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Suramin inhibits Zika virus replication by interfering with virus attachment and release of infectious particles

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Abstract

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

Highlights

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

1. Introduction

Zika virus (ZIKV) is a flavivirus that was first isolated in 1947 in Uganda (Dick et al., 1952) and is primarily transmitted by *Aedes* mosquitos. Up to 2007, it received little attention, as only sporadic outbreaks were reported and these were not linked to serious disease. ZIKV has gained attention after the 2007 outbreak on Yap island and the 2013 epidemic in French Polynesia. Research efforts and public concern were further sparked by the massive epidemic in Brazil and many other South- and Central-American countries, which uncovered a link between ZIKV infection and serious foetal neurodevelopmental defects and neurological complications in adults.

About 80% of ZIKV infections are asymptomatic. In combination with ZIKV persistence in semen and the potential for sexual transmission, this poses a risk to women that are pregnant or trying to conceive. Symptomatic ZIKV infections are commonly characterized by low-grade fever, skin rash, conjunctivitis, and general malaise. However, the enormous scale of the recent epidemics has also revealed uncommon but more serious consequences, like the Guillan-Barré syndrome and foetal neurodevelopmental defects, like microcephaly. These could be due to ZIKV's ability to infect and persist in several immune-privileged tissues, like the central nervous system, placenta and testis (Miner and Diamond, 2017).

ZIKV has a 10.7 kb ssRNA genome of positive polarity, which is 5' capped and lacks a poly(A) tail. The replicative cycle begins with binding to receptor and co-receptors, like AXL and TIM1 (Liu et al., 2016; Meertens et al., 2017), followed by entry through receptor-mediated endocytosis, uncoating and translation of the genome into a single polyprotein. The latter is processed by cellular and viral (NS3) proteases to yield the structural proteins C, prM and E and 7 nonstructural proteins, which are responsible for RNA replication in association with modified endoplasmic reticulum (ER) membranes and interactions with the host, including counteracting innate immune responses. Newly synthesized genomes, together with C, prM and E, are assembled into immature virions that bud into the ER lumen. These pass through the secretory pathway during which prM cleavage by the host protease furin leads to maturation, before particles are released into the extracellular space.

There are no registered vaccines against ZIKV and efforts to identify inhibitors of ZIKV replication have been initiated only recently, for example in the form of two large-scale compound screens that yielded surprisingly few common hits (Adcock et al., 2017; Barrows et al., 2016; Cheng et al., 2016; Dallmeier and Neyts, 2016; Xu et al., 2016). Considering the generally low success rate in antiviral

drug development, it is crucial to identify a large number of ZIKV inhibitors in cell culture and elucidate their mode of action, in order to have sufficient lead compounds with the potential to advance further towards clinical development.

Suramin is an approved anti-parasitic drug that also blocks the replication of a variety of viruses (see (Albulescu et al., 2015) and references therein), including arboviruses like chikungunya virus (CHIKV) (Albulescu et al., 2015; Henss et al., 2016; Ho et al., 2015) and dengue virus (Chen et al., 1997). Animal experiments have indicated that suramin can be used for preventing and treating enterovirus-71 and CHIKV infections (Kuo et al., 2016; Ren et al., 2014).

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

2. Materials and Methods

2.1. Cells, viruses and compounds

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

2.2. Cytopathic effect (CPE) reduction assay

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

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

Plaque assays were performed as described (Van Boheemen et al, submitted). Briefly, after a 1-min centrifugation at 13,000xg, virus-containing cell culture supernatants were 10-fold serially diluted in medium and 500-µL volumes were adsorbed for 2 h on confluent Vero cell monolayers in 6-well clusters. The inoculum was removed and an overlay of 1.2% Avicel RC-581 (FMC BioPolymer) in DMEM, 2% FCS, 25 mM HEPES, and antibiotics, was added. After a 5-day incubation, cells were fixed with formaldehyde, stained with crystal violet and plaques were counted. For PRNT, $3.2x10^4$ PFU of ZIKV were incubated with various dilutions of heat-inactivated patient serum (against ZIKV SL1602) or a control serum for 1h at 37°C. Afterwards 250-µL volumes of 10-fold serial dilutions were adsorbed for 2 h on confluent Vero cell monolayers in 12-well clusters. An overlay was placed on top and plaques were visualized after 5 days as described for the plaque assay.

