Title: New insights into the mechanism of action of the cyclopalladated complex - **CP2** in *Leishmania*: Calcium Dysregulation, Mitochondrial Dysfunction and Cell Death

Angela M. A. Velásquez,[a,b] Paula J. Bartlett,[b] Irwin A. P. Linares,[c] Thais G. Passalacqua,[a] Daphne D. L. Teodoro,[a] Kely B. Imamura,[a] Stela Virgilio,[d] Luiz R. O. Tosi,[d] Aline de L. Leite,[e] Marilia A. R. Buzalaf,[e] Jecika M. Velasques,[f] Adelino V. G. Netto,[f] Andrew P. Thomas,[b] Marcia A. S. Graminha[a]#*

[a]São Paulo State University (Unesp), School of Pharmaceutical Sciences, Araraquara, São Paulo, Brazil.

[b]Department of Pharmacology, Physiology and Neuroscience, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ, USA.

[c] Department of Chemistry, São Carlos Institute of Chemistry – IQSC, University of São Paulo (USP), São Carlos-SP, Brazil

[d]Department of Cellular and Molecular Biology, and Pathogenic Bioagents, Ribeirão Preto Medical School, University of São Paulo (USP), University of São Paulo, Ribeirão Preto-SP, Brazil

[e]Laboratory of Biochemistry, Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo (USP), Bauru-SP, Brazil

[f]São Paulo State University (Unesp), Institute of Chemistry, Araraquara, São Paulo, Brazil.

Running Title: The mechanism of action of **CP2** in *Leishmania*

SUPPLEMENTARY MATERIAL

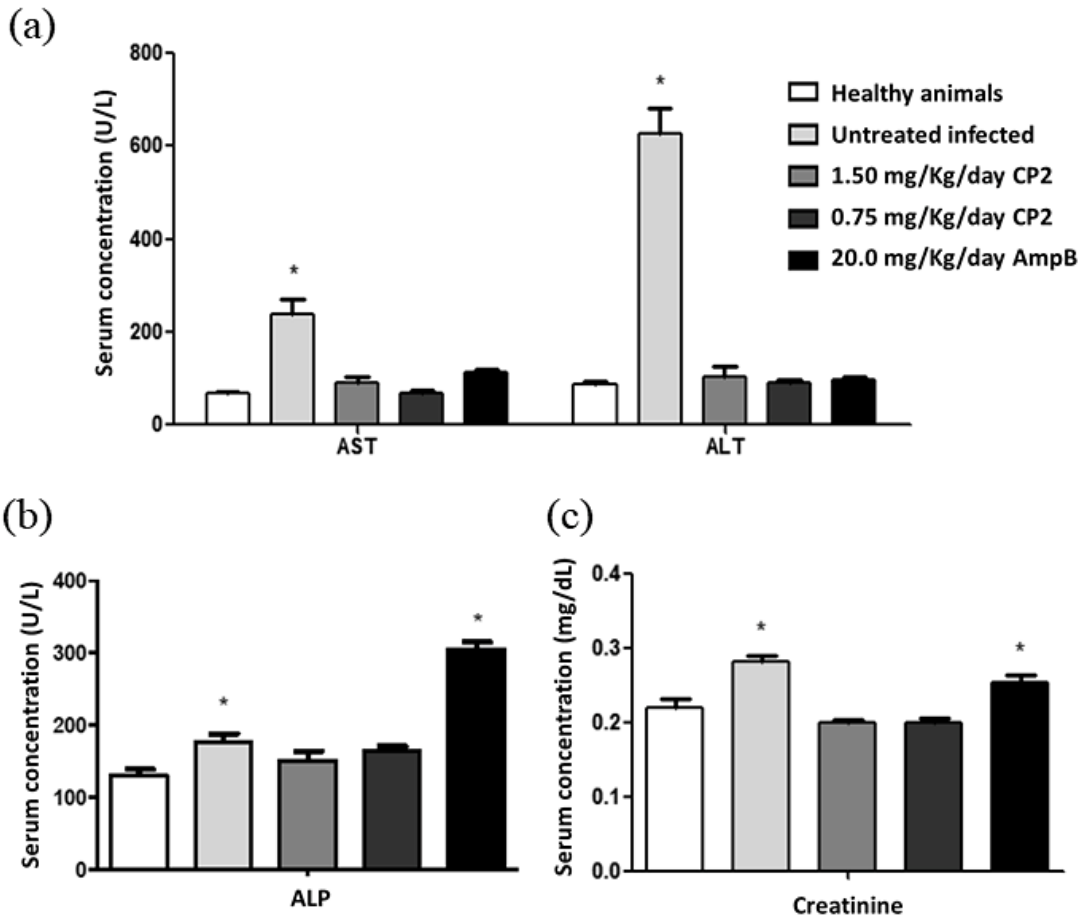


Fig. S1. Serum concentration of biomarkers of liver and renal function in hamsters noninfected and infected with *Leishmania infantum* and treated with 1.50 mg/kg/day or 0.75 mg/kg/day of **CP2** and Amphotericin B – AmpB. (a) AST and ALT levels; (b) ALP and (c) creatinine levels. The data are expressed as mean plus the SD. *: statistically significant difference with the healthy animals (noninfected animals) ($p < 0.05$). Reference values: AST: 20-150 UI/L, ALT: 20 – 128 U/L; ALP: 50 – 186 U/L; creatinine: 0.6 – 1.4 mg/dL (1). AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase.

