Suramin action in African trypanosomes involves a RuvB-like DNA helicase

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\textbf{A B S T R A C T}

Suramin is one of the oldest drugs in use today. It is still the treatment of choice for the hemolymphatic stage of African sleeping sickness caused by \textit{Trypanosoma brucei rhodesiense}, and it is also used for surra in camels caused by \textit{Trypanosoma evansi}. Yet despite one hundred years of use, suramin’s mode of action is not fully understood. Suramin is a polypharmacological molecule that inhibits diverse proteins. Here we demonstrate that a DNA helicase of the pontin/ruvB-like 1 family, termed \textit{T. brucei} RuvBL1, is involved in suramin resistance in African trypanosomes. Bloodstream-form \textit{T. b. rhodesiense} under long-term selection for suramin resistance acquired a homozygous point mutation, isoleucine-312 to valine, close to the ATP binding site of \textit{T. brucei} RuvBL1. The introduction of this missense mutation, by reverse genetics, into drug-sensitive trypanosomes significantly decreased their sensitivity to suramin. Intriguingly, the corresponding residue of \textit{T. evansi} RuvBL1 was found mutated in a suramin-resistant field isolate, in that case to a leucine. RuvBL1 (Tb927.4.1270) is predicted to build a heterohexameric complex with RuvBL2 (Tb927.4.2000). RNAi-mediated silencing of gene expression of either \textit{T. brucei} RuvBL1 or RuvBL2 caused cell death within 72 h. At 36 h after induction of RNAi, bloodstream-form trypanosomes exhibited a cytokinesis defect resulting in the accumulation of cells with two nuclei and two or more kinetoplasts. Taken together, these data indicate that RuvBL1 DNA helicase is involved in suramin action in African trypanosomes.

1. Introduction

Suramin is an enigmatic molecule. It has a molecular weight of 1300 Da, carries six negative charges at physiological pH, and it is not orally bioavailable – yet despite this lack of drug-like properties, suramin has been in use as an anti-infective agent for over a century in humans as well as in veterinary medicine. Its primary indications are human African trypanosomiasis (HAT) and surra in camels (Giordani et al., 2016); suramin was also used for the treatment of onchocerciasis, caused by the nematode \textit{Onchocerca volvulus} (Hawking, 1978). Suramin is a polypharmacological molecule that inhibits dozens of different enzymes and receptors (Voogd et al., 1993). Its potential applications in medicine are manifold (Wiedemar et al., 2020), ranging from antiparasitic, antiviral, and anticancer chemotherapy to the use as an antidote for snakebite (Murakami et al., 2005) or as a new treatment option for autism (Naviaux et al., 2017). Paradoxically, we know more about the mechanisms of action of suramin for these repurposed applications than for its primary indication, African trypanosomiasis. \textit{Trypanosoma brucei} subspecies and \textit{Trypanosoma evansi} cause neglected tropical diseases affecting humans and livestock. Human African trypanosomiasis is largely under control thanks to successful programs against sleeping sickness and tsetse fly vector control, and new drugs fed into the development pipeline, in particular fexinidazole as a new, oral treatment for \textit{T. b. gambiense} infections (Dickie et al., 2020; Franco et al., 2020). The animal trypanosomiases, in contrast, remain a serious problem for agriculture, causing huge economic losses and hindering development (Giordani et al., 2016). \textit{T. evansi} is not restricted to tsetse flies for transmission and thus has a wider geographical distribution than \textit{T. brucei}. This makes it a problem not only for Africa but also for countries in Asia, South America, and potentially Europe (Desquesnes et al., 2013). Suramin is still being used to treat first-stage (i.e. hemolymphatic) \textit{T. b. rhodesiense} infections in humans, and it was used...
extensively for *T. evansi* infections in livestock until suramin resistance became widespread (El Rayah et al., 1999; Zhou et al., 2004).

The molecular nature of the suramin target(s) in African trypanosomes has been the subject of many studies. Suramin impairs oxygen consumption and ATP production in *T. brucei* bloodstream forms (Fairlamb and Bowman, 1980), and it inhibits several of the glycolytic enzymes of *T. brucei*: hexokinase, aldolase, glycero-3-phosphate dehydrogenase, and phosphoglycerate kinase (Willson et al., 1993). Suramin was also shown to inhibit other trypanosomal enzymes such as glycerocephosphate oxidase (Fairlamb and Bowman, 1977), serine oligo-peptidase (Morty et al., 1998), and RNA editing ligase (Zimmermann et al., 2016). If all these enzymes are susceptible to suramin, which is the primary target? It is still currently unclear which of the *T. brucei* enzymes that are inhibited by suramin are responsible for the cytotoxic effect of the drug. These are expected (i) to be essential for the proliferation of bloodstream-form trypanosomes and (ii) to be affected in suramin-resistant mutants.

We have previously attempted to elucidate the mechanism of action of suramin through the generation of resistant *T. brucei* mutants. In vitro selection at high concentrations of suramin (25-fold the IC₅₀) readily produced derivatives with a 100-fold lower susceptibility to suramin (Wiedemar et al., 2018). However, this approach provided insights into the uptake mechanisms of suramin rather than its targets (Wiedemar et al., 2019). The suramin-resistant trypanosomes expressed a specific variant surface glycoprotein (VSG) variant termed VSG₈₅, which binds suramin with high affinity (Zeelen et al., 2021) and provides an alternative endocytosis route to the known endocytosis pathways via either ISG75 (Alsford et al., 2012) or low-density lipoprotein (Vansterkenburg et al., 1993). Here we have further selected VSG₈₅ expressing *T. brucei* for high-level suramin resistance, which has led to the identification of a DNA helicase as a likely new target of suramin in African trypanosomes.

