

1 **A replicative *in vitro* assay for drug discovery against *Leishmania donovani***

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10 Running Head: Replicative *in vitro* assay against *Leishmania donovani*

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14 **ABSTRACT**

15 The protozoan parasite *Leishmania donovani* is the causative agent of visceral
16 leishmaniasis, a disease potentially fatal if not treated. Current available treatments have
17 major limitations and new and safer drugs are urgently needed. In recent years,
18 advances in high throughput screening technologies have enabled the screening of
19 millions of compounds to identify new antileishmanial agents. However, most of the
20 compounds identified *in vitro* did not translate their activity when tested in *in vivo*
21 models, highlighting the need to develop more predictive *in vitro* assays. In the present
22 work, we describe the development of a robust replicative, high content, *in vitro*
23 intracellular *L. donovani* assay. Horse serum was included in the assay media to replace
24 standard foetal bovine serum to completely eliminate the extracellular parasites derived

25 from the infection process. A novel phenotypic *in vitro* infection model has been
26 developed complemented with the identification of the proliferation of intracellular
27 amastigotes measured by EdU incorporation.

28 *In vitro* and *in vivo* results for miltefosine, amphotericin B and the selected compound 1
29 have been included to validate the assay.

30

31 **Introduction**

32 The leishmaniasis are a complex of diseases, with visceral and cutaneous
33 manifestations caused by protozoan parasites of the genus *Leishmania*. Visceral
34 leishmaniasis (VL) has been the main focus for drug R&D over the past two decades,
35 due to the large disease burden in East Africa and South Asia (1) and potential patient
36 death if not treated. For VL, there has been progress in treatment over the past decade
37 with clinical evidence for efficacy of, and registration for use of oral miltefosine,
38 paromomycin and the liposomal formulation of amphotericin B (AmBisome™) in
39 South Asia (2), as well as combinations of these standard drugs (3). The need for new
40 drugs to treat VL remains, as (i) miltefosine is the only approved oral treatment but
41 requires 28 days of treatment and potential teratogenicity limits its use (4), (ii)
42 paromomycin requires 21 days of treatment and intramuscular administration
43 ([http://www.dndi.org/diseases-projects/diseases/vl/current-treatment/current-treatment-](http://www.dndi.org/diseases-projects/diseases/vl/current-treatment/current-treatment-vl.html)
44 [vl.html](http://www.dndi.org/diseases-projects/diseases/vl/current-treatment/current-treatment-vl.html)) and (iii) liposomal amphotericin B formulations, which have successful cure
45 rates with a single dose (5), require intravenous infusion, have a high cost if not donated
46 and the requirement for cold storage, limiting use in countries where the disease is
47 endemic (6). As part of the drive to find new treatments there has been a re-focus on the
48 assays and models used to identify and develop new molecules as antileishmanial drugs.
49 For *in vitro* screens and assays, this has ranged from the need to develop methods, that:

- 50 (i) Are adaptable to and enable high throughput screens against the replicative
51 intracellular – macrophage amastigote stage of *Leishmania donovani*, one of
52 the causative species of VL (7).
- 53 (ii) Include high throughput technologies that enable the collection of more
54 information compared to the traditionally used assays based on manual
55 counting and reporter genes (8, 9). For example, High Content Screening
56 (HCS) systems that permit the screening of large sets of compounds using
57 imaging techniques that also capture information about compounds' toxicity
58 against host cells and mode of action (10, 11) have been applied to
59 antileishmanial drug discovery (12-17).

60 In this paper, we describe methods to overcome some of critical issues related to
61 reproducibility and biological relevance and to the replication of the intracellular
62 parasite.

63 The role of replication rate of intracellular amastigotes on interpretation of data from
64 assays is often ignored. In vivo we know that in the *L. donovani* mouse model the
65 parasite load in the liver increased 20-fold over the initial eight days (18) and in the *L.*
66 *donovani* hamster model the parasite burden increased more than 6 logs in the spleen
67 and 4 logs in the liver over the 56 days of the study (19). Recent experiments reported a
68 doubling time of two days in an *ex vivo* splenic explant model system established 21
69 days post infection developed by the same group (20). We determined the replication
70 rate of intracellular amastigotes in our assay using an adaptation of a classical
71 nucleotide analogue incorporation assay (21) to enable visual identification of cells
72 actively replicating within macrophage vacuoles.

73

74 **MATERIALS AND METHODS**

75

76 **Cell Lines**

77 THP-1 cells (human monocytic leukemia) were made available by GSK-Biological
78 Reagents and Assay Development Department (BRAD, Stevenage, UK) and were
79 maintained in RPMI media (Life-Technologies) supplemented with 1.25 mM Pyruvate
80 (Life-Technologies), 2.5 mM Glutamine (Life-Technologies), 25 mM HEPES (Life-
81 Technologies) and 10% heat inactivated FBS (Gibco).

82 *Leishmania donovani* (MHOM/SD/62/1SCL2D, LdBOB) expressing green fluorescence
83 protein (GFP) (14) was kindly provided by Manu de Rycker, University of Dundee,
84 UK. Axenic amastigotes were maintained at 37°C, 5% CO₂ in media containing 15 mM
85 KCl solution (Invitrogen), 10 mM KH₂PO₄ (Merck), 136 mM KH₂PO₄ (Merck), 0.5
86 mM MgSO₄ (Sigma-Aldrich), 24 mM NaHCO₃ (Invitrogen), 25 mM Glucose (Sigma-
87 Aldrich), 1mM L-Glutamine (Invitrogen), 1xRPMI Vitamin Solution (Sigma-Aldrich),
88 10 μM Folic Acid (Sigma-Aldrich), 100 μM Adenosine (Sigma-Aldrich), 5mg/L Hemin
89 (Sigma-Aldrich), 1xRPMI Amino Acid solution (Sigma-Aldrich), 25 mM MES,
90 0.0004% Phenol Red and 20% Heat Inactivated FBS (Gibco) in Milli-Q water. The
91 selection antibody Nourseothricin (Jena Bioscience) was regularly added to the cultures
92 of amastigotes. Promastigotes were maintained at 30°C in M199 Media (Sigma Aldrich)
93 supplemented with 25mM HEPES (Invitrogen), 12mM NaHCO₃ (Invitrogen), 1mM L-
94 Glutamine (Invitrogen), 1xRPMI Vitamin Solution (Sigma-Aldrich), 10μM Folic Acid
95 (Sigma-Aldrich), 100μM Adenosine (Sigma-Aldrich), 5mg/L Hemin and 10% Heat
96 Inactivated FBS (Gibco) (14).