2.4. ZIKV RNA analysis

RNA was isolated from ZIKV-infected cells using TriPure isolation reagent (Roche) according to the manufacturer's instructions. ZIKV RNA was isolated from the medium of infected cells using the Qiamp Viral mini kit (Qiagen) with AVL lysis buffer spiked with equine arteritis virus (EAV) as internal control, as described previously (Scholte et al., 2015). ZIKV RNA copy numbers were determined by an in-house internally-controlled TaqMan multiplex RT-qPCR, using PGK1 as a reference gene, as

described (Manuscript in preparation). Briefly, the detection of ZIKV RNA was done with the primer pair Fw 5'-AATGGCAGTCAGTGGAGATG/ Rv 5'-ACTCTTGTGTGTCCTTCCTAAC and a FAM-labelled probe (5'-6FAM-ATAGGTTTGCACATGCCCTCAGGT-3'-BHQ_1) using the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems) and the CFX384 Touch™ Real-Time PCR Detection System. Known quantities of *in vitro* transcribed RNA were used to generate calibration curves for absolute quantification of copy numbers. Each sample was analyzed in triplicate.

2.5. Immunofluorescence assay

Vero cells (50,000 per well) were seeded on coverslips in 24-well clusters. The next day they were infected with ZIKV or mock-infected and treated with various concentrations of suramin. At 26 h p.i. cells were fixed with 3% paraformaldehyde in PBS, and processed for immunofluorescence microscopy as previously described (Scholte et al., 2013). ZIKV-specific proteins were visualized with a 1:1000 dilution of the SL1602 patient antiserum, and double-stranded RNA was stained with mouse monoclonal antibody J2 (English & Scientific Consulting). Detection of primary antibodies was done with 1:500 dilutions of goat anti-human Alexa488 and donkey anti-mouse Cy3.

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

Confluent Vero cells (75 cm^2) were infected with ZIKV at an MOI of 0.05 or mock infected and incubated for 3 days. Medium was replaced with protein-labeling medium (Met- and Cys-free DMEM) containing 88 µCi of ^{35}S-labelled Met and Cys (0.0176 µCi/µL) and cells were incubated for 15 h. The culture supernatants were collected and cellular debris was removed by centrifugation for 10 min at 233 x g. Unincorporated label was removed by pelleting virions through a cushion of 20% sucrose in TESV buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM NaCl) by ultracentrifugation for 2 h at 45,000 rpm and 4°C using a Beckmann SW55 rotor. After removal of the supernatant and sucrose layer, the virus pellet was washed with TESV buffer, and resuspended in 0.6 mL of TESV. Aliquots of labelled virus were stored at -80°C. For binding assays, Vero cells seeded in 24-well clusters were incubated with 4 µL ($3.2x10^4$ PFU) of radioactive ZIKV for 2 h at 4°C in a total volume of 125 µL. After removal of the inoculum and washing three times with PBS, cells were lysed in 200 µL of 100 mM Tris-HCl pH 6.8, 8% SDS (w/v), 40 mM DTT, and 40% glycerol to quantify cell-bound radioactivity

using a Beckman-Coulter LS6500 Multi-Purpose scintillation counter and Ultima Gold™ scintillation liquid.

2.8. Statistical analysis

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

3. Results

3.1. Suramin inhibits ZIKV in CPE reduction assays

To determine the anti-ZIKV activity of compounds, we have set up CPE reduction assays with Vero cells, which were treated with serial dilutions of compound, followed by mock-infection or ZIKV-infection at an MOI of 0.05. After a 4-day incubation, colorimetric viability assays were performed to quantify the protective effect of a compound against ZIKV-induced CPE. Publications on compounds with demonstrated anti-ZIKV activity were not available at the time we set up our assay (3/2016) and therefore we included mycophenolic acid (MPA), known to inhibit a broad-spectrum of viruses, as a positive control. MPA had an EC_{50} of 0.42 µM and a CC_{50} of over 250 µM in our assay and its antiviral effect has also been demonstrated by others ((Adcock et al., 2017; Barrows et al., 2016). Suramin protected Vero cells from ZIKV-induced cell death with an EC_{50} value of 39.8 µM (supplemental Fig. S1). The CC_{50} of suramin was 1.9 mM, resulting in a selectivity index (SI) of 48.

