



# Snapshot Profiling of the Antileishmanial Potency of Lead Compounds and Drug Candidates against Intracellular *Leishmania donovani* Amastigotes, with a Focus on Human-Derived Host Cells

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**ABSTRACT** This study characterized the *in vitro* potencies of antileishmanial 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 of the host cells tested.

**KEYWORDS** drug potency, host cell, *Leishmania donovani*

Parasites of the genus *Leishmania* are causative agents of neglected tropical diseases (NTDs) known as the leishmaniasis. 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 *Leishmania donovani* or *Leishmania infantum* (2) and is 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 regions where VL is endemic, 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 setup 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 among the determinants of directly acting drugs, which need to accumulate in infected host cells to exert their antileishmanial effects, and immunomodulatory agents, which affect cellular pathways to kill intracellular parasites indirectly. The 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 antileishmanial drug sodium stibogluconate (SSG) (13). Hence, the current study was undertaken to characterize the potencies of current lead compounds and drug candidates against intracellular *L. donovani* amastigotes in a panel of different host cells.

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

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VL-2098 (15), and the (*R*) and (*S*) enantiomers of PA-824 (16). Notably fexinidazole has entered clinical trials for VL ([www.dndi.org](http://www.dndi.org)). Since SSG (pentavalent antimony) is a prodrug 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 (London School of Hygiene and Tropical Medicine [LSHTM] breeding colony), and human peripheral blood mononuclear cells (PBMCs) harvested from heparinized blood collected from adult human donors were prepared as described previously (13). Autologous plasma was centrifuged for 30 min at  $2,000 \times g$  at 20°C and stored at 4°C for the duration of the experiment. Mononuclear cells were resuspended in RPMI 1640 medium plus penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% autologous plasma and differentiated at 37°C and 5% CO<sub>2</sub> for a total of 6 days with the addition of fresh medium after 3 days. Selected assays used monocytes obtained through positive immunomagnetic selection with CD14 MicroBeads (MACS; Miltenyi Biotec) following the manufacturer's recommendations. The CD14-positive monocyte-enriched fraction was resuspended in RPMI 1640 medium plus 10% heat-inactivated fetal bovine serum (hi-FBS), 100 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF) (R&D Systems, UK), and penicillin (100 U/ml)-streptomycin (100 µg/ml) and differentiated at 37°C and 5% CO<sub>2</sub> 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 to 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 were 100%. Host cells, plated in Lab-Tek 16-well chamber slides (Fisher Scientific, UK) at a density of  $4 \times 10^4$  cells/well, were infected with *Leishmania donovani* (MHOM/ET/67/HU3) amastigotes harvested from Rag-1-knockout (B6) mice (LSHTM breeding colony) as described previously (13). Infected cultures were exposed to 6 point (4 point when limited by cell number) serial compound dilutions (2-fold and 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 were evaluated as described previously (13). The percentage of infected cells was used to estimate 50% effective concentration (EC<sub>50</sub>) and 90% effective concentration (EC<sub>90</sub>) 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 to 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) was included as a standard drug in selected assays. Nitroheterocyclic drugs were synthesized at the University of Dundee as described previously (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 was obtained from GSK (UK). Aqueous stock solutions of SSG, potassium antimonyl tartrate trihydrate, and miltefosine were prepared as described previously (13), and those of other compounds were prepared in dimethyl sulfoxide (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 license PPL70/6997). For blood donations, consenting volunteers were recruited through an anonymous blood dona-

**TABLE 1** Characterization of cell type-dependent potency of antimonials in primary human macrophages and differentiated THP-1 cells

