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

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ABSTRACT

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

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

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

Introduction

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

(i) Are adaptable to and enable high throughput screens against the replicative intracellular – macrophage amastigote stage of *Leishmania donovani*, one of the causative species of VL (7).

(ii) Include high throughput technologies that enable the collection of more information compared to the traditionally used assays based on manual counting and reporter genes (8, 9). For example, High Content Screening (HCS) systems that permit the screening of large sets of compounds using imaging techniques that also capture information about compounds' toxicity against host cells and mode of action (10, 11) have been applied to antileishmanial drug discovery (12-17).

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

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

MATERIALS AND METHODS

Antimicrobial Agents and Chemotherapy

Cell Lines

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

Leishmania donovani (MHOM/SD/62/1SCL2D, LdBOB) expressing green fluorescence protein (GFP) (14) was kindly provided by Manu de Rycker, University of Dundee, UK. Axenic amastigotes were maintained at 37ºC, 5% CO_2 in media containing 15 mM KCl solution (Invitrogen), 10 mM KH_2PO_4 (Merck), 136 mM KH_2PO_4 (Merck), 0.5 mM $MgSO_4$ (Sigma-Aldrich), 24 mM $NaHCO_3$ (Invitrogen), 25 mM Glucose (Sigma-Aldrich), 1mM L-Glutamine (Invitrogen), 1xRPMI Vitamin Solution (Sigma-Aldrich), 10 µM Folic Acid (Sigma-Aldrich), 100 µM Adenosine (Sigma-Aldrich), 5mg/L Hemin (Sigma-Aldrich), 1xRPMI Amino Acid solution (Sigma-Aldrich), 25 mM MES, 0.0004% Phenol Red and 20% Heat Inactivated FBS (Gibco) in Milli-Q water. The selection antibody Nourseothricin (Jena Bioscience) was regularly added to the cultures of amastigotes. Promastigotes were maintained at 30ºC in M199 Media (Sigma Aldrich) supplemented with 25mM HEPES (Invitrogen), 12mM $NaHCO_3$ (Invitrogen), 1mM L-Glutamine (Invitrogen), 1xRPMI Vitamin Solution (Sigma-Aldrich), 10µM Folic Acid (Sigma-Aldrich), 100µM Adenosine (Sigma-Aldrich), 5mg/L Hemin and 10% Heat Inactivated FBS (Gibco) (14).

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

The intra-macrophage assay was adapted from de Rycker *et al.* (14) and Peña *et al*. (16). THP-1 cells were grown in CELLMASTER roller bottles (Greiner cat. # 680048) at an initial seeding concentration of $2x10^5$ cells/mL for 72 h. Cells were visually inspected with an optical microscope and counted with a CASY Counter (model TT, Roche). Cells were differentiated in a 225 cm^3 T-FLASK (80 ml) in the presence of 30 nM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) at a final concentration of $6x10^5$ cells/mL. Following 24h incubation at 37°C, 5% CO_2, differentiation was visually confirmed checking the confluence of the differentiated adherent monolayer and PMA-containing media was removed washing twice with complete growth media, taking care of not disrupting the cell layer.

Each T-flask containing differentiated THP-1 cells was infected with 80 mL of a suspension of $6x10^6$ parasites/mL in THP-1 complete growth media without PMA and incubated additional 24h. The media was removed and the cell monolayer washed with PBS. The infected cells were harvested by treatment with a solution of 0.25% (w/v) trypsin/EDTA in PBS and seeded in assay plates ($1.6x10^5$ cells/mL, 50 µl/well) in assay media, containing RPMI media supplemented with 2% Heat Inactivated Horse Serum (Gibco) or Foetal Bovine Serum (Gibco), 25 mM $NaHCO_3$ (Invitrogen™) and 30 nM PMA using a Multidrop Combi dispenser (Thermo Scientific). A parallel culture of uninfected differentiated THP-1 cells was treated as described for infected cells and used as control for 100% compound response. Assay plates were incubated at 37°C, 5% CO_2 for the time required for the assay and then fixed with 4% formaldehyde for 30 min at room temperature adding 50 µl of 8% (v/v) formaldehyde solution (Sigma-Aldrich) in PBS to each well containing 50 µl of media. After fixation, cells were washed twice with 100 µL PBS using an EL406 multi well platewasher (BioTek), stained with 30 µl of a solution of DAPI (10µg/mL) and 0.1% (v/v) Triton X-100 in PBS for 30 min at

