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In Vitro Screening Test Using *Leishmania* Promastigotes Stably Expressing mCherry Protein

Paola Vacchina, Miguel A. Morales

Eck Institute for Global Health, Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana, USA

Transgenic *Leishmania major* and *Leishmania donovani* axenic promastigotes constitutively expressing mCherry were used for *in vitro* antileishmanial drug screening. This method requires minimal sample manipulation and can be easily adapted to automatic drug tests, allowing primary high-throughput screenings without the need for expensive and sophisticated instruments.

Protozoan parasites of the genus *Leishmania* are the causative agents of a wide spectrum of human and animal diseases. The clinical manifestations of *Leishmania* infections range from lesions of the skin and mucous membranes to lethality, the latter caused by visceral species, including *Leishmania donovani* (1). *Leishmania* species cause morbidity and mortality throughout large areas of the Old and New World. Leishmaniasis is an emerging disease with an annual incidence of 2 million cases, and more than 12 million people are infected in over 80 countries where the disease is endemic (2), causing 80,000 deaths per year, and 350 million people are at risk of infection and disease. The increased prevalence of *Leishmania*-human immunodeficiency virus (HIV) coinfection is the major reason for the recent emergence of leishmaniasis in the Western world (3).

Since their discovery in the 1940s, the highly toxic pentavalent antimonials [Sb(V)] have been the primary first-line treatment for all types of leishmaniasis in most parts of the world. However, the increasing frequency of relapse in leishmaniasis patients is forcing the use of other chemotherapeutic agents, including amphotericin B, isethionate pentamidine, paromomycin, and miltefosine (4, 5). Unfortunately, the majority of these antiparasitic drugs present severe side effects, there is no guarantee of cure, and some of them are frequently accompanied by emergence of drug resistance (6–8).

Alternative treatments are needed; however, advances in drug discovery approaches have not been satisfactory. Although the use of axenic promastigote cultures of *Leishmania* as a primary screening method has being validated (7–11), the traditional approaches employed to determine the drug effect in culture present several shortcomings. Microscopic counting is prone to error, time-consuming, and requires trained personnel; the use of tetrazolium salts such as MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] to measure cell viability is labor-intensive, and results are not always reliable (12). The more sensitive and less labor-intensive colorimetric resazurin-based method may present variable metabolic behavior under different cell culture conditions. Furthermore, it is not appropriate to perform kinetic experiments due to the significant cytotoxicity that results from prolonged exposure to the reagent (13–15). Other disadvantages of these metabolic assays are the requirement to incubate the substrate with viable cells at 37°C for a sufficient time to generate a measurable signal. This may lead to undesirable artifacts resulting from interactions between the reagent, the tested drug, and the biochemistry of the cells (16). On the basis of these considerations, there is a strong need to develop appropriate screening technologies. The use of fluorescent reporter genes such as those coding for green fluorescent protein (GFP) (17–19) and the red versions red fluorescent protein (RFP) (20, 21) and mCherry (21, 22) have considerably facilitated the screening and testing of antimicrobial agents against axenic amastigotes and intracellular amastigotes, allowing both *in vitro* and real-time visualization *in vivo*. Although animal models are well established for large-scale primary drug screening, its practical use is limited due to high costs.

Here, we introduce a simple fluorometric method for primary drug screening using red fluorescent promastigotes. We generated *Leishmania major* and *L. donovani* parasite cultures stably expressing the mCherry gene, whose product is a red monomeric fluorescent protein derived from the nowadays obsolete tetrameric *Discosoma* protein DsRED and presents an excitation maximum at 587 nm (587_{ex}) and an emission maximum at 610 nm (610_{em}). mCherry is constitutively expressed in the cytoplasm, presents high photostability, is resistant to photobleaching, and does not require posttranslational modifications like GFP does. It matures very rapidly, allowing fluorescence detection shortly after the gene is expressed, which is appropriate for real-time analysis (12, 23).

