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Note

A new, rapid and sensitive bioluminescence assay for drug screening on *Leishmania*

Lucie Paloque [a], Nicolas Vidal [b], Magali Casanova [a], Aurélien Dumètre [a], Pierre Verhaeghe [c], Daniel Parzy [a], Nadine Azas [a,*]

[a] Infections Parasitaires, Transmission, Physiopathologie et Thérapeutique, UMR MD3, Faculté de Pharmacie, Aix-Marseille Université, 27 Boulevard Jean Moulin, CS 30064, 13385 Marseille Cedex 05, France
[b] Institut de Chimie Radicalaire, UMR CNRS 7273, Faculté des Sciences de St Jérôme, Aix Marseille Université, 13397 Marseille Cedex 20, France
[c] Equipe Pharmacochimie Radicalaire, Institut de Chimie Radicalaire, UMR CNRS 7273, Faculté de Pharmacie, Aix-Marseille Université, 27 Boulevard Jean Moulin, CS 30064, 13385 Marseille Cedex 05, France

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ABSTRACT

We validated a new method, based on luciferine/luciferase bioluminescence, for drug screening on promastigotes of different *Leishmania* species. Results obtained with this new, rapid, reproducible, and reliable method are in good accordance with results obtained by the conventional MTT assay. This bioluminescence assay has a lower detection limit.

Leishmaniasis are parasitic diseases encountered in 98 countries, mainly in tropical and sub-tropical regions, but also in the Mediterranean area (WHO, 2012). These infections are caused by a protozoan of the *Leishmania* genus. This parasite exists in two morphological stages: extracellular flagellated promastigotes in the digestive tract of their sandfly vector and non-motile intracellular amastigotes in cells of the mononuclear phagocytic system of their mammalian host (Olivier and Gregory, 2008; Sharlow et al., 2010). Leishmaniasis depend on the *Leishmania* species, and range from benign cutaneous leishmaniasis to fatal visceral leishmaniasis. There are an estimated 14 million people infected by leishmaniasis worldwide with an annual incidence of 2 million, however the number of cases is certainly underestimated (WHO, 2010).

The main drugs used against leishmaniasis are pentavalent antimonials, liposomal amphotericin B, pentamidine, miltefosine and paromomycin (Croft and Coombs, 2003). Few other drugs are at present in phase of clinical trials: sitamaquine, imiquimod and antifungal azoles (WHO, 2010). However, the serious side effects of most of these drugs, their high cost, as well as the emergence of resistance lead to the urgent need of new therapeutics (Croft et al., 2006; Maltezou, 2010) and to verify, in a therapeutic context, the sensitivity of clinical isolates to antileishmanial drugs.

Different methods are used for screening the antileishmanial activity of new potential drugs: colorimetric assays based on the mitochondrial reduction of AlamarBlue® (Mikus et Steverding, 2000) or of tetrazolium salts MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (adapted from Mosmann, 1983), XTT (Williams et al., 2003) or MTS (Berg et al., 1994), and assays using transgenic parasites expressing fluorescent protein or producing luciferin-luciferase-based luminescence (Dube et al., 2009). Most of these methods are time consuming and some of them, like the reported gene techniques, can't be used on clinical isolates.

ATP from viable cells can be quantified by measuring the light produced from the firefly luciferase-catalyzed luciferin-ATP reaction, in order to assess cell viability as well as drug cytotoxicity on several prokaryotic or eukaryotic cells (Selan et al., 1992; Luque-Ortega et al., 2001; Lafond et al., 2010), but not on non-genetically modified *Leishmania* parasites. In the present study, we designed an ATP-bioluminescence assay for measuring the effects of drugs on the ATP production of *Leishmania* promastigotes, without using transgenic parasites. According to validation guidelines (ICH, 2005; Ederveen, 2010), the following parameters were determined for bioluminescence method validation and

* Corresponding author. Tel.: +33 491 835 564; fax: +33 491 835 537.
E-mail addresses: lupaloque@hotmail.com (L. Paloque), yeleniko@yahoo.fr (N. Vidal), magali.casanova@univ-amu.fr (M. Casanova), aurelien.dumetre@univ-amu.fr (A. Dumètre), pierre.verhaeghe@univ-amu.fr (P. Verhaeghe), d.parzy@free.fr (D. Parzy), nadine.azas@univ-amu.fr (N. Azas).