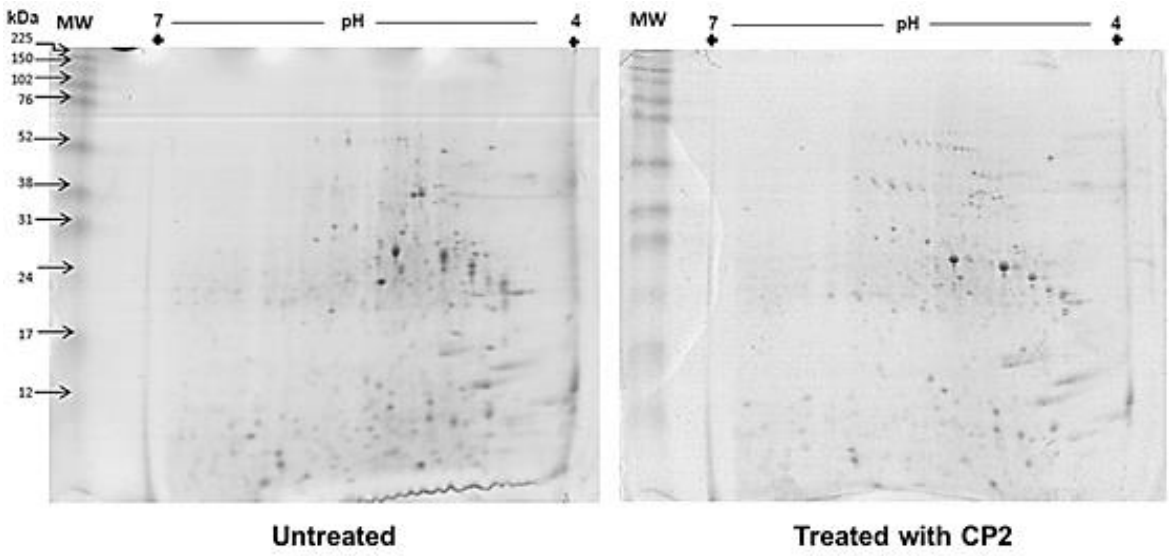


Fig. S2. Representative 2D gels of soluble proteins from *Leishmania amazonensis*. 12.5% SDS-PAGE gel stained with Coomassie blue from *L. amazonensis* promastigotes protein extracts (61.5 µg of protein) (treated and untreated with **CP2**). Range pH 4 to 7. MW: Full range Amersham Rainbow Marker, GE.

TABLE S1 Identifications of spots with differences in abundance between promastigote forms of *Leishmania amazonensis* untreated and treated with cyclopalladated complex, **CP2**.

spot [#]	***L. mexicana (strain MHOM/GT/2001/U1103)* (Proteoma ID UP000007259)**		**Protein Score**	**% Coverage**	**Fold**	**p < 0.5**	**pI**	**MW (KDa)**
	ID[I]	**Accession Number**						
5 NT	Putative mitochondrial Ribonucleoprotein p18	LMXM_15_0275	17,239.45	48.3	2	0.08	5.09	9
5 T			5,282,292	31.9				
9 NT	Peroxidoxin (Tryparedoxin peroxidase)	LMXM_23_0040	22,504.93	69.5	4	0.2	5.40	19
9 T			15,252.9	69.5				
15 NT	Putative heat-shock protein hsp70	LMXM_28_2770	19,639.22	29.2	9	0.16	5.02	23
15 T			6,620.35	27.7				
21 NT	Tubulin beta chain	LMXM_08_1171	20,650.04	40.9	4	0.05	4.41	20
21 T			3,204,158	25.1				
29 NT	Uncharacterized protein	LMXM_36_6760	13,120.50	54.7	4	$5.5\ E^{-03}$	5.08	22
29 T			19,669.89	64.7				
33 NT	Tubulin alpha chain	LMXM_13_0300_1	2,291,276	19.7	3	$2.7\ E^{-03}$	4.93	26
33 T			9,652,642	34.9				
39 NT	Putative translation elongation factor 1-beta	LMXM_33_0840	14,410.28	39.7	11	$1.60\ E^{-04}$	5.34	29

39 T			29,411.30	27.4				
40 NT	Protein disulfide-isomerase, PDI	LMXM_36_6940	10,848.11	62.2	3	0.14	4.80	40
40 T			18,882.04	66.1				
41 NT	Cytochrome c oxidase subunit IV	LMXM_12_0670	11,389.83	66.4	2	2.3 E^{-03}	5.63	31
41 T			17,035.66	77.6				
43 NT	Putative carboxypeptidase	LMXM_32_2540	9,001,966	57.7	2	0.05	5.31	36
43 T			2,217.23	43.9				
44 NT	Putative calreticulin	LMXM_30_2600	3,599.968	31.1	7	0.03	4.14	52
44 T			3,232.07	27.3				
45 NT	Tubulin alpha chain	LMXM_13_0280	39,404.96	38.7	2	3.4 E^{-03}	5.08	37
45 T			31,938.27	32.1				
52 NT	Trypanothione reductase	LMXM_05_0350	3,617.80	49.5	6	2.25 E^{-04}	5.64	40
52 T								
59 NT	Putative calreticulin	LMXM_30_2600	2,548,968	27.3	2	4.01 E^{-04}	4.53	52
59 T								
61 NT	Elongation factor 1-alpha	LMXM_17_0080	7,055.58	18	Absent in the treated	8.0 E^{-03}	6.58	6
63 NT	Putative 10 kDa heat shock protein, HSP-10	LMXM_26_0620	13,049.80	49	Absent in the treated	0.035	5.56	7

