.

2. Materials and Methods

2.1. Cells, viruses and compounds

Vero cells (ATCC CCL-81) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 8% fetal calf serum (FCS), 100 IU/mL of penicillin and 100 µg/mL of streptomycin at 37°C in 5% CO_2. ZIKV SL1602 was isolated in Leiden, the Netherlands, in 2016 from an infected traveler returning from Suriname (van Boheemen et al, submitted). Vero cells were seeded (150,000 cells or 75,000 cells/well, respectively) in 12-well or 24-well clusters 24 h prior to infection with ZIKV (MOI of 3) in EMEM with 25 mM HEPES, 2% FCS, 2 mM L-glutamine, and antibiotics (EMEM/2%FCS). After 2 h, the inoculum was removed, cells were washed 3 times with PBS after which they were maintained in EMEM/2%FCS. All experiments with ZIKV were performed in our BSL-3 facility. Suramin (Sigma) was dissolved in MilliQ to yield a 25 mM stock and mycophenolic acid (Sigma) was dissolved in ethanol to yield a 10 mM solution.

2.2. Cytopathic effect (CPE) reduction assay

CPE reduction assays with Vero cells were performed by seeding cells at a density of 5,000 cells/well in 96-well clusters, 24 h before compound treatment and infection. The next day, 1.5-fold or 2-fold serial dilutions of the compound were added to the cells, followed by 500 PFU/well of ZIKV in a total volume of 150 µL. Each concentration was tested in quadruplicate and each assay plate contained the following controls: no cells, uninfected&untreated cells, infected&untreated cells and infected&solvent-treated cells. After 4 days, 30 µL/well of CellTiter 96® Aqueous Non-Radioactive Cell Proliferation reagent (Promega) was added. After a 3-h incubation, reactions were stopped and virus was inactivated by adding 30 µL of 37% formaldehyde, followed by measuring the absorption at 490 nm. Viability assays on uninfected cells were performed in parallel to determine the CC_{50}. Data were normalized to untreated uninfected cells and EC_{50} and CC_{50} values were calculated with Graph-Pad Prism 7 (see section 2.8).

2.3. ZIKV titration and plaque reduction neutralization titer (PRNT) assay

Plaque assays were performed as described (Van Boheemen *et al*, submitted). Briefly, after a 1-min centrifugation at 13,000xg, virus-containing cell culture supernatants were 10-fold serially diluted in medium and 500-µL volumes were adsorbed for 2 h on confluent Vero cell monolayers in 6-well clusters. The inoculum was removed and an overlay of 1.2% Avicel RC-581 (FMC BioPolymer) in DMEM, 2% FCS, 25 mM HEPES, and antibiotics, was added.

After a 5-day incubation, cells were fixed with formaldehyde, stained with crystal violet and plaques were counted. For PRNT, $3.2x10^4$ PFU of ZIKV were incubated with various dilutions of heat-inactivated patient serum (against ZIKV SL1602) or a control serum for 1h at 37°C. Afterwards 250-µL volumes of 10-fold serial dilutions were adsorbed for 2 h on confluent Vero cell monolayers in 12-well clusters. An overlay was placed on top and plaques were visualized after 5 days as described for the plaque assay.

2.4. ZIKV RNA analysis

RNA was isolated from ZIKV-infected cells using TriPure isolation reagent (Roche) according to the manufacturer's instructions. ZIKV RNA was isolated from the medium of infected cells using the Qiamp Viral mini kit (Qiagen) with AVL lysis buffer spiked with equine arteritis virus (EAV) as internal control, as described previously [16]. ZIKV RNA copy numbers were determined by an in-house internally-controlled TaqMan multiplex RT-qPCR, using PGK1 as a reference gene, as described (Manuscript in preparation). Briefly, the detection of ZIKV RNA was done with the primer pair Fw 5'-AATGGCAGTCAGTGGAGATG/ Rv 5'-ACTCTTGTGTGTCCTTCCTAAC and a FAM-labelled probe (5'-6FAM-ATAGGTTTGCACATGCCCTCAGGT-3'-BHQ_1) using the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems) and the CFX384 Touch™ Real-Time PCR Detection System. Known quantities of *in vitro* transcribed RNA were used to generate calibration curves for absolute quantification of copy numbers. Each sample was analyzed in triplicate.

2.5. Immunofluorescence assay

Vero cells (50,000 per well) were seeded on coverslips in 24-well clusters. The next day they were infected with ZIKV or mock-infected and treated with various concentrations of suramin. At 26 h p.i. cells were fixed with 3% paraformaldehyde in PBS, and processed for immunofluorescence microscopy as previously described [16]. ZIKV-specific proteins were visualized with a 1:1000 dilution of the SL1602 patient antiserum, and double-stranded RNA was stained with mouse monoclonal antibody J2 (English & Scientific Consulting). Detection of primary antibodies was done with 1:500 dilutions of goat anti-human Alexa488 and donkey anti-mouse Cy3.

2.6. Production of ^{35}S-labeled ZIKV and binding assays

Confluent Vero cells (75 cm^2) were infected with ZIKV at an MOI of 0.05 or mock infected and incubated for 3 days. Medium was replaced with protein-labeling medium (Met- and Cys-free DMEM) containing 88 µCi of ^{35}S-labelled Met and Cys (0.0176 µCi/µL) and cells were incubated for 15 h. The culture supernatants were collected and cellular debris was removed by centrifugation for 10 min at 233 x g. Unincorporated label was removed by pelleting virions through a cushion of 20% sucrose in TESV buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM NaCl) by ultracentrifugation for 2 h at 45,000 rpm and 4°C

using a Beckmann SW55 rotor. After removal of the supernatant and sucrose layer, the virus pellet was washed with TESV buffer, and resuspended in 0.6 mL of TESV. Aliquots of labelled virus were stored at -80°C. For binding assays, Vero cells seeded in 24-well clusters were incubated with 4 µL ($3.2x10^4$ PFU) of radioactive ZIKV for 2 h at 4°C in a total volume of 125 µL. After removal of the inoculum and washing three times with PBS, cells were lysed in 200 µL of 100 mM Tris-HCl pH 6.8, 8% SDS (w/v), 40 mM DTT, and 40% glycerol to quantify cell-bound radioactivity using a Beckman-Coulter LS6500 Multi-Purpose scintillation counter and Ultima Gold™ scintillation liquid.

2.8. Statistical analysis

GraphPad Prism 7 was used for EC_{50}, IC_{50} and CC_{50} calculations by non-linear regression and for statistical analyses by one-way ANOVA with Dunnett's multiple comparison test.

3. Results

3.1. Suramin inhibits ZIKV in CPE reduction assays

To determine the anti-ZIKV activity of compounds, we have set up CPE reduction assays with Vero cells, which were treated with serial dilutions of compound, followed by mock-infection or ZIKV-infection at an MOI of 0.05. After a 4-day incubation, colorimetric viability assays were performed to quantify the protective effect of a compound against ZIKV-induced CPE. Publications on compounds with demonstrated anti-ZIKV activity were not available at the time we set up our assay (3/2016) and therefore we included mycophenolic acid (MPA), known to inhibit a broad-spectrum of viruses, as a positive control. MPA had an EC_{50} of 0.42 µM and a CC_{50} of over 250 µM in our assay and its antiviral effect has also been demonstrated by others ([6, 8]. Suramin protected Vero cells from ZIKV-induced cell death with an EC_{50} value of 39.8 µM (supplemental Fig. S1). The CC50 of suramin was 1.9 mM, resulting in a selectivity index (SI) of 48.

3.2. Suramin reduces ZIKV RNA accumulation and infectious progeny titers in cell culture

To confirm the antiviral effect of suramin treatment observed in the CPE reduction assays, a dose-response experiment was performed with ZIKV-infected Vero cells (MOI 3) that were treated with 25 to 400 µM of suramin (or 2.5 µM MPA as positive control) from 2 h prior to infection up to 44 h p.i. Suramin treatment caused a convincing dose-dependent reduction in intracellular ZIKV RNA levels (Fig. 1A), yielding a 3-log decrease in ZIKV genome copies after treatment with 400 µM of suramin. An even stronger effect was observed on the production of infectious progeny (Fig.1B). No infectious particles could be detected at the highest suramin dose tested, although the presence of 40 µM residual suramin in the 10-1 dilution of the samples used for titration by plaque assay may have increased the limit of detection to 100 PFU/mL for samples treated with 400 µM suramin. Nonetheless, even

at 50 μM, suramin treatment caused a more than 2-log reduction in progeny virus titers. MPA treatment only modestly inhibited RNA synthesis and virus production, yielding a reduction of about 1-log in both assays.

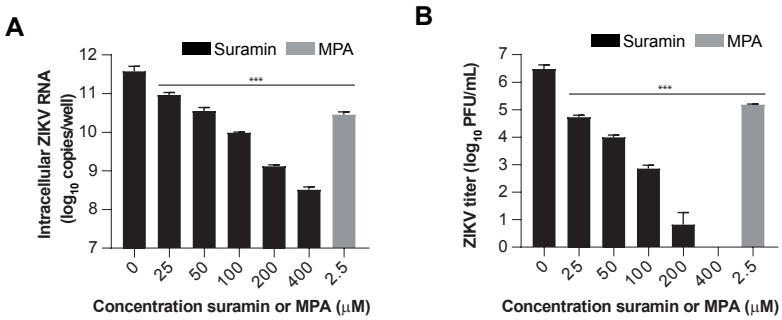

Figure 1. Effect of suramin on the accumulation of intracellular ZIKV RNA and infectious progeny titers. A. ZIKV-infected Vero cells were treated with 25 to 400 μM suramin from 2 hours prior to infection till 44 h p.i. Untreated and mock-infected cells were included as controls and the inhibitory effect of suramin was compared to that of 2.5 μM MPA. Total RNA was isolated from cells (n=3) at 44 h p.i. and intracellular ZIKV RNA copy numbers were determined by RT-qPCR using a standard curve of *in vitro* transcribed ZIKV RNA for absolute quantification. B. ZIKV titers in the medium of suramin-treated cells (n=3) were determined by plaque assay. Significant differences are indicated by * (***, $p<0.0001$).

3.3. Suramin inhibits the attachment of ZIKV to host cells

A time-of-addition experiment was performed to determine which step(s) of the ZIKV replication cycle are inhibited by suramin. The compound was added to Vero cells to a final concentration of 200 μM either two hours before infection, at the time of infection, or at various time points post infection, after which the compound remained present until sample collection at the end of the experiment (Fig. 2). Suramin had the strongest inhibitory effect on the accumulation of intracellular ZIKV RNA when added prior to or together with the virus (Fig. 2, samples -2 and 0), resulting in a ~100-fold reduction in the amount of intracellular ZIKV RNA. When the compound was added 30 min or more after adding the virus, the inhibitory effect on the accumulation of intracellular ZIKV RNA diminished rapidly. Immunofluorescent staining of ZIKV-infected Vero cells treated with 25-200 μM suramin from 2 h before till 2 h after addition of the virus revealed that the compound caused a dose-dependent reduction in the number of infected cells reaching 95% at 200 μM (Fig. 3A). The remaining ZIKV-positive cells appeared not to be protected by suramin treatment as their signal intensity in immunofluorescence microscopy resembled that of untreated cells (see inset of Fig. 3A). These remaining ZIKV-positive cells are most likely responsible for the residual production of infectious virus that was observed when cells were treated with 200 μM suramin from -2 to 2 h p.i. (Fig. 3B). Nonetheless, we concluded that suramin treatment during the early steps of the replication cycle (binding/entry) only, still resulted in an up to 100-fold and dose-dependent reduction of progeny virus titers (at 26 h p.i.).

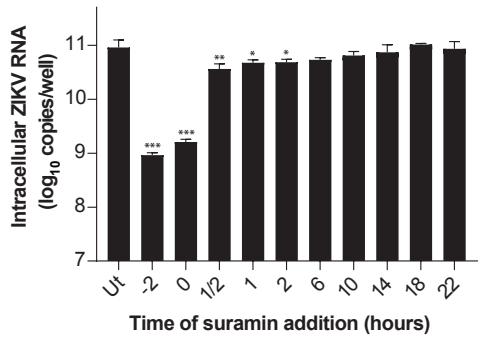

Figure 2. Effect of suramin on ZIKV replication in time-of-addition assay
ZIKV-infected Vero cells were treated with 200 μM of suramin starting 2 h before infection until 26 h p.i. Untreated (Ut) and mock-infected cells were included as controls. Total RNA was isolated from cells at 26 h p.i. and intracellular ZIKV RNA copy numbers were determined by RT-qPCR (n=3). Significant differences compared to untreated infected cells are indicated by * (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

To test whether suramin affects ZIKV attachment to host cells, we have produced ^{35}S-labeled ZIKV for use in virus binding assays. The radioactive ZIKV was incubated with Vero cells for 2 h at 4°C, which should only allow binding, while preventing endocytosis/entry. After washing, cells were lysed and the amount of cell-bound radioactivity was determined by scintillation counting. Vero cell-associated radiolabel was readily detected when ^{35}S-labeled ZIKV was used in this assay, while hardly any radiolabel was bound when Vero cells were incubated with a virus-free control sample that had been prepared by ^{35}S-labeling of mock-infected cells (Fig. 3C). To determine whether inhibition of binding could be measured in this assay, ^{35}S-labeled ZIKV was incubated with a neutralizing antiserum from a ZIKV-infected patient prior to the binding assay (Fig. 3C). This serum completely neutralized the virus at 1:5 (and higher) dilutions in plaque reduction neutralization assays (Fig. 3C, inset). When the binding assay was performed with virus that was neutralized with a 1:5 antiserum dilution, a 50% reduction in cell-bound radiolabel was observed. It remains unclear whether the remaining amount of radioactivity resulted from cell-bound radiolabeled virus or from radiolabeled host proteins that might be absent in the ^{35}S-labeled control sample obtained from mock-infected cells as the latter had not undergone the ZIKV-induced CPE. Nonetheless, the assay allowed us to measure decreases in ZIKV binding. When various suramin concentrations were tested in this assay, we observed a dose-dependent inhibition of ^{35}S-ZIKV binding of up to 50% (Fig. 3D), the same residual level of labeling measured when using pre-neutralized virus (Fig. 3C). Taken together these results indicate that suramin treatment affects binding of ZIKV to the host cell.

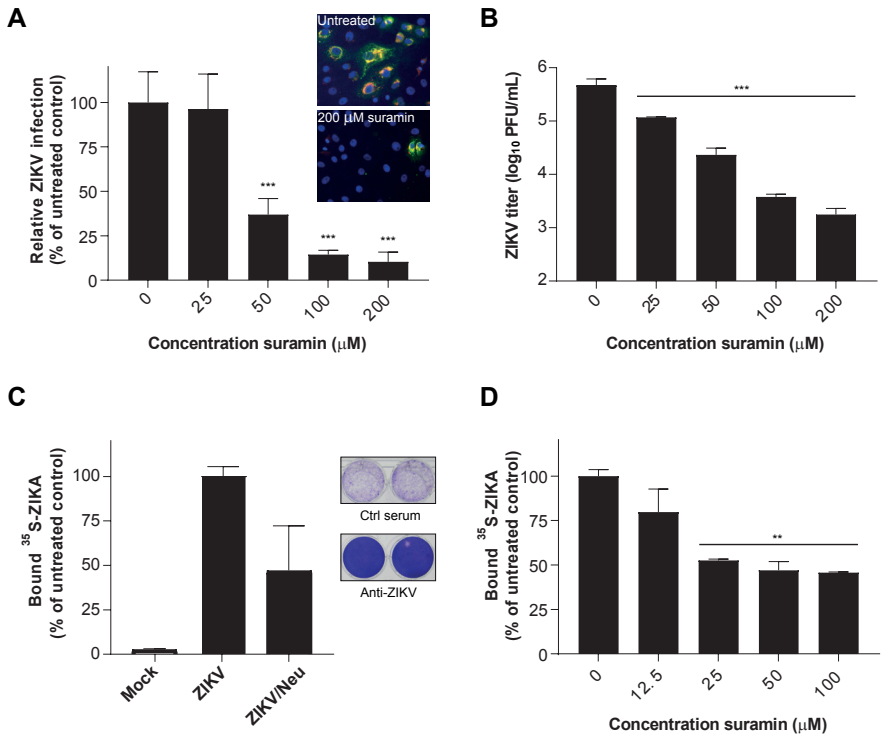

Figure 3. Effect of suramin on ZIKV attachment to host cells
A. ZIKV-infected Vero cells were treated with 25 to 200 µM of suramin, starting 2 hours prior to the addition of virus till 2 h p.i. At 26 h p.i. cells were fixed and analyzed by immunofluorescence microscopy. Quantification of the percentage of infected cells was performed by determining the number of dsRNA (red) and ZIKV protein (green) positive cells in two images acquired per sample. Results are presented as the number of infected cells (%) relative to the untreated control. The inset shows typical micrographs of untreated cells and cells treated with 200 µM of suramin only during the early steps of infection. B. ZIKV titers in the medium of Vero cells that were treated with 25 to 200 µM of suramin from -2 to 2 h p.i. Supernatants were harvested at 26 h p.i. from two independent infections performed in duplicate, and ZIKV titers were determined by plaque assay (2 replicates per supernatant). C. ZIKV binding assay. ^{35}S-labeled ZIKV was incubated with Vero cells for 2 h at 4°C. A virus-free control sample that had been prepared by ^{35}S-labeling of mock-infected cells was included as a negative control (Mock). ^{35}S-labeled ZIKV that was neutralized (ZIKV/Neu) with a patient immune serum (see inset for PRNT results) was included as a control for reduced binding. After extensive washing the remaining cell-bound radioactivity was quantified by scintillation counting (n=2). D. Effect of suramin on ZIKV binding. ^{35}S-labeled ZIKV was incubated for 2 h at 4°C with Vero cells without suramin or in the presence of 12.5 to 100 µM suramin. After extensive washing the remaining cell-bound radioactivity was quantified by scintillation counting (n=2). Significant differences compared to untreated infected cells are indicated by * (**, p<0.01; ***, p<0.001).