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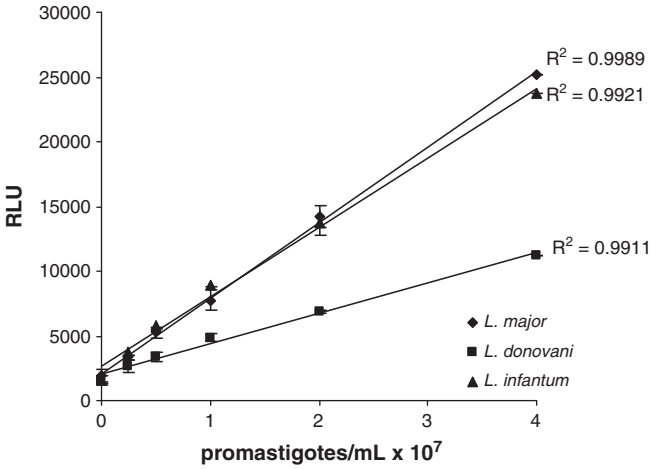

Fig. 1. Dosage by bioluminescence of ATP produced by promastigotes of different *Leishmania* species (◆, *L. major*; ■, *L. donovani*; ▲, *L. infantum*). This figure shows a linear relationship between promastigote concentration and RLU (Relative Light Unit) of the luciferine/luciferase reaction. Mean of three independent experiments. R^2, correlation coefficient.

compared to the reference method MTT: linearity, detection limit (i.e. sensitivity), lowest quantitation limit, precision (i.e. intermediate precision and repeatability) and accuracy.

Leishmania species used in this study were *L. major* MHOM/IL/80/MON-103, *L. infantum* MCAN/ES/98/LLM-877 and *L. donovani* MHOM/IN/00/DEVI (CNR *Leishmania* Montpellier, France). Promastigotes were grown at 27 °C in 5 mL of Schneider's medium (Gibco) supplemented with 2 mM L-glutamine, antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and heat-inactivated foetal calf serum (20% v/v). Experiments were performed with logarithmic phase promastigotes seeded in sterile 96-well plates in duplicate, in concentrations ranging from 1.10^4 to 4.10^7 parasites/mL in a final volume of 100 µL per well. For the MTT assay, plates were centrifuged at 900 g for 10 min and the supernatant was removed. After adding MTT (0.5 mg/mL in RPMI 1640 medium, 100 µL/well), promastigotes were incubated for 6 h at 27 °C, time required for reduction of tetrazolium salts to insoluble formazan. The plates were subsequently centrifuged at 900 g for 10 min and the supernatant was removed. The formazan pellet was dissolved in 100 µL of DMSO and the absorbance measured in a plate reader at 570 nm. For the bioluminescence method, 20 µL of each well was transferred in sterile white 96-well plate containing 80 µL of the parasite culture medium per well. The luciferin/luciferase reagent Promafax (Yelen, France) was prepared according to the manufacturer's instructions. Then, 100 µL of this reagent was added per well. Luminescence was measured in a luminometer (Tecan infinite F200) with the following settings: wait timer 3 min, shaking (duration 5 s, amplitude 3 mm), and luminescence integration time 500 ms. The luminescence signal was expressed in relative light unit (RLU).

