

Supplementary Information

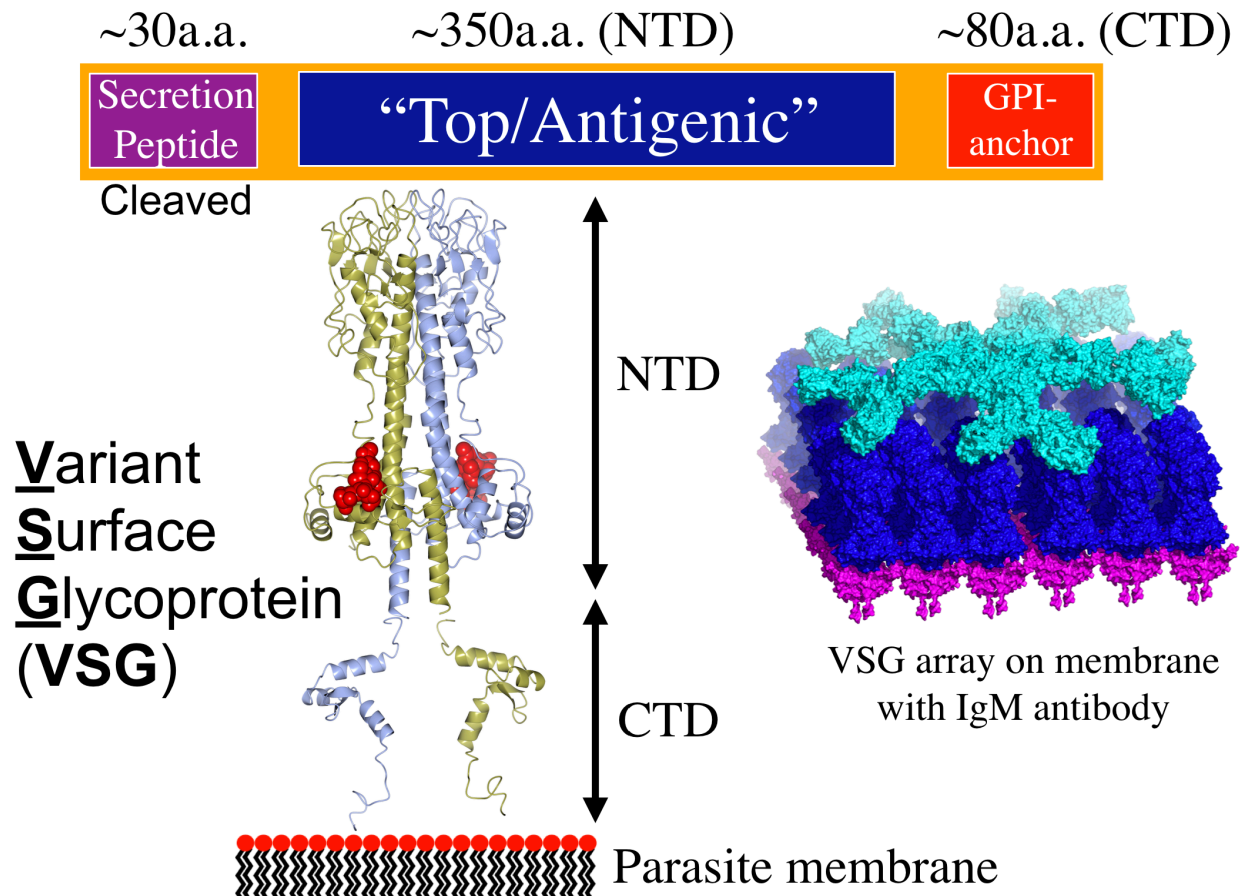
A Parasite Coat Protein Binds Suramin to Confer Drug Resistance

Johan Zeelen^{1*}, Monique van Straaten^{1*}, Joseph Verdi^{1,2}, Alexander Hempelmann¹, Hamidreza Hashemi², Kathryn Perez³, Philip D. Jeffrey⁴, Silvan Hälg⁵, Natalie Wiedemar^{5,6}, Pascal Mäser^{5,6}, F. Nina Papavasiliou², C. Erec Stebbins^{1†}

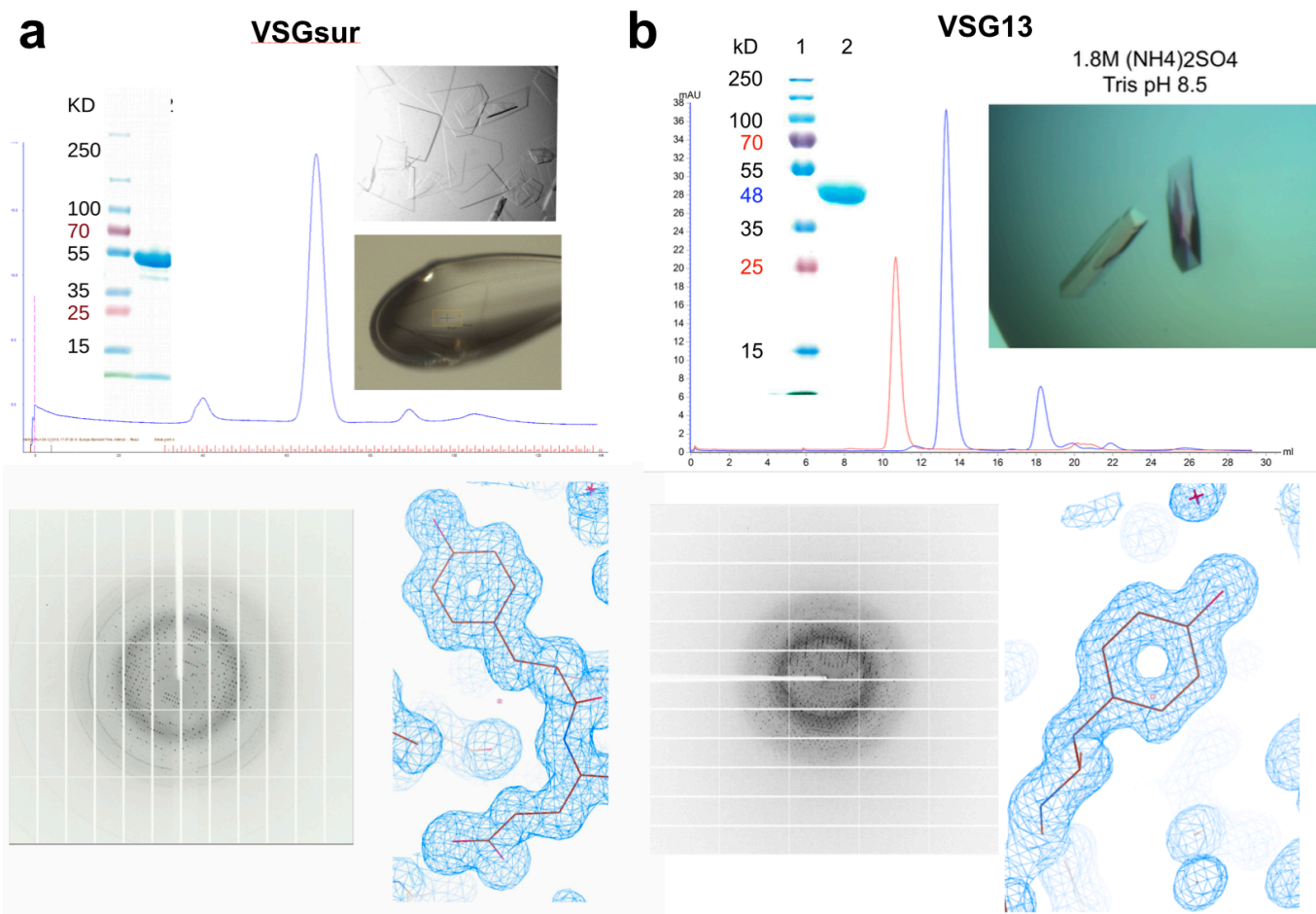
¹Division of Structural Biology of Infection and Immunity, German Cancer Research Center ²Division of Immune Diversity, German Cancer Research Center, Heidelberg, Germany. ³Protein Expression and Purification Core Facility, EMBL Heidelberg, Meyerhofstraße 1, Heidelberg, Germany ⁴Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA ⁵University of Basel, Basel CH-4001, Switzerland ⁶Swiss Tropical and Public Health Institute, Basel CH-4002, Switzerland.