3.4. Suramin treatment also reduces the release of infectious progeny

Analysis of the infectious virus titers in the supernatant of samples from a time-of-addition experiment revealed that suramin not only affected an early step of the replication cycle, but also reduced progeny titers when added at later stages (Fig. 4A). When suramin treatment was started at 2 h post entry, it had no significant effect on intracellular viral RNA levels,

suggesting it does not interfere with RNA replication (Fig. 2A), while a ~100-fold reduction of infectious progeny released into the medium was observed (Fig. 4A). To confirm that suramin also has a late effect on the release or maturation of viral particles, we treated ZIKV-infected cells with 25-200 μM of suramin from 14 to 26 h p.i. As expected, the number of infected cells (based on immunofluorescence microscopy) did not change (results not shown) by this late suramin treatment, while there was a dose-dependent decrease of up to 35-fold in the amount of infectious progeny released (Fig. 4B).

To further corroborate this effect on virion biogenesis, a time-of-addition experiment was performed, in which cells where infected and subsequently treated with 200 μM suramin starting from 2, 14, 18, or 22 h p.i. until the medium was harvested at 26 h p.i. A consistent ~10-fold reduction in the amount of released genome copies was observed, whether suramin treatment was initiated at 2 h p.i. or as late as 22 h p.i. (Fig. 4C, black bars). However a stronger, ~100-fold reduction in released infectious progeny titers was observed when suramin treatment was initiated at 2 h p.i. (Fig. 4C, gray bars). The latter effect was less pronounced (~10-fold) when suramin treatment was initiated late in the replication cycle (18 or 22 h p.i.). These results suggest that suramin affects both the total number of released virions (genome copies) and their infectivity (PFU).

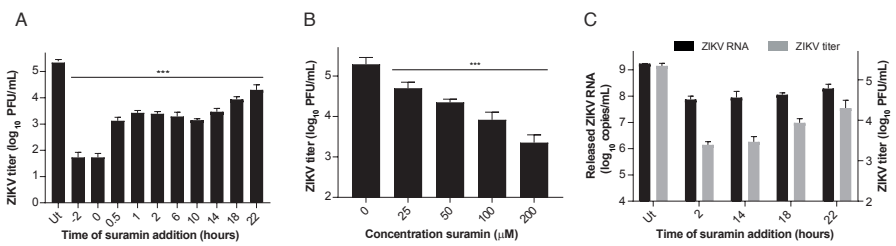

Figure 4. Effect of suramin on the production of infectious ZIKV progeny

A. Time-of-addition-assay. ZIKV-infected Vero cells were treated with 200 μM of suramin from -2 to 26 h p.i. Untreated (Ut) and mock-infected cells were included as controls. At 26 h p.i. supernatants were harvested in triplicate and virus titers were determined by plaque assay. B. ZIKV-infected Vero cells were treated with 25 to 200 μM suramin from 14 to 26 h p.i. Untreated and mock-infected cells were included as controls. At 26 h p.i., supernatants were harvested from two independent infections performed in duplicate and analyzed by plaque assay (n=4). C. ZIKV-infected Vero cells were treated with 200 μM of suramin starting at 2, 14, 18, and 22 h p.i. Untreated (Ut) and mock-infected cells were included as controls. At 26 h p.i., supernatants were harvested and virus titers were analyzed by plaque assay (n=3). Viral RNA was isolated from the supernatants and copy numbers were determined by RT-qPCR (n=3). Significant differences compared to untreated infected cells are indicated by * (**, $p<0.01$; ***, $p<0.001$).

4. Discussion

Here we demonstrate that the approved drug suramin has anti-ZIKV activity by interfering with viral attachment, as well as the release of infectious progeny from ZIKV-infected cells. In CPE reduction assays suramin had an EC_{50} value of 39.8 μM and a SI of 48. In this assay, MPA had an EC_{50} of 0.42 μM, similar to values reported recently by others [6, 8]. In low-

MOI CPE reduction assays MPA appeared to inhibit ZIKV more efficiently than suramin, but in the context of a high-MOI infection, treatment with 2.5 μM MPA led to a mere 10-fold reduction in the accumulation of intracellular ZIKV RNA and infectious ZIKV titers in the medium (Fig. 1). In contrast, treatment of cells infected at a high MOI with 50 μM of suramin, while also reducing intracellular ZIKV RNA accumulation by 10-fold, yielded a ~300-fold reduction in the amount of infectious progeny (Fig. 1B). MPA's limited effect on high-MOI ZIKV infections, its immunosuppressive properties and its contraindicated use during pregnancy makes it an unattractive lead to follow.

Time-of-addition experiments and binding experiments with radioactive ^{35}S-labeled ZIKV at 4°C suggested that suramin affects viral attachment to the host cell.
The inhibition of attachment might be due to suramin's effect on virions, as was previously reported for DENV [13] and enterovirus A71 [17], for which suramin was proposed to have a 'neutralizing effect' that blocks the interactions of these viruses with cellular heparan sulfate, and the P-selectin ligand, respectively. Suramin was suggested to interact with surface-exposed positively charged residues in the DENV E protein that are part of the glycosaminoglycan-binding (GAG-binding) sites, thereby interfering with attachment to the host cell [13]. It was recently demonstrated that binding to GAGs likely also plays a role in ZIKV attachment [18].

Besides interacting with the virion, suramin could also have an effect on host cell (co) receptors, as it was shown to abolish the interaction of several cellular receptors with their ligands [19-21]. Binding of suramin to cellular (glyco)proteins, like the proposed ZIKV receptors AXL [3, 22] or glycoprotein TIM1 [23] could interfere with ZIKV attachment.
Suramin treatment also inhibited the release of infectious progeny from ZIKV-infected cells, not only by lowering the number of genomes released, but also by reducing the specific infectivity (Fig. 4).

This post-entry effect might be due to binding of the negatively charged suramin to one of ZIKV's RNA-binding proteins, as suramin was shown to inhibit Dengue and hepatitis C virus helicase activity [24, 25], norovirus RNA-dependent RNA polymerase activity [26, 27], reverse transcriptase activity of various retroviruses [28], including HIV [29], and the activity of alphavirus replication complexes [10]. Since we did not observe inhibition of ZIKV RNA synthesis in infected cells, an effect on NS5 appears unlikely. Suramin might inhibit the helicase activity of ZIKV NS3 or might affect packaging by binding to positively charged residues on the capsid protein. This would result in accumulation of genomes in the cell, while virus release would be diminished. The E protein, prM, NS1, NS2A, NS4A or/and NS4B [130] or furin could also be targets of suramin, with consequences for virion assembly, glycosylation of ZIKV proteins and/or maturation of the virion, lowering the infectivity of released virions. ZIKV prM, E, and NS1 proteins contain

potential N-glycosylation sites [31, 32] and glycosylation of the E proteins of ZIKV and West Nile Virus is important for virion maturation and infectivity [33, 34]. Suramin is a highly charged molecule that, complexed with serum albumin, can be taken up by cells through endocytosis. The concentration of albumin determines whether suramin mainly accumulates in the lysosomal compartment or in the Golgi apparatus, mitochondria and nucleolus [35]. The latter might cause a generalized negative effect on the secretory pathway that would also affect virion biogenesis.
Studies on suramin-related compounds revealed that the six sulfonate groups as well as the symmetry of the molecular structure are crucial elements for suramin's anti-CHIKV activity [10]. Addition of sulfonate groups and a higher level of branching increased the compound's anti-enterovirus activity [17], suggesting the molecule can be further optimized.

Because of the increased and geographically expanding incidence, unnoticed infections in combination with sexual transmission, and potentially severe consequences of mother-to-child transmission, ZIKV infection continues to be a major public health concern, for which therapeutics are urgently needed. Suramin has been approved for use in humans and has been used safely and successfully in animal models for treating several virus infections. Future studies should evaluate the efficacy of suramin on ZIKV replication in animal models and determine whether suramin or related compounds have the potential to advance into further clinical development for treatment of ZIKV infection.

Acknowledgments
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Supplemental figures

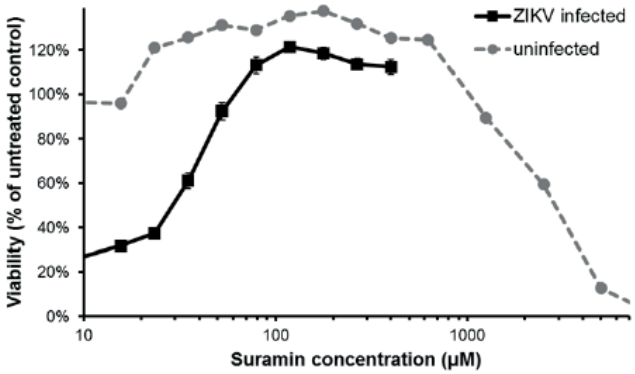

Figure S1. Protective effect of suramin on viability of ZIKV-infected Vero cells in CPE reduction assay (black squares). The effect of suramin on the viability of uninfected cells (grey circles) was measured to determine the CC_{50} of suramin.

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Chapter 5

Suramin inhibits chikungunya virus replication by interacting with virions and blocking the early steps of infection

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Abstract

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus that can cause a debilitating disease that is primarily characterized by persistent joint pain. CHIKV has been emerging globally, while neither a vaccine nor antiviral medication is available. Suramin, a drug used to treat parasitic infections, was previously shown to inhibit CHIKV replication. In this study we aimed to obtain more detailed insight into its mechanism of action. We found that suramin interacts with virions and can inhibit virus binding to cells. It also appeared to inhibit post-attachment steps of the infection process, likely by preventing conformational changes of the envelope glycoproteins required for fusion and the progression of infection. Following selection and genotyping of suramin-resistant mutants, the substitutions N5R and H18Q in the E2 glycoprotein were reverse engineered in order to understand their role. Indeed, suramin-resistant viruses carrying these two E2 mutations appeared to be able to overcome the post-attachment inhibitory effect of suramin. Conversely, a virus with a G82R mutation in E2, which renders the virus dependent on the interaction with heparan sulfate for its entry, was more sensitive to suramin than wild-type virus. Using molecular modelling studies, we predicted the potential suramin binding sites on the mature spikes of the chikungunya virion. We conclude that suramin interferes with CHIKV entry by interacting with the E2 envelope protein, which can inhibit attachment and interfere with conformational changes required for fusion.

Keywords: anti-CHIKV treatment, suramin, E2 envelope protein, attachment, fusion, drug repurposing

1. Introduction

Chikungunya virus (CHIKV) is a re-emerging alphavirus that is transmitted by *Aedes* sp. mosquitoes and has caused several large outbreaks in the past 15 years. Before 2013 CHIKV was circulating mainly in Africa and Asia [1], but following its introduction into the Caribbean it has now become endemic in Latin America as well [2]. Acute CHIKV infection is associated with fever, rash, muscle pain, and general malaise. Furthermore the virus often causes a debilitating joint pain that can last for months to years [3]. Prophylactic or therapeutic treatment for CHIKV infections is still not available on the market, and vector control measures do not provide the ultimate solution [4]. Although progress is being made in CHIKV vaccine and antiviral drug development [3, 5-7], we would still be poorly prepared in the face of a new CHIKV epidemic.

We and others have previously shown that the antiparasitic drug suramin inhibits CHIKV replication by targeting an early step in the viral replication cycle [8-10]. Moreover, the compound also inhibits CHIKV replication in a mouse model [11]. These findings show the potential for the (off-label) therapeutic use of suramin for the treatment of chronic chikungunya fever, and possibly also for its prophylactic use during severe CHIKV outbreaks.

CHIKV has a 11.8-kb single-stranded (ss) RNA genome of positive polarity, which is capped, poly-adenylated and packaged into an icosahedral nucleocapsid that is surrounded by an envelope containing 80 projections (spikes), each consisting of three E1-E2 (envelope proteins) heterodimers [12]. The E2 protein is involved in the interaction with the host cell and therefore is an important determinant of pathogenicity, cellular tropism [13, 14], and immunogenicity [15, 16]. The viral replication cycle begins with attachment of the virion to the cell surface through interactions with glycosaminoglycans, such as heparan sulfate [17]. Subsequent binding to a receptor, like the recently identified Mxra8 protein [18] will lead to uptake of the virion via receptor-mediated endocytosis [19]. Endosomal acidification causes structural rearrangements in the virion that induce E1 protein-mediated fusion of the viral envelope with the endosomal membrane [20]. This leads to nucleocapsid release and its disassembly in the cytoplasm to liberate the RNA genome. Subsequently, the genome is translated into a polyprotein that is processed into the non-structural proteins nsP1 to 4 that (together with host factors) assemble into membrane-associated replication and transcription complexes (RTCs). The structural proteins (capsid, E3, E2, 6k/TF and E1) are expressed from a subgenomic RNA in the form of a second polyprotein. After autoproteolytic release of the capsid protein, the remainder of the structural polyprotein traffics through the secretory pathway, during which it is cleaved by host cell proteases and undergoes post-translational modifications like glycosylation. Ultimately, the mature envelope proteins will reach the plasma membrane. Here, interaction between nucleocapsids and the cytoplasmic sides of the envelope proteins are essential for the budding process and the formation of new virus particles [21].

The work presented here provides insight into the mode-of-action of suramin, through specific analysis of virus binding and fusion, and by selecting and characterizing suramin-resistant CHIKV variants, which contained N5R and H18Q mutations in the envelope protein E2. Moreover, we found that a virus with the G82R mutation in E2, which renders the virus dependent on heparan sulfate binding for infectivity and was implicated in attenuation of vaccine strain 181/25 [6], was more sensitive to suramin than wild-type CHIKV. Molecular docking studies provided more insight into suramin's inhibitory activity, since the compound was predicted to bind to virus particles at positions that could interfere with conformational changes in the envelope proteins that need to occur during entry.

2. Materials and Methods

2.1. Cells, compounds, and viruses.

Vero E6 and BHK21 cells were grown in DMEM or BHK medium, respectively, supplemented with 8% fetal calf serum (FCS) and penicillin/streptomycin. BS-C-1 cells were cultured as previously described [22]. Suramin, chloroquine and ammonium chloride were purchased from Sigma and ^{3}H-suramin from Hartmann Analytic. CHIKV LS3 (KC149887) and Semliki Forest virus strain SFV4 (KP699763.1) were launched from full-length cDNA clones. All studies with live CHIKV were performed in biosafety cabinets in BSL-3 facilities.

2.2. Preparation of ^{35}S-labeled viruses and purification of virus stocks.

^{35}S-labeled CHIKV and SFV were produced in Vero E6 cells as described before [23]. To remove non-incorporated label, the cell culture supernatant was subjected to pelleting through a sucrose cushion by ultracentrifugation in a SW41 Ti rotor (at ~200,000g for 2h). Virus pellets were resuspended in 1xTESV buffer (20mM Tris-HCl pH 7.4, 1mM EDTA, 100mM NaCl), before aliquoting and storage at -80°C.

2.3. Virus attachment assay. To study virus binding

^{35}S-CHIKV or ^{35}S-SFV samples (1x10^{4} CPM) were incubated with Vero E6 cells for 1h at 4°C, in the presence or absence of suramin, followed by washing and lysis in 4x dye-free Laemmli sample buffer (LSB). The amount of bound radioactivity in samples was quantified by liquid scintillation counting with a Beckman-Coulter LS6500 Multi-Purpose scintillation counter and Ultima Gold™ scintillation liquid. The binding of fluorescently labeled CHIKV (DiD-CHIKV) to BS-C-1 cells was assessed by fluorescence microscopy, as previously described [22].

2.4. Bulk fusion assay.

Pyrene-labelled CHIKV and liposomes containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin and cholesterol in a molar ratio of 1:1:1:1.5 were used in a bulk fusion assay as previously described [19].

