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

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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).

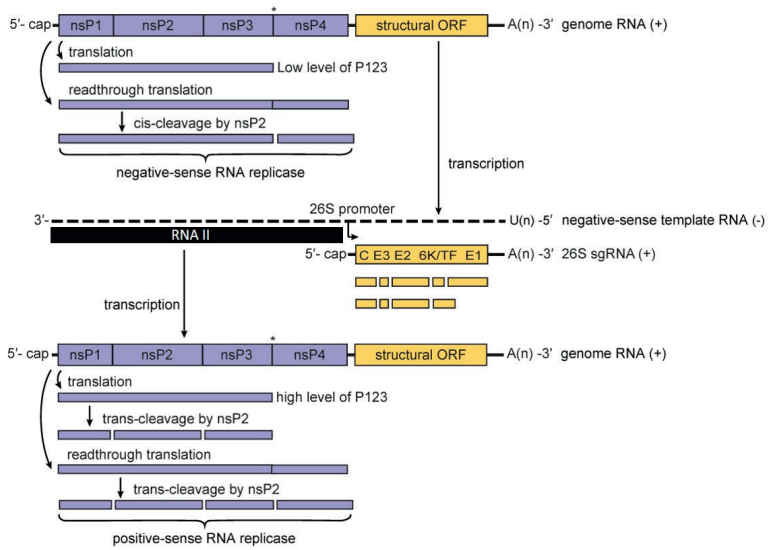
The role of RNAII in alphavirus replication has not been investigated in detail. It could be merely a byproduct from complexes that were engaged in genome synthesis and became blocked/stalled when reaching a region of active transcription at the subgenomic promoter region. From an evolutionary point 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).

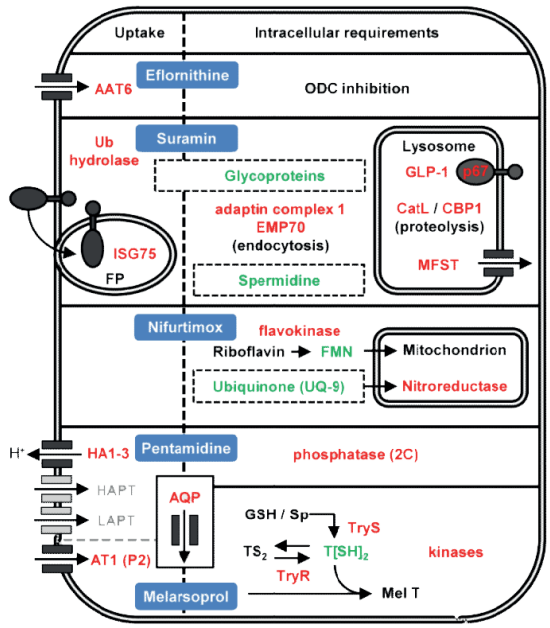


Figure 2. Schematic summary of RIT-seq determinants of suramin, and other drugs, efficacy in *T. brucei*. Depicted in red and green are proteins and metabolites linked to drug activity. For suramin, the proposed course of events would be binding to ISG75, and subsequent accumulation in lysosomes via 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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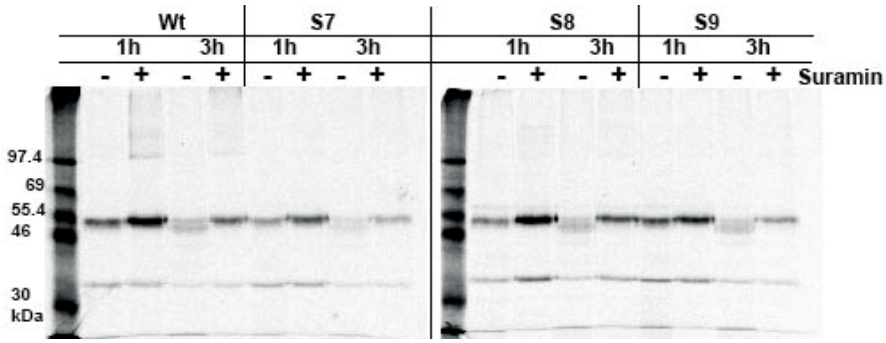
Appendix



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