room temperature and washed additional two times with 50 µL PBS. Finally, 50 µL of PBS were added to each well, plates were sealed and stored at 4ºC until analysis.

Image analysis

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

Compounds and assay plates

Amphotericin B and miltefosine were purchased from Sigma Aldrich. Compound 1 was available from the GSK collection of compounds (Table 1).

Pre-dispensed assay plates (Greiner µclear black, 384-well) were prepared by adding 250 nL of compounds dissolved in 100% DMSO or 250 nL of DMSO to each well by using an Echo® liquid handler (Labcyte Inc.). Eleven-point one in three dilution curves were generated from a top concentration of 50 µM.

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

Data analysis

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

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

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

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

Biosafety and animal use

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

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

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

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

EdU Incorporation

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

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

In vivo* activity against *L. donovani

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

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

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

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

Pharmacokinetic studies

Experimental compounds were administered to BALB/c female mice (25 g weight) by oral gavage at 50 mg/kg dose at a volume of 20 ml/kg. All mice were treated during the fed state. Drugs were administered as 10% 70:30 Tween80: EtOH/ddH_2O suspensions and the blood sampling scheme was: 15, 30 and 45 minutes, 1, 1.5, 2, 3, 4, 8 and 24 hours. At each time-point, 10 µL of blood were taken from the lateral tail vein from three animals. LC-MS was used for the establishment of compound concentration in blood with a sensitivity of LLQ = 1–5 ng/ml in 25 ml blood. The concentration of each drug was calculated in the peripheral total blood compartment. The non-compartmental

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

RESULTS

Assay development

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

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

Effect of horse serum on extracellular amastigotes

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

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

Effect of HS on *L. donovani* intracellular amastigotes

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

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

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

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

Edu incorporation

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

After the optimization of the experimental conditions, the incorporation of EdU over time by infected THP-1 cells maintained in HS-containing media was determined adding 50 µM of EdU 24 hours after plating and measuring EdU incorporation in 12-hour lapses from 24 to 72 hours post plating. The number of amastigotes per macrophage was determined in both the GFP and in the EdU channel. Not all amastigotes incorporated EdU during the course of infection but the incorporation rate 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 pEC_{50} (pEC_{50}= -Log (EC_{50}))
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 pEC_{50} 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 EC_{50} value. The exposure data were sufficiently favorable to warrant
316 further *in vivo* testing.

317

In vivo antileishmanial activity

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

To validate this *in vitro* assay, two reference drugs, amphotericin B and miltefosine and the GSK compound 1 were tested in the intra-macrophage assay in the presence of FBS or HS and using an *in vivo* animal model, allowing for a comparison of the *in vitro* and *in vivo* activities. The *in vitro* activities of amphotericin B and miltefosine were in accordance with previous reports and no significant difference between their activity in the presence of FBS or HS was observed. Compound 1 was selected as a proof of concept study, as it showed a pEC_{50} value higher than amphotericin B in the presence of FBS (pEC_{50}=7.8) but was inactive when tested in the presence of HS (pEC_{50}<4.3). When tested *in vivo*, amphotericin B and miltefosine confirmed their activity, reducing the parasite burden by 99.52% and 77.23% respectively. In contrast, compound 1 was inactive when administered orally, reducing the parasite burden by 20.93% only. Since compound 1 possesses lead-like physicochemical properties (34) (Table 1) and reasonable bioavailability in mice in terms of Cmax and AUC (Table 2), we propose that factors other than pharmacokinetics might contribute to the lack of efficacy in the