64 NT	Tubulin alpha chain	LMXM_13_0280	12,585.71	21.8	Absent in the treated	4.39 E^{-05}	5.07	8
66 NT	Tubulin alpha chain	LMXM_13_0280	14,615.03	10.6	Absent in the treated	8.10 E^{-05}	6.06	8
71 NT	Tubulin alpha chain	LMXM_13_0280	21,735.83	23.2	Absent in the treated	9.4 E^{-03}	5.43	10
72 NT	Tubulin alpha chain	LMXM_13_0280	13,070.50	19.3	Absent in the treated	4.37 E^{-04}	6.40	10
76 NT	Tubulin beta chain	LMXM_08_1171	10,318.76	36.6	Absent in the treated	8.3 E^{-03}	4.60	11
78 NT	Putative heat-shock protein hsp70	LMXM_28_2770	4,412,818	23.3	Absent in the treated	5.95 E^{-04}	5.29	11
82 NT	Tubulin alpha chain	LMXM_13_0280	13,789.17	31.4	Absent in the treated	0.001	4.64	13
84 NT	Tubulin alpha chain	LMXM_13_0280	2,357,408	20.6	Absent in the treated	8.69 E^{-04}	4.59	16
88 NT	Peroxidoxin (Tryparedoxin peroxidase)	LMXM_23_0040	19,809.79	63.7	Absent in the treated	7.28 E^{-04}	5.69	20
104 NT	Putative heat-shock protein hsp70	LMXM_28_2770	12,713.07	35	Absent in the treated	3.3 E^{-03}	5.48	24
111 NT	Putative heat-shock protein hsp70	LMXM_28_2770	7,316,579	27.4	Absent in the treated	3.2 E^{-03}	4.98	25
116 NT	Tubulin beta chain	LMXM_08_1171	49,924.61	45.6	Absent in the treated	5.0 E^{-03}	4.98	26
123 NT	60S acidic ribosomal protein P0	LMXM_27_1380	2,450,133	21.1	Absent in the treated	1.7 E^{-03}	4.74	28
124 T	Heat shock protein 83-1, HSP83	LMXM_32_0312	3,216,727	17.1	Absent in the treated	2.5 E^{-03}	5.03	29
129 NT	ATP synthase subunit beta	LMXM_25_1170	17,231.98	58.3	Absent in the treated	2.72 E^{-08}	4.88	29
131 NT	Elongation factor 2	LMXM_36_0180	4,936.26	23.4	Absent in the treated	9.33 E^{-04}	5.70	31
153 NT	Putative heat shock protein	LMXM_18_1370	5,724,801	33.3	Absent in the treated	9.50 E^{-05}	4.91	50

155 NT	Putative heat shock 70-related protein 1, mitochondrial, mHSP-70-1	LMXM_29_2550	6,643.28	44.6	Absent in the treated	2.9 E^{-03}	5.58	53
159 NT	Putative glucose-regulated protein 78, GRP78	LMXM_28_1200	13,739.33	48.9	Absent in the treated	2.8 E^{-02}	5.22	55
178 T	Tubulin alpha chain	LMXM_13_0280	14,579.73	19.7	Absent in the untreated	2.73 E^{-04}	5.91	9
179 T	Tubulin alpha chain	LMXM_13_0280	11,120.45	10.6	Absent in the untreated	7.33 E^{-04}	5.99	9
181 T	Tubulin beta chain	LMXM_08_1171	7,961,711	36.3	Absent in the untreated	2.5 E^{-03}	4.31	10
183 T	Tubulin beta chain	LMXM_08_1171	11,653.01	36.6	Absent in the untreated	6.59 E^{-04}	4.46	11
185 T	Peroxidoxin (Tryparedoxin peroxidase)	LMXM_23_0040	13,540.69	66.4	Absent in the untreated	7.19 E^{-04}	5.63	19
190 T	Tubulin alpha chain	LMXM_13_0280	4,511,121	18.8	Absent in the untreated	4.03 E^{-04}	5.19	22
191 T	Tubulin beta chain	LMXM_08_1171	23,298.46	42.2	Absent in the untreated	2.79 E^{-04}	4.42	22
195 T	Tubulin alpha chain	LMXM_13_0280	17,885.20	22.6	Absent in the untreated	1.81 E^{-03}	5.14	23
196 T	Tubulin beta chain	LMXM_08_1171	24,353.90	39.5	Absent in the untreated	1.23 E^{-04}	4.64	24
201 T	Tubulin beta chain	LMXM_08_1171	60,965.22	52.8	Absent in the untreated	1.86 E^{-05}	4.84	25
202 T	Tubulin beta chain	LMXM_08_1171	18,868.88	50.3	Absent in the untreated	2.34 E^{-03}	4.75	25
203 T	Tubulin alpha chain	LMXM_13_0280	28,689.78	20.4	Absent in the untreated	2 E^{-02}	5.07	26
207 T	ATP synthase subunit beta	LMXM_25_1170	14,692.52	46.9	Absent in the untreated	6.13 E^{-04}	4.76	29
212 T	ATP synthase subunit beta	LMXM_25_1170	17,265.15	64	Absent in the untreated	1.96 E^{-05}	5.08	39
216 T	ATP synthase subunit beta	LMXM_25_1170	19,263.57	67.6	Absent in the untreated	2.72 E^{-04}	5.06	46

