Miltfosine is effective against *Candida albicans* and *Fusarium oxysporum* nail biofilms *in vitro*

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Onychomycosis is a fungal nail infection that represents ~50 % of all nail disease cases worldwide. Clinical treatment with standard antifungals frequently requires long-term systemic therapy to avoid chronic disease. Onychomycosis caused by non-dermatophyte moulds, such as *Fusarium* spp., and yeasts, such as *Candida* spp., is particularly difficult to treat, possibly due to the formation of drug-resistant fungal biofilms on affected areas. Here, we show that the alkylphospholipid miltfosine, used clinically against leishmaniasis and cutaneous breast metastases, has potent activity against biofilms of *Fusarium oxysporum* and *Candida albicans* formed on human nail fragments *in vitro*. Miltfosine activity was compared with that of commercially available antifungals in the treatment of biofilms at two distinct developmental phases: formation and maturation (pre-formed biofilms). Drug activity towards biofilms formed on nail fragments and on microplate surfaces (microdilution assays) was evaluated using XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assays, and drug effects on fingernail biofilms were analysed by scanning electron microscopy (SEM). For *F. oxysporum*, miltfosine at 8 μg ml⁻¹ inhibited biofilm formation by 93 %, whilst 256 μg ml⁻¹ reduced the metabolic activity of pre-formed biofilms by 93 %. Treatment with miltfosine at 1000 μg ml⁻¹ inhibited biofilm formation by 89 % and reduced the metabolic activity of pre-formed *C. albicans* biofilms by 99 %. SEM analyses of biofilms formed on fingernail fragments showed a clear reduction in biofilm biomass after miltfosine treatment, in agreement with XTT results. Our results show that miltfosine has potential as a therapeutic agent against onychomycosis and should be considered for *in vivo* efficacy studies, especially in topical formulations for refractory disease treatment.

INTRODUCTION

Onychomycosis is a nail infection caused by fungal pathogens and represents up to 50 % of all nail diseases worldwide (Ghannoum et al., 2000). Importantly, one in five patients with human immunodeficiency virus infection develops onychomycosis (Gupta et al., 2000), and other predisposing factors include immunosuppressive drug therapy, diabetes mellitus, peripheral vascular disease, trauma to aged nails, *Tinea pedis* infection and prolonged contact with water and detergents (Jayatilake et al., 2009).

Onychomycosis can be caused by dermatophytes or non-dermatophyte moulds (NDMs), and also by yeasts. In the northern hemisphere, the disease is mainly caused by dermatophytes, predominantly from the species *Trichophyton rubrum* and *Trichophyton mentagrophytes* (Foster et al., 2004; Ghannoum et al., 2000; Kaur et al., 2008; Nenoff et al., 2014). In contrast, in warmer and more humid countries, the incidence of onychomycosis caused by NDMs, such as *Fusarium* spp., or yeasts, such as *Candida* spp., is higher than that of the dermatophyte-borne disease (de Araújo et al., 2003; Chi et al., 2005; Morales-Cardona et al., 2014). Among yeast species, *Candida albicans* and *Candida parapsilosis* are the most commonly implicated in onychomycosis (Foster et al., 2004; Ghannoum et al., 2000), and *Candida* spp. are found in up to 75 % of cases of fingernail infections (Foster et al., 2004).

Clinical treatment of yeast- and dermatophyte-borne onychomycosis can be achieved using a variety of available antifungal drugs, such as polyenes (amphotericin B), triazoles (fluconazole, itraconazole, voriconazole), imidazoles (clotrimazole, ketoconazole, miconazole), morpholines (amorolfine) and allylamines (terbinafine) (Jayatilake et al., 2009; Jo Siu et al., 2013; Shemer, 2012). Nevertheless, the side effects associated with therapy, the frequent requirement for long-term drug administration and the refractory nature of the disease severely limit the effectiveness of
currently available antifungals against onychomycosis. Systemic administration of both itraconazole and fluconazole is effective against Candida disease (Elewski, 1998; Parlak et al., 2006), but complete cure requires long-term therapy. Whilst the efficacy of conventional antifungal drugs such as azoles and allylamines is well documented for dermatophyte- and yeast-borne onychomycoses, this is not the case for disease caused by NDMs. In fact, Fusarium spp. are resistant in vitro to many of the antifungal compounds licensed for the treatment of fungal infections, including terbinafine, itraconazole and voriconazole (Alastruey-Izquierdo et al., 2008; Bueno et al., 2010), and Fusarium nail infections, in the clinic, are refractory to common antifungals used against dermatophytes, such as terbinafine and itraconazole (Baudraz-Rosselet et al., 2010; Lurati et al., 2011). Traditional topical or systemic antifungal therapies used in the treatment of onychomycosis caused by dermatophytes are also less effective against nail infections caused by yeasts, including Candida spp. (Figueiredo et al., 2007). Thus, the development of new compounds with higher antifungal activity and improved safety is paramount for the effective treatment of fungal nail infections.

