Snapshot profiling of anti-leishmanial potency of lead compounds and drug candidates against intracellular *L. donovani* amastigotes with focus on human derived host cells

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Running title: Potency of anti-leishmanials in human host cells

Abstract

This study characterised *in vitro* potencies of anti-leishmanial agents against intracellular *Leishmania donovani* amastigotes in primary human macrophages, obtained with or without CD14-positive monocyte enrichment, phorbol 12-myristate 13-acetate (PMA) differentiated THP-1 cells and mouse peritoneal exudate macrophages (PEMs). Host cell dependent potency was confirmed for pentavalent and trivalent antimony. Fexinidazole was inactive against intracellular amastigotes across the host cell panel. Fexinidazole sulfone, (*R*)-PA-824, (*S*)-PA-824 and VL-2098 displayed similar potency in all host cells tested.

Parasites of the genus *Leishmania* are causative agents of Neglected Tropical Diseases (NTDs) known as the leishmaniases. In the host, parasites survive and multiply as intracellular amastigotes in the parasitophorous vacuole of primarily tissue-resident macrophages (1). Main disease manifestations include visceral leishmaniasis (VL) (2) and cutaneous leishmaniasis (CL) (3). VL is caused by infection with *L. donovani* or *L. infantum* (2) and estimated to cause more than 50 000 deaths per year (4). Limitations of current chemotherapeutics include the need for long treatment courses, variable treatment responses between endemic regions, safety concerns and lack of drug stability in hot climates (5, 6). With increased support for drug research and development for NTDs the last decade has seen increased efforts in drug discovery for leishmaniasis. This was accompanied by the set-up of high-throughput, high-content platforms to screen compounds against intracellular *Leishmania* amastigotes in mammalian host cells (7-9). Different mammalian cells are used for this purpose. However, host cell properties are amongst the determinants of directly acting drugs, which need to accumulate in infected host cells to exert their anti-leishmanial effects, and immunomodulatory agents, which affect cellular pathways to kill intracellular parasites indirectly. Involvement of host cell transporters has been demonstrated in drug accumulation and treatment outcome for antimonials (10, 11) and miltefosine (12). The nature of the host cell has been shown to impact on the *in vitro* potency of the standard anti-leishmanial drug sodium stibogluconate (SSG) (13). Hence, the current study was undertaken to characterise potencies of current lead compounds and drug candidates against intracellular *L. donovani* amastigotes in a panel of different host cells.

Selection of host cells was focussed on human derived cells to ensure relevance to clinical use. Peritoneal exudate mouse macrophages (PEMs) were included as they have an established role in anti-leishmanial drug evaluations. Compounds profiled included the nitroheterocyclic drugs fexinidazole and its sulfone metabolite (14), VL-2098 (15) and the

(*R*) and (*S*) enantiomers of PA-824 (16). Notably fexinidazole has entered clinical trials for VL (www.dndi.org). Since SSG (pentavalent antimony) is a pro-drug and requires conversion to the trivalent form (17), we included both oxidation states in the current study.

THP-1 cells (ATCC-TIB-202, LGC Ltd., Teddington, UK), PEMs, harvested from BALB/c or CD-1 mice (LSHTM breeding colony), and human peripheral blood mononuclear cells (PBMCs), harvested from heparinised blood collected from adult human donors, were prepared as described (13). Autologous plasma was centrifuged for 30 minutes at 2,000 x g at 20°C and stored at 4°C for the duration of the experiment. Mononuclear cells were re-suspended in RPMI 1640 medium plus penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% autologous plasma and differentiated at 37°C, 5% CO_2 for a total of 6 days with addition of fresh medium after 3 days. Selected assays used monocytes obtained through positive immunomagnetic selection with CD14 MicroBeads (MACS; Miltenyi Biotec), following the manufacterer's protocol. The CD14-positive monocyte-enriched fraction was re-suspended in RPMI 1640 medium plus 10% hi-FBS, 100 ng/ml recombinant human M-CSF (R&D Systems, UK) and penicillin (100 U/mL) / streptomycin (100 mg/mL) and differentiated at 37°C, 5% CO_2 for 6 days. Prior to infection cells were washed with fresh medium containing no antibiotic. Depending on the final number of cells obtained, PBMC derived macrophages from 2-3 individual donors were either combined or plated separately for drug potency evaluations. At the time of drug addition cells obtained without CD14 selection were > 85% macrophages as estimated by morphological appearance in Giemsa-stained preparations and those obtained with positive CD14 selection 100%. Host cells, plated in Lab-tek 16-well chamber slides (Fisher Scientific, UK) at a density of 4 x 10^4 cells/well, were infected with *Leishmania donovani* (MHOM/ET/67/HU3) amastigotes, harvested from Rag-1-knockout (B6) mice (LSHTM breeding colony), as described (13). Infected cultures were exposed to 6 point (4 point when limited by cell number) serial compound dilutions (2-fold, 3-fold for

