COLORIMETRIC ASSAY FOR SCREENING COMPOUNDS AGAINST LEISHMANIA AMASTIGOTES GROWN IN MACROPHAGES

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Abstract. An estimated 12 million persons throughout the world suffer from the protozoan disease leishmaniasis. Current treatments have liabilities including poor activity against some forms of leishmaniasis, toxicity, or the need for parenteral administration. Higher throughput methods to screen chemical compounds are needed to facilitate the search for new antileishmania drugs. In the mammalian host, Leishmania parasites exist as amastigotes that replicate within macrophages. Therefore, an in vitro screening assay using intramacrophage amastigotes most closely represents the natural infection. We have transfected strains of Leishmania major and Leishmania amazonensis with the β-lactamase gene, which catalyzes a colorimetric reaction with the substrate nitrocefin. The growth of these β-lactamase–expressing Leishmania within macrophages was quantified in 96-well plates using an optical density plate reader, thus simplifying the methodology for scoring inhibitor assays. This simple and relatively inexpensive colorimetric assay helps improve throughput for screening compounds for antileishmania activity.

INTRODUCTION

The opportunity for discovering new therapeutics to treat leishmaniasis has never been better with the recent completion of the Leishmania Genome Project and progress toward characterizing the transcriptome, proteome, and protein structures of this pathogen (http://www.sgpp.org). This explosion of information converges with modern high-throughput methods of screening compounds against molecular targets. During the next decade, it is likely that many thousands of molecules will be screened against leishmanial drug targets, and a significant subset of inhibitors will be suitable for follow-up testing against Leishmania parasites grown in culture. Unfortunately, low-throughput screening assays against the Leishmania cultures pose a major obstacle to rapid identification of lead antileishmania compounds.

Current techniques for screening of Leishmania cultures involve various compromises. Testing promastigotes can be done rapidly, as with any cells that grow in suspension culture. However, promastigotes are not the relevant life-cycle stage for mammalian Leishmania infection, and correlation of data from promastigotes and amastigotes is unreliable. Axenic amastigotes can also be screened efficiently, but by removing the parasite from its intracellular niche, the assay does not test for penetration of the compound into the host cell nor for activity in the peculiar environment of the macrophage phagolysosome. In addition, axenic amastigotes may have different metabolic processes than intracellular amastigotes. The preferable method for in vitro testing of compounds against Leishmania amastigotes involves growing amastigotes in mammalian macrophages. However, quantifying the growth of intracellular amastigotes is usually done by direct visual examination of stained cells on glass microscope slides. This method is labor intensive, subjective, and incompatible with high-throughput screening. A more efficient method for quantifying growth of intracellular Leishmania amastigotes would help remove the drawbacks of current screening methods.

The use of reporter genes to monitor intracellular proliferation of microorganisms has been effectively applied for bacteria, viruses, and other parasites. Such methods produce objective quantitative data, increase throughput, and decrease manual labor. A variety of reporter genes have been effectively used in biological screens including green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), β-galactosidase, firefly luciferase, and alkaline phosphatase. A multimeric form of GFP has been expressed in Leishmania amazonensis; however, the application for drug screening is limited to promastigotes. A useful method employing the luciferase reporter gene has also been developed, but a luciferase system may have certain drawbacks. First, the luminescent readout is transient, and mixing of the sample and the reagent needs to be timed with entering samples into the luminometer. Second, the reagents for running the luciferase assay and plate luminometers are expensive. Leishmania parasites have also been engineered to express β-galactosidase, but high background activity from host macrophages prevents the use of this reporter system in amastigote-macrograph drug screening assays (Buckner FS, unpublished data).

Here we report the use of the β-lactamase reporter gene for quantifying Leishmania amastigotes in macrophages grown in microtiter plates. The β-lactamase gene was integrated into the rRNA region of the genome, thereby allowing for high-level stable expression of the enzyme. A colorimetric readout (yellow to red) using the relatively inexpensive reagent, nitrocefin (Figure 1), was quantified by directly placing the culture plates into an enzyme-linked immunosorbent assay (ELISA) plate reader. Strains of L. major and L. amazonensis were engineered for this assay. The method will facilitate relatively high-throughput screening of these strains against chemical libraries with potential antileishmania activity.

