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Leishmania is not prone to develop resistance to tamoxifen

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ABSTRACT

Tamoxifen, an antineoplastic agent, is active *in vitro* and *in vivo* against the parasitic protozoa *Leishmania*. As part of our efforts to unravel this drug's mechanisms of action against the parasite and understand how resistance could arise, we tried to select tamoxifen-resistant *Leishmania amazonensis*. Three different strategies to generate tamoxifen resistant mutants were used: stepwise increase in drug concentration applied to promastigote cultures, chemical mutagenesis followed by drug selection and treatment of infected mice followed by selection of amastigotes. For amastigote selection, we employed a method with direct plating of parasites recovered from lesions into semi-solid media. Tamoxifen resistant parasites were not rescued by any of these methods. Miltefosine was used as a control in selection experiments and both stepwise selection and chemical mutagenesis allowed successful isolation of miltefosine resistant mutants. These findings are consistent with a multi-target mode of action to explain tamoxifen's leishmanicidal properties. Considering that drug resistance is a major concern in anti-parasitic chemotherapy, these findings support the proposition of using tamoxifen as a partner in drug combination schemes for the treatment of leishmaniasis.

1. Introduction

Leishmaniasis is a complex of vector-borne infectious diseases transmitted by sand flies and caused by protozoan parasites of the genus *Leishmania*. The disease is widespread in the world in tropical and subtropical regions (Alvar et al., 2012). Recent data indicate that approximately 0.2–0.4 million and 0.7 to 1.2 million cases of visceral and cutaneous leishmaniasis respectively occur each year (Alvar et al., 2012).

The limited drug arsenal available for the treatment of leishmaniasis presents numerous shortcomings, such as toxicity, the need for prolonged and mostly parenteral medication and price. Furthermore, failure has been reported for most drugs used in the treatment of the disease (Bryceson, 2001; Croft, 2001) and, in some settings, this failure has been attributed to drug resistance. Widespread resistance to antimonials is found in the Northeast of India (Sundar, 2001; Sundar et al., 2001) and there is great concern on the selection of parasites resistant to miltefosine (Cojean et al., 2012; Rijal et al., 2013), the first oral drug used in the treatment of leishmaniasis. For all these reasons, there is an urgent need to discover and develop new antileishmanial drugs as well as strategies to protect current and future therapies from the threat of resistance.

Drug resistance is related to the ability of pathogens to circumvent the effects of drugs and is due to the genetic adaptability that enables the selection of appropriate strategies against these drugs (Vanaerschot et al., 2013). Selection of drug resistant parasites has been used for the identification of drug resistance genes in parasitic protozoa and has also contributed to understanding the mechanisms of action of some of these therapeutic agents (Ouellette et al., 2004; Muller and Hemphill, 2011; Alsford et al., 2013). In *Leishmania*, this strategy has been applied *in vitro* using stepwise increase in the concentration of the drug of interest, followed by the use of different molecular techniques for the analysis of differentially expressed genes/proteins and for identification of point mutations in drug resistant parasites compared to wild-type lines (Berg et al., 2013; Vanaerschot et al., 2013). The

Abbreviations: TM, tamoxifen; MNNG, *N*-methyl-*N*-nitroso-*N*′-nitroguanidine; MT, miltefosine transporter; PBS, phosphate buffered solution; NBD-PC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine.

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dihydrofolate reductase/thymidilate synthase was the first gene identified in a methotrexate selected resistant line of *Leishmania major* using this strategy (Beverley et al., 1984, 1986). Stepwise increase in drug concentration has also been applied to the selection of miltefosine resistant mutants and this phenotype has been linked to point mutations on a P-type ATPase, known as miltefosine transporter (MT), responsible for the translocation of phospholipids across the plasma membrane (Perez-Victoria et al., 2003; Coelho et al., 2012, 2014).

We have previously reported that tamoxifen, a selective oestrogen receptor modulator, is active *in vitro* and *in vivo* against *Leishmania* species (Miguel et al., 2007, 2008, 2009). We have demonstrated that tamoxifen's antileishmanial activity is not dependent on the interaction with oestrogen receptors (Bonano et al., 2014) but the precise antileishmanial mechanism of action is still uncertain. The aim of this work was to apply drug selection experimental protocols to *Leishmania amazonensis* with the objective of developing parasite lines that would be suitable to the characterization of tamoxifen's mechanism of action.

2. Material and methods

2.1. Drugs

Tamoxifen, tamoxifen citrate, miltefosine and *N*-methyl-*N*-nitroso-*N*′-nitroguanidine (MNNG) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions of tamoxifen and miltefosine (10 mM) were prepared in ethanol or sterile water, respectively. MNNG was diluted in DMSO at a concentration of 10 mg/mL. Tamoxifen citrate was used for the treatment of BALB/c mice infected with *L. amazonensis*, prepared daily with saline.