*These authors contributed equally to this work.

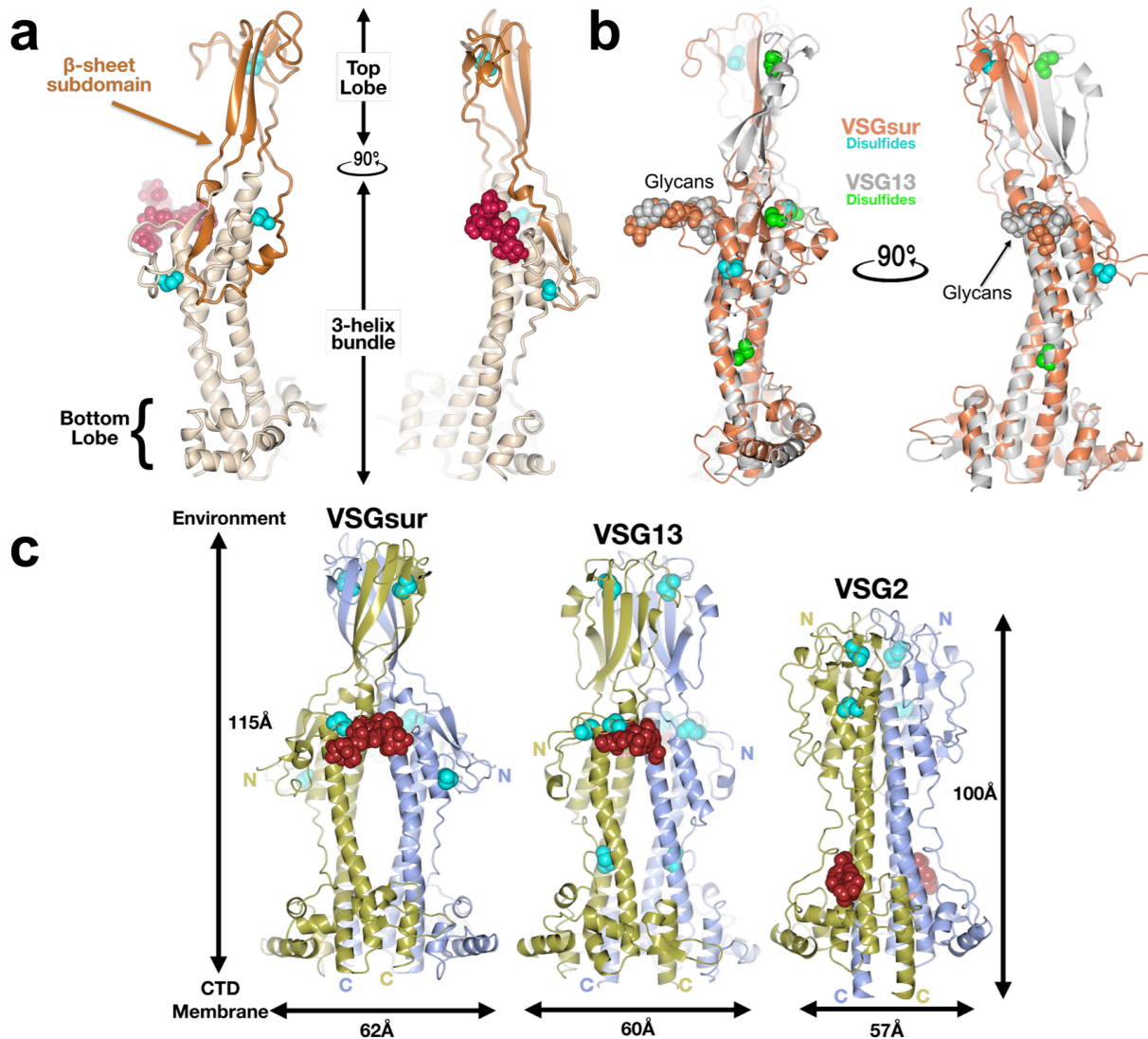
†Correspondence to: e.stebbins@dkfz-heidelberg.de



Supplementary Fig. 1: Domain structure of the VSG proteins. The top bar shows a schematic of the domain structure of the VSG coat proteins with rough amino acid size associated with each. NTD is the N-terminal domain and CTD is the C-terminal domain. Underneath is the structure of VSG1 schematically represented by a composite model of N and C-terminal domain structures (PDB IDs 5LY9 and 5M4T, respectively), illustrated over a cartoon of the parasite membrane. On the right is a model of an array of VSG proteins (NTD in blue, CTD in magenta) on the surface and interacting with a hypothetical model of immunoglobulin M (IgM shown in cyan).



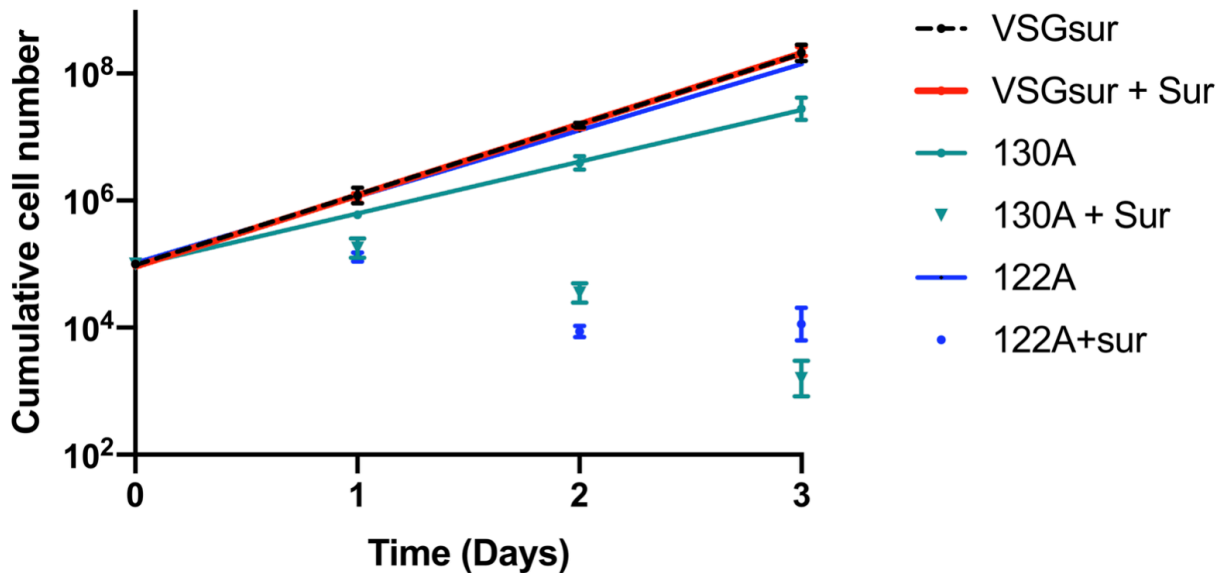
Supplementary Fig. 2: Purification, crystallization, and representative electron density of VSGsur and VSG13. Summary of various steps in the crystallographic structural solution of VSG13 and VSGsur. **(a)** Panels showing the gel filtration chromatogram (Superdex 200, Methods) of purified VSGsur NTD and a coomassie stained SDS-PAGE gel of the final material used for crystallization. Images of cryo-crystals grown in hang-drops and cooled crystals in the X-ray beam, a diffraction image, and final model 2Fo-Fc electron density contoured at 1σ . **(b)** Panels included are: the gel filtration chromatogram (Superdex 200, Methods) of purified VSG13 NTD (native in blue and reductively methylated in red, the latter running larger as perhaps a tetramer but occurring as a dimer in the asymmetric unit of the crystal), a coomassie stained SDS-PAGE gel of the final material used for crystallization, images of crystals prior to harvesting in hanging drops, a diffraction image, and final model 2Fo-Fc electron density contoured at 1σ .