MATERIALS AND METHODS

Chemicals. Nitrocefin and CENTA were purchased from Calbiochem (La Jolla, CA). Sodium stibogluconate for injection (Pentostam, Wellcome Foundation, London, UK) was obtained from the Centers for Disease Control (Atlanta, Georgia). Nourseothricin was purchased from Sigma (St. Louis, MO). Amphotericin B for injection was purchased from Pharma-Tek, Inc. (Huntington, New York). The azole antifungal drugs were provided in pure powder form from the antibiotic testing laboratory in Clinical Microbiology at the University of Washington Medical Center. The azole compounds were quality tested against National Committee
Fixed in methanol for 5 minutes, and stained with Giemsa (Sigma) for microscopic analysis. Total number of amastigotes in 100 macrophages were counted.

β-lactamase expression vector. pIR1SAT was kindly provided by S. Beverley (Washington University). The plasmid sequence and restriction map are available via the Internet: http://www.microbiology.wustl.edu/dept/fac/beverley/DNA/exp/B354120pIR1SAT.doc. When linearized, this vector integrates into the Leishmania genome to replace one copy of the SSU rRNA gene. This location is transcribed by pol1, therefore high levels of expression occur. In addition, the SSU gene is highly conserved among Leishmania species, allowing a single vector to be used in different species.

The β-lactamase gene was cut from the vector, blam(-)/pUC (PanVera, Madison, WI) using XbaI and Xmal, and subcloned into expression site of pIR1SAT using the same enzymes. Correct cloning was confirmed by DNA sequencing of the resulting plasmid, pIR1SAT-BLA. This plasmid was digested with SvaI, which linearized it in preparation for electroporation.

Transfection of Leishmania. L. major and the L. amazonensis strains were electroporated using the same conditions. Mid-log promastigotes (~1 × 10^7/mL) were collected by centrifugation and washed once with PBS + 2% glucose. The cells were resuspended in cold electroporation buffer (NaH2PO4 0.7 mM, KCl 5 mM, NaCl 137 mM, Hepes 21 mM, and glucose 6 mM), centrifuged, and resuspended in electroporation buffer at 2 × 10^6 cells/mL. A volume of 0.4 mL cells was mixed with 10 μg of linearized DNA and placed in a 2 mm gap BTX electroporation cuvette (BTX Inc., San Diego, CA). The cuvette was chilled on ice for 10 minutes then electroporated using an ElectroCell Manipulator 600 (BTX Inc.) set at 480 V, 13 μF, and 500 μF. The electroporated cuvette was placed on ice for 10 minutes, then the cells were transferred to a flask with 10 mL of promastigote culture medium. Two days post-electroporation, nourseothricin 50 μg/mL was added to select for parasites expressing the streptothricin acetyltransferase (SAT) gene. Robust cultures of nourseothricin-resistant cells arose approximately 10–14 days after the electroporation. The cell populations were cloned by limiting dilution in 96-well plates.

Mouse infections. Because Leishmania parasites that are grown in laboratory conditions tend to lose virulence, we infected BALB/c mice with the β-lactamase–expressing clones. Mice were injected subcutaneously at the base of the tail with 1 × 10⁶ stationary-phase promastigotes in a volume of 100 μL of RPMI-1640. Mice were monitored for lesion development (approximately 2 weeks for L. amazonensis and 3–4 weeks for L. major). When a nodule of ~0.8 cm was present, mice were killed, the lesion was excised and then manually ground to a suspension between the rough surfaces of sterilized glass microscope slides. The material was cultured in promastigote medium, and the resulting cultures were frozen in numerous aliquots. Serially passaged promastigote cultures were replaced monthly to avoid outgrowth of culture-adapted avirulent subpopulations.

