Anti-Acanthamoeba efficacy and toxicity of miltefosine in an organotypic skin equivalent

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Objectives: Acanthamoebae can cause infections of several organs, including eye, skin, lung and brain. Except for Acanthamoeba keratitis, these infections are linked to immunodeficiency. Treatment is generally problematic, due to the lack of sufficiently effective and also easily manageable drugs. In a previous study we discovered that miltefosine (hexadecylphosphocholine) is highly active against Acanthamoeba spp. in vitro. The aim of the current study was to evaluate the suitability of miltefosine for the topical treatment of Acanthamoeba infections.

Methods: Storage life and time dependency, susceptibilities of opportunistic bacterial and fungal pathogens, and synergistic and adverse effects of combinations with other anti-Acanthamoeba substances were determined. Moreover, an organotypic skin equivalent was adapted for investigating the penetration of acanthamoebae into the epidermis and the human tissue tolerability of miltefosine.

Results: It was shown that miltefosine can be stored as a 2 mM stock solution and also as a 50 mM dilution over a period of 12 months at 4°C without any loss of activity. Efficacies against staphylococci and Candida albicans were established. Acanthamoebae were able to penetrate the skin equivalent within 24 h. This penetration was prevented by treatment with miltefosine, while miltefosine treatment was well tolerated by the skin equivalent.

Conclusions: Miltefosine has been approved for oral and topical treatment of leishmaniasis and may also be a promising candidate for the topical treatment of Acanthamoeba infections.

Keywords: amoebae, emerging diseases, immunosuppression, infection models, treatment

Introduction

Acanthamoeba spp. are the causative agents of Acanthamoeba keratitis (AK), on the one hand, and of disseminating infections such as skin lesions, pneumonia, and granulomatous amoebic encephalitis (GAE), occurring mainly in immunocompromised individuals, on the other. The treatment of Acanthamoeba infections is still problematic due to the lack of sufficiently effective and also easily manageable drugs. AK is usually treated with a combination of cationic antiseptics such as chlorhexidine, which inhibit membrane function, and with aromatic diamidines such as propanidide isethionate (Brolene®), which inhibit DNA synthesis. These antiseptics have to be applied hourly for 3 days and subsequently up to six times a day. This treatment has generally proved to be very successful, but early onset of treatment is crucial. Moreover, resistance against propanidide does occur and in a recent case persistent amoebae were observed after 1 year of topical treatment with chlorhexidine.
opportunistic Acanthamoeba infections there is currently no drug of choice and only few patients have survived.1–3 Miltefosine (Impavido®), an alkylphosphocholine, has known activity against different protozoan parasites,6–8 and has been approved for oral and topical treatment of visceral and cutaneous leishmaniasis, respectively. In previous studies several groups have demonstrated its high efficacy against Acanthamoeba spp. and other amphizoic amoebae in vitro.11–13 Due to the lack of an established medication, topical miltefosine has recently been applied for the treatment of Acanthamoeba skin lesions, leading to a complete healing of the lesions within 6 weeks.14

The aim of the current study was to evaluate the suitability of miltefosine for the topical treatment of Acanthamoeba infections in more depth. Therefore, firstly, storage life and time dependency of the efficacy of different concentrations of miltefosine against trophozoites and cysts of Acanthamoeba were assessed in a microtitre plate system. Secondly, the susceptibility of important opportunistic pathogens, and synergistic and adverse effects of combinations of miltefosine with established anti-Acanthamoeba substances were determined. Thirdly, an organotypic skin equivalent was adapted for investigating the penetration of acanthamoebae into the epidermis and the human tissue tolerability of anti-Acanthamoeba-effective concentrations of miltefosine.

Materials and methods

Amoeba culture

Acanthamoeba castellanii strain 1BU, genotype T4 (ATCC PRA-105), was used throughout the study. This strain had been isolated from a corneal specimen of a patient who had developed a severe keratitis in the left eye.15 This strain was chosen because it displays pronounced pathogenicity-related physiologic properties and a strong cytopathic effect in vitro, and it has been shown to be susceptible to miltefosine in a previous study.11,15 The amoebae were grown on agar plates coated with a lawn of Escherichia coli and, subsequently, fresh axenic cultures were obtained before each experiment as described previously.15 Briefly, cysts were harvested from 7-day-old plate cultures using sterile cotton-tipped applicators, washed three times in sterile saline, transferred into 3% HCl and incubated at room temperature overnight in order to kill the bacteria. After another washing step the cysts were transferred into proteose–peptone–yeast extract–glucose medium and incubated at 30°C. The trophozoites were harvested from 72-h-old cultures by centrifugation at 500 g for 7 min. The cells were resuspended in sterile medium and PBS, respectively, and subsequently counted in a Bürker–Türk haemocytometer and brought to a concentration of 10⁶ cells/mL.