infection model, such as poor pharmacodynamics at the site of action. In particular, we suggest it could be linked to its lack of activity in the *in vitro* horse serum intra-macrophage assay, in contrast with the high pEC_{50} value obtained when a media containing FBS was used (AM/MAC output). Several reasons could explain the lack of activity of compound 1 in HS: compound structure related properties, the lack of activity against intracellular replicating amastigotes in horse serum, or the compound could be active only against the extracellular amastigotes forms found in presence of FBS. Even though the exact mode of action of compound 1 has not been clarified, the correlation between the results obtained in the *in vitro* horse serum intra-macrophage assay and the *in vivo* mouse model seem to suggest that the *in vitro* results obtained with horse serum translate to the *in vivo* animal model and that this assay mimics an *in vivo L. donovani* infection more accurately than the same assay with FBS. In fact, the standard drugs miltefosine and amphotericin B were active in *in vitro* and *in vivo* assays and compound 1 was inactive both *in vitro* when horse serum was used, irrespectively of the output used for the determination of its pEC_{50}, and *in vivo.*

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

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

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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

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)
#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

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).

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

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.

FIGURES LEGENDS

Fig.1 A) Average number of THP-1 cells (6 fields) in assay media containing 2% FBS, 10% FBS, 2% HS, 10% FBS. 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

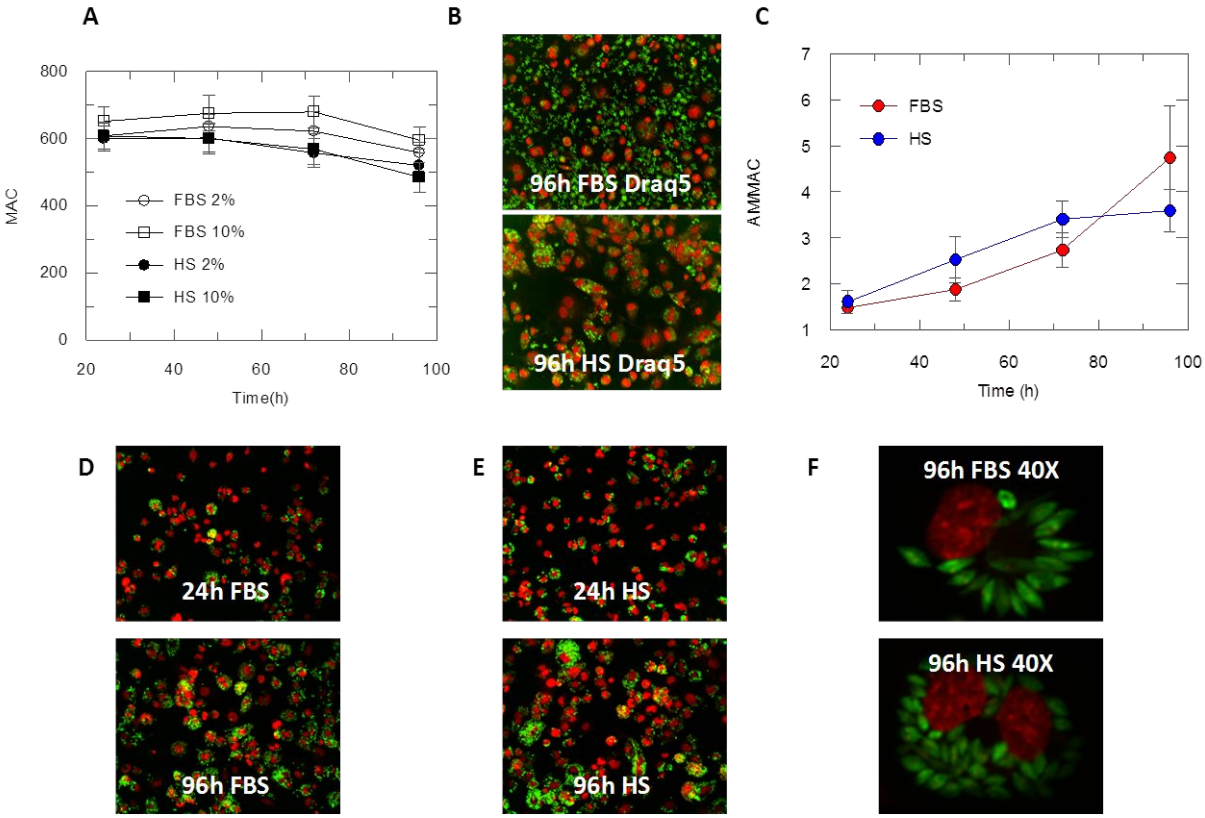
are represented, final percentage of infection 86% in FBS and 78% in presence of HS. D) THP-1 cells (DAPI, red) infected with *L. donovani* (green) in the presence of FBS 2% at 24 and 96h (20x, air objective). E) THP-1 cells (DAPI, red) infected with *L. donovani* (green) in the presence of HS 2% at 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, 0µM). 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 50µM. 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 EC_{50} value for compound 1.



