The Q_i site of cytochrome *b* is a promiscuous drug target in *Trypanosoma cruzi* and *Leishmania donovani*

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Running title: Cyt *b* - a promiscuous drug target in kinetoplastids

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Supplementary figures

Supplementary tables

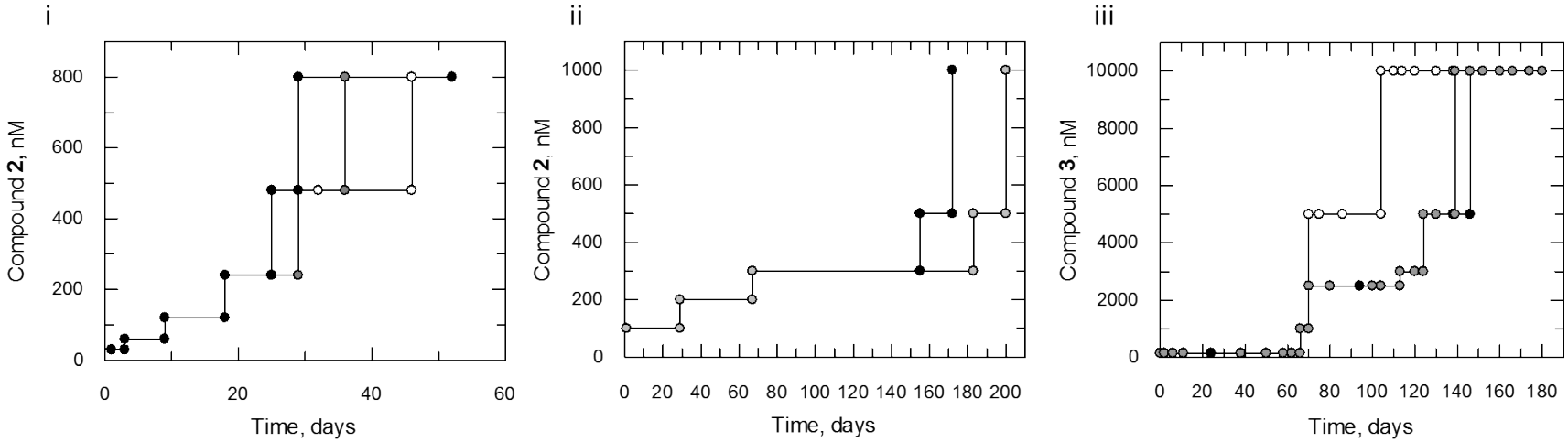

Figure S1 - **Resistance generation *in vitro***. Schematic representation of the generation of compound **2**-resistant cell lines in *L. donovani* (promastigotes) (i) and *T. cruzi (*epimastigotes) (ii) and with compound **3** in *T. cruzi* (iii). Each passage of cells in culture (circles) is indicated with clones I, II and III indicated in black, white and grey, respectively.

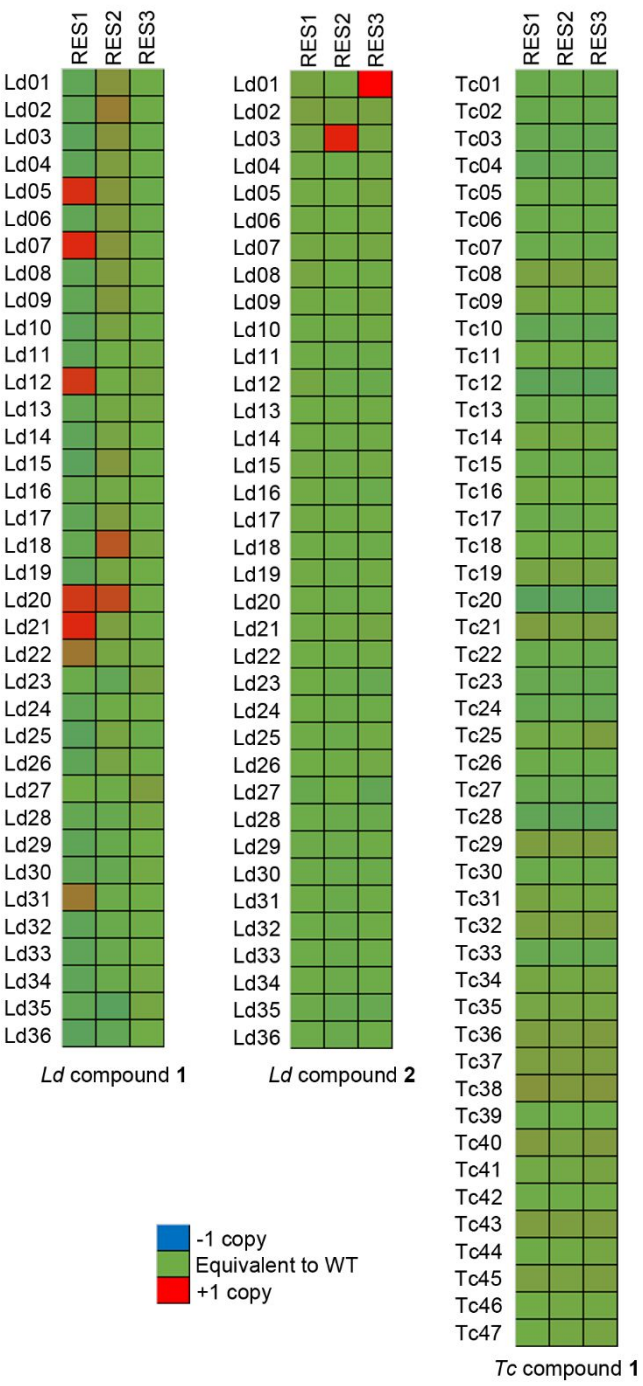

Figure S2 - Copy number variation (CNV) analysis for *L. donovani* (*Ld*) and *T. cruzi* (*Tc*) clones resistant to compounds **1** and compound **2**.