217 T	Putative glucose-regulated protein 78, GRP78	LMXM_28_1200	17,257.46	48.2	Absent in the untreated	1.80 E^{-04}	5.20	55
218 T	Putative glucose-regulated protein 78, GRP78	LMXM_28_1200	6,912,567	50.6	Absent in the untreated	1.69 E^{-04}	5.16	55

[#] Spot number; [I] Primary IDs correspond to proteins supported by the higher number of unique peptides and coverage value (http://www.uniprot.org/proteomes/?query=Leishmania&sort=score). NT: Spot of parasites not treated with **CP2** (untreated control), T: Spot of parasites treated with **CP2**.

1. qPCR Analysis

1.1 Primers Design

The primers were designed based on the 3' region of the genes (Table 1) using the IDT Integrated DNA Technologies software tools (https://www.idtdna.com/pages) and Primer Express version 3.0.1 (http://www.downloadcollection.com/primer_express.htm).

TABLE S2 Primers used for amplification of the *Leishmania amazonensis* genes.

Gene	Accession number	Primer sequence (5'–3')	Product size (bp)
Tryparedoxin peroxidase (Tryp Redox)	LMXM_23_0040	F: GAGATTGCTCGTGACTATGG R: AGGTCATTGATCGTTGCG	110
Calreticulin	LINJ_31_2670/ LMXM_30_2600	F: GCGCCAAACAATACGTACCA R: AACGACCCTTCCTGGATGTG	59
Protein disulfide-isomerase (PDI)	LMXM_36_6940	F: GGTGGCCAAGAGCTTCGA R: CGTTCGTAGTGGCGTCCAT	62
kDNA	Nicolas et al. (2)	F: CCTATTTTACACCAACCCCCAGT R: GGGTAGGGGCGTTCTGCGAAA	120

F (forward) and R (reverse).

1.2 Total RNA extraction

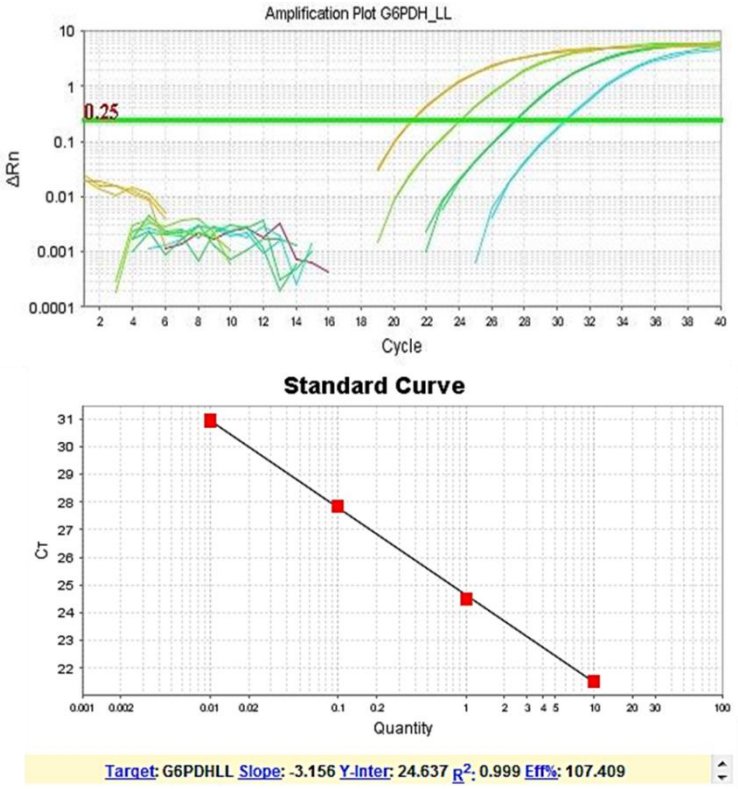
Total RNA from *L. amazonensis* ($5x10^6$ promastigotes mL^{-1}), treated and untreated with **CP2** (13.3 μmol L^{-1}), was extracted using Direct-zol™ RNA MiniPrep (Zymo Research), according to the manufacturer's instructions. Briefly, promastigotes were washed with PBS 1X after 72 h of growth in presence and absence of **CP2** and the pellet was resuspended in Trizol 600 μL. RNA was treated with DNase/RNase, resuspended in DEPC-treated water and stored at -80^0C. The final RNA concentration was determined by absorbance reading at 260/280 nm using a NanoDrop Lite Spectrophotometer (Thermo Scientific). cDNA synthesis was performed using 5 μg of total RNA and the 3′Race System for Rapid Amplification of cDNA ends kit (Life

