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Rapid fluorescent assay for screening drugs on *Leishmania* amastigotes

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ABSTRACT

A rapid fluorescent viability assay employing alamarBlue was optimized for use with *Leishmania* axenic amastigotes, the stage of the parasite responsible for disease pathology. The activity of two protein kinase inhibitors, Staurosporine and H-89, as well as Amphotericin B, on promastigotes and amastigotes of *Leishmania donovani* and *Leishmania tropica* was compared. Both protein kinase inhibitors inhibited promastigote growth at lower concentrations than amastigotes, while the GI_{50} for Amphotericin B on both stages was similar. This assay only requires a limited number of axenic amastigotes (50,000 cells/well) and can be used to rapidly screen large chemical or natural product libraries for activity against amastigotes.

1. Introduction

Leishmania, protozoan parasites belonging to the Trypanosomatida family, cause three main forms of human diseases: cutaneous, mucocutaneous and visceral leishmaniasis, as well as visceral disease in dogs. The leishmaniases are endemic in 88 countries, responsible for disease in approximately 12 million people and threaten 350 million people worldwide. An estimated 1–2 million new cases occur annually (WHO, 2007, Desjeux, 2004, Berman, 2005). First line drugs such as Pentostam, Amphotericin B and its liposomal derivatives are generally toxic, expensive and/or require hospitalization (Berman, 2005). Resistance, especially to *Leishmania donovani* that causes fatal visceral disease, is an increasing problem and new drugs are urgently needed (Berman, 2005, Lira et al., 1999, Croft and Coombs, 2003). Several methods are available to screen potential compounds for activity on *Leishmania* and include counting parasites, labeling with radioactive nucleotides, colorimetric metabolic indicators, flow cytometry or transgenic parasites (Fumarola et al., 2004, Sereno et al., 2007). However many studies still measure leishmanicidal activity using promastigotes, the extracellular stage of the parasite found in the sandfly vector, which are easy to grow in culture, rather than amastigotes, the intracellular stage that resides in macrophages and causes disease. Assays based on staining and counting parasites in infected macrophages are laborious, time consuming and difficult to scale up into a system for high-throughput screening (HTS) (Fumarola et al., 2004, Sereno et al., 2007). On the other hand, HTS of drugs on promastigotes is inherently inaccurate, since compounds active only on amastigotes, the intracellular stage, will be missed (Ephros et al., 1999).

We have optimized an *in vitro* fluorescent microplate assay based on the reduction of alamarBlue for use with axenic amastigotes. Axenic amastigotes grown under environmental conditions that mimic the intracellular stage of the parasite are metabolically and antigenically similar to tissue amastigotes (Gupta et al., 2001). The alamarBlue viability assay was optimized using amastigotes of *L. donovani* and *Leishmania tropica* that cause visceral and cutaneous leishmaniasis, respectively. It is rapid, reliable and simple and does not require cell lysis, washing or extraction steps. Furthermore, it can be used to develop inexpensive HTS assays for the routine screening of new anti-leishmanial drug candidates.

2. Materials and methods

2.1. Parasites

Leishmania used in this study were *L. donovani* MHOM/SD/1962/1S-Cl2D and *L. tropica* MHOM/IS/1990/LRC-L590.

Promastigotes were grown at 26 °C in complete medium: Medium-199 (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mM L-glutamine, 100 μM adenosine, 23 μM folic acid, antibiotics (100 IU penicillin G and 100 μg/ml streptomycin), 1×BME vitamin mix, 25 mM 2-(N-morpholino) ethanesulfonic acid (MES), 4.2 mM NaHCO3 and heat-inactivated fetal calf serum (fcs, 10% v/v) adjusted to pH 6.8.

Axenic amastigotes of *L. donovani* were grown as described by Debrabant et al. (2004) in complete RPMI 1640 containing 20% fcs at

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37 °C, pH 5.5. Axenic amastigotes of *L. tropica* were cultured like *L. donovani* with two modifications: first, only 10% v/v fcs was used and second, the amastigotes were grown at 36 °C without CO_2.