β-lactamase assay. Promastigotes were tested for β-lactamase activity after washing cells once in PBS. Promastigotes were resuspended in PBS containing 0.1% Nonidet P-40 (Sigma) and nitrocefin 100 μM. Nonidet P-40 lysed the cells, thus resulting in higher rate of conversion of the substrate. Pilot experiments determined that 100 μM nitrocefin pro-
vided sufficient signal to be visually observable and detectable by optical density (OD) readings. Catalysis of nitrocefin to its red product was quantified at OD₄₉₀, on a 96-well plate reader. CENTA was used at 50 μM and its product was quantified at OD₄₉₀.

Amastigotes grown in macrophages were quantified for β-lactamase activity by first removing the medium (by gentle pipetting), then adding nitrocefin 100 μM in PBS with 0.1% Nonidet P-40. The plates were incubated at 37°C for 4 hours and then read at an OD of 490 nm.

**Compound screening assay.** As described above, macrophages were seeded into 96-well plates at 5 × 10⁵/well, then infected the next morning with stationary-phase β-lactamase expressing promastigotes at 5 × 10⁵/well. The following morning, the media was aspirated with a pipette (removing many extracellular promastigotes), and fresh media (200 μL) containing dilutions of the compounds under study was added. The cells were incubated at 34°C for 96 hours then developed with nitrocefin as described above.

Growth of the amastigotes was represented as a percentage of growth observed in the absence of inhibitors. Under the conditions described, very few extracellular promastigotes remained in the cultures.

**Data analysis.** The statistics and graphics software, Prism (GraphPad Software Inc., San Diego, CA), was used for all analyses.

**RESULTS**

**Reporter gene expression in Leishmania.** The β-lactamase gene was cloned into pIR1SAT and electroporated into *L. major* and *L. amazonensis* promastigotes. The parasite population that survived selection with nourseothricin was confirmed to have β-lactamase activity. The nitrocefin substrate was converted from yellow to a red color (measured at OD₄₉₀) within 30 minutes in the presence of 10⁻⁵ promastigotes in 0.5 mL of PBS. The reaction occurred approximately twice as fast if a detergent (0.1% NP 40) was added to lyse the parasites. CENTA, another yellow-colored substrate, was also efficiently metabolized in the presence of β-lactamase–expressing parasites. However, CENTA is converted to a gold color that is not as easily appreciated by the naked eye as the red reaction product of nitrocefin. We developed all subsequent experiments using nitrocefin as substrate.

Serial dilutions of β-lactamase–expressing *Leishmania* promastigotes showed a nearly linear relationship between the number of parasites and β-lactamase activity over a range of 20,000 to 500,000 parasites (Figure 2). The activity levels were similar between *L. amazonensis* and *L. major*. There is minimal background activity in untransfected *Leishmania*.

**β-lactamase expression by Leishmania amastigotes.** The promastigotes were allowed to enter stationary-growth phase and were infected onto monolayers of murine peritoneal macrophages. The parasites efficiently infected the macrophages and converted to amastigotes; intracellular proliferation was evident over the course of 3–5 days. No quantitative differences in the degree of parasitism were observed comparing untransfected and β-lactamase–expressing strains, indicating that the genetic manipulations nor the β-lactamase protein had no significant effect on the *in vitro* phenotype of the parasites.

The performance of the β-lactamase–expressing *Leishmania* amastigotes was compared with untransfected amastigotes in a drug screening experiment using the conventional antileishmania drug sodium stibogluconate. There was no significant difference in the dose response to this drug as determined by microscopic counting of intracellular amastigotes fixed on glass slides stained with Giemsa (Figure 3). Growth inhibition of the β-lactamase parasites in the presence of sodium stibogluconate was quantified using the substrate nitrocefin and scored at OD₄₉₀. The results of this assay closely match the results from counting the amastigotes microscopically (Figure 3).

It was observed that the conversion of nitrocefin to its red-colored product occurred nonspecifically if the reaction was performed directly in the *Leishmania* culture medium (RPMI without phenol red, 10% FBS, and penicillin/streptomycin). This was apparently a consequence of factors in the serum causing hydrolysis of the substrate. The problem was avoided by removing the media at the end of the growth assay and replacing it with developing buffer (free of serum). Washing of the wells was avoided so as not to disturb the macrophage monolayer.