antimonials) or assay medium (untreated controls) for 3 days (no medium change) or 5 days (medium change after 3 days). Each concentration and control was tested in quadruplicate. Upon termination of the assay slides were prepared and data evaluated as described (13). Percentage of infected cells was used to estimate EC_{50} and EC_{90} values as the clinically most relevant read out. Intracellular burden in untreated controls was determined by counting the number of amastigotes in 50 infected host cells per well. Experiments were carried out in a direct comparative assay design in which different host cell types were infected at the same time with the same batch of parasites and exposed to dilutions prepared from the same stock solution of compounds. This approach was chosen to ensure that any variation in drug potency between different cell types could be attributed to cell type rather than day-to-day differences in parasite or drug preparation. Structurally related compounds were tested in parallel in the same experiment and miltefosine (Zentaris GmbH, Germany) included as standard drug in selected assays. Nitroheterocyclic drugs were synthesised at the University of Dundee as described (14, 16). VL-2098 was prepared in a single step from 4-(trifluoromethoxy)phenol and (*R*)-2-bromo-1-((2-methyloxiran-2-yl)methyl)-4-nitro-1*H*-imidazole using a modification of the published synthesis of delamanid (OPC-67683) (18). Potassium antimonyl tartrate trihydrate (trivalent antimony) was obtained from Sigma, UK and SSG from GSK, UK. Aqueous stock solutions of SSG, potassium antimonyl tartrate trihydrate and miltefosine were prepared as described previously (13), those of other compounds in dimethylsulfoxide (Sigma, UK).

Experiments involving animals were approved by the Animal Welfare and Ethics Review Board at LSHTM and performed under license in accordance with the Animals (Scientific Procedures) Act 1986 (UK Home Office Project Licence PPL70/6997). For blood donations consenting volunteers were recruited through an anonymous blood donation

system. Approval for blood donations and the specific study was given by the LSHTM Ethics Committee (reference numbers 5520 and 6404).

At the EC_{50} level both pentavalent and trivalent antimony were more potent against *L. donovani* amastigotes in primary human macrophages compared to differentiated THP-1 cells, by factors of 21 to >100. The difference in SSG's potency between these two cell types is consistent with previous observations (13). As different methodologies exist for the generation of primary human macrophages (10, 13, 19) and cells obtained from total PBMC fractions by plastic adherence may contain lymphocyte and platelet contaminations (20) we wanted to rule out that the methodology used affected our conclusion. Hence we additionally evaluated SSG's potency in macrophages generated from CD-14 positive enriched monocytes. Again SSG was more potent in primary human macrophages compared to differentiated THP-1 cells tested in parallel, with up to 20 fold differences at the EC_{50} level. Also, SSG displayed anti-leishmanial activity in primary human macrophages already after 3 days exposure when either isolation method was used. Data is summarised in Table 1. In macrophages obtained from CD-14 positive enriched monocytes EC_{90} values were consistently higher than those estimated in macrophages obtained from total PBMC fractions. However, it should be noted that a systematic comparison of SSG's potency between the two cell isolation procedures was outside the scope of this study. Since the *in vitro* potency of SSG has been shown to decrease with increasing infection levels (21) it is important to note that infection levels in macrophages obtained from CD-14 positive enriched monocytes or differentiated THP-1 cells were not higher than those in macrophages obtained from total PBMC fractions (Supplementary Table 1).

