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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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# 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  $\sim 5 \mu\text{M}$ ). The compound inhibited replication of various CHIKV isolates in cell culture with an  $EC_{50}$  of  $\sim 80 \mu\text{M}$  ( $CC_{50} > 5 \text{ mM}$ ) 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 hepatitis 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  $^{32}\text{P}$ -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  $^{32}\text{P}$ -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  $4 \times 10^6$  cells in 0.4 mL PBS and 4  $\mu\text{g}$  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  $\mu\text{F}$ , cells were transferred to T-75 flasks with pre-warmed medium, followed by a 10-h incubation at 37 °C.

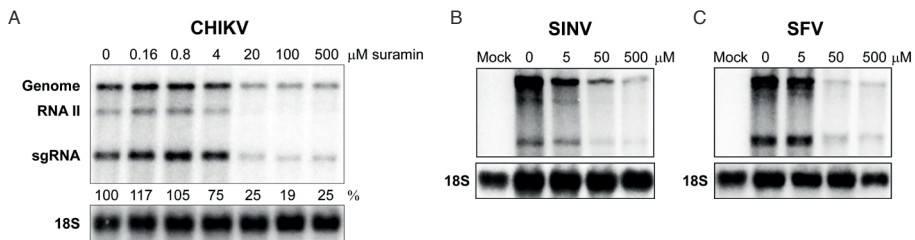
#### 2.7 Statistical analysis

Graph-Pad Prism 5.01 was used for  $\text{EC}_{50}$ ,  $\text{IC}_{50}$  and  $\text{CC}_{50}$  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  $[\alpha\text{-}^{32}\text{P}\text{-CTP}]$  into viral RNA, which was severely impaired by suramin in a dose-dependent manner, with an  $\text{IC}_{50}$  of approximately 5  $\mu\text{M}$  (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  $^{32}\text{P}$ -CTP remained even in the presence of 500  $\mu\text{M}$  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 <sup>32</sup>P-labeled reaction products were analyzed by denaturing agarose gel electrophoresis and phosphorimaging. 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

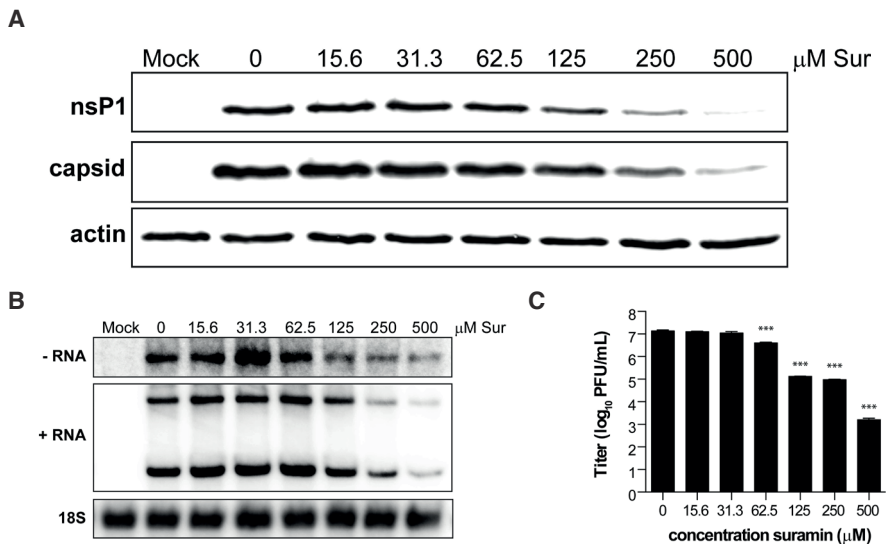
To determine the antiviral efficacy of suramin in cell culture, Vero E6 cells were infected with different CHIKV strains and treated with serial dilutions of the compound in a CPE protection assay. Viability assays on uninfected cells were performed in parallel to determine the CC<sub>50</sub>. The EC<sub>50</sub> 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<sub>50</sub> values are ~15 times higher than the IC<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> was still above 5 mM)