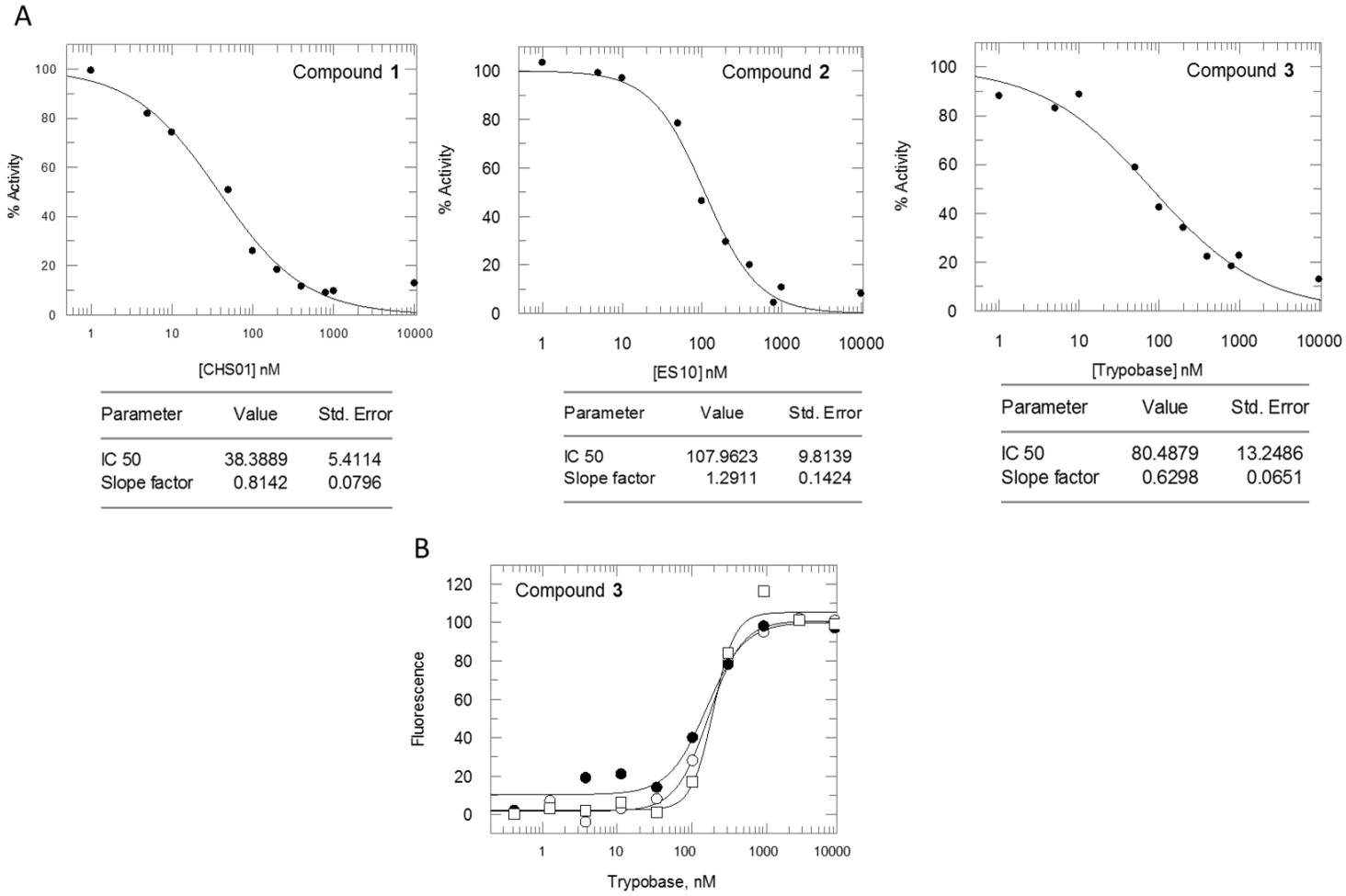

Figure S3 – Representative complex III and O_2 consumption assay data. (A) Representative individual IC_{50} curves generated from complex III assays (see Materials and Methods for details). The data shown for compounds **1** and **2** were determined in *L. donovani* lysate**.** Data shown for compound 3 was determined in *T. cruzi* lysate. (B) Representative IC_{50} curves generated in O_2 consumption assays with *T. cruzi* epimastigotes (see Materials and Methods for details). Data represents three biological replicates.

