**Supporting Information**

# Antileishmanial chemotherapy through clemastine fumarate mediated inhibition of the *Leishmania* inositol phosphorylceramide synthase

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*Contents (12 pages)*

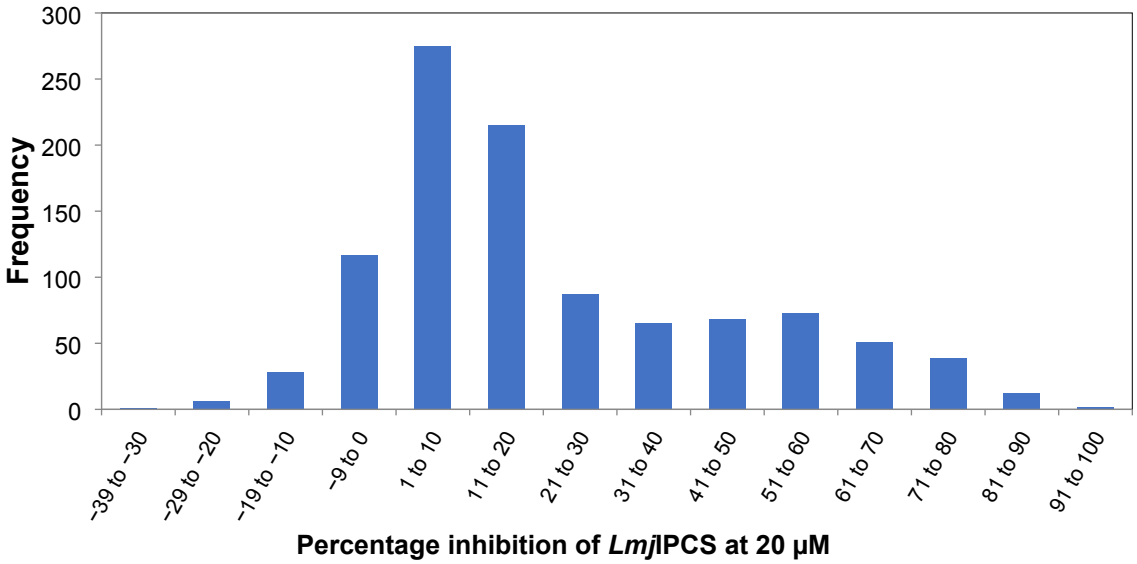


**Figure S1. Percentage inhibition frequency distribution of *Lmj*IPCS by NINDS set at 20 µM.** *Lmj*IPCS inhibition was determined using the 96-well plate formatted assay as described, with fluorescent NBD-C6-ceramide as the acceptor substrate (5 µM), and bovine phosphatidylinositol (PI; 100µM) as the donor substrate. Compounds were diluted in DMSO at 20 µM. IPC was quantified in relation to an NBD-C6-ceramide standard curve. All plates were run in triplicate with average Z-factors for all plates= 0.79 (min value ≥ 0.65).

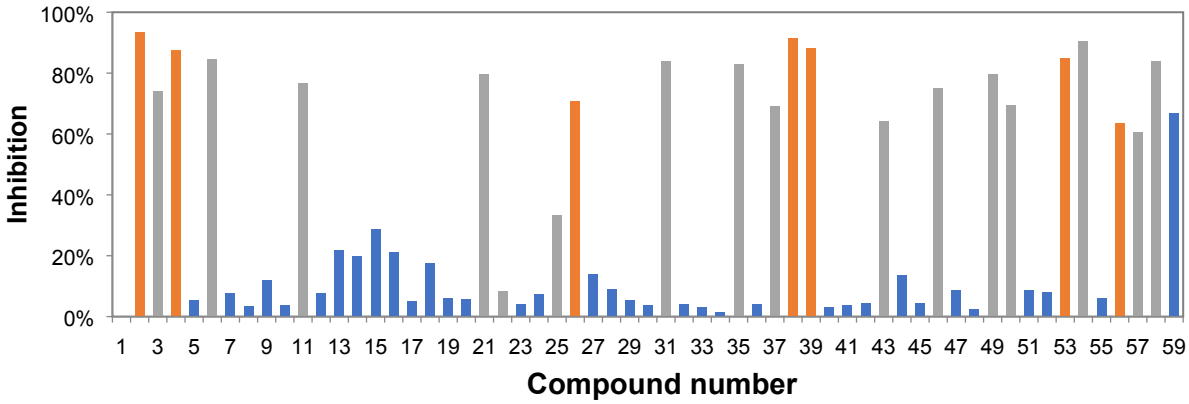


**Figure S2 *L. major* promastigote growth inhibition of compounds from NINDS Set**. *L. major* promastigotes were incubated with the 57 most active *Lmj*IPCS inhibitors, at 10 µM, for 72h. Then parasite viability was assessed fluorimetrically by addition of resazurin solution to the culture. Lane 1: no inhibitor; Lane 59: amphotericin B control; Lanes 2, 4, 26, 38, 39, 53 and 56 (orange bars) are known non-selective cytotoxic compounds; Lane 22 is the known antileishmanial pentamidine **9**.