97

98 ***In vitro* intra-macrophage *L. donovani* assay**

99 The intra-macrophage assay was adapted from de Rycker *et al.* (14) and Peña *et al.* (16).
100 THP-1 cells were grown in CELLMASTER roller bottles (Greiner cat. # 680048) at an
101 initial seeding concentration of 2×10^5 cells/mL for 72 h. Cells were visually inspected
102 with an optical microscope and counted with a CASY Counter (model TT, Roche).
103 Cells were differentiated in a 225 cm^3 T-FLASK (80 ml) in the presence of 30 nM of
104 phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) at a final concentration of 6×10^5
105 cells/mL. Following 24h incubation at 37°C , 5% CO_2 , differentiation was visually
106 confirmed checking the confluence of the differentiated adherent monolayer and PMA-
107 containing media was removed washing twice with complete growth media, taking care
108 of not disrupting the cell layer.

109 Each T-flask containing differentiated THP-1 cells was infected with 80 mL of a
110 suspension of 6×10^6 parasites/mL in THP-1 complete growth media without PMA and
111 incubated additional 24h. The media was removed and the cell monolayer washed with
112 PBS. The infected cells were harvested by treatment with a solution of 0.25% (w/v)
113 trypsin/EDTA in PBS and seeded in assay plates (1.6×10^5 cells/mL, 50 μl /well) in assay
114 media, containing RPMI media supplemented with 2% Heat Inactivated Horse Serum
115 (Gibco) or Foetal Bovine Serum (Gibco), 25 mM NaHCO_3 (Invitrogen™) and 30 nM
116 PMA using a Multidrop Combi dispenser (Thermo Scientific). A parallel culture of
117 uninfected differentiated THP-1 cells was treated as described for infected cells and
118 used as control for 100% compound response. Assay plates were incubated at 37°C , 5%
119 CO_2 for the time required for the assay and then fixed with 4% formaldehyde for 30 min
120 at room temperature adding 50 μl of 8% (v/v) formaldehyde solution (Sigma-Aldrich)
121 in PBS to each well containing 50 μl of media. After fixation, cells were washed twice
122 with 100 μL PBS using an EL406 multi well platewasher (BioTek), stained with 30 μl
123 of a solution of DAPI (10 μg /mL) and 0.1% (v/v) Triton X-100 in PBS for 30 min at

124 room temperature and washed additional two times with 50 μ L PBS. Finally, 50 μ L of
125 PBS were added to each well, plates were sealed and stored at 4°C until analysis.

126

127 **Image analysis**

128 Automated image analysis was performed with an image analysis algorithm developed
129 on Acapella[®] High Content Imaging and Analysis Software (PerkinElmer). THP-1 cells
130 count (MAC) and average number of amastigotes per macrophage (AM/MAC) were
131 calculated for each well, using the building blocks included in the analysis program.
132 Briefly, the nuclei and cytoplasm for each macrophage were selected using DAPI stain.
133 Amastigotes were detected as spots using the GFP signal and were filtered using area
134 and roundness. In EdU incorporation experiments (*ibid.*), the number of parasites'
135 nuclei that were labeled was used to determine the incorporation of the thymidine
136 analogue in the nuclei. Images were taken with a High-Content Screening System
137 (Opera QEHS, Perkin Elmer) with a 20x air objective, acquiring a minimum of four
138 fields per well. Two or three sequential images were taken for each well exciting at 405
139 nm (DAPI), 488 nm (GFP) and 635 nm (EdU).

140

141 **Compounds and assay plates**

142 Amphotericin B and miltefosine were purchased from Sigma Aldrich. Compound 1 was
143 available from the GSK collection of compounds (Table 1).
144 Pre-dispensed assay plates (Greiner μ clear black, 384-well) were prepared by adding
145 250 nL of compounds dissolved in 100% DMSO or 250 nL of DMSO to each well by
146 using an Echo[®] liquid handler (Labcyte Inc.). Eleven-point one in three dilution curves
147 were generated from a top concentration of 50 μ M.

148 Plates were stored at -20°C until use and allowed to equilibrate at room temperature
149 before addition of the cell suspension.

150

151 **Data analysis**

152 Data were normalized to percentage biological response by using positive (i.e. highest
153 response represented by non-infected cells, R_{Ctrl2}), or negative (i.e. lowest response
154 achieved in the absence of any testing compound, R_{Ctrl1}) controls by using the following
155 equation (Equation 1):

$$\%Response = \frac{|R_{Ctrl1} - R_x|}{|R_{Ctrl1} - R_{Ctrl2}|} \cdot 100$$

156 where R_x is the assay response measured for each compound X. R_{Ctrl1} and R_{Ctrl2} were
157 included in each assay plate and calculated as the average of the replicates.

158 Assay performance statistics, such as signal to background ratio and Z' (22) were
159 calculated using templates in ActivityBase XE (IDBS, Guilford, Surrey, UK). Activities
160 were expressed as pEC_{50} ($pEC_{50} = -\text{Log } EC_{50} \text{ (M)}$). Values of pEC_{50} were obtained
161 using the ActivityBase XE nonlinear regression function in the full curve analysis
162 bundle to fit the 4-parameter logistic equation.

163

164 **Biosafety and animal use**

165 Experimental procedures with *L. donovani* were carried out following standard
166 operating procedures in compliance with biosafety level 3 regulations (BSL3). THP-1
167 cells were treated according to GSK policies for the manipulation of human biological
168 samples.

169 The protocols used for animal studies were approved by the Diseases of the Developing
170 World (DDW-GSK) ethical committee. The animal research complied with Spanish and

171 European Union legislation (European directive 86/609/EEC) on animal research and
172 GlaxoSmithKline 3R policy on the care and use of animals: Replacement, Reduction
173 and Refinement.

174 Additional *in vivo* experiments were carried out at the London School of Hygiene &
175 Tropical Medicine. These were performed under licence, issued by the UK Home Office
176 Animal (Scientific Procedures) act 1986 and EU Directive 2010/63/EU.

177

178 **EdU Incorporation**

179 THP-1 cells were differentiated, infected and seeded in 384-wells plates as previously
180 described and incubated in horse serum-containing assay media. For the optimization of
181 EdU (Click-iT® Plus Alexa Fluor® 647 Picolyl Azide Toolkit, Lifetech) conditions (13,
182 23), concentrations ranging from 1 to 100 μ M were added to different wells at time 0
183 and every 12 hours for 72 hours, when cells were fixed with 4% formaldehyde for 30
184 minutes.

185 For intracellular amastigotes replication experiments, EdU was added 24 hours after
186 plating to a final concentration of 50 μ M in 1% DMSO. Plates were fixed every 12
187 hours from 0 to 72 hours post EdU addition with 4% formaldehyde for 30 min. EdU
188 detection was performed following manufacturer's indications and cells were stained
189 with DAPI as previously described. Controls of GFP signal quenching and EdU positive
190 spots detection in the absence of EdU in infected and uninfected cells were included in
191 each experiment.

192

193 ***In vivo* activity against *L. donovani***

194 Sodium stibogluconate sensitive (SSG) *L. donovani* (MHOM/ET/67/HU3) amastigotes
195 were isolated from donor RAG1.B6 mouse. Freshly isolated parasites were re-
196 suspended in RPMI1649 media at a concentration of 1×10^8 /ml.