L. major promastigotes (MHOM/JL/80/Friedlin) and *L. donovani* promastigotes (MHOM/SD/62/1S-CL2D) were maintained at 27°C in M199 medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biolabs). The mCherry gene (711 bp) was obtained from the pmCherry-N1 vector (Clontech, Palo Alto, CA) by PCR amplification (LongAmp *Taq*; NEB). The PCR product was purified and cloned into pGEMT (Promega) to create the construct mCherry-pGEMT and further subcloned into the *Leishmania* expression vector pLEXSY-Hyg2 (Jena Bioscience) (Fig. 1A). Five micrograms of the resulting expression plasmid mCherry-pLEXSY was digested with SwaI. The linearized expression cassette was electroporated as described previously into *L. major* and *L. donovani* log-phase cultures, and transfected parasites were selected in the presence of hygromycin B (Sigma) at a final concentration of 75 μg/ml (24). Integration into the chro-

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Address correspondence to Miguel A. Morales, miguel.morales@nd.edu.
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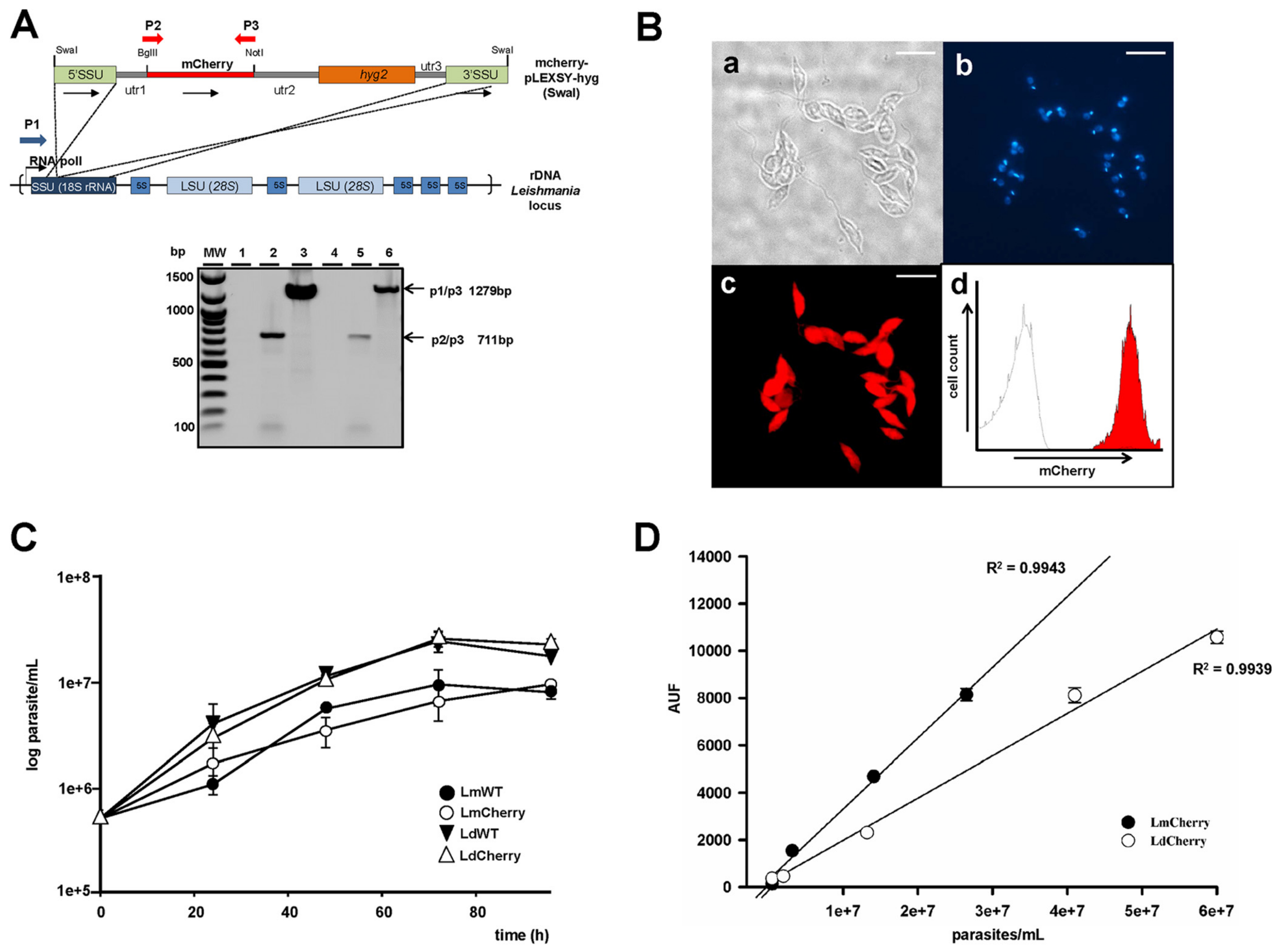

