Supplemental Methods

Axenic *L. donovani* assay

Compounds to be tested were pre-dispensed into white, clear bottom 384 well plates (Greiner). For potency determinations, ten-point one in three dilution curves were generated using a Perkin Elmer Janus liquid handling robot. The top concentration was 50 µM and on each plate a control curve of Amphotericin B was included. LdBOB axenic amastigotes were added to all wells containing compounds (250 cells per well, 50µl) using a Thermo Wellmate dispenser. Media only was dispensed into control columns. After a 68 h incubation at 37 °C under 5% CO_2 in a humidified incubator, resazurin was added to each well at a final concentration of 0.5 mM and the plates were incubated for a further 4 h. Plates were then sealed with clear film and resorufin fluorescence was detected using a Perkin Elmer Victor 3 plate reader with excitation at 528 nm and emission at 590 nm.

Intracellular *L. donovani* assay

For intramacrophage screening, compounds were pre-dispensed into 384 well sterile intermediary plates (Greiner) using a Matrix Platemate Plus with 384 well pipetting head (1 µl per well). Potency plates were prepared as for the axenic assay. The following controls were included on all plates: no effect control: DMSO, maximum effect control: amphotericin B (final concentration 2 µM). To the intermediary plates, 100 µl of THP-1 media was added and plates were shaken for >5 min to ensure complete mixing. THP-1 cells (8,000 per well, 50 µl) were plated into black clear-bottom 384 well plates (Corning) in presence of 10 nM PMA. After 20 min at room temperature, the plates were incubated at 37 °C under 5% CO_2 in a humidified

incubator for 75 h. The cells were then washed with 450 µl sterile phosphate buffered saline (PBS) supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin (PBS-A) using an EL406 multi well platewasher (BioTek) and amastigotes (eGFP expressing LdBOB axenic amastigotes) were added to all wells at a multiplicity of infection of 5 (40,000 amastigotes per well). After 40 min at room temperature, plates were returned to the incubator. Amastigotes were incubated in the presence of macrophages for 16 h. Any remaining extracellular amastigotes were subsequently removed with an overflow wash of 1 ml PBS-A per well (wash buffer is being aspirated from the top of the well as it is being dispensed) followed by addition of 25 µl of the compound pre-dilutions using a Matrix Hydra DT pipetting station. The final dilution of each compound was 200-fold. Plates were incubated for 72 h and then washed (250 µl PBS-A) and fixed (4 % (v/v) formaldehyde-PBS, 30 min, room temperature). After fixation, the wells were washed with 250 µl PBS, stained (10 µg ml^{-1} DAPI, 0.4 µg ml^{-1} HCS Cellmask Deep Red in PBS + 0.1% (v/v) Triton X-100, 30 min, room temperature) and washed with 250 µl PBS. Finally, PBS + 0.05% (v/v) thimerosal was added to the wells, the plates were sealed and imaged on a high-content microscope (GE IN Cell 1000 or GE IN Cell 2000) using a 10x objective. Image analysis was carried out with GE IN Cell Analyzer 1000 Workstation using the “Multi Target Analysis” module. Settings for segmentation were as follows: nuclei: minimum area: 142.384 μm^2, sensitivity: 81, method: top-hat; cells: characteristic area: 2500 μm^2, sensitivity: 60, method: multiscale top-hat [1]; organelles (amastigotes): granule size 1 – 3, 3 scales, sensitivity: 90, detection in entire cell. For each well, THP-1 cell count and average number of amastigotes per cell were reported.

MRC-5 assay

MRC-5 cells were harvested by treatment with 0.05% (w/v) trypsin plus 0.48 mM EDTA for 5 min and diluted to $2.5x10^4$ ml^{-1}. Aliquots (40 µl) of the cell suspension were plated in columns 1 – 22 of white, clear-bottom 384 well plates (Greiner) and plates were allowed to rest at room temperature for >1h before moving to 37 °C, 5% CO_2 in a humidified incubator. Media only was dispensed into control columns. After 24 h, compounds were added to the cell plates directly using a Labcyte ECHO 550 acoustic dispenser (250 nl Potency plates were prepared as for the axenic assay. On each plate a control curve of doxorubicin was included. After compound addition, the plates were incubated for 68 h. The resazurin assay was carried out as for the axenic amastigote *Leishmania* assay.

Replication Assessment

For determination of axenic amastigote growth, cells were plated as described above with one plate for each time point (0, 26, 31, 49, 55, 73 h). At each time point, cells from a total of 4 or 6 wells were harvested, fixed and counted in a CASY cell counter (Roche).

For determination of intracellular amastigote replication the assay was set-up as described above, but instead of fixing one plate at 72 h, a plate was fixed at each of the following time-points: 24, 48, 72, 96 and 168 h. After the last time point the plates were stained, imaged and analysed.