2. Material and methods

2.1. Trypanosoma spp. strains

Trypanosoma b. rhodesiense STIB900, c1 was a fresh clone of STIB900 (Wiedemar et al., 2018), which derived from STIB704 isolated in 1982 from a male patient in St. Francis Hospital, Ifakara, Tanzania. Trypanosoma b. brucei 2T1 is a derivative of Lister427 and is widely used for reverse genetics (Alsford et al., 2005). Trypanosoma evansi Westry2 and STIB779 were isolated from camels in Western Sudan in 1995 and Kenya in 1979, respectively (El Rayah et al., 1999).

2.2. Cell culture

Parasites were cultivated at 37 °C, 5% CO₂ in Iscove’s Modified Dulbecco’s Medium supplemented according to Hirumi (Hirumi and Hirumi, 1989) and with 15% heat-inactivated horse serum for *T. b. rhodesiense* or 10% heat-inactivated fetal calf serum for *T. b. brucei* 2T1. The selection for suramin resistance was carried out under constant drug pressure with gradually increasing concentrations from 1.1 μM to 18.9 μM suramin over the course of one year (Fig. S1).

2.3. Drug testing

Drug efficacy was determined in vitro with the Alamar Blue assay (Riaz et al., 1997). Serial drug dilutions were prepared in a 96-well plate, and parasites were added to a concentration of 2 × 10⁹ cells/mL for STIB900 and 1 × 10⁸ cells/mL for 2T1. After 68 h of incubation, resazurin was added at a concentration of 11.4 μg/mL, and the plates were incubated for 2 to 4 more hours. The fluorescence was quantified with a SpectraMax reader (Molecular Devices) and SoftMax Pro 5.4.5 Software. GraphPad Prism 6.0 was used to fit dose-response curves (non-linear regression model, variable slope, four parameters, lowest value set to zero) and to calculate the IC₅₀ values.

2.4. Isolation of nucleic acids, polymerase chain reaction

Genomic DNA for PCR and Sanger sequencing was isolated from approximately 1 × 10⁹ bloodstream-form *T. brucei*, grown in vitro, and washed once with PBS, using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s protocol. The DNA was eluted in 200 μl milliQ water. PCR was carried out with the KAPA HiFi PCR Kit (KAPA Biosystems) in a total volume of 25 μL. Primers for amplification and sequencing of Tb927.4.1270 a934g were Tb927.4.1270-F-01 and Tb927.4.1270-R-01-BamHI; all primers are listed in Supplementary Table S1. Genomic DNA for genome sequencing of *T. evansi* was isolated from cells propagated in mice (female NMRI, 22–25 g). The trypanosomes were collected from euthanized mice by heart puncture, separated from the blood with a diethylaminoethyl cellulose column prepared from Whatman DEAE cellulose (DE52 pre-swollen, 3W4057-200) (Lanham and Godfrey, 1970), and the genomic DNA was isolated by chloroform/phenol extraction.

RNA was isolated from approximately 1 × 10⁸ cells grown to a density of 1 × 10⁶ cells/mL using the RNAeasy Mini Kit (Qiagen), including an on-column DNase I treatment (Qiagen). The RNA was isolated from each cell line in triplicates from independent cultures. For RT-PCR, T. b. brucei 2T1 lines (RuvBL1 RNAi, RuvBL2 RNAi) were grown for 24 h in the absence or presence (1 μg/mL) of tetracycline. For RNA-Seq, the four T. b. rhodesiense STIB900 lines (c1, c1_sur1, c1_sur1_3500, c1_sur1_18900) were grown in parallel without suramin.

2.5. RNA-seq and analysis

Sequencing libraries for RNA-Seq were prepared separately for each replicate using the TruSeq Stranded mRNA Library preparation kit (Illumina). 126-nucleotide single-end sequencing was carried out on an Illumina HiSeq 2500 machine. The quality of the reads was determined with fastqc (Andrews, S., 2010. FastQC A Quality Control Tool for High Throughput Sequence Data [Online]. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ last accessed 02/08/2023). Since the quality was high, the reads were mapped untrimmed to the T. b. brucei TREU927 reference sequence (TriTrypDB-38), supplemented with the Lister427 bloodstream expression sites (Hertz-Fowler et al., 2008) plus VSG⁹⁰⁰ (GenBank MF093646) and VSG⁸⁵⁰ (GenBank MF093647), with the Burrows-Wheeler Aligner (Li and Durbin, 2009) using the default settings. The SAM-files were converted to BAM-files using SAMtools (Li et al., 2009) and sorted and indexed with Picard (“Picard Toolkit.” Broad Institute, GitHub Repository. https://broadinstitute.github.io/picard/last accessed 02/08/2023); read duplicates were marked, and read group identifiers added with Picard as well. Deviations from the reference sequence (variants) were called with the Genome Analysis Toolkit (GATK) version 4.0.7.0 haplotypetcaller (McKenna et al., 2010) in gvcf-mode. The individual g.vcf-files were combined into one using GATK combinegvcfs and genotyped using GATK genotypegvcfs to obtain a VCF-file with all the identified variants. Variant annotation was calculated using snpEff version 4.3T (Cingolani et al., 2012) based on the TREU927 reference annotation (TriTrypDB-38) complemented with the genes of the Lister427 bloodstream expression sites, VSG⁹⁰⁰ and VSG⁸⁵⁰. Variants were excluded that laid within highly diverse regions with a low quality of alignment, were wrongly called due to small differences in allele frequencies, or were within VSG genes or pseudogenes. Sequencing data were deposited in the European Nucleotide Archive (accession PRJEB51200).

