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Flow Cytometric Screening for Anti-Leishmanials in a Human Macrophage Cell Line

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Abstract

High-throughput drug screening methods against the intracellular stage of *Leishmania* have been facilitated by the development of in vitro models of infection. The use of cell lines rather than primary cells facilitates these methods. Peripheral blood mononuclear cell (PBMC) derived macrophages and THP-1 cells were infected with stationary phase *egfp* transfected *Leishmania amazonensis* parasites and then treated with anti-leishmanial compounds. Drug activity was measured using a flow cytometric approach, and toxicity was assessed using either the MTT assay or trypan blue dye exclusion. Calculated EC₅₀'s for amphotericin B, sodium stibogluconate, and miltefosine were 0.1445±0.0005 µg/ml, 0.1203±0.018 mg/ml, and 26.71 µM using THP-1 cells, and 0.179±0.035 µg/ml 0.1948±0.0364 mg/ml, and 13.77±10.74 µM using PBMC derived macrophages, respectively. We conclude that a flow cytometric approach using *egfp* transfected *Leishmania spp.* can be used to evaluate anti-leishmanial compounds against the amastigote stage of the parasite in THP-1 cells with excellent concordance to human PBMC derived macrophages.

Keywords

Leishmania amazonensis; parasite; amastigote; drug screening; THP-1; flow cytometry

Introduction

Although there are as many as 12 million cases of leishmaniasis worldwide (WHO, 1984), the therapeutic armamentarium for the disease is limited, and the drugs in use are all associated with undesirable side effects. High throughput drug screening facilitates the rapid testing of chemical libraries for compounds with anti-leishmanial activity. However, the complex life-cycle of the parasite involves an extracellular and an intracellular stage with differing metabolic profiles. It is the intracellular stage, or amastigote form, that produces human disease. Screening of drugs against this stage has been facilitated by the availability of *in vitro* models

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of infection using macrophages from human peripheral blood, mouse peritoneal cavity, or cell lines.

The sources of macrophages from animals and from immortalized cell lines for the *in vitro* models have their respective merits, and the variability among them may contribute to their differences in susceptibility to *Leishmania* infection. Monocytic cell lines readily provide a homogeneous population of macrophages, in comparison to those collected from animals or humans, e. g. primary human peripheral blood monocyte (PBMC) derived macrophages (Auwerx, 1991; Prieto et al., 1994; Matthews et al., 2001). The latter are less homogeneous and also known to vary with the sources and from one batch to another. Monocytic cell lines of human origin include U937 and THP-1, which resemble tissue macrophages to various extents (Auwerx, 1991; Prieto et al., 1994; Matthews et al., 2001). The THP-1 cell line, derived from the blood of a human with acute monocytic leukemia, has been shown to support the infection by promastigotes of several *Leishmania* species (Ogunkolade et al., 1990). *L. donovani* and *L. infantum* were found to be as susceptible to anti-leishmanials in these cells as in mouse peritoneal macrophages (Gebre-Hiwot et al., 1992), except when different lipid formulations of amphotericin B were used. (Yardley and Croft, 2000).

We report here that the THP-1 cell line is a suitable surrogate for human PBMC derived macrophages for the *in vitro* screening of antileishmanial compounds against *L. amazonensis* using flow cytometry.

Methods

Culture of parasites

Leishmania mexicana amazonensis transfected episomally with a modified p6.5 plasmid encoding *egfp* and a tunicamycin resistance gene was used (Kawazu et al., 1997). The *egfp* transfected strain was maintained in culture at 24°C in M199 medium plus 10% fetal bovine serum and 10 µg/ml tunicamycin. Stationary phase promastigotes were harvested and washed in phosphate buffered saline prior to use.

Culture of macrophages and cell lines

Peripheral blood mononuclear cells were collected using standard Ficoll gradients from healthy volunteers obtained after informed consent (UCSD IRB# 051038). THP-1 cells were maintained in culture at 2×10^5 – 1×10^6 cells/mL in RPMI 1640 with 10% heat-inactivated fetal bovine serum at 37°C in humidified atmosphere with 5% CO₂. Cells were stimulated with autologous platelet poor serum or 20 nM phorbol myristic acid (PMA) for PBMC and THP-1 cells respectively, prior to infection with promastigotes.

Quantification of intracellular infection

Parasites were added to wells containing macrophages at a ratio of 10:1 in fresh media. Cells were placed in a 34°C incubator. After 1 day, cells were washed to remove extracellular parasites and then anti-leishmanial compounds were added. After further incubation for 1–4 additional days (optimized according to cell type and anti-leishmanial compound used), cells were harvested from wells using Cell Stripper (Mediatech Inc., Manassas, VA), washed and then fixed in 1% EM grade formaldehyde. Fixed cells were analyzed on a Becton Dickinson FACSCanto® cytometer (BD, Franklin Lakes, NJ) with an excitation wavelength of 488 nm and an emission filter of 530/30 nm. Five thousand cells were analyzed for each measurement using the FACSDiva® software (BD).