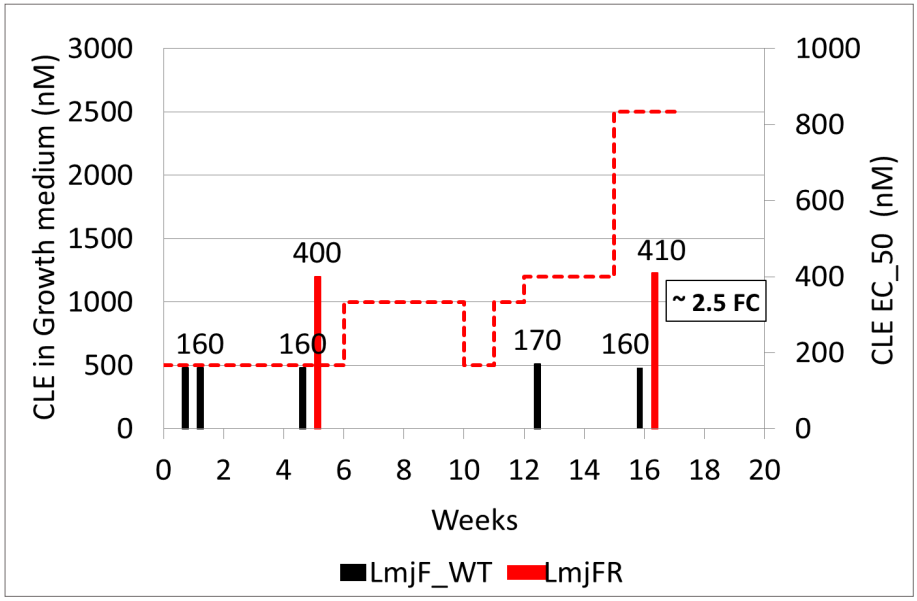


**Figure S3. Selection for clemastine-resistance and $EC_{50}$ values in *L. major* promastigotes**. Left-hand *y*-axis is the amount of clemastine (CLE) added (red dotted line) in the culture medium; right-hand *y*-axis shows the $EC_{50}$ values of clemastine at each time point in the parental wild type (LmjF_WT, black bars) and the resistant lines (LmjFR, red bars). See Selection of independent clones for Clemastine resistance section in Material and Methods for a full description.