Supplementary Fig. 3: Comparison of the VSG NTDs

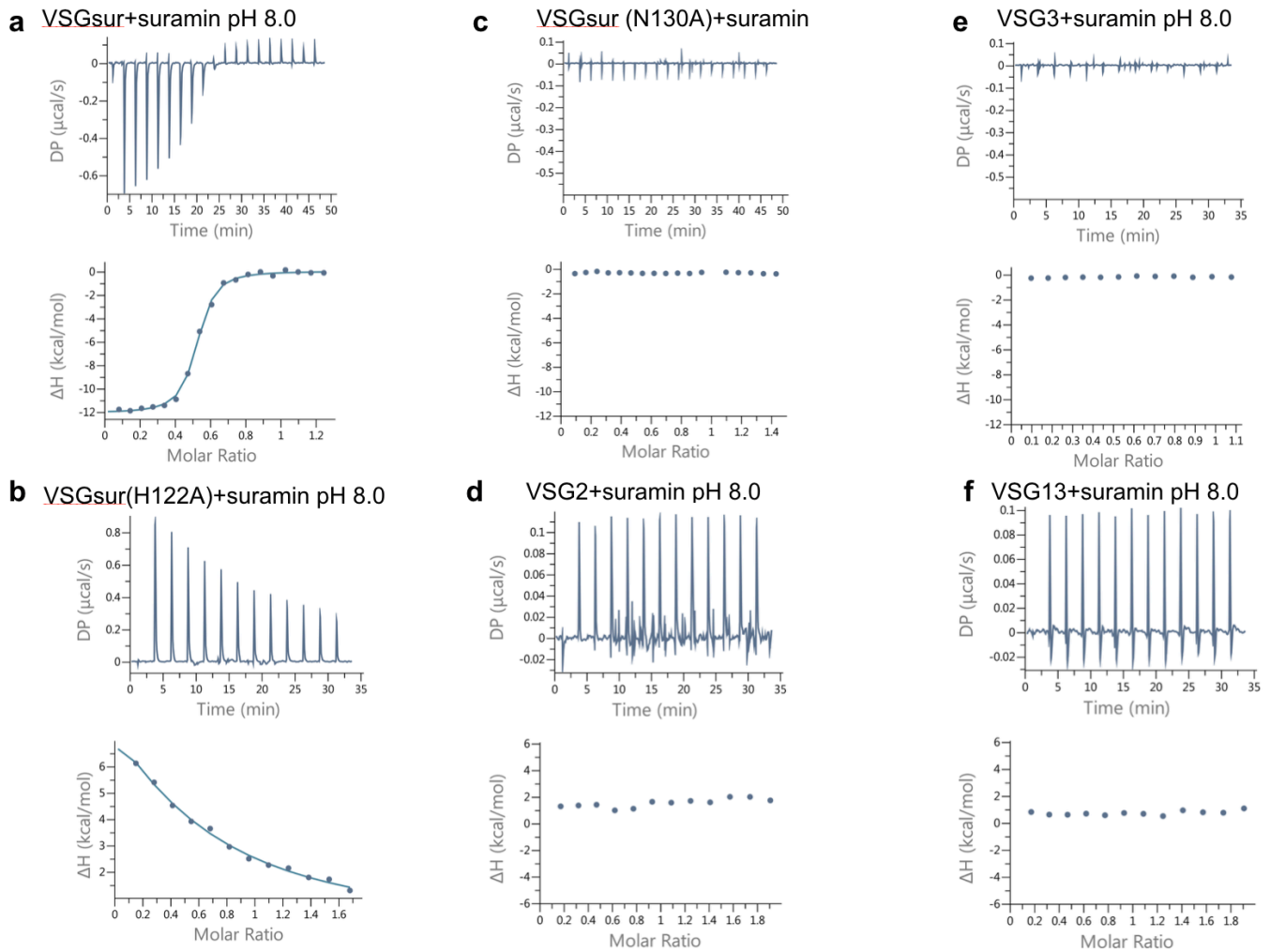
(a) A single monomer of the VSGsur NTD is shown as a ribbon diagram. The β -sheet subdomain that forms the top lobe is colored in orange and the rest of the structure in beige. The N-linked glycan is shown as red space-filling spheres, the cysteine disulfide atoms as space-filling cyan spheres. **(b)** Structural alignment of monomers of VSGsur (orange) and VSG13 (gray) with corresponding glycans and disulfides shown in space filling representation and colored the same as the protein to which they are linked. The alignment produces an overall root-mean-square deviation of 1Å for the conserved portions of the structure (calculated over 220 C α positions that primarily encompass the three-helix bundle and elements of the bottom lobe). **(c)** Comparison of the structures of VSGsur, VSG13, and VSG2. The N-linked glycans are displayed as red space-filling atoms and the disulfide bonds are shown in cyan. Approximate dimensions of the molecules are noted, as well as the directions toward the external environment and toward the C-terminal domain (CTD) and plasma membrane of the trypanosome.

WT and Mutant VSGsur growth curves



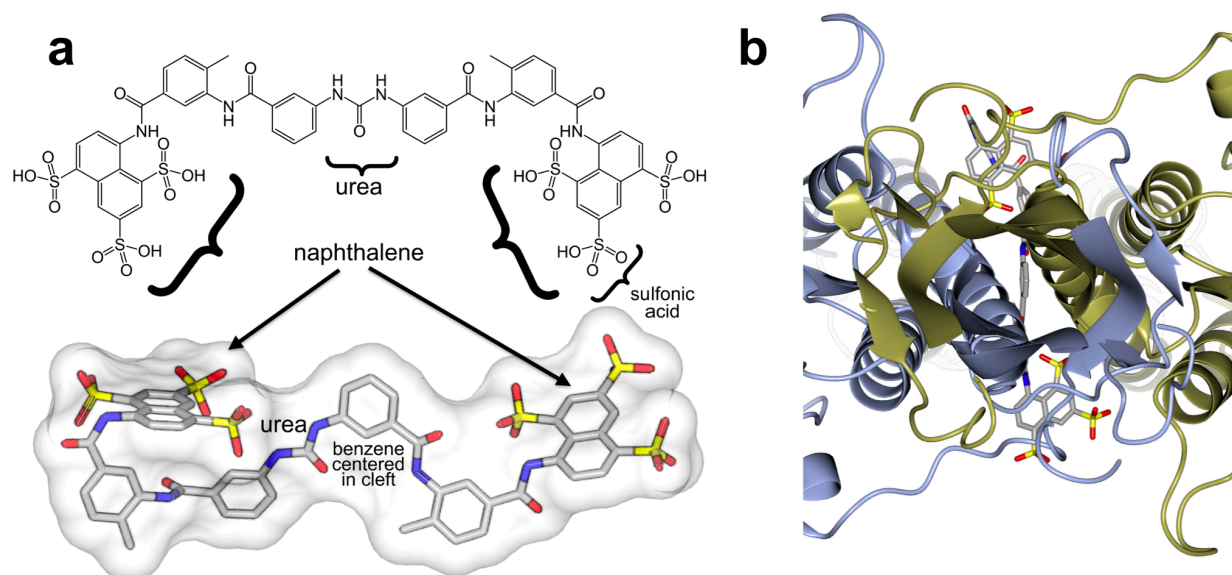
Supplementary Fig. 4: Growth curves of VSGsur and VSG mutants.

Strains expressing VSGsur and VSGsur mutants were grown with and without suramin (incubation of 0.7 μ M suramin for 24 h, Methods). The cell densities were determined by cell counting using a Neubauer hemocytometer. This procedure was repeated for 3-4 days in a row. Analysis was performed with GraphPad Prism, using a nonlinear regression model for curve fitting (Exponential growth with log).



Supplementary Fig. 5: Sample ITC results.