Virus	EC <sub>50</sub> (μM)	CC <sub>50</sub> (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  $\mu\text{M}$ ) 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  $\mu\text{M}$  suramin (Fig. 2A). The accumulation of CHIKV RNA and positive-stranded RNA (+RNA) was also severely impaired at concentrations of 125  $\mu\text{M}$  suramin or higher (Fig. 2B). The production of infectious CHIKV was strongly inhibited, leading to a 4-log reduction when 500  $\mu\text{M}$  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.



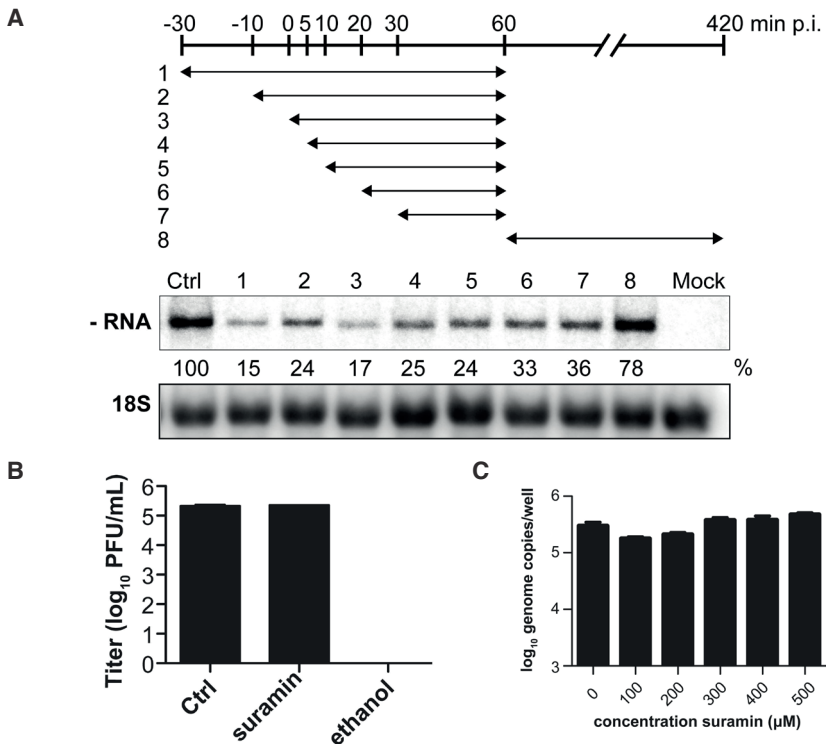
**Figure 2.** Effect of suramin on CHIKV replication (A) Western blot analysis of nsP1 and capsid protein levels in CHIKV-infected Vero E6 cells (MOI 1) that were treated with suramin at the concentrations indicated above the lanes and analyzed at 12 h p.i. Actin was used as loading control. (B) CHIKV 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 ( $\pm$  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  $\mu\text{M}$  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  $\mu\text{M}$  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.



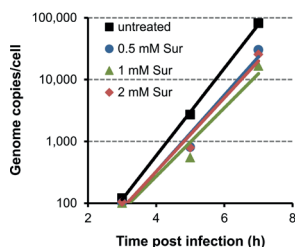
**Figure 3.** Effect of suramin on early steps of the CHIKV replication cycle (A) Vero E6 cells were infected with CHIKV (MOI 5) and were left untreated (Ctrl) or were treated with 500  $\mu\text{M}$  suramin during the intervals schematically indicated for each sample. At 60 min p.i., cells were washed extensively and incubated in medium without suramin (sample 1-7) or with 500  $\mu\text{M}$  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  $\mu\text{M}$  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  $^{\circ}\text{C}$  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  $\mu$ M 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.