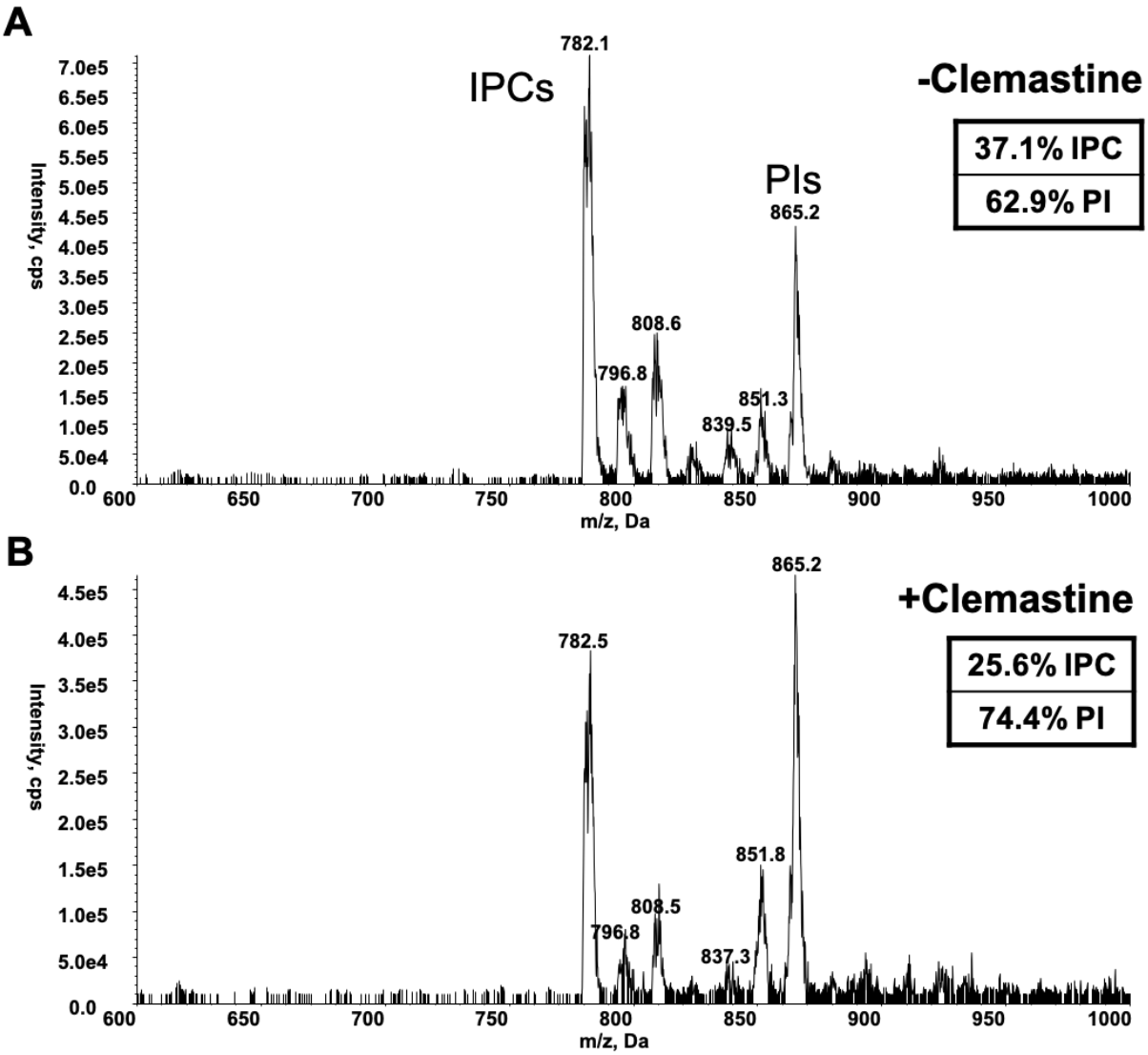


**Figure S4 Phospholipid analysis of *L. major* in absence and presence of clemastine.** Negative ion mode ESI-MS/MS (600–1000 m/z) precursors of m/z 241 scans to detect inositol-containing phospholipids in total lipid extracts from *L. major* in the (a) absence or (b) presence of clemastine (10 μM) for 3.75 h. IPCs: inositol phosphorylceramides and PIs: phosphatidylinositols. Representative spectra of one of three independent biological replicates. Inset panels display relative percentage values obtained for the PI and IPC pools, determined via GC-MS quantitative inositol quantification.