Resistance to conventional treatment means that onychomycosis often becomes chronic due to incomplete pathogen elimination. Careful monitoring and treatment of severely compromised patients is essential, because chronically infected sites may act as fungal reservoirs and may lead to fungal cellulitis or even to systemic infections (Bourgeois et al., 2008; Widmer et al., 2012; Ramage et al., 2001a, 2008). Also, these are particularly relevant onychomycosis pathogens in warmer countries, including Brazil, where the chronic nature of the disease may be linked to the limited access of antifungal drugs to the thick biomass of fungal cells and extracellular material found in affected areas, which led to the suggestion that onychomycosis should be considered a biofilm-based disease (Burkhart et al., 2002). Biofilms are heterogeneous microbial communities of cells, typically with altered phenotypes relative to planktonic cells, adhered to a biotic or abiotic surface and embedded in a polymeric extracellular matrix (Donlan & Costerton, 2002). It is notoriously difficult to fully eliminate fungal biofilms (Douglas, 2003; Martinez & Fries, 2010; Ramage et al., 2012), which represent reservoirs of pathogens in catheters and prosthetic devices, leading to continuous patient reinfection (Baillie & Douglas, 2000). Hence, the presence of thick fungal masses with extracellular material, the firm adherence of fungi to the nail plate on onychomycosis sites, the presence of dormant fungal elements and the difficulty in eradicating the disease suggest that onychomycoses may involve fungal biofilm formation (Burkhart et al., 2002; Nusbaum et al., 2012); however, the presence of biofilms in onychomycosis sites has not been formally established.

The alkylphospholipid miltefosine (hexadecylphosphocholine), currently used for the treatment of visceral leishmaniasis and in topical formulations against cutaneous breast metastases (Dorlo et al., 2012; Leonard et al., 2001), has a broad-spectrum fungicidal activity in vitro (Biswas et al., 2013; Borba-Santos et al., 2015; Tong et al., 2007; Vila et al., 2013; Widmer et al., 2006). We showed previously that miltefosine is active against C. albicans biofilms formed in vitro on the surface of central venous catheters (Vila et al., 2013). Given the need for novel therapy against onychomycosis and the ‘biofilm-like’ nature of this disease, we evaluated the effect of miltefosine against fungal biofilms formed over human fingernails using an in vitro model of nail biofilm formation. The onychomycosis agents C. albicans and Fusarium oxysporum were chosen for this study because in vitro biofilm formation by these species (on various abiotic surfaces) is well described (Chandra et al., 2001, 2005; Imamura et al., 2008; Mukherjee et al., 2012; Ramage et al., 2001a, 2008) and is well established in our laboratory (Vila et al., 2013, 2015b). These are particularly relevant onychomycosis pathogens in warmer countries, including Brazil, where the study was performed. In this country, the frequent use of ‘open’ footwear exposes feet to soil-dwelling fungi, such as F. oxysporum (Buot et al., 2010; Morales-Cardona et al., 2014), whilst the common practice of complete cuticle removal during manicure (often performed without adequate cleaning and sterilization) exposes nails to C. albicans infections. We show here that miltefosine is active against nail biofilms formed by both of these onychomycosis agents.

METHODS

Micro-organisms. Two reference strains from the American Type Culture Collection were selected for this study: C. albicans ATCC 24433 (collected from a human nail infection) and F. oxysporum ATCC 48112 (site of infection not registered). Both strains were maintained in Sabouraud dextrose agar (SDA) at 4 °C and stocks were kept for no longer than 30 days. Prior to use in experiments, F. oxysporum was subcultured in SDA for 10 days at 28 °C and C. albicans was subcultured twice on Sabouraud dextrose broth for 24 h at 35 °C. Then, F. oxysporum conidia were harvested by filtration (from colonies gently scraped from the agar) and washed in 0.1 M phosphate buffer saline (PBS, pH 7.2), and C. albicans yeast cells in liquid cultures were harvested by centrifugation and washed twice in PBS.

Antifungal agents. Miltefosine (Cayman Chemical) and fluconazole (Pfizer) were kept as 2000 μg ml⁻¹ stock solutions in sterile distilled water, and amphotericin B, terbinafine, itraconazole and voriconazole (all obtained from Sigma-Aldrich) were kept as 1600 μg ml⁻¹ stocks in DMSO. Antifungal agent stocks were kept at −20 °C, all drugs were diluted immediately before use and final DMSO concentrations did not exceed 0.14 %.

Planktonic susceptibility assays

MIC. MIC values for the treatment with antifungal agents were determined for planktonic cells (susceptions of C. albicans yeast or F. oxysporum conidia) using the broth microdilution assays published by the Clinical and Laboratory Standards Institute (CLSI, 2008a, b) in the documents M27-A3 (for C. albicans) and M38-A2 (for F. oxysporum). Minimum concentrations that inhibited fungal growth relative to controls were determined by visual inspection, and inhibition of 5 and 90 % of fungal growth (IC₅₀ and IC₉₀, respectively) was confirmed by spectrophotometric readings (OD₄₉₀) in a SpectraMAX 340 microtitre plate reader (Molecular Devices). The percentage of inhibition was calculated using the following equation: 100−(A−A₀) / A₀ × 100, where A is the optical density of wells containing antifungal agent and C is the optical density of control wells.