197 On day 0 female BALB/c mice (20 g; Charles River, Margate, UK) were infected
198 intravenously by the lateral tail vein with 2×10^7 amastigotes (0.2 ml inoculum) and
199 randomly assorted into four groups of five members.

200 Drug treatment started 7 days post infection and continued until day 11. Groups were
201 treated with either (i) vehicle only, orally, twice daily for 5 days, (ii) miltefosine
202 (Paladin Inc., Canada), 12 mg/Kg, orally, once daily for 5 days, (iii) with AmBisome
203 (Gilead, USA) at 1mg/Kg intravenously for 3 days (day 7, 9 and 11 post infection), and
204 (iv) compound 1 at 50 mg/Kg, orally, twice for 5 days.

205 At day 14 post infection, all animals were sacrificed and the parasite burden was
206 determined microscopically on Giemsa stained liver smears after methanol fixation. The
207 number of amastigotes per 500 cells was counted microscopically (X100, oil
208 immersion) and the parasite load normalized to untreated controls.

209

210 **Pharmacokinetic studies**

211 Experimental compounds were administered to BALB/c female mice (25 g weight) by
212 oral gavage at 50 mg/kg dose at a volume of 20 ml/kg. All mice were treated during the
213 fed state. Drugs were administered as 10% 70:30 Tween80: EtOH/ddH₂O suspensions
214 and the blood sampling scheme was: 15, 30 and 45 minutes, 1, 1.5, 2, 3, 4, 8 and 24
215 hours. At each time-point, 10 μ L of blood were taken from the lateral tail vein from
216 three animals. LC-MS was used for the establishment of compound concentration in
217 blood with a sensitivity of LLQ = 1–5 ng/ml in 25 ml blood. The concentration of each
218 drug was calculated in the peripheral total blood compartment. The non-compartmental

219 data analysis was performed with WinNonlin 5.0 (Pharsight) and supplementary
220 analysis was performed with GraphPad Prism (GraphPad Software).

221

222 **RESULTS**

223 **Assay development**

224 In this intra-macrophage system the infection process was performed “in bulk” prior to
225 the dispensation of the cell suspension in the assay plates, to eliminate any possible intra
226 well variation and to increase the robustness of the assay.

227 Copies of identical plates were prepared to allow fixing and staining at different time
228 points and plotting of the growth curve. Cells were fixed with formaldehyde prior to
229 DAPI staining. DAPI was used to detect the nucleus of THP-1 cells and GFP to detect
230 intracellular amastigotes using the image analysis algorithm described in materials and
231 methods. When performed for large scale screening of compounds, the assay had an
232 average throughput of 40 plates/run (two runs/week-240,000 wells/week) and the
233 average Z' calculated at 96h using the AM/MAC output was 0.59 ± 0.12 .

234

235 **Effect of horse serum on extracellular amastigotes**

236 The presence of extracellular parasites was determined by visual inspection of the plates
237 at each time point. The assay media used reduced serum level, 2% serum instead of
238 10% normally in the complete growth media for culture of THP-1 cells, to minimize the
239 growth of extracellular parasites. Neither the presence of HS nor the reduced quantity of
240 FBS significantly affected the THP-1 counts (Fig.1A). When cells were incubated with
241 FBS, an increase in the extracellular parasites load could still be seen over the four days
242 of incubation. In contrast, the few extracellular parasites present after seeding in the
243 presence of HS-containing media, were killed within few hours of incubation. This

244 difference could not be recorded when cells were stained with DAPI since extracellular
245 parasites were removed with the washing steps required to remove the dye after
246 staining. This was overcome by the use of Draq5, a nuclear dye that can be added with
247 formaldehyde in a single step and does not need to be washed out. Figure 1B illustrates
248 the difference in the content of extracellular parasites when infected THP-1 cells were
249 incubated for four days in the presence of FBS or HS (Draq 5 staining).

250

251 **Effect of HS on *L. donovani* intracellular amastigotes**

252 The number of amastigotes per host cell (AM/MAC) at each time point (24h, 48h, 72h,
253 96h) was plotted to determine the growth of the intracellular amastigotes, both in the
254 presence of FBS and HS (Fig.1C). Differentiated THP-1 cells do not replicate, therefore
255 the increase in the total number of amastigotes was not influenced by the increase of the
256 number of host cells (24, 25).

257 In the presence of FBS 2%, the number of AM/MAC increased on average from 1.5 to
258 4.7 over 96h. When HS was used in the assay media at the same concentration, the
259 AM/MAC increased from 1.6 to 3.6 over the 96 h of incubation, with a linear increment
260 in the initial 72 hours post plating. When FBS was used, the presence of extracellular
261 parasites and the potential of host cell reinfection prevented the replication rate to be
262 accurately evaluated. At the same time, the use of HS ensured the elimination of any
263 extracellular parasites after few hours of incubation, removing possible influence of re-
264 infection in the observed increase and allowed any observed growth to be attributed to
265 intracellular replication. Figures 1D and 1E show infected THP-1 cells fixed and stained
266 with DAPI 24h and 96h post plating. These experiments were carried out in the
267 presence of 0.5% DMSO, that is the concentration found in each well when compounds
268 are screened. This concentration did not significantly affect either the number of host

269 cells or the replication of intracellular amastigotes when compared to a parallel
270 experiment without DMSO (data not shown).

271 It was also observed that the shape of the intracellular parasite was influenced by the
272 serum used. In presence of FBS the intracellular amastigotes were elongated (having
273 similarity with extracellular amastigotes) while they were more round and amastigote-
274 like when incubated in the presence of HS (Fig.1F), an observation previously made
275 (26).

276

277 **Edu incorporation**

278 The optimal EdU concentration and exposure time were initially determined. THP-1
279 cultures infected with *L. donovani* amastigotes were incubated with increasing amounts
280 of EdU for different periods of time in a single experiment that was processed at once to
281 detect the EdU incorporated into amastigotes' DNA. Analysed images showed that
282 amastigotes were able to significantly incorporate EdU with an increasing and sustained
283 rate when exposed to 50 μ M EdU for at least 12 hours (Fig. 2A); the incorporation rate
284 achieved a plateau after 72 hours of exposition without parasite number reduction and
285 thus without apparent toxic effects. Uninfected cultures and cultures with no exposure
286 to EdU were included as technical detection controls.

287 After the optimization of the experimental conditions, the incorporation of EdU over
288 time by infected THP-1 cells maintained in HS-containing media was determined
289 adding 50 μ M of EdU 24 hours after plating and measuring EdU incorporation in 12-
290 hour lapses from 24 to 72 hours post plating. The number of amastigotes per
291 macrophage was determined in both the GFP and in the EdU channel. Not all
292 amastigotes incorporated EdU during the course of infection but the incorporation rate
293 was consistent with the increase of intracellular parasite burden, reaching 40% parasites

294 labelled as proliferating and demonstrating that the increase in the number of
295 amastigotes per macrophage is to be attributed to replication (Fig. 2B).