Chemicals

Miltefosine was purchased from Cayman Chemical (Tallinn, Estonia) and prepared as a 2 mM stock solution in 5% ethanol. Propamidine isethionate (Brolene®) was purchased from Sanofi–Aventis (Vienna, Austria) and chlorhexidine was purchased from Sigma–Aldrich (Vienna, Austria).

Storage life and time dependency of the efficacy of miltefosine

Storage life and time dependency of the efficacy of the 2 mM stock solution and of a 50 μM dilution in PBS, both stored at 4°C, were assessed in monthly intervals over a period of 12 months by comparing with freshly prepared solution in a 96-well microtitre system using trophozoites and cysts of Acanthamoeba strain 1BU. Each well was seeded with 10⁴ trophozoites or cysts. The log-reduction of amoeba cells was recorded after 0.5, 24, 48 and 72 h by counting the survivors in a Bürker–Türk haemocytometer. Viability was determined by Trypan Blue exclusion, and 100% eradication was confirmed by transferring 100 μL of the suspension to an E. coli-coated agar plate and recording amoebal growth for 14 days.

Synergistic and antagonistic effects

Synergistic and adverse effects of combinations of miltefosine with propamidine isethionate and chlorhexidine were evaluated in a microtitre system as described above. Miltefosine was used at concentrations of 10, 20, 40, 80, 160 and 320 μM. Co-treatment assays were performed in parallel with 0.1% propamidine isethionate and 0.02% chlorhexidine, respectively. Efficacy was recorded after 0.5, 1, 1.5 and 24 h.

Efficacy against opportunistic pathogens

The susceptibility of bacteria and yeasts to miltefosine was evaluated in suspension tests. Therefore, standard qualitative suspension tests16 were carried out with Staphylococcus aureus (DSM 799), Enterococcus hirae (DSM 3320) and Candida albicans (DSM 1386) at concentrations of ~1.5 × 10⁸ cfu/mL. The efficacies of 10, 25, 75, 100, 150 and 200 μM miltefosine were assessed at exposure times of 1, 6, 12, 18 and 24 h. All tests were performed with and without addition of 0.03% albumin, and 0.9% NaCl was used as a growth control.

Toxicity against human cells

The toxicity of miltefosine to human keratinocytes was evaluated and compared with the tolerabilities of 0.02% and 0.1% chlorhexidine by the tetrazolium reduction assay EZ4U (Biomedica GmbH, Vienna, Austria), a cytotoxicity assay. The assay was performed according to the manufacturer’s instructions.

Topical activity of miltefosine against Acanthamoeba in a skin equivalent

In vitro reconstructed skin equivalents were generated as described previously with slight modification.17 Briefly, a collagen suspension containing fibroblasts was poured into cell culture inserts. After gelation at 37°C in a humidified atmosphere for 2 h, the gels were equilibrated and 1.3 × 10⁶ keratinocytes suspended in 2 mL of keratinocyte growth medium were added. After overnight incubation the medium was removed, putting the keratinocytes at the air–liquid interface (day 0). Skin equivalents were cultivated in serum-free keratinocyte-defined medium supplemented with 1.3 mM calcium, 10 μg/mL transferrin, 50 μg/mL ascorbic acid and 0.1% BSA (Sigma–Aldrich, Vienna, Austria). Assays were performed in 6-well plates. A 20 μL suspension of 10⁴ trophozoites in PBS or 20 μL of PBS alone was inoculated onto the centre of each skin equivalent and the plates were incubated at 34°C. A parallel setup was treated with 50 μM miltefosine 1 h after inoculation of amoebae. This concentration was chosen because although some Acanthamoeba strains are susceptible to even considerably lower concentrations, concentrations of ≥40 μM have been proven to be generally amoebicidal in previous studies.11–13 The following controls were included: blank; application of 20 μL of PBS without amoebae; and
application of 50 μM miltefosine without amoebae. Punches were taken after 6, 24 and 48 h, and subjected to immunostaining. Briefly, a polyclonal anti-Acanthamoeba antiserum was produced by immunization of a rabbit with Acanthamoeba whole cell antigen. Tissue sections were incubated with anti-Acanthamoeba antiserum, washed, stained with FITC-linked antirabbit antibodies and counterstained with Hoechst 33258. A control staining was performed with haematoxylin & eosin (HE).

All tests were performed at 34°C, which corresponds to the human skin and eye temperature, under sterile conditions, and were repeated in two independent biological repeats.

All experiments were conducted in accordance with the Declaration of Helsinki and national and institutional standards.