Minimum fungicidal concentration (MFC). MFC values (i.e. the lowest concentrations of test compounds that produced no fungal
growth) were determined by transferring a 10 μl aliquot of each sample treated with concentrations higher than the visual MIC into drug-free SDA plates and incubating at 35 °C for 48 h (for C. albicans) or 28 °C for 10 days (for F. oxysporum). Drug effects were considered ‘fungicidal’ when the MFC value was fourfold or less than the MIC value. Above this value, the antifungal effect was considered ‘fungistatic’ (Pfaller et al., 2004).

Biofilm susceptibility assays. Antifungal susceptibility tests were performed on biofilms at two distinct developmental phases (formation and maturation), based on a protocol described previously (Pierce et al., 2008), with some modifications. For biofilm formation on microplate surfaces, aliquots of 100 μl C. albicans yeast cells (10^7 c.f.u. ml^-1) or F. oxysporum conidia (10^6 conidia ml^-1) suspensions were transferred into each well and plates were incubated for 1.5 h at 36 °C (‘adhesion phase’) under constant agitation (150 r.p.m.). Then, cell suspensions were gently aspirated and 100 μl freshly prepared RPMI 1640 supplemented with 2% glucose was added to each well. For C. albicans biofilm experiments, media were also supplemented with 20% FBS (Gibco). Plates were then incubated for 24 (C. albicans) or 48 h (F. oxysporum) at 36 °C under constant agitation (150 r.p.m.) to allow biofilm growth. Biofilms were treated with drugs either immediately after the adhesion phase (Group BF: biofilms treated during formation) or later in biofilm development (Group PF: pre-formed biofilms treated 24 or 48 h post-adhesion, for C. albicans and F. oxysporum, respectively. For Group PF biofilms, the medium was changed to (100 μl fresh medium per well) immediately after the adhesion phase. All biofilms were treated by the addition of 100 μl twofold concentrated solutions of the following antifungal agents: amphotericin B (0.03–16 μg ml^-1), miltefosine (2–1000 μg ml^-1), fluconazole (2–1000 μg ml^-1), voriconazole (0.5–256 μg ml^-1) or terbinafine (0.5–256 μg ml^-1). After drug addition, plates were incubated for 24 (C. albicans) or 48 h (F. oxysporum) at 35 °C under agitation (150 r.p.m.). Then, biofilms were subjected to an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay (see below), and minimum biofilm inhibitory concentrations (BICs) that reduced the absorbance of samples from biofilms under formation (BFIC50 and BFIC90) or pre-formed biofilms (PFIC50 and PFIC90) (for Group BF or PF biofilms, respectively). Nail sections containing biofilms were then processed for SEM as described previously (Vila et al., 2015b). Briefly, nails were washed in 0.01 M PBS (pH 7.2), and then fixed in 2.5% glutaraldehyde and 4% formaldehyde, in 0.1 M cacodylate buffer, for 1 h at room temperature. Subsequently, nails were washed in the same buffer, post-fixed in 1% osmium tetroxide and 1.25% potassium ferrocyanide for 2 h, and dehydrated in a series of ethanol solutions of increasing concentration (30, 50, 70, 90, 100 % and ‘ultradry’ ethanol) for 30 min at each concentration. Then, samples were critical point dried in CO2, coated with gold and observed in a Quanta 250 scanning electron microscope (FEI).

RESULTS

Miltefosine is active against C. albicans and F. oxysporum planktonic cells

Current therapy against onychomycosis often fails to achieve complete cure and drug resistance may be due, at least in part, to the presence of fungal biofilms, where cells have increased resistance to therapy (Burkhart et al., 2002). Recently, we showed that treatment with the antimicrobial drug miltefosine is effective against fungal biofilms formed in clinical catheters in vitro (Vila et al., 2013).

As an initial step to examine the susceptibility of the onychomycosis agents C. albicans and F. oxysporum to miltefosine, planktonic forms of these fungi were treated in vitro with this drug and the effect of miltefosine was compared with that of a panel of standard antifungal agents (currently used to treat onychomycosis in the clinic). Susceptibility assays showed that the selected strains of both species were susceptible to miltefosine. For C. albicans, miltefosine at 0.5 μg ml^-1 inhibited 90% of planktonic cell growth, and this susceptibility profile was similar to those of amphotericin B and fluconazole (Table 1). In contrast, terbinafine had reduced antifungal activity (IC90 > 16 μg ml^-1) against C. albicans yeasts and voriconazole had the lowest IC90 values (0.06–0.125 μg ml^-1; Table 1)