2.6. Whole genome sequencing

DNA Sequencing libraries were prepared using Illumina’s KAPA Hyper Prep Kit, and paired-end 126 nucleotide sequencing was carried out on an Illumina HiSeq 2500 machine. Raw sequencing reads were mapped to the *T. evansi* reference genome STIB805 (TriTrypDB-46) using the Burrows-Wheeler Aligner (Li and Durbin, 2009). The SAM-files
were converted to BAM-files using SAMtools (Li et al., 2009), then sorted to BAM-files using SAMtools (Li et al., 2009), then sorted and indexed using Picard (Picard Toolkit) Broad Institute, GitHub Repository. https://broadinstitute.github.io/picard/ last accessed 02/08/2023. The aligned sequencing reads were visualized with the Integrative Genomics Viewer (Robinson et al., 2011; Thorvaldsdottir et al., 2013) to inspect the candidate genes that had been detected in the T. b. rhodesiense STIB900 derivatives. Sequencing data were deposited in the European Nucleotide Archive (accession PRJEB51200).

2.7. Reverse genetics with T. brucei

The construct for the introduction of the mutation a934g into RuvBL1 was synthesized (GenScript, Netherlands). It contained the last 704 nucleotides of the Tb927.4.1270 coding sequence including the mutation, the 3’UTR, an α tubulin mRNA processing site, a blasticidin resistance gene, an α tubulin mRNA processing site, and the 215 nucleotides downstream of Tb927.4.1270. For the generation of homozygous mutants, the blasticidin resistance gene was exchanged with a neomycin resistance gene. The construct was amplified by PCR (primers Tb927.4.1270-F-02-HindIII and Tb927.4.1270-R-02; Table S1) and purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). 3 to 4 × 10⁶ cells were transfected with 3–8 μg of DNA in 100 μl bloodstream form transfection buffer (Schumann Burkard et al., 2011) using program Z-001 on the Amaxa Nucleofector (Lonza). Limiting dilutions of the parasites were prepared in 48-well plates. After one day, selection antibiotics were added: blasticidin (Invivogen) at 5 μg/mL for T. b. rhodesiense and at 10 μg/mL for 2T1 cells; G418 (Invivogen) initially at 3 μg/mL, reduced to 1 μg/mL after picking of clones 4–5 days after transfection.

For overexpression of RuvBL1 and RuvBL2, a DNA fragment containing both coding sequences with an interspaced α tubulin mRNA processing site and flanked with HindIII and BamHI sites was synthesized (GenScript, Netherlands) with re-coded internal HindIII sites to allow cloning. For simultaneous overexpression of RuvBL1 and RuvBL2, the fragment was directly cloned into a pRPa plasmid. For overexpression of RuvBL1 alone, the re-coded RuvBL1 sequence was amplified by PCR (primers Tb927.4.1270-F-01 and Tb927.4.1270-R-02; Table S1) and purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). 3 to 4 × 10⁶ cells were transfected with 3–8 μg of DNA in 100 μl bloodstream form transfection buffer (Schumann Burkard et al., 2011) using program Z-001 on the Amaxa Nucleofector (Lonza). Limiting dilutions of the parasites were prepared in 48-well plates. After one day, selection antibiotics were added: blasticidin (Invivogen) at 5 μg/mL for T. b. rhodesiense and at 10 μg/mL for 2T1 cells; G418 (Invivogen) initially at 3 μg/mL, reduced to 1 μg/mL after picking of clones 4–5 days after transfection.

For inducible knock-down of RuvBL1 or RuvBL2 expression, the stem-loop pRP-ISC MSCI/2 plasmid was used to generate two RNAi constructs. Optimal targeting sequences (i.e. nts 483–1076 for RuvBL1 and 236–749 for RuvBL2) as identified by the RNAi tool (Redmond et al., 2003) were amplified by PCR with overhang primers (Tb927.4.1270-F-03 and Tb927.4.1270-R-03-BamHI), digested, purified, and cloned into a pRPa plasmid. All constructs were checked by Sanger sequencing. 4 × 10⁶ 2T1 cells were transfected with approximately 5 μg of SacI/SalI digested and purified plasmid. The parasites were selected with 5 μg/mL hygromycin B Gold (Invivogen). Overexpression was induced with 1 μg/mL tetracycline (Sigma-Aldrich).

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2.8. cDNA synthesis and quantitative PCR

Complementary DNA was synthesized with the SuperScript™ III Reverse Transcriptase kit (Invitrogen) using an oligo(dT)₁₅ primer (Promega). Quantitative PCR (primers Tb927.4.1270-F-01 and Tb927.4.1270-R-04; Tb927.4.2000-F-01 and Tb927.4.2000-R-01) was carried out in duplicates using Fast SYBR® Green Master Mix (Applied Biosystems) in a StepOnePlus real-time PCR System (Applied Biosystems). Data were analyzed with StepOne Software v2.3. Telomerase reverse transcriptase (TERT) was used as a housekeeping gene for normalization with the ΔCt method.