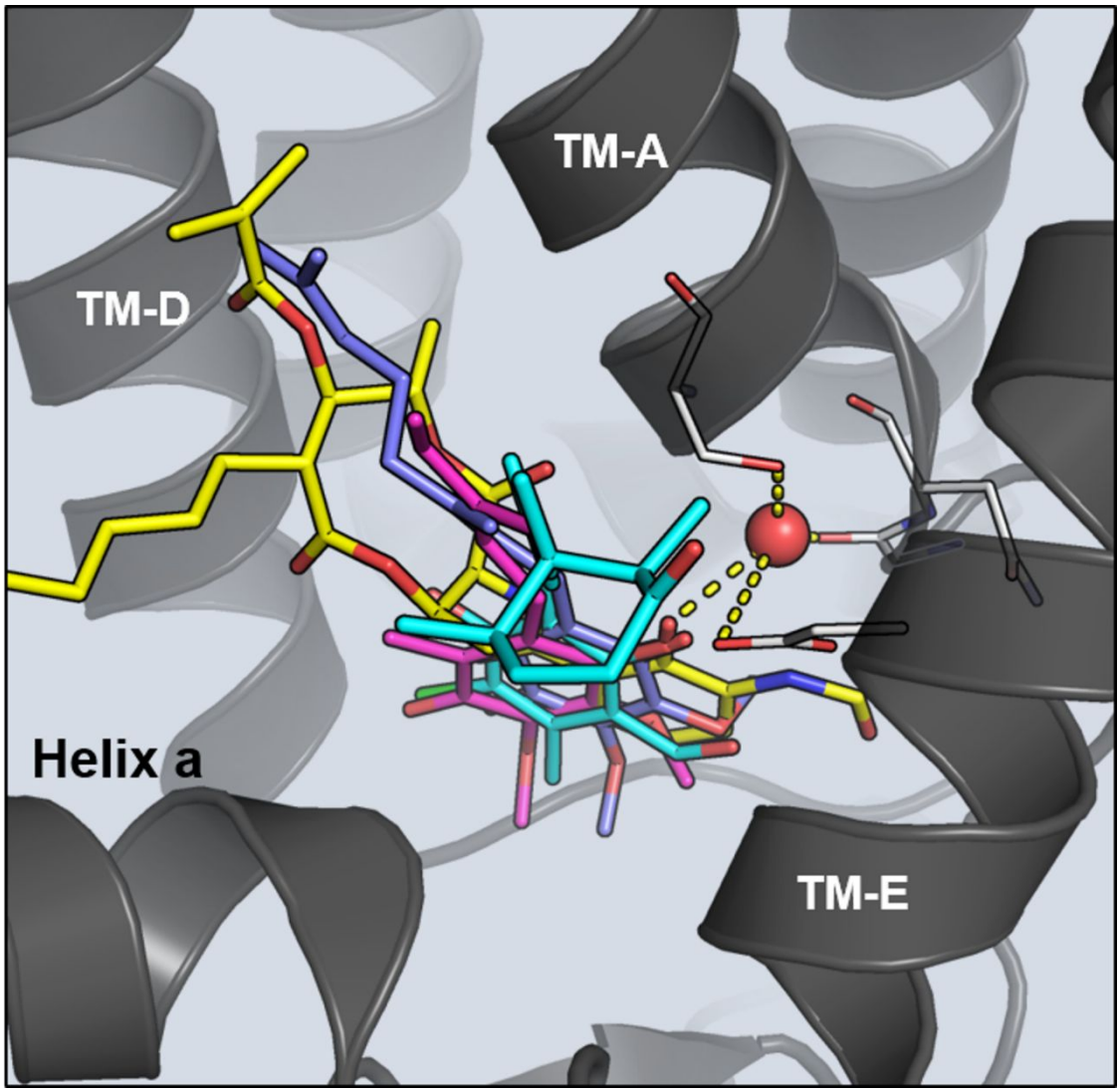

Figure S4 - Conserved water molecule in different X-ray structures of cytochrome *b* in complex with ligands binding to the Q_i site. The water molecule (red sphere) was explicitly considered during the docking calculations of antimycin A and compounds **1**, **2** and **3**. The ligands are displayed as sticks: ubiquinone (violet and magenta, PDB 1NTZ and 3L70); ascochlorin (cyan, PDB 3H1L); antimycin A (yellow, PDB ID 1PPJ).