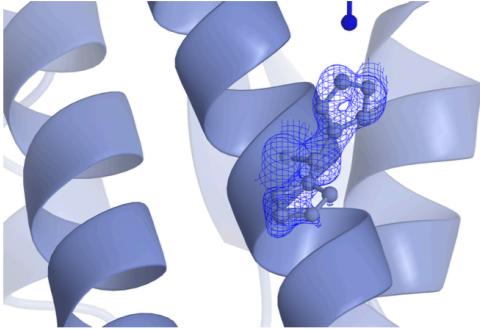
ITC data for suramin binding to each VSG protein. The upper panels contain the baseline corrected raw data, and the lower panel contains the peak-integrated, concentration normalized data for the heat of reaction vs. molar ratio of suramin per VSG protein. **(a)** VSGSur, pH 8.0: 300 μ M suramin was titrated into 46 μ M VSG Sur pH 8., fitted with a single binding site model to calculate a K_d of 234 \pm 28 nM and N of 0.49 \pm 0.03 **(b)** VSGSur H122A: 450 μ M suramin was titrated into 51.1 μ M VSGSur H122A. A K_d was not fit to the data, although it is clear that the mutation negatively affected the binding affinity. **(c)** VSGSur N130A: 300 μ M suramin was titrated into 40 μ M VSGSur N130A. No binding was detected. **(d)** VSG2: 200 μ M Suramin was titrated into 20 μ M VSG2 protein. No binding was detected. **(e)** VSG3: 300 μ M suramin was titrated into 53.1 μ M VSG3. No binding was detected. **(f)** VSG13: 200 μ M Suramin was titrated into 20 μ M VSG132 protein. No binding was detected.



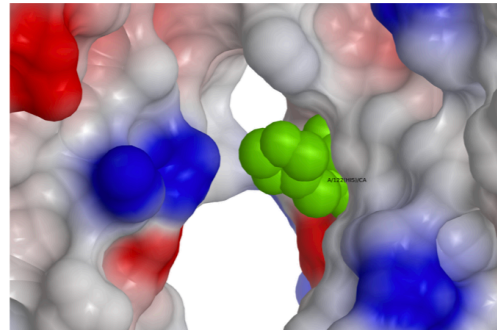
Supplementary Fig. 6: Two-fold axis of dimer symmetry for VSGsur-suramin complex.

(a) The top drawing illustrates the chemical structure of suramin with several of its functional groups denoted, whereas the bottom renders the drug as found in the protein structure with a transparent molecular surface shown. Oxygen atoms are shown in red, nitrogen in blue, and carbon gray. **(b)** Ribbon diagram of VSGsur (one monomer blue and the other gold) looking down the two-fold axis of symmetry for the dimer. Suramin is shown as a ball-and-stick chemical representation as in (a). In the center of the rotational axis for the dimer, one of the suramin benzene rings is visible.