Anti-leishmanial potencies of the nitroheterocyclic compounds (*R*)-PA-824, (*S*)-PA-824 and VL-2098 displayed less than 3-fold differences against amastigotes in primary human macrophages compared to differentiated THP-1 cells at the EC_{50} and EC_{90} level after

3 days of compound exposure. Previously reported differences in anti-leishmanial activity between the two enantiomers of PA-824 (16) were confirmed in both human derived host cells. Larger variations between assays were observed for fexinidazole sulfone, resulting in 2-10 fold differences at the EC_{50} level. However, variable quality of dose response curve fits, as checked visually, was noted between experiments. Fexinidazole was inactive at concentrations up to 80 µM in both human derived cell types. Data is summarised in Table 2. Due to the absence of apparent host cell dependent drug action no further investigation was carried out for nitroheterocyclic compounds in macrophages generated from CD-14 positive enriched monocytes. Estimation of the steepness of the dose response curves through Hill slopes gave values of >1 for VL-2098, (*R*)-PA-824 and (*S*)-PA-824 and <1 for fexinidazole sulfone. Levels of infection in human derived host cells were similar and increased over the course of the experiments, but differed in PEMs (Supplementary Table 2). Hence, potencies against *L. donovani* amastigotes in PEMs are reported in Table 2 without direct comparisons to the human derived cells. Potencies were in agreement with values reported in the literature (14-16).

Cell lines are often used over primary cells due to ease of culture and an argument of homogeneity. PMA differentiated THP-1 cells are widely used in anti-leishmanial drug research, but different stimulation conditions are reported (7, 8, 10, 22). The host cell's ability to sustain infection with *Leishmania* parasites and comparison of potency of reference compounds to other host cells and assay formats has been the focus in developing protocols for anti-leishmanial drug evaluation using differentiated THP-1 cells. However it has been shown that certain cell characteristics, including lysosomal structures, differ when different stimulus conditions are used (23). Of note, lowering extracellular oxygen tension from 18% to 5% O_2 has also been shown to affect PMA induced THP-1 cell differentiation and function (24). So far, these effects have not been explored in anti-leishmanial drug research.

Using primary human macrophages derived from individual blood donors we found drug potencies to be consistent between different donors (Fig. 1), but monocyte isolation requires a more dedicated approach than standard cell culture and macrophage yields are less predictable.

In summary, we show that antimonials are a class of compounds where the choice of host cell affects drug potency under the conditions tested and provide potency profiles of current anti-leishmanial lead and drug candidates in human derived host cells, including primary macrophages. Antimonials have been shown to cause oxidative stress and activation of *L. donovani* infected host cells *in vitro* with generation of ROS, NO and TNF-alpha and subsequent killing of intracellular parasites (25, 26). Drug transporters at the host cell level have been linked to clinical responses to antimonial treatment and drug resistance (10, 11, 27). Modulation of gene expression profiles by SSG has also been demonstrated *in vitro* and increased levels of glutathione were measured in SSG treated compared to untreated host cells (28). It is possible that differences in the response to oxidative stress, production of cytokines, expression of drug transporters or a combination of these factors between different host cells account for the host-cell dependent phenotype of antimonial drug action.

To enable evidence based host cell choice in anti-leishmanial drug research systematic functional characterisation of the different cell types and their cell-parasite interactions are needed. Finally, the lack of anti-leishmanial activity of fexinidazole in all three cell types tested underlines the importance of drug metabolism for successful treatment outcomes with this drug.

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Conflicts of interest: none

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Figure legends

Table 1. Characterisation of cell type dependent potency of antimonials in primary human macrophages and differentiated THP-1 cells.