2.5. ^{3}H-suramin-virus binding assay.

Purified virus particles prepared in 1xTESV buffer (containing only trace amounts of protein from the culture medium) were incubated with 0.5x10^{6} CPM of ^{3}H-suramin, for 1h at 37°C. The unbound suramin was removed by gel-filtration using P30-Microbiospin columns (BioRad) according to the manufacturer's instructions; virions in the flow-through were lysed in dye-free LSB and bound radioactive suramin was quantified by scintillation counting as described under 2.3.

2.6. Reverse genetics.

The mutations listed in Table 1 were introduced into the pMALS2L (G588A, A979G, G980A, T5645C) and pMALS2R (A8554G, C8595A) plasmids [24] using QuickChange site-directed mutagenesis (Stratagene, USA). Subsequently, single or combined mutations were transferred to the CHIKV LS3 plasmid by using unique BamHI, XmaI, AgeI, XhoI and SfiI restriction sites. The reverse-engineered mutant CHIKV mutants were launched via *in vitro* transcription (Epicentre) and RNA transfection of BHK-21 cells as described previously [24]. After 24 to 48 h, when extensive cytopathic effects (CPE) had occurred, the virus-containing supernatants were harvested and used to produce passage 1 virus stocks on Vero E6 cells, which were used in subsequent experiments. These virus stocks were verified by Sanger sequencing of the full genome to confirm the presence of the introduced mutations and absence of other mutations. Only in the case of G980A (in nsP1) we observed rapid reversion to the wild-type genotype.

2.7. RNA isolation and RT-qPCR.

Total cellular RNA was isolated by lysing the cells in LiDS/LET as previously described [24] or in TRIpure reagent according to the manufacturer's (Invitrogen) instructions. To measure genome copy numbers an internally controlled TaqMan quantitative RT-PCR assay was used [24].

2.8. Cytopathic effect (CPE) reduction assay.

CPE reduction assays were basically performed as previously described [8], except that an MOI of 0.05 was used and the incubation period was 72h for CHIKV mutants S4.1, S4.3, S5 or 96h for wt CHIKV and mutants S2.1, S2.3, S3, S7, S8 and S9, or 120h for mutants S2.2 and G82R. After performing a colorimetric viability assay, absorption was measured at 450 nm using an EnVision Multilabel Plate Reader (PerkinElmer, US).

2.9. Plaque number reduction assay.

To study virus entry, Vero E6 cells were incubated with approximately 100 PFU of CHIKV for 1h at 37°C in the presence of a range of suramin concentrations. After removing the inoculum, the cell monolayer was washed and overlay medium containing 1.2% Avicel RC-581 (FMC BioPolymer) in DMEM, 2% FCS, 25mM HEPES, and penicillin/streptomycin was added. After three days, the cell monolayers were fixed with 3% formaldehyde in PBS solution and plaques were stained and counted. To study attachment, suramin treatment and virus uptake were done for 1h at 4°C, and 1000 PFU were used in order to detect approx. 100 plaques in the untreated wells, as low temperature diminished virus binding.

2.10. Molecular modelling.

Molecular Operating Environment (MOE) 2018.10 [25] and Maestro [26] software was used. The CHIKV E2-E1 glycoprotein heterodimer (PDB ID 3N42) and trimeric complex (PDB ID 3J2W) were preprocessed using the Schrödinger Protein Preparation Wizard by assigning bond orders, adding hydrogens, and performing a restrained energy minimization of the added hydrogens using the OPLS_2005 force field. The missing residues of E2 (1-6) were manually introduced and the downstream docking processes are described in more detail in the Supplemental information. The electrostatic potential surface was obtained using the Surfaces and Maps tool in MOE after splitting the molecule in multiple chains. Figures were prepared with MOE.

2.11. Statistics.

GraphPad Prism 8 was used as previously described [8, 23] for EC_{50} determination by non-linear regression. The statistical analysis was performed with one-way ANOVA using Dunnett's multiple comparison test (Fig. 1 A, B, C).

3. Results and discussion

3.1. Suramin inhibits viral attachment and fusion by interacting with the chikungunya virion

Several experiments were carried out to analyze the impact of suramin on the early events of infection, i.e. virus attachment, internalization, and fusion. ^{35}S or DiD-labeled CHIKV [22] were used in virus binding assays, in the presence or absence of suramin, at 4°C. Active endocytosis does not occur at temperatures below 18°C [27] and therefore suramin is expected to remain in the extracellular environment. Virus attachment to the cell surface probably involves electrostatic interactions with the GAGs or other plasma membrane factors (receptors, adhesion molecules) and infections can be synchronized by placing cells at 4°C. By directly measuring the amount of radioactively- or fluorescently-labeled virus in the presence of increasing concentrations of compound, we found that suramin inhibits CHIKV attachment (Fig. 1A and B) in two distinct cell lines and with two different experimental readouts. Binding of ^{35}S-labelled SFV was even more strongly inhibited by suramin (supplemental figure S1A), which is in line with suramin's previously observed lower EC_{50} for SFV compared to CHIKV [8]. In addition, suramin (30 μM) completely inhibited pyrene-labeled CHIKV from fusing with liposomal membranes at pH ~5.5 in the absence of host proteins, as shown in figure 1C [19]. Because suramin is known to bind to positive charges on the surface of proteins, we suspect that suramin binds directly to the envelope proteins, thereby preventing the conformational changes that are required for fusion. In contrast to Ho *et al.*, who studied the effect of suramin on surface-expressed envelope proteins in insect cells that were subsequently triggered to fuse at low pH, we studied the effect of suramin on membrane fusion in the context of whole virions. To confirm the interaction of suramin with virions, we incubated purified CHIKV (lacking serum proteins as these are known to strongly bind suramin; [28] with ^{3}H-labeled suramin. Compared to a control supernatant from mock-infected cells (which bound 3000 CPM of

suramin) purified CHIKV bound over 30,000 CPM of ^{3}H-suramin (Fig. 1D), confirming that the compound does interact with virus particles. ^{3}H-suramin also interacted with SFV (Fig. S1C), and more specifically with the (native) E proteins on the surface of intact virions, since treatment with proteinase K or heat denaturation severely decreased the quantity of bound radiolabeled suramin. For other viruses like enterovirus A71 (EV-A71) and human immunodeficiency virus 1 (HIV-1) it was also found that suramin blocked their access to cellular receptors by directly interacting with virions [29, 30].

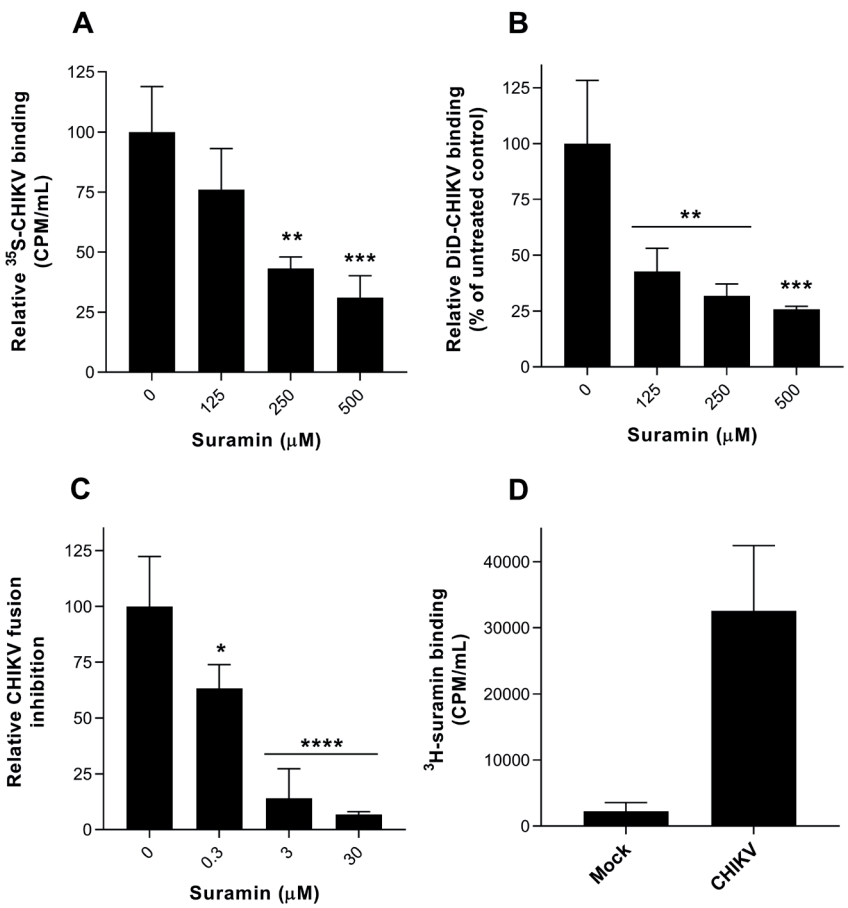

Figure 1. The effect of suramin on the early events of CHIKV infection. (A) The binding of ^{35}S-labelled CHIKV to Vero E6 cells in the presence or absence of suramin was determined at 4°C by scintillation counting of remaining radioactivity in cellular lysates obtained after extensive washing (average +/- SD; n=3). (B) Binding of DiD-labeled CHIKV to suramin-treated BS-C-1 cells, analyzed by fluorescent microscopy, in the presence of increasing concentrations. (C) Fusion of pyrene-labeled CHIKV in a bulk fusion assay with liposomes, triggered by lowering the pH, in the presence of increasing suramin concentrations (n=5 and 3, for untreated and treated samples, respectively). (D) Binding of ^{3}H-labeled suramin to CHIKV (purified virus was used to exlude interference by serum proteins). The control used in this assay was culture medium from uninfected cells that was treated the same way as when purifying virus (n=3). The data represent the means ± the SD and significant differences are indicated with * (****p < 0.001, ***p < 0.005, **p < 0.01, *p < 0.05 and ns as not significant).

Surprisingly, when SFV particles were pre-attached to cells (at 4°C, in the absence of compound) and treated with suramin at 37°C, only a modest ~20% inhibition in virus uptake was observed, regardless of the suramin concentrations tested (Fig. S1B). Hence it is possible that at physiological temperature, pre-attached viruses cannot be displaced by the compound or the entry process might be too fast to be inhibited by suramin. When evaluating these direct binding studies, it should be noted that it remains unclear what fraction of the bound virions will actually lead to a productive infection.
The inhibitory effect of suramin on the binding of virus particles to the cell surface could be due to its direct interaction with virions and/or with cellular receptors.

The direct binding assays with radiolabeled or fluorescently labeled viral particles clearly showed that suramin inhibits binding/fusion of both CHIKV and SFV (Fig. 1 and S1). This is in contrast with our earlier study [8], in which we concluded that suramin inhibits an early step, but not attachment. In that study, we relied on an RT-qPCR assay to quantify cell-bound CHIKV. However, this is an indirect measurement and the majority of the detected RNA molecules do not represent infectious particles, as we and others found that genome copy to PFU ratios of commonly used virus stocks are generally over 1000:1 [31, 32]. At least in part, this is due to the fact that virus stocks are generally harvested when extensive cytopathic effect (CPE) has occurred, which leads to the release of viral RNA not associated with infectious virus particles, e.g. in the form of naked RNA, nucleoprotein complexes or nucleocapsids. Therefore, we no longer consider RT-qPCR an appropriate assay to study CHIKV binding when standard, non-purified CHIKV specimens are used.
To evaluate whether the effect of suramin on CHIKV binding could be reliably measured by an improved RT-qPCR-based assay, we have used a PEG-precipitated virus stock to improve the CHIKV RNA to PFU ratio. After titration on Vero E6 cells, the ratio ranged from 7632:1 in the non-purified stock to 68:1 in the PEG-precipitated stock. Clearly, even after this procedure, the number of CHIKV RNA molecules still strongly outnumbers the number of infectious particles. Subsequently, we repeated the qPCR-based measurement of virus binding at 4°C in the presence and absence of suramin (Fig. S2), for both non-purified and PEG-precipitated virus stocks. While for the former there was an unexpected increase in detected CHIKV RNA in the presence of suramin, the PEG-precipitated stock showed a slight decrease, confirming that the quality of the virus stock strongly influences the experimental outcome and conclusions.

3.2. Suramin-resistant CHIKV variants acquired mutations in the envelope protein E2
Repeated passaging of CHIKV in the presence of increasing suramin concentrations that do not fully inhibit replication (from 25 up to 300 μM) yielded variants that could grow to titers above 105 PFU/mL in the presence of 150 μM suramin. By passage 5 (P5) and passage 7, drug concentrations of 150 and 300 μM, respectively, were tolerated, concentrations that reduced wild-type virus titers by at least 2 logs [8]. Genotyping of the P5 virus revealed the

presence of three nonsynonymous mutations in the CHIKV nsP1, nsP3 and E2 proteins. Two passages later (P7), the suramin-resistant variant had acquired several additional mutations in the same 3 proteins (Table 1).

Table 1. Mutations in CHIKV resulting from serial passaging in the presence of increasing (suboptimal) concentrations of suramin.

Mutation in	**At P5 (150 µM suramin)**		**At P7 (300 µM suramin)**	
	Nucleotide substitution	Amino acid mutation	Nucleotide substitution	Amino acid mutation
nsP1	G588A	R171Q	G588A A979G G980A	R171Q T301K G302R
nsP3	U5645C	opal524R		
E2	A8554G	N5R	A8554G C8595A	N5R H18Q

To pinpoint which of these mutations is/are responsible for the suramin-resistant phenotype, they were all reverse engineered into our CHIKV full-length cDNA clone [24], either alone or in combination. For each of the reverse-engineered viruses, the plaque phenotype and sensitivity to suramin were determined and for several mutants the growth kinetics were also compared.

Viruses with the R171Q and opal524R mutations (CHIKV mutants S4.1, S4.3 and S5) were found to produce larger plaques on Vero E6 cells, suggesting they could be linked to cell culture adaptation. The apparent 'suramin resistance' (a maximum EC_{50} increase of 1.5-fold) of some of these mutants was more likely due to their accelerated growth and increased CPE, rather than to a specific resistance to the compound (Fig. 3A). Both R171Q and opal524R had been previously reported in CHIKV isolates such as MADOPY1, StBI and StVE (GenBank accession numbers KP003808.1, KP003811.1, and KP003810.1 respectively) which all had been passaged in cell culture prior to sequencing [33]. The mutation opal524R in the nsP3-coding region was reported also by Mounce *et al.* in the context of resistance selection against the compound DFMO [34], alongside with other nsP mutations. In the case of CHIKV and other alphaviruses (SFV, ONNV), evolutionary pressures have maintained both variants (stop and arginine codon) as it offers a fitness advantage when switching between vertebrate and invertebrate hosts [35-37]. The combination of the R171Q and opal524R mutations was also found independently in other CHIKV isolates (Kovacikova *et al*, manuscript in preparation), and it seems that these merely reflect adaptation to repeated passaging in mammalian cells.

In contrast, the T301K mutation in nsP1, causing a small-plaque phenotype, and the two mutations in E2 (N5R and H18Q) that did not alter the plaque phenotype (Fig. 2A), have to our knowledge not been previously identified in other isolates. However, the T301 residue was found to be changed to an I in the CHIKV 181/25 vaccine strain, that was attenuated by repeated

passaging in MRC-5 cells, and was accompanied by mutations T12I and G82R in E2, C42F in 6K and A404V in E1 [38]. It was later proven that T301I was not related to the attenuated phenotype of the vaccine strain, but that the two E2 mutations were responsible [39].

Reverse engineering of the N5R and H18Q mutations in E2 produced CHIKV variants with an increased tolerance to suramin (Fig. 2A), as an increase in EC_{50} was observed when both mutations were present (S5 and S9 in Fig. 2A). This suggested that the two E2 mutations cause the suramin-resistant phenotype, identifying E2 as the target of the compound.