F. oxysporum conidia were less sensitive to miltefosine than C. albicans, with IC50 and IC90 values of 0.5 and 1 μg ml^-1, respectively, whereas IC90 values of 0.125 and 0.5 μg ml^-1 were obtained for the standard antifungal drugs amphotericin B and voriconazole, respectively (Table 1). However, terbinafine and itraconazole had reduced antifungal activity against F. oxysporum (Table 1). The MFC of miltefosine and amphotericin B was two- to fourfold higher than the IC90 for C. albicans and F. oxysporum planktonic cells, and the MFC of voriconazole was also two- to fourfold higher than the IC90 for C. albicans (Table 1). These results suggest that miltefosine has fungicidal activity against planktonic forms of C. albicans and F. oxysporum, according to the classification proposed by Pfaller et al. (2004), which defines respectively. The activity of miltefosine on nail biofilms was quantified using an XTT assay or visualized by scanning electron microscopy (SEM; see below).

SEM. For SEM, biofilms on nail fragments were treated with miltefosine as described above, using concentrations above and below the BFIC90 or PFIC90 (for Group BF or PF biofilms, respectively). Nail sections containing biofilms were then processed for SEM as described previously (Vila et al., 2015b). Briefly, nails were washed in 0.01 M PBS (pH 7.2), and then fixed in 2.5% glutaraldehyde and 4% formaldehyde, in 0.1 M cacodylate buffer, for 1 h at room temperature. Subsequently, nails were washed in the same buffer, post-fixed in 1% osmium tetroxide and 1.25% potassium ferrocyanide for 2 h, and dehydrated in a series of ethanol solutions of increasing concentration (30, 50, 70, 90, 100 % and ‘ultradry’ ethanol) for 30 min at each concentration. Then, samples were critical point dried in CO2, coated with gold and observed in a Quanta 250 scanning electron microscope (FEI).
Table 1. Susceptibility of C. albicans yeast cells and F. oxysporum conidia to miltefosine, compared with that of the standard antifungals amphotericin B, fluconazole, itraconazole, voriconazole and terbinafine

Planktonic cell susceptibility was determined by visual inspection (100 % inhibition) and by turbidity (OD492) as described in Methods.

<table>
<thead>
<tr>
<th>Antifungal drug</th>
<th>Visual inspection</th>
<th>OD492 IC50</th>
<th>MFC</th>
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<tbody>
<tr>
<td>C. albicans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miltefosine</td>
<td>0.5</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.125</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0.25</td>
<td>0.25</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.125–0.25</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>&gt;16</td>
<td>1–4</td>
<td>&gt;16</td>
</tr>
<tr>
<td>F. oxysporum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miltefosine</td>
<td></td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.25</td>
<td>0.06–0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>&gt;16</td>
<td>16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.125–0.25</td>
<td>ND</td>
<td>0.125</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>&gt;16</td>
<td>ND</td>
<td>16</td>
</tr>
</tbody>
</table>

ND, Not determined because it was below the detection level of the microdilution assay used.

that an agent has fungicidal activity when the MFC is up to two- to fourfold higher than the MIC.

Miltefosine is active against C. albicans and F. oxysporum biofilms formed on microplate surfaces

To determine the minimal concentrations of miltefosine and standard antifungal drugs that inhibit biofilm formation and affect pre-formed biofilms, we adapted in vitro microdilution assays previously described by Mukherjee et al. (2012) for Fusarium spp. and by Pierce et al. (2008) for C. albicans.

For C. albicans, treatment with miltefosine at 125 μg ml⁻¹ reduced biofilm formation growth/viability (BFIC90) by 90 % and reduced the growth/viability of pre-formed biofilms (PFIC90) by 50 %, whilst treatment with miltefosine at 250 μg ml⁻¹ reduced the growth/viability of pre-formed biofilm cells (PFIC90) by 90 %. In contrast, treatment with fluconazole (at up to 2000 μg ml⁻¹) did not affect either forming or mature C. albicans biofilms (Table 2). Amphotericin B was the most active drug against C. albicans biofilms, at both developmental stages, with a BFIC90 of 4 μg ml⁻¹ and a PFIC90 of 12 ± 4 μg ml⁻¹ (Table 2).

For F. oxysporum, a similar antibiofilm effect was observed for miltefosine and amphotericin B, with 6 ± 2 μg ml⁻¹ of the drug inhibiting biofilm formation by 90 % (BFIC90) and 12 ± 4 μg ml⁻¹ reducing the growth/viability of pre-formed biofilms (PFIC90) by 90 % (Table 2).

In agreement with the results obtained for planktonic cells, terbinafine and voriconazole showed low anti-Fusarium biofilm activity, even towards the early stages of biofilm development (Table 2). Terbinafine also failed to inhibit biofilm formation or affect pre-formed C. albicans biofilms (Table 2). Despite the good antifungal activity observed for planktonic cells, voriconazole showed only limited antibiofilm effect against both phases of C. albicans biofilm development (Table 2). Given the high IC90 (>16 μg ml⁻¹; Table 1) obtained for itraconazole against F. oxysporum in planktonic assays, this drug was not included in antibiofilm assays for this species.