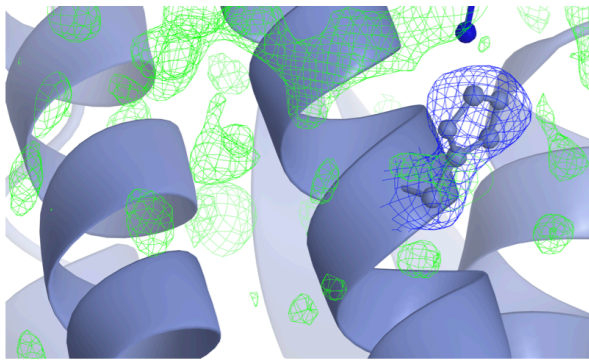
a Two H122 conformations



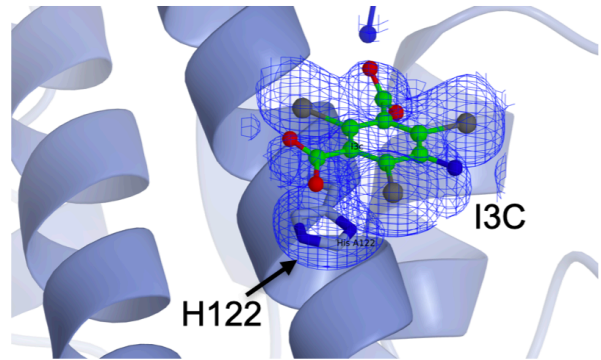
c Closed conformation surface



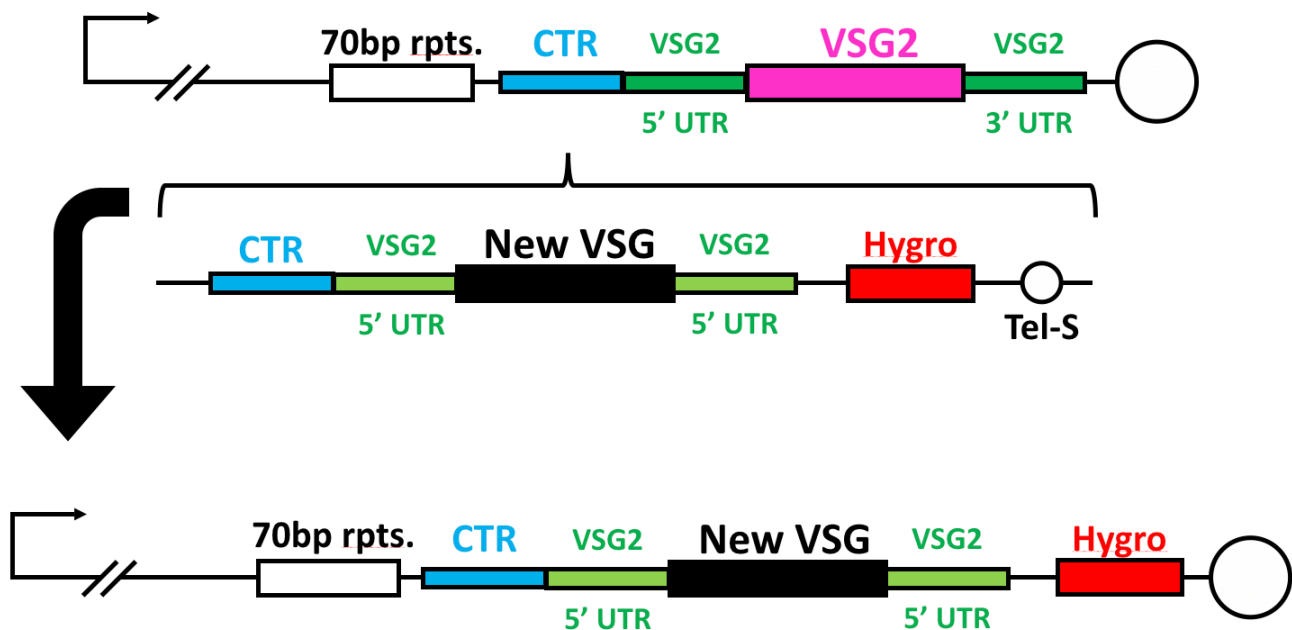
b
Closed conformation H122 density



d
I3C Position and Density



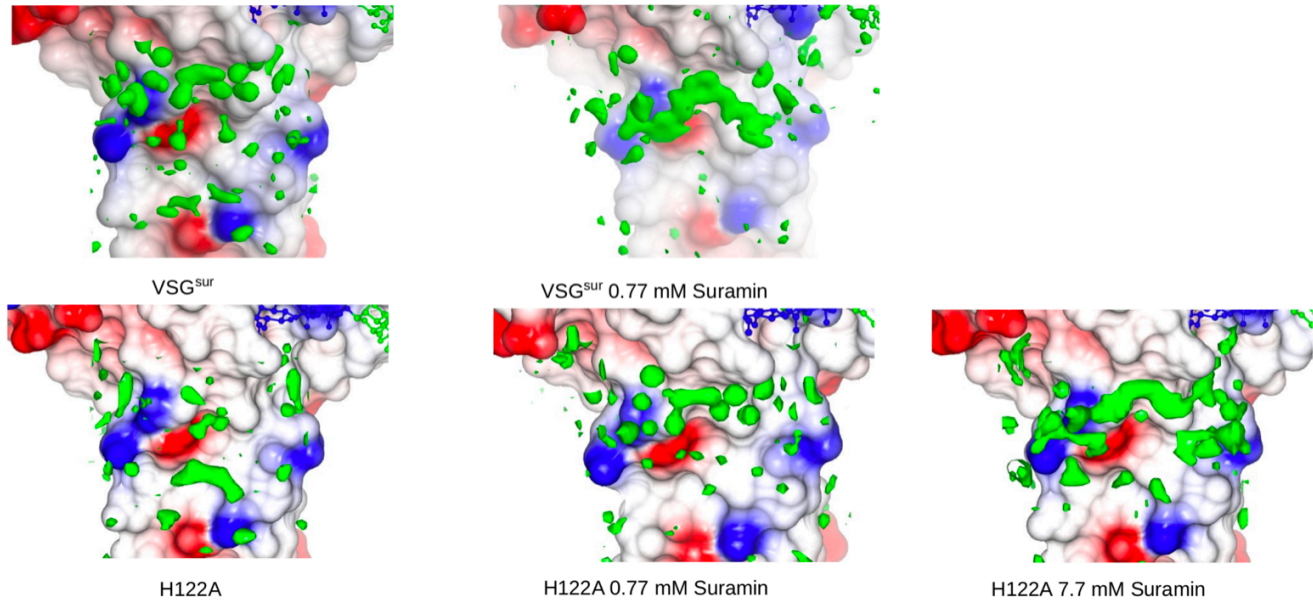
Supplementary Fig. 7: H122 “open” and “closed” conformations. (a) Two H122 conformations in the native crystals structure of VSGsur with corresponding electron density (b) Closed conformation electron density of H122 (c) Closed conformation surface of VSGsur (d) Position of H122 and the I3C group used in phasing the crystal structure with corresponding electron density.



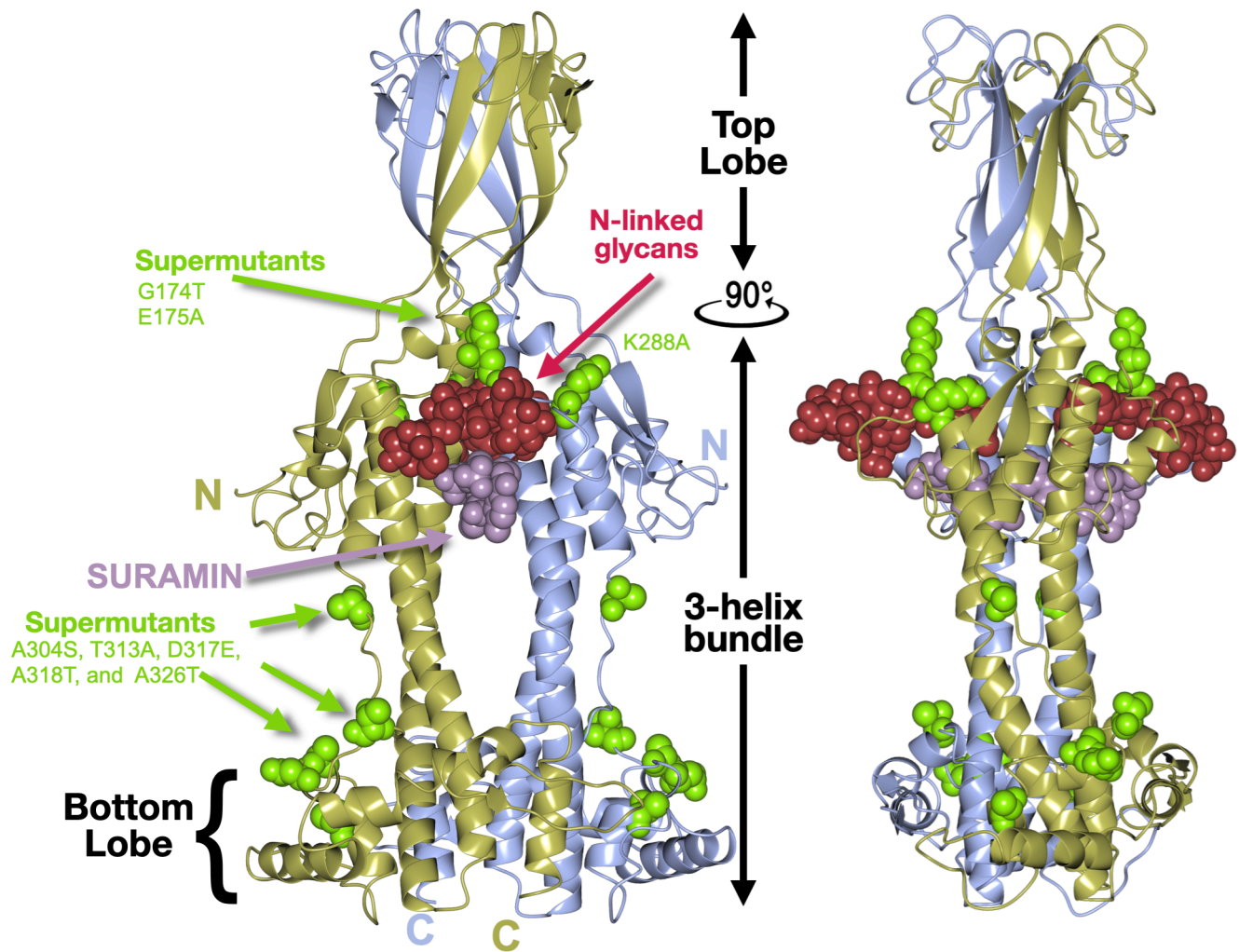
Supplementary Fig. 8: Creation of VSGsur and VSGsur mutant expressing strains

The same construct was used multiple times to generate different VSG-expressing cell lines. The top schematic shows the endogenous sub-telomeric expression site in 2T1 cells, which express VSG2 (pink) with its endogenous UTRs (dark green). The vector used to integrate new VSGs was adapted from Pinger et al.¹, wherein the homology directed integration of a novel VSG ORF (black) flanked by the UTRs of VSG2 is mediated by 3' telomere seeds (Tel-S) and 5' homology to the upstream co-transposed region (CTR – teal). Transfected cells are identified by screening for the integration of a hygromycin selection cassette (red) that is expressed via read-through transcription driven by the subtelomeric promoter.

Panels of WT and H122A with suramin soaks

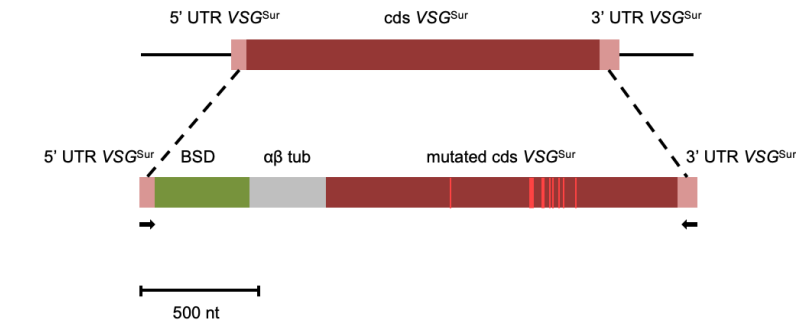


Supplementary Fig. 9: Panels of WT and H122A with suramin soaks. Molecular surface of one monomer in the VSG_{sur} dimer shown colored by electrostatic potential (white is neutral, blue is positive/basic, and red is acidic/negatively charged). Electron density in the suramin binding site is shown in green.