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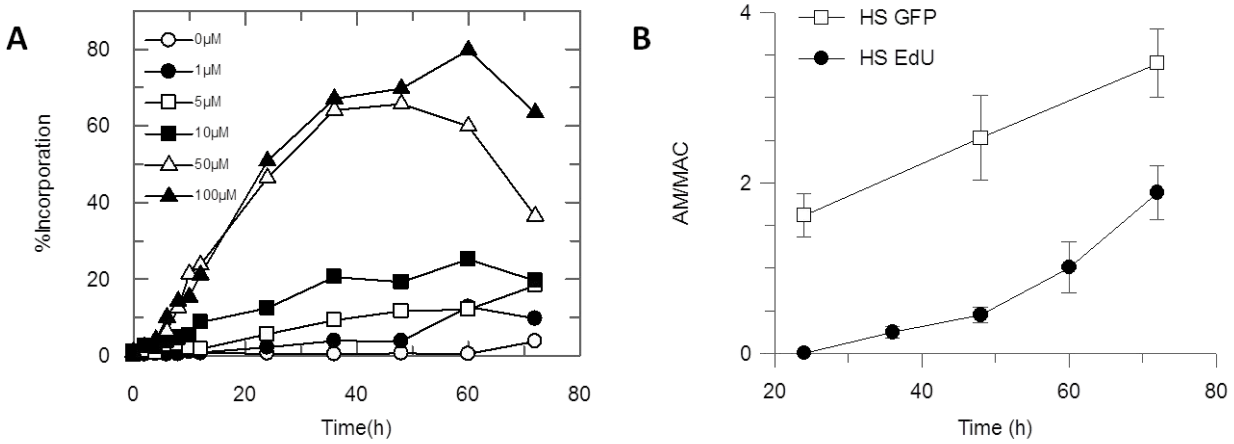