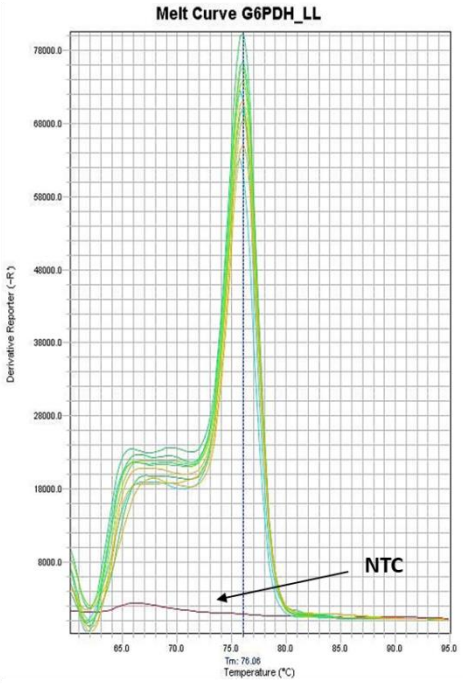
Technologies), and an Oligo(dT)18 Primer, according to previously established methodology (3). Finally, cDNAs were treated with RNase H, nanodrop quantified and stored at -20°C until use for qPCR analysis. All samples were made in triplicate.

1.3 Real-time PCR

Quantitative PCR reactions (qPCR) were performed using the SYBR® Green Master Mix Fast Kit (Applied Biosystems®) in a StepOnePlus™ machine (Applied Biosystems®, Foster City, CA, USA) according to the manufacturer's instructions. Each qPCR reaction contained 5 µL of 1X SYBR™ Green Master Mix, 2 µL of 100 pmol of each primer (forward and reverse), and 1 µL of 10 ng of each cDNA sample. Negative controls were performed without cDNA templates. Amplifications were performed with initial denaturation at 95ºC for 20 s followed by 40 denaturation cycles at 95ºC for 15 s, annealing at 60ºC for 1 min, and extension at 95ºC for 15 s. Each sample was analyzed in triplicate. Melt curve analysis of each reaction was performed after completion of the amplification protocol to determine the specificity of the reaction. A 10-fold dilution series of genomic DNA was used for standard curve construction to determine the efficiency of each target, according to the equation $E = 10^{(-1/S)-1}$, where S was the slope of the standard curve generated from 10-fold serial dilutions. The Ct and melting temperature (Tm) data were expressed as the mean ± SD based on three measurements and the relative quantification was made by the ΔΔCt method (4). The Ct values for the *Leishmania* kDNA reference gene were used for data normalization (2). The *Leishmania* glucose-6-phosphate dehydrogenase (G6PD) reference gene was also validated in order to determine the best reference gene for data analysis.

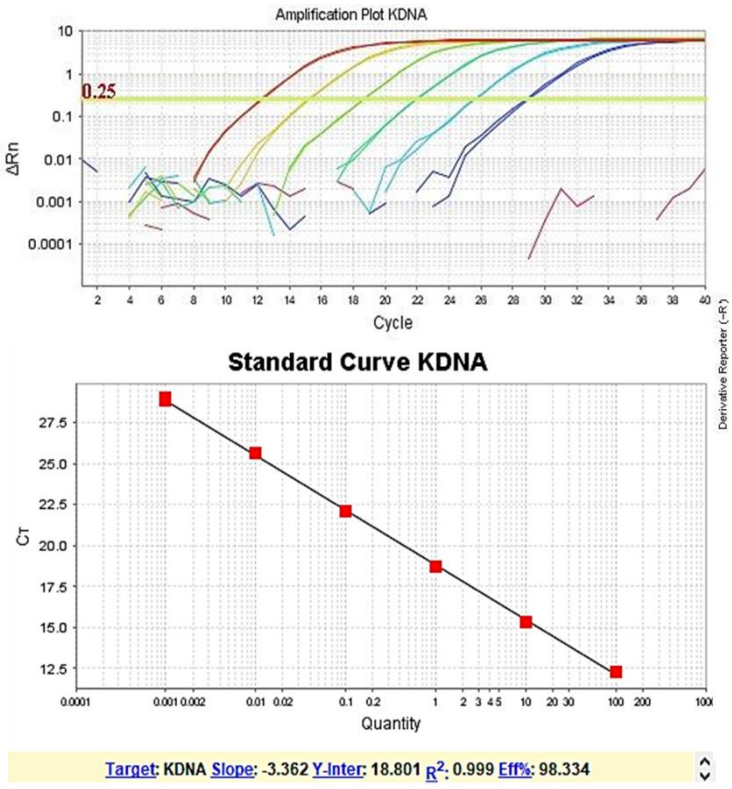
93 (a)