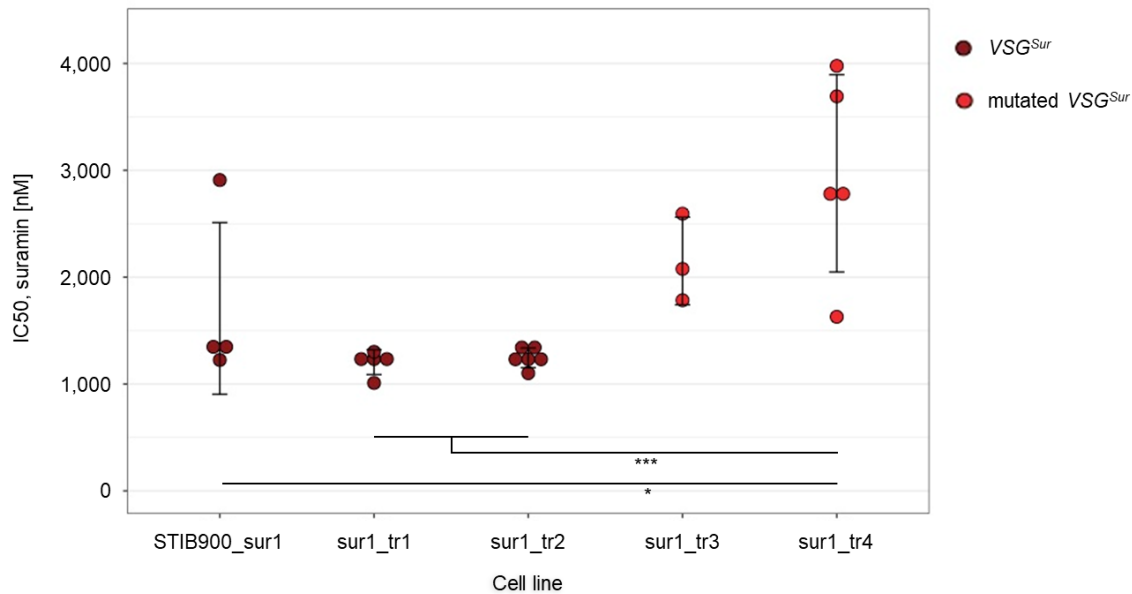


Supplementary Fig. 10: “Supermutations” mapped to VSGsur. Ribbon diagrams of VSGsur/suramin co-crystal structure in two orientations. Mutations discovered in “supersur” VSGsur mutants with heightened resistance to suramin are shown as green space filling atoms. Suramin and the N-linked glycan of VSGsur are depicted as space filling atoms in purple and crimson respectively.

a

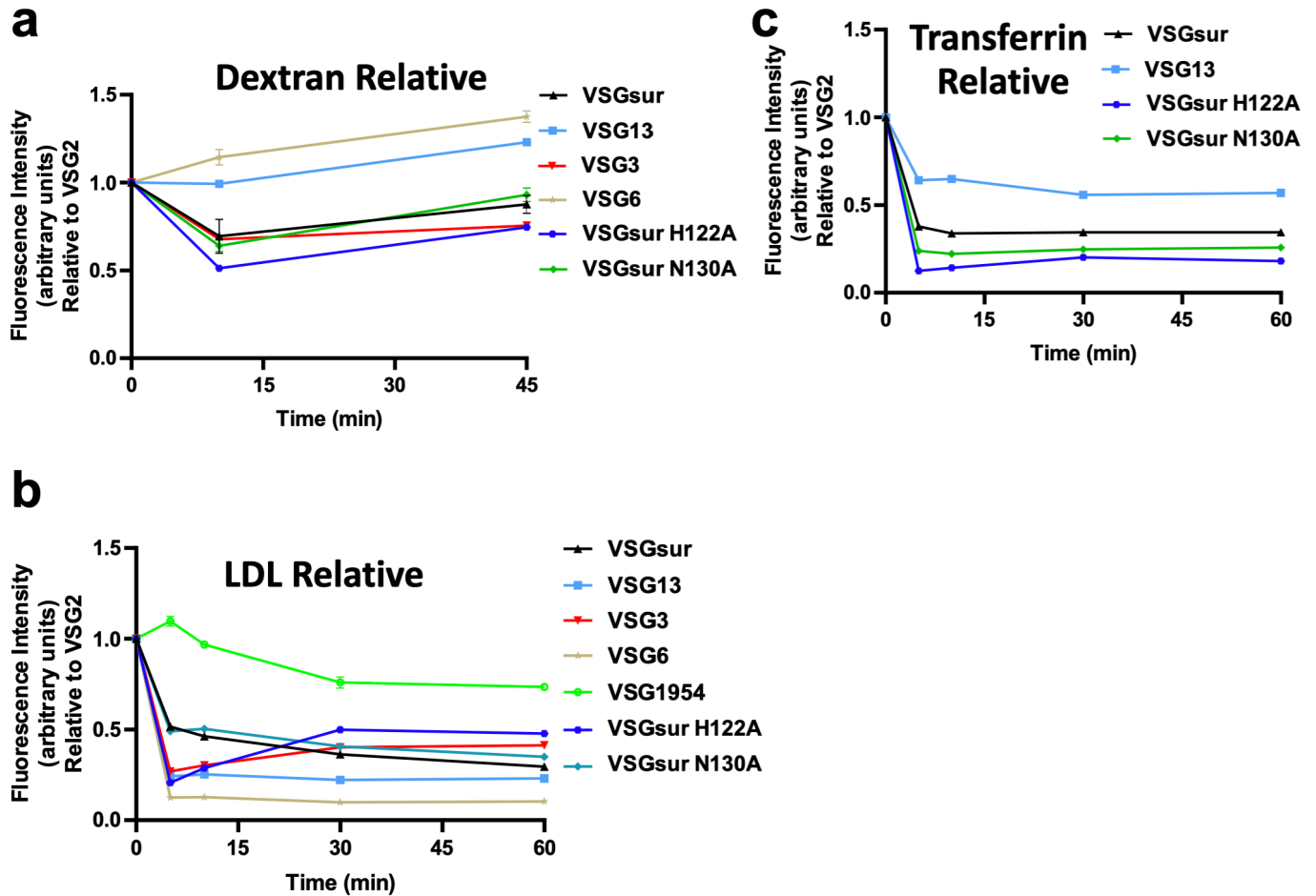


b



Supplementary Figure 11. Replacement of VSG_{sur} with "supermutant" VSG_{sur} at the active expression site of *T. b. rhodesiense* STIB900_sur1.

(a) Construct for gene replacement; mutations in the VSG_{sur} coding sequence (dark red) are shown in light red, arrows indicate primer binding sites. BSD, blasticidine resistance gene; $\alpha\beta$ tub, $\alpha\beta$ tubulin splice site. (b) 50% inhibitory concentrations of the transfected clones and the parent (sur1) as measured with Alamar Blue assays. The pseudoclonal lines sur1_tr1 and sur1_tr2 still expressed VSG_{sur}, while the pseudoclonal lines sur1_tr3 and sur1_tr4 expressed the mutant version. The scatter plots represent mean \pm standard deviation. Significant differences are indicated (Tukey's multiple comparisons test).



Supplementary Fig. 12: Endocytosis rates by different VSGs relative to VSG2

Alexa 488-dextran (a), bodipy-LDL (b), and Alexa 488-transferrin (c) endocytosis by *T. b. brucei* 2T1 cells expressing a variety of different VSG genes. Due to technical limitations, each cell line could not be analyzed simultaneously within each experiment for each ligand. Therefore, VSG2 was used as a control in all experiments, allowing the calculation of each cell line's relative uptake rate of each ligand as compared to the uptake rate of each ligand observed by VSG2 expressing cells within each separate experiment. Each of these graphs therefore represent the combined experimental results from 2 to 3 separate experiments. Cell lines with a relative fluorescence intensity below 1 at a given time point have less efficient endocytic rates compared to VSG2 expressing cells, and vice versa. All graphs share the same Y axis. Error bars when present denote the range (N=2 experimental replicates for each cell line at every time point), while error bars were left out when they were smaller than the size of the data point and therefore not visible.