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

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

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

3.3. Suramin inhibits the attachment of ZIKV to host cells

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

To test whether suramin affects ZIKV attachment to host cells, we have produced ^{35}S-labeled ZIKV for use in virus binding assays. The radioactive ZIKV was incubated with Vero cells for 2 h at 4°C, which should only allow binding, while preventing endocytosis/entry. After washing, cells were lysed and the amount of cell-bound radioactivity was determined by scintillation counting. Vero cell-associated radiolabel was readily detected when ^{35}S-labeled ZIKV was used in this assay, while hardly any radiolabel was bound when Vero cells were incubated with a virus-free control sample that had been prepared by ^{35}S-labeling of mock-infected cells (Fig. 3C). To determine whether inhibition of

binding could be measured in this assay, ^{35}S-labeled ZIKV was incubated with a neutralizing antiserum from a ZIKV-infected patient prior to the binding assay (Fig. 3C). This serum completely neutralized the virus at 1:5 (and higher) dilutions in plaque reduction neutralization assays (Fig. 3C, inset). When the binding assay was performed with virus that was neutralized with a 1:5 antiserum dilution, a 50% reduction in cell-bound radiolabel was observed. It remains unclear whether the remaining amount of radioactivity resulted from cell-bound radiolabeled virus or from radiolabeled host proteins that might be absent in the ^{35}S-labeled control sample obtained from mock-infected cells as the latter had not undergone the ZIKV-induced CPE. Nonetheless, the assay allowed us to measure decreases in ZIKV binding. When various suramin concentrations were tested in this assay, we observed a dose-dependent inhibition of ^{35}S-ZIKV binding of up to 50% (Fig. 3D), the same residual level of labeling measured when using pre-neutralized virus (Fig. 3C). Taken together these results indicate that suramin treatment affects binding of ZIKV to the host cell.

3.4. Suramin treatment also reduces the release of infectious progeny

Analysis of the infectious virus titers in the supernatant of samples from a time-of-addition experiment revealed that suramin not only affected an early step of the replication cycle, but also reduced progeny titers when added at later stages (Fig. 4A). When suramin treatment was started at 2 h post entry, it had no significant effect on intracellular viral RNA levels, suggesting it does not interfere with RNA replication (Fig. 2A), while a ~100-fold reduction of infectious progeny released into the medium was observed (Fig. 4A). To confirm that suramin also has a late effect on the release or maturation of viral particles, we treated ZIKV-infected cells with 25-200 μM of suramin from 14 to 26 h p.i. As expected, the number of infected cells (based on immunofluorescence microscopy) did not change (results not shown) by this late suramin treatment, while there was a dose-dependent decrease of up to 35-fold in the amount of infectious progeny released (Fig. 4B).

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

latter effect was less pronounced (~10-fold) when suramin treatment was initiated late in the replication cycle (18 or 22 h p.i.). These results suggest that suramin affects both the total number of released virions (genome copies) and their infectivity (PFU).

4. Discussion

Here we demonstrate that the approved drug suramin has anti-ZIKV activity by interfering with viral attachment, as well as the release of infectious progeny from ZIKV-infected cells. In CPE reduction assays suramin had an EC_{50} value of 39.8 μM and a SI of 48. In this assay, MPA had an EC_{50} of 0.42 μM, similar to values reported recently by others (Adcock et al., 2017; Barrows et al., 2016). In low-MOI CPE reduction assays MPA appeared to inhibit ZIKV more efficiently than suramin, but in the context of a high-MOI infection, treatment with 2.5 μM MPA led to a mere 10-fold reduction in the accumulation of intracellular ZIKV RNA and infectious ZIKV titers in the medium (Fig. 1). In contrast, treatment of cells infected at a high MOI with 50 μM of suramin, while also reducing intracellular ZIKV RNA accumulation by 10-fold, yielded a ~300-fold reduction in the amount of infectious progeny (Fig. 1B). MPA's limited effect on high-MOI ZIKV infections, its immunosuppressive properties and its contraindicated use during pregnancy makes it an unattractive lead to follow.

Time-of-addition experiments and binding experiments with radioactive ^{35}S-labeled ZIKV at 4°C suggested that suramin affects viral attachment to the host cell.