2.2. Optimization of alamarBlue assay and drug-screening

Effect of alamarBlue incubation time on fluorescence was examined by diluting *L. donovani* and *L. tropica* promastigotes or axenic amastigotes (8×10^6 to 10^2 parasites/well) in complete medium appropriate for each stage. Parasites at each concentration (250 µl/well) were aliquoted in triplicate into 96-well flat-bottom plates (NUNC™, Denmark) and alamarBlue (25 µl/well, AbD Serotec, UK) was added. The plates were read (λex=544 nm; λem=590 nm) after increasing incubation periods up to 72 h using a fluorescent microplate reader (Fluoroskan Ascent FL, Finland).

Standard conditions for the axenic amastigote viability assay were established essentially as described above with the following changes. *L. donovani* and *L. tropica* amastigotes (2×10^6 to 10^2 parasites/well) were diluted in complete medium appropriate for each stage. Each concentration was aliquoted on 96-well plates in triplicate and cultured 24 h under conditions appropriate for each stage and species.

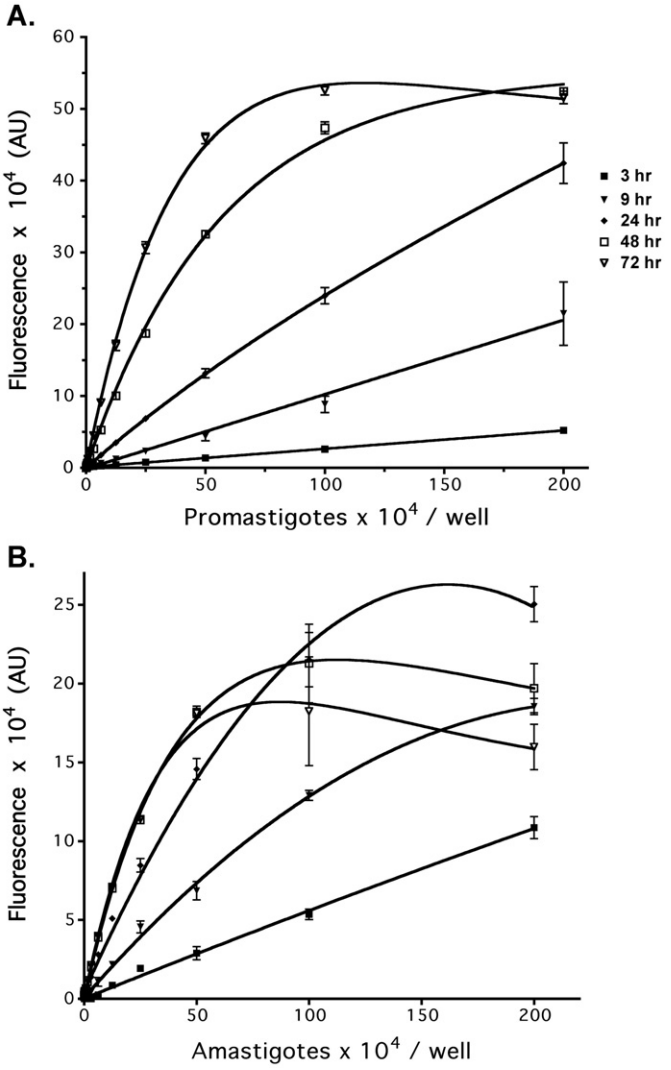

Fig. 1. Effect of incubation time with alamarBlue on the fluorescent standard curves produced by promastigotes and amastigotes during growth in culture. Increasing concentrations of promastigotes (panel A) or amastigotes (panel B) were incubated directly with alamarBlue (10% v/v) at either 26 °C or 37 °C, respectively, and the fluorescence measured (λex=544 nm; λem=590 nm) at the indicated times.