Supplementary Table 1: Crystallographic Statistics for Wild Type VSGsur

	VSGsur	VSGsur+I3C	VSGsur + 0.77mM suramin
Data Collection			
Beamline	BESSY MX 14.1	BESSY MX 14.2	SLS X06DA
Processing software	XDSAPP	XDSAPP	go.pi
Wavelength (Å)	0.9184	2.066	1.0
Resolution range (Å)	48.05 -1.21 (1.25-1.21)	44.15-1.92 (1.99-1.92)	47.87-1.86 (1.93 -1.86)
Space group	P 21 21 2	P 21 21 2	P 1 21 1
Unit Cell <i>a</i> , <i>b</i> , <i>c</i> (Å)	47.0 71.0 130.4	46.96 70.89 129.65	52.69 79.22 118.00
Unit Cell α , β , γ (°)	90 90 90	90 90 90	90 90.78 90
Total reflections	747748 (26978)	507768 (5366)	545357 (55408)
Unique reflections	122558 (7073)	27999 (845)	81516 (8077)
Multiplicity	6.1 (3.8)	18.1 (6.4)	6.7 (6.8)
Completeness (%)	91.49 (53.39)	82.57 (25.54)	99.91 (99.77)
Mean <i>I</i> / σ (<i>I</i>)	8.57 (0.38)	13.99 (0.68)	12.25 (2.32)
Wilson B-factor	15.43	30.05	
<i>R</i> -merge	0.09566 (2.578)	0.1762 (1.899)	0.1091 (0.759)
<i>R</i> -meas	0.1044 (2.988)	0.1813 (2.055)	0.1185 (0.8029)
<i>R</i> -pim	0.04114 (1.487)	0.04182 (0.7539)	0.04537 (0.3039)
CC1/2	0.998 (0.174)	0.995 (0.380)	0.997 (0.741)
CC*	0.999 (0.544)	0.999 (0.742)	0.999 (0.923)
Refinement			
Refinement reflections	122545 (7073)	27993 (845)	81516 (8077)
R-free reflections	2100 (121)	1124 (34)	3910 (372)
R-work	0.174 (0.377)	0.235 (0.333)	0.195 (0.297)
R-free	0.184 (0.348)	0.256 (0.382)	0.227 (0.337)
CC(work)	0.964 (0.477)	0.917 (0.588)	0.910 (0.719)
CC(free)	0.971 (0.494)	0.933 (0.588)	0.891 (0.597)
No. of atoms	3305	2781	6654
macromolecules	2799	2481	5707
ligands	83	77	296
solvent	423	233	651
Protein residues	377	335	758
RMS(bonds)	0.01	0.008	0.008
RMS(angles)	1.41	1.04	0.94
Ramachandran favored (%)	98.36	98.46	97.33
Ramachandran allowed (%)	1.37	1.54	2.54
Ramachandran outliers (%)	0.27	0	0.13
Rotamer outliers (%)	0	0.80	0.84
Clashscore	3.3	1.60	5.54
Average B-factor (Å ²)	32.2	45.96	21.46
macromolecules	31.14	46.05	20.52
ligands	32.9	49.86	32.26
solvent	39.11	43.55	24.82
Number of TLS groups		7	16

Highest-resolution shell statistics are in parentheses.

Supplementary Table 2: Crystallographic Statistics for VSG13

	VSG13	VSG13 + 0.5mM NaBr
Data Collection		
Beamline	ESRF ID29	Diamond i03
Processing software	iMosflm /CCP4 DIALS	iMosflm/CCP4 DIALS
Wavelength (Å)	1.0	0.9198
Resolution range (Å)	48.14 -1.378 (1.428- 1.378)	52.55-1.56 (1.616-1.56)
Space group	C 1 2 1	C 1 2 1
Unit Cell <i>a</i> , <i>b</i> , <i>c</i> (Å)	73.717 68.341 156.903	74.1477 68.4071 157.759
Unit Cell α , β , γ (°)	90 92.548 90	90 92.1646 90
Total reflections	465941 (45521)	432679 (21591)
Unique reflections	155980 (15141)	109295 (8780)
Multiplicity	3.0 (3.0)	4.0 (2.4)
Completeness (%)	97.09 (94.49)	97.22 (78.32)
Mean <i>I</i> / σ (<i>I</i>)	8.07 (1.15)	18.77 (0.09)
Wilson B-factor	18.5	19.34
<i>R</i> -merge	0.04926 (0.6997)	0.1892 (4.641)
<i>R</i> -meas	0.05999 (0.8474)	0.2143 (5.751)
<i>R</i> -pim	0.03368 (0.4707)	0.09891 (3.314)
CC1/2	0.995 (0.84)	0.983 (0.06)
CC*	0.999 (0.955)	0.996 (0.336)
Refinement		
Refinement reflections	155980 (15092)	109082 (8755)
R-free reflections	1636 (159)	2019 (164)
R-work	0.2176 (0.4012)	0.2263 (0.3773)
R-free	0.2409 (0.4511)	0.2325 (0.3552)
CC(work)	0.949 (0.864)	0.901 (0.233)
CC(free)	0.947 (0.844)	0.900 (0.200)
No. of atoms	5782	5808
macromolecules	5256	5142
ligands	105	128
solvent	421	538
Protein residues	701	695
RMS(bonds)	0.011	0.009
RMS(angles)	1.12	0.95
Ramachandran favored (%)	97.67	98.09
Ramachandran allowed (%)	2.33	1.91
Ramachandran outliers (%)	0	0.00
Rotamer outliers (%)	0.71	0.00
Clashscore	2.81	4.23
Average B-factor (Å ²)	31.18	32.93
macromolecules	30.99	31.85
ligands	33.83	40.09
solvent	32.83	41.47
Number of TLS groups	0	28

Highest-resolution shell statistics are in parentheses.

Supplementary Table 3: Crystallographic Statistics for Mutant VSGsur

	VSGsur H122A	VSGsur H122A + 0.77mM suramin	VSGsur H122A + 7.7mM suramin
Data Collection			
Beamline	Diamond i03	SLS X06DA	SLS X06DA
Processing software	XIA2/DIALS (CCP4)	XIA2/DIALS (CCP4)	go.pi
Wavelength (Å)	0.9763	1.0	1.0
Resolution range (Å)	48.05 -1.47 (1.523-1.47)	38.08-1.75 (1.81-1.75)	39.16-1.66 (1.72-1.66)
Space group	P 21 21 2	P 21 21 2	P 21 21 2
Unit Cell <i>a</i> , <i>b</i> , <i>c</i> (Å)	47.0158 70.9113 130.677	46.90 70.95 130.44	46.94 71.03 130.61
Unit Cell α , β , γ (°)	90 90 90	90 90 90	90 90 90
Total reflections	995504 (99575)	588342 (59570)	686746 (66206)
Unique reflections	75170 (7360)	44210 (4304)	52136 (5028)
Multiplicity	13.2 (13.5)	13.3 (13.8)	13.2 (13.2)
Completeness (%)	99.28 (94.57)	98.94 (97.44)	99.79 (98.15)
Mean <i>I</i> / σ (<i>I</i>)	12.12 (0.44)	16.67 (1.15)	19.44 (1.49)
Wilson B-factor	23.6	26.28	24.19
<i>R</i> -merge	0.09745 (2.95)	0.1224 (2.281)	0.0916 (1.719)
<i>R</i> -meas	0.1014 (3.067)	0.1273 (2.366)	0.0953 (1.788)
<i>R</i> -pim	0.02776 (0.8326)	0.03457 (0.6261)	0.0261 (0.4861)
CC1/2	1 (0.414)	0.999 (0.503)	1 (0.559)
CC*	1 (0.766)	1 (0.818)	1 (0.847)
Refinement			
Refinement reflections	74634 (6960)	44206 (4304)	52131 (5028)
R-free reflections	3654 (349)	2211 (215)	2605 (251)
R-work	0.2008 (0.3851)	0.178 (0.325)	0.185 (0.302)
R-free	0.2236 (0.3853)	0.207 (0.325)	0.208 (0.320)
CC(work)	0.959 (0.713)	0.9579 (0.725)	0.950 (0.787)
CC(free)	0.946 (0.633)	0.959 (0.726)	0.950 (0.738)
No. of atoms	3150	3067	2988
macromolecules	2749	2680	2644
ligands	83	72	61
solvent	318	315	283
Protein residues	368	359	356
RMS(bonds)	0.006	0.015	0.013
RMS(angles)	1.21	1.23	1.19
Ramachandran favored (%)	98.9	99.43	98.86
Ramachandran allowed (%)	1.1	0.57	1.14
Ramachandran outliers (%)	0	0	0
Rotamer outliers (%)	2.46	0.36	0
Clashscore	4.43	0.73	0.93
Average B-factor (Å ²)	38.44	39.22	38.91
macromolecules	38.26	38.90	38.86
ligands	39.85	36.81	30
solvent	39.6	42.47	41.31
Number of TLS groups	9	5	

Highest-resolution shell statistics are in parentheses.