### Results

#### Stability, efficacy and toxicity of miltefosine

No reduction of efficacy was observed over the period of 12 months, neither in the stock solution nor in the dilution, when stored at 4°C. The efficacies were unaltered compared with those that had been observed with a freshly prepared miltefosine solution in a previous study.\(^{11}\) At all 12 timepoints (1–12 months), all three preparations of 50 μM miltefosine—the one stored as a 50 μM dilution in PBS, the one prepared from a stored 2 mM stock solution in 5% ethanol and the one prepared from a fresh 2 mM stock solution in 5% ethanol—were equally effective against Acanthamoeba, resulting in a 100% killing of trophozoites and a 3 log reduction of cysts in all three repeats. The trophozoites exhibited extensive vacuolization, rounded up and detached from the cavity bottom. Killing of the trophozoites was reached within 30 min in all experiments. Excystation of cysts was entirely inhibited.

Miltefosine was effective against S. aureus and C. albicans, whereas it was relatively ineffective against E. hirae. However, compared with Acanthamoeba spp, longer treatments were needed to achieve comparable efficacies. The most susceptible was S. aureus, being completely killed within 12 h after treatment with 10 μM miltefosine, while for the total reduction of E. hirae and C. albicans 24 h treatments with concentrations of 75 and 25 μM, respectively, were needed (Table 1). At the very short exposure time of 1 h none of the test organisms was killed. After 6 h, S. aureus was totally killed by 75 μM miltefosine, whereas the same concentration was effective against C. albicans only after 18 h. However, C. albicans was killed by a 12 h treatment with 150 μM miltefosine. In all cases, 0.03% albumin as an organic load diminished the efficacy of miltefosine.

No statistically significant synergistic or adverse effects were observed with combinations with either 0.1% propamidine isethionate or 0.02% chlorhexidine. Efficacies of miltefosine at the investigated periods of treatment were comparable to those without the addition of propamidine isethionate and chlorhexidine.

It was shown that the toxicity of miltefosine is significantly lower than the toxicity of chlorhexidine, a standard drug used for treatment of AK. After 1 h of treatment, cells remained relatively unaffected by 50 μM miltefosine (Figure 1), a concentration already 100% amoebicidal within the given time frame.\(^{11}\) The higher toxicity of chlorhexidine was even more pronounced after 2 h of treatment, where the toxicity of the lower concentration of chlorhexidine (0.02%) was comparable to treatment with 1000 μM miltefosine, while treatment with 50 μM miltefosine was well tolerated by the cells.

Acanthamoebae penetrate the stratum corneum of skin equivalents, which is inhibited by miltefosine treatment

It was shown that an Acanthamoeba infection and its therapy can be monitored by the newly established organotypic skin equivalent model. The untreated skin equivalent (blank) behaved like ‘normal’ skin: basal keratinocytes resided on the collagen matrix, and differentiating keratinocytes and upper cornified layers were clearly visible after 48 h (Figure 2a). The PBS control (Figure 2b) demonstrates that although, of course, the upper cornified layers were much less pronounced and the cells were slightly bloated (which can be explained simply by the liquid), the skin equivalent grew normally and remained unaffected by the application of PBS. Acanthamoebae that were topically applied onto the cornified envelope of the skin equivalent, however, readily penetrated and destroyed the cornified layers and migrated into the skin equivalent, as visible in HE stain (Figure 2d, arrows; control: Figure 2c). The migration of the amoebae into the skin equivalent is shown in Figure 2(e–h). Intercalation of acanthamoebae between the corneocytes was detected after 6 h and first ‘lesions’ were visible after 24 h (Figure 2f, shown in detail in Figure 2h). Then the amoebae

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**Table 1.** Efficacy of miltefosine against S. aureus, E. hirae and C. albicans: qualitative suspension test using ~1.5 × 10\(^7\)/mL and 0.9% NaCl as a growth control

<table>
<thead>
<tr>
<th></th>
<th>S. aureus DSM 799</th>
<th>E. hirae DSM 3320</th>
<th>C. albicans DSM 1386</th>
<th>Control NaCl</th>
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<tbody>
<tr>
<td>Miltefosine</td>
<td>1 h 6 h 12 h 18 h 24 h</td>
<td>1 h 6 h 12 h 18 h 24 h</td>
<td>1 h 6 h 12 h 18 h 24 h</td>
<td>1 h 6 h 12 h 18 h 24 h</td>
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<td>100 μM</td>
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<td>75 μM</td>
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<td>+ – – – – – – –</td>
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<td>50 μM</td>
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<td>25 μM</td>
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<td>10 μM</td>
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<td>+ – – – – – – –</td>
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+ , Growth (turbidity); – , no growth.
S. aureus and C. albicans skin equivalent. It was shown that miltefosine remains stable for a stock solution, but also as