Antimonial	Expt <sup>a</sup>	Days <sup>b</sup>	PBMC-derived mφ <sup>c</sup>		Differentiated THP-1 cells		PBMC-derived mφ (CD14 <sup>+</sup> ) <sup>d</sup>	
			EC <sub>50</sub> (μg Sb/ml [95% confidence interval])	EC <sub>90</sub> (μg Sb/ml [95% confidence interval])	EC <sub>50</sub> (μg Sb/ml [95% confidence interval])	EC <sub>90</sub> (μg Sb/ml [95% confidence interval])	EC <sub>50</sub> (μg Sb/ml [95% confidence interval])	EC <sub>90</sub> (μg Sb/ml [95% confidence interval])
Sb <sup>v</sup>	1	3	5.39 (4.68–6.11)	10.22 (9.59–10.86)	ND <sup>e</sup>	ND	ND	ND
Sb <sup>v</sup>	2	5	5.17 (4.62–5.71) <sup>f</sup>	11.20 (9.48–12.92) <sup>f</sup>	108.76 (53.52–164.00)	>200	ND	ND
		5	2.43 (1.66–3.20) <sup>f</sup>	10.27 (4.14–16.39) <sup>f</sup>				
Sb <sup>iii</sup>	2	5	0.10 (0.09–0.11) <sup>f</sup>	>0.17 <sup>f</sup>	>10	>10	ND	ND
		5	0.08 (0.07–0.08) <sup>f</sup>	>0.17 <sup>f</sup>				
Sb <sup>v</sup>	3	3	ND	ND	>900	>900	15.08 (11.70–18.46) <sup>g</sup>	>30 <sup>g,h</sup>
	3	5	ND	ND	117.62 (84.91–150.34)	>900	11.11 (8.58–13.65) <sup>f,g</sup>	>30 <sup>f,g,i</sup>
	3	5	ND	ND			6.01 (5.59–6.42) <sup>f</sup>	>30 <sup>f,j</sup>

<sup>a</sup>Experiment numbers indicate parallel testing of antimonials in assays with the same number. Miltefosine was included as a positive control and displayed EC<sub>50</sub>/EC<sub>90</sub> values (95% confidence intervals in parentheses) of 7.97 μM (4.47 to 11.47)/>20 μM in differentiated THP-1 cells and 1.61 μM (1.24 to 1.98)/>5 μM in PBMC-derived macrophage (mφ) in experiment 1. In experiment 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).

<sup>b</sup>Days indicate the number of days of continuous drug exposure.

<sup>c</sup>PBMC-derived mφ refers to macrophages obtained without positive (CD14) selection.

<sup>d</sup>PBMC-derived mφ (CD14 positive [CD14<sup>+</sup>]) refers to macrophages obtained from positively selected (CD14<sup>+</sup>) monocytes.

<sup>e</sup>ND, not determined.

<sup>f</sup>Cells were derived from two individual blood donors.

<sup>g</sup>Cells were derived from the same blood donor.

<sup>h</sup>Percentage inhibition at 30 μg Sb/ml was 51.6%.

<sup>i</sup>Percentage inhibition at 30 μg Sb/ml was 73.3%.

<sup>j</sup>Percentage inhibition at 30 μg Sb/ml was 77.8%.

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

At the EC<sub>50</sub> level, both pentavalent and trivalent antimony were more potent against *L. donovani* amastigotes in primary human macrophages than in differentiated THP-1 cells by factors of 21 to >100. The difference in the potency of SSG 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 the potency of SSG in macrophages generated from CD14-positive enriched monocytes. Again, SSG was more potent in primary human macrophages than in differentiated THP-1 cells tested in parallel, with up to 20-fold differences at the EC<sub>50</sub> level. Also, SSG already displayed antileishmanial activity in primary human macrophages after 3 days of exposure when either isolation method was used. Data are summarized in Table 1. In macrophages obtained from CD14-positive enriched monocytes, EC<sub>90</sub> values were consistently higher than those estimated in macrophages obtained from total PBMC fractions. However, a systematic comparison of the potencies of SSG 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 CD14-positive enriched monocytes or differentiated THP-1 cells were not higher than those in macrophages obtained from total PBMC fractions (data not shown).