296

297 ***In vitro* activity**

298 The activity of amphotericin B and miltefosine in FBS-containing media was in
299 accordance with previously reported data (12), showing a pEC₅₀ (pEC₅₀= -Log (EC₅₀))
300 equal to 7.17 and 6.56 respectively in the amastigotes/cell output. Both compounds
301 maintained their activity when tested in the presence of HS (Fig.3).

302 Compound 1 (Table 1) was assayed as part of the high throughput screening campaign
303 against the kinetoplastids *L. donovani*, *T. cruzi* and *T. brucei* (16). This compound,
304 when tested in the FBS containing media, exhibited a pEC₅₀ of 7.8 in the intra-
305 macrophage assay as measured by the number of amastigotes/cell. Measuring the
306 percentage of infected macrophages, the compound showed no significant activity, with
307 a maximum asymptote of 40%. When the compound was assayed in the presence of HS
308 it was found to be inactive by both parameters (Fig.4).

309

310 **Pharmacokinetic studies**

311 Compound 1 was administered to the mice by oral gavage in a single dose for 5 days
312 and no signs of pain, distress or local or systemic toxicity was observed. Values for
313 AUC (Area Under the Curve) and plasma compound concentrations at peak and trough
314 are given in Table 2. The values for AUC were high enough to ensure activity related to
315 the calculated EC₅₀ value. The exposure data were sufficiently favorable to warrant
316 further *in vivo* testing.

317

318 ***In vivo* antileishmanial activity**

319 Amphotericin B (AmBisome), miltefosine and compound 1 were tested on *L. donovani*
320 infected BALB/c mice. AmBisome and miltefosine were active *in vitro* both in the
321 presence of FBS or HS. *In vivo* they decreased the parasite burden of the 99.52% and
322 77.23% at 1 mg/kg i.v. and 12 mg/kg p.o. respectively, in accordance with previously
323 reported data (27). In contrast, compound 1 at two daily doses of 50mg/kg only reduced
324 the parasite burden of the 20% after 5 days of treatment (Table 3).

325

326 **DISCUSSION**

327 Drug discovery for antileishmanial compounds has recently been focused on phenotypic
328 rather than target based screens, due to the limited number of fully validated targets and
329 issues of confirming on-target effects of active compounds (28, 29). However, the *in*
330 *vitro* activity of test compounds frequently does not translate to *in vivo* activity,
331 underlining the need for the development of new and more predictive *in vitro* assays
332 adaptable to a high throughput screening.

333 It has been demonstrated that the activity of antileishmanial drugs is host cell dependent
334 (30). Primary host cells mimic the biological situation more accurately but are not
335 compatible with the needs of a high throughput screen. Instead, immortalized human
336 monocytic THP-1 cells, that can be differentiated into macrophage-like cells, are able to
337 develop and sustain *L. donovani* infections (24, 31). Different high content screening
338 assays using PMA-differentiated THP-1 cells infected with either promastigotes or
339 amastigotes have been developed, confirming their suitability as *L. donovani* hosts (12-
340 14, 16).

341

342 In comparison to traditional assays that provide information mainly on parasite
343 viability, the use of HCS technologies permits the assessment of potential toxicity
344 against the host cells and to observe morphological changes that can provide useful
345 information to understand the mode of action of the compounds of interest (10). In our
346 assay THP-1 cells were differentiated and infected “in bulk” and dispensed into assay
347 plates containing the compounds to be tested as previously described (16). The use of
348 cells that have been differentiated and infected “in bulk” assured a homogenous
349 distribution of the infection throughout the plates, strongly reducing inter well
350 variability, and eliminated the need of using intermediate plates loaded with test
351 compounds.

352 One limitation of this protocol is that it does not allow any wash steps after the
353 dispensation of cells in the plate and that would remove extracellular parasites derived
354 from the infection process or from the rupture of host cells during trypsinization or
355 dispensing. This can be problematic as axenic amastigotes are adapted to grow in
356 culture with an average doubling time of 6 hours and thus, after infection, any parasite
357 that is not phagocytized by a host cell can grow over the incubation period and re-infect
358 neighboring hosts. In addition, the pH of the assay media is higher than the pH of the
359 culturing media and could contribute to the differentiation of the amastigotes to an
360 intermediate form of the parasite similar to promastigotes. The primary objective in
361 antileishmanial drug discovery is to identify compounds able to interfere with the
362 growth and survival of the intracellular parasites rather than acting on the extracellular
363 parasites. As the presence of HS in the media was found to kill extracellular parasites
364 within a few hours of incubation, HS was included in the assay media in order to
365 prevent the growth and establishment of an extracellular culture, without affecting the
366 viability of the hosts or of the intracellular amastigotes. The use of HS-containing media

367 allowed to reduce the number of washing steps following infection and ensured the
368 elimination of any extracellular parasites deriving from a mechanical rupture of the host
369 cell within a few hours following initial infection. We have also observed that, in the
370 presence of HS, the intracellular parasites assumed a round shape, characteristic of the
371 amastigote stage, whereas they were more elongated when incubated with FBS. The
372 ability of HS to kill extracellular parasites and to push the differentiation of intracellular
373 amastigotes towards a more amastigote-like form are in accordance with what
374 previously reported by Frothingham and Lehtimaki (26).

375 The antiparasitic effect of serum components has already been described. The
376 trypanolytic factor present in human serum is responsible for the inability of
377 *Trypanosoma brucei brucei* to infect humans (32). In the case of *Leishmania* and horse
378 serum there is no evidence of a similar mechanism. However, it is known that horse
379 serum is less rich in nutrients and growth factors compared to foetal bovine serum and
380 this could contribute to the observed effect.

381 When FBS-containing media was used in this assay, the presence of extracellular
382 parasites, and hence the simultaneous contribution of replication and re-infection to the
383 observed increase of the number of amastigotes/macrophages over time, did not permit
384 us to conclusively establish the replication rate. A previous report on the doubling time
385 of intracellular amastigotes in the presence of FBS and in absence of extracellular
386 parasites, extrapolated a replication rate of approximately 12 days from the 7 days
387 growth curve (14). In the assay developed in this work, when HS was included in the
388 media, the number of amastigotes/macrophages doubled from 24h to 72h and, since no
389 extracellular parasites were visible, it was possible to attribute this proliferation solely
390 to the replication of the intracellular parasite, as demonstrated with the EdU
391 incorporation assay. The replication observed in this horse serum intra-macrophage

392 assay was lower than the one observed in the *in vivo* mouse model (18) or hamster
393 model (19), but was similar to that observed in the *ex vivo* splenic explant culture from
394 hamster infected with *L. donovani* described by Osorio *et al.* (20), where the number of
395 amastigotes/macrophage doubled in the first 48 hours post plating. The two-day
396 doubling time we observed in the *in vitro* system described in the present work, is also
397 in accordance with the doubling time observed by other groups when THP-1 cells were
398 infected with *L. donovani* promastigotes (12, 31). Even if results obtained in different
399 assays using different strains are difficult to compare, the fact that we observed and
400 were able to quantify the replication of intracellular parasites in the horse serum *in vitro*
401 system, is of importance for the development of more predictive *in vitro* assays (7)..