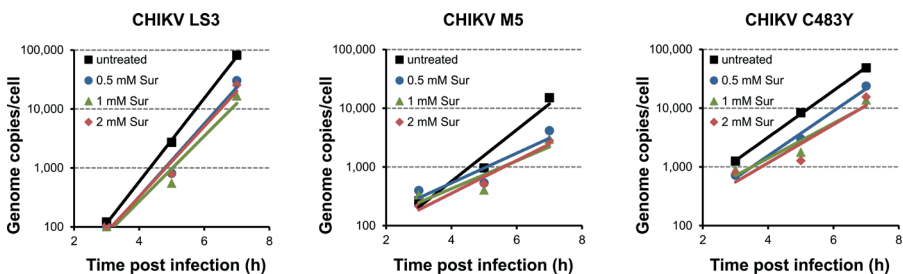


**Figure 4.** Effect of suramin on the kinetics of CHIKV RNA accumulation in cell culture.

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  $\mu$ M were found for CHIKV M5 and CHIKV C483Y, respectively, compared to 41  $\mu$ M for the parental CHIKV LS3. In a CPE protection assay with Vero E6 cells  $EC_{50}$  values of 72 and 61  $\mu$ M 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  $\mu$ M). 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.



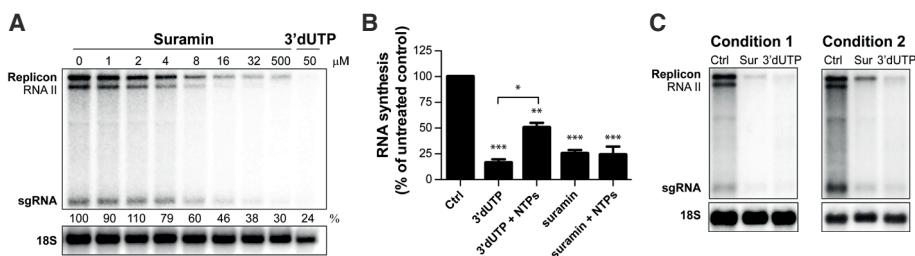
**Figure 5.** Effect of suramin on the kinetics of CHIKV RNA accumulation of wt CHIKV and two mutants that are resistant to ribavirin and favipiravir. Vero E6 cells were infected with CHIKV LS3, CHIKV M5 or CHIKV C483Y at an MOI of 3 and at 1 h p.i. the inoculum was removed, cells were extensively washed 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  $\mu$ M (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 <sup>32</sup>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 <sup>32</sup>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, <sup>32</sup>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. <sup>32</sup>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  $\mu$ M. 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  $\sim 62$   $\mu$ M, which is slightly better than suramin dissolved in water (79  $\mu$ M). 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)	$EC_{50}$ ( $\mu$ M)	$CC_{50}$ ( $\mu$ M)
#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	$\sim 100$
#PC3-Br1-sur.2	3.0	-	1.0	0.2	$\sim 100$	$\sim 100$
#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	$\sim 50$
#PC9-Br1-sur.2	9.0	-	1.0	0.2	ND	$\sim 50$

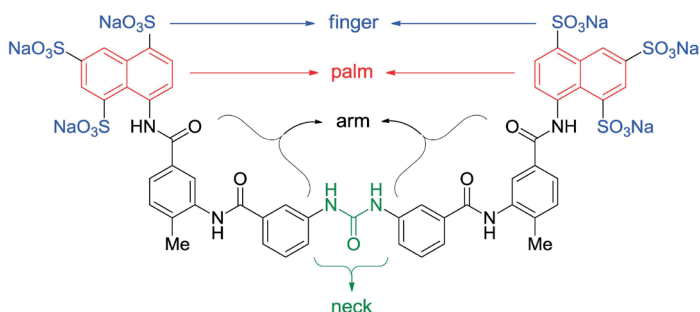
ND:  $EC_{50}$  not determined due to low  $CC_{50}$ . PC: phosphatidylcholine, DDAC: distearyldimethylammonium chloride, DDAB: dimethyl-dioctadecylammonium bromide.

