In Vivo Monitoring of Intracellular ATP Levels in *Leishmania donovani* Promastigotes as a Rapid Method To Screen Drugs Targeting Bioenergetic Metabolism

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Parasitic protozoa from the genus *Leishmania* are the causative agents for the variety of clinical manifestations of leishmaniasis, a disease with an annual incidence of 2 million people worldwide according to the World Health Organization (http://www.who.int/emc/diseases/leish/leisdisl.html). Treatment currently relies exclusively on chemotherapy. The development of both new drugs and fast low-cost tests for their screening are required due to the growing incidence of drug resistance.

A method for the rapid screening of drugs targeting the bioenergetic metabolism of *Leishmania* spp. was developed. The system is based on the monitoring of changes in the intracellular ATP levels of *Leishmania donovani* promastigotes that occur in vivo, as assessed by the luminescence produced by parasites transfected with a cytoplasmic form of *Phothinus pyralis* luciferase and incubated with free-membrane permeable β-luciferin analogue β-luciferin-1-[4,5-dimethoxy-2-nitrophenyl] ethyl ester. A significant correlation was obtained between the rapid inhibition of luminescence with parasite proliferation and the dissipation of changes in mitochondrial membrane potential (ΔΨm) produced by buparvaquone or plumbagin, two leishmanicidal inhibitors of oxidative phosphorylation. To further validate this test, a screen of 14 standard leishmanicidal drugs, using a 50 μM cutoff, was carried out. Despite its semiquantitative properties and restriction to the promastigote stage, this test compares favorably with other bioenergetic parameters with respect to time and cell number requirements for the screening of drugs that affect mitochondrial activity.

MATERIALS AND METHODS

Construction of the expression vector pX63NEO-3Luc. *Phothinus pyralis* luciferase gene (lac) with a mutation in the last three amino acids was a kind gift from T. Aeschicher and I. Vorberg (Max Planck Institut für Biologie, Tübingen, Germany) (21). The luciferase gene was inserted at the BamHI restriction site of the polylinker of the *Leishmania* expression vector pX63NEO (17). Insert orientation was checked by electrophoretic analysis of the resulting fragments after digestion with *BglII* and *Asp718*. The plasmid was purified using Qiagen Spin Miniprep kit (Qiagen, Hilden, Germany), and its purity was assessed by agarose electrophoresis.

**Parasites.** *Leishmania donovani* promastigotes (MHOM/SD/00/1S-2D), kindly provided by S. Turco, Kentucky University, were transfected with pX63NEO-3Luc containing the luciferase mutated gene, either in the right (3-Luc strain) or the reversed (Neo strain) orientation by electroporation, according to the method of Lebowitz (16). Parasites were selected by growth in RPMI 1640 medium (Gibco, Paisley, United Kingdom) supplemented with 10% Hanks-Glc. 2 mM D-glutamine, 100 U of uniciline per ml, 48 mg of gentamicin per ml, and 30 μg of geneticin (G-418; Gibco) per ml (RPMI-HIFCS) at pH 7.2, a medium which was also used for parasite maintenance.

Promastigotes were harvested at late exponential phase and washed twice in Hanks buffer supplemented with 10 mM D-glucose (pH 7.2) at 4°C (Hanks-Glc).

**Drugs.** A total of 14 drugs, summarized in Table 1, were tested. Buparvaquone, plumbagin, and lawsone, all naphthoquinone derivatives were used as positive controls (6, 7, 19), while the other compounds were tested blind. Drug
TABLE 1. Comparison of the drug effect on some viability parameters of 3-Luc L. donovani promastigotes

<table>
<thead>
<tr>
<th>Drug</th>
<th>ED50 (μM) (95% CI)*</th>
<th>Luminescence</th>
<th>Proliferation</th>
<th>ΔΨm</th>
<th>ΔΨm</th>
<th>ΔΨm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buparvaquone</td>
<td>0.04 (0.07–0.02)</td>
<td>0.03 (0.04–0.02)</td>
<td>0.05 (0.07–0.04)</td>
<td></td>
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<tr>
<td>Plumbagin</td>
<td>1.15 (1.15–0.8)</td>
<td>2.99 (7.1–1.26)</td>
<td>1.79 (2.2–1.4)</td>
<td></td>
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</tr>
<tr>
<td>1–4 Naphthoquinone</td>
<td>1.12 (1.4–0.9)</td>
<td>0.82 (0.92–0.73)</td>
<td>0.23 (0.4–0.1)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pyronaridine</td>
<td>2.3 (4.5–1.2)</td>
<td>0.7 (1.12–0.43)</td>
<td>2.9 (3.6–2.3)</td>
<td></td>
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<tr>
<td>Juglone</td>
<td>0.43 (0.51–0.36)</td>
<td>0.59 (1.56–0.22)</td>
<td>0.39 (1.5–0.07)</td>
<td></td>
<td></td>
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<tr>
<td>Menadione</td>
<td>8.38 (11.1–6.3)</td>
<td>6.74 (11.41–3.9)</td>
<td>4.82 (6.1–3.8)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lawsone</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lapachol</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mepacrineb</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mepacrinec</td>
<td>17.5 (25.3–12.1)</td>
<td>0.84 (0.99–0.7)</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Diminazene aceturate</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nifurtimox</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
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<tr>
<td>Atovaquone</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
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<tr>
<td>Meglumine antimionate</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
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<td></td>
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<tr>
<td>Pentamidine</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>0.72 (0.8–0.65)</td>
<td></td>
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* ED50 values were estimated by the Litchfield and Wilcoxon procedure. The 95% confidence interval (CI) values are included in parentheses. Assays were carried out as described in Materials and Methods.