Experiment (Expt.) numbers indicate parallel testing of antimonials in assays with the same number, days indicates the number of days of continuous drug exposure, PBMC derived mΦ refers to macrophages obtained without positive (CD14) selection, PBMC derived mΦ ($CD14^+$) refers to macrophages obtained from positively selected ($CD14^+$) monocytes. EC_{50} / $_{90}$ values are given in µg Sb/mL with 95% confidence intervals in brackets, - not determined.

[a]Cells were derived from two individual blood donors.

[b]Cells were derived from the same blood donor.

[c,d,e] Percentage inhibition at 30 µg Sb/ml was 51.6%, 73.3% and 77.8% respectively.

Miltefosine was included as positive control and displayed EC_{50} / $_{90}$ values (95% confidence intervals in brackets) of 7.97 µM (4.47 – 11.47) / >20 µM in differentiated THP-1 cells and 1.61 µM (1.24 – 1.98) / >5 µM in PBMC derived mΦ in expt 1. In expt. 2 respective values were 1.64 µM (1.35 – 1.93) / 7.77 µM (6.42 – 9.12) and 1.42 µM (1.38 – 1.45) / 3.92 µM (2.78 – 5.07).

Table 2. Potency of nitroheterocyclic compounds against intracellular *L. donovani* amastigotes in three different host cell types.

Experiment (Expt.) numbers indicate parallel testing of respective compounds in assays with the same number. EC_{50} / $_{90}$ values are given in µM with 95% confidence intervals in brackets. Data is representative of 2 - 4 separate directly comparative experiments.

- not determined, N.O. not obtained.

[a]Cells were derived from two individual blood donors.

[b]PEMs were harvested from CD-1 mice in this assay and BALB/c mice in all other assays.

Miltefosine was included as positive control in expt. 4 and displayed EC_{50} values **(**95% confidence intervals in brackets) of 8.58 µM (6.39 – 10.76) in differentiated THP-1 cells, 2.67 µM (1.78 – 3.57) in PBMC derived mΦ and 1.19 µM (0.83-1.54) in PEMs.

Figure 1. Comparison of EC_{50} values between primary human macrophages obtained from different blood donors.

Data is given for 3 day compound exposures to fexinidazole sulfone (A), (*R*)-PA-824 (B), (*S*)-PA-824 (C), VL-2098 (D) and for 5 day exposures to SSG (E). Symbols represent results with cells from individual blood donors (full circles) or with cells pooled from 2-3 individual blood donors (full triangles).

Table 1.

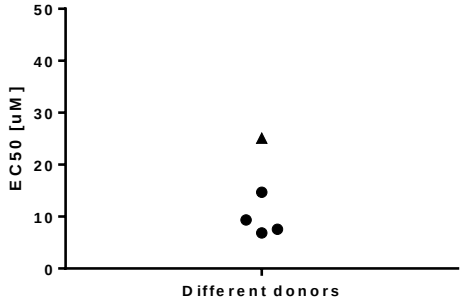
	Expt.	Days	PBMC derived mΦ		Differentiated THP-1 cells		PBMC derived mΦ ($CD14^+$)	
			EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}
Sb^V	1	3	5.39 (4.68-6.11)	10.22 (9.59-10.86)	-	-	-	-
Sb^V	2	5	5.17 (4.62-5.71)[a]	11.20 (9.48-12.92)[a]	108.76 (53.52-164.00)	>200	-	-
	2	5	2.43 (1.66-3.20)[a]	10.27 (4.14-16.39)[a]				
Sb^{III}	2	5	0.10 (0.09-0.11)[a]	>0.17[a]	>10	>10	-	-
	2	5	0.08 (0.07-0.08)[a]	>0.17[a]				
Sb^V	3	3	-	-	>900	>900	15.08 (11.70-18.46)[b]	>30[b,c]
	3	5	-	-	117.62 (84.91-150.34)	>900	11.11 (8.58-13.65)[a,b]	>30[a,b,d]
	3	5	-	-			6.01 (5.59-6.42)[a]	>30[a,e]

Table 2.