Supplementary Data 1. Codon optimised eGFP sequence

ATGGTCAGCAAGGGCGAGGAGCTGTTCACGGGCGTGGTGCCGATCCTGGTGGAG

CTGGACGGCGACGTGAACGGCCACAAGTTCTCGGTGTCGGGCGAGGGCGAGGGC

GACGCCACGTACGGCAAGCTGACGCTGAAGTTCATCTGCACGACGGGCAAGCTG

CCGGTGCCGTGGCCGACGCTGGTGACGACGCTGACGTACGGCGTGCAGTGCTTA

GCCGCTACCCGGACCACATGAAGCAGCACGACTTCTTCAAGAGCGCGATGCCGG

AGGGCTACGTGCAGGAGCGCACGATCTTCTTCAAGGACGACGGCAACTACAAGA

CGCGCGCGGAGGTGAAGTTCGAGGGCGACACGCTGGTGAACCGCATCGAGCTGA

AGGGCATCGACTTCAAGGAAGACGGCAACATCCTGGGCCACAAGCTGGAGTACA

ACTACAACAGCCACAACGTGTACATCATGGCGGACAAGCAGAAGAACGGCATCA

AGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTGGCCG

ACCACTACCAGCAGAACACGCCGATCGGCGACGGCCCGGTGCTGCTGCCGGACA

ACCACTACCTGAGCACGCAGTCGGCCCTGAGCAAGGACCCGAACGAGAAGCGCG

ACCACATGGTGCTGCTGGAGTTCGTGACGGCGGCGGGCATCACGCTGGGCATGG

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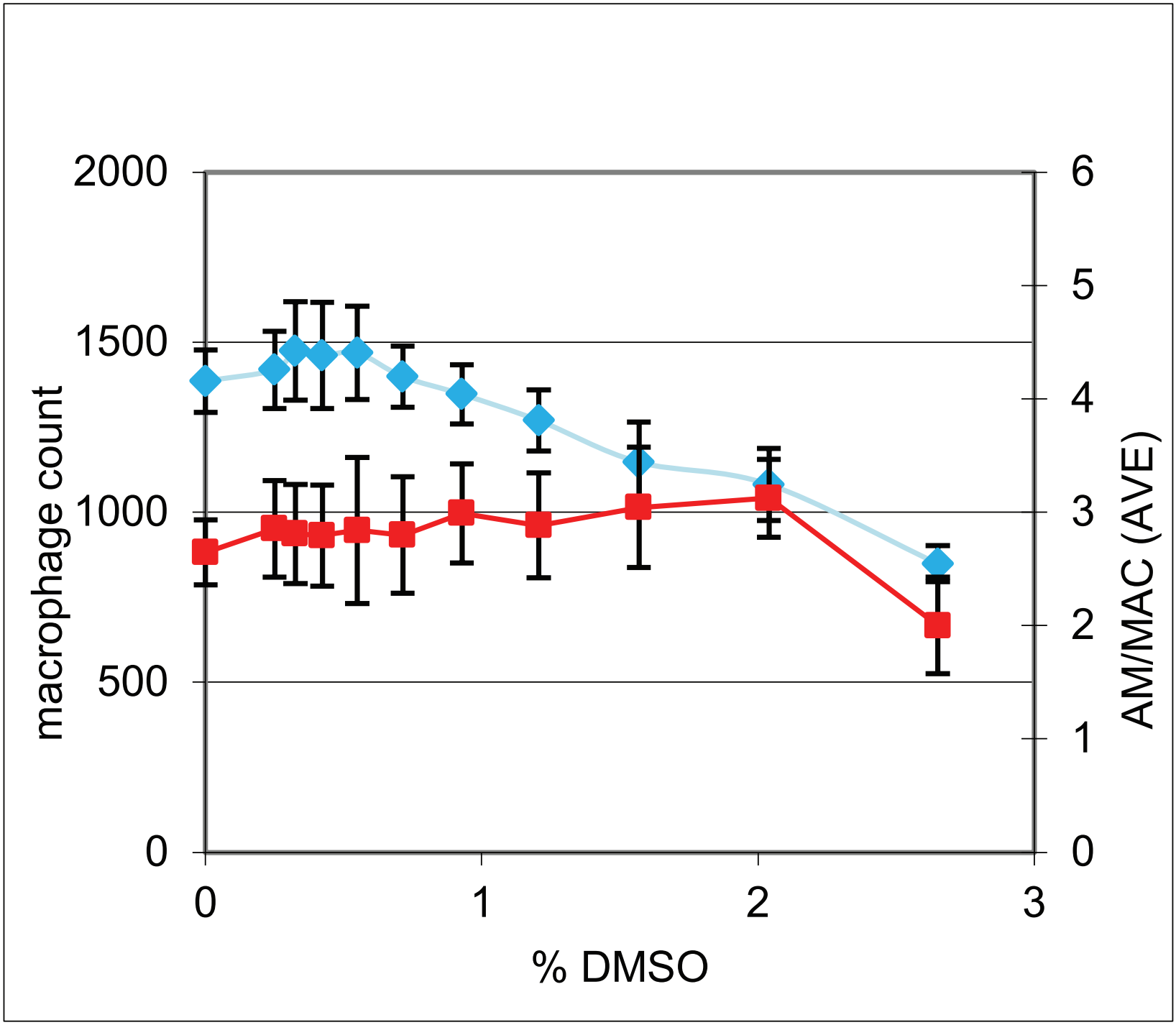
References

1. Serra JP (1982) Image analysis and mathematical morphology. London ; New York: Academic Press.



Supplementary Figure 1: DMSO tolerance

The indicated DMSO concentrations were tested in the intracellular *Leishmania* assay. The blue points indicate the number of macrophages counted and the red squares show the average number of amastigotes per macrophage. Averages and standard deviations of eight replicates are given.