402 De Muylder *et al.* described the use of a media containing 5% HS and 5% FBS to wash
403 differentiated THP-1 cells after infection with *L. donovani* promastigotes (12). The
404 choice of use of HS in the washing media was not discussed in this report, but,
405 considering that differentiation and infection were performed in wells, it appears that
406 HS was chosen to assist in the elimination of the extracellular parasites after infection,
407 prior to compounds' addition. In the same report, it appears that HS was not included in
408 the assay media and the effects of horse serum on the replication and appearance of
409 intracellular amastigotes were not characterised.

410 DNA synthesis rate is highly up-regulated during the replication process representing a
411 good biomarker for proliferation. The incorporation of thymidine analogues during the
412 active S-phase in dividing cells has been widely used as a molecular biomarker for
413 proliferation (21). BrdU has been previously used to qualitatively identify the
414 intracellular amastigotes as a replicating population, following THP-1 infection with *L.*
415 *donovani* promastigotes (13). In the present work, to confirm that the increase in the
416 number of amastigotes/macrophages observed when HS was included in the assay

417 media was attributable to replication, the EdU pycolil-azide combined methodology was
418 used, allowing the identification of those amastigotes that have entered into S-phase
419 while infecting macrophages, without compromising GFP fluorescence and amastigote
420 identification. The increase of EdU incorporation over time specifically identifies
421 proliferation events that take place within the macrophages, since the addition of EdU
422 after 24 hours of incubation with horse serum ensured that only intracellular parasites
423 would have been exposed to the thymidine analogue. The EdU incorporation rate was
424 similar to the estimated replication rate based on direct counting. The detection of non-
425 labelled parasites after long incubation periods suggests there might be a non-dividing
426 subpopulation of amastigotes, in accordance with observations by Kloehn *et al.* in
427 murine *L. mexicana* lesions (33).

428 To validate this *in vitro* assay, two reference drugs, amphotericin B and miltefosine and
429 the GSK compound 1 were tested in the intra-macrophage assay in the presence of FBS
430 or HS and using an *in vivo* animal model, allowing for a comparison of the *in vitro* and
431 *in vivo* activities. The *in vitro* activities of amphotericin B and miltefosine were in
432 accordance with previous reports and no significant difference between their activity in
433 the presence of FBS or HS was observed. Compound 1 was selected as a proof of
434 concept study, as it showed a pEC₅₀ value higher than amphotericin B in the presence of
435 FBS (pEC₅₀=7.8) but was inactive when tested in the presence of HS (pEC₅₀<4.3).
436 When tested *in vivo*, amphotericin B and miltefosine confirmed their activity, reducing
437 the parasite burden by 99.52% and 77.23% respectively. In contrast, compound 1 was
438 inactive when administered orally, reducing the parasite burden by 20.93% only. Since
439 compound 1 possesses lead-like physicochemical properties (34) (Table 1) and
440 reasonable bioavailability in mice in terms of C_{max} and AUC (Table 2), we propose
441 that factors other than pharmacokinetics might contribute to the lack of efficacy in the

442 infection model, such as poor pharmacodynamics at the site of action. In particular, we
443 suggest it could be linked to its lack of activity in the *in vitro* horse serum intra-
444 macrophage assay, in contrast with the high pEC₅₀ value obtained when a media
445 containing FBS was used (AM/MAC output). Several reasons could explain the lack of
446 activity of compound 1 in HS: compound structure related properties, the lack of
447 activity against intracellular replicating amastigotes in horse serum, or the compound
448 could be active only against the extracellular amastigotes forms found in presence of
449 FBS. Even though the exact mode of action of compound 1 has not been clarified, the
450 correlation between the results obtained in the *in vitro* horse serum intra-macrophage
451 assay and the *in vivo* mouse model seem to suggest that the *in vitro* results obtained
452 with horse serum translate to the *in vivo* animal model and that this assay mimics an *in*
453 *vivo* *L. donovani* infection more accurately than the same assay with FBS. In fact, the
454 standard drugs miltefosine and amphotericin B were active in *in vitro* and *in vivo* assays
455 and compound 1 was inactive both *in vitro* when horse serum was used, irrespectively
456 of the output used for the determination of its pEC₅₀, and *in vivo*.

457 This is, to our knowledge, the first report on the inclusion of horse serum in the assay
458 media for the whole assay, not only to completely remove the extracellular parasites and
459 impede their growth over the incubation period, but also to increase the replication rate
460 of the intracellular amastigotes from the 12 days observed with FBS (14) to 2 days.

461 The activity of the test compounds *in vivo* correlated with what observed *in vitro* in the
462 intra-macrophage horse serum assay. Although the causes of the different *in vitro*
463 activities of compound 1 in FBS and HS are still not clear, these results suggest that the
464 assay here described is a right step towards the development of a translational *in vitro*
465 assay and represents an incentive for the deeper investigation of its application in
466 antileishmanials drug discovery.

467

468 **ACKNOWLEDGMENTS**

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470 Training Network “ParaMet” (grant 290080). Rosario Díaz and Fernando Aguilar were
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472 Jimenez-Alfaro and SMTech Department for supplying compounds pre-dispensed in
473 microtiter plates and managing compound logistics. We thank Maria Marco Martin,
474 Ignacio Cotillo and Emilio Alvarez for the technical assistance in the experiments and
475 discussion of the results. We thank the DMPK department for its work in PK/PD studies
476 and LAS department for its support in the animal facilities. This research was conducted
477 in collaboration with the Drugs for Neglected Diseases initiative (DNDi) and we
478 acknowledge both financial support and permission to include the *in vivo* results for
479 compound 1. We are also indebted to Manu de Rycker and scientists from Dundee
480 University Drug Discovery Unit for providing the eGFP strain.

481

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585

586

587 **TABLES**

MW	419.228
MF	C17H15Cl2F3N4O
SMILES	FC(F)(F)c1nc(Nc2cc(Cl)cc(Cl)c2)ncc1C(=O)NC1CCCC1
aring	2
clogp	4.602
hba	3
hbd	2
heavy	27
tpsa	66.91

588

589 **Table 1** Physicochemical properties of compound 1. MW: Molecular weight, MF:

590 Molecular formula, aring: number of aromatic rings, clogp: calculated partition-

591 coefficient between n-octanol and water, hba: hydrogen-bond acceptor, hbd: hydrogen-

592 bond donor, heavy: number of heavy atoms (no hydrogen atoms), tpsa: total polar

593 surface area.