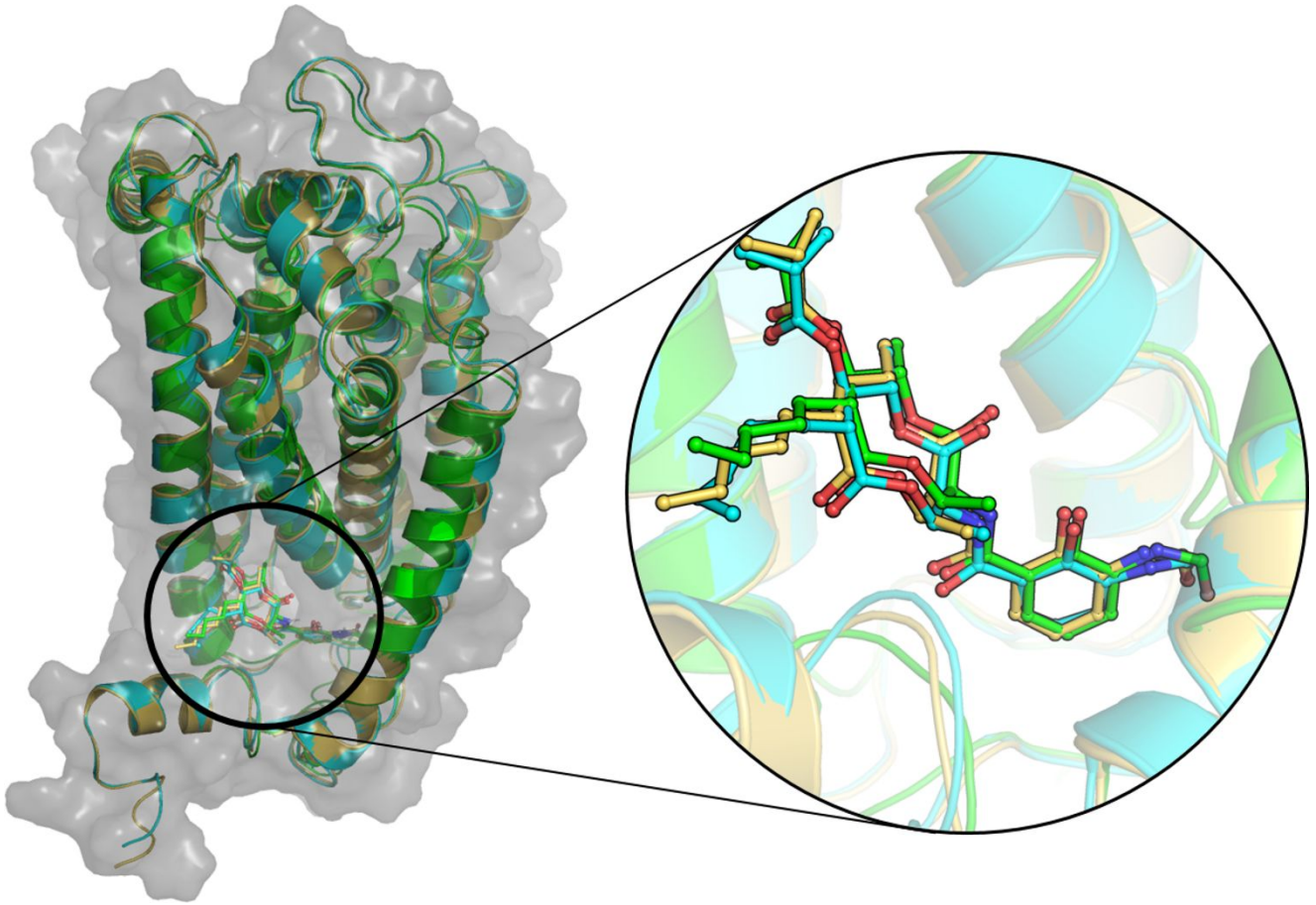

Figure S5 - Binding mode of antimycin A in the cytochrome *b* Q_i site.
Left. Structural alignment of the X-ray crystal structure of cytochrome *b* from chicken (PDB ID 3H1I, in green) with the homology models of cytochrome *b* from *L. donovani* (yellow) and *T. cruzi* (cyan). **Right.** Close-up view into the Q_i site of cytochrome *b* displaying antimycin A (in sticks): avian (in green), *L. donovani* complex model (in yellow) and *T. cruzi* complex model (in cyan).