### 3.9. Essential moieties in the structure of suramin

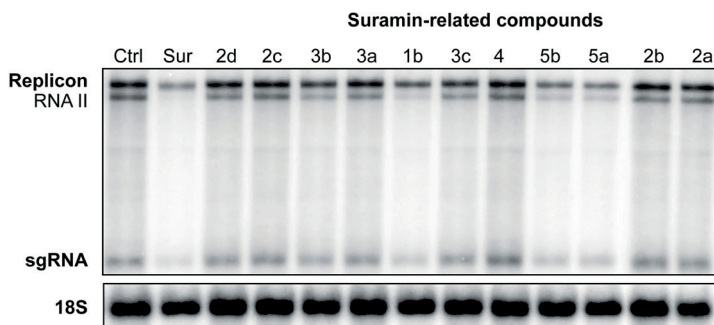
Suramin is a symmetric molecule (Fig. 7A; 1a in Table 3) with in the center a urea (NH-CO-NH) functional group as the “neck”. Suramin also contains two benzene rings with amide linkers on each side as the “arms” and possesses two naphthalene rings as the

A

#### Suramin structure



B



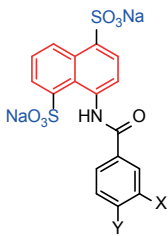
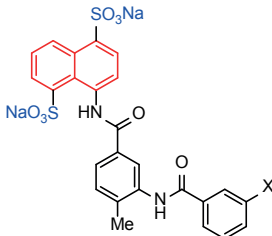
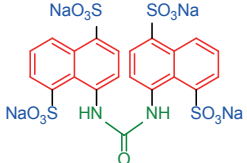
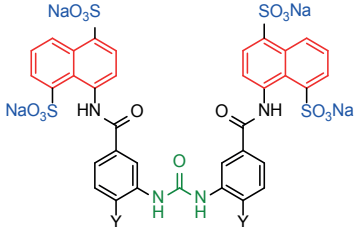
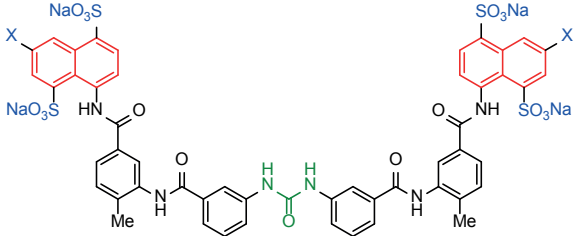
**Figure 7.** Effect of suramin-related molecules on CHIKV RNA synthesis.

(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  $\mu$ M) than tetrasulfonate 1b ( $EC_{50}$  200  $\mu$ M) 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 <sub>2</sub>	H	2
	b	NO <sub>2</sub>	H	2
	c	NH <sub>2</sub>	Me	2
	d	NO <sub>2</sub>	Me	2
	a	NH <sub>2</sub>	—	2
	b	NO <sub>2</sub>	—	2
	c	NHC(=S)OEt	—	2
	—	—	—	4
	a	—	H	4
	b	—	Me	4
	a	SO <sub>3</sub> Na <sub>3</sub>	—	6
	b	H	—	4



palm	arm	neck	Effect on RNA synthesis	EC <sub>50</sub> (μM)	CC <sub>50</sub> (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