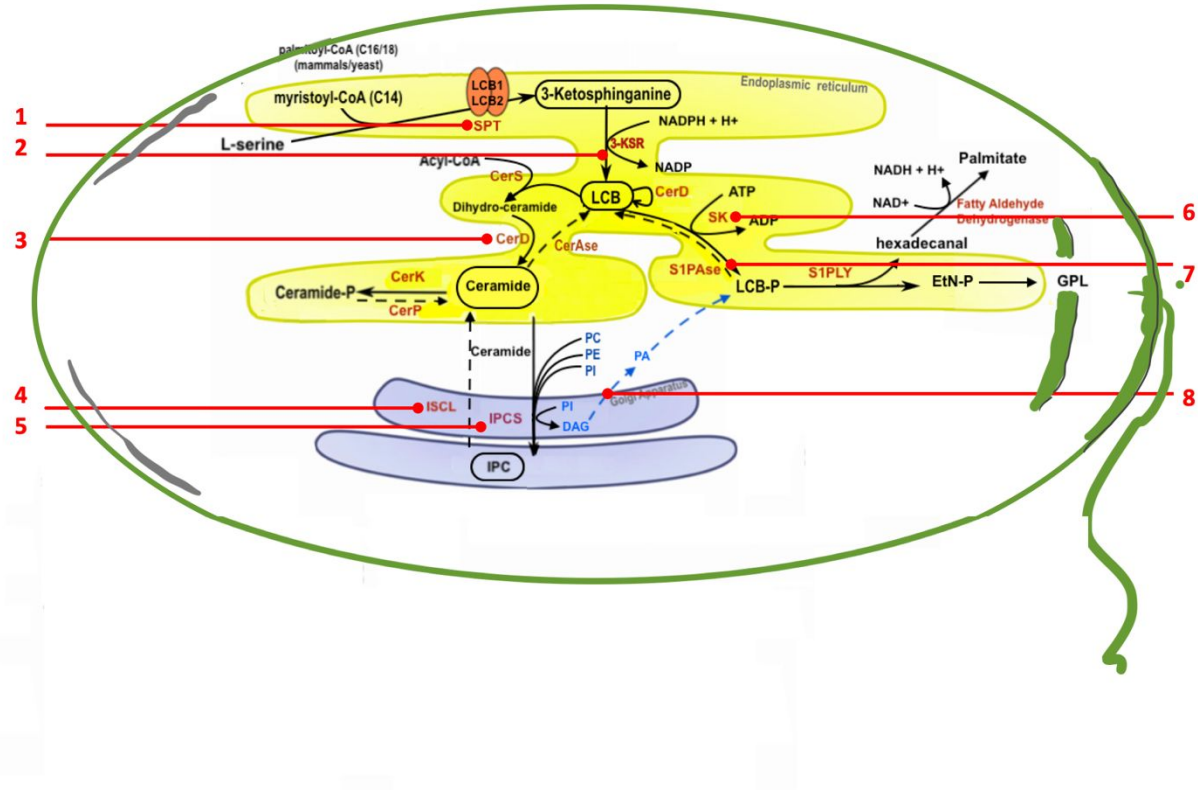


**Figure S5 SNPs identified in sphingolipid-genes in two CleR individual lines of *L. major* promastigotes. Numbers (red) highlight the eight genes in which SNPs were found.** SPT (1); 3-KSR (2); CerD (3); ISCL (4); IPCS (5); SK (6); S1PAse (7); PAF (8); CerS; CerK; CerP; S1PLY. See Table 1 for a full description of the gene names. PC: phosphatidylcholine, PE: phosphatidylethanolamine, PI; phosphatidylinositol, DAG: diacylglycerol.