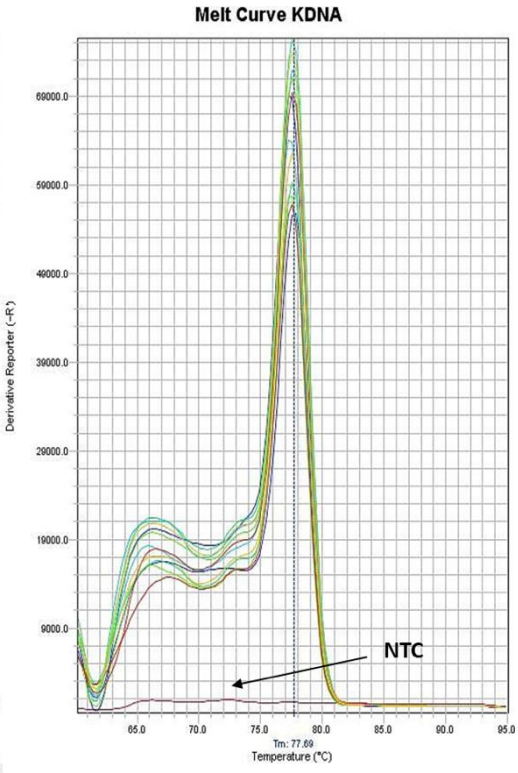

Amplification Plot G6PDH_LL
0.25
ΔRn
Cycle
Standard Curve
Ct
Quantity
Target: G6PDHLL Slope: -3.156 Y-Inter: 24.637 R2: 0.999 Eff%: 107.409


Melt Curve G6PDH_LL
Derivative Reporter (-R')
NTC
Tm: 76.06
Temperature (°C)

94

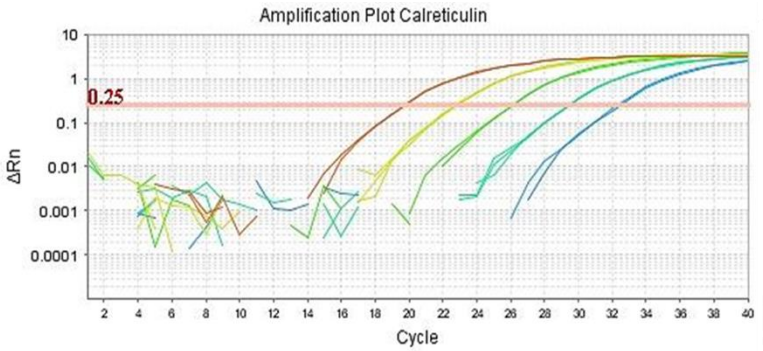
95 (b)

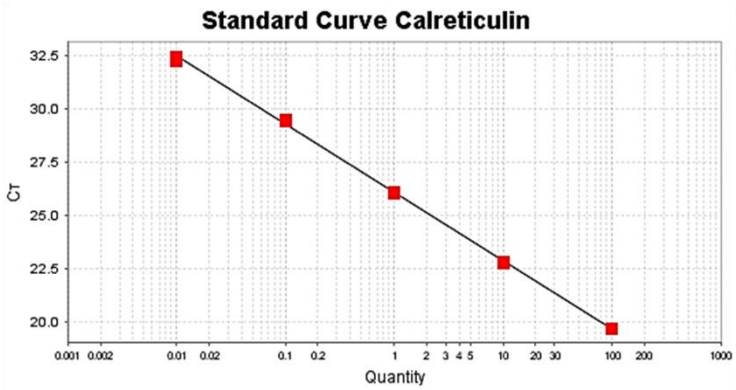

Amplification Plot KDNA
0.25
ΔRn
Cycle
Standard Curve KDNA
Ct
Quantity
Target: KDNA Slope: -3.362 Y-Inter: 18.801 R2: 0.999 Eff%: 98.334

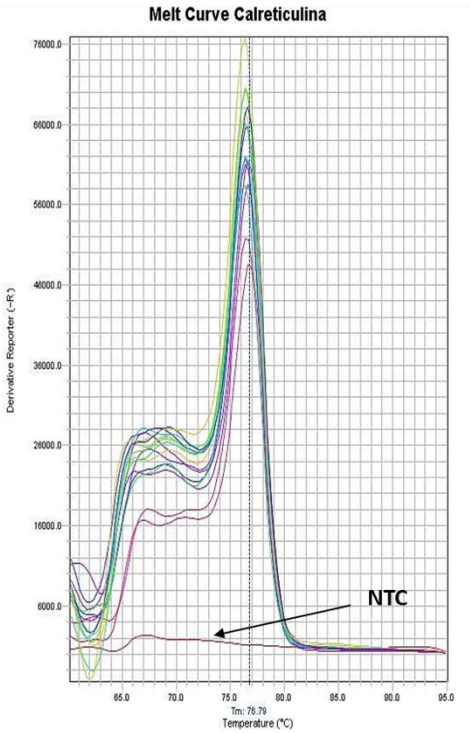

Melt Curve KDNA
Derivative Reporter (-R')
NTC
Tm: 77.69
Temperature (°C)

96

(c)