2.9. DAPI staining and phenotype scoring

T. b. brucei 2T1 RuvBL1 and RuvBL2 RNAi parasites, uninduced or induced for 24 h and 36 h with 1 μg/mL tetracycline, were spun down for 5 min at 800 g, washed once in 1x Voorhees-modified PBS (vPBS; 157 mM NaCl, 3 mM KCl, 16 mM Na₂HPO₄, 3 mM KH₂PO₄, 46 mM sucrose, 10 mM glucose, pH 7.6), and resuspended in 1% FFA (EMS). The cells were incubated for 2 min on ice, centrifuged for 5 min at 2000 g, and the pellet was resuspended in 1x PBS. Cells were loaded on microscopy slides (Hecht Assistent), allowed to adhere for 10 min, then permeabilized in ice-cold MeOH for 30 min. Nuclei and kDNAs were stained for 2 min with 10 μg/mL DAPI (Sigma), washed, mounted with Vectashield® antifade mounting medium, and covered with coverslips (Duran). Cells were imaged with a Leica DM5000B microscope equipped with a Leica K5 Camera using the LAS v4.9 software. Images were processed with ImageJ Fiji. At least 150 cells per condition, in triplicates, were counted.

2.10. Homology modelling

Homology models of TbRuvBL1 (Tb927.4.1270) and TbRuvBL2 (Tb927.4.2000) were built with Phyre2 (Kelley et al., 2015) using Saccharomyces cerevisiae RuvBL1 (pdb ID 6GEN) as template, yielding confidence scores of 100%, respectively. Structural alignments were performed, and structural models visualized in PyMOL (Schrödinger and DeLano, 2020).

2.11. Phylogeny

Amino acid sequences were acquired from different taxonomic groups by Blastp searches (Altschul et al., 1990) using T. brucei RuvBL1 as the query. Multiple sequence alignment (Edgar, 2004), construction of a Neighbor-Joining tree (Saitou and Nei, 1987), and bootstrapping (Felsenstein, 1985) were done with MegaX (Kumar et al., 2018). The alignment was trimmed at the N- and C-terminus for overlapping sequences. Distances were computed using the JTT matrix (Jones et al., 1992). The alignment in Fig. S3 was made with Clustal Omega (Sievers et al., 2011) and edited with Jalview version 2.1.1.4 (Waterhouse et al., 2009).

3. Results

3.1. Selection of a 1000-fold suramin resistant T. b. rhodesiense mutant

From a fresh clone of our drug testing reference strain, T. b. rhodesiense STIB900_c1, we had previously generated several suramin-resistant lines that are resistant because they express VSG™, a peculiar variant surface glycoprotein that binds suramin (Wiedema et al., 2018, 2019; Zeelen et al., 2021). One of these lines, T. b. rhodesiense STIB900_c1_sur1, was subjected to further selection. Bloodstream-form cultures were continuously exposed to sublethal concentrations of suramin over a period of one year. Starting from 1 μM, the concentration of suramin was gradually increased to 18.9 μM as the trypanosomes were losing their susceptibility (Fig. S1A). The finally obtained line, T. b. rhodesiense STIB900_c1_sur1_18900, had a 50% inhibitory concentration (IC₅₀) for suramin of 11.0 μM, more than a thousand-fold higher than that of the parental clone T. b. rhodesiense STIB900_c1 (Fig. S1B). Parasite growth during the selection process was significantly slowed down at the highest suramin concentrations, explaining the discrepancy between IC₅₀ and the highest concentration used for selection (upon removal of the drug, parasite growth returned to normal rates). With progressing suramin resistance, the trypanosomes also lost their susceptibility to the related molecule trypan blue, albeit to a lesser extent than observed for
suramin (Fig. S1B). No alteration was observed regarding the sensitivity to the reference drugs melarsoprol and pentamidine (Fig. S1B).

3.2. Candidate genes for suramin resistance and mutations therein

Given the role that VSG can play in suramin resistance (Wiedemar et al., 2018, 2019; Zeelen et al., 2021), we first determined which VSG was expressed by the suramin-resistant lines. For this purpose, the VSG coding regions were amplified by PCR from reverse-transcribed mRNA. As reported previously (Wiedemar et al., 2018), *T. b. rhodesiense* STIB900_c1_sur1 had switched from VSG<sup>900</sup> to VSG<sup>Sur</sup>. The same VSG<sup>Sur</sup> was expressed also by the intermediate line *T. b.*

