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Development of *Leishmania donovani* stably expressing DsRed for flow cytometry-based drug screening using chalcone thiazolyl-hydrazone as a new antileishmanial target

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ABSTRACT

Green fluorescent protein produces significant fluorescence and is extremely stable, however its excitation maximum is close to the ultraviolet range and thus can damage living cells. Hence, *Leishmania donovani* stably expressing DsRed were developed and their suitability for flow cytometry-based antileishmanial screening was assessed by evaluating the efficacies of standard drugs as well as newly synthesised chalcone thiazolyl-hydrazone compounds. The DsRed gene was successfully integrated at the 18S rRNA locus of *L. donovani* and transfectants (LdDsRed) were selected using hygromycin B. Enhanced expression of DsRed and a high level of infectivity to J774A.1 macrophages were achieved, which was confirmed by fluorescence microscopy and flow cytometry. Furthermore, these LdDsRed transfectants were utilised for development of an in vitro screening assay using the standard antileishmanial drugs miltefosine, amphotericin B, pentamidine and paromomycin. The response of transfectants to standard drugs correlated well with previous reports. Subsequently, the suitability of this system was further assessed by screening a series of 18 newly synthesised chalcone thiazolyl-hydrazone compounds in vitro for their antileishmanial activity, wherein 8 compounds showed moderate antileishmanial activity. The most active compound **5g**, with ca. 73% splenic parasite reduction, exerted its activity via generating nitric oxide and reactive oxygen species and inducing apoptosis in LdDsRed-infected macrophages. Thus, these observations established the applicability of LdDsRed transfectants for flow cytometry-based antileishmanial screening. Further efforts aimed at establishing a high-throughput screening assay and determining the in vivo screening of potential antileishmanial leads are required.

1. Introduction

Treatment of visceral leishmaniasis with the available antileishmanial drugs is far from ideal and a search is underway at a global level for the development of a new, safe, effective and inexpensive drug for the control of visceral leishmaniasis. The drug development process requires appropriate screening assays in order to search out new lead molecules. However, there are several drawbacks with the previously used in vitro screening assays based on either microscopic counting or use of radioactive nucleotides or dye-based methods, which necessitate the development of rapid, convenient and reliable screening assays. The advent of reporter genes with a readily measurable phenotype allows its application as a promising tool for studying disease progression, drug screening methods and allows live imaging. Various reporter genes such as chloramphenicol acetyltransferase, β-galactosidase, secreted alkaline phosphatase, firefly luciferase, green fluorescent protein (GFP), glucuronidase and red fluorescent protein (RFP) have been studied to screen antileishmanial compounds [1]. Of these, fluorescent reporter genes, which are easily distinguishable from the endogenous cell background [1], offer a better choice. Amongst the entire reporter genes studied so far, mostly GFP transfectants of *Leishmania* parasites have been developed [2–5], out of which only stable transfection of the enhanced GFP (EGFP) reporter has been found suitable both for in vitro and in vivo infection studies [2,6,7]. Although in our laboratory GFP-expressing parasites are routinely used for antileishmanial compounds [3], this reporter gene has many drawbacks, such as a very low signal-to-noise ratio due to a high level of autofluorescence of GFP. Moreover, although the native GFP produces significant fluorescence and is extremely stable, the excitation

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maximum is close to the ultraviolet range and thus can damage living cells [8]. Furthermore, in order to assess the mechanism of action of any antileishmanial compound, GFP actually limits the freedom to use many other important dyes such as CFSE, DAF-2DA, DCFDA, FITC etc. that fall in the same wavelength range. Lately, RFP has been integrated into some *Leishmania* spp. [9,10] to study various biological functions [8,11]. RFP-expressing parasites certainly free the dominantly preferred 520/40 nm channel, which provides us an edge to include a few more dyes in the staining panel for flow cytometry experiments. In addition, based on the improved photostability and high quantum yield [9], DsRed was considered suitable for our studies through flow cytometry. These characteristics make DsRed an ideal candidate for fluorescence imaging, particularly for multicolour experiments involving GFP and its variants. It has a maximum emission of 579 nm with a 557 nm excitation wavelength but can also be excited by a standard 488 nm laser, allowing DsRed to be used with laser-based confocal microscopes and flow cytometers [9]. Although these transgenic parasites are used for many purposes in cell biology, their biological fitness such as infectivity, fluorescence level and suitability for in vitro screening assays has not been properly characterised.

In the present study, transgenic *Leishmania donovani* stably expressing DsRed-Express-N1 were generated and their infectivity to J774A.1 macrophages was assessed. Consequently, the applicability of this stable transfectant in a host cell-based antileishmanial screening assay using flow cytometry was assessed employing standard antileishmanial drugs. Furthermore, to establish the appropriateness of DsRed transfectants in identifying lead compounds, a new chemical series of chalcone thiazolyl-hydrazone hybrids was evaluated for their antileishmanial efficacy. In addition, the aptness of the system was also checked to simultaneously gather more information on the drugs' effect on various biochemical parameters.