FIG 1 (A) Integration of the mCherry-pLEXSY expression cassette into the *Leishmania* 18S rRNA locus previously linearized with SwaI. The black arrow indicates the start of transcription from the rRNA genes. Hygromycin B (*hyg2*) is used as a selection marker. P1 (blue arrow) and P2 and P3 (red arrows) indicate the positions where primers anneal (upper panel). Diagnostic PCR was performed to corroborate genomic integration. A band of 711 bp was obtained from mCherry-positive clones of *L. major* (lane 2) and *L. donovani* (lane 5). Integration of mCherry-pLEXSY into the *L. major* and *L. donovani* genomic DNA appeared as a 1.3-kbp fragment, not obtained from WT strains (lanes 1 and 4). Lane MW, molecular size markers. (B) mCherry detection by epifluorescence microscopy and flow cytometry analysis. Bright-field (a), NucBlue fixed cell stain (b), and red fluorescent (c) images of *L. donovani* expressing mCherry are shown. Scale bar, 10 μm. (d) Histograms of fluorescence intensity of WT (dotted line) and transfected (red filled) *L. donovani* promastigotes. (C) Growth curves of WT and transfected *L. major* and *L. donovani* parasites. (D) Quantification of mCherry fluorescence. The graph shows a linear relationship between the number of promastigotes and fluorescence AUF of mCherry. R^2, correlation coefficient. Results represent the average of three independent experiments.

mosomal 18S rRNA locus (*ssu*) was confirmed by PCR. A primer pair including one primer hybridizing within the expression cassette (P3) and another hybridizing to an *ssu* sequence not present on the plasmid was used (Fig. 1A). Additional PCRs that involved mCherry-specific primers (P2 and P3) were performed. A band of 1,279 kbp confirmed the integration of the gene, and a band of 711 bp was also obtained for mCherry-positive clones. Primer sequences are available upon request.

mCherry expression in the two *Leishmania* species was tested by epifluorescence microscopy (Nikon 90/NikonAZ 100). Cells were washed with phosphate-buffered saline (PBS), fixed in 4% formaldehyde, and subsequently stained with NucBlue fixed cell stain (Life Technologies) to visualize the nucleus and kinetoplast (magnification, ×60). Transgenic parasites showed normal DNA content and bright cytoplasmic red fluorescence, indicating that the fixation step did not quench mCherry fluorescence, making this cell line extremely valuable (Fig. 1B). The high level of fluorescence detected was maintained in the axenic amastigote stage (data not shown). mCherry expression was also analyzed in wild-type (WT) and transgenic promastigotes in a Beckman Coulter FC500 MPL flow cytometer (Fig. 1B). Morphometric profiles of transfected parasites were comparable to the WT ones (data not shown). To examine parasite fitness, the growth rate of transfected cultures was measured and compared to that of WT strains. Parasites were seeded in triplicate (5×10^5 cells/ml), and the concentrations of *L. major* and *L. donovani* WT (LmWT and LdWT) cultures and *L. major* and *L. donovani* mCherry-pLEXSY (LmCherry and LdCherry) cultures were assessed daily by direct microscopic counting until the parasites reached stationary phase. No differences were observed in parasite growth rates (Fig. 1C). Additionally, to evaluate culture fluorescence in relation to parasite concentration, the fluorescence of each culture was measured

TABLE 1 EC_{50}s of reference antileishmanial drugs obtained with CellTiter-Blue test ($555_{ex}/580_{em}$) or by mCherry quantification ($518_{ex}/605_{em}$) with WT and transfected parasites