The ATP-bioluminescence assay allowed us to determine, for each *Leishmania* species, a linear relationship between promastigote concentration and RLU values (Fig. 1), with correlation coefficients >0.99. Overall, the detection limit (DLx) and lowest quantitation limit (QLx) obtained with this new method were 3.7 to 52 lower than with the MTT method (Fig. 2 and Table A.1). The repeatability of the new method, obtained from three independent experiments realised by one operator, was defined by a coefficient of variation (CV) ≤0.14, ≤0.23 and ≤0.12 for *L. major*, *L. donovani* and *L. infantum* respectively (Fig. 3A). The intermediate precision, determined from six independent experiments realised by two independent operators (3 experiments each), was defined by CV ≤0.21, ≤0.3 and ≤0.04 for *L. major*, *L. donovani* and *L. infantum* respectively (Fig. 3B). So, determination of linearity, detection limit, lowest quantitation limit, repeatability and intermediate

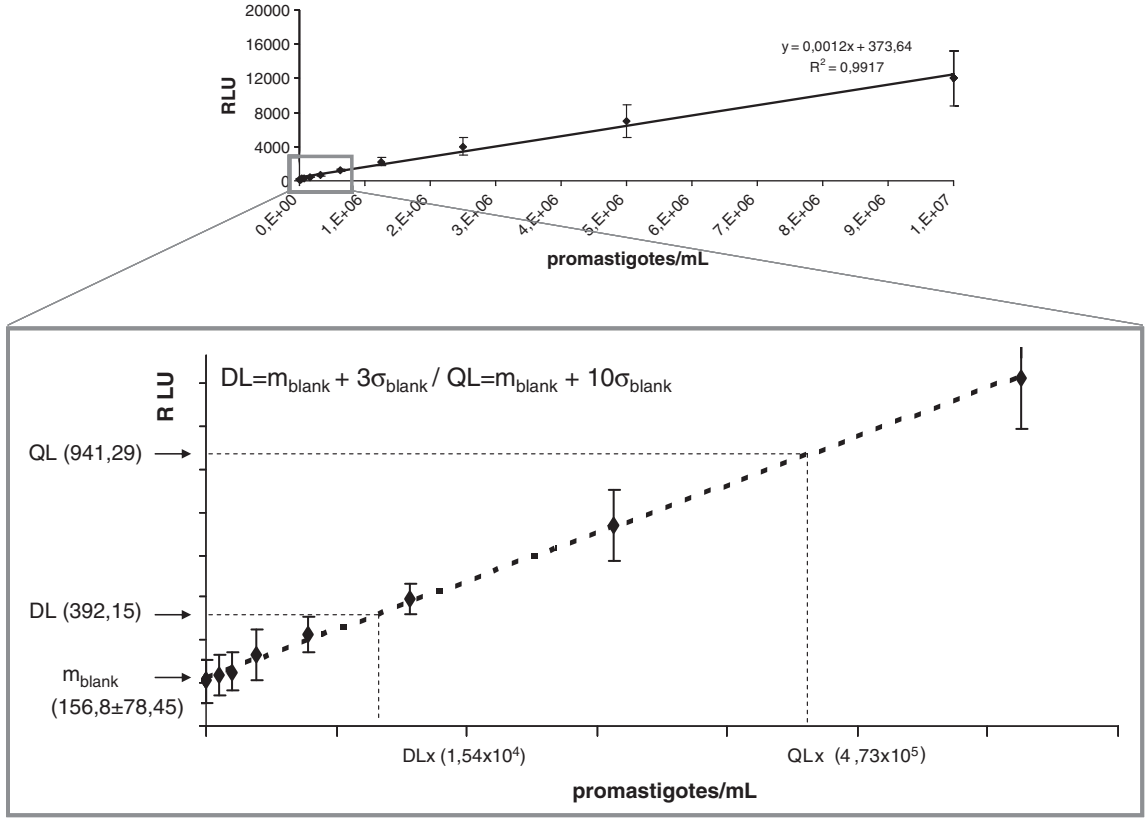

Fig. 2. Calculation of detection limit (DL/DLx) and lowest quantitation limit (QL/QLx) of the new bioluminescence assay on linear relationship between promastigote concentration and RLU, for *Leishmania major*. The RLU values are the mean of five independent experiments.