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. 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 RNA synthesis with an IC50 of ~5 µM. The compound inhibited replication of various CHIKV isolates in cell culture with an EC50 of ~80 µM (CC50 >5 mM) and was also active against Sindbis virus and Semliki Forest virus. *In vitro* experiments hinted that suramin interferes with (re)initiation of RNA synthesis. Time-of-addition studies suggested that suramin also interferes with an early post-attachment step in infection, possibly entry. Favipiravir- or ribavirin-resistant CHIKV (nsP4) mutants did not exhibit cross-resistance to suramin, suggesting different modes 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 structure activity relationship. The antiviral effect of suramin containing liposomes was also analyzed. Its approved status makes it worthwhile to explore the use of suramin to treat and/or prevent CHIKV infections.

1. Introduction

Chikungunya virus (CHIKV) is a mosquito-borne arthrogenic alphavirus that has infected millions of people since its re-emergence in 2005. In November 2013, CHIKV emerged in the Caribbean (Weaver, 2014; Weaver and Lecuit, 2015), 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 (Thiberville et al., 2013), there are still no registered drugs on the market for treating CHIKV infections.

Suramin is a symmetrical sulfonated naphtylamine compound that was approved for the treatment of parasitic infections in 1921, while about 60 years later, its anti-cancer and antiviral potential were discovered (Liu and Zhuang, 2011; Voogd et al., 1993). Suramin was described as the first reverse transcriptase inhibitor for HIV (Mitsuya et al., 1984) and the compound was also shown to inhibit several RNA viruses (Ellenbecker et al., 2014; Jiao et al., 2013; Wang et al., 2014). Recently, suramin was identified as an inhibitor of norovirus RdRp activity by virtual screening and biochemical assays with purified enzymes (Mastrangelo et al., 2012; Tarantino et al., 2014). We therefore assessed the effect of suramin on CHIKV RNA synthesis using our recently established *in vitro* assay with isolated RTCs (Albulescu et al., 2014). Suramin inhibited CHIKV RNA synthesis and our studies with CHIKV-infected cells revealed that suramin also inhibits an early step in CHIKV infection. We describe here the inhibition of CHIKV replication by suramin through two independent mechanisms and provide more insight into the structure-activity relationship. Finally, the preparation, charcterization and in vitro assay, of liposomal formulations for suramin were reported, as novel delivery system for ameliorate the administration of the compound.

2. Material and Methods

2.1. Cell lines, viruses and virus titration.

Vero E6 and BHK-21 cells were cultured as described in (Scholte et al., 2013). Infectious clone-derived CHIKV LS3 and strain ITA07-RA1 have been previously described (Scholte et al., 2013). 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 (Delang et al., 2014) and CHIKV C483Y is identical to LS3 except for a C483Y mutation in nsP4 that renders it resistant to ribavirin (Coffey et al., 2011). 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 (Scholte et al., 2013). All experiments with CHIKV were performed in the Leiden University Medical Center biosafety level 3 facility.

2.2. Compounds

Suramin was from Santa Cruz and Sigma and 3'dUTP from TriLink. Suramin analogs 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 milliQ. Suramin-containing liposomes were prepared as previously described (Mastrangelo et al., 2014).

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 (Scholte et al., 2013).

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 (Albulescu et al., 2014) using RTCs isolated from VeroE6 cells infected with CHIKV, SINV or SFV4 or BHK-21 cells transfected with CHIKV replicon RNA (see 2.7).

2.5. RNA isolation and analysis

RNA isolation from infected cells, denaturing agarose gel electrophoresis, detection of ^{32}P-RNA and viral RNA by hybridization with (strand-) specific probes have been described previously (Albulescu et al., 2014; van Hemert et al., 2008). CHIKV genome copy numbers were determined by internally-controlled TaqMan multiplex RT-qPCR as described(Scholte et al., 2015).

2.6. SDS-PAGE and Western blotting

Detection of CHIKV proteins by Western blotting was done using procedures and antisera that were described previously (Scholte et al., 2015; Scholte et al., 2013).

2.7. 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 (Fros et al., 2010) 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.

2.8 Statistical analysis

Graph-Pad Prism 5.01 was used for statistical analysis and EC50, IC50 and CC50 determination.