SUPPLEMENTARY METHODS

1. Construction of pHH-VSG^{Sur}-hyg plasmid:

A switched variant of VSG termed VSG^{Sur} that emerged from *Trypanosoma brucei rhodesiense* culture under suramin selection has been reported to correlate with suramin resistance (Wiedemar *et al.*, Molecular Microbiology, 2018). A DNA sequence encoding the VSG^{Sur} (GenBank: MF093647.1) was codon-optimized and synthesized as a pUC19 clone (BioCat, Germany). VSG^{Sur} DNA was PCR amplified from pUC19-VSG^{Sur} plasmid using Q5 DNA polymerase (New England Biolabs) and the following primers:

VSG^{Sur}-pHH-F1: CGACACGTACGCGGCATGCAAGCCGTAACACGC

VSG^{Sur}-pHH-R1: GAAATTTGAGGGGGGAAATTAAGCAAAAATGCAAGCAAAAGAGG

Also, a puromycin-resistant knock-in vector pHH-VSG3-PAC (PCT/EP2019/079063) was linearized by PCR using the following primers:

pHH-VSG2.3'-UTR-F1: TTTCCCCCTCAAATTTCCCCCTCC

pHH-VSG2-CTR-R1: ATGCCGCGTACGTGTCG

The VSG^{Sur} and pHH knock-in vector amplicons were assembled using HiFi® DNA Assembly Master Mix (New England Biolabs) according to the manufacturer's instructions, to replace VSG3 with VSG^{Sur}. To replace puromycin resistance with hygromycin, a hygromycine gene was PCR amplified from pHD789 plasmid (Irmer and Clayton, Nucleic Acids Research, 2001) using the following primers:

Hyg-pHH-F1: GCTCTAGAAGTAGTCAGCTTACCATGAAAAAGCCTGAACTCACCGCGAC

Hyg-pHH-R1: TGGGCAGGATCGATCCCTACTCTATTCTTTGCCCTCGGACGAGTG

Also, the pHH knock-in vector excluding puromycin gene was linearized using the following primers:

Aldolase-3'-UTR-F2: GGATCGATCCTGCCCATTTGGCTTTTCCCTTGTCTCGTG

Actin-5'-UTR-R2: AGCTGACTAGTTCTAGAGCTTATTTTATGGCAGCAACGAGACCTTAC

Finally, the hyg gene and pHH knock-in vector amplicons were assembled using HiFi® DNA Assembly Master Mix (New England Biolabs) according to the manufacturer's instructions.

2. Generation of a *T. brucei* Lister427 Clone Expressing VSG^{Sur}:

A *T. brucei brucei* cell line expressing VSG2, termed 2T1 (Alsford and Horn, Molecular Biochemical Parasitology, 2008), was transfected with EcoRV-linearized pHH-VSG^{Sur}-hyg plasmid to replace VSG2. Briefly, 2x10⁷ cells were electroporated with 10 µg DNA in 100 µl of a home-made Tb-BSF buffer (Burkard *et al.*, Molecular and Biochemical Parasitology, 2011) using Amaxa Nucleofector (Lonza), program X-001. After incubation in non-selective HMI-9 medium for 6 hours, hygromycin was added at 5 µg/ml and the

cells were grown for 6 days at 37°C, 5% CO₂. Single clones isolated by serial dilution were screened for a VSG2-negative phenotype by FACS analysis using an APC-conjugated anti-VSG2 mouse mAb.

3. Generation of pHH-VSG vector for VSG Knock-in *T. brucei*:

Genomic DNA was extracted from VSG2-expressing 2T1 cells using DNAzol® reagent (Life Technologies) according to the manufacture's instruction. The VSG2 gene plus its upstream co-transposed region (CTR) and downstream telomere region were PCR amplified using Q5 DNA polymerase (New England Biolabs) and the following primers:

VSG2-CTR-F2: GAAGGCAGCGGAAAGTGTGCCAATGC

Tb427-tel-R2: AACACCTTAATCCGAAACACC

Also, pUC19 vector (Life Technologies) was linearized by PCR using the following primers:

pUC19-tel-F1: GATATCTCTAGAGTCGACCTGCAGGCATGCAAGC

pUC19-CTR-R2: ACTTTCCGCTGCCTTCGATATCGGATCCCCGGGTACCG

Additionally, telomere seeds were amplified by PCR from pSY-37F1D-CTR-BSD plasmid (Pinger *et al.*, Nature Communications, 2017) using the following primers:

pSY.tel-F1: ACGGTGTTTCGGATTAAGGCCGCGGAATTCGATTAGG

pSY.tel-R1: AGGTCGACTCTAGAGATATCGGATCCACTAGCTAGTGATTAAC

Finally, the above mentioned amplicons were assembled to make pHH-VSG2-Tel plasmid using HiFi® DNA Assembly Master Mix (New England Biolabs) following the manufacturer's instructions. In order to insert a puromycin resistance cassette, pHH-VSG2-Tel was linearized by PCR using the following primers:

pHH-VSG2-Tel-F1:

ATGGGGGGATATTAGACTTAGGCTTAGGATTAGGATTAGGATTAGGTTAATTTTTTCTCTTT
TTTTTTAACTCACACCTCTATCCTG

pHH-VSG2-Tel-R1: GCTTGCATGCCGCGTTCGTG

Also, a puromycin resistance cassette was PCR amplified using the following primers:

pHH-PAC-F1: GGATTAGGCACAGCAAGGTCTTCTGAAATTCATGT

pHH-PAC-R1: CTAAGTCTAATATCCCCCATTTTCTTCTTTTACATCA

The PCR amplicons from pHH-VSG2-Tel vector, and the puromycin cassette were assembled to make pHH-VSG2-PAC plasmid using HiFi® DNA Assembly Master Mix (New England Biolabs) following the manufacturer's instructions. To replace puromycin resistance with hygromycin, a hygromycine gene was

PCR amplified from pHD789 plasmid (Irmer and Clayton, Nucleic Acids Research, 2001) using the following primers:

Hyg-pHH-F1: GCTCTAGAAGCTAGTCAGCTTACCATGAAAAAGCCTGAACTCACCGCGAC

Hyg-pHH-R1: TGGGCAGGATCGATCCCTACTCTATTTCCTTTGCCCTCGGACGAGTG

Also, the pHH knock-in vector excluding puromycin gene was linearized using the following primers:

Aldolase-3'-UTR-F2: GGATCGATCCTGCCCATTTGGCTTTTCCCTTGTCTCGTG

Actin-5'-UTR-R2: AGCTGACTAGTTCTAGAGCTTATTTTATGGCAGCAACGAGACCTTAC

Finally, the hyg gene and pHH knock-in vector amplicons were assembled using HiFi® DNA Assembly Master Mix (New England Biolabs) according to the manufacturer's instructions.

Alamar Blue assays

Serial drug dilutions were prepared on a 96-well plate and the parasites were added to a final concentration of 2×10^4 cells/ml. After incubation for 68 hours, resazurin was added to a concentration of 11.4 µg/ml. The fluorescence of viable cells was determined after 3-4 hours with a SpectraMax reader (Molecular Devices) and SoftMax Pro 5.4.5 Software. Fitting of the dose-response curves (non-linear regression model, variable slope; four parameters, lowest value set to zero) and calculation of the IC50 values were carried out with GraphPad Prism 6.00.