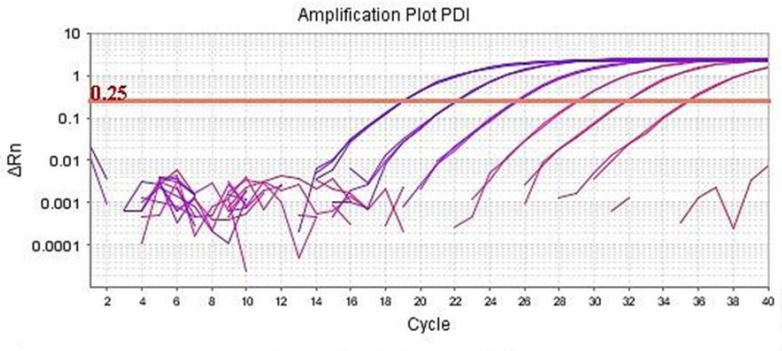


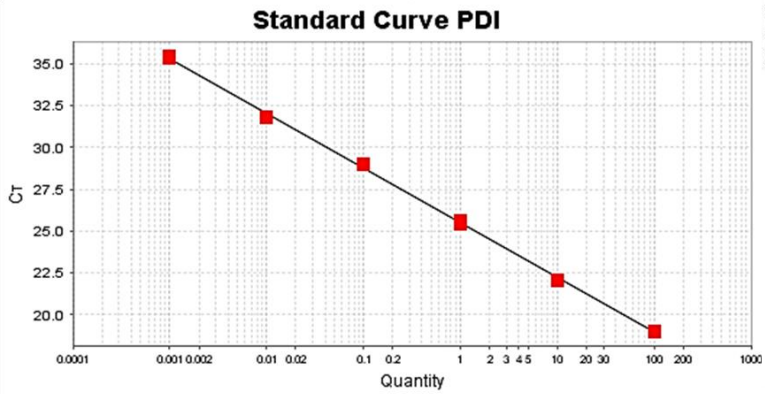


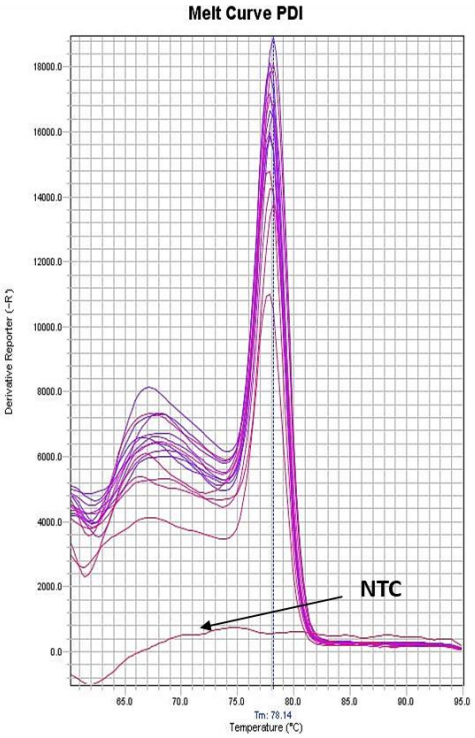


Target: calrreticulina Slope: -3.203 Y-Inter: 26.073 R^2: 0.999 Eff%: 105.21

(d)







Target: PDI Slope: -3.284 Y-Inter: 25.488 R^2: 0.999 Eff%: 101.595

(e)