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**Fig. 1. In situ introduction of mutated RuvB-like helicase 1 enhances suramin resistance.** (A) Genomic context and the construct for reverse genetics, which consists of the last 704 nucleotides of the coding sequence and the 3′UTR of RuvBL1, a βα-tubulin mRNA processing site, a blasticidin resistance gene (BSD), an αβ-tubulin mRNA processing site; and 309 nt homologous region downstream of the gene. The location of the mutation a934g, as part of the Val<sup>312</sup> codon, is marked with a black line and an asterisk. (B) Suramin sensitivities of parental *T. b. rhodesiense* STIB900-c1-sur1, a RuvBL1-Val<sup>312</sup> transfectant thereof, and the suramin selected line STIB900-c1-sur1_3500 represented as 50% inhibitory concentrations (IC<sub>50</sub>). N = 3 independent IC<sub>50</sub> assays, each consisting of 2 technical replicates. Bar plots represent the mean, values of the individual assays are shown as small squares, and error bars represent standard deviations. P-values as calculated with Tukey’s multiple comparison test, *p < 0.05; **p < 0.001; ***p < 0.0001. (C) Suramin sensitivities of *T. b. brucei* 2T1 clones after single or double transfection with mutated RuvBL1. The clones after the first transfection were either heterozygous for the mutated helicase (D8 and A6; gray) or had remained wildtype (B7 and D4; white). After the second transfection, the clones were either homozygous (A6_A6, A6_B4, and A6_E5; black) or had remained heterozygous (A6_D8 and A6_F4; gray). Bar plots represent the mean, values of the individual assays are shown as square datapoints, and error bars represent standard deviations. N = 4 independent IC<sub>50</sub> assays, each consisting of 2 technical replicates, N = 8 for 2T1 and clone A6. Significant differences between groups (p < 0.001) as calculated with One-Way ANOVA followed by Tukey’s multiple comparison test are indicated with lowercase letters (a, b, c).
rhodensiense_c1_sur1_3500 (Fig. S1C). The final line of T. b. rhodesiense_c1_sur1_18900, however, had acquired 14 point mutations in the expressed VSG<sub>Sur</sub>, 8 of which were non-synonymous. These mutations and their enhancing effect on suramin resistance were reported elsewhere (Zeelen et al., 2021).

Aiming to identify further genetic mutations associated with suramin resistance, in particular mutations of genes other than VSG, we performed RNA-Seq of the sensitive parental clone (T. b. rhodesiense STIB900_c1), its VSG<sub>Sur</sub>-expressing derivative (STIB900_c1_sur1), and the two derivatives with higher-level suramin resistance (STIB900_c1_sur1_3500 and STIB900_c1_sur1_18900). All cultures were grown in the absence of suramin. Mapping of the obtained reads revealed three non-synonymous mutations in non-VSG genes that had emerged during the selection process. None of these variants are annotated as known nucleotide polymorphisms in the TriTryp SNP database (tritrypdb.org) (Aslett et al., 2010).

Two of the polymorphisms were heterozygous (Fig. S1C). The first was an insertion at nucleotide 798 in the gene for a putative phosphotransferase 2C (Tb927.11.760), leading to a frameshift from amino acid 267 onwards. However, fewer than 30% of the RNA-Seq reads carried this mutation, so it was not further investigated. The second was a missense mutation (t1064c) in the gene for an ADAM Cysteine-Rich Domain containing protein of the YagE family (Tb927.11.3700), resulting in the mutation leucine to proline at position 355. However, the mutation was present only in T. b. rhodesiense STIB900_c1_sur1_18900 (Fig. S1C), and the T. brucei YagE orthologue is not essential in bloodstream-form trypanosomes as determined by the genome-wide RNAi screen (Alsford et al., 2011). Therefore, again, the gene was not further investigated here.

The third mutation was the most interesting because it was heterozygous in the intermediate line T. b. rhodesiense STIB900_c1_sur1_3500 and homozygous in the final line STIB900_c1_sur1_18900 (Fig. S1C). This was confirmed by PCR on genomic DNA followed by Sanger sequencing. The mutation was a missense variant (a934g) in the RuvB-like 1 DNA helicase gene (Tb927.4.1270; herein called RuvB1L), resulting in the amino acid substitution of isoleucine-312 with valine (I312V) in the T. brucei RuvB1L orthologue.

3.3. In situ mutation of RuvB1L in T. brucei decreases suramin sensitivity

First, we investigated the effect of the RuvB1L I312V mutation in the genetic background it originally appeared in. The parental line T. b. rhodesiense STIB900_c1_sur1, which is suramin resistant but homozygous for wild-type RuvB1L (Fig. S1), was transfected with a construct encoding for the I312V mutant of RuvB1L (Fig. 1A). This gave rise to only one positive clone that had the construct correctly integrated into the RuvB1L locus by homologous recombination. The transfected clone was heterozygous for the mutation as determined by PCR and Sanger sequencing, and it exhibited a 2.2-fold increase in IC<sub>50</sub> for suramin as compared to untransfected T. b. rhodesiense STIB900_c1_sur1 (Fig. 1B). The IC<sub>50</sub> shift was slightly lower than the 2.9-fold increase observed for T. b. rhodesiense STIB900_c1_sur1_3500 (Fig. 1B), the line in which the heterozygous mutation I312V in RuvB1L had emerged during suramin selection (Fig. S1). Thus, there might be additional factors contributing to suramin resistance in the suramin-selected line.

To test the effect of the mutation I312V in a neutral genetic background, the same construct (Fig. 1A) was transfected into T. b. brucei 2T1 bloodstream forms (Alsford et al., 2005). This generated two positive clones that were heterozygous for the mutation. In addition, two clones were obtained which had integrated the selective marker (the blasticidin S deaminase gene) but not the mutation in RuvB1L, presumably due to a recombination event downstream of codon 312. These two clones were used as additional controls. The IC<sub>50</sub> values for suramin in the heterozygous RuvB1L I312V mutants were 1.8-fold higher than those of the controls (Fig. 1C), while no significant alteration was observed regarding the susceptibility to trypan blue, melarsoprol, or pentamidine (Suppl. Fig. S2).