594

Animal_ID	Actual Dose	Cmax (ng/mL)	Cmax_D (kg*ng/mL/mg)	AUCall (h*ng/mL)	AUCINF_obs (h*ng/mL)	AUCINF_D_obs (h*kg*ng/mL/mg)
#1	50	227.0	4.5	401.0	438.1	8.8
#2	50	154.0	3.1	339.7	358.4	7.2
#3	50	95.9	1.9	227.6	238.8	4.8
Average		159.0	3.2	322.8	345.1	6.9
SD		65.7	1.3	87.9	100.3	2.0

595

596 **Table 2** Blood pharmacokinetic parameters in Balb/c mice after single oral gavage
597 administration of compound 1 (actual dose= 50 mg/kg). DNAUC and Cmax/D, dose
598 normalized value of AUC and Cmax (AUC or Cmax/Experimental dose).

599

Tested compound	Administration	% inhibition	95% C.I.
AmBisome	1 mg/kg i.v. (D7, D9 and D11)	99.52	0.28
Miltefosine	12 mg/kg p.o. x 5 days	77.23	12.41
Compound 1	50 mg/kg BID x 5 days	20.93	8.34

600

601 **Table 3** Activity of amphotericin B, miltefosine and compound 1 against Balb/c mice
602 infected with *L. donovani* HU3 (N=5). i.v.= intravenous, p.o.=oral, BID (“bis in die”)=
603 twice a day, C.I.=confidence interval.

604

605 FIGURES LEGENDS

606 **Fig.1** A) Average number of THP-1 cells (6 fields) in assay media containing 2% FBS,
607 10% FBS, 2% HS, 10% FBS. B) THP-1 cells (DRAQ5, red) infected with *L. donovani*
608 (green) in the presence of FBS 2% or HS 2% at 96h (20x, air objective. C) Evolution of
609 the number of amastigotes per macrophages (AM/MAC) in the presence of HS 2%
610 (blue) or FBS 2% (red) and 0.5% DMSO. Number of amastigotes per total macrophages

611 are represented, final percentage of infection 86% in FBS and 78% in presence of HS.

612 D) THP-1 cells (DAPI, red) infected with *L. donovani* (green) in the presence of FBS

613 2% at 24 and 96h (20x, air objective). E) THP-1 cells (DAPI, red) infected with *L.*

614 *donovani* (green) in the presence of HS 2% at 24 and 96h (20x, air objective). F) THP-1

615 cells (DAPI, red) infected with *L. donovani* (green) in the presence of FBS 2% or HS

616 2% at 96h (40x, water objective).

617

618 **Fig. 2** A) Percentage of intracellular *L. donovani* amastigotes incorporating EdU (100,

619 50, 10, 5, 1, 0 μ M). B) Number of amastigotes per macrophage in infected THP-1 cells

620 processed for EdU detection. Amastigotes were detected as GFP positive spots (white

621 squares) or EdU (50 μ M) positive spots (black circles).

622

623 **Fig. 3** Dose response curves of amphotericinB-FBS (A), miltefosine-FBS (B),

624 amphotericinB-HS (C) and miltefosine-HS (D). Curves were generated from 11 points,

625 1/3 dilutions at a maximum concentration of 50 μ M, Data are presented as mean and SD,

626 4 replicates.

627

628 **Fig. 4** Dose response curves of compound 1 tested in the presence of FBS (squares) or HS

629 (circles). Curves were generated from 11 points, 1/3 dilutions at a maximum concentration of

630 50 μ M. Data are presented as mean and SD, 4 replicates.

631

632 **Fig. 5.** Whole blood levels in BALB/c mice after single oral gavage administration of

633 compound 1 (actual dose= 50 mg/kg) The dotted line represents the EC₅₀ value for

634 compound 1.

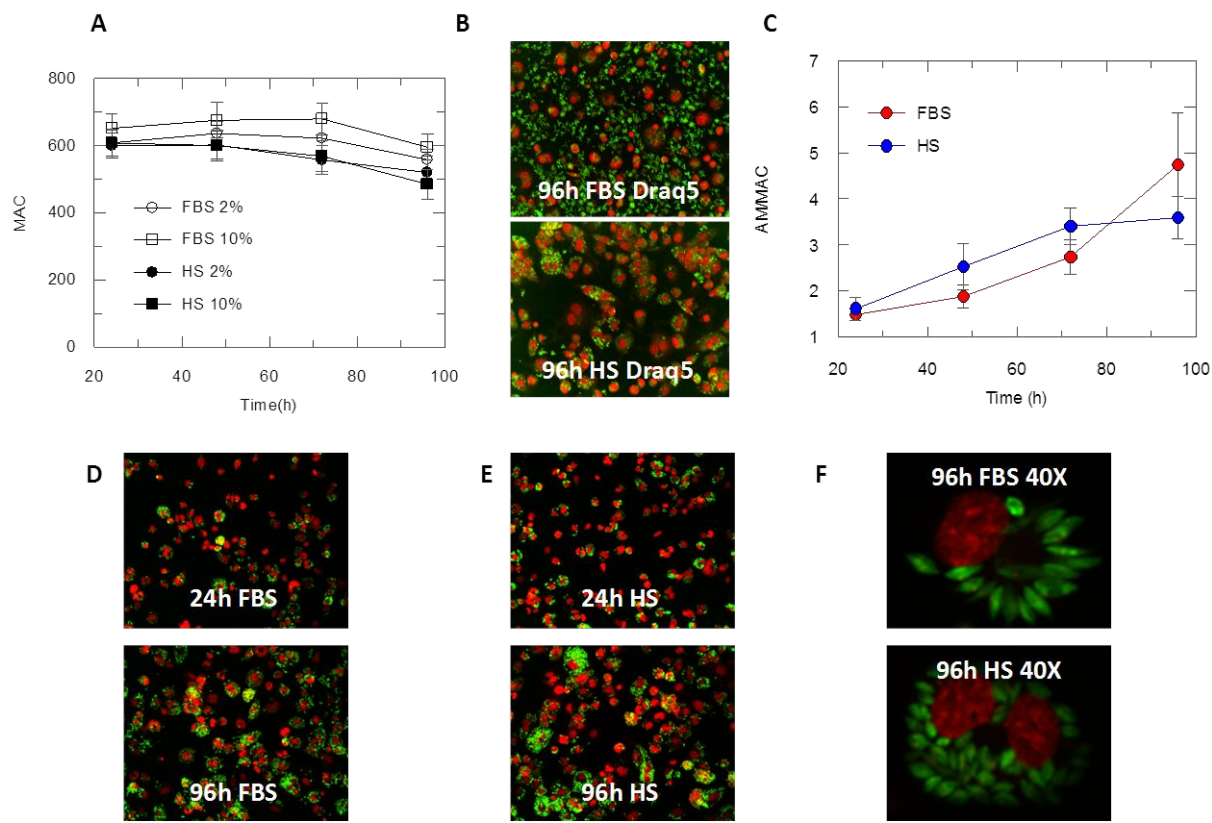


Fig.1 A) Average number of THP-1 cells (6 fields) in assay media containing 2% FBS, 10% FBS, 2% HS, 10% HS. B) THP-1 cells (DRAQ5, red) infected with *L. donovani* (green) in the presence of FBS 2% or HS 2% at 96h (20x, air objective). C) Evolution of the number of amastigotes per macrophages (AM/MAC) in the presence of HS 2% (blue) or FBS 2% (red) and 0.5% DMSO. Number of amastigotes per total macrophages are represented, final percentage of infection 86% in FBS and 78% in presence of HS. D) THP-1 cells (DAPI, red) infected with *L. donovani* (green) in the presence of FBS 2% at 24 and 96h (20x, air objective). E) THP-1 cells (DAPI, red) infected with *L. donovani* (green) in the presence of HS 2% at 24 and 96h (20x, air objective). F) THP-1 cells (DAPI, red) infected with *L. donovani* (green) in the presence of FBS 2% or HS 2% at 96h (40x, water objective).