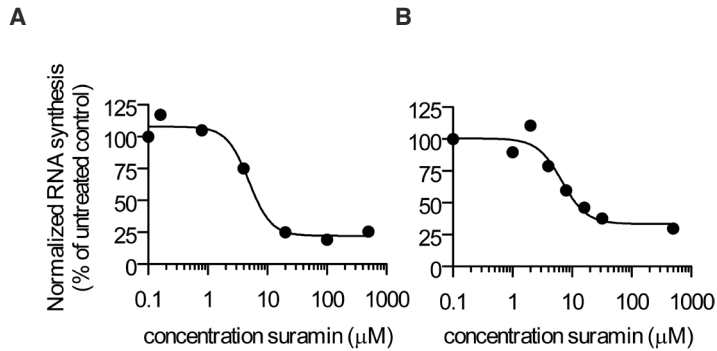
This work was supported by the EU-FP7 EUVIRNA (#264286) and SILVER (#260644) grants, as well as by the Ministry of Science and Technology of R.O.C. (# NSC 103-2923-I-008-001 and MOST 103-2113-M-007-018-MY3) and the National Central University, Taiwan (# 103G603-14). We are grateful to the LUMC biosafety 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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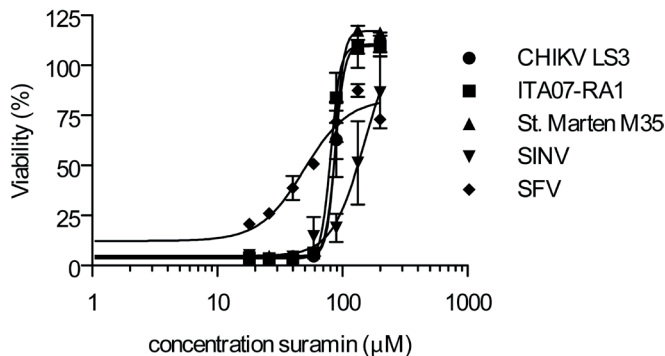
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## Supplemental Figures

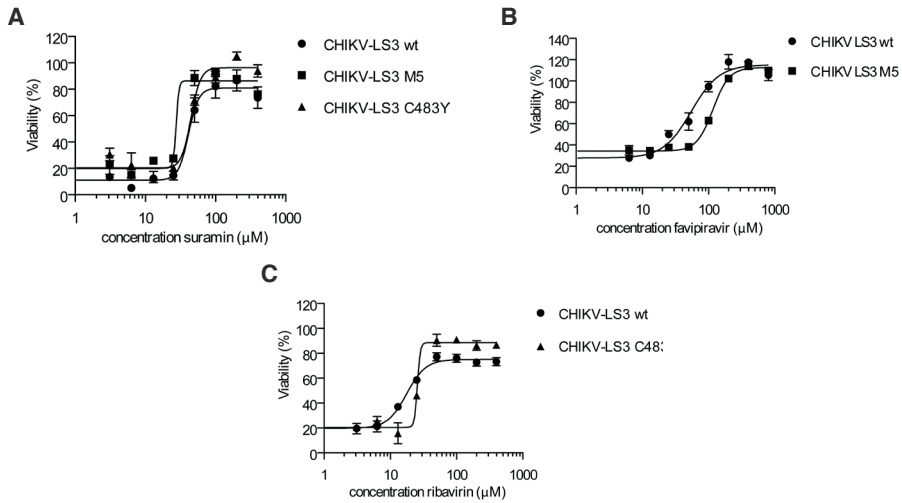


**Figure S1.** Quantification of the effect of suramin on alphavirus RNA synthesis *in vitro*.

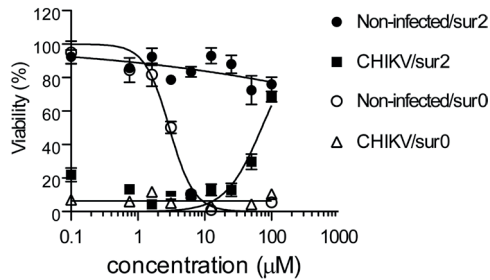
*In vitro* RNA synthesis assays were performed with RTCs, either isolated from CHIKV-infected Vero E6 cells (A) or from BHK-21 cells transfected with a CHIKV replicon (B), in the presence of increasing concentrations of suramin. RNA synthesis, i.e. the amount of incorporated  $^{32}\text{P}$ -CTP, was 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  $\text{IC}_{50}$  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.  $\text{EC}_{50}$  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.