A

CHIKV strain	Genotype	Plaque phenotype	EC50 (μM) +/- SD
wild type (WT)			60 +/- 0.7
S 2.1	nsP1: R171Q		65 +/- 34
S 2.2	nsP1: T301K		< 15
S 2.3	nsP1: R171Q nsP1: T301K		87 +/- 10
S 3	nsP3: opal524R		82 +/- 5
S 4.1	nsP1: R171Q nsP3: opal524R		83 +/- 15
S 4.3	nsP1: R171Q nsP1: T301K nsP3: opal524R		65 +/- 8
S 5	nsP1: R171Q nsP1: T301K nsP3: opal524R E2: N5R E2: H18Q		168 +/- 8
S 7	E2: N5R		104 +/- 0.7
S 8	E2: H18Q		95 +/- 19
S 9	E2: N5R E2: H18Q		105 +/- 21

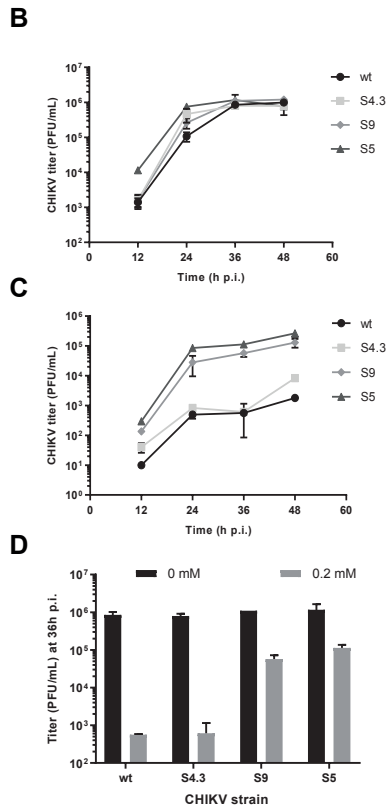

Figure 2. Characterization and suramin sensitivity of reverse engineered CHIKV variants. (A) Mutations identified in suramin-resistant CHIKV mutants (Table 1) were reverse-engineered (individually or in combinations) into infectious cDNA clone CHIKV LS3. Plaque morphology (in the absence of suramin) and EC_{50} (mean, n=4) for suramin are shown for each of the recombinant viruses. The values were determined from at least two independent experiments. For S2.2, the EC_{50} could not be determined because in this assay the mutant did not cause CPE with the same kinetics as the other strains. (B), (C) Replication kinetics of CHIKV mutants S4, S5, S9 and wt virus were compared during infection of Vero E6 cells in the absence (B) or presence of 0.2 mM suramin (C). At several time-points p.i., culture supernatants were harvested and infectious virus titers were determined by plaque assay (n=2). (D) Side-by-side comparison of the 36h p.i. titers of various mutants and wt virus grown in the absence (N.T., not treated) or presence of 0.2 mM suramin (n=2). All experiments were performed in Vero E6 cells and the data represent mean ± the SD.

The replication kinetics of relevant mutants, S4.3 (containing mutations in nsPs only), S9 (containing only the E2 mutations), and S5 (containing all mutations) were slightly faster than that of the wt control, reaching slightly higher titers by 24 h p.i., but similar titers by 36 h p.i. (Fig. 2B). Despite its faster replication, S4 is as sensitive to suramin treatment as the wt virus, with a 3-log reduction in titers upon treatment with 200 μM suramin. The S9 and S5 mutants, on the other hand, exhibited a titer reduction of only 1 log (Fig. 2C and D). These findings indicate that the nsP mutations (the S4 variant) were not involved in suramin resistance and that the E2 mutations alone were responsible for the resistant phenotype.

3.3. The N5R and H18Q mutations in E2 enhance CHIKV entry

To understand how the E2 mutations affect CHIKV infectivity in the presence of suramin, we analyzed the early steps (attachment, uptake, fusion) of infection, using a plaque number reduction assay in which virus and compound were present only during the first hour. We also reverse engineered a virus with a G82R mutation in E2, as this mutation was previously shown to render CHIKV fully dependent on HS binding for infection and is responsible for the attenuated phenotype of CHIKV vaccine strain 181/25 [13, 40]. Among all E2 mutant viruses tested, the G82R mutant was extremely sensitive to suramin, as its uptake and infectivity were almost abolished in the presence of 50 μM suramin, whereas this concentration merely caused a 40% reduction for wt virus (Fig. 3A). The S7 and S9 mutants were indeed more resistant to suramin than wt virus, as their entry was less inhibited by the presence of 50 μM suramin. In the presence of 200μM suramin, the uptake of wt virus, mutant S7 and mutant S8 was reduced to 40% of that of untreated cells, while the uptake of mutant S9 remained at ~60%, confirming that the presence of both E2 mutations leads to an increased resistance. The binding of mutant S9 at 4°C, however, was inhibited to the same extent by suramin as that of wt virus, suggesting the N5R and H18Q mutations do not offer resistance to the compound during the attachment step (Fig. 3B). This finding was corroborated by a time-of-addition assay where the pre-treatment of cells with suramin inhibited wt and mutant S9 to the same extent. However, if treatment was started at the moment of infection or later, the S9 mutant replicated better than the wt virus (supplemental figure S3). Therefore, it seems that the two mutations allow the virus to overcome the inhibitory effect of suramin at a post-attachment step, such as fusion.

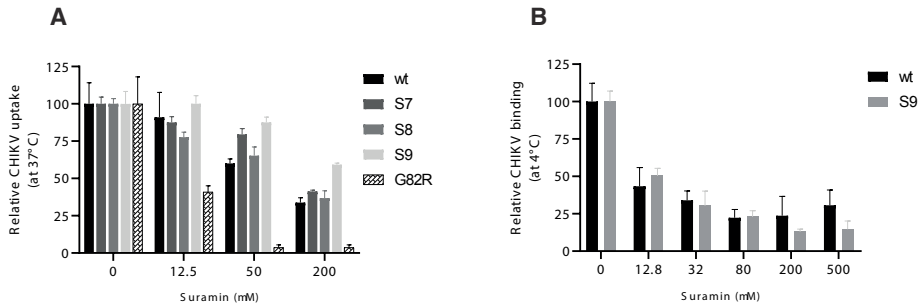

Figure 3. The effect of E2 mutations on suramin-resistance and the early steps of infection. (A) Virus uptake and infectivity were determined based on a PRNT-like assay. Approx. 100 PFU of wt CHIKV and mutants S7, S8, S9 and G82R were incubated with Vero E6 cells for 1h in the presence or absence of increasing suramin concentrations. Afterwards the inoculum was removed, the monolayers were washed with PBS and overlay medium was added. After a 3-day incubation, the cells were fixed and plaques were stained and counted. (B) The plaque number reduction assay was used to analyze synchronized attachment of wt CHIKV and variant S9 at 4°C, in the presence and absence of suramin. After binding for 1h in the cold, the inoculum and suramin were removed and replaced with overlay medium without suramin, and the rest of the procedure was performed as described under (A). The data represent the means ± the SD (n=3).

3.4. Molecular modelling predicts suramin to bind between two adjacent E2 proteins in a mature spike

The observations on the involvement of E2 mutations in suramin resistance prompted us to explore the interaction between the viral envelope protein and compound in more detail, by using a molecular docking approach. We employed a molecular docking approach using suramin and the E2 protein structure on its own, in the form of the E1-E2 heterodimer or as present within a mature CHIKV spike. Our initial attempts of docking suramin to an isolated E2 protein structure or to the E1-E2 heterodimer (PDB ID 3N42) revealed that the compound could interact with a region lacking a clear secondary structure (a groove between domains A and C and linked to the flexible N-terminal part of E2). The N5R mutation that was implicated in suramin resistance maps to this flexible region in the N-terminal domain of E2. Based on this predicted binding, the introduction of negative charges at position 6 and 160 (F6D, T160D) were expected to repel the binding of suramin to that area. To test whether this was indeed the case, these mutations were reverse engineered. The recombinant viruses had a plaque size similar to the wt CHIKV, but in CPE reduction assays they were more or equally sensitive to suramin, with EC_{50} of 54.5 µM (for F6D) and 16.7 µM (for T160D), compared to 55.5 µM of wt CHIKV. Previously Ho *et al.* have also docked suramin to the E1-E2 heterodimer and predicted that suramin would bind in a region between the two proteins [9].

In a more recent publication, a structure for the mature CHIKV spike was obtained by modeling the crystal structures of the E1 and E2 proteins into the cryo-EM image of a CHIKV VLP [41]. Due to this novel information, we now consider the earlier docking

studies to be less representative for the natural situation, since in the context of a virion the suggested suramin binding sites would not be exposed in mature spikes, which are formed by E1-E2 heterotrimers. Therefore, to refine our understanding of the possible electrostatic interactions between suramin and the surface of CHIKV, we employed a molecular docking approach based on the more relevant model of the envelope heterotrimer.

In this model, suramin (depicted in yellow) interacts with a very flexible loop in the N-terminal region of one E2 molecule, while it extends towards the middle of domain A of an adjacent E2 (Fig. 4A). Moreover, several suramin molecules are predicted to bind the same spike. Because domain A is involved in receptor recognition, as well as being the target of neutralizing antibodies, it has a pivotal role in the viral replicative cycle [14, 16, 18, 42].

By analyzing the charge distribution on the surface of the CHIKV spike, it became clear that the N-terminal loop of E2 harbors positive charges (K at positions 3 and 10). By acquiring the mutation N5R, there was a clear increase in the positive charges (differences indicated with black rectangles in Fig. 4C and D), probably leading to a stronger interaction with suramin and perhaps pulling it away from another area of the spike. Counterintuitively, because one would expect that a resistance mutation would prevent the interaction with suramin, we speculate that the N5R mutation actually attracts the compound thereby changing the binding mode. Perhaps the N5R mutation directs suramin away from the center of domain A, which is known to be involved in receptor recognition and is also the target of neutralizing antibodies [15, 18].
According to our molecular modelling prediction, the H18Q mutation, which is located in a region across from the E1 fusion loop, might enhance the effect of the N5R mutation by stabilizing the flexible N-terminal loop to achieve a better interaction with the compound or could cause other unpredicted structural changes in the heterotrimer leading to a decreased affinity for suramin. Additionally, H18Q could also facilitate fusion by aiding in the conformational rearrangements required for exposure of the fusion loop. Combined, the N5R and H18Q mutations might change the binding geometry of suramin, perhaps sidetracking it from the core of the E1/E2 heterodimer/spike and/or facilitating fusion (Fig. 4C).

Our hypothesis that suramin is attracted to the center of domain A is further supported by the observation that the CHIKV variant that is completely dependent on heparan sulfate interactions for infectivity [13, 40] has a G82R mutation in E2. In CPE-based assays, the G82R mutant is more sensitive to suramin (EC_{50} <15 μM) than wt CHIKV. Interestingly, residue G82 is located at the center of the spike, where the A domains of the three E2 subunits are found (highlighted in red in Fig. 4A), and maps to the area that interacts with MXRA8,

a receptor which was recently found to promote CHIKV entry [18]. The introduction of a positive charge at position 82 of E2 could increase the affinity for suramin at the center of the spike and block cell attachment.

These results suggest that suramin has more impact on the infection when it is attracted to the center of the spike, while more distant positive charges could direct it away from this important region, allowing attachment and interaction with specific receptors, which could explain why the observed the suramin-resistance mutations emerged.

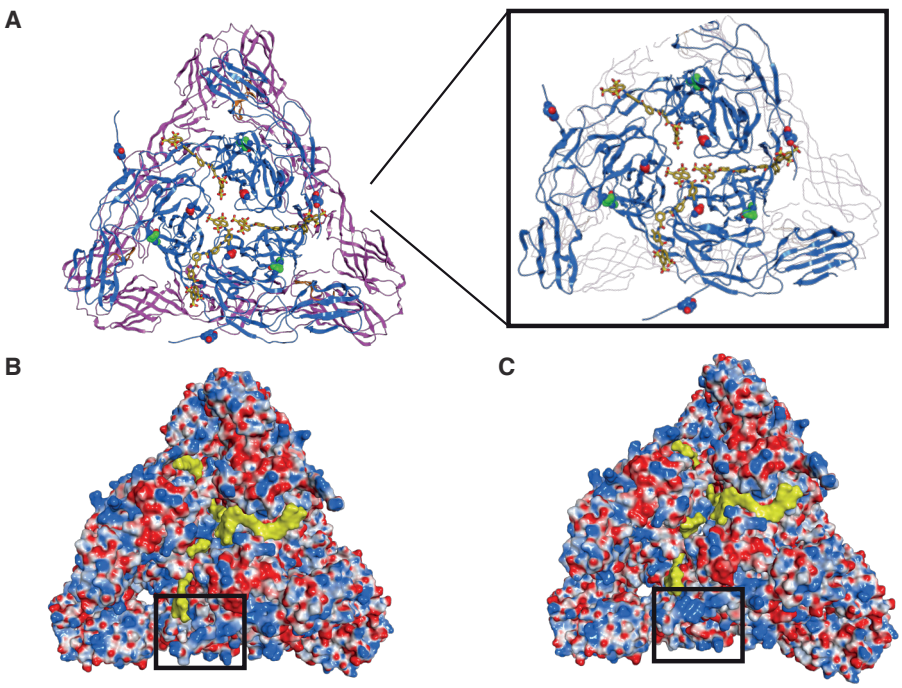

Figure 4. Molecular docking of suramin to a mature CHIKV spike. (A) The top view of a full wt E1-E2 heterotrimer (PDB ID 3J2W). The E2 proteins are represented as blue ribbon, the E1 as purple ribbon and the fusion loop as orange ribbon; the N5 and H18 residues are represented with carbon atoms in blue and green, respectively, and residue G82 with carbon atoms in red, belong to E2. Suramin is represented in yellow (3 molecules, carbon atoms and molecular surface). In the right black rectangle there is a clearer view of the spike core. (B) Electrostatic potential (Coulombic surface coloring) of the heterotrimer of wt CHIKV. The black rectangle marks the N-terminal domain of one E2 protein, where the positive charges are found. (C) Electrostatic potential (Coulombic surface coloring) of the heterotrimer of the N5R/H18Q mutant, CHIKV S9. The black rectangle highlights the N-terminal domain of E2 showing an increase in positive charges (blue molecular surface). For presentation purposes, the transmembrane and C-terminal segments of the E1 and E2, which interact with capsid proteins seen in (A), were removed.

4. Conclusions

The aim of our study was to understand how suramin inhibits the early steps of the replicative cycle of CHIKV and other alphaviruses. We have shown that suramin can interact with CHIKV *in vitro*, and inhibits attachment of the virus to the host cell. Moreover, it could prevent conformational rearrangements in the viral spike glycoproteins that are required for fusion. We were able to select suramin-resistant CHIKV variants and demonstrated that the N5R and H18Q mutations in E2 were responsible for resistance. These mutations did not offer the virus a major advantage during the binding to cells in the presence of suramin. The benefit of these mutations appears to play a role in overcoming suramin's inhibitory effects during later stages of entry, perhaps allowing the suramin-bound (mutant) spikes to undergo conformational changes required for fusion and progression of the infection. Although CHIKV is able to acquire resistance mutations to the compound, suramin is still an interesting drug candidate as the level of resistance is rather low and required repeated passaging. Additionally, suramin protected human primary dermal fibroblasts from CHIKV-induced CPE with an EC_{50} of approx. 95 μM , proving its efficiency in a more relevant cell model for arbovirus infection.

Regarding its use in humans, suramin could be explored as prophylactic in the context of an outbreak, since it is a compound with one of the longest known half-lives in humans [43]. In previous clinical studies, concerning the treatment of AIDS and certain types of cancer, multiple and serious side effects were attributed to the long-term use of suramin [44]. However, this concerned seriously ill patients and long-term treatment. Such an extended regimen is not required for the treatment of parasitic infections for which suramin has proven to be effective and much better tolerated. Therefore, we believe that also for the short-term treatment or prevention of chikungunya virus infections, suramin would be an interesting drug to evaluate.

5. Acknowledgements

We thank Laura A. Wolters for technical assistance and Dr. Igor Sidorov for bioinformatics advice. We would also like to thank Dr. Adriaan H. de Wilde for fruitful discussions and feedback. SF and AB acknowledge support from the Life Science Research Network Wales grant no. NRNPGSep14008, an initiative funded through the Welsh Government's Ser Cymru program. ICA, EJS, MJvH acknowledge the support of the EU-FP7 EUVIRNA grant (No. 264286).

Appendix 1 – Supplemental materials and methods

Molecular modeling – in depth description of section 2.10

All molecular docking studies were performed on a Viglen Genie Intel®CoreTM i7-3770 vPro CPU@ 3.40 GHz x 8 running Ubuntu 14.04. Molecular Operating Environment (MOE) 2018.10 [25] and Maestro [26] were used as molecular modelling software. The CHIKV E2-E1 glycoprotein heterodimer and trimeric complex structures were downloaded from the PDB data bank (http://www.rcsb.org/; PDB code 3N42 and 3J2W). The proteins were preprocessed using the Schrödinger Protein Preparation Wizard by assigning bond orders, adding hydrogens and performing a restrained energy minimization of the added hydrogens using the OPLS_2005 force field. The missing N-terminal residues at the start of the E2 protein (residues 1-6) were manually introduced and energy minimized. Suramin structure was built with MOE and then prepared using the Maestro LigPrep tool by energy minimizing the structures (OPLS_2005 force filed), generating possible ionization states at pH 7±2, generating tautomers and low-energy ring conformers. SiteMap tool in Maestro was used to individuate a potential binding area in proximity of the important mutations, N5R and H18Q. The selected area was used as binding site for the molecular docking studies. For the trimeric complex, three different, one for each heterodimer, 36 Å docking grids (inner-box 10 Å and outer-box 46 Å) were prepared using as centroid a threonine residue localized at the spatial center between the N5 and H18 of two adjacent E2 subunits. In the case of the isolated E2 unit, the same binding area for grid generation was defined selecting as centroid a dummy atom, manually positioned at equal distance from N5 and H18. Molecular docking studies were performed using Glide SP precision keeping the default parameters and setting 5 as number of output poses per input ligand to include in the solution. The output database was saved as mol2 file. The docking results were visually inspected for their ability to bind the active site. The electrostatic potential surface was obtained using the Surfaces and Maps toll in MOE after splitting the molecule in multiple chains. Figures were prepared with MOE.

