Uptake of the antileishmania drug tafenoquine follows a sterol-dependent diffusion process in *Leishmania*

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Objectives: The present study was designed to elucidate the mechanism of tafenoquine uptake in *Leishmania* and its sterol dependence.

Methods: Because tafenoquine is a fluorescent compound, spectrofluorimetric analysis allowed us to monitor its uptake by *Leishmania* promastigotes and intracellular amastigotes, and to evaluate the effect of temperature, energy and H+ gradient on drug entry. The influence of sterols on tafenoquine uptake in *Leishmania* parasites was determined in experiments using sterol-depleting agents such as methyl-β-cyclodextrin or cholesterol oxidase.

Results: Tafenoquine exhibited fast entry kinetics into *Leishmania* in an energy-independent, but pH- and temperature-dependent, non-saturable process. Furthermore, sterol depletion decreased tafenoquine uptake.

Conclusions: These findings suggest that *Leishmania* takes up tafenoquine by a diffusion process and that decreases in membrane sterol content may induce a decrease in drug uptake.

Keywords: 8-aminoquinolines, tafenoquine uptake, plasma membrane fluidity, sterol depletion

Introduction

Leishmaniasis, which is caused by the parasitic protozoan *Leishmania*, has a wide variety of clinical manifestations, ranging from self-healing cutaneous lesions to visceral disease, with an annual incidence of more than 2 million new cases. Treatment of this disease relies exclusively on chemotherapy, limited to a few first-line drugs, including miltefosine, paromomycin, amphotericin B deoxycholate and lipid amphotericin B, along with pentavalent antimonials, although the last group are no longer effective in endemic areas in Bihar (India) due to drug resistance. The limited number of active drugs has prompted the WHO to recommend combination therapy in order to extend the life expectancy of these compounds. New drugs, including the 8-aminoquinolines, are being developed as part of the search for cheaper oral treatments for visceral leishmaniasis. The 8-aminoquinoline scaffold has been extensively used in the development of antiprotozoal drugs, typically as antimalarial compounds. The most recent application for these drugs is the use of sitamaquine and tafenoquine as alternative leishmanicidal compounds; these two drugs are at different stages of introduction because of differences in their haematological toxicity (primarily metahaemoglobinemia and haemolysis).

Tafenoquine, an 8-aminoquinoline analogue of primaquine that is in clinical trials for the treatment and prevention of *Plasmodium* infections, may be a good candidate for an alternative leishmaniasis therapy. Indeed, tafenoquine has shown potent in vivo leishmanicidal activity by affecting mitochondrial activity in *Leishmania* parasites, leading to an apoptosis-like death process; however, the precise mechanism of tafenoquine uptake in *Leishmania* remains unknown. The present article is focused on elucidating the mechanism of tafenoquine uptake in *Leishmania* and its sterol dependence.

Methods

*Leishmania strain and culture*

*Leishmania major* (MHOM/JL/80/Friedlin) promastigotes were grown at 28 °C in RPMI 1640 modified medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% heat-inactivated fetal bovine serum (HIFBS) (Invitrogen). Intracellular amastigotes were obtained as described previously. Briefly, macrophages differentiated from THP-1 cells were
infected with late-stage promastigotes at a ratio of 1:10 macrophages/parasites. Infected macrophages were maintained for 96 h at 37°C and 5% CO₂ in RPMI 1640 medium plus 10% HIFBS. Amastigotes were harvested from macrophages selectively lysed with 0.0125% SDS in PBS (1.2 mM KH₂PO₄, 8.1 mM NaH₂PO₄, 130 mM NaCl and 2.6 mM KCl, pH 7).