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 (Mastrangelo et al., 2012; Tarantino et al., 2014), we set out to study its effect on CHIKV RNA synthesis using our recently established *in vitro* assay. This assay measures the incorporation of ^{32}P-CTP into viral RNA and we found that suramin severely impaired the *in vitro* RNA-synthesizing activity of RTCs isolated from CHIKV-infected cells in a dose-dependent manner, with an IC50 of approximately 5 µM (Fig 1A). Suramin also inhibited the *in vitro* activity of RTCs obtained from SINV- (Fig. 1B) or SFV-infected cells (Fig. 1C), suggesting that suramin has a broad-spectrum inhibitory effect on alphavirus RNA synthesis. A small fraction of the RNA synthesizing

activity appeared to be 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.

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 CC50. The EC50 values for our infectious clone-derived CHIKV LS3, a natural isolate from Italy (ITA07-RA1) and a CHIKV strain from the Caribbean outbreak (STM35) were 75-80 µM (Table 1). The EC50 values are ~15 times higher than the IC50 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 CC50 of suramin in our system was higher than 5 mM, yielding a selectivity index (SI) of >60 for CHIKV and SFV. In a plaque reduction assay, in which suramin was only present for 1 h during infection, the concentration of suramin that reduced the number of CHIKV plaques by 50% was determined to be 80 µM (data not shown).

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 CHIKV replication cycle. Vero E6 cells were pretreated with various concentrations of suramin up to 500 µM (until the lysis step), infected with CHIKV, and lysed for analysis at 12 h p.i. The expression of nsP1 and capsid protein was reduced by suramin 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 by suramin, leading to a 4-log reduction when 500 µM of the compound was present (Fig. 2C). The observed reduction of -RNA levels and (consequently) that of +RNA, nonstructural and structural proteins and infectious virus in this single cycle analysis suggests that suramin affects an early step of the replication cycle.

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

To determine which step of the CHIKV replication cycle is inhibited by suramin, 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, 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, after which they were 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. 3B). When suramin was added very early, not later than 30 minutes p.i., it strongly reduced CHIKV replication, as indicated by the ~90% 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 an ~20% reduction in -RNA levels (Fig. 3A, samples 7 & 8). These results suggest that -besides its effect on RNA synthesis- suramin also inhibits a very early step of the CHIKV replicative cycle, possibly attachment or entry.

To test whether suramin has a negative (virucidal) effect on the infectivity of virions, 10^5 PFU of CHIKV were subjected to a 30-min incubation with 500 µM suramin or 70% ethanol (positive control for virucidal activity) and the (remaining) infectious virus titer was analyzed by plaque assay. Compared to the untreated control (Ctrl), suramin caused no drop in the infectious titer, while ethanol completely abolished infectivity (Fig. 3B). This demonstrates that suramin has no virucidal effect and also suggests that it does not interact with CHIKV particles, or only very transiently with low affinity. 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 minutes in medium with various concentrations of suramin, after which the cells were washed 5 times with ice cold PBS. The amount of bound virus was quantified by RT-qPCR analysis of total RNA collected immediately after the last washing step (Fig. 3C). Suramin had no significant effect on the amount of bound virus and therefore does not appear to interfere with attachment of CHIKV, but likely at a later post-attachment step such as entry. The compound might interfere with 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 cell culture, we analyzed the kinetics of the accumulation of CHIKV genomic RNA in infected cells that were treated with various high doses of suramin added 1 h after infection. Figure 4 shows that post-infection treatment with suramin leads to slower kinetics of RNA synthesis *in vivo*, leading to ~1-log 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 vivo*, 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 sgRNA (data not shown), suggesting that besides its effect on entry, suramin also inhibits RNA synthesis *in vivo*. However, the effect on RNA synthesis appeared to be much weaker *in vivo* compared to effect on the early step (entry), which might be due to the poor uptake/intracellular availability of suramin.

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

We determined the suramin sensitivity of CHIKV (nsP4) mutant virus CHIKV M5, which is resistant to favipiravir (Delang et al., 2014) and of CHIKV C483Y, which has a C483Y mutation in nsP4 that renders it resistant to ribavirin (Coffey et al., 2011). In a CPE protection assay EC50 values of 72 and 61 μM were found for CHIKV M5 and CHIKV C483Y, suggesting that these mutants are even slightly more sensitive to suramin than the wt parent virus CHIKV LS3 (EC50 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). Like 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.