b The luminescence assay was done according to the standard protocol.

c The promastigotes were preincubated 4 h prior to the luminescence assay.

RESULTS

Optimization of the bioluminescence assay. The levels of luciferase in transfected promastigotes were estimated by enzymatic activity as (2.52 ± 0.27) × 10^3 copies/promastigote. This value is 10 times higher than that obtained for the native glycosomal form of the enzyme (data not shown).

Both free d-luciferin and its neutral ester (DMNPE-luciferin) were compared as luminescence substrates (Fig. 1). Upon addition of the substrate, the luminescence increased rapidly and dependent on substrate concentration between 2.5 and 25 μM. At 25 μM the luminescence obtained with DMNPE-luciferin was approximately four times higher than that with d-luciferin (Fig. 1). The maximum level was reached ca. 10 min after DMNPE-luciferin addition, followed by slow decay for at least 1 h.

When assayed at 25 μM DMNPE-luciferin, luminescence was directly proportional to the cell number (Fig. 1, inset). Luciferase activity depleted total ATP content by only 3.8% ± 1.5% from its initial value of 15 nmol/mg of protein (1 mg of protein = 3.6 × 10^3 promastigotes).

Treatment of parasites with 10 mM 2-dGlc (a competitive inhibitor of glycolysis) or with 2 mM KCN (as inhibitor of the respiratory chain) led to luminescence decreases of 22 and 85%, respectively, and to a decrease of 97% when these inhibitors were added together.
Effects of naphthoquinones on promastigote bioluminescence and other viability parameters. To remove false negatives caused by a direct inhibition of luciferase, drugs were tested in vitro at their highest concentration on purified luciferase. None of the drugs produced a significant in vitro inhibition (≤5%). Buparvaquone and plumbagin inhibited parasite luminescence in vivo at nanomolar and micromolar concentrations (Table 1) but not the natural hydroxynaphthoquinone lawsone, reported previously as inactive (1, 19).

A good correlation was obtained for the EC_{50} of buparvaquone (an inhibitor of respiratory chain) and plumbagin (uncoupler of oxidative phosphorylation) for parasite proliferation, DC_{m}, and luminescence (Table 1). Lawsone was inactive in the three systems (EC_{50} ≥50 μM) (Table 1).

Validation of the luminescence test with other drugs. A set of 14 drugs was assayed blind in the luminescence assay using an initial cutoff of 50 μM. In these tests only six compounds inhibited luminescence (Fig. 2). Plumbagin, 1-4 naphthoquinone, pyronaridine, and menadione were active as inhibitors of luminescence at a micromolar concentration, whereas buparvaquone and juglone were active at a nanomolar concentration range. As above, a good correlation between luminescence decrease with the other two parameters was obtained (Table 1).

When the same set of drugs was tested for inhibition of promastigote proliferation, another two drugs (mepacrine and pentamidine), inactive at 50 μM by the standard luminescence test, were highly inhibitory (Table 1). Nevertheless, either when the pentamidine concentration was increased to 100 μM or when the parasites were preincubated with 50 μM mepacrine for 4 h prior to DMNPE-luciferin addition, the luminescence levels were decreased to 25.5% ± 7.8% and 28.2% ± 4.3%, respectively, compared to the control parasites. This result suggested a slow accumulation of mepacrine, confirmed by the decrease in the oxygen consumption rates of parasites permeabilized with digitonin, making mitochondria accessible to the drug. The control rate for intact parasites (8.45 nmol O_2 min^{-1} × [10^6 cells]^{-1}) was not modified at 10 μM mepacrine, whereas in permeabilized parasites a decrease of 26.5% was observed at this concentration.

DISCUSSION

Microorganisms transfected with P. pyralis luciferase have been extensively used to improve proliferation assays in slow-dividing organisms, such as Mycobacterium tuberculosis (13, 20, 33). However, the potential of using the internal expression of luciferase as a probe to monitor in vivo changes in metabolism affecting intracellular ATP levels have not been fully explored in higher eukaryotes (15) or parasitic protozoa.

