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Suramin inhibits chikungunya virus replication through multiple mechanisms

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ABSTRACT

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

1. Introduction

Chikungunya virus (CHIKV) is a mosquito-borne arthritogenic alphavirus that has infected millions of people since its re-emergence in 2005. In November 2013, CHIKV emerged in the Caribbean (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 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 De Clercq, 2015; Liu and Zhuang, 2011; Voogd et al., 1993). It was shown that suramin had anti-reverse transcriptase activity against tumor-inducing viruses (De Clercq, 1979) and it was actually the first documented HIV reverse transcriptase inhibitor that was tested in human patients (Broder et al., 1985), but the compound's side effects outweighed the clinical benefit due to the required long term treatment (Kaplan et al., 1987). A later study revealed that suramin's anti-HIV-1 activity *in vivo* was

Abbreviations: CHIKV, chikungunya virus; SINV, Sindbis virus; SFV, Semliki Forest virus; Sur, suramin; nsP, nonstructural protein; RdRp, RNA-dependent RNA polymerase; RTCs, replication and transcription complexes; CPE, cytopathic effect; CC_{50}, half maximal cytotoxic concentration; EC_{50}, half maximal effective concentration in cell culture; IC_{50}, half maximal inhibitory concentration *in vitro*; 3′dUTP, 3′-deoxyuridine-5′-triphosphate.

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actually due to its inhibitory effect on the interaction between the viral gp120 and the CD4 receptor (Schols et al., 1990). 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 (Aguilar et al., 1999), cytomegalovirus (Baba et al., 1993), human hepatitis B virus (Schulze et al., 2007), hepatitis delta virus (Petcu et al., 1988), hepatitis C virus (Garson et al., 1999), dengue virus (Chen et al., 1997), several bunyaviruses (Crance et al., 1997; Ellenbecker et al., 2014; Iqbal et al., 2000; Jiao et al., 2013), norovirus-like particles (Tamura et al., 2004) and enterovirus 71 (Wang et al., 2014), for which the antiviral activity of suramin was also confirmed in an animal model (Ren et al., 2014). In recent *in vitro* studies suramin was identified as a hepatis C virus and dengue virus helicase inhibitor (Basavannacharya and Vasudevan, 2014; Mukherjee et al., 2012) and also as a norovirus RdRp inhibitor by virtual screening and biochemical assays with purified enzymes (Mastrangelo et al., 2012; Tarantino et al., 2014). 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 (Albulescu et al., 2014). 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 (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 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 (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 LS3, SINV or SFV4 or BHK-21 cells transfected with CHIKV replicon RNA (see 2.6).

2.5. CHIKV protein and RNA analysis

RNA isolation from infected cells, denaturing agarose gel electrophoresis, detection of ^{32}P-RNA or viral RNA by hybridization with (strand-) specific probes have been described previously (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). Detection of CHIKV proteins by SDS–PAGE and Western blotting was done using procedures and antisera that were described previously (Scholte et al., 2013, 2015).

2.6. Transfection of cells with CHIKV replicon RNA

Freshly trypsinized BHK-21 cells were transfected by electroporation using 4×10^6 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.

2.7. Statistical analysis

Graph-Pad Prism 5.01 was used for EC_{50}, IC_{50} and CC_{50} determination by non-linear regression and for statistical analysis performed with one-way ANOVA with Dunnett's (Fig. 2C) or Bonferroni's (Fig. 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 (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 that is based on the RNA-synthesizing activity of RTCs isolated from CHIKV-infected cells. This assay measures the incorporation of [α]^{32}P-CTP into viral RNA, which was severely impaired by suramin in a dose-dependent manner, with an IC_{50} of approximately 5 μM (Fig. 1A, Supplemental Fig. S1A). Suramin also inhibited the *in vitro* activity of RTCs derived from SINV-(Fig. 1B) or SFV-infected cells (Fig. 1C), suggesting that it is a broad-spectrum inhibitor of alphavirus RNA synthesis. A small fraction of the RNA-synthesizing activity appeared refractory to the inhibitory effect of suramin, as some residual incorporation of ^{32}P-CTP remained even in the presence of 500 μM of the compound.

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

To determine the antiviral efficacy of suramin in cell culture, Vero E6 cells were infected with different CHIKV strains and treated with serial dilutions of the compound in a CPE protection assay. Viability assays on uninfected cells were performed in parallel to determine the CC_{50}. The EC_{50} values for infectious clone-derived CHIKV LS3, a natural isolate from Italy (ITA07-RA1) and a Caribbean CHIKV strain (STM35) were 75–80 μM (Table 1, Supplemental Fig. S2). The EC_{50} values are ~15 times higher than