Homozygous T. b. brucei mutants were generated after exchanging the blasticidin resistance marker of the construct with the neomycin S transferase gene, transflecting one of the heterozygous transgenic clones, and selection for clones that were resistant to blasticidin as well as G418. Among the five positive clones, three were homozygous for valine-312, while two had retained heterozygosity. The homozygous mutants exhibited a further 1.4-fold increase of IC<sub>50</sub> as compared to the heterozygous mutants, and a 2.4-fold increase of IC<sub>50</sub> as compared to parental T. b. brucei 2T1 (Fig. 1C). Again, the sensitivity to trypan blue, melarsoprol, and pentamidine remained unaffected (Fig. S2). Thus, the mutation I312V in the RuvB1L orthologue of T. brucei decreases sensitivity specifically for suramin. This indicates that the T. brucei RuvB1L helicase is involved in the mode of action of suramin.

3.4. A T. evansi suramin-resistant field isolate carries a similar mutation in RuvB1L helicase

Suramin resistance is a major problem in the control of surra in camels (El Rayah et al., 1999; Zhou et al., 2004). We generated whole-genome sequencing data of a suramin-resistant T. evansi field isolate, Westry2 (El Rayah et al., 1999), which we inspected for the orthologues of the identified candidate genes. In particular, we looked for non-synonymous deviations from the reference sequence T. evansi STIB805 (Carnes et al., 2015) that were not present in a suramin-sensitive T. evansi isolate, STIB779 (El Rayah et al., 1999). The RuvB1L sequences of the suramin-sensitive T. evansi STIB779 and STIB805 (TevSTIB805.4.1310) were identical to that of T. brucei. Intriguingly, however, T. evansi Westry2 carried a heterozygous mutation in the RuvB1L gene at exactly the same position as the laboratory-selected T. b. rhodesiense line, i.e. at codon 312. The mutation was a934t, leading to an amino acid replacement from isoleucine to leucine (I312L).

3.5. Isoleucin-312 is located close to the active site of RuvB1L

Tb927.4.1270 shares 65% sequence identity with Saccharomyces cerevisiae RuvB-like 1 DNA helicase. However, despite a high degree of sequence conservation among the eukaryotic RuvB-like 1 proteins, the orthologues from the kinetoplastids form a clearly distinct branch in the phylogenetic tree (Fig. 2A). RuvB1L forms a heterohexameric complex with the paralogous RuvB-like DNA helicase RuvB2L. The corresponding homolog in T. brucei, Tb927.4.2000, is encoded 170 kb upstream of RuvB1L on chromosome 4. The RuvB1L complex facilitates ATP-dependent nucleosome sliding as part of larger complexes with a function in nucleosome remodelling in eukaryotes. In yeast, the two distinct chromatin remodelling complexes, INO80 and SWR1, are well characterized (Gerhold and Gasser, 2014). A SWR1-like remodeller complex in T. brucei was described recently (Vellmer et al., 2022). Homology models of RuvB1L and RuvB2L were built with high confidence using S. cerevisiae orthologues (pdb 6GEN) (Willholt et al., 2018) as template. I312 in the RuvB1L structural model (labeled in orange in Fig. 2B) is located downstream of the ATP-binding domain, six residues apart from the Walker A motif (Matias et al., 2006). The latter motif is highly conserved (Fig. 2C, Suppl. Fig. S3) and has, together with the Walker B motif, a key role in ATP binding and hydrolysis by coordinating the $\beta$ and $\gamma$ phosphates of ATP and the water activating magnesium ion (Wendler et al., 2012). Bound ATP and the I312 side chain are more than 23 Å apart, rendering it unlikely but not impossible, that suramin, whose structure spans approximately 30 Å, interacts directly with the ATP binding site. Superimposing the homology models to the RuvB1L and RuvB2L structural models of S. cerevisiae revealed that the Walker B motif is involved in the mode of action of suramin.
Fig. 2. Phylogenetic analysis of *T. brucei* RuvB-like and homology model. (A) Neighbor-Joining phylogenetic tree of RuvB-like helicases from different taxonomic groups showing how the kinetoplastid sequences form a distinct clade (blue numbers, percent positives of 1000 rounds of bootstrapping; asterisk, presumed root based on the prokaryotic outgroup; scale bar, number of amino acid substitutions per site; for the complete sequence alignment see Fig. S3). (B) Homology model of TbRuvBL1 (Tb927.4.1270) in cartoon representation with Ile312 drawn in orange sticks. ATP (sticks) and Mg²⁺ (sand colored sphere) are superimposed from the *S. cerevisiae* RuvBL1 structure (pdb ID 6GEN) (Willhoft et al., 2018). (C) Section of a multiple sequence alignment of RuvB-like helicases showing how Ile312 is conserved across species (the full alignment is in Fig. S3). The location of Walker B motif (hhhhDE) (Hanson and Whiteheart, 2005; Matias et al., 2006) and the nucleotide interacting sensor 1 motif are indicated. (D) Homology model of the heterohexameric complex of TbRuvBL1 (Tb927.4.1270, blue) and TbRuvBL2 (Tb927.4.2000, red) in cartoon representation, top and side view. The side-chain atoms of Ile312 are drawn as orange balls, ATP and Mg²⁺ are superimposed and shown as in (B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
implicated in substrate sensing (Erzberger and Berger, 2006).