Parasite	EC_{50} (mean ± SD)					
	Miltefosine (μM)		Amphotericin B (nM)		Isethionate pentamidine (μg/ml)	
	CellTiter-Blue test	mCherry fluorescence	CellTiter-Blue test	mCherry fluorescence	CellTiter-Blue test	mCherry fluorescence
LmWT	11.47 ± 0.86		16.77 ± 2.33		4.73 ± 0.78	
LmCherry	10.54 ± 0.43	12.99 ± 0.77	14.44 ± 4.80	16.73 ± 0.53	5.67 ± 1.98	4.98 ± 0.31
LdWT	13.14 ± 0.73		20.81 ± 1.27		0.74 ± 0.11	
LdCherry	16.74 ± 3.95	16.67 ± 2.67	22.40 ± 2.62	17.89 ± 3.06	0.83 ± 0.34	0.75 ± 0.02

($518_{ex}/605_{em}$) daily for 4 days and subsequently plotted against parasite concentration (Fig. 1D). The background fluorescence was subtracted from the fluorescence of each sample. Results demonstrated that the two strains presented a linear relationship between promastigote number (parasites/ml) and the quantified fluorescence (arbitrary units of fluorescence [AUF]), with correlation coefficients of >0.99. While the linear response for *L. major* was between 5×10^5 and 3×10^7 parasites/ml, *L. donovani* showed a wider range, between 5×10^5 and 6×10^7 parasites/ml.

The antileishmanial effects of the reference compounds miltefosine (Sigma), amphotericin B (Sigma), and isethionate pentamidine (Sigma) were evaluated in transfected parasites and compared with those in WT cultures. The 50% effective concentration (EC_{50}) was defined as the concentration of drug required to reduce by 50% the initial parasite concentration. Culture viability was assessed by using the fluorometric resazurin-based reference method CellTiter-Blue (Promega) (25, 26). Briefly, 1×10^6 cells/ml were seeded in a 96-well plate and incubated in the presence of increasing drug concentrations for 72 h at 27°C along with appropriate solvent controls. Afterwards, 20 μl of CellTiter-Blue reagent was added to 100 μl of culture. After 4 h at 37°C, fluorescence was measured ($555_{ex}/580_{em}$) using a Typhoon FLA 9500 laser scanner (GE Healthcare) and analyzed with ImageQuant TL software (GE Healthcare). Table 1 shows the EC_{50}s obtained. For the three compounds tested, no differences in antileishmanial activities were detected, indicating that transfection and constitutive mCherry expression did not affect drug efficacy. Finally, we evaluated the use of mCherry fluorescence quantification in order to assess cell viability in transgenic *Leishmania* promastigotes. LmCherry and LdCherry cultures (1×10^6 cells/ml, 200 μl/well in a 96-well plate) were incubated in the presence of increasing drug concentrations. After the incubation period (72 h at 27°C), fluorescence was quantified ($518_{ex}/605_{em}$) and EC_{50}s were determined (Table 1). Data reported here show good accordance between EC_{50}s obtained with the resazurin-based method and by direct measurement of fluorescent cultures.

To our knowledge, this is the first report in which mCherry-transfected *L. donovani* promastigotes were used as a tool to perform primary drug screenings. Previous work using *Leishmania major* (21) and *Leishmania infantum chagasi* (22) introduced the use of mCherry as a reporter gene to monitor *in vitro* and *in vivo* infections. The aim of this work is to validate an alternative method for primary drug screening on promastigotes of different *Leishmania* species. The proposed approach is inexpensive and does not require the use of host cell-based assays or animal models. Our results confirm that this approach is rapid and reliable and is in good accordance with results obtained by the standard resazurin-based assay. Although the procyclic promastigote form is not the infective stage and differences in susceptibility to drugs have been previously addressed, the implementation of a low-cost method with few manipulation steps is highly valuable in reducing the extent of drug libraries. Remarkably, this method requires only a microplate reader in order to be implemented and can be easily adapted to automatic drug testing, allowing primary high-throughput screenings without the need for expensive and sophisticated instruments. This last feature is particularly appealing to laboratories with limited resources.

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