**Stability of β-lactamase expression.** The parasite populations were cloned by limiting dilution, and individual clones were determined to have nearly identical levels of β-lactamase activity, indicating a homogeneous population. Promastigote clones that were propagated without nourseothricin selection pressure did not lose β-lactamase activity during a period of > 2 months. Clones were used to infect individual mice at the base of the tail. After 2–4 weeks, the lesions were excised, and the parasites were grown in tissue culture. These parasites were used to infect macrophages, and the degree of infection was quantified after 5 days using nitrocefin. There was no loss of β-lactamase activity after passage through mice. In fact, the activity was higher for the *L. amazonensis* strain after mouse passage probably due to more efficient infection of macrophages (Figure 4).

**Compound screening.** The colorimetric screening assay was used to screen a panel of antimycotic compounds that are recognized to have antileishmania activity. Each compound
was tested in three independent assays against the two β-lactamase-expressing *Leishmania* strains (Table 1). Variability between assays (shown by standard errors) was almost never worse than one dilution. (Assays were done in serial dilutions of two.) ED₅₀ values for the reference compounds, sodium stibogluconate and amphotericin B, were comparable to published values for amastigotes of similar *Leishmania* strains (Table 1).

Itraconazole and clotrimazole were the most potent azole antifungal compounds tested against these strains of *Leishmania*. Fluconazole and voriconazole had ED₅₀ values greater than the highest dilution tested (10 μg/mL).

**DISCUSSION**

This *Leishmania* reporter gene system was designed to have characteristics to optimize its utility in amastigote-macrophage drug screening assays. The reporter gene was integrated into the genome to ensure its stable expression even without antibiotic (nourseothricin) selection pressure. This is important because the parasites are necessarily cultured without antibiotic selection pressure when cultured in macrophages. The stability of expression was demonstrated by passing the parasites through mice (2–4 week infection) and observing β-lactamase expression in the recovered parasites (Figure 4). In addition, by preparing parasite clones, homogeneous expression levels were ensured. It was not necessary to maintain cloned promastigotes in nourseothricin, as stable levels of β-lactamase were observed in culture for over 8 weeks.

Next, a sufficient level of expression of the reporter gene was necessary to generate a detectable readout with the numbers of parasites present in a microtiter plate. By integrating the β-lactamase gene into the rRNA region of the genome using the pIRISAT vector, transcription should be driven by RNA polymerase I, thus leading to high levels of expression. Levels of β-lactamase were sufficient to detect between 20,000 and 500,000 parasites in a linear fashion in a 2-hour assay with nitrocefin (Figure 2). These numbers of parasites correspond with typical numbers of cells growing in a single well of a 96-well plate. Greater sensitivity of the assay could be achieved with longer incubation times.

The specificity of the assay appeared to be excellent with no significant β-lactamase present in host macrophages (Figure 2). However, pilot experiments showed that it would not be possible to add the substrate directly to the amastigote-macrophage culture medium because of factors in the medium that led to metabolism of the substrate. It was determined that the bovine serum was responsible for converting nitrocefin to its red product. This problem was solved by using

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<th>L. major</th>
<th>L. amazonensis</th>
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<tr>
<td>NaSb</td>
<td>38.3 ± 4.4</td>
<td>20 ± 5.0</td>
</tr>
<tr>
<td>Amphotericin B†</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.01</td>
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<tr>
<td>Itraconazole</td>
<td>0.25 ± 0.11</td>
<td>1.06 ± 0.35</td>
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<tr>
<td>Clotrimazole</td>
<td>0.28 ± 0.14</td>
<td>0.69 ± 0.17</td>
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<tr>
<td>Ketoconazole</td>
<td>0.67 ± 0.29</td>
<td>1.01 ± 0.05</td>
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<tr>
<td>Miconazole</td>
<td>0.88 ± 0.42</td>
<td>0.80 ± 0.16</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>&gt; 10</td>
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<tr>
<td>Voriconazole</td>
<td>&gt; 10</td>
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* Published ED₅₀ values for sodium stibogluconate (NaSb) for *L. major* and *L. amazonensis* are 33 and 16 μg/mL, respectively.
† Published ED₅₀ values of amphotericin B for *L. major* and *L. mexicana* are 0.05 and 0.14 μg/mL, respectively.