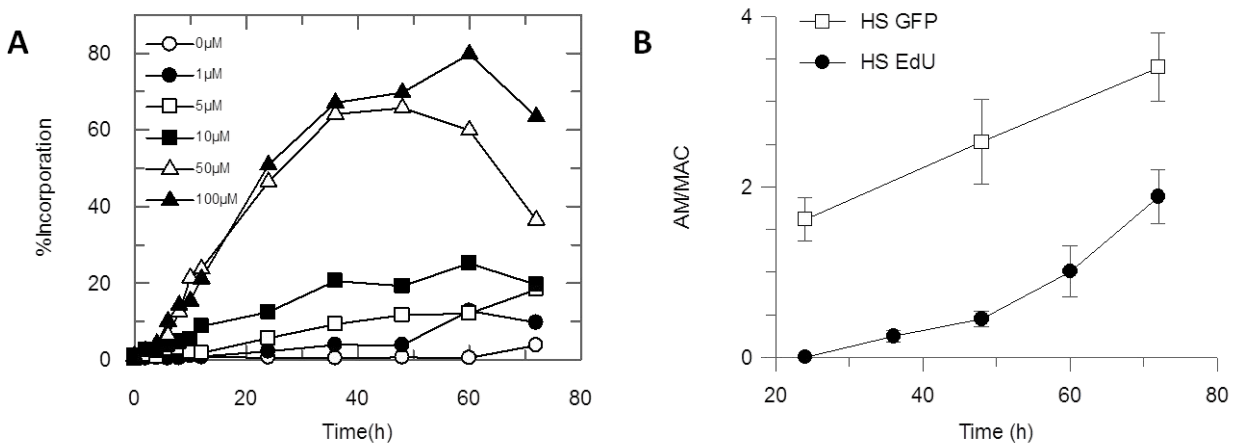


Fig. 2 A) Percentage of intracellular *L. donovani* amastigotes incorporating EdU (100, 50, 10, 5, 1, 0 μM). B) Number of amastigotes per macrophage in infected THP-1 cells processed for EdU detection. Amastigotes were detected as GFP positive spots (white squares) or EdU (50 μM) positive spots (black circles).

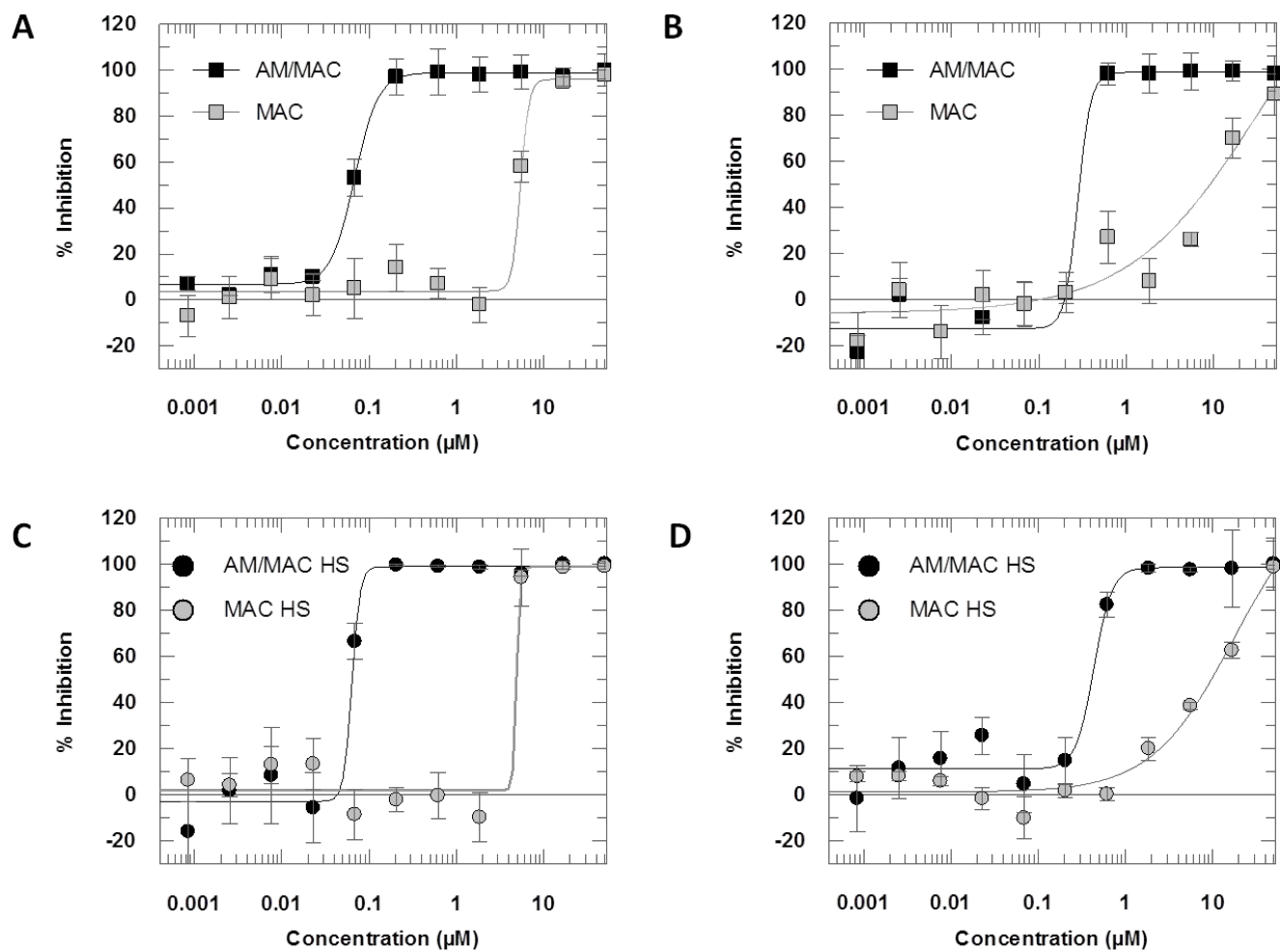


Fig.3 Dose response curves of amphotericinB-FBS (A), miltefosine-FBS (B), amphotericinB-HS (C) and miltefosine-HS (D). Curves were generated from 11 points, 1/3 dilutions at a maximum concentration of 50µM. Data are presented as mean and SD, 4 replicates.