**Tafenoquine uptake**

Promastigotes (2 × 10⁷ cells/mL) were treated with 5 μM tafenoquine for different times (1, 3, 5, 8, 10 and 15 min) or with different tafenoquine concentrations (0.2, 0.5, 1, 2, 4, 6, 8, 10 and 20 μM) for 1 min in culture medium at 28°C. For tafenoquine uptake experiments in amastigotes, 10⁷ cells/mL were treated with 10 μM tafenoquine for different times (1, 5 and 15 min) in culture medium (pH 5.5) at 37°C and 5% CO₂. Parasites were then washed with the same medium followed by PBS and re-suspended in 10% SDS. Tafenoquine accumulation was determined fluorimetrically by recording an emission spectrum in the range 360–460 nm upon excitation at 340 nm using an Amino-Bowman Series 2 spectrometer as described previously.⁸

**Effects of temperature, energy and H⁺ gradient on tafenoquine uptake**

These experiments were performed as described previously.⁹ Briefly, promastigotes (2 × 10⁷ cells/mL) or amastigotes (10⁷ cells/mL) were incubated for 10 min with 5 μM tafenoquine at 28 and 4°C or with 10 μM tafenoquine at 37 and 4°C, respectively, in HEPES-buffered saline (HBS; 21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄ and 6 mM glucose, adjusted to pH 7.4 for promastigotes or pH 5.5 for amastigotes), and the amount of drug incorporated into the cells was determined as described above. For energy depletion studies, promastigotes or amastigotes were pre-incubated for 30 min with 5 mM 2-deoxy-D-glucose and 20 mM NaN₃ at 28 or 37°C, respectively, in HBS buffer without glucose. H⁺-gradient dependence was determined in parasites pre-treated with 10 μM of the ionophores nigericin and monensin in HBS for 10 min at 28 or 37°C.

**Preparation of parasite surface membrane-enriched fraction**

Leishmania promastigotes (10⁷ cells/mL) in lysis buffer (10 mM Tris–HCl, 2 mM EDTA and 25 μg/mL leupeptin, pH 8.0) were disrupted for 45 min in a pre-chilled, high-pressure cavitation. The parasite lysates were centrifuged at 1000 g to eliminate cell debris, then the supernatant was centrifuged at 8000 g at 4°C for 30 min to obtain parasite surface membrane-enriched fractions. The supernatant was removed and the pellets were washed twice with 10 mM HEPES/145 mM NaCl, pH 7.4, buffer before being re-suspended in the same buffer. Alkaline phosphatase tartrate-resistant activity was determined as a control for membrane-enriched fractions, as described previously.¹⁰

**Anisotropy of parasite plasma membrane**

Leishmania plasma membrane samples (0.14–0.15 mg/mL) in 10 mM HEPES/145 mM NaCl, pH 7.4, buffer were incubated with 1,6-diphenylhexa-1,3,5-triene (DPH) or 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) probes in N,N′-dimethylformamide (DMF) in the dark for 30 min at a 1/2500 probe/protein weight ratio. The final DMF concentration in the membrane suspension was always <0.05%. The slit widths for both excitation and emission were 5 nm and the integration time was 1 s. Steady-state fluorescence anisotropy (r), as defined by Lackowicz,¹¹ was determined by measuring the vertical and horizontal components of the fluorescence emission with excitation polarized vertically. The excitation wavelength for DPH and TMA-DPH was 360 nm, with emission being monitored at 430 nm. Temperture ramps were done from 4 to 40°C in steps of 0.5°C, with an equilibration time at each temperature of 30 s and a heating rate of 30°C/min. The data point at each temperature was the average of ten 1 s anisotropy measurements.

**Tafenoquine uptake after sterol depletion in L. major**

Promastigotes (10⁷ cells/mL) were pre-incubated with 10 mM methyl-β-cyclodextrin (MCD) or 1 U/mL cholesterol oxidase (CH-OX) for 1 h in culture medium without HIFBS at 4°C in order to deplete sterols from the plasma membrane, as described previously.¹² Similar experiments with intracellular amastigotes (10⁷ cells/mL) were determined using 10 mM MCD in culture medium (pH 5.5). After washing twice with PBS, parasites were stained with the sterol dye marker filipin.¹³ Leishmania parasites were incubated on ice with 10 μg/mL filipin for 10 min in culture medium without HIFBS and washed twice with PBS, and the sample fluorescence was determined by recording an emission spectrum (400–600 nm) upon excitation at 350 nm. After sterol depletion, promastigotes and amastigotes were incubated, respectively, with 5 μM tafenoquine at 28°C and 10 μM at 37°C for 15 min in culture medium. Tafenoquine accumulation was measured as described above.