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. In this biosafe system suramin also inhibited RNA synthesis with an IC50 of ~5 μM (Fig. 6A). The inhibitory effect of

nucleoside analogs can be reversed by adding an excess of NTPs (Albulescu et al., 2014), 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 target the NTP binding pocket.

As can be seen in figures 1 and 6C, we observed that even at very high doses of suramin some incorporation of ^{32}P-CTP into viral RNA remained. We hypothesized that this might be because complexes already involved in RNA synthesis (interacting with the template) are not sensitive to inhibition by suramin, which would then mainly inhibit (re)initiating RTCs. To test our hypothesis, we allowed a reaction 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 30 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" generated non-radioactive products that are not detected. Merely preincubating RTCs with suramin for 15 min before starting the *in vitro* reaction did not have the same effect (Fig. 6C, condition 2).

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 suramin for their efficacy to inhibit CHIKV replication in CPE protection assays (Table 2). The negatively charged suramin was indeed bounded in cationic liposomes in order to develop formulations, exhibiting decreased drug-related toxicity, enhanced cellular uptake, and possible higher accumulation in macrophage-rich organs.

Control liposomes without suramin exhibited a relatively high cytotoxicity, while suramin-containing liposomes were less cytotoxic, with CC50 values of 50-100 µM. Formulation L3 inhibited CHIKV replication with an EC50 of ~62 µM, which is slightly better than suramin dissolved in water. The L3 formulation is an interesting starting point for further optimization to improve the efficacy of suramin.

3.9. Structure activity relationship

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 contains two benzene rings with amide linkers on each side as the “arms” and possesses two naphthalene rings as the “palms” and six sulfonate groups as the “fingers”. Table 3 lists eleven suramin-related compounds (1–5), that were tested for their ability to inhibit CHIKV RNA synthesis *in vitro* (Fig. 7B; Table 3) and to inhibit CHIKV replication *in vivo* in a CPE protection assay (Table 3). These molecules include suramin derivative 1b, which possess a very similar structure to that of suramin except it only has four sulfonate fingers. The two symmetric analogs 4 and 5 have four fingers, two palms, and a neck, but compound 4 had no arms, while compound 5 had two short arms. Asymmetric compounds 2a–d and 3a–c1 are synthetic intermediates, each of which possessed two fingers and one palm only. None of these compounds had a neck.

Examination of the biological activities of compounds 1–5 (Table 3) indicates that CHIKV RNA synthesis was inhibited by compounds 1a, 1b, 5a and 5b. The presence of a neck, two arms, two palms, and 4–6 fingers in all of these compounds appears to be an important feature for inhibition of the RTC. Analog 4, which lacks arms did not inhibit RNA synthesis. Also asymmetric compounds 2a–d and 3a–c, which only have one arm, were inactive regardless of the length of the arm. Therefore, a neck and two arms are essential to their capability of inhibiting the CHIKV RNA synthesis.

Suramin (1a) with six sulfonate groups exhibited greater anti-CHIKV activity (EC50 80 μM) in cell culture than tetrasulfonate 1b (EC50 200 μM). These results indicate that the number of sulfonate fingers plays a role in the antiviral effect, likely the one that inhibits the early step (entry) of the CHIKV replication cycle. Apparently, a compound with more fingers performs better than its analog with fewer fingers. Also the length/conformation of the spacer consisting of the arms and neck appear to be important for the antiviral activity *in vivo*.

Among all sulfonates 1–5, suramin turned out to be the best candidate as the potential anti-CHIKV drug. It possesses long arms with lipophilicity that could interact with lipids and might aid in crossing the cell membrane. Meanwhile, the multiple sulfonate fingers therein offer hydrophilicity for allowing it to dissolve in water. The amphiphilic properties (1a) and the unique framework of neck–arm–palm–finger associated with the sulfonates 1a and 1b make these two compounds stand out in the development of new drugs against CHIKV.

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 another, earlier but post-attachment, step of the CHIKV replicative cycle, likely viral entry. Suramin appears to inhibit (re)initiation of CHIKV RNA synthesis, maybe by interfering with binding of the template RNA. The structure activity relationship was analyzed, which did not yield more effective compounds, but provided insight into the (different) structural elements that are important for both inhibitory activities of suramin. Inhibition of CHIKV replication through two different mechanisms makes it worthwhile to further explore the therapeutic potential of suramin, especially in novel (liposome) formulations.