The inhibition of attachment might be due to suramin's effect on virions, as was previously reported for DENV (Chen et al., 1997) and enterovirus A71 (Nishimura et al., 2015), for which suramin was proposed to have a 'neutralizing effect' that blocks the interactions of these viruses with cellular heparan sulfate, and the P-selectin ligand, respectively. Suramin was suggested to interact with surface-exposed positively charged residues in the DENV E protein that are part of the glycosaminoglycan-binding (GAG-binding) sites, thereby interfering with attachment to the host cell (Chen et al., 1997). It was recently demonstrated that binding to GAGs likely also plays a role in ZIKV attachment (Kim et al., 2017).

Besides interacting with the virion, suramin could also have an effect on host cell (co)receptors, as it was shown to abolish the interaction of several cellular receptors with their ligands (Coffey et al., 1987; Waltenberger et al., 1996; Wu et al., 2016). Binding of suramin to cellular (glyco)proteins, like

the proposed ZIKV receptors AXL (Meertens et al., 2017; Richard et al., 2017) or glycoprotein TIM1 (Tabata et al., 2016) could interfere with ZIKV attachment.

Suramin treatment also inhibited the release of infectious progeny from ZIKV-infected cells, not only by lowering the number of genomes released, but also by reducing the specific infectivity (Fig. 4).

This post-entry effect might be due to binding of the negatively charged suramin to one of ZIKV's RNA-binding proteins, as suramin was shown to inhibit Dengue and hepatitis C virus helicase activity (Basavannacharya and Vasudevan, 2014; Mukherjee et al., 2012), norovirus RNA-dependent RNA polymerase activity (Mastrangelo et al., 2012; Tarantino et al., 2014), reverse transcriptase activity of various retroviruses (De Clercq, 1979), including HIV (Chandra et al., 1985), and the activity of alphavirus replication complexes (Albulescu et al., 2015). Since we did not observe inhibition of ZIKV RNA synthesis in infected cells, an effect on NS5 appears unlikely. Suramin might inhibit the helicase activity of ZIKV NS3 or might affect packaging by binding to positively charged residues on the capsid protein. This would result in accumulation of genomes in the cell, while virus release would be diminished. The E protein, prM, NS1, NS2A, NS4A or/and NS4B (Garcia-Blanco et al., 2016) or furin could also be targets of suramin, with consequences for virion assembly, glycosylation of ZIKV proteins and/or maturation of the virion, lowering the infectivity of released virions. ZIKV prM, E, and NS1 proteins contain potential N-glycosylation sites (Berthet et al., 2014; Kuno and Chang, 2007) and glycosylation of the E proteins of ZIKV and West Nile Virus is important for virion maturation and infectivity (Li et al., 2006; Mossenta et al., 2017). Suramin is a highly charged molecule that, complexed with serum albumin, can be taken up by cells through endocytosis. The concentration of albumin determines whether suramin mainly accumulates in the lysosomal compartment or in the Golgi apparatus, mitochondria and nucleolus (Baghdiguian et al., 1996). The latter might cause a generalized negative effect on the secretory pathway that would also affect virion biogenesis.

Studies on suramin-related compounds revealed that the six sulfonate groups as well as the symmetry of the molecular structure are crucial elements for suramin's anti-CHIKV activity (Albulescu et al., 2015). Addition of sulfonate groups and a higher level of branching increased the compound's anti-enterovirus activity (Nishimura et al., 2015), suggesting the molecule can be further optimized.

Because of the increased and geographically expanding incidence, unnoticed infections in combination with sexual transmission, and potentially severe consequences of mother-to-child transmission, ZIKV infection continues to be a major public health concern, for which therapeutics are

urgently needed. Suramin has been approved for use in humans and has been used safely and successfully in animal models for treating several virus infections. Future studies should evaluate the efficacy of suramin on ZIKV replication in animal models and determine whether suramin or related compounds have the potential to advance into further clinical development for treatment of ZIKV infection.