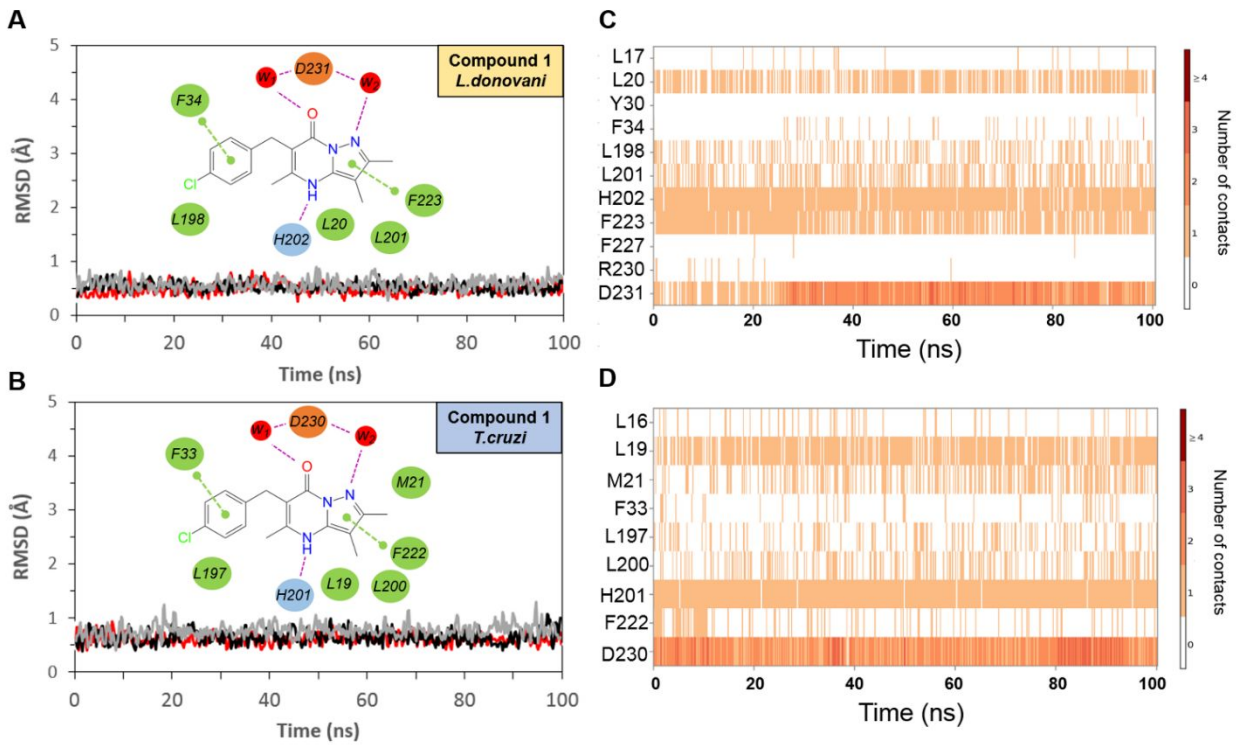

Figure S6 - Structural analysis of compound 1 – cytochrome *b* interactions. (A and B) Time evolution of the ligand root mean square deviation (RMSD) during the MD simulation of compound **1** bound to the Q_i site of cytochrome *b* from *L. donovani* (A) and *T. cruzi* (B). Each plot corresponds to an independent replica (red, black and grey lines). The most relevant ligand-protein interactions are displayed above the RMSD plot. Hydrophobic residues are highlighted in green, acidic residues in orange, polar residues in blue and water molecules in red. (C and D) Time evolution of key ligand-protein contacts during the MD simulations of the cytochrome *b* from *L. donovani* (C) and *T. cruzi* (D) in complex with compound **1**.

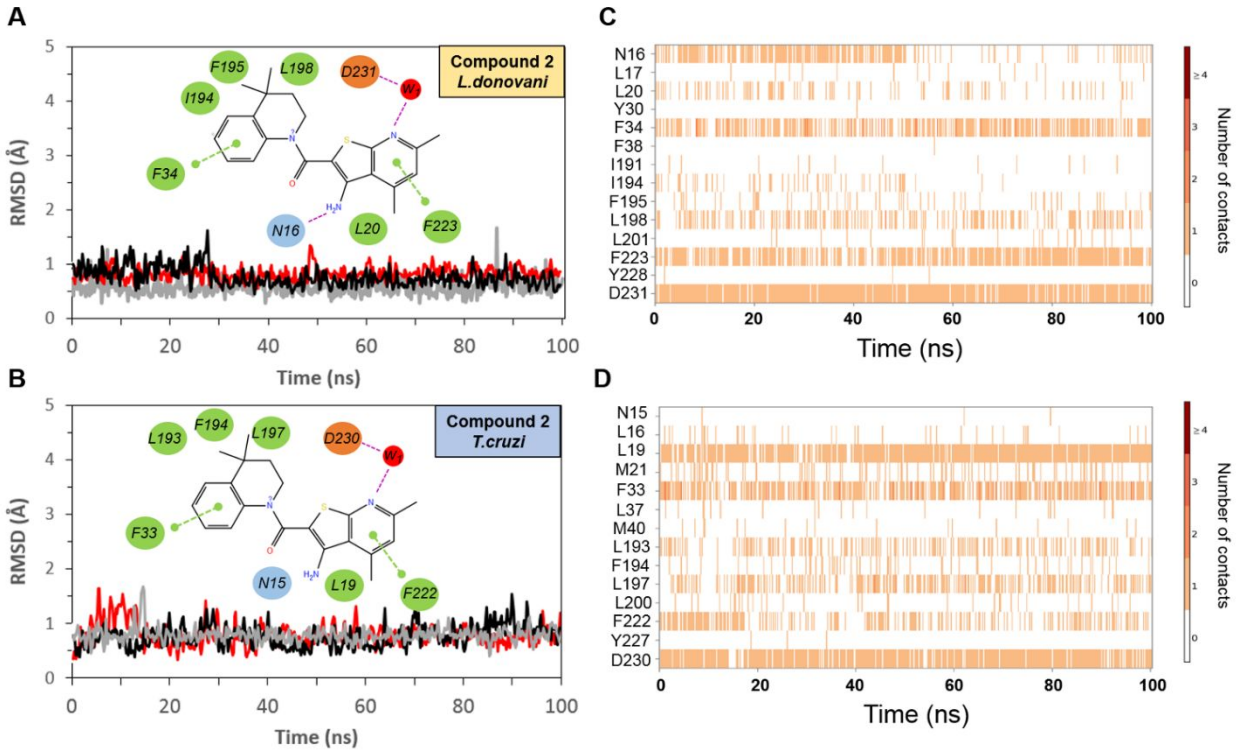

Figure S7 - Structural analysis of compound 2 – cytochrome *b* interactions. (A and B) Time evolution of the ligand root mean square deviation (RMSD) during the MD simulation of compound **2** bound to the Q_i site of cytochrome *b* from *L. donovani* (A) and *T. cruzi* (B). Each plot corresponds to an independent replica (red, black and grey lines). The most relevant ligand-protein interactions are displayed above the RMSD plot. Hydrophobic residues are highlighted in green, acidic residues in orange, polar residues in blue and water molecules in red. (C and D) Time evolution of key ligand-protein contacts during the MD simulations of the cytochrome *b* from *L. donovani* (C) and *T. cruzi* (D) in complex with compound **2**.