Appendix 2 – Supplemental figures S1, S2, S3

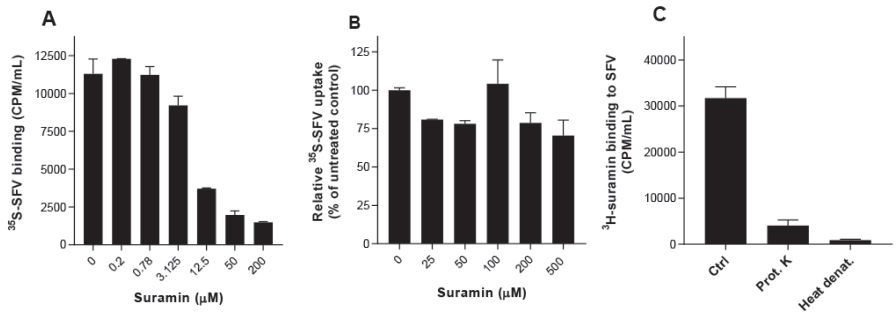

Figure S1. Effect of suramin on the early events of SFV infection. (A) Binding of ^{35}S-labelled SFV to Vero E6 cells, at 4°C, in the presence of 4-fold serial dilutions of suramin. (B) after a 30-min pre-attachment of virus at 4°C in the absence of the compound, the unbound particles were removed by washing with PBS. Subsequently, medium with suramin was added to the cells followed by incubation at 37°C for 30 min. Cell-associated radiolabeled virus was quantified by cell lysis and liquid scintillation counting. (C) ^{3}H-suramin was incubated with purified SFV (in the absence of serum proteins) followed by treatment with proteinase K for 15 min at 37°C or by heat denaturation for 5 min at 95°C, followed by quantification of suramin binding as described for panel B. The average +/-SD is shown; n=2.

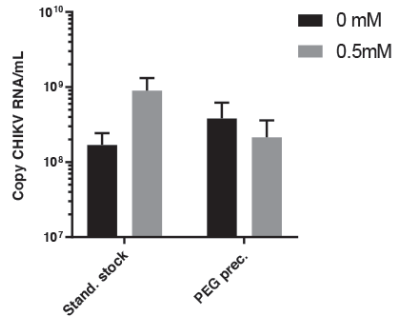

Figure S2. Effect of suramin on CHIKV uptake analyzed by RT-qPCR. The effect of treatment with 0.5 mM suramin during viral uptake (MOI=1) using either a non-purified, standard CHIKV stock (Stand. stock) or a PEG-precipitated stock of the same virus (PEG prec.). After 1h p.i. at 4°C the compound and inoculum (which had been added simultaneously) were washed away with PBS and the CHIKV RNA copy numbers in the cell lysates were analyzed by RT-qPCR (n=2).

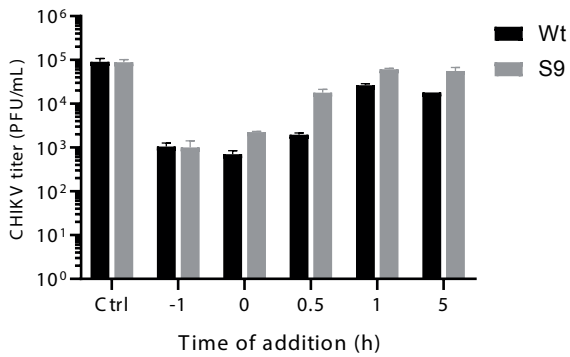

Figure S3. The effect of E2 mutations on suramin-resistance analyzed through a time-of addition assay. Replication of wt and S9 in the presence of 400 μM suramin, added at various time points prior and after the start of the infection, was determined by measuring the titer of infectious CHIKV at 10 h p.i.

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Chapter 6

General discussion

CHIKV is a serious human pathogen for which antiviral drugs are still not available. The research described in this thesis aimed to advance our knowledge on CHIKV replication and to contribute to the development of much-needed inhibitors of CHIKV infection. Following the development of an *in vitro* assay to study CHIKV replication, this tool was used to study the mode of action (MoA) of antiviral compounds (chapter 2) and suramin was identified as a potent inhibitor of viral RNA synthesis. However, we discovered that in cell culture, suramin's antiviral activity was mainly due to inhibition of CHIKV binding/entry, and to a lesser extent virus release (chapters 3 and 5). Suramin was also found to inhibit binding/entry and virion biogenesis of Zika virus (ZIKV), a recently emerged flavivirus that caused massive epidemics and serious manifestations, such as malformations in newborns and Guilain-Barre syndrome in adults (chapter 4). Due to its ability to form electrostatic interactions with positive charges on proteins, suramin may block the contact between virions and their (co)receptors, by interacting with either virus or receptor, or with both. In chapter 5, using radioactively-labelled suramin, it was clearly shown that the compound interacts with CHIKV and SFV particles, more specifically with their envelope proteins. Additionally, suramin could interfere with cell attachment and/or the structural changes required for fusion. Suramin-resistant CHIKV variants were selected, which contained mutations in the E2 envelope protein (involved in receptor interactions), supporting the idea that suramin blocks the early steps of the infectious cycle. Below, these findings are further discussed in the context of findings related to other viruses that are inhibited by suramin.

1. An *in vitro* system for CHIKV replication and its applications

The goal of this research project was to gain more insight into the replication mechanism of CHIKV and to develop assays for identifying inhibitors of CHIKV RNA synthesis.

To develop *in vitro* assays for CHIKV RNA synthesis, two approaches were followed. The first concerned the reconstruction of active complexes using recombinant nsP4, the viral RNA polymerase, expressed in and purified from bacteria, and a preparation of nsP123 isolated from mammalian cells. The second approach concerned the (semi)purification of membrane-associated viral replication and transcription complexes (RTCs) from CHIKV-infected cells. The activity of such complexes was tested in assays that measure the incorporation of radiolabeled CTP into viral RNA products.

The first approach seemed promising as it had been described for Sindbis virus (SINV) nsP4. Initially Tomar and collaborators were able to purify only D97nsP4 (the core catalytic domain), because the full-length protein was prone to degradation. However, the truncated protein only showed *in vitro* terminal adenylyl-transferase (TATase) activity (1). The N-terminal domain of nsP4 is very important for interactions with the other nsPs to control RNA synthesis (1, 2), but the purification of full-length nsP4 is challenging because of its instability, which is caused by the N-terminal Tyr residue that renders it a target for rapid

proteasomal degradation via the N-end rule pathway (3). These issues could potentially be avoided by expressing nsP4 N-terminally linked to another protein that can be removed after the purification step. Using 6xHis-SUMO as the N-terminal tag, Rubach *et al.* were successful in purifying full-length SINV nsP4 in significant amounts, without reporting any proteolytic degradation (4). They were able to show that nsP4 retained the TATase activity, and additionally had *in vitro* -RNA synthesizing activity, when supplemented with a BHK-21 cell membrane fraction containing the polyprotein nsP123 (in a form that could not be cleaved internally).

We have expressed nsP4 of CHIKV, in a recombinant form preceded by 6xHis-SUMO. This protein could be purified and also displayed terminal transferase activity in an *in vitro* assay (conference abstract (5)). Unfortunately, the expression and purification procedures did not yield sufficient amounts of pure CHIKV nsP4 for extensive characterization in enzymatic assays (or crystallization studies), due to massive degradation and insolubility. In the future, other nsP4 expression systems should be explored, e.g. baculovirus-driven expression in insect cells, which could be considered a more natural situation for the expression of arbovirus proteins, than bacteria. More recently, a truncated version of CHIKV nsP4 (nsP4-D118) was purified from bacteria, but the characterization of its *in vitro* TATase activity and detergent tolerance did not provide new information with respect to what had been described for SINV 13 years earlier (6).

While these earlier studies (1, 4) were very useful for optimizing the purification strategies for alphavirus polymerases and understanding the minimal requirements for their *in vitro* activity, they did raise some major concerns, since they relied on T7 RNA polymerase driven expression. As pointed out by Lehmann *et al.*, T7 RNA polymerase can be a notorious contaminant in this type of assays, which can lead to false-positive results (7). Although the active site mutant nsP4 was also tested, controls using 'empty bacteria', only expressing the T7 RNA polymerase were lacking in the papers cited above.

Due to the unsolved technical issues and problems with the 'reconstitution system' based on pure nsP4 and nsP123, we decided to (semi)purify active RTCs from CHIKV-infected cells. This strategy had already been successfully applied in our laboratory for several nidoviruses (8, 9). An *in vitro* system for studying the RNA synthesis of SINV had also been developed, but the experimental design heavily relied on the T7 RNA polymerase-driven expression. As mentioned above, those results should be interpreted with caution, since proper controls such as lysates from cells infected with recombinant vaccinia viruses expressing only the T7 RNA polymerase were lacking (10).

Chapter 2 describes in detail the purification of CHIKV RTCs from infected mammalian cells, the optimization of an *in vitro* replication assay (IVRA) to study RNA replication and its application to evaluate (direct acting) inhibitors.

CHIKV RTCs were harvested at a timepoint (6 h p.i.), when they were most active in +RNA synthesis (genomic and subgenomic RNA). Newly synthesized RNA was detected by the incorporation of ^{32}P-CTP and reaction products were stable in this system, being protected from the activity of cytoplasmic nucleases perhaps due to association with cellular membranes (11), capsid proteins or polysomes. This was concluded after comparing the half-life of the RNA already present in the isolated RTCs with an RNA transcript introduced in the system, which was rapidly degraded. In addition, it has also been shown that newly introduced RNA templates are not used by pre-assembled SFV replication complexes, possibly due to their sequestration in spherules (12).

Surprisingly, in addition to the CHIKV genome and subgenomic RNA, another +ssRNA molecule was consistently detected (see figure 1 below), both in infected cell lysates as well as after synthesis of CHIKV RNA in the IVRA. This newly (re)discovered RNA species, termed RNAII, corresponded to the 5'-proximal ~7.5kb of the genome, up to the subgenomic promoter region (Psg). An RNA similar to the one we found for CHIKV has been described for SINV in 1997 and was named RNAII, as it is part of the replicative form II (RFII) (13). Earlier publications from the 1970's on SFV and SINV also mentioned the presence of other ssRNA besides the genome and subgenomic mRNA (14, 15). When we examined figures in publications from the late 1980's (16), we could also observe RNAII, although it was misidentified in the text. RNAII is likely visible in several other older publications, but often was ignored or mislabeled. A recent example is a publication on the importance of non-structural polyprotein processing and nsPs for SINV pathogenesis, in which the authors present pictures of gels (Fig 3 C, D) in which RNAII is clearly present (separated from the genomic and subgenomic RNA), but not indicated (17). In addition, in the same paper, another SINV-specific RNA can be noticed below the sgRNA, which becomes more abundant later in infection. We have observed a similar RNA in CHIKV-infected cells (Chapter 2, fig 1a), but this species is not detected as a product of the IVRA, although this might be due to its small size and limited incorporation of radiolabel (below the detection level).

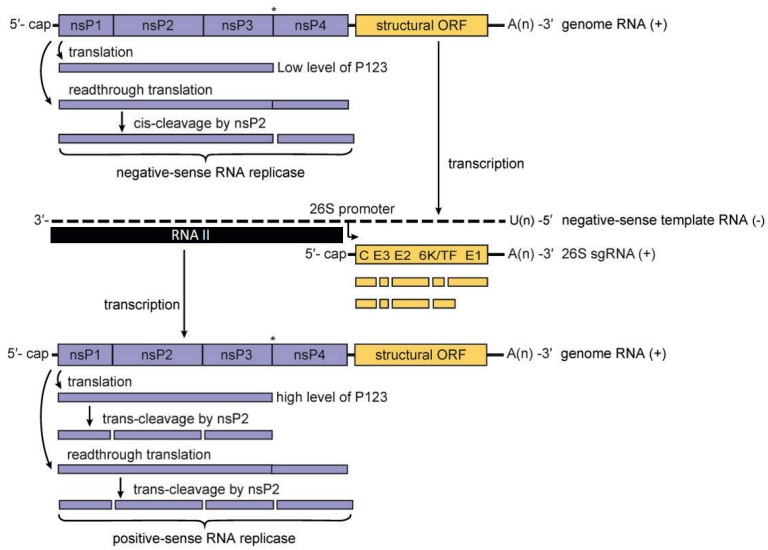
The role of RNAII in alphavirus replication has not been investigated in detail. It could be merely a byproduct from complexes that were engaged in genome synthesis and became blocked/stalled when reaching a region of active transcription at the subgenomic promoter region. From an evolutionary point of view, this assumption does not appear to make sense, considering how well-regulated the rest of the replication cycle is. It also remains to be determined if RNAII is capped and/or polyadenylated, and whether it may function as an mRNA for nsP synthesis.

I would favor the idea that RNAII is produced in order to drive the predominant synthesis of sgRNA at later time points in infection, by forming a dsRNA region in the preceding 5' part, hence directing the RTCs to only transcribe the single-stranded region of the negative sense RNA. Of course, this raises many questions worthy of investigation. For example, is

RNAII present in comparable amounts as -RNA? Which type of RTC is responsible for its synthesis? Could it perhaps be the short-lived nsP1-nsP23-nsP4 complex? Another open question is whether RNAII is exported from the spherules that contain the RTCs, since all other +RNAs are released into the cytosol. Whether the synthesis of RNA II is indeed connected to the production of sgRNA could be explored using seco-pregnane steroids, which have been shown to specifically block sgRNA synthesis for SINV and other viruses (18).

In order to investigate the nucleotide requirements of several CHIKV fidelity mutants, another research group has applied an *in vitro* assay somewhat similar to ours, which relied on ^{32}P-UTP incorporation (19). However, their assay optimization is not described and genomic RNA synthesis was hardly visible. This is making it difficult to reliably assess whether genomic and subgenomic RNAs are truly produced de novo in this system, and that the incorporation did not merely result from 'end labeling' of pre-existing RNA molecules. The fact that these authors did not observe RNAII raises further concerns about RTC activity and reaction products. Because of the discrepancies between the results obtained with the two assays, it is important to make a comparison concerning how they were designed to understand where the dissimilarities might stem from. Stapleford *et al.* harvested RTCs at the moment when – in our hands - they are hardly active anymore. Prior to setting up the IVRA, we first used metabolic labeling to determine when RTCs were most active and harvested at that particular moment. Also, why the authors used an MOI of only 1 for infection and then harvested the RTCs at 16 h p.i. is not clear. We used high MOIs to achieve a synchronized single-cycle infection and thus maximize the quantity of active complexes. The choice of [α^{32}P]-UTP as the radiolabeled nucleotide in the IVRA of Stapleford *et al.* is also questionable, since this can produce false-positive results due to UTP incorporation driven by host terminal transferases (8), which is why we opted to use radiolabeled CTP in our assays. Lastly, it is unclear why the authors chose to separate the isolated RNA in non-denaturing agarose gels, as it gave poor and uncertain results, due to the absence of loading controls. Though the work of Stapleford *et al.* extends beyond their *in vitro* CHIKV replication assay, it is regrettable that they did not consult the recent literature concerning this topic.

The observations concerning RNAII underline that the replication of alphaviruses is more complex than presented in most articles and text books. Therefore, further investigation is required as the potential role of the additional RNAs in the replication cycle, pathogenesis and dissemination in the insect and mammalian host remains unclear.



Fiure 1. Schematic representation of CHIKV replication, updated to include RNAII.

Besides the opportunity to study more fundamental aspects of CHIKV RNA synthesis in depth, our IVRA can also be used as a tool to evaluate CHIKV inhibitors, e.g. to determine whether they are active in their present form and do not require further metabolic processing (assuming this step would be performed extracellularly or in living cells). Additionally, the IVRA could be used to determine if compounds (e.g. identified by cell-based screening) directly affect viral RNA synthesis, by targeting one or more proteins of the RTC or its co-opted host factors. The assay would also allow for screening of compounds that are cytotoxic or poorly taken up by cells, obviously followed by medicinal chemistry-driven efforts to improve their activity and obtain derivatives suitable for use in cells, and ultimately humans.