**Statistical analysis**

Statistical comparisons between groups were performed using Student’s t-test. Differences were considered significant at a level of P<0.05.

**Results and discussion**

The present work aimed to examine the mechanism of tafenoquine uptake in the protozoan parasite Leishmania; tafenoquine is a promising leishmanicidal oral drug with less haematological toxicity than sitamaquine. The tafenoquine concentrations used in these experiments were designed considering the EC₅₀ value of tafenoquine in L. major promastigotes (2.24 ± μM), as previously described.⁹ The uptake of tafenoquine at 28°C reached saturation in about 5 min (Figure 1a); similar behaviour was observed at 4°C, but with lower accumulation than at 28°C (Figure 1a). Similar results were obtained in amastigotes incubated at 37°C (Figure 1a). Furthermore, the initial rate of tafenoquine uptake was not saturable at up to at least 20 μM tafenoquine (Figure 1b). No differences in tafenoquine uptake were observed in the ATP-depleted parasites (Figure 1c), although tafenoquine uptake was found to be 50% lower at 4°C than at 28°C (P<0.05) for promastigotes and 60% lower at 4°C than at 37°C for amastigotes (P<0.05) (Figure 1c). In summary, tafenoquine shows fast entry kinetics into Leishmania parasites in a protein- and energy-independent, but temperature-dependent, non-saturable process. Furthermore, the accumulation of weakly basic aminooquinolines such as chloroquine and, more recently, sitamaquine seems to be pH-dependent.¹⁴,¹⁵ This finding was confirmed by the observation of a significant reduction in tafenoquine accumulation only in promastigotes pre-incubated with the ionophores monensin and nigericin (P<0.05) (Figure 1c), which enable the exchange of sodium and potassium ions with protons, respectively, thereby affecting the pH gradient in the cell. No differences in drug accumulation
were observed in amastigotes after ionophore treatment due to the acidic pH of the incubation medium (pH 5.5). Finally, fluorescence anisotropy studies showed significant differences between the anisotropy values for the measurements at 4 and 40°C (Figure 1d and e). Since the anisotropy values for TMA-DPH and DPH give an estimate of their free rotation in the lipid bilayer, higher fluorescence anisotropy should correspond to a decrease in membrane fluidity. This could explain the lower tafenoquine uptake observed at 4°C (with lower fluidity). Consequently, tafenoquine appears to cross the plasma membrane via a diffusion process driven by its chemical potential gradient and the pH gradient, as well as being influenced by the lipophilicity of this compound (XlogP3 = 5.4; http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=115358).

The plasma membrane is the site where the drug is taken up, and any modification to its composition or dynamics is therefore...
likely to have an impact on drug–membrane interactions, especially when the drug must be solubilized into the lipid bilayer before it can be taken up. To study the influence of sterols on tafenoquine uptake in Leishmania parasites, we first examined whether treatment with MCD or CH-OX induced changes in the sterol content of L. major, using Filipin fluorescence to monitor sterol depletion. The relationship between sterol content and plasma membrane fluidity has been described in a previous study, showing that a decrease in sterol content in plasma membrane fluidity has been described in a previous study. Sterol depletion resulted in a significant 26% reduction in tafenoquine uptake in promastigotes after MCD treatment (Figure 2a). Furthermore, sterol depletion induced by MCD and CH-OX did not have a significant effect on parasite viability, as observed in amastigotes after MCD treatment (Figure 2a). In amastigotes, a 45% reduction in tafenoquine uptake was observed after MCD treatment (P < 0.05) (Figure 2b). These results can be explained by the extent of sterol extraction observed in Figure 2(a). These results were consistent with possible drug entry through lipid raft domains, as previously described for the uptake of other antileishmania drugs. However, recent studies have shown that sitamaquine uptake in Leishmania parasites is a sterol-independent diffusion process. Thus, there is significant variability in the uptake phenomena of different compounds included in the 8-aminoquinoline family.

In conclusion, our results suggest that tafenoquine uptake in Leishmania follows a sterol-dependent diffusion process.

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Transparency declarations
None to declare.

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