**Figure 3.** Correlation of colorimetric and microscopic quantification of *L. major* (A) or *L. amazonensis* (B) amastigotes grown in macrophages. Growth inhibition of amastigotes in the presence of sodium stibogluconate (NaSb) dilutions was determined to be very similar using either detection method. (*L. major* ED₅₀ = 40 to 80 μM; and *L. amazonensis* ED₅₀ = 20 to 40 μM). Each point represents the mean of triplicate measurements with SE values ± 7%. The drug was tested in 3-fold serial dilutions. WT = wild type; BLA = β-lactamase.

**Figure 4.** Persistent expression of the β-lactamase gene after passage of *Leishmania* parasites through mice. Parasites were recovered from skin lesions of mice after 2 weeks (*L. amazonensis*) or 4 weeks (*L. major*) and expanded in promastigote culture. Parasites from before the mouse infection were compared with parasites recovered from mice for infectivity of macrophages. The level of infection was quantified by β-lactamase activity 4 days after infection of the macrophages. Bars show the mean ± SEM. OD₄₉₀ = optical density at 490 nm.
a multichannel pipette to fully remove the medium from the plate, then adding substrate in a serum-free buffer. Washing the plate was not necessary and possibly deleterious to the accuracy of the assay.

There were no apparent biological consequences of transformation of the β-lactamase gene into the two strains of *Leishmania*. In principle, deleterious effects could occur either by disruption of genomic architecture or by the presence of this foreign protein. *In vitro* growth of promastigotes (not shown) and amastigotes was unaltered by expression of β-lactamase. In addition, transfected parasites produced normal-appearing lesions in BALB/c mice. It is theoretically possible that the presence of a foreign gene might alter the sensitivity of the strains to some inhibitors, but we feel that this risk is unlikely.

Most importantly, the assay needed to produce results that accurately reflected the actual growth of *Leishmania* amastigotes in the murine macrophages. As shown in Figure 3, scoring the parasite growth with the colorimetric assay demonstrated very close correlation with microscopic quantification of the intracellular amastigotes. The ED<sub>50</sub> results for two established antileishmania drugs, sodium stibogluconate and amphotericin B, obtained using the colorimetric assay were comparable to published results by other groups using the same or different strains (Table 1). Interassay consistency was very good. Within individual assays, dilutions were performed in triplicate or quadruplicate with well-to-well variability consistently < 20%. One area that required special attention was the very careful maintenance of the *Leishmania* cultures, as occasionally poor infection of macrophages would occur with promastigotes that had remained too long in stationary phase.

The utility of the assay was demonstrated in a screen of sixazole antifungal drugs. Of note, itraconazole appears to have the greatest potency of the orally used azole drugs, whereas fluconazole and voriconazole have weak *in vitro* activity. Itraconazole has been shown to have promise in a pilot study of patients with mucocutaneous leishmaniasis,<sup>19</sup>20<sup> Fluconazole has also been shown to be effective as an antileishmania drug; however, this is thought to be due the fact that fluconazole is concentrated in the skin.<sup>21</sup> The data in this paper indicate that fluconazole is far from the most potent ofazole drugs. Future efforts to identify compounds with similar tissue distribution characteristics to fluconazole but greater potency might lead to more efficacious antileishmania therapy.

An area of future improvement of this colorimetric assay will be to use murine peritoneal macrophages with monocyte-macrophage cell lines, such as J774 cells. Eliminating the need for murine peritoneal macrophages would dramatically reduce costs and would improve throughput using this system. Experiments investigating this future direction are underway. Additional strains of *Leishmania*, including *L. donovani*, are being generated to express the β-lactamase gene.

In summary, a colorimetric assay for quantifying the growth of *L. major* or *L. amazonensis* grown in macrophages was developed by expressing the β-lactamase gene in these strains. The assay is simple to perform, and the reagents are relatively inexpensive. The results can be quantified using a standard optical density plate reader. The results of drug inhibition assays using this colorimetric method closely match the results of quantifying the parasites microscopically. This colorimetric assay may be helpful for laboratories involved in screening large numbers of compounds for antileishmania activity.

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