2. Suramin inhibits the activity of CHIKV RTCs *in vitro*

Using our IVRA, the first non-nucleosidic compound identified as an inhibitor of CHIKV RNA synthesis was suramin, a compound marketed for the treatment of parasitic infections. It also inhibited the activity of SINV and SFV RTCs, as well as the activity of RTCs isolated from cells transfected with CHIKV RNA replicon-s, with a similar IC_{50} as found for the complexes obtained from infected cells (see Chapter 3). The major advantage of using an IVRA based on replicon RTCs is the absence of virus particles, which thus offered a biosafe alternative for studying the structure-activity relationship of several suramin-related compounds. Based on this assay, we could conclude that the symmetry of the compound and the presence of a high number of sulfate groups (negative charges) are a pre-requisite for inhibition of CHIKV replication. Interestingly, using the replicon

RTCs-based IVRA, we could also show that suramin did not compete with NTPs, and that its inhibitory mechanism appeared to be the blocking of re-initiation of RNA synthesis (on negative strand templates).
Initially, suramin was identified in a molecular docking screen for compounds that bind to the active site of the norovirus RdRp (20), which represents a conserved region among the polymerases of RNA viruses. This was the main reason why it was chosen for testing in the IVRA, and its inhibitory effect on RNA synthesis, presumably by blocking the RdRp of CHIKV (nsP4), was confirmed (Chapter 3). Because suramin can interact with many positively-charged (RNA-binding) protein surfaces, many other viral or host proteins could (also) be the target of suramin (summarized in the introduction of Chapter 3 and discussed in more details below, at point 5). In the IVRA, an (additional) effect on nsP2, which has NTPase, 5'-RNA triphosphatase and helicase activity cannot be excluded, as suramin was also shown to inhibit HCV NS3 helicase activity (21). The NTPase and 5' RNA phosphatase activities of CHIKV nsP2 depend on a pH of 7 or higher and the presence of Mg^{2+} and NTPs (22), conditions which are met in our IVRA and would therefore support these activities. Thus, it is highly possible that suramin targets both nsP4 and nsP2, while inhibiting CHIKV RNA synthesis.

Furthermore, it is important to exclude that suramin binds and inhibits the activity of creatine phosphokinase (CPK), which is part of the crucial energy regenerating system (relying on creatine phosphate and creatine phosphokinase). Using a separate assay, to monitor only the activity of CPK in the presence of suramin (a typical IVRA reaction, but without the RTCs), we could confirm that suramin does not inhibit the synthesis of ATP by the energy regenerating system (results not shown). Hence, the CP/CPK system is not among the targets of suramin in the IVRA.

Results obtained using IVRAs should obviously be confirmed in cultured cells or *in vivo* (if it is known). For example, certain nucleoside analogs, even if supplied in their active form (i.e. tri-phosphorylated), may not show an inhibitory effect on RNA synthesis in an IVRA, because they exert a dual effect in living cells on both host and virus. For example, ribavirin triphosphate can inhibit virus replication indirectly by reducing GTP pools (23, 24), but also by being incorporated into the newly produced RNA and causing lethal mutagenesis (25).

3. In cell culture, suramin inhibits multiple steps of the CHIKV replication cycle
To confirm the inhibitory activity of suramin that was found in the IVRA, we analyzed its effect in cell-based assays for CHIKV infection. Using a dose-response assay we confirmed that suramin inhibits CHIKV replication in a dose-dependent manner, as reduced RNA and protein levels were observed, and a decrease in infectious progeny was observed. These observations were validated in parallel by another group, using CHIKV strain S27 in

BHK21 cells (26). Their work supports part of our results presented in chapters 3 and 5, and therefore strengthens our conclusions. The following paragraphs summarize our results from Chapters 3 and 5 and compare them with the findings of Ho *et al.*

In Chapter 3, suramin was shown to have an antiviral effect in CPE reduction assays with several CHIKV isolates and related alphaviruses SINV and SFV. Ho *et al.* have performed a similar analysis with several CHIKV strains (S27 and three Asian strains from Singapore, Indonesia, and Malaysia) on BHK-21, U2OS, and MRC-5 cells. Their EC_{50} estimations were much lower than ours, which might be due to the strains used in their study. However, the discrepancy is more likely caused by their viability assay which is based on crystal violet staining and OD measurement, which in our experience is less accurate than colorimetric cell viability assays like the MTS assay. Surprisingly, the authors did use such a commercial WST-1 viability assay to determine the CC_{50} of suramin, but for unclear reasons did not use the same assay in the CPE reduction assay to determine the EC_{50}. EC_{50} and CC_{50} determination should be performed in parallel and with the same assay. to avoid discrepancies and inconsistencies.

To identify the step of the replication cycle that is targeted by suramin, time of compound addition experiments were performed. Despite some differences in experimental setup and readout, both our experiments (Chapter 3) and those by Ho *et al.* demonstrated that suramin blocks CHIKV replication more efficiently when it is used prior to infection or at the moment the virus is added to the cells. These results imply that suramin might interfere with virus binding/entry, e.g. by blocking the interaction with receptor or co-receptor (by binding to the sites on the virus surface required for receptor recognition/interaction, or vice versa, by saturating the virus-binding sites on the receptors). In addition, Ho *et al.* have shown that suramin treatment at 2 or 6 h p.i. did not affect intracellular levels of CHIKV RNA, while the extracellular levels were 1 log lower, suggesting that suramin also interferes with the release of newly formed particles. In contrast to the effect on RNA synthesis found in the IVRA, suramin mainly inhibits an early step of the replication cycle in cell-based assays. We could demonstrate some effect on RNA synthesis also in infected cells, but this would normally be overshadowed by the early effect on binding/entry. One complicating factor is that the large size and negative charges of suramin likely will hamper its cellular uptake (27). Because of the anticipated low intracellular concentration, suramin will most likely not have a direct effect on the activities of the nsPs in the infected cell. In infected cells the effect on RNA synthesis is limited, compared to the major effect suramin has on the entry step – either by inactivating the virus, blocking its attachment to the cells surface or fusion with the host membranes or several of these processes.

We demonstrated that ^{3}H-suramin binds to intact CHIKV and SFV particles, more specifically to the proteins on the envelope surface (chapter 5). We have shown that this binding does not have a virucidal effect and this was confirmed by Ho *et al.* Therefore, we

presume suramin does not bind to virus particles irreversibly, and most likely the compound will dissociate in media supplemented with FCS, as serum albumin was shown to have a high affinity for suramin (28). Using a direct approach based on radiolabeled or fluorescent CHIKV, we demonstrated that suramin inhibits virus attachment to cells (Chapter 5), in assays performed at 4°C, a condition that would prevent endocytosis. Our findings were supported by those of Ho *et al.*, who have used a PRNT-based assay. Consequently, virus attachment is at least one of the steps that is targeted by suramin. In Chapter 5, a bulk fusion assay was employed, which revealed that suramin also inhibits fusion with liposomal membranes. This might be because the compound blocks the structural changes required for the two membranes to fuse. Our experimental setup allowed a more direct, specific measurement of fusion, compared to the insect cell-based expression of structural proteins used by Ho *et al.*, which demonstrated that suramin appeared to inhibit low pH-induced cell fusion (similarly to a neutralizing antibody). In conclusion, the interaction of suramin with CHIKV appears to interfere with attachment as well as fusion.

Ho *et al.* also described a minor inhibitory effect of suramin on CHIKV release, and they have shown that high suramin concentrations block the release of virus, without affecting intracellular viral RNA levels. These authors claimed that virus budding was affected, leading to reduced extracellular transmission. An alternative explanation would be that the newly released particles are less infectious, perhaps by suramin inhibiting the proper maturation of structural proteins during their post-translational trafficking along the secretory pathway. In support of this idea, the activity of several lysosomal enzymes is inhibited by suramin (29, 30), which was also shown to accumulate in other low-pH cellular compartments with a low pH, such as the trans-Golgi network (31, 32).

We have used molecular modeling to identify potential suramin binding sites on the virion surface. In Chapter 5, we predicted that one heterotrimeric CHIKV surface projection could bind several suramin molecules towards its top. The ligand would stretch from the N-terminal disordered region of one E2 protein towards the middle of domain A of an adjacent E2 molecule. In this manner, one arm of suramin, with its negative charges, blocks the region of domain A that interacts with glycosaminoglycans (GAGs) or specific receptors. This region was recently found to be the target of two neutralizing antibodies (NAbs) that block virus entry and egress, and for which escape mutations at residues W64 and G95 were found (33). The W64G substitution in E2 offered an escape from neutralization by both NAbs, but left the virus with an attenuated phenotype in mice.

Ho and collaborators have also used molecular docking in an attempt to predict suramin binding sites, but they have modelled suramin on a single E1-E2 heterodimer and consequently their results predict suramin to bind to a region between the two proteins

that is not exposed to the environment in the actual trimeric surface projection/spike. Therefore, it is unlikely that the surface they suggest to be the suramin-binding site will have much relevance for the inhibition of virus entry.
In Chapter 3, we concluded that suramin did not block virus attachment to the cell surface, while in Chapter 5, the results obtained with the radiolabeled or fluorescent CHIKV clearly demonstrated inhibition of attachment. This apparent discrepancy was due to the fact that we initially used an indirect measurement, the quantification of viral RNA by RT-qPCR to measure bound virus particles, whereas in Chapter 5 we relied on direct measurements of labelled viruses. The problem with the RT-qPCR-based method can be attributed to the commonly used virus stocks, which have genome copy to PFU ratios that are over 1000:1, due to the moment of harvesting when extensive CPE has occurred and intracellular viral RNA has likely been released into the medium as well (our own observations and those of (34, 35)). Besides providing a more direct measure for the binding of intact, envelope-labelled viruses, the ^{35}S-virus preparations are further purified to remove unincorporated label and other contaminants (naked RNA, nucleocapsids). Thus, we assume that the lack of effect found in Chapter 3, was due to this technical issue (i.e. the bulk of detected RNA did not represent infectious particles) that masked the inhibition of binding by suramin. We therefore think the experiments with labeled virions represent the actual situation more accurately and concluded that suramin does inhibit virus attachment. This was corroborated by additional RT-qPCR based experiments with improved purification of virus stocks, which however still had RNA copy:PFU ratios of ~80:1, but did reveal a modest inhibitory effect of suramin.

While binding experiments with radiolabeled CHIKV at 4°C clearly demonstrated that suramin inhibited attachment, we obtained more puzzling results when we studied the effect of suramin on virus binding and uptake at 37°C (results shown in the Appendix, left side). At this higher temperature, we found an increased amount of radiolabeled envelope proteins at 1 h p.i. in lysates of cells treated with suramin compared to untreated cells. Because the cells are metabolically inactive at 4°C, we suspect that suramin then blocks the electrostatic interactions between CHIKV and GAGs, the negatively charged co-receptors/attachment factors, which would lead to release of virus during the washing steps. Under physiological conditions (37°C), when endocytosis occurs, treatment with suramin might not inhibit attachment so strongly, because the viral attachment dynamics are much faster at this higher temperature. At 37°C suramin might have an inhibitory effect later in the infectious cycle, for example on the fusion step in the endosome.

In infected cells, the envelope proteins of radiolabeled virions display a clear degradation pattern when total cell lysates are compared at 1 and 3 h p.i. This might be due to endosomal degradation of virion-associated envelope proteins or degradation of post-fusion envelope

proteins that ended up in membranes that would further progress through the endosomal pathway. However, these proteins remained stable in suramin-treated samples. There are four possible explanations for these observations:
CHIKV particles remained attached to the plasma membrane and did not enter via endocytosis, thus remaining resistant to degradation in the endo-lysosomal pathway.
CHIKV particles reached the endosomes, but the envelope proteins were not degraded because suramin inhibited the responsible proteases or blocked endosomal maturation.
CHIKV particles were taken up by endosomes, but their conformation was 'frozen' by suramin, preventing membrane fusion and rendering the envelope proteins resistant to degradation.
With the bold assumption that, once in the endosome, CHIKV envelope projections require enzymatic cleavage in order to expose the fusion loop (as is the case for the spike proteins of coronaviruses), suramin might block the endosomal enzyme required for the fusion step.

4. Mutations in the CHIKV E2 protein lead to suramin resistance

To determine the target of suramin, we have selected for suramin-resistant CHIKV variants. The reverse genetics studies described in Chapter 5 demonstrated that the mutations N5R and H18Q in the E2 protein were both responsible for suramin resistance. These mutations are in regions of E2 that are not highly conserved between viruses of the SFV clade. To our knowledge, these were the first reported mutations that cause some resistance to suramin for any virus. The N5R mutation is located in a flexible loop with a nearby positive charge (K3), and the R could potentially orient itself towards areas/ligands with negative charges. The H18 residue is not surface exposed at all and is thought to be involved in E2-E1 contacts in the p62/E1 crystal, together with the preceding amino acid, L16 (36).
Individually, the suramin-resistance mutations offer little resistance, but when combined, the S9 virus performs better in the presence of suramin in CPE and PRNT-like assays, and it also shows improved replication kinetics. The N5R and H18Q mutations of S9 did not offer the virus an advantage during the attachment step, but apparently offered an advantage at a later stage of entry, perhaps during fusion of the viral envelope with the host membrane.

Besides the E2 mutations that were shown to be responsible for suramin-resistance, mutations in several nsPs were also detected after repeated passaging of CHIKV in the presence of the compound. These mutations, R171Q and T301K in nsP1 and the opal-R in nsP3, as discussed in chapter 5, seem to be merely non-specific cell culture adaptations. In a study concerning SINV, repeated passaging of the virus in BHK-21 cells gave rise to adaptive mutations, mainly in E2 (S1R, D70K and S114R), which rendered the virus attachment dependent on heparan sulfate (HS) for infection (37). However, in an assay with liposomes containing lipid-conjugated heparin there was no difference in the low pH-induced fusion activity of wt virus and the adapted heparin-binding virus (38). These

findings indicate that whether SINV strains interact with HS or not at neutral pH, they are all capable of fusion with membranes under acidic conditions and perhaps this could be a characteristic of alphaviruses.

While analyzing mutations in CHIKV E2 that were previously reported in the literature, we discovered that the G82R mutation in E2 makes the virus dependent on HS (39). G82R, the most important factor that causes attenuation of CHIKV 181/25 (40), is the same mutation that was independently selected for in MRC-5 cells treated with the antiviral compound arbidol, which is marketed as a broad-spectrum antiviral drug for the treatment of respiratory infections (41). This was likely not noted before as the authors referred to this mutation as G407R, based on the amino acid numbering of the full-length structural polyprotein instead of E2 alone. We were unable to reproduce the results of Delogu *et al.* in our CPE-reduction assays, and found that arbidol was quite toxic and provided little protection against CHIKV infection (unpublished results). Therefore, we suspect that the outcome of the resistance-passaging of Delogu *et al.* merely reflected adaptation of CHIKV to more efficient infection of MRC-5 cells (even in the presence of arbidol). In our plaque assays, CHIKV E2-G82R had a small-plaque phenotype in Vero E6 cells, while virus titers were not affected. This combination might be explained by a restricted cell-to-cell spread (maybe HS expression is less abundant in Vero E6 cells). Strikingly, in MRC-5 cells the E2-G82R mutant virus had a large-plaque phenotype, suggesting enhanced cell-to-cell spread (results not shown). G82R, but also wt CHIKV reached higher titers in MRC-5 cells than on Vero E6 cells and it would be interesting to study whether this is due to differences in the GAG abundance/expression patterns. As mentioned above, adaptation of CHIKV to HS-binding in cell culture is expected to direct the selection of mutations that increase the number of positively charged residues at the surface of the E2 protein (37, 40). This is corroborated by the observation that the G82R mutation attenuates CHIKV because it renders virus infectivity dependent on HS (39, 42). Due to their location, it is unlikely that the N5R and H18Q mutations that cause suramin-resistance, are a result of cell culture adaptation, since neither of these mutations is located in the region targeted by neutralizing antibodies (33) or directly involved in receptor-binding (43).

To understand if any of the mutations acquired by passaging CHIKV in the presence of suramin offers an advantage in other cell types (treated or not with suramin), we compared various reverse-engineered mutants in CPE-based assays (results not shown). Wild-type virus and all tested mutants produced extensive CPE in Vero E6 cells, except for the T301K virus. In the presence of suramin, only the mutant viruses with both E2 mutations replicated and caused CPE in Vero E6 cells. The E2 G82R mutation caused an extreme sensitivity to suramin and it was the only mutant that exhibited sensitivity to suramin in all tested cell lines (Vero E6, HeLa, MRC-5, BHK21), implying that suramin interferes with its attachment to the cell surface via HS. Interestingly, the G82R and N5R mutants were the only two variants that caused CPE in HeLa cells (in the absence of suramin),

suggesting that also the N5R mutation has an effect on interactions with HS or another GAG, and thereby modulates infectivity in a cell type-dependent way. In MRC-5 cells, most CHIKV variants caused extensive CPE and did not respond to suramin treatment, with the exception of the G28R mutant. It seems that CHIKV wt and the S9 mutant with the two suramin-resistance mutations in E2 share a similar HS-independent entry mechanism and a complete resistance to suramin treatment in MRC-5 cells. Consequently, the selected suramin-resistant mutations seem to be specific for the situation in Vero E6 cells treated with suramin – and might represent an escape mechanism that allows faster entry in the presence of suramin in this cell type.