Miltefosine is active against C. albicans and F. oxysporum biofilms formed on human fingernail fragments

The results of microdilution assays showed that miltefosine is active against biofilms of both C. albicans and F. oxysporum formed on microplate surfaces. To determine whether miltefosine is also active against onychomycosis agents forming biofilms in a nail environment, we used a model of fungal biofilm formation on human nail fragments (Vila et al., 2015b). In this model, fungal biofilms are formed in vitro on the surface of sterilized human nail fragments of ~0.5 cm in length (donated by volunteers with no previous history of nail infection).

Biofilms at different developmental stages (Groups BF and PF) were treated with miltefosine at concentrations of 4, 40, 200 and 1000 μg ml⁻¹ for C. albicans, and 2, 4 and 8 μg ml⁻¹ for F. oxysporum. These concentrations were selected to include values below and above the BFIC90 and PFIC90 values determined for biofilms formed on microplate surfaces (using the microdilution assay; Table 2). Biofilm growth/viability on fingernails was significantly inhibited in the presence of miltefosine at concentrations similar to the BFIC90 values obtained using the microdilution assay for both C. albicans (BFIC90 of 125 μg ml⁻¹, P<0.01; Fig. 1a) and F. oxysporum (BFIC90 4 μg ml⁻¹,
**Table 2. Susceptibility of *C. albicans* and *F. oxysporum* biofilms to miltefosine compared with that of the standard antifungals amphotericin B, fluconazole, itraconazole, voriconazole and terbinafine**

The effects of drugs on biofilms formed on microplate surfaces were quantified using an XTT reduction assay as described in Methods. B\textsubscript{fIC}\textsubscript{50} and B\textsubscript{fIC}\textsubscript{90} concentrations that inhibited biofilm formation by reducing cell growth/viability by 50 and 90 %, respectively; P\textsubscript{fBIC}\textsubscript{50} and P\textsubscript{fBIC}\textsubscript{90} concentrations that reduced pre-formed biofilm viability/growth by 50 and 90 %, respectively.

<table>
<thead>
<tr>
<th>Antifungal drug</th>
<th>Biofilm formation (Group BF)</th>
<th>Pre-formed biofilm (Group PF)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>B\textsubscript{fIC}\textsubscript{50}</td>
<td>B\textsubscript{fIC}\textsubscript{90}</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miltefosine</td>
<td>ND</td>
<td>125</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>1–2</td>
<td>4</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>1–4</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miltefosine</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>4</td>
<td>8–16</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>64–256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>ND</td>
<td>8</td>
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</tbody>
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ND, Not determined because it was below the detection level of the microdilution assay used.

\(P<0.01;\) Fig. 1b). In addition, concentrations eight- or two-fold higher than the B\textsubscript{fIC}\textsubscript{90} for *C. albicans* (1000 \(\mu\)g ml\(^{-1}\)) and *F. oxysporum* (8 \(\mu\)g ml\(^{-1}\)) led to a dramatic reduction of the growth/viability of biofilms \((P<0.0001\) and \(P<0.01,\) respectively; Fig. 1a, b).

Treatment with miltefosine at 200 \(\mu\)g ml\(^{-1}\) (1.6-fold higher than the P\textsubscript{fBIC}\textsubscript{90}) significantly reduced the growth/viability of pre-formed biofilms \((P<0.01),\) whilst 40 \(\mu\)g ml\(^{-1}\) had no inhibitory effect on the growth/viability of biofilm cells (Fig. 1c). In addition, when pre-formed biofilms were treated with miltefosine at 1000 \(\mu\)g ml\(^{-1}\), the growth/viability of biofilm cells was reduced by 99 % \((P<0.0001;\) Fig. 1c).

Pre-formed *F. oxysporum* biofilms grown over fingernails were also highly susceptible to miltefosine, and treatment with concentrations ≥ 64 \(\mu\)g ml\(^{-1}\) (fourfold higher than the P\textsubscript{fBIC}\textsubscript{90}) led to a significant reduction in the growth/viability of biofilm cells \((P<0.0001;\) Fig. 1d).

**Miltefosine inhibits the establishment of nail biofilms of *C. albicans* and *F. oxysporum***

The output of the XTT assay (whether on plate surfaces or nail fragments) reflects different potential drug effects combined, including changes in the metabolic activity of biofilm cells, growth reduction and cell death. Thus, to examine in more detail the activity of miltefosine against fungal biofilms formed on human nails, we analysed treated nail biofilms by SEM.