Drug Screening

The activity of amphotericin B, miltefosine and pentavalent antimony was screened using infected THP-1 cells and primary monocyte derived macrophages (MDM) and the EC₅₀, CC₅₀, and selective index were determined for each of the therapeutic condition applied to both cell types. Six to eight serial dilutions of each of the anti-leishmanial agents in media were added to the wells containing infected cells. Starting concentrations were: 1) amphotericin B - 0.8 µg/ml followed by serial 2 fold dilutions, 2) sodium stibogluconate – 33.33 mg/ml followed by serial 3 fold dilutions, and 3) miltefosine – 100 µM followed by serial 3 fold dilutions. The untreated cells received an equivalent amount of media in place of diluted drug. The EC₅₀ was defined as the concentration of drug which reduced the infection rate to 50% of that seen in the untreated cells based on GFP fluorescence. All assays were performed in three separate experiments for both cell types, and the results from the two of three experiments with the best infection rates in each cell line were chosen for analysis. Samples were normalized to untreated controls, and then a non-linear regression curve fit with a sigmoidal dose-response curve with variable slope model was employed in Graphpad PRISM® to calculate the EC₅₀ and CC₅₀.

Cytotoxicity Assay

The CC₅₀ was defined as the concentration of drug which reduced the viability of the macrophages such that only 50% of the cells survived when compared to the untreated group. The MTT assay, which measures the reduction of the yellow MTT dye to purple formazan by living cells, was used to assess cytotoxicity of amphotericin B and miltefosine to macrophages. However, since sodium stibogluconate itself was found to affect the MTT assay, the standard trypan blue dye exclusion test was used for that drug. In this method, viable (clear) and non-viable (blue) cells were counted using a hemocytometer to determine cell viability in percent. All cytotoxicity assays were performed in duplicate. CC₅₀ was calculated using Graphpad PRISM® as described above.

Selective Index Calculation

The selective index(SI) was calculated by determining the ratio of the EC₅₀ to the CC₅₀.

Results

Flow cytometric evaluation of infected cells for GFP fluorescence revealed infection rates equivalent to $44.1 \pm 16.3\%$ and $63.8 \pm 12.9\%$ for untreated PBMC derived macrophages and untreated THP-1 cells, respectively. The mean EC₅₀, CC₅₀, and SI calculated for each anti-leishmanial compound in each cell line is shown in Table 1. The effect of anti-leishmanial compounds on this infection is demonstrated by Figure 1, which is a representative set of histograms demonstrating that the median fluorescence intensity, as a measure of infection, decreased with increasing concentrations of amphotericin B. Amphotericin B was found to be slightly more effective than sodium stibogluconate in the human macrophages (EC₅₀ of 0.179 ± 0.035 µg/ml and 0.1948 ± 0.0364 mg/ml, respectively), and slightly less effective in THP-1 cells (EC₅₀ of 0.1445 ± 0.0005 µg/ml and 0.1203 ± 0.018 mg/ml respectively) while miltefosine was much less active in both cell types. Amphotericin B had a much larger selective index(SI) than did either sodium stibogluconate, or miltefosine in both human macrophages (SI of 355.9, 32.34, and 6.13, respectively) and THP-1 cells (SI of 105.2, 38.24, and 1.51 respectively). Measurement of infection of the EC₅₀ of amphotericin B was also performed using standard Giemsa staining of cells with same conditions used for the flow cytometry experiments. An infection rate of 46% was seen, and a EC₅₀ of 0.074 µg/ml was calculated, similar to what was seen using our flow cytometric system.

Discussion

The drug susceptibilities determined in this *in vitro* system were comparable between the THP-1 cell line and isolated human macrophages, and correlated very well with those reported in the literature (Yardley and Croft, 2000; Ayres et al., 2008; Varela et al., 2009). The flow cytometric method used reported previously for screening anti-leishmanial compounds against the clinically relevant amastigote stage of GFP-transfected *Leishmania (Viannia) panamensis* (Varela et al., 2009) in the U937 cell line. In that report CD33 was used as a marker for macrophages and level of GFP fluorescence used as a measure of parasitic infection, and the authors found that two anti-leishmanial compounds, meglumine antimoniate and amphotericin B gave EC₅₀'s of 20 and 0.078 µg/ml respectively, which were comparable to those previously determined by conventional methods with animal-derived macrophages. (Yardley and Croft, 2000).