2. Materials and methods

2.1. Parasites, cell lines and animals

L. donovani strain Dd8 was used for generating DsRed transfectant promastigotes. Parasites were grown in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with $NaHCO_3$, glucose, HEPES (Merck, Mumbai, India), gentamicin (Sigma-Aldrich), 10% heat-inactivated fetal bovine serum (hi-FBS) (Gibco™; Thermo Fisher Scientific, Waltham, MA), 50 U/mL penicillin plus 50 mg/mL streptomycin (Sigma-Aldrich) at 25 °C [12,13]. The mouse macrophage (MΦ) cell line J774A.1 (American Type Culture Collection, Manassas, VA) was maintained in DMEM medium (Sigma-Aldrich) supplemented with $NaHCO_3$, glucose, HEPES, gentamicin, 10% hi-FBS, 50 U/mL penicillin and 50 mg/mL streptomycin in 75-cm^2 culture flasks (Nunc®; Thermo Fisher Scientific) at 37 °C and 5% CO_2. In vivo studies were performed in outbred hamsters (40–45 g) of both sexes.

2.2. Synthesis of chalcone thiazolyl-hydrazone-based hybrids

Details of the synthesis of chalcone thiazolyl-hydrazones and intermediate compounds are provided in the Supplementary material.

2.3. Construction of pLEXSY-DsRed cassette and transfection into L. donovani

The 678-bp coding region for DsRed was amplified by PCR using the vector template DsRed-Express-N1 (Clontech, Saint-Germain-en-Laye, France) with the forward primer 5′-AGATCTATGGATAG CACTGAGAACGTCATCAAGCC-3′ and the reverse primer 5′-GCTAGCCTACTGGAACAGGTGGTGGCG-3′, which have *Bgl*II and *Nhe*I restriction sites, respectively. The amplified product was first cloned into pTZ57R/T (TA) vector (Fermentas/Thermo Fisher Scientific) and then into the pLEXSY-*hyg2* expression vector (Jena Bioscience, Jena, Germany) using *Bgl*II and *Nhe*I restriction enzymes (Thermo Fisher Scientific). Furthermore, the DsRed-integrated pLEXSY vector was digested with the restriction enzyme *Swa*I (Thermo Fisher Scientific) and the large fragment (5.7 kb) was used to obtain parasites expressing the DsRed open reading frame (ORF). Parasites expressing DsRed were obtained by transfection of *L. donovani* with the pLEXSY-DsRed construct by electroporation as described previously [3]. Briefly, ca. 300 μL of parasite suspension (1×10^8 parasites/mL) was placed into a 2-mm electroporation cuvette (Bio-Rad, Hercules, CA) and was mixed with 20 μg of the pLEXSY-DsRed construct previously digested with the restriction enzyme *Swa*I and precipitated in ethanol, and was incubated for 10 min at 4 °C. The mixture was electroporated using a Gene Pulser (Bio-Rad) carried out by one pulse of 450 V/cm and 500 μF with minimum resistance. Electroporated parasites were transferred to culture flasks (Nunc) containing 5 mL of complete RPMI medium supplemented 20% FBS. Selection of transfectants was initiated 24 h after electroporation using 10 μg/mL hygromycin B (HygB) and gradually increased up to 200 μg/mL. Following initial drug selection, growth was optimised (100 μg/mL) and HygB was removed from the culture. Stable transfectants were designated as LdDsRed and were further cultured without HygB.

2.4. Confirmation of L. donovani-DsRed (LdDsRed) transfectants

Genomic DNA from cultured wild-type (WT) and LdDsRed parasites was purified using a DNA Isolation Kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. Homologous recombination of the DsRed ORF gene at the 18S rRNA locus of *L. donovani* promastigotes resulting in transgenic clones was confirmed by PCR amplification and western blot analysis both using monoclonal and polyclonal antibodies.

The fluorescence of transfected LdDsRed parasites both in promastigotes and in intracellular amastigotes was visualised using a fluorescence microscope (Nikon, Melville, NY) [3,14]. Flow cytometry-based analysis of LdDsRed promastigotes and infected MΦs was performed using a Cytomics FC 500 MPL Flow Cytometer (Beckman-Coulter Life Sciences, Indianapolis, IN) using an argon laser (excitation at 557 nm and emission at 587 nm). LdDsRed promastigotes as well as uninfected and infected MΦs (1×10^6 cells/mL) were suspended in 500 μL of phosphate buffer saline (PBS) and were analysed by flow cytometry [7]. Data were analysed using Kaluza Flow Cytometry Analysis Software (Beckman-Coulter). The percentage of infected MΦs and their mean fluorescence intensity (MFI) were measured with respect to controls.

2.5. In vitro infectivity of LdDsRed parasites

Assessment of the in vitro infectivity of LdDsRed parasites was carried out using the J774A.1 MΦ cell line. Cells were washed with PBS and 1×10^5 cells/mL was incubated for 24 h at 37 °C and 5% CO_2 in 24-well cell culture plates (Nunc). Subsequently, cells were infected with LdDsRed promastigotes at 1:10 and 1: 20 (MΦ:parasite cell ratio) multiplicity of infection (MOI). Following incubation for 12, 24, 36 and 48 h at 37 °C, extracellular LdDsRed promastigotes (non-phagocytosed) were removed by two to three successive washings with medium. Furthermore, infected MΦs were detached using 0.1% trypsin–ethylene diamine tetra-acetic acid (EDTA) solution and were then analysed by flow cytometry.

2.6. Suitability of intracellular LdDsRed amastigotes for in vitro antileishmanial screening

For in vitro drug screening against intracellular amastigotes, LdDsRed promastigote-infected J774A.1 MΦs were treated with