SEM images show that *C. albicans* control biofilms grown in vitro over human fingernails covered the entire surface of nail fragments and had the typical architecture of mature *C. albicans* biofilms, comprising a dense network of interconnected hyphae, pseudo-hyphae and budding yeasts (Fig. 2a, b). Treatment with miltefosine at 4 \(\mu\)g ml\(^{-1}\) did not inhibit biofilm development (Fig. 2c, d), but 40 \(\mu\)g ml\(^{-1}\) led to a clear reduction in the biofilm biomass found on nail surfaces (Fig. 2e). SEM images confirmed that treated cells appeared healthy, although more hyphae than yeasts were observed (Fig. 2f). Treatment with miltefosine at 200 \(\mu\)g ml\(^{-1}\) inhibited biofilm formation almost completely, resulting in ‘clean’ nails with only a few lumps of cells, which appeared damaged (Fig. 2g, h, white arrows). Most cells remained in the yeast and pseudo-hypha phases (Fig. 2h).

Control *F. oxysporum* biofilms grown in vitro over human fingernails consisted of dense networks of interconnected hyphae and covered the entire surface of nail fragments (Fig. 3a, b). Treatment of biofilms under formation with miltefosine at 2 or 4 \(\mu\)g ml\(^{-1}\) reduced biofilm development significantly, with only relatively smaller areas of the nail fragment being covered by biofilms (Fig. 3c, e). Additionally, we observed subtle morphological alterations on treated hyphae, whose surface appeared wavy in some regions (Fig. 3d, f, white arrows) when compared with the control (Fig. 3b). The presence of miltefosine at 8 \(\mu\)g ml\(^{-1}\) inhibited biofilm formation almost completely (Fig. 3g), and the remaining cells appeared highly damaged and/or collapsed (Fig. 3h).

**Miltefosine reduces the metabolic activity and disrupts *C. albicans* and *F. oxysporum* pre-formed biofilms on human fingernails**

To evaluate the effects of miltefosine on pre-formed (i.e. mature) biofilms, nail fragments containing biofilms formed
for 24 (C. albicans) or 48 h (F. oxysporum) were treated with four concentrations of miltefosine (4, 40, 200 and 1000 µg ml⁻¹ for C. albicans or 32, 64, 256 and 1024 µg ml⁻¹ for F. oxysporum) and analysed by SEM after 24 or 48 h of treatment (for C. albicans and F. oxysporum, respectively).

At 48 h post-adhesion, C. albicans control mature biofilms covered a large proportion of the total area of nail surfaces and were composed mostly of interconnected hyphae with considerably less numerous budding yeasts (Fig. 4a, b). Treatment with miltefosine at 4 µg ml⁻¹ did not affect pre-formed F. oxysporum biofilms when compared with untreated controls as determined by SEM and XTT assays (not shown). In contrast, treatments with miltefosine at 40 or 200 µg ml⁻¹ reduced the biofilm biomass on nail fragments to only a few ‘clumps’ (Fig. 4c, e). However, no surface alterations could be observed in these few biofilm cells (Fig. 4d, f). Interestingly, despite the similarity between the structures of C. albicans biofilms treated with miltefosine at 40 and 200 µg ml⁻¹ as observed by SEM, the metabolic assay showed that treatment with 200 µg ml⁻¹ (1.6-fold higher than the PFBIC₅₀) significantly reduced the metabolic activity of biofilm cells (P<0.01; Fig. 1c), whilst 40 µg ml⁻¹ had no inhibitory effect on the metabolic activity of biofilm cells (Fig. 1c). SEM images of biofilms treated with miltefosine at 1000 µg ml⁻¹ showed clear nail fragments, with no associated biofilms (Fig. 4g, h).

In agreement with the XTT results showing that mature nail biofilms of F. oxysporum were highly susceptible to miltefosine treatment at concentrations ≥64 µg ml⁻¹ (Fig. 1d), we observed that treatment with these concentrations led to the elimination of biofilms from fingernail surfaces (Fig. 5c, e, g), with only damaged (Fig. 5d) or collapsed (Fig. 5f, h) cells remaining. Concentrations of miltefosine <64 µg ml⁻¹ were also tested (8, 16 and 32 µg ml⁻¹), but these did not affect pre-formed F. oxysporum biofilms, as determined by SEM and XTT analyses (data not shown).

**DISCUSSION**

Onychomycoses share some of the main characteristics of biofilms: the thick biomass of cells covered by extracellular
Fig. 2. SEM analysis of *C. albicans* biofilms formed *in vitro* on the surface of human nail fragments (a, b) in the absence (untreated control), or in the presence of miltefosine at concentrations of (c, d) 4, (e, f) 40 or (g, h) 200 μg ml⁻¹. Treatment
started immediately after the adhesion phase (Group BF, treated during formation). The highest drug concentration (200 µg ml$^{-1}$) inhibited C. albicans nail biofilm formation in vitro (g, h) when compared with controls (a, b). Treatment with 200 µg ml$^{-1}$ resulted in cell damage (g, h, white arrows). Bar, 3 mm (a, c, e, g) and 20 µm (b, d, f, h).