Our data (Table 1) are consistent with, albeit not identical to, those previously reported for *L. amazonensis* amastigotes in murine peritoneal macrophages, i.e. CC₅₀'s of 30.4, 0.5, and 16.0 µg/ml for glucantime, amphotericin B and miltefosine, respectively (Ayres et al., 2008). Differences in experimental conditions make absolute comparisons difficult.

We also calculated a therapeutic index (SI) for each of the three drugs. The SI values calculated after day 1 of therapy for amphotericin B and sodium stibogluconate were fairly high in human macrophages (356 and 32 respectively). Interestingly, other groups have found that the peak concentration of sodium stibogluconate during standard clinical therapy (10 mg/kg of Sb) resulted in peak serum concentrations of 0.008–0.01 mg/ml which is well below the EC₅₀ we calculated in this study (Chulay et al., 1988; al et al., 1995). On the other hand, work by Atkinson and Bennett showed that standard doses of amphotericin B (0.3 mg/kg on days 1 and 2 followed by increasing doses to 0.5 mg/kg) during clinical therapy are expected to maintain a serum level of this drug above 0.2 µg/ml, which was just above our calculated EC₅₀ (Atkinson, Jr. and Bennett, 1978). We did notice that during our experiment a longer miltefosine exposure was required in human macrophages to reduce the infection. Therefore for miltefosine, we treated infected human macrophages with drug for 4 days in attempt to improve the therapeutic effect, but significant toxicity against the macrophages was seen. The calculated SI for miltefosine was very low (1.51 for THP-1 cells, and 6.13 for PBMC derived macrophages), suggesting a narrow therapeutic window. Miltefosine's mode of action against *Leishmania* was postulated to be via interference with its cellular carrier systems and/or ether-lipid biosynthesis leading to induction of apoptotic DNA fragmentation in the parasites. Miltefosine is known to be teratogenic to mammalian cells, and it is likely that there is some induction of apoptosis in the macrophages as well (Verma and Dey, 2004). Nonetheless several large scale clinical trials of miltefosine performed in India have shown excellent anti-leishmanial efficacy, and tolerable side effects. A recent pharmacokinetic study performed on Dutch soldiers returning from Afghanistan with cutaneous leishmaniasis and treated with a 28 day course of miltefosine (150 mg daily), found a median concentration during the last week of treatment of 3.08 mg/ml (Dorlo et al., 2008) which is close to the CC₅₀ of 75 uM calculated in our system. It is clear from the human pharmacokinetic studies that *in vitro* models can be used to find lead compounds, but are not necessarily predictive of the effective serum drug levels for clinical application.

We conclude that *Leishmania* species, such as *L. amazonensis*, episomally transfected to produce GFP with the p6.5 expression vector, can be used to evaluate anti-leishmanial compounds against the amastigote stage of the parasite in THP-1 cells with excellent concordance to human PBMC derived macrophages. Although this *in vitro* system is not fully predictive of plasma levels required for clinical responses, the method should facilitate the screening of anti-leishmanial drugs for promising lead compounds against clinically relevant amastigotes.

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Figure 1.

Histograms of THP-1 cells cultured with *L. amazonensis* for A) untreated cells, and B) treated with 0.1 µg/ml of amphotericin B, C) treated with 0.8 µg/ml of amphotericin B, D) treated with 0.39 µM miltefosine, E) treated with 100 µM miltefosine, F) treated with 0.4 µg/ml of sodium stibogluconate, and G) treated with 50 µg/ml of sodium stibogluconate. These histograms contain two peaks corresponding to uninfected cells on the left and infected cells on the right. Increasing concentrations of amphotericin B, miltefosine, and sodium stibogluconate demonstrate shifting of the population so that the right peak diminishes in size and the left peak increases in size. Of note, for miltefosine, cells were harvested at 4 days after drug exposure as opposed to 1 or 2 days (depending on cell type) with amphotericin B and sodium stibogluconate. Thus, the histogram with the low level miltefosine exposure demonstrating increased shift to the right had undergone an additional two days in culture before harvest.

EC₅₀, CC₅₀, and Selective Index (SI) of sodium stibogluconate, amphotericin B and miltefosine in THP-1 cells and human PBMC derived macrophages

Table 1

	THP-1 cell			Human Macrophage		
	EC ₅₀	CC ₅₀	SI	EC ₅₀	CC ₅₀	SI
Sodium stibogluconate (mg/ml)	0.1203±0.018	4.6±1.1	38.24	0.1948±0.0364	6.3±2.8	32.34
Amphotericin B (µg/ml)	0.1445±0.0005	15.2±0.7	105.2	0.179±0.035	63.7±18.2	355.9
Miltefosine (µM)	26.71	40.5±12.0	1.51	13.77±10.74	84.5±6.9	6.13