This is due to the substrate limitation caused by the poor
membrane permeability of \( \alpha \)-luciferin at neutral pH and sequestration of luciferase into peroxosomes. We have improved the system and applied it to screen leishmanicidal drugs in a fast- and cost-effective manner by using a free-membrane permeable neutral caged luciferin ester, hydrolyzed once inside the cytoplasm (5, 24), and parasites transfected with a mutated version of the \( P. \) pyralis luciferase gene that retained the enzyme in the cytoplasm (21), with a much better yield in luminescence than the native glycosomal form of the enzyme.

Luminescence output is proportional to both the luciferase substrate concentration and the cell number. According to our measurements of total ATP levels, the slow decay observed after the maximum is not produced by ATP depletion by the luminescence reaction but is more likely caused by luciferase inhibition by the end products of \( \alpha \)-luciferin (8).

The standard protocol is biased toward the identification of drugs affecting mitochondrial ATP production rather than inhibitors of glycolysis. This is in agreement with our results on luminescence inhibition by KCN and 2-DGlc, with the correlation between the \( ED_{50} \) for luminescence and \( \Delta \Psi_{\text{mt}} \), and with previous reports in the literature (1, 22), where minimal energetic requirements can only be fulfilled by oxidative phosphorylation and not by glycolysis. Putative inhibitors of the last pathway would be identified indirectly through the depletion of pyruvate, provided that \( \alpha \)-glucose was the sole source of carbon and that all the other metabolic intermediates from glycolysis or the Krebs cycle or the internal carbohydrate reservoirs have undergone a strong depletion.

Inhibition of luminescence correlated well (\( \alpha = 0.01 \)) with the leishmanicidal activity of naphthoquinones and hydroxynaphthoquinones (6, 7, 14, 19). Atovaquone and meglumine antimonials were not detected since they mainly affect the amastigote stage (7, 17). In general, the inhibition of proliferation was always higher than the inhibition of luminescence at the same drug concentration. A likely explanation for this is that the loss of parasite viability will require a significant but not total depletion of ATP levels, as seen in similar experiments with rat hepatocytes (15).

The luminescence assay failed to detect two known leishmanicidal drugs, pentamidine and mepacrine. Mepacrine was described as an inhibitor of topoisomerase II and protein synthesis with rat hepatocytes (15). Atovaquone and meglumine antimonials can only be fulfilled by oxidative phosphorylation and not by glycolysis. Putative inhibitors of the last pathway would be identified indirectly through the depletion of pyruvate, provided that \( \alpha \)-glucose was the sole source of carbon and that all the other metabolic intermediates from glycolysis or the Krebs cycle or the internal carbohydrate reservoirs have undergone a strong depletion.

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The luminescence assay failed to detect two known leishmanicidal drugs, pentamidine and mepacrine. Mepacrine was described as an inhibitor of topoisomerase II and protein synthesis (11) rather than as a fast effector on the energetic metabolism of the parasite. Even so, luminescence is affected after longer incubation with mepacrine, possibly by an indirect pathway. Pentamidine was reported as an inhibitor of polyamine biosynthesis and \( \delta \)DNA replication (2); however, it also inhibits oxidative phosphorylation on \( L. \) major promastigotes in digitonin-permeabilized parasites at 200 \( \mu \)M (23), a concentration four times higher than our 50 \( \mu \)M cutoff. In fact, 100 \( \mu \)M pentamidine inhibited luminescence in agreement with this earlier study.

The assay can also uncover effects on bioenergetic metabolism as a putative secondary target for drugs. Pyronaridine, reported to be a DNA polymerase II inhibitor in \( P. \) falciparum (4), produced a considerable inhibition of luminescence, and a possible inhibition on luciferase was excluded by in vitro assays on parasite lysates. This is also confirmed by the good correlation obtained in our assay with bioenergetic parameters directly related to ATP production in mitochondria, such as the membrane potential \( \Delta \Psi_{\text{mt}} \).

An intrinsic limitation of the method is its restriction to the promastigote, the only stage where the expression of genes inserted into the pX63NEO vector reached good levels (17). Since new expression vectors for amastigotes are being improved (3), this strategy should be explored further. However, assays in infected macrophages will be limited by the access of \( \alpha \)-luciferin into the parasitophorous vacuole. The system has advantages over other methods since it requires a lower number of cells than that required for measuring oxygen consumption rate and since it is less time-consuming than measurement of the \( \Delta \Psi_{\text{mt}} \). This assay is also simple and easy to automate. Although initially designed for fast-acting drugs affecting ATP production, its utility can be extended to the slow-accumulating drugs, as previously discussed, and for other compounds that cause the reduction of ATP levels, such as membrane-active molecules.

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REFERENCES