2.2. Parasites and cells

L. amazonensis (MHOM/BR/1973/M2269) promastigotes were grown in medium 199 (Sigma–Aldrich) supplemented with 10% heat-inactivated fetal calf serum, 0.25% hemin, 12 mM $NaHCO_3$, 50 U/mL penicillin and 50 μg/mL streptomycin at 25 °C. Amastigotes were obtained from infected mice, as described (Arruda et al., 2008). In brief, female BALB/c mice (4–5 week-old) were infected with 10^6 stationary-phase parasites injected subcutaneously in the right hind footpad. After 8–12 weeks, lesions were removed and homogenized in phosphate-buffered saline (PBS); the suspension was cleared of cell debris by centrifugation at 50 g for 8 min. The supernatant containing amastigotes was recovered and washed three times in PBS. Amastigotes were counted in a Neubauer hemocytometer.

MCF-7 breast cancer cell line was cultured in DMEM supplemented with L-glutamine, glucose and 10% fetal bovine serum (Life Technologies). This line was maintained in exponential growth phase by sub-culturing twice weekly in 25-cm^2 flasks at 37 °C and 5% CO_2. For sub-culturing, media was removed from the flasks, cells were washed with PBS and then detached by incubation with 2 mL of Trypsin/EDTA solution (Vitrocell Embriolife, Campinas, Brazil) for 5–10 min followed by inactivation with DMEM. Cells were counted and resuspended in growth media at 10^5 cells/mL.

2.3. Drug selection in promastigotes

Selection of resistant parasites was initiated using different concentrations of tamoxifen (2, 4, 6, 8 and 12 μM) and 10 μM of miltefosine. For miltefosine, the drug was increased using a stepwise selection until they were resistant to 150 μM. Selection was performed with at least three successive passages for each dose (Coelho et al., 2014). For tamoxifen, parasites were kept for at least 40 passages in the presence of 12 μM tamoxifen (around 250 days of treatment) after previous treatment with 8 μM of tamoxifen for 10 passages.

2.4. Mutagenesis in vitro

Mutagenesis was applied to *L. amazonensis* wild-type promastigotes (5×10^6 parasites/mL in M199 medium) that were initially treated with 3 μg/mL of MNNG (Sigma–Aldrich) for a period of 4 or 24 h at 25 °C in a total of at least 250 mL of liquid medium, as described (Iovannisci and Ullman, 1984). Mutagenized parasites were then washed three times with PBS and resuspended in fresh medium at a concentration of 5×10^6 parasites/mL. Viability post-treatment was evaluated by cell counting and once the cell cultures started to grow, mutagenized parasites were submitted to selection in the presence of 20 μM tamoxifen or 70–75 μM miltefosine. Selection was performed in liquid or semi-solid 199 medium. Parasites were seeded in 150 mL of M199 at a density of 2×10^6 parasites/mL or plated in semi-solid medium at concentration of 4×10^7 parasites/plate in a total of at least 20 plates.

2.5. Drug selection in amastigotes

Mice were infected as described above. Five weeks after infection, treatment with tamoxifen was initiated. Infected animals received intraperitoneal injections of 30.4 mg tamoxifen citrate/kg/day (equivalent to 20 mg/kg/day tamoxifen) for 15 days. Sixty days after the end of treatment, mice were euthanized and amastigotes were purified from lesions. Amastigotes recovered from infected mice treated or not with tamoxifen were counted and then plated in medium 199 containing 1% of agar (Invitrogen Corporation, NY, USA) and 0.6 μg/mL of biopterin (Sigma–Aldrich). A total of 5×10^6 amastigotes were directly plated in triplicate, in plates containing 20, 30 or 50 μM of tamoxifen or in the absence of drug.

2.6. Parasite viability

Susceptibility to tamoxifen, miltefosine or MNNG was evaluated by a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT, Sigma–Aldrich) viability test assay as previously described (Zauli-Nascimento et al., 2010). Briefly, promastigotes (2×10^6 per well) in M199 were incubated in the presence of increasing concentrations of drug (tamoxifen, 2–128 μM; miltefosine, 3–200 μM or MNNG, 1.5–96 μg/mL assayed at a 2-fold dilution) for 24 h. MTT cleavage was measured in a microplate reader (POLARstar Omega, BMG Labtech, Ortenberg, Germany) with a test wavelength of 595 nm and a reference wavelength of 690 nm. Assays were performed in triplicate and results are expressed as the mean and standard deviation (SD) of at least three independent experiments.

2.7. Uptake of fluorescent phosphocholine

Log-phase promastigotes were labelled with NBD-PC (Molecular Probes) as described (Coelho et al., 2014). Briefly, parasites were labelled with 10 μM NBD-PC for 30 min. After washing, parasites were resuspended in PBS for flow cytometry analysis. Labelled parasites were analysed at room temperature using Guava EasyCyte Mini Flow Cytometer System (Millipore). Data from 5000 cells, defined by gating at data acquisition, was collected and analysed using CytoSoft version 4.2.1 software (Guava Technologies) and FlowJo version 9.4.9 software (Tree Star, Ashland, Oregon).