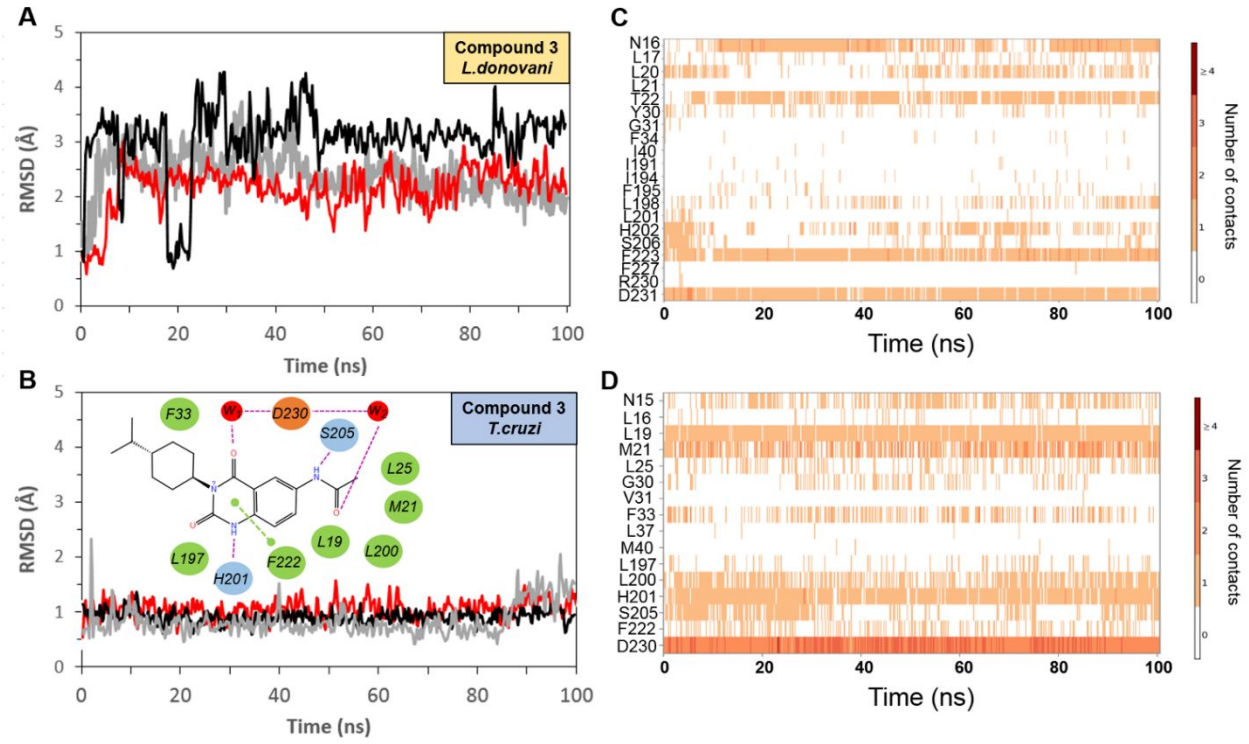

Figure S8 - Structural analysis of compound 3 – cytochrome *b* interactions. Time evolution of the ligand root mean square deviation (RMSD) during the MD simulation of compound **3** bound to the Q_i site of cytochrome *b* from *L. donovani* (A) and *T. cruzi* (B). Each plot corresponds to an independent replica (red, black and grey lines). The most relevant ligand-protein interactions are displayed above the RMSD plot. Hydrophobic residues are highlighted in green, acidic residues in orange, polar residues in blue and water molecules in red. (C and D) Time evolution of key ligand-protein contacts during the MD simulations of cytochrome *b* from *L. donovani* (C) and *T. cruzi* (D) in complex with compound **3**.