Acknowledgments

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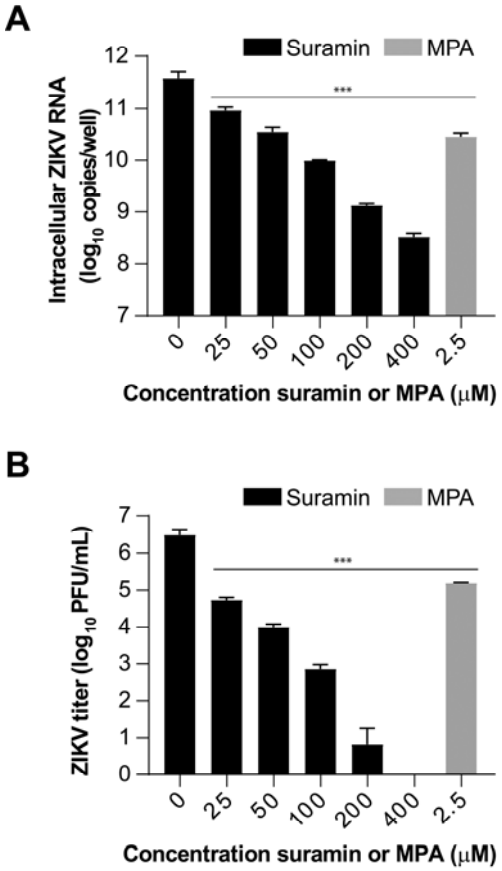

Figure 1. Effect of suramin on the accumulation of intracellular ZIKV RNA and infectious progeny titers. A. ZIKV-infected Vero cells were treated with 25 to 400 µM suramin from 2 hours prior to infection till 44 h p.i. Untreated and mock-infected cells were included as controls and the inhibitory effect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

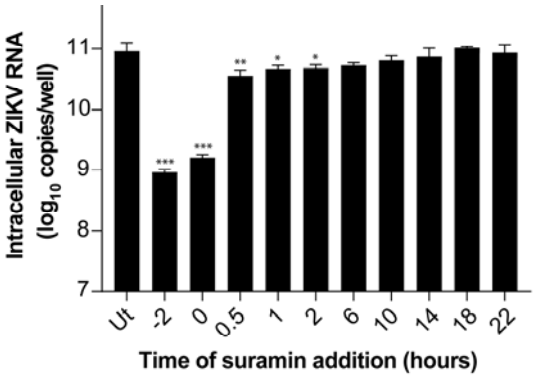

Figure 2. Effect of suramin on ZIKV replication in time-of-addition assay
ZIKV-infected Vero cells were treated with 200 µM of suramin starting 2 h before infection until 26 h p.i. Untreated (Ut) and mock-infected cells were included as controls. Total RNA was isolated from cells at 26 h p.i. and intracellular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