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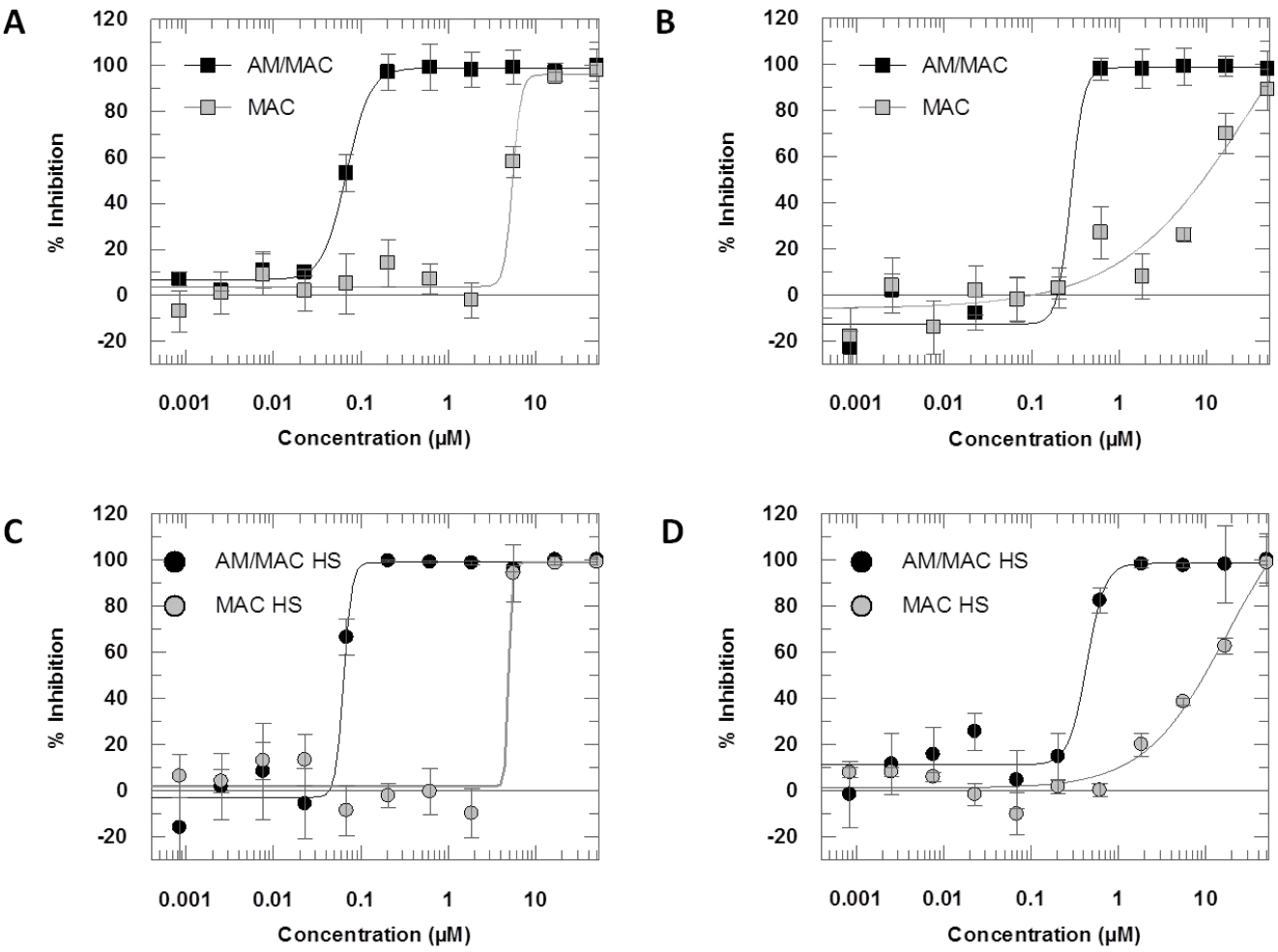