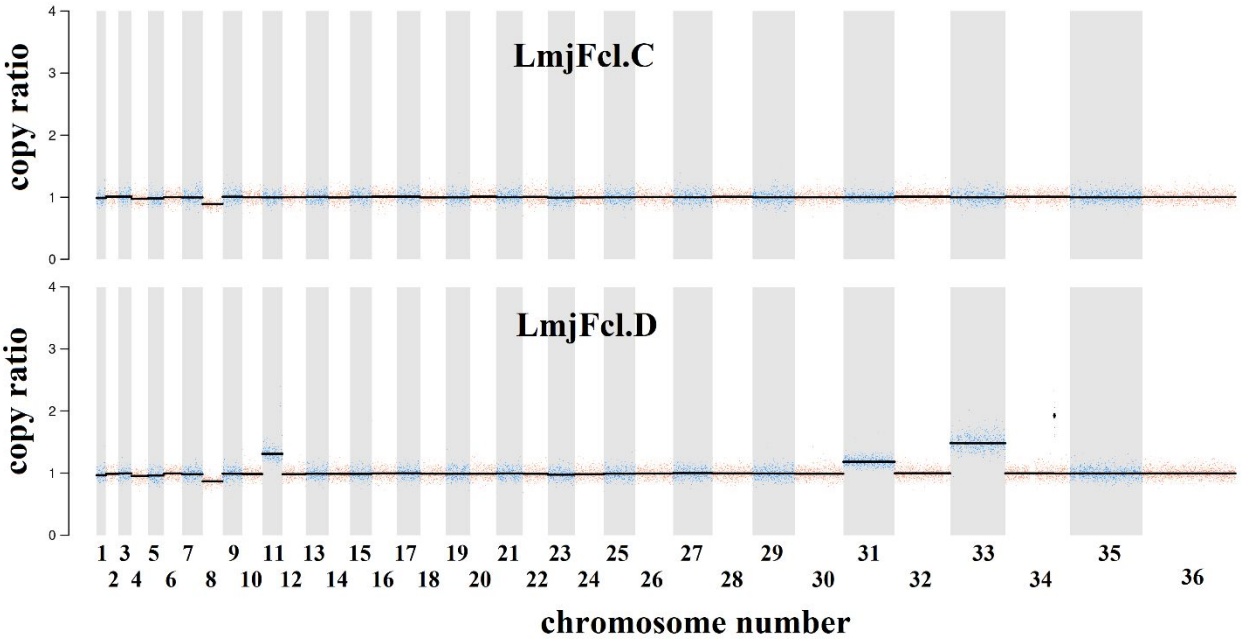


**Figure S6 Copy Number Variation (CNVs) found in in two individual clones of *Leishmania major* promastigotes.** The copy ratio median (thick black lines) is shown in log2 ratio (*y-axis*) for all the chromosomes (*y-axis*) of clones cl.C (top panel) and cl.D (bottom panel). See Selection of independent clones for Clemastine resistance section in Material and Methods for a full description. Table S4 (excel file) provides CNV values in both clones.

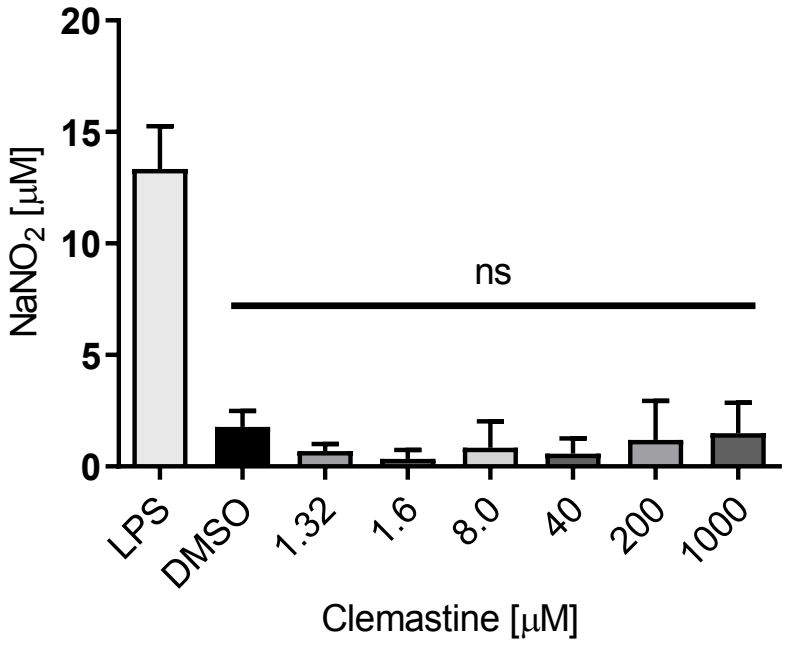


**Figure S7 Nitric oxide production by macrophage after clemastine treatment.** BMDM were incubated with serial dilutions of clemastine for 48 h at 37 °C. The concentration of nitric oxide released on the culture supernatant were measured by GRIESS method. Data values are representative of one of three independent biological replicates with standard error (bars). Changes in relation to DMSO (One-way ANOVA) were statistically non-significant (ns).

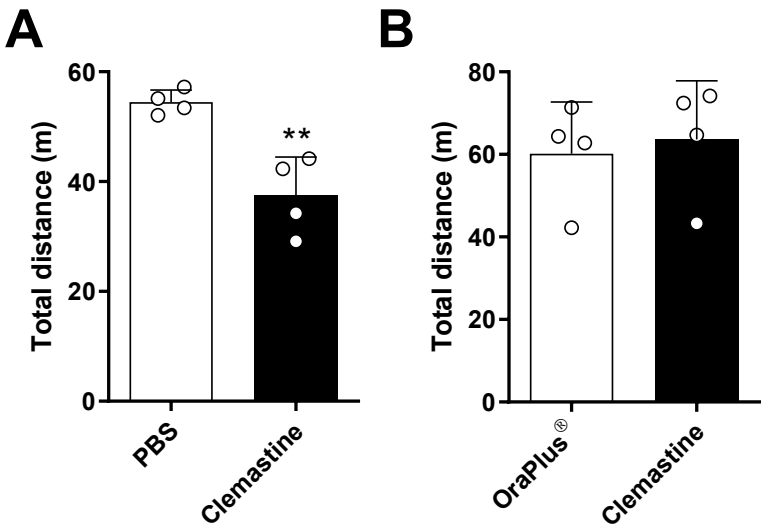


**Figure S8 Clemastine intraperitoneally reduces mice mobility.** BALB/c mice were injected intraperitoneally(**A**) with clemastine (11.65 mg $kg^{-1}$) or PBS, or by oral route (**B**)with clemastine (134 mg $kg^{-1}$) or OraPlus. Then, mice were allowed to freely move for 30 min in an open field arena. The total distance was recorded and quantified using the ANY-maze software. Values are represented by mean ± SEM. Statistically significant values (One-way ANOVA, $P<0.05$, 95% Confidence Interval) are shown with stars: ** $p \leq 0.01$.