material (which results in poor antifungal penetration), drug-resistant behaviour and the ability to function as a reservoir of pathogenic micro-organisms, favouring chronic infection (Burkhart et al., 2002; Nusbaum et al., 2012). Burkhart et al. (2002) suggested that dermatophytes (i.e. onychomycosis caused by dermatophytes) are often refractory to standard oral antifungals due to the existence of fungal biofilms in infected sites. Here, we show that fungal biofilms formed on human nail fragments in vitro are sensitive to the alkylphospholipid miltefosine—a drug already used for the treatment of parasitic diseases (leishmaniasis) and active against C. albicans biofilms formed in vitro (Vila et al., 2013). These results are important given the poor efficacy of the current treatment regimens for onychomycosis. Also, patients with renal or hepatic diseases may not be able to use systemic antifungals against onychomycosis, reinforcing the notion that alternative topical agents—such as miltefosine, available as topical (as well as oral) commercial formulations—are urgently needed, especially for the treatment of nail infections caused by NDMs and yeasts.

Tong et al. (2007) have shown that several dermatophytes (including T. rubrum and T. mentagrophytes) are highly susceptible to miltefosine in planktonic form, but no biofilm susceptibility assays have yet been published. Dermatophytes, such as T. rubrum and T. mentagrophytes, are the most common causative agents of onychomycosis in the north-east hemisphere (Foster et al., 2004; Ghannoum et al., 2000; Kaur et al., 2008; Nenoff et al., 2014), but in the southern hemisphere Fusarium and Candida have shown an emerging incidence rate in onychomycosis (de Araújo et al., 2003; Chi et al., 2005; Morales-Cardona et al., 2014). Fusarium and Candida were selected as models for this work based on their high incidence in the southern hemisphere and poor responsiveness to commercially available antifungal drugs.

The use of nail fragments as a substrate for the in vitro adherence of Candida was first described by Oliveira et al. (2010), but robust biofilms were not observed. The in vitro nail biofilm model used here generates dense biofilms of both C. albicans and F. oxysporum (Figs 2–5), and is likely to be useful in other pre-clinical studies of drug activity against onychomycosis.

Candida nail infections are usually treated with oral administration of fluconazole and itraconazole. Here, we demonstrate that fluconazole treatment does not inhibit C. albicans biofilm formation in vitro; also, it does not affect pre-formed biofilms, even at concentrations up to 1000 µg ml$^{-1}$ (Table 2). These data are in agreement with those previously showing that concentrations of fluconazole >256 (Kuhn et al., 2002; Mukherjee et al., 2003) and >1024 µg ml$^{-1}$ (Ramage et al., 2002), respectively, were necessary to reduce 50 % of C. albicans biofilm growth/viability. Our results may explain the resistance of Candida nail infections to treatment with azoles and the need for long periods of treatment to achieve total fungal elimination (Jayatilake et al., 2009). Overall, our data support the notion that biofilm formation during onychomycosis contributes to increase drug resistance to antifungals (Burkhart et al., 2002).

In the present work, miltefosine was active against F. oxysporum biofilms at concentrations between 4 and 16 µg ml$^{-1}$ (Table 2), whilst the standard antifungals (itraconazole, voriconazole and terbinafine) had little or no activity against F. oxysporum biofilms in vitro (Table 2), in line with reports showing that Fusarium spp. onychomycosis is resistant to terbinafine and itraconazole in the clinic (Baudraz-Rosselet et al., 2010; Lurati et al., 2011).

Our experiments showed that miltefosine is more effective than azoles (fluconazole, itraconazole and voriconazole) and terbinafine against both forming and pre-formed C. albicans and F. oxysporum biofilms (Table 2). This is an important finding because fluconazole is the reference antifungal treatment for onychomycosis caused by C. albicans (Jayatilake et al., 2009), and itraconazole, voriconazole and terbinafine are in the treatment of NDM nail infections. Although better antibiofilm activity than that of miltefosine was demonstrated by amphotericin B for both species using the in vitro microdilution assay (for biofilms formed on microplate surfaces), nail infections respond slowly to this drug, as it binds tightly to serum proteins and does not penetrate tissues readily (Jayatilake et al., 2009). Miltefosine has good oral bioavailability (82–95 %) and distributes to all tissues, accumulating mainly in the liver, lungs, kidneys and spleen. Although miltefosine has significant side effects (mainly gastrointestinal) when administered orally; it is considerably less toxic than amphotericin B (Borb-Santos et al., 2015; Widmer et al., 2006).