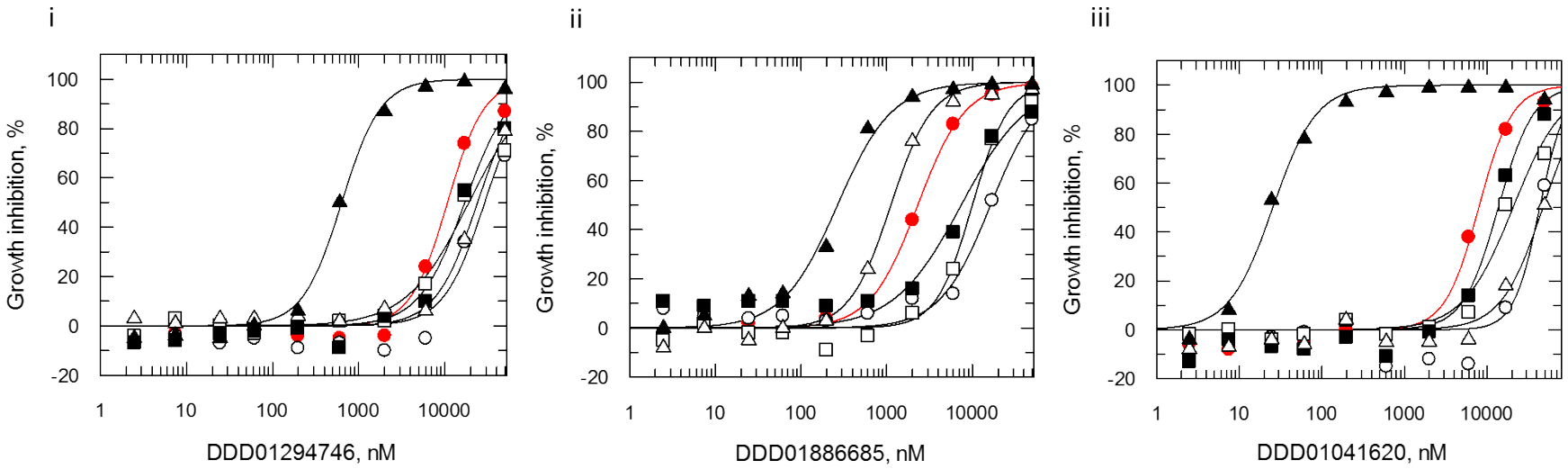

Figure S9 - Cytochrome *b* resistant panel screen. Test compounds were screened against wild-type *T. cruzi* epimastigotes (red) and also five cell lines bearing representative mutations within cytochrome *b*. Resistant line R1, open circles; R2, open squares; R3, closed squares; R4, open triangles and R5, closed triangles. Data represents the mean of duplicate technical replicates.

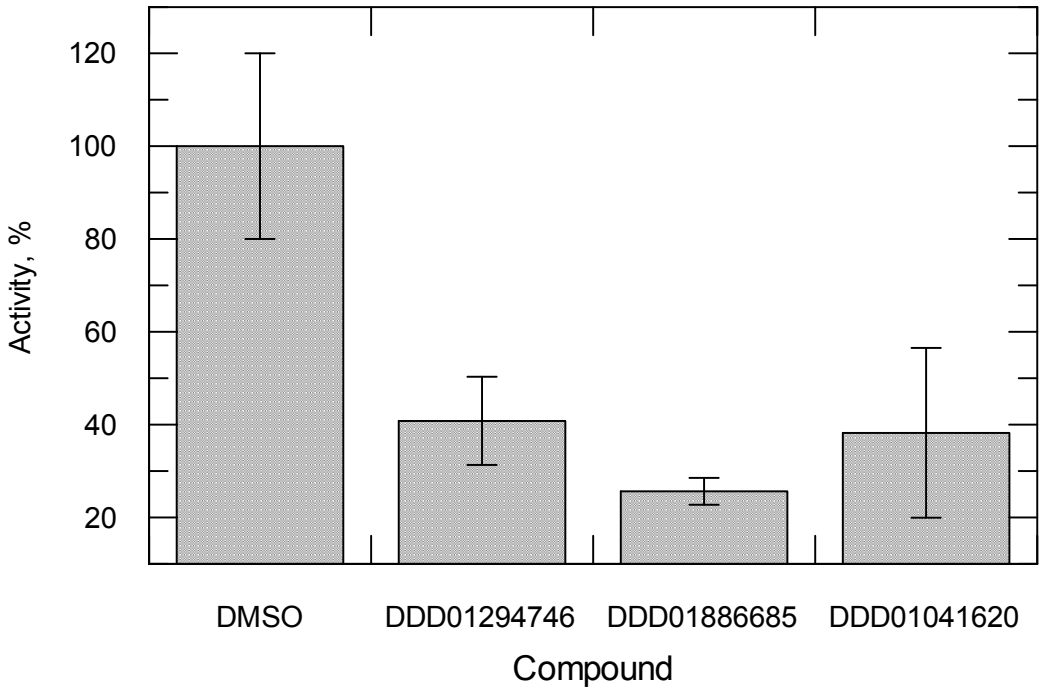

Figure S10 - Complex III assays with compounds identified via the cytochrome *b* resistant panel screen. Lysates enriched with mitochondria isolated from *T. cruzi* epimastigotes were incubated with test compounds (20 μM) or DMSO for 8 min prior to initiation of the complex III assay by the addition of the pseudo substrate decylubiquinol. Complex III activity in the presence of test compounds was determined and compared to activity in the presence of DMSO. Data represents the weighted mean ± standard deviation of triplicate technical replicates and is representative of the data from three biological replicates.