3.6. Overexpression of RuvB-like helicases has no significant effect on suramin sensitivity

Inhibition of a target protein by a drug can, in certain cases, be compensated for by overexpression of the target itself. To test whether overproduction of RuvBL1 helicase affects suramin sensitivity, we transfected T. b. brucei 2T1 cells with a pRPa-based construct for tetracycline-inducible overexpression of RuvBL1. Three positive transfectants were investigated. Induction with tetracycline (1 μg/mL) led to an increase in RuvBL1 mRNA levels of 2.2–3.0 fold as measured by qPCR, but the suramin sensitivity in the induced cells was not altered as compared to uninduced cells (Suppl. Figs. S4A and S4B). Overproduction of RuvBL1 alone might not have an effect because the protein is predicted to build a heteromeric complex together with RuvBL2 (Fig. 2D). To simultaneously overexpress both genes, we made a construct containing RuvBL1 and RuvBL2 separated by an α tubulin mRNA processing site. Again, T. b. brucei 2T1 cells were transfected and three positive clones were obtained and investigated. Overexpression of the target genes was confirmed in principle, but it happened to a much lower extent than for RuvBL1 alone: after induction by tetracycline, the mRNA levels of RuvBL1 and RuvBL2 only rose 1.3- to 1.6-fold and 1.3- to 1.5-fold, respectively (Suppl. Fig. S4C). The tetracycline-induced cells had a slightly higher IC$_{50}$ to suramin than the uninduced cells for all three clones, but the difference was not statistically significant (Suppl. Fig. S4D). So overall, the experiments linking overexpression of RuvBL orthologues in T. brucei to suramin sensitivity were not conclusive.

The expression levels of the RuvBL orthologues were only slightly increased on the transcriptome level and might be further regulated on the translational and post-translational levels. Even if it were possible to reach high-level overexpression of RubB1L1 and RubB2L2, this would unlikely result in the overproduction of a functional complex. Additional factors are necessary for function and proper folding to the predicted ring-like structure (Abrahao et al., 2021).

3.7. The RuvBL1 and RuvBL2 helicases are both independently essential for T. brucei

If RuvBL1 is indeed a drug target, then it has to be essential for the proliferation of bloodstream-form trypanosomes. Using pRPa-iSL-based plasmids (Alsford et al., 2005), we generated two stem-loop RNAi cell lines for tetracycline-inducible silencing of RuvBL1 or RuvBL2 expression in bloodstream-form T. brucei. Both lines showed a strong down-regulation of RuvBL1 and RuvBL2 expression: 24 h after the addition of tetracycline (1 μg/mL), the mRNA levels of either gene had dropped to below 20% of the original value as determined by qPCR (Fig. 3A). Both individual genetic knock-downs led to a rapid slowdown of growth within the first 24 h, which culminated in growth arrest and cell death within three days after induction (Fig. 3B). These observations confirmed the results of the high-throughput RNAi screen in T. brucei (Alsford et al., 2011), in which RuvBL1 and RuvBL2 were both categorized as essential genes for bloodstream-form parasites.

3.8. Downregulation of either RuvBL1 or RuvBL2 blocks cytokinesis

Due to the rapid cell growth arrest upon down-regulation of either RuvB-like helicase, we investigated the DNA content of the RNAi lines in the short time window before the trypanosomes were dying. The cell lines were induced with 1 μg/mL tetracycline for 24 h and 36 h, fixed, and 4′,6-diamidino-2-phenylindole (DAPI)-stained to visualize the nuclei (N) and the kinetoplasts (K; the DNA-containing structure within the single mitochondrion). The parental line (T. b. brucei 2T1), the uninduced, and the induced RNAi cell lines were analyzed by light microscopy regarding their DNA content. Compared to the typical wildtype phenotypes in the parental line (mostly 1K1N cells with one kinetoplast and one nucleus, plus some cells with 2K1N and 2K2N; black bars in Fig. 4A), a marked decrease of the 1K1N and 2K1N phenotypes was observed in the tetracycline-induced lines (gray bars in Fig. 4A) in favor of a clear accumulation of 2K2N cells. At 36 h post-induction, the 1K1N cells had dropped from 80% of the total population to about 20% in the RuvBL1 RNAi line, and from 76% to 30% in the RuvBL2 RNAi line. At the same time, the 2K2N cells had increased from 7% to 37% in the RuvBL1

Fig. 3. RuvBL1 and RuvBL2 are essential for bloodstream-form T. brucei. (A) Steady-state gene expression levels as determined with qPCR at 24 h post tetracycline (tet) induction (n = 3, with 2 technical replicates). Ct values were normalized with the housekeeping gene TERT. (B) Representative growth curves of uninduced (-tet) and induced clone (+tet) compared to the parental line T. b. brucei 2T1 (n = 3). Upper panel, transfectant clone D3 – RuvBL1 RNAi; lower panel, transfectant 6B – RuvBL2 RNAi.
RNAi line and from 10% to 35% in the RuvBL2 RNAi line (Fig. 4A). Moreover, in both induced cell lines, we observed a considerable percentage of cells with non-canonical phenotypes such as >2K2N and 1K2N (Fig. 4). At 36 h post-induction, the populations were composed of 20% and 15% of >2K2N in the RuvBL1 and RuvBL2 RNAi lines, respectively, and 18% and 15% of 1K2N cells. The dramatic accumulation of cells with more than two nuclei and multiple kinetoplasts suggested that down-regulation of either RuvB-like helicase interferes with cytokinesis, which was confirmed by fluorescence microscopy (Fig. 4B).