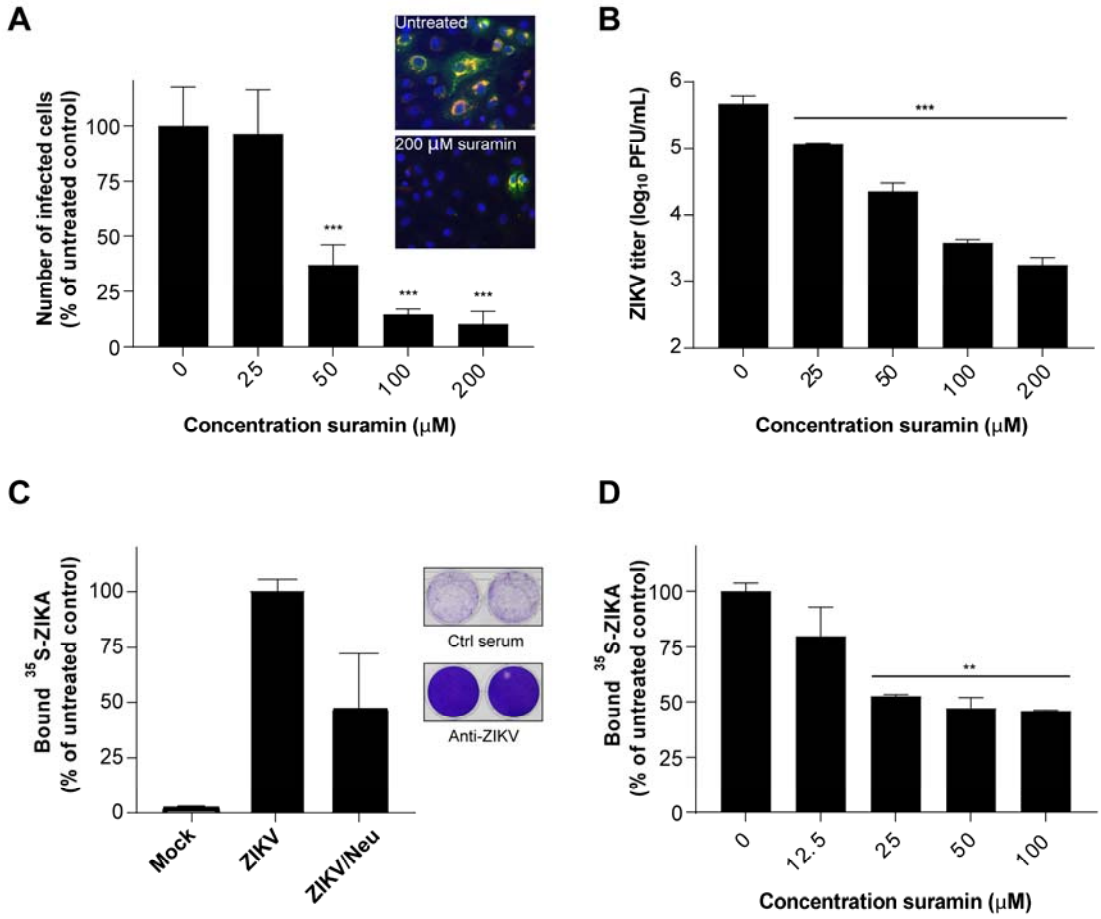

Figure 3. Effect of suramin on ZIKV attachment to host cells

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

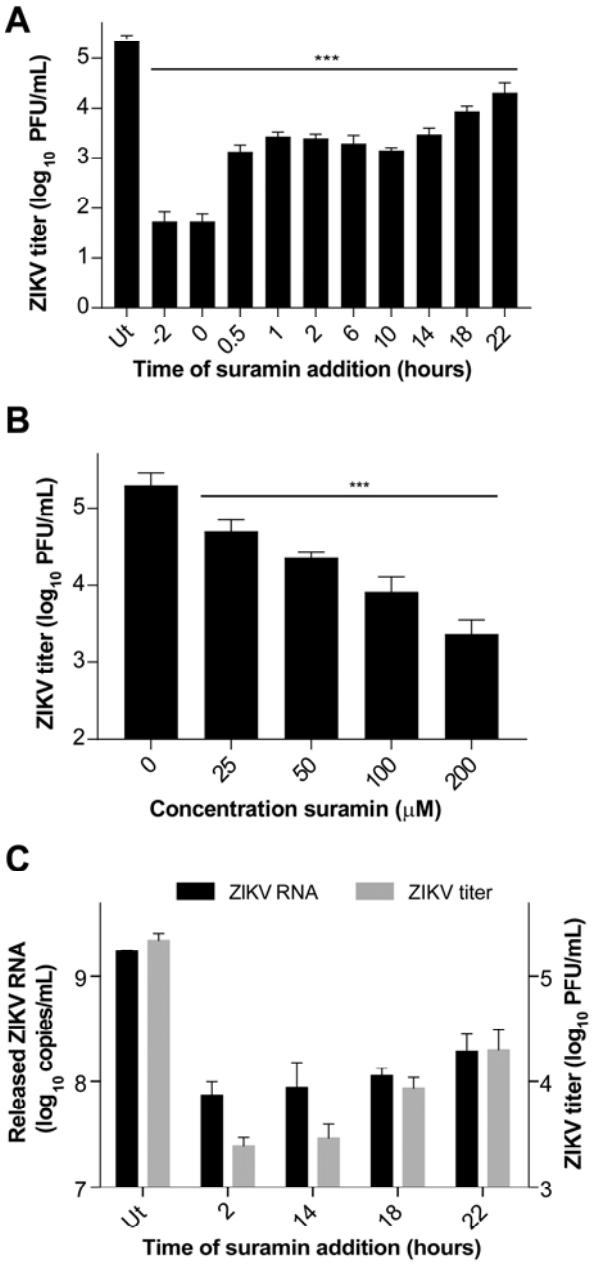

Figure 4. Effect of suramin on the production of infectious ZIKV progeny

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

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