Table S1 – Whole genome sequencing for *L. donovani* clones resistant to compound **1**. Summary of read counts and coverage of sequencing (i). Summary of significant SNPs identified in compound **1**-resistant clones (ii).

i

Cell line	Number of reads	Read length	Percentage mapped	Fold coverage	Gain of SNP heterozygosity	Gain of SNP homozygosity
WT	36675180	100	87.98	88	-	-
RES1	36620390	100	84.93	85	1	1
RES2	36764126	100	86.46	87	1	1
RES3	36619010	100	85.05	85	1	1

ii

Chromosome	Nucleotide position	Reference	Mutation	Amino acid change	Compound 1			Gene name
					R1	R2	R3	
Homozygous mutations (coding regions only)								
Kinetoplast	9139	G	C	Gly37Ala	1/1	1/1	0/0	*cytochrome b*
Kinetoplast	9694	G	T	Cys222Phe	0/0	0/0	1/1	*cytochrome b*
Heterozygous mutations (coding regions only)								
24	688650	C	G	Arg565Pro	0/1	0/0	0/0	LdBPK_241820.1; SET domain containing protein
25	378147	C	A	Gln381Lys	0/0	0/0	0/1	LdBPK_251030.1; hypothetical
35	1178869	C	A	Ala125Ser	0/0	0/1	0/0	LdBPK_352870.1; major facilitator superfamily

Table S2 - Whole genome sequencing for *T. cruzi* clones resistant to compound **1**. Summary of read counts and coverage of sequencing (i). Summary of significant SNPs identified in compound **1**-resistant clones (ii).

(i)

Cell line	Number of reads	Read length	Percentage mapped	Fold coverage	Gain of SNP heterozygosity	Gain of SNP homozygosity
WT	36050370	100	46.09	46	-	-
RES1	36359988	100	58.48	58	1	1
RES2	36353662	100	64.19	64	0	1
RES3	36438792	100	59.48	59	0	1

(ii)

Chromosome	Nucleotide position	Reference	Mutation	Amino acid change	Compound 1			Gene name
					R1	R2	R3	
Homozygous mutations (coding regions only)								
Kinetoplast	4742	C	T	Leu197Phe	1/1	1/1	1/1	*cytochrome b*
Heterozygous mutations (coding regions only)								
Kinetoplast	2098	C	T	-	0/1	0/0	0/0	NADH dehydrogenase subunit 8 (ND8) pre-edit

Table S3 – Assessment of compounds 1 and 2 in complex III assays with lysates prepared from WT and resistant *L. donovani* cell lines. Lysates enriched with mitochondria isolated from *T. cruzi* epimastigotes were incubated with test compounds (20 µM) or DMSO for 8 min prior to initiation of the complex III assay by the addition of the pseudo substrate decylubiquinol. Complex III activity in the presence of test compounds was determined and compared to activity in the presence of DMSO. Fold-changes in sensitivity compared to wild-type are in parentheses.

Cell lines	IC_{50} values, nM	
	Compound **1**	Compound **2**
WT	44 ± 7	99 ± 13
Compound **1** RES1	220 ± 51 (5)	
Compound **2** RES3		4200 ± 500 (42)

Data represents the weighted mean ± SD of at least three biological replicates (n≥3).

Table S4 – Whole genome sequencing for *L. donovani* clones resistant to compound **2**. Summary of read counts and coverage of sequencing (i). Summary of significant SNPs identified in compound **2**-resistant clones (ii).

(i)

Cell line	Number of reads	Read length	Percentage mapped	Fold coverage	Gain of SNP heterozygosity	Gain of SNP homozygosity
WT	38367592	100	81.37	86	-	-
RES1	35899862	100	82.41	81	1	1
RES2	36102782	100	80.19	79	0	1
RES3	35915250	100	83.71	82	0	1

(ii)

Chromosome	Nucleotide position	Reference	Mutation	Amino acid change	Compound 2			Gene name
					R1	R2	R3	
Homozygous mutations (coding regions only)								
Kinetoplast	9121	G	C	Gly31Ala	0/0	1/1	0/0	*cytochrome b*
Kinetoplast	9648	T	C	Ser207Pro	1/1	0/0	0/0	*cytochrome b*
Kinetoplast	9708	T	A	Phe227Ile	0/0	0/0	1/1	*cytochrome b*
Heterozygous mutations (coding regions only)								
4	1272440	G	A	Cys179Tyr	0/1	0/0	0/0	LdBPK_150110.1; hypothetical

Table S5 - Assessment of compound potency against wild-type and resistant *L. donovani* cell lines in intra-macrophage assays. The potency of compounds **1** and **2** were assessed against WT parasites and also resistant clones generated by exposure to compounds **1** and **2**. Compound DDD01012232, an established divalent cation chelator [58], was also assessed as a negative control. Cell lines demonstrating resistance or cross-resistance relative to wild-type are highlighted in blue while those demonstrating hypersensitivity are highlighted in grey. Fold-changes in sensitivity compared to wild-type are in parentheses.

Compound ID	***Leishmania* EC_{50}, µM**		
	WT	Compound **1** RES1	Compound **2** RES1
DDD01012232 (CON)	0.1	0.1	0.2
Compound **1**	0.1	1.3 (11)	0.02 (6)
Compound **2**	0.4	0.03 (13)	4.4 (11)

Data represents the mean ± SD of at least two technical replicates.