In rats, serum concentrations of miltefosine reached 110 mM (44.83 µg ml$^{-1}$) after 2 weeks of daily dosing with 10 mg kg$^{-1}$ (Widmer et al., 2006). This concentration is 40-fold higher than the IC$_{50}$ values reported here for the treatment of C. albicans and F. oxysporum in vitro (Table 1), and the IC$_{50}$ values reported previously for several fungal species causing invasive mycoses (Widmer et al., 2006). This concentration is also 10-fold higher than that capable of inhibiting F. oxysporum biofilm growth/viability by 90 % (4 µg ml$^{-1}$), in our in vitro nail biofilm model (Fig. 1b). However, the miltefosine serum concentration achieved in treated rats (Widmer et al., 2006) is slightly lower than that required...
Fig. 3. SEM of *F. oxysporum* biofilms formed in vitro on the surface of human nail fragments (a, b) in the absence (untreated control), or in the presence of miltefosine at concentrations of (c, d) 2, (e, f) 4 or (g, h) 8 µg ml⁻¹. Treatment started immediately after the adhesion phase (Group BF, treated during formation). Whilst all concentrations tested reduced biofilm biomass, treatment with 8 µg ml⁻¹ led to total inhibition of biofilm growth (g, h). Bar, 3 mm (a, c, e, g) and 50 µm (b, d, f, h).
to inhibit *C. albicans* biofilm formation and to reduce the viability of pre-formed biofilms of both *C. albicans* and *F. oxysporum* species (200 and 64 μg ml⁻¹, respectively) in our *in vitro* nail biofilm model (Figs 1–5). Even if oral administration of miltefosine turns out to be ineffective due to inadequate plasma concentrations of the drug, topical formulations of this compound may still be useful in the treatment of onychomycosis.

**Fig. 4.** SEM of *C. albicans* biofilms formed *in vitro* on the surface of human nail fragments, and then (a, b) left untreated (control) or treated with miltefosine at (c, d) 40, (e, f) 200 or (g, h) 1000 μg ml⁻¹ for 24 h. Treatment started 24 h after the end of the adhesion phase (Group PF, pre-formed biofilms, treated during maturation). Whilst all concentrations tested reduced biofilm biomass, treatment with 1000 μg ml⁻¹ led to total elimination of pre-formed biofilms (g, h). Treatment with 40 and 200 μg ml⁻¹ led to subtle morphological alterations on treated hyphae, whose surface appeared wavy in some regions (d, f, white arrows). Bar, 3 mm (a, c, e, g) and 20 μm (b, d, f, h).
Fig. 5. SEM of *F. oxysporum* biofilms formed *in vitro* on the surface of human nail fragments and then (a, b) left untreated (control) or treated with miltefosine at (c, d) 64, (e, f) 256 or (g, h) 1024 μg ml⁻¹ for 48 h. Treatment started 48 h after the
Treatment with miltefosine at 1000 (C. albicans) or 8 μg ml\(^{-1}\) (F. oxysporum) at the early stages (after the adhesion of the cells to the nails) prevented biofilm growth by 89 and 93%, respectively. However, the effects of drugs on pre-formed biofilms – expected to resemble established onychomycosis infections more closely – are likely to be more relevant for the translation of our results to the clinic. Treatment with miltefosine at 200 μg ml\(^{-1}\) reduced the growth/viability of C. albicans biofilms by 52%, whilst treatment with 256 μg ml\(^{-1}\) reduced the growth/viability of F. oxysporum biofilms by 93% (Fig. 1c, d). However, when concentrations close to 1 mg ml\(^{-1}\) were added, pre-formed biofilms of both C. albicans and F. oxysporum were completely eliminated (reduction of 99% in growth/viability) from fingernail fragments (Figs 1, 4 and 5). These data are very encouraging because miltefosine concentrations as high as 2 mg ml\(^{-1}\) showed no toxic effect on the oral mucosa of mice in previous experiments by our group (Vila et al., 2015a).

Miltefosine is a phosphatidylcholine analogue and therefore is inserted in the cell membrane, potentially disrupting the function of membrane-associated proteins (Biswas et al., 2014; Zuo et al., 2011). In addition, in Saccharomyces cerevisiae planktonic cells, miltefosine is concentrated in the inner mitochondrial membrane, where it interrupts the membrane potential in the electron transport sequence and triggers signalling to apoptosis-like cell death through the activation of metacaspase MCA1 of S. cerevisiae (that is also present in C. albicans) (Biswas et al., 2014; Zuo et al., 2011). In this proposed mechanism of action, the antifungal effect of miltefosine is closely related to mitochondrial function.

In our biofilm model, after treatment with miltefosine at 40 μg ml\(^{-1}\), C. albicans biofilm cells were able to reduce XTT to formazan complexes to the same extent as control cells. Alone, this result could lead to the conclusion that this concentration did not affect biofilm cells. However, SEM images demonstrated substantial morphological alterations in the biofilms treated with miltefosine at 40 μg ml\(^{-1}\), showing that those cells were no longer as healthy as control cells. Total inhibition of mitochondrial activity was reached for both fungal biofilms at higher concentrations of miltefosine, as described above.

**CONCLUSIONS**

Taken together, our data show that miltefosine is effective against both developmental phases (formation and maturation) of C. albicans and F. oxysporum biofilms formed on human nail fragments in vitro. Miltefosine activity reduces biofilm formation and biomass, and also the viability of pre-formed biofilms. These results confirm that miltefosine is a promising antifungal drug that should be considered for further analysis in efficacy tests in vivo. Additionally, this work shows that the in vitro model of nail biofilms used here can be applied successfully to the pre-clinical testing of novel onychomycosis agents.

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