4. Discussion

In bacteria, the RuvB-like DNA helicases are part of the resolvasome, a multiprotein complex that mediates branch migration during homologous recombination of DNA and resolution of the four-way Holliday junction (Wyatt and West, 2014). As such, RuvB-like helicase has a DNA-binding domain and an ATPase domain. It forms a hexameric ring around the DNA and functions as an ATP-driven motor that promotes heteroduplex formation (Wyatt and West, 2014). RuvB-like DNA helicases have been well studied in bacteria (Iwasaki et al., 1991; Shinagawa et al., 2001). In eukaryotes, the proteins RuvB-like 1 and RuvB-like 2 are also called pontin and reptin (Jha and Dutta, 1999), respectively, and they have acquired additional functions including DNA repair and chromatin remodelling (Dauden et al., 2021; Tammana and Tammana, 2017). A chromatin remodelling complex from T. brucei, which contains TbRuvBL1 and TbRuvBL2, has been described recently (Vellmer et al., 2022). There is to our knowledge only one experimental study from trypanosomatids, where LmRUVBL1 and LmRUVBL2 from Leishmania major were recombinitely (co-)expressed. The purified proteins formed an elongated heterodimer with ATPase activity in vitro, but they did not form a ring (Abrahao et al., 2021).

Here we provide forward as well as reverse genetic evidence that RuvBL1 DNA helicase from T. brucei is involved in suramin resistance and a likely target of suramin: the point mutation I312V in RuvBL1 emerged heterozygously in T. b. rhodesiense upon suramin pressure and turned homozygous during further selection. Consistently, the introduction of the mutation I312V in situ to RuvBL1 of T. b. brucei caused a 1.8-fold (heterozygous) to 2.4-fold (homzygous) increase in the IC50 to suramin. These resistance phenotypes were not very strong, but given the polypharmacology of suramin, any mutation in just one of its targets is expected to have only a moderate effect, as the other physiologically relevant targets prevent a stronger resistance on the cellular level (so regarding the evolution of drug resistance in the field, suramin’s polypharmacology is an asset). However, the fact that the mutation of RuvBL1 significantly lowered the susceptibility to suramin, suggests that the DNA helicase is a target of suramin in T. brucei. Further, this hypothesis is in agreement with the published findings that (i) suramin inhibits DNA helicases from Dengue virus (Basavannacharya and Vasudev, 2014), hepatitis C virus (Mukherjee et al., 2012), and SARS-CoV2 virus (Zeng et al., 2021); and (ii) exposure of bloodstream-form T. brucei to suramin caused a cytokinesis phenotype (Thomas et al., 2018) similar to the one observed upon RNAi-mediated down-regulation of RuvBL1. Functional assays to corroborate a direct interaction between T. brucei RuvBL1 helicase and suramin will be challenging, requiring the presence of RuvBL2 and possibly further proteins of the complex, or even a DNA substrate.

Mutation of RuvBL1 isoleucine-312 was also detected in a suramin-resistant field isolate of T. evansi, in that case to leucine. T. evansi is a subspecies of T. brucei (Lai et al., 2008) that is mechanically transmitted by biting flies and is no longer dependent on the tsetse fly host. Thus the parasite has spread across the tropical regions globally (Desquesnes et al., 2013), and cases were reported even in southern Europe (Desquesnes et al., 2008; Tamarit et al., 2010). The resistant T. evansi strain Westry 2 was isolated from an infected camel in the Republic of the Sudan. It had an IC50 to suramin of over 40 μg/mL (28 μM) as determined in a short-term [3H]-hypoxanthine incorporation assay (El Rayah et al., 1999). This isolate had not been adapted to in vitro culture, preventing its further characterization. Given the polypharmacological nature of suramin, drug resistance is more likely to arise by transport phenotypes (increased export or decreased import) than by target site mutation, as for example in the case of VSGsur expression. VSGsur-mediated suramin resistance, however, would not be sustainable in the field where the parasites must undergo immune escape by changing their surface coat regularly. In contrast, the mutation of the conserved isoleucine-312 in a field isolate indicates RuvBL1 relevance for suramin.
resistance in the field, warranting further investigation of RuB1L1 in *T. evansi*.

Sequence analysis and the TbRuB1L1 homology model place I312 in close vicinity to the active site and motifs with crucial residues engaging in ATP binding and hydrolysis, this suggests the possibility of inhibition via suramin-binding to the respective region. Further, I312 is located near the pore of the hexameric assembly upstream of the sensor 1 motif (Fig. 2B and C), a region likely interacting with a DNA substrate. Here, suramin binding could interfere with substrate interaction or even trigger substrate sensing-dependent, perpetual ATPase activity. However, 1, 2 and 10 V mutations are considered conservative, retaining a hydrophobic, rather than bulky side chain, rendering a direct effect on suramin binding less likely. It is also possible that the I312 mutation leads to a structural rearrangement that, in turn, affects interaction with suramin. Altogether, the precise molecular mechanism of suramin interaction with TbRuB1L1 remains to be investigated.

Finally, our findings render RuB1L1 a potential drug target. It was recently proposed as a novel target for antimalarials (Khusana et al., 2021). The fact that (i) RNAi-mediated silencing of RuB1L1 expression in bloodstream-form T. brucei was rapidly lethal, and (ii) the RuB1L1 orthologues from kineoplastids form a distinct phylogenetic clade, qualify RuB1L1 as a target in drug development for trypanosomal diseases.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2023.09.003.

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Appendix A. Supplementary data

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