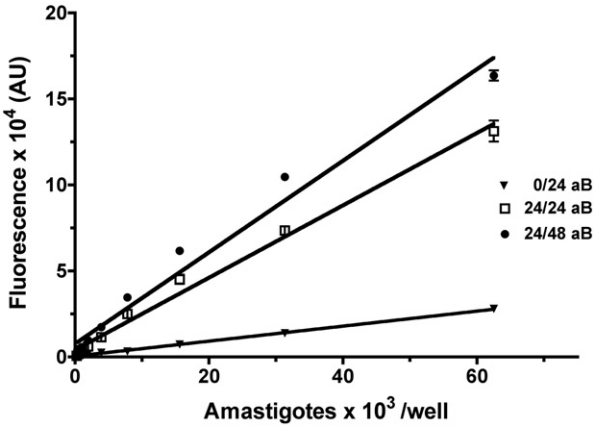

Fig. 2. Comparison of pre- and post-incubation times on the fluorescence produced by amastigotes in the alamarBlue viability assay. Increasing concentrations of axenic amastigotes were incubated directly with alamarBlue (10% v/v) for 24 h (0/24 aB) and the fluorescence measured (λex=544 nm; λem=590 nm). Alternatively, the same parasites were first cultured for 24 h prior to adding alamarBlue and then incubated for an additional 24 or 48 h (24/24 aB or 24/48 aB) before measuring the fluorescence as above.

AlamarBlue was added (10% v/v) and the plates read 24 h later using a fluorescent microplate reader as above.

Final drug-screening assay was carried out as follows. Promastigotes (2×10^6 cells/ml) or axenic amastigotes (4×10^5 cells/ml) were aliquoted in triplicate (125 µl/well) into 96-well flat-bottom plates containing each drug diluted in the appropriate complete medium (125 µl/well, containing 1% DMSO final concentration). Staurosporine, H-89 and Amphotericin B were obtained from Sigma-Aldrich (St. Louis, MO). Complete medium containing DMSO was used as a negative control and the parasites with or without drugs were incubated either at 26 °C (promastigotes), 36 °C (*L. tropica* axenic amastigotes) or 37 °C (*L. donovani* axenic amastigotes). Twenty-four hours later alamarBlue (25 µl/well) was added, the plates were incubated for an additional 24 h and the fluorescence read as described above. Data was analyzed using Prism 4 (GraphPad Software, San Diego, CA).

3. Results

3.1. Optimization of fluorescent viability assay for axenic amastigotes

The incubation times and concentrations of *L. donovani* promastigotes and axenic amastigotes were compared by adding alamarBlue to parasite cultures and measuring the change in fluorescence over time (Fig. 1). A direct correlation between fluorescence and parasite number was seen for both promastigotes and amastigotes. However the range of parasite concentrations showing a linear correlation with fluorescence decreased, as the time period of incubation for either stage with alamarBlue increased (Fig. 1). Over short incubation periods (3, 9 and 24 h) good linear correlation (r^2=0.997, 0.908 and 0.985, respectively) between promastigotes and fluorescence (Fig. 1A) was seen for most of the concentrations examined (100 to 2×10^6 cells/well), though already by 24 h some deviation from linearity is apparent at promastigote concentrations $>2\times10^6$/well (data not shown). Longer incubation times with alamarBlue, 48 and 72 h, resulted in a further increase in the slope of the curve allowing for better discrimination at lower parasite concentrations $<5\times10^5$/well, but a complete loss in linearity at higher parasite concentrations. The fluorescence plateaued or even decreased slightly at 48 and 72 h when concentrations $>10^6$ promastigotes/well were used. A decrease in the fluorescence of alamarBlue has been reported when either long incubation times or excess cells are used in assays with this viability indicator (O'Brien et al., 2000). Therefore, viability assays that are carried out for >24 h with alamarBlue in the medium require lower promastigote concentrations between 6×10^4 to 2.5×10^5 cells/well.