Antileishmanial 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 those in differentiated THP-1 cells at the EC<sub>50</sub> and EC<sub>90</sub> levels after 3 days of compound exposure. Previously reported differences in antileishmanial 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- to 10-fold differences at the EC<sub>50</sub> level. However, variable quality of dose response curve fits, as checked visually, was noted between experiments. Fexinidazole was inactive at concentrations of up to 80 μM in both

**TABLE 2** Potency of nitroheterocyclic compounds against intracellular *L. donovani* amastigotes in three different host cell types<sup>a</sup>

Drug	Expt <sup>b</sup>	Differentiated THP-1 cells		PBMC-derived mφ		PEMs	
		EC <sub>50</sub> (μM [95% confidence interval])	EC <sub>90</sub> (μM [95% confidence interval])	EC <sub>50</sub> (μM [95% confidence interval])	EC <sub>90</sub> (μM [95% confidence interval])	EC <sub>50</sub> (μM [95% confidence interval])	EC <sub>90</sub> (μM [95% confidence interval])
(R)-PA-824	1	2.91 (2.77–3.04)	6.56 (4.66–8.47)	4.59 (4.15–5.03) <sup>c</sup>	5.76 (5.25–6.27) <sup>c</sup>	1.16 (0.89–1.42)	5.58 (3.25–7.92)
	1			2.63 (2.37–2.89) <sup>c</sup>	4.92 (4.36–5.47) <sup>c</sup>		
	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) <sup>d</sup>	4.55 (3.46–5.64) <sup>d</sup>
(S)-PA-824	1	27.83 (24.95–30.71)	>40	38.62 (37.53–39.70)	>40	13.03 (8.05–18.02)	NO <sup>e</sup>
	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) <sup>d</sup>	NO <sup>d</sup>
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	ND <sup>f</sup>	ND	0.39 (0.36–0.42)	0.56 (0.52–0.61)	0.23 (0.21–0.24) <sup>d</sup>	0.45 (0.35–0.56) <sup>d</sup>
	3	ND	ND	0.27 (0.21–0.33)	0.41 (0.39–0.43)	ND	ND
Fexinidazole	3	>80	>80	>80 <sup>c</sup>	>80 <sup>c</sup>	>80	>80
	3			>80 <sup>c</sup>	>80 <sup>c</sup>		
Fexinidazole sulfone	1	3.15 (2.06–4.25)	>20	7.57 (6.72–8.43) <sup>c</sup>	>40 <sup>c</sup>	5.59 (5.21–5.96)	>20
	1			6.85 (4.56–9.13) <sup>c</sup>	>40 <sup>c</sup>		
	3	8.43 (7.38–9.49)	>40	9.37 (4.66–14.07) <sup>c</sup>	>80 <sup>c</sup>	10.44 (6.88–14.00)	>40
	3			14.70 (10.73–18.68) <sup>c</sup>	>80 <sup>c</sup>		
	4	2.24 (1.42–3.06)	>80	20.94 (17.02–24.86)	>80	10.27 (5.64–14.90)	>20

<sup>a</sup>Data is representative of 2 to 4 separate directly comparative experiments.

<sup>b</sup>Experiment numbers indicate parallel testing of respective compounds in assays with the same number. Miltefosine was included as a positive control in experiment 4 and displayed EC<sub>50</sub> values (95% confidence intervals in parentheses) of 8.58 μM (6.39 to 10.76) in differentiated THP-1 cells, 2.67 μM (1.78 to 3.57) in PBMC-derived mφ, and 1.19 μM (0.83 to 1.54) in PEMs.

<sup>c</sup>Cells were derived from two individual blood donors.

<sup>d</sup>PEMs were harvested from CD-1 mice in this assay and BALB/c mice in all other assays.

<sup>e</sup>NO, not obtained.