		Differentiated THP-1 cells		PBMC derived mΦ		PEMs	
Drug	**Expt.**	**EC_{50}**	**EC_{90}**	**EC_{50}**	**EC_{90}**	**EC_{50}**	**EC_{90}**
(*R*)-PA-824	1	2.91 (2.77-3.04)	6.56 (4.66-8.47)	4.59 (4.15-5.03)[a]	5.76 (5.25-6.27)[a]	1.16 (0.89-1.42)	5.58 (3.25-7.92)
	1			2.63 (2.37-2.89)[a]	4.92 (4.36-5.47)[a]		
	2	4.01 (3.94-4.09)	8.39 (6.93-9.58)	5.06 (4.55-5.57)	7.27 (6.46-8.09)	2.26 (2.09-2.44)[b]	4.55 (3.46-5.64)[b]
(*S*)-PA-824	1	27.83 (24.95-30.71)	>40	38.62 (37.53-39.70)	>40	13.03 (8.05-18.02)	N.O.
	2	27.90 (25.32-30.49)	50.82 (44.66-26.97)	30.57 (26.73-34.41)	37.82 (35.88-39.77)	13.53 (11.67-15.39)[b]	N.O.
VL-2098	1	0.22 (0.21-0.23)	0.36 (0.32-0.41)	0.32 (0.31-0.34)	0.48 (0.45-0.50)	0.23 (0.20-0.26)	0.45 (0.37-0.54)
	2	-	-	0.39 (0.36-0.42)	0.56 (0.52-0.61)	0.23 (0.21-0.24)[b]	0.45 (0.35-0.56)[b]
	3	-	-	0.27 (0.21-0.33)	0.41 (0.39-0.43)	-	-
Fexinidazole	3	>80	>80	>80[a]	>80 [a]	>80	>80
	3			>80 [a]	>80 [a]		
Fexinidazole sulfone	1	3.15 (2.06-4.25)	>20	7.57 (6.72-8.43)[a]	>40[a]	5.59 (5.21-5.96)	>20
	1			6.85 (4.56-9.13)[a]	>40[a]		
	3	8.43 (7.38-9.49)	>40	9.37 (4.66-14.07)[a]	>80[a]	10.44 (6.88-14.00)	>40
	3			14.70 (10.73-18.68)[a]	>80[a]		
	4	2.24 (1.42-3.06)	>80	20.94 (17.02-24.86)	>80	10.27 (5.64-14.90)	>20

Figure 1.

A



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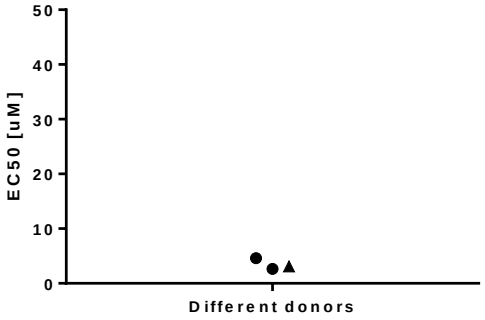

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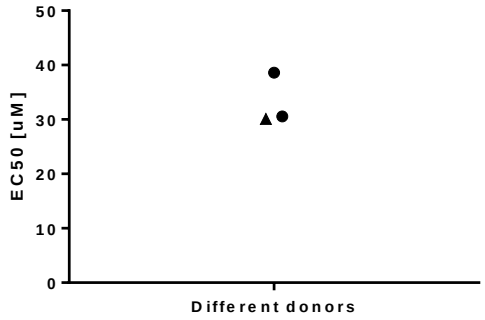

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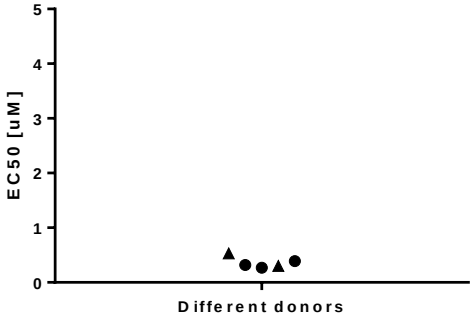

E