**Table S1 Results of screening of NINDS set against L.major (primary assay) and Lmj IPCS (secondary assay)**

Available as a separate Excel File

**Table S2 Relative abundance of metabolites (LCMS) in wild type *L. major* promastigotes treated with clemastine (10 μM) for 12 h**.

| **Metabolite (mass/retention time)** | **Formula** | **Log fold-change (WTTx vs WTCx)** | **Adjusted P-value** |
|---|---|---|---|
| L-serine (106.0499/713.26) | $C_3H_7NO_3$ | -0.13 | ns |
| Sphinganine (302.3053/683.32) | $C_{18}H_{39}NO_2$ | 1.63 | 0.0009 |
| Ceramide (d33:0) (526.5192/155.9) | $C_{33}H_{67}NO_3$ | 2.32 | 0.0026 |
| Ceramide (d36:0) (568.5663/169.03) | $C_{36}H_{73}NO_3$ | 2.13 | 0.0008 |
| Ceramide (d34:0(2OH)) (556.5296/155.73) | $C_{34}H_{69}NO_4$ | 1.36 | 0.0031 |
| Ceramide (d36:0(2OH)) (584.5610/155.73) | $C_{36}H_{73}NO_4$ | 1.25 | 0.0052 |

Fold changes (Log FC, P< 0.05) are the peak intensity relative to untreated cells (N=4). Statistically significant values (P<0.05, 95% Confidence Interval) are shown, ns is statistically non-significant. Data were processed with PiMP pipeline.[14]

**Table S3 Polymorphisms identified in two individual clones of *Leishmania major* promastigotes.**

| **Species** | ***L. major*** | | | | |
|---|---|---|---|---|---|
| **Clones** | **cl.C** | **AF** | **cl.D** | **AF** | **Total** |
| **IGV** | 15,399 | 0.02-0.75 | 10,150 | 0.02-0.63 | **25,549** |
| **(%)** | 52.05 | | 34.3 | | **86.35** |
| **CDS** | 2,449 | 0.02-0.27 | 1,587 | 0.02-0.40 | **4,036** |
| **(%)** | 8.27 | | 5.36 | | **13.64** |
| **Missense ™** | 2,024 | 0.02-0.27 | 1,263 | 0.02-0.40 | **3287** |
| **(%)** | 6.84 | | 4.27 | | **11.11** |
| **Silent** | 414 | 0.02-0.25 | 316 | 0.03-0.24 | **730** |
| **(%)** | 1.4 | | 1.07 | | **2.47** |
| **TOTAL (n)** | 17,848 | 0.02-0.75 | 11,737 | 0.02-0.63 | **29,585** |
| **(%)** | 60.29 | | 39.64 | | **99.93** |

IGV: US/DS-, IG-, non-coding transcripts (exons) or splice region- variants. CDS: missense (non-synonymous), silent (synonymous), start/stop lost/gained -variants; RNA, translation sites, frameshift-, initiator codon- or splice region-variants. ™ Silent mutations were excluded.

**Table S4 Copy number variation (ploidy) in whole genome sequencing data of clemastine resistant clones of Leishmania major**

Ploidy Log-2 ratios (Figure S6) were calculated as described in Material and Methods. Haploid ratio is the ratio of length-normalised per gene coverage for a given gene compared to the median of the parental chromosome of that gene. Available as a separate Excel File

**Table S5 Wild type *Leishmania major* promastigotes treated with Clemastine.**

| Clemastine (μM) | Swollen cells (%) observed at four time points (hours) | | | |
|---|---|---|---|---|
| | **24** | **20** | **16** | **12** |
| **20** | >95% | >50% | >50% | >50% |
| **10** | ~95% | ~50% | ~50% | ~20% |
| **5** | ~90% | ~40% | ~40% | ~20% |
| **0** | 0% | 0% | 0% | 0% |

Drug exposure (≤ 24 hours and <10 μM) showed no change in cell density. Exposure time (10 μM) longer than 24 hours (i.e. 42 hours) showed a significant reduction in parasite density from $30x10^6$ to $6.2x10^6$ cells per mL, in untreated and treated cells, respectively.