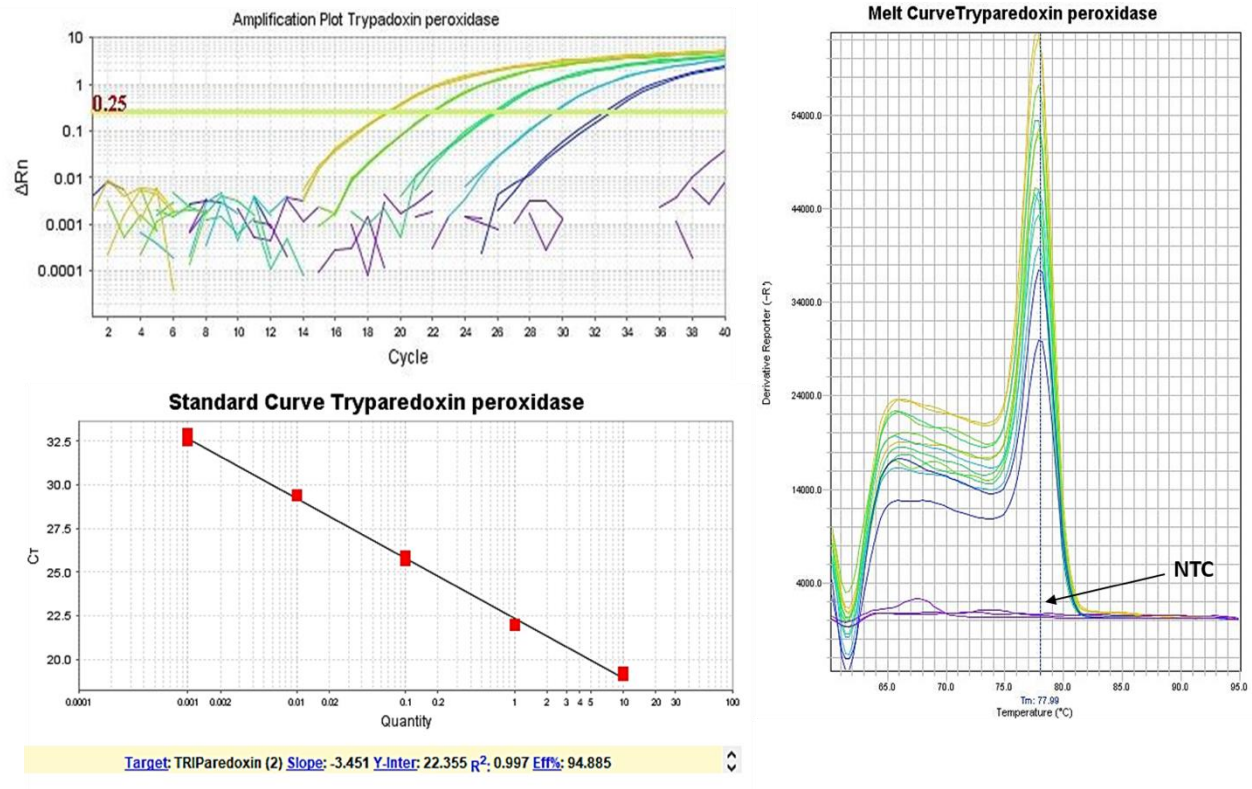


Fig. S3. Melting and standard curves of (a) glucose-6-phosphate dehydrogenase (G6PD, reference gene), (b) kinetoplast DNA (kDNA, reference gene), (c) tryparedoxin peroxidase (Tryp. Redox.), (d) calreticulin, and (e) protein disulfide-isomerase (PDI) of *Leishmania amazonensis.* A 10-fold dilution series of genomic DNA was used for standard curve construction to determine the efficiency of each target according to the equation $E = 10^{(-1/S)-1}$, where S was the slope of the standard curve generated from 10-fold serial dilutions.

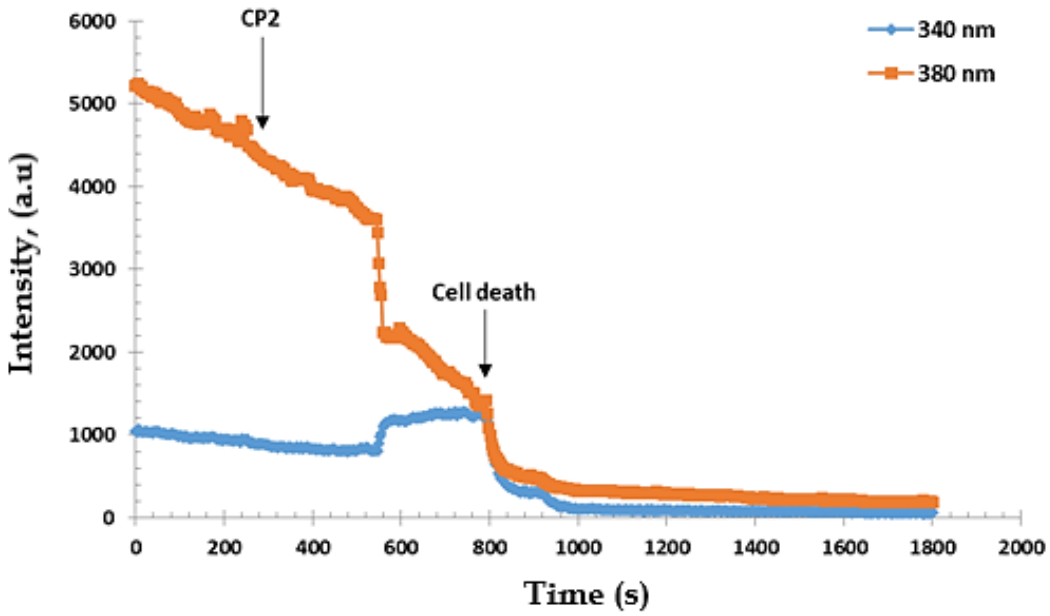


Fig. S4. Representative trace of effect of **CP2** on the $[Ca^{2+}]_i$ of *Leishmania mexicana*. The parasites were cultivated until the mid-log growth phase and then loaded with 5 µmol L^{-1} Fura-2/AM in a loading buffer containing 1.3 mmol L^{-1} of $CaCl_2$. Changes in $[Ca^{2+}]_i$ were measured with Fura-2 excitation at 340/380 nm and emission at >510 nm and are plotted as the 340 nm (blue) and 380 nm (orange) signals. Increases in $[Ca^{2+}]_i$ are reflected in increases in the ratio of 340 nm to 380 nm fluorescence. The rapid decrease in fluorescence at both wavelengths reflects loss of the dye from the cells due to loss of cell viability, and was taken as an endpoint of cell death.

References

1. Washington IM, Hoosier G Van. The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents. Suckow MA, Stevens KA, Wilson RP, editors. Elsevier; 2012. 1288 p. http://doi.org/10.1016/C2009-0-30495-X

2. Nicolas L, Prina E, Lang T, Milon G. Real-time PCR for detection and quantitation of Leishmania in mouse tissues. J Clin Microbiol. 2002;40(5):1666–

9. http://doi.org/10.1128/JCM.40.5.1666

3. Nogueira CT, Del Cistia ML, Urbaczek AC, Jusi MM, Velásquez AMA, Machado RZ, et al. Potential application of rLc36 protein for diagnosis of canine visceral leishmaniasis. Mem Inst Oswaldo Cruz. 2018;113(3):197–201. http://doi.org/10.1590/0074-02760170171

4. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real- Time Quantitative PCR and the 2-ΧΧCT Method. METHODS. 2001;408(25):402–8. http://doi.org/10.1006/meth.2001.1262