Suramin was originally developed to treat Trypanosoma infections, but its exact MoA has not been clarified (44). Nevertheless, *T. brucei* strains with increased resistance to suramin could be selected, but only in haemolymphatic stage parasites and not in the case of the procyclic forms produced in insects (45). Using RNAi target sequencing (RIT-seq), followed by RNAi screens, Alsford *et al.* were able to shed more light on how the anti-trypanosomal activity of drugs (suramin included) was actually induced (46). In the case of suramin multiple targets surfaced, linked to its uptake or inhibitory activity, and eight were selected for further investigation. Knockdown of the endomembrane protein MFST (major facilitator superfamily transporter) the lysosomal cathepsin-L like protease (Cat-L) lead to a clear increase in EC_{50} for suramin. Other identified proteins that affected the sensitivity towards suramin were: a bloodstream stage-specific invariant surface glycoprotein (ISG75), lysosomal proteins (CBP1peptidases, p67 and Golgi/lysosomal protein-1, GLP-1), several spermidine and N-acetylglucosamine (NAG) biosynthetic enzymes, and all subunits of the adaptin complex (AP) 1, which is involved in clathrin-mediated endocytosis. The proposed mechanism-of-action for suramin is summarized in Figure 2. Although it had been shown previously that suramin resistance was linked to downregulation of endocytosis, another study demonstrated it was connected to antigenic variation of trypanosomal surface glycoproteins (out of 2000 types, only one is expressed), as suramin treatment led to the emergence of a *T. brucei* form with a surface glycoprotein (VSGsur) that caused resistance to suramin treatment (47).

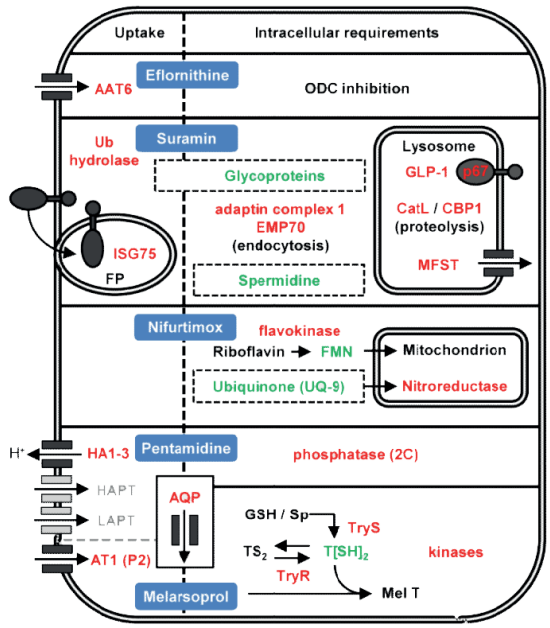


Figure 2. Schematic summary of RIT-seq determinants of suramin, and other drugs, efficacy in *T. brucei*. Depicted in red and green are proteins and metabolites linked to drug activity. For suramin, the proposed course of events would be binding to ISG75, and subsequent accumulation in lysosomes via the flagellar pocket (FP) and endosomes. From there MFST could transport suramin into the cytosol. (Modified from (46)).

5. Suramin has broad-spectrum antiviral activity

Another important pathogen that is sensitive to suramin is ZIKV, a flavivirus endemic to Africa, that re-emerged and at the beginning of 2015 caused a massive epidemic in South America with devastating neurodevelopmental outcomes for newborns from infected mothers. Briefly, as described in Chapter 4 of this thesis, we have shown that ZIKV SL1602 (a clinical isolate) is sensitive to suramin using several assays adapted from our CHIKV studies. For ZIKV and CHIKV the compound seems to have a similar MoA, affecting virus entry steps as well as the release of infectious particles. The replicative cycles of alpha- and flaviviruses have similarities, such as GAG-dependent attachment to the plasma membrane and entry via receptor mediated endocytosis (RME). ZIKV attachment is mediated by electrostatic interactions of positively-charged amino acids of the E protein with negatively-charged GAGs at the cell surface, having preferably long-chained and highly-sulfated HS (48, 49). There are also significant differences between the replicative cycles of alpha- and flaviviruses, like the mechanism of virion biogenesis. In the case of flaviviruses this relies on budding into the ER lumen (not at the plasma membrane as for CHIKV) and maturation during transit through the secretory pathway. Suramin could accumulate in the trans-Golgi network and hence impair the activity of enzymes (like furin or glycosyltransferases) required for the maturation of new ZIKV particles, or it could block virus release, through an unknown mechanism.

While we were investigating suramin's MoA against ZIKV, another research group made similar discoveries (50). Their findings in part corroborated ours, leading to the conclusion that suramin did not "inactivate viral particles, but interfered with virus adsorption, entry and post-infection events". However, some of their results appear to differ from ours and other previously published studies, but this might be due to their un-conventional experimental setup and the fact that also their controls behave different than published elsewhere in literature.

Suramin's inhibitory activity against the alphaviruses CHIKV, SFV and SINV (this thesis, (26, 51)), as well as against flaviviruses ZIKV (this thesis, (50)), DENV (52), BVDV (53), and HCV (54) appears to be based on a similar primary mechanism, i.e. the compound blocking the access to cell surface attachment factors. The bunyaviruses RVFV (55) and SFTSV (56) are inhibited by suramin at several steps during infection, but the main mechanism seems to be through the interaction with the nucleocapsid protein (N) and interfering with budding into the Golgi apparatus, which is crucial for particle assembly.

Suramin's spectrum extends beyond the alpha-, flavi- and bunyaviruses mentioned above. It can also inhibit DNA viruses, retroviruses and other RNA viruses, independent of whether they are enveloped or non-enveloped.
DNA viruses targeted by suramin at their entry step include HSV-1, CMV and duck hepatitis B virus (57-59). Suramin also inhibits entry of retroviruses, and HIV-1 was actually the first viral pathogen for which suramin's antiviral activity was evaluated in human patients (60, 61). Rous sarcoma virus is also sensitive to suramin, which blocks virus uptake or uncoating (59).

Suramin was shown to also inhibit non-enveloped viruses of the *Picornaviridae* family, more specifically the causative agent of hand foot and mouth disease, EV-A71 (62, 63). Interestingly, suramin's spectrum of activity was restricted to type A enteroviruses, and its MoA depended on competition with sulfated receptors for a binding site at the 5-fold vertex of the EV-A71 capsid, blocking virus attachment to cells (64).
Suramin also inhibits ebola virus (EBOV), another important enveloped RNA virus that has received a lot of public interest due to the recent serious epidemics in Africa. Suramin treatment also affected an early step of the EBOV infectious cycle in cell culture, as demonstrated with EBOV envelope glycoprotein pseudo-typed lentiviral vectors (51).
Preliminary (unpublished) results from our group have shown that suramin can also inhibit MERS-CoV (isolate EMC2012) and SARS-CoV (isolate Frankfurt-1) in CPE-based assays using Vero cells, with EC_{50}s of 50 and 100 µM, respectively. Even though the two coronaviruses have specific protein receptors (DPP4 and ACE2) for entering their target cells, MERS-CoV can also use attachment factors conjugated with sialic acid residues, which contribute to the negatively charged environment at the cell surface. Presumably by binding to the viral surface, suramin could block this interaction in Vero cells (65).

In theory suramin could also affect the enzymatic activity of non-structural proteins (nsPs) by due to its high affinity for positively charged regions, such as those present in viral proteins interacting with negatively charged molecules, such as RNA. In cell culture, the effect on intracellular nsPs would likely be limited, since the compound accumulates in intracellular compartments and not in the cytosol or the replication organelles where most of those viral proteins would exert their activity.
In summary, we can conclude that suramin inhibits a wide variety of enveloped and non-enveloped RNA and DNA viruses, likely through a common mechanism that involves interfering with the electrostatic interactions between viruses and attachment factors at the cell surface.

6. Therapeutic strategies for CHIKV infections and outlook

As mentioned earlier, CHIKV causes a highly debilitating disease, for which vaccines or antiviral therapy are currently not on the market. Treatment of patients is mainly supportive at the moment (e.g. through the use of analgesics/painkillers). The development of a vaccine against CHIKV is of the utmost importance to prevent further spread of the virus and large epidemics as we have seen in the recent past. Antiviral compounds would be required, to treat people that have been already infected and are struggling with the persistent painful consequences (chronic arthritis for e.g.) of CHIKV infection. Compared to vaccines, antiviral compounds might be cheaper, easier to administer, stockpile, distribute and more suitable for certain target groups (that cannot be vaccinated) and emergency usage in new outbreak situations (to curb the outbreak). Considering the enormous costs involved in bringing a new drug to the market, broad-spectrum antivirals and repurposed compounds already on the market for other indications are of particular interest.

Suramin, a drug that has already been used for over 100 years to treat parasitic infections, was shown to have antiviral activity against CHIKV by us and others (Chapters 3 and 5 of this thesis, (26)). The drug was synthesized for the treatment of the early stages of trypanosomiasis and is still in use today and is even offered for free by the WHO in trypanosomiasis endemic areas. A course of suramin treatment for trypanosomiasis costs 27$, and it comprises five 1-g intravenous doses, administered over 2 weeks, with minimal side effects. In November 2018, a Dutch tourist returning from Malawi developed trypanosomiasis and required urgent suramin treatment, which was not available in the Netherlands or Belgium and had to be imported from the Tropical institute in Basel. This illustrates that suramin has rightfully been placed on the WHO list of essential medicines, which each country should have available.

Suramin has not been tested in clinical trials for the treatment of CHIKV infections, but its efficacy was shown in a CHIKV mouse model (66). It has also been shown to be effective in mice and adult Rhesus monkeys for the treatment of EV-A71 infections, suggesting that suramin is a promising compound for the prevention and treatment of hand foot and mouth disease (63).
A highly efficient way to prevent drug-resistance during antiviral therapy of (rapidly mutating) RNA viruses, is the simultaneous use of multiple drugs with different viral targets, as exemplified by the combination of simeprevir and sofosbuvir to cure HCV infections or or the use of several multi-class combination drugs like Prezcobix, or Evotaz to control HIV infection (67, 68).

A dual-agent treatment, combining suramin and zanamivir (an anti-influenza drug), was tested *in vitro* against human parainfluenza infection (hPIV-3), which can cause serious respiratory illness in infants (69). The authors have shown that lower concentrations of both compounds can be used when they are combined, leading to higher levels of inhibition by simultaneously interacting with the haemagglutinin-neuraminidase (HN) protein. This exemplifies that combinatorial repurposing approaches of approved drugs can be a fast and rewarding way to develop new antiviral therapies.

Recently, it was shown that treatment of U2OS cells with a combination of suramin and epigallocatechin-3-gallate synergistically inhibited the replication of the African CHIKV strain S27 (70). Combining suramin with inhibitors that specifically target the activities of CHIKV nsPs might be a promising strategy towards the development of a combination treatment for CHIKV infections. Potential candidates are an inhibitor that targets the methyltransferase activity of CHIKV nsp1 (71) and favipiravir (72), a broad spectrum drug targeting the RdRp activity of CHIKV. Compounds that stimulate the host's natural antiviral mechanisms, e.g. by inducing viperin expression (73), could also be considered for combination treatment.
Hopefully the antiviral strategies mentioned above can be applied to treat patients and contain CHIKV epidemics. Nevertheless, both vaccines and antiviral drugs should be considered as pillars of a coordinated strategy during epidemics, regardless of the pathogen at hand (74).

Concluding remarks

This thesis describes the quest for compounds targeting alphavirus replication, which started with the development of an *in vitro* assays to study RNA replication and identify compounds with antiviral potential, but took an unexpected turn.
Suramin, which directly blocked CHIKV RNA synthesis in the *in vitro* assay, turned out to have a different mode-of-action in cell-based assays, a story with some parallels to early studies on the effect of suramin on HIV-1 infection (75).

In cell culture suramin primarily inhibits CHIKV binding and fusion with host membranes. Besides being an anti-parasitic drug with anti-cancer properties, suramin also inhibits a variety of viruses, and the work described in this thesis has demonstrated that its antiviral spectrum extends to alphaviruses and ZIKV.

Viruses have always been part of our existence, shaping human evolution and continuing to do so, probably even on a bigger scale, due to increased globalization, travel, changes in land use and expansion of human activities into previously uninhabited areas. Climate change and the rise in temperatures will lead to expansion of the distribution of insect vectors and likely will increase the incidence of outbreaks of "once tropical" diseases caused by DENV, ZIKA, CHIKV, *Plasmodium* sp., *Vibrio cholerae* etc. The design of better vaccines and development of new antiviral strategies, including those based on drug repurposing should enhance our preparedness for preventing and treating these infections.

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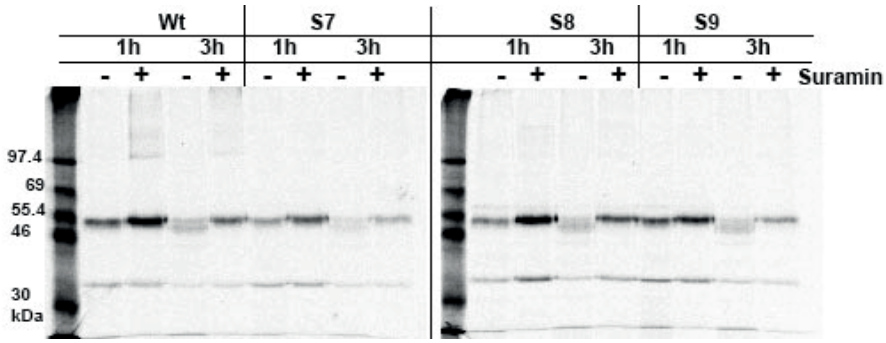
Appendix



Effect of suramin on ^{35}S-CHIKV uptake, at 37°C.

At 1 and 3 h p.i. of Vero E6 cells with CHIKV wt and S9, in the presence or absence of suramin, whole cell lysates were prepared in Laemmli sample buffer. Afterwards ^{35}S-labelled CHIKV proteins were separated by SDS-PAGE and visualized by phosphor imaging with a Typhoon scanner.

List of Commonly Used Abbreviations

CHIKV – Chikungunya virus
ZIKV – Zika virus
SINV – Sindbis virus
SFV – Semliki Forest virus
RTC – replication and transcription complex
RNA – ribonucleic acid
nsP – non-structural protein
RME – receptor mediated endocytosis
IVRA – *in vitro* replication assay
MoA – Mode of action
WT – wild-type

Summary

This thesis is focused on understanding the particularities of Chikungunya virus (CHIKV) replication, and the mechanism(s) by which it can be inhibited by suramin, a compound with a broad spectrum of activity.

Chikungunya virus (CHIKV) is a member of the *Togaviridae* family, and it can be transmitted to humans through the bite of infected mosquitoes. In the past, this virus, of African origin, has caused large epidemics in Asia, but the most recent one took place in 2014-2015 in a new territory, the Americas, where it caused over 1 million suspected and confirmed cases. The infection is manifested through acute joint and muscle pain (that can last for years), fever, and rash. To this day, there is no approved vaccine or treatment for CHIKV infection. A much broader introduction regarding CHIKV and its genome organization, protein function, replication, pathogenesis and preventive or therapeutic strategies can be found in **Chapter 1**, the general introduction of the thesis. Soon after the CHIKV epidemic was over, another virus, also mosquito-borne and originating from Africa, continued to cause serious health problems in Central and South America. This was the Zika virus (ZIKV), a member of the *Flaviviridae* family. Though ZIKV mostly causes asymptomatic infections or mild disease characterized by low fever, rash, conjunctivitis, and malaise, the epidemics in the Americas have also linked ZIKV infection to fetal malformations like microcephaly and to Guillain-Barré syndrome in adults. Besides this, previously unrecognized routes of mother-to-child and sexual transmission were uncovered. Similarly, as in the case of CHIKV, no preventive or therapeutic strategy (vaccines or drugs) for treatment of ZIKV infection are available on the market. The compound suramin had been marketed for the treatment of parasitic infections, but its anti-cancer and antiviral potential were also discovered in the last 40 years. Therefore, we sought to test if suramin could inhibit the replication of CHIKV and ZIKV.

Chapter 2 describes the development of an *in vitro* replication assay (IVRA) that relies on CHIKV replication/transcription complexes isolated from infected cells. This assay 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. While optimizing the assay, we identified a new RNA species that is produced during CHIKV infection, alongside the genomic and subgenomic RNA that are required for the production of specific proteins involved in the replication and virus production process, but also in interaction with/manipulation of the host cell. The new species was called RNAII, similarly to the one identified for the CHIKV-related Sindbis virus (SINV) in 1997 by Wielgosz and Huang. It is suspected to direct the replication/transcription complexes to later in infection predominantly produce the subgenomic RNA, which is required for the production of structural proteins that are used for progeny virus assembly.