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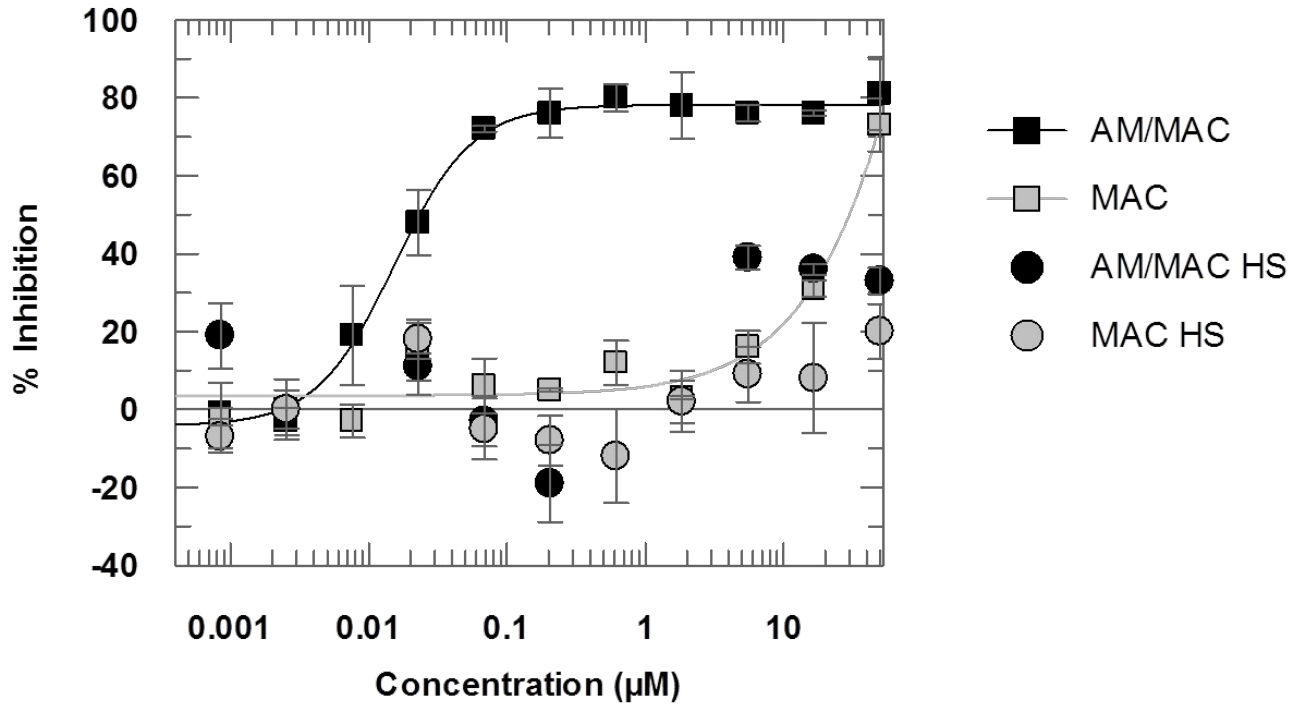

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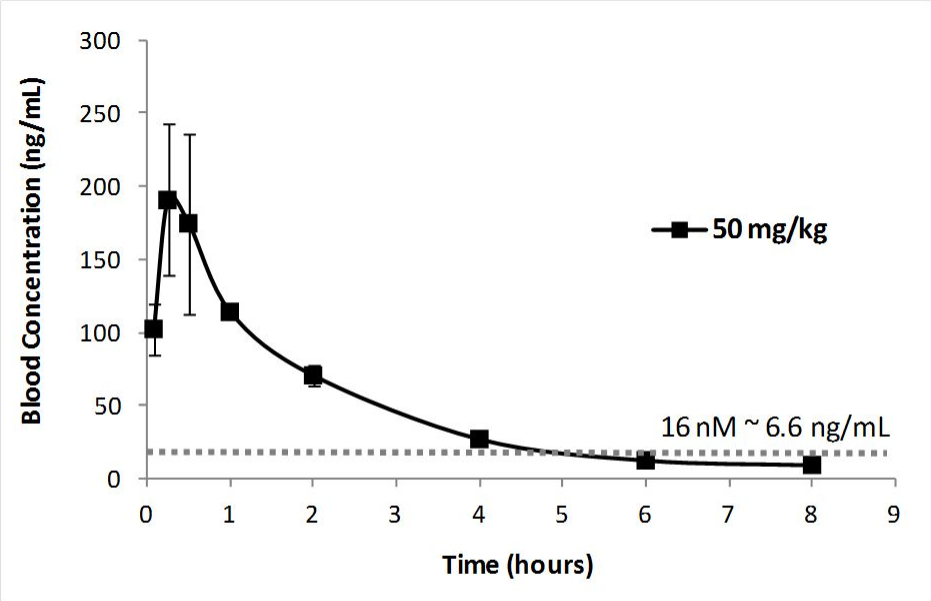

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