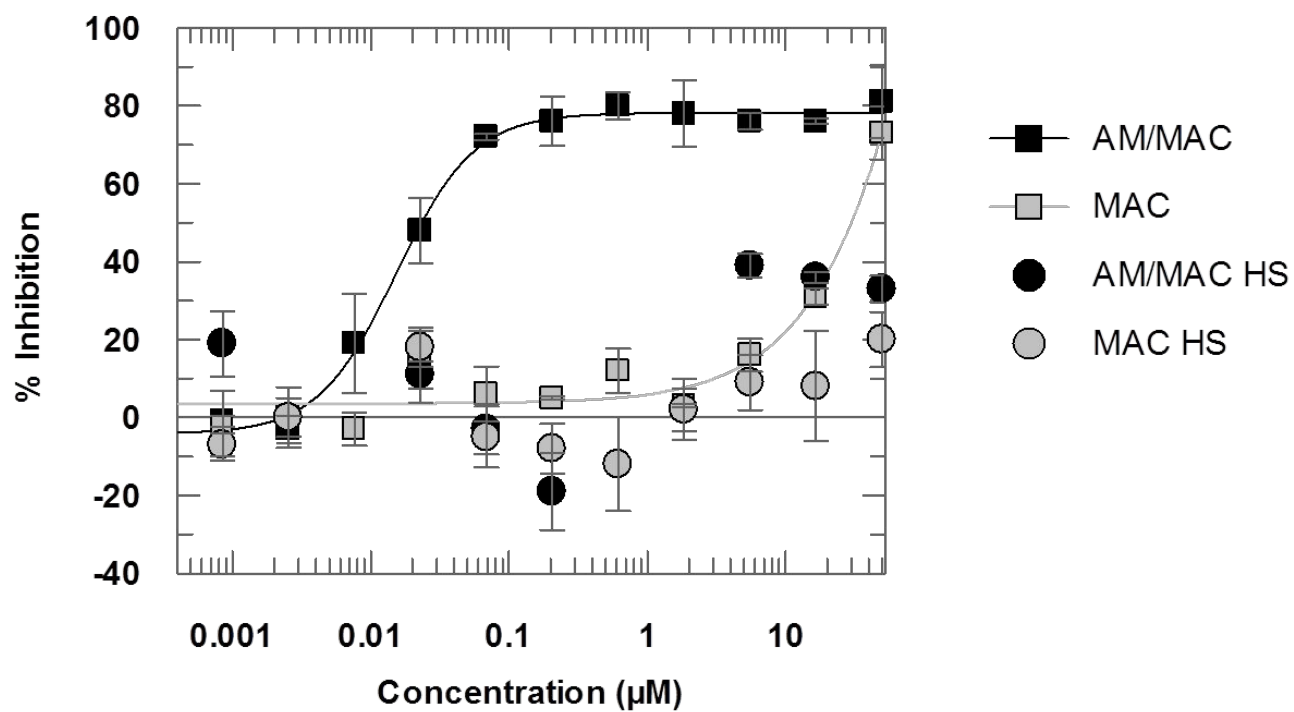


Fig.4 Dose response curves of compound 1 tested in the presence of FBS (squares) or HS (circles). Curves were generated from 11 points, 1/3 dilutions at a maximum concentration of 50 μM . Data are presented as mean and SD, 4 replicates.

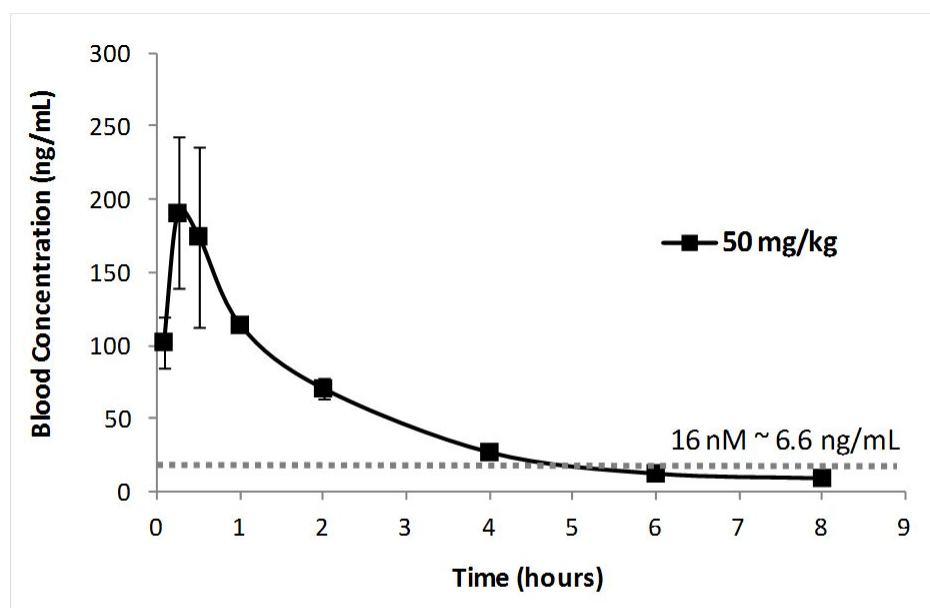


Fig. 5. Whole blood levels in BALB/c mice after single oral gavage administration of compound 1 (actual dose= 50 mg/kg) The dotted line represents the EC₅₀ value for compound 1.

MW	419.228
MF	C17H15Cl2F3N4O
SMILES	<chem>FC(F)(F)c1nc(Nc2cc(Cl)cc(Cl)c2)ncc1C(=O)NC1CCCC1</chem>
aring	2
clogp	4.602
hba	3
hbd	2
heavy	27
tpsa	66.91

Table 1 Physicochemical properties of compound 1. MW: Molecular weight, MF: Molecular formula, aring: number of aromatic rings, clogp: calculated partition-coefficient between n-octanol and water, hba: hydrogen-bond acceptor, hbd: hydrogen-bond donor, heavy: number of heavy atoms (no hydrogen atoms), tpsa: total polar surface area.

Animal_ID	Actual Dose	Cmax (ng/mL)	Cmax_D (kg*ng/mL/mg)	AUCall (h*ng/mL)	AUCINF_obs (h*ng/mL)	AUCINF_D_obs (h*kg*ng/mL/mg)
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Table 2 Blood pharmacokinetic parameters in Balb/c mice after single oral gavage administration of compound 1 (actual dose= 50 mg/kg). DNAUC and Cmax/D, dose normalized value of AUC and Cmax (AUC or Cmax/Experimental dose).

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Miltefosine	12 mg/kg p.o. x 5 days	77.23	12.41
Compound 1	50 mg/kg BID x 5 days	20.93	8.34

Table 3 Activity of amphotericin B, miltefosine and compound 1 against Balb/c mice infected with *L. donovani* HU3 (N=5). i.v.= intravenous, p.o.=oral, BID (“bis in die”)= twice a day, C.I.=confidence interval.