Using the IVRA, in **Chapter 3** we have identified suramin as a potent inhibitor of CHIKV replication. However, cell-based assays revealed that suramin's main mode-of-action is dependent on the inhibition of an early step of the replicative cycle, namely virus entry into the host cell. In addition, several suramin-related compounds were analyzed, and though these compounds were not more effective, they provided insight into the structural elements (symmetry of the molecule and the presence of negative charges) that are important for both inhibitory activities of suramin observed *in vitro* and in cell culture.

The antiviral effect of suramin is very broad, and in **Chapter 4** we show that it also inhibits the replication of the re-emerging ZIKV, by interfering both with its entry and biogenesis of progeny virions.

Subsequently, we explored how suramin can inhibit CHIKV entry steps. **Chapter 5** describes the identification of the CHIKV E2 envelope glycoprotein as the target of suramin and mode-of-action studies that suggest that by interacting with E2 suramin blocks virus attachment to cells, and subsequent fusion of the particle with cellular membranes. CHIKV can become more resistant to suramin by acquiring mutations in the E2 protein. The amino acid substitutions that we found were N5R and H18Q, which allowed the virus to replicate much better in the presence of suramin, as compared to the wild-type virus. However, a known CHIKV mutant, with a G82R substitution in E2 that adapts the virus for infecting mammalian cells (by interacting with a molecule on their cell surface, heparan sulfate), was more sensitive to suramin.

Lastly, **Chapter 6** summarizes and discusses the key findings presented in this thesis. Their implications in the context of the broader literature are presented, followed by a general discussion and conclusion.

Samenvatting

Titel: Het remmen van chikungunya-virus replicatie - Inzichten in de replicatie van het chikungunya-virus en de antivirale activiteit van suramin

Dit proefschrift is gericht op het beter begrijpen van de moleculaire biologie en replicatie van het chikungunya-virus (CHIKV), en het mechanisme waarmee suramine, een geneesmiddel met een breed spectrum, dit proces kan remmen.
Chikungunya-virus (CHIKV) is een lid van de *Togaviridae*-familie en kan op mensen worden overgedragen door de beet van geïnfecteerde muggen. Het virus is van Afrikaanse origine en heeft in het verleden grote epidemieën in Azië veroorzaakt, maar de meest recente uitbraak vond plaats in 2014-2015 in een nieuw gebied, Amerika, waar het meer dan 1 miljoen vermoedelijke en bevestigde gevallen veroorzaakte. De infectie manifesteert zich door acute gewrichts- en spierpijn (die jaren kan duren), koorts, en uitslag. Tot op heden is er geen goedgekeurd vaccin of behandeling voor CHIKV-infectie. Een meer uitgebreide inleiding met betrekking tot CHIKV en de virale genoomorganisatie, eiwitfuncties, replicatie, pathogenese en preventieve of therapeutische strategieën is te vinden in **hoofdstuk 1**, de algemene inleiding van het proefschrift. Kort nadat de CHIKV-epidemie voorbij was, begon een ander virus, ook door muggen overgebracht en afkomstig uit Afrika, ernstige gezondheidsproblemen te veroorzaken in Midden- en Zuid-Amerika. Dit was het Zika-virus (ZIKV), een lid van de *Flaviviridae*-familie. Hoewel ZIKV meestal slechts asymptomatische infecties of milde ziekte veroorzaakt, gekenmerkt door lage koorts, uitslag, conjunctivitis en malaise, hebben de epidemieën in Amerika ZIKV-infectie ook in verband gebracht met foetale misvormingen zoals microcefalie en met het Guillain-Barré-syndroom bij volwassenen. Daarnaast werden eerder niet-herkende routes van moeder op kind transmissie en seksuele overdracht ontdekt. Net als in het geval van CHIKV, is er voor ZIKV infectie geen preventieve of therapeutische strategie (vaccins of geneesmiddelen) op de markt. Het geneesmiddel suramine was eerder op de markt gebracht voor de behandeling van parasitaire infecties, maar in de afgelopen 40 jaar is ontdekt dat het ook antikanker en antivirale potentie heeft. Daarom hebben we getest of suramine de replicatie van CHIKV en ZIKV *in vitro* kan remmen.
In **hoofdstuk 2** wordt de ontwikkeling van een *in vitro* replicatie assay (IVRA) beschreven die was gebaseerd op CHIKV-replicatie/transcriptiecomplexen die werden geïsoleerd uit geïnfecteerde cellen. Deze test kan worden gebruikt om CHIKV-RNA-synthese te bestuderen, en ook om remmers van dit proces te identificeren en om hun werkingsmethoden te bestuderen. Tijdens het optimaliseren van de test werd een nieuwe RNA-soort geïdentificeerd die tijdens CHIKV-infectie wordt geproduceerd, naast het genomische en subgenome RNA dat nodig is voor de productie van specifieke eiwitten die betrokken zijn bij de virale replicatie en virusproductie, maar ook bij interacties met en manipulatie van de gastheercel. De nieuwe soort is RNAII genoemd, naar analogie van

een vergelijkbaar RNA dat in 1997 door Wielgosz en Huang werd geïdentificeerd voor het aan CHIKV-verwante Sindbis-virus (SINV). Er wordt vermoed dat dit RNA de replicatie/transcriptiecomplexen dirigeert om later in infectie hoofdzakelijk het subgenome RNA te produceren, wat is vereist voor de productie van de structurele eiwitten die worden gebruikt voor de assemblage van nieuwe virusdeeltjes.

Met behulp van de IVRA hebben we in **hoofdstuk 3** suramine geïdentificeerd als een krachtige remmer van CHIKV replicatie. Uit analyses van geïnfecteerde cellen bleek echter dat de werking van suramine afhankelijk is van de remming van een vroege stap van de virale cyclus, namelijk het binnenkomen van het virus in de gastheercel. Verder werden verschillende aan suramine gerelateerde verbindingen getest. Hoewel deze verbindingen niet effectiever waren, gaven ze wel inzicht in de structurele elementen (symmetrie van het molecuul en de aanwezigheid van negatieve ladingen) die belangrijk zijn voor de beide remmende activiteiten van suramine die zijn waargenomen *in vitro* en in celkweek.

Het antivirale effect van suramin is zeer breed en in **hoofdstuk 4** laten we zien dat het ook de replicatie van het nieuw opkomende virus ZIKV kan remmen, door de opname van virus toegang en de biogenese van nieuwe virions te verstoren.

Vervolgens probeerden we te begrijpen hoe suramine de vroege fase van CHIKV infectie kan remmen. **Hoofdstuk 5** beschrijft de identificatie van het CHIKV E2 envelop glycoproteïne als het doelwit van suramine. Mechanistische studies suggereren dat suramine door de interactie met E2 de virushechting aan cellen blokkeert, en de daaropvolgende fusie van het virion met cellulaire membranen. CHIKV kan resistenter worden tegen suramine door mutaties in het E2-eiwit te verwerven. De relevante aminozuursubstituties die we vonden waren N5R en H18Q, waardoor het virus veel beter kon repliceren in de aanwezigheid van suramine, in vergelijking met het wildtype virus. Echter, een bekende CHIKV-mutant met een G82R substitutie in E2, die het virus beter geschikt maakt voor het infecteren van zoogdiercellen (door interactie met een molecuul op hun celoppervlak, heparaan sulfaat), bleek gevoeliger voor suramine.

Tenslotte vat **hoofdstuk 6** de belangrijkste bevindingen van dit proefschrift samen. Hun implicaties in de context van de bredere literatuur worden gepresenteerd, gevolgd door een algemene discussie en conclusie.

Rezumat

Titlu: Inhibarea replicării virusului Chikungunya - Perspective asupra replicării acestui virus și modul de acțiune al compusului suramin

Această lucrare este axată pe înțelegerea particularităților replicării virusului Chikungunya (CHIKV) și a mecanismelor prin care acest proces poate fi inhibat de suramin, un compus cu spectru larg de activitate.

CHIKV este un membru al familiei *Togaviridae* care se transmite la oameni prin mușcătura țânțarilor infectați. În trecut acest virus de origine africană a provocat mari epidemii în Asia, dar cea mai recenta epidemie a avut loc între 2014-2015 in America, unde a cauzat peste 1 milion de cazuri confirmate. Persoanele infectate manifestă dureri articulare și musculare acute (care pot dura ani întregi), febră și erupții cutanate. În prezent nu există niciun vaccin sau tratament aprobat pentru infecția cu CHIKV. O introducere mult mai amplă în ceea ce privește CHIKV, organizarea genomului său, funcțiile proteinelor virale, replicarea, patogeneza și strategiile preventive sau terapeutice poate fi găsită în **Capitolul 1**, introducerea generală a tezei. La scurt timp după terminarea epidemiei cauzate de CHIKV, un alt virus, de asemenea transmis de țânțari și originar din Africa, a continuat să provoace grave probleme de sănătate în America Centrală și de Sud. Acesta este virusul Zika (ZIKV), un membru al familiei *Flaviviridae*. Deși ZIKV cauzează predominant infecții asimptomatice sau boli fără complicații caracterizate prin febră ușoară, erupții cutanate, conjunctivită și stare de rău, epidemiile din America au asociat, de asemenea, infecția cu ZIKV de malformații fetale, precum microcefalia, și sindromul Guillain-Barré la adulți. În plus, au fost descoperite rutele de transmitere a infecției nerecunoscute anterior, precum de la mamă la copil și prin contact sexual. În mod similar, precum în cazul CHIKV, nu există pe piață o strategie preventivă sau terapeutică (vaccinuri sau medicamente) împotriva infecției cu ZIKV. Cercetările din ultimii 40 de ani au arătat ca suramin, un compus comercializat pentru tratamentul infecțiilor parazitare, are activitate anticanceroasă, dar și antivirală. Prin urmare, am dorit să testăm dacă acest compus ar putea inhiba replicarea celor două virusuri, CHIKV și ZIKV.

Capitolul 2 descrie dezvoltarea unui sistem de replicare *in vitro* (IVRA) care se bazează pe complexe de replicare/transcripție, aparținând virusului CHIKV, izolate din celulele infectate. Acest test poate fi utilizat pentru a studia sinteza ARN-ului viral, precum și pentru a identifica compuși care inhibă acest proces și a le studia modul de acțiune. În timpul optimizării acestui sistem, am identificat un nou tip de ARN care este produs în timpul infecției cu CHIKV, alături de ARN-ul genomic și subgenomic, specii/soiuri/tipuri care sunt necesare pentru sinteza proteinelor virale implicate în procesul de replicare sau în producția de virus nou, precum și în interacțiunea cu celula gazdă. Acest nou tip de RNA a fost numit RNAII, similar cu cel identificat de Huang și Wielgosz (1997) pentru virusul Sindbis (SINV), un virus înrudit cu CHIKV. Suspectăm că acest nou ARN dirijează mai

târziu în cursul infecției complexele de replicare/transcripție spre a produce predominant ARN subgenomic, necesar pentru generarea de proteine structurale care sunt apoi utilizate în asamblarea noilor particule virale.

Folosind acest sistem de replicare *in vitro*, în **capitolul 3** am identificat compusul suramin ca fiind un puternic inhibitor al replicării CHIKV. Cu toate acestea, testele bazate pe celule in cultură au demonstrat că suramin are ca principal mod de acțiune inhibarea unei etape timpurii a ciclului replicativ, și anume intrarea virusului în celula gazdă. În plus, au fost analizați mai mulți compuși înrudiți cu suramin și, deși acești compuși nu au fost mai eficienți, au oferit o perspectivă asupra elementelor structurale (simetria moleculei și prezența sarcinilor negative) care sunt importante pentru activitățile inhibitoare ale compusului suramin observate atât *in vitro*, cât și în culturi de celule mamaliene.

Efectul antiviral al suraminului are un spectru foarte larg, iar în **capitolul 4** arătăm că inhibă, de asemenea, și replicarea virusului ZIKV, interferând atât cu intrarea sa în celula gazdă, cât și cu biogeneza virionilor descendenți.

Ulterior, am explorat cum suraminul poate inhiba etapele prin care CHIKV intră în celula gazdă. **Capitolul 5** descrie identificarea glicoproteinei E2, expusă la suprafața anvelopei virale a lui CHIKV, ca una din țintele compusului. Experimentele din acest capitol sugerează că prin interacțiunea suraminului cu E2 este blocată atașarea virusului la membrana celulei gazdă, precum și fuziunea ulterioară a particulei cu membranele celulare (în urma endocitozei particulelor). CHIKV poate deveni mai rezistent la acțiunea compusului prin dobândirea de mutații în proteina E2. Substituțiile de aminoacizi N5R și H18Q, au permis virusului să se reproducă mult mai bine în prezența suraminului, în comparație cu virusul original. Cu toate acestea, un mutant CHIKV cunoscut în literatură, având substituția G82R în E2 care adaptează virusul pentru infectarea celulelor mamaliene (prin interacțiunea cu o moleculă de pe suprafața celulară a acestora, heparan sulfat), a fost mai sensibil la suramin.

Spre final, **capitolul 6** rezumă principalele concluzii ale acestei lucrări și discută implicațiile acestora în contextul literaturii recente, și se încheie cu o concluzie generală.

Curriculum Vitae

Irina Cristina Albulescu (née Florea), was born on December 25th 1985, in Petroşani, Hunedoara county, Romania. She attended the National College "Mihai Viteazul" in Ploiesti, Prahova County, between 2000-2004, and graduated with a chemistry-biology profile. She continued with her Bachelor studies in Biochemistry (2004-2008) at the University of Bucharest, followed by a Master program in Biochemistry and Molecular Biology (2008-2010) at the same university. From December 2007, Irina began working at the Institute of Cellular Biology and Pathology (ICBP), under the supervision of Dr. Dorin Alexandru, and later of Dr. Anca Gafencu and Dr. Adrian Manea, where she also performed her Bachelor's and Master's thesis projects. During the four years spent at the ICBP, she was promoted from research assistant to scientific researcher.

In 2012, she moved to the Netherlands and volunteered as a scientist in the lab of Dr. Frank van Kuppeveld at Nijmegen Center for Molecular Life Sciences (NCMLS). Soon after, she obtained a PhD studentship in the lab of Dr. Eric Snijder at the Leiden University Medical Center (LUMC), under the direct supervision of Dr. Martijn van Hemert. In addition, she was also an early stage researcher (2012-2015) in the European Initial Training Network EUVIRNA. Her research was focused on host factors involved in the replication of alphaviruses and identification of compounds with antiviral activity. As part of her PhD research project, Irina performed an industrial training stage at Janssen Infectious Diseases in Beerse (Belgium), under the supervision of Dr. Florence Herschke. Between October 2016 and June 2018, she was involved in the EU-funded ZIKAlliance project, still under the supervision of Dr. Martijn van Hemert, working on the characterization of a ZIKV clinical isolate and the identification of antiviral compounds targeting ZIKV. From July 2018 until January 2019 she continued to work at the LUMC under the direct supervision of Dr. Marjolein Kikkert on the EU-funded Zoonoses Anticipation and Preparedness Initiative (ZAPI) project, which concerned the development of a yellow fever virus 17D-based vaccine platform.

In May 2019, Irina has re-joined the group of Dr. Frank van Kuppeveld at the Faculty of Veterinary Medicine in Utrecht and is now involved in the development of vaccination strategies that provide broad protection against antigenically variable pathogens.

List of Publications

Pietilä MK, **Albulescu IC**, Hemert MJV, Ahola T. Polyprotein Processing as a Determinant for *in vitro* Activity of Semliki Forest Virus Replicase. Viruses. 2017 Oct; 9(10).

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ERRATA SHEET

This errata sheet lists oversights for the doctoral thesis of Irina Cristina Albulescu, titled "Targeting alphavirus replication – Insights into the chikungunya virus replication and the antiviral activity of suramin *in vitro*", Leiden University Medical Center, 2019, ISBN 97894-93184-16-9 (printed and pdf).

Location	**Oversight**
Page 11, Figure 1	Missing the citation of "the creative commons license, as CC-BY-SA 4.0 https://creativecommons.org/licenses/by-sa/4.0/"
Page 14. Figure 3	Missing the text "Copyright Massachusetts Medical Society", after "adapted from [2]".
Page 18, Figure 4	Missing the citation of "the creative commons license, as CC-BY-SA 4.0 https://creativecommons.org/licenses/by-sa/4.0/"
Page 122, Figure 1	Missing the citation of "Adapted from Adapted from https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/ *Togaviridae*, under the creative commons license, as CC-BY-SA 4.0 https://creativecommons.org/licenses/by-sa/4.0/"