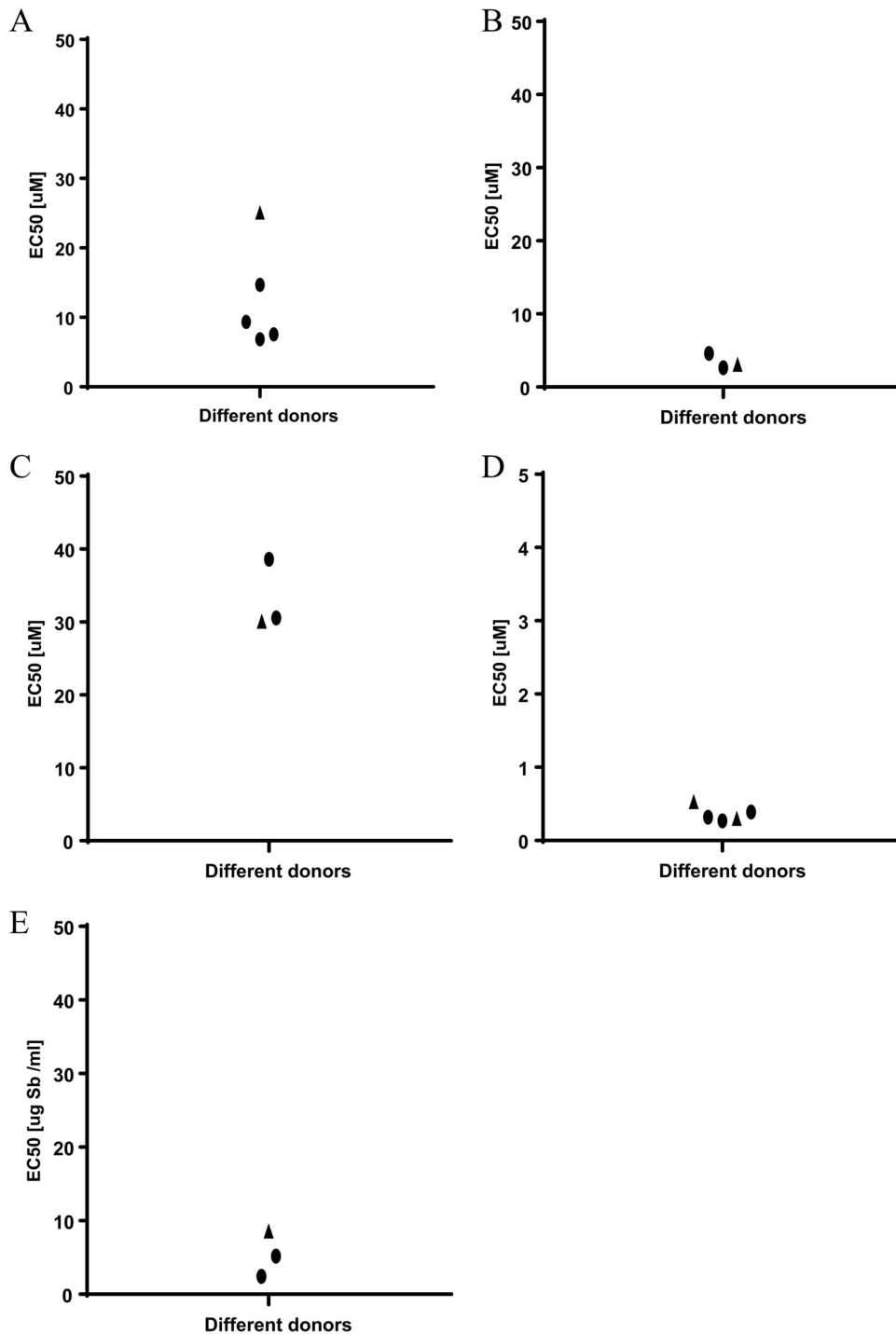
<sup>f</sup>ND not determined.

human-derived cell types. Data are summarized 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 CD14-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 (data not shown). 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. Phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells are widely used in antileishmanial 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 the potencies of reference compounds to other host cells and assay formats have been the focus in developing protocols for antileishmanial 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). Lowering extracellular oxygen tension from 18% to 5% O<sub>2</sub> has also been shown to affect PMA-induced THP-1 cell differentiation and function (24). So far, these effects have not been explored in antileishmanial drug research.

Using primary human macrophages derived from individual blood donors, we found drug potencies to be consistent between different donors (Fig. 1); however, 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 antileishmanial 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 the generation of reactive oxygen species (ROS), nitric oxide (NO), and tumor necrosis factor alpha



**FIG 1** Comparison of EC<sub>50</sub>s between primary human macrophages obtained from different blood donors. Data are given for 3-day 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 (filled circles) or with cells pooled from 2 to 3 individual blood donors (filled triangles).

(TNF-α) 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 cells compared to untreated host cells (28). It is possible that differences in the response to oxidative stress, production of cytokines, expression of drug transport-

ers, 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 antileishmanial drug research, systematic functional characterization of the different cell types and their cell-parasite interactions are needed. Finally, the lack of antileishmanial 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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We declare no conflicts of interest.

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