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TABLES

Table 1. Antiviral activity of suramin against various alphaviruses in cell culture. *EC50 values were determined in CPE reduction assays and the average and standard deviation of 2 independent experiments, performed in quadruplicate are listed.*

Virus	EC50 (µM)
CHIKV LS3	79 ± 11.6
CHIKV ITA07-RA1	76 ± 7
CHIKV STM35	79 ± 12.9
SINV	141 ± 18.3
SFV	40 ± 10

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)	EC50 (µM)	CC50 (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	57	~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	~ 100	~ 50
#PC9-Br1-sur.2	9.0	-	1.0	0.2	~ 100	~ 50

ND: EC50 not determined due to low CC50.

Table 3. ***Structure of suramin-related compounds and their effect on CHIKV replication in vivo and RNA synthesis in vitro*.**

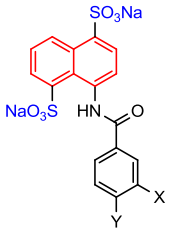
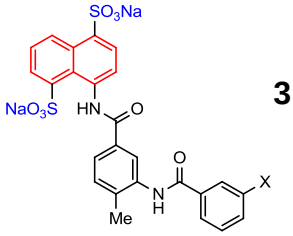
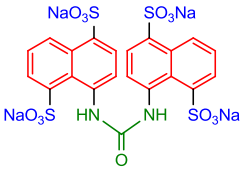
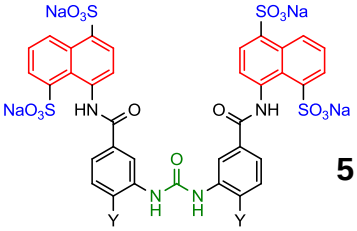
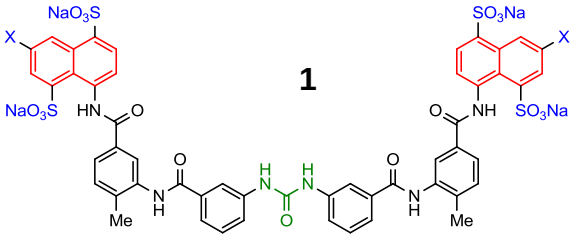
Compound structure	label	x=	y=	finger	palm	arm	neck	Effect on RNA synthesis	EC50	CC50
 **2**	**a**	NH_2	H	2	1	short × 1	0	-	739	>800
	b	NO_2	H	2	1	short × 1	0	-	>800	>800
	c	NH_2	Me	2	1	short × 1	0	-	>800	>800
	d	NO_2	Me	2	1	short × 1	0	-	>800	>800
 **3**	**a**	NH_2	—	2	1	long × 1	0	-	420	>800
	b	NO_2	—	2	1	long × 1	0	-	>800	>800
	c	NHC(=S)OEt	—	2	1	long × 1	0	-	>800	>800
 **4**	—	—	—	4	2	0	1	-	>800	>800
 **5**	**a**	—	H	4	2	short × 2	1	+	403	>800
	b	—	Me	4	2	short × 2	1	+	>800	>800
 **1**	**a** (suramin)	SO_3Na	—	6	2	long × 2	1	+	79	>800
	b	H	—	4	2	long × 2	1	+	210	>800

FIGURE LEGENDS

Fig1. Effect of suramin on alphavirus RNA synthesis *in vitro*.

In vitro RNA synthesis assays with RTCs isolated from 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.

Figure 2. Effect of suramin on CHIKV replication

(**A**) Western blot analysis of nsP1 and capsid protein expression 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 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.

Fig 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 extensively washed and they were 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. (**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 number of bound CHIKV genome copies per well was determined by internally controlled multiplex RT-qPCR.

Fig 4. Effect of suramin on the kinetics of CHIKV RNA accumulation *in vivo.*

Vero E6 cells were infected with CHIKV (MOI 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.

Fig 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.

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 520 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 not (condition 2) RNA synthesis. ^{32}P-CTP was absent during the first 30 min, but was present during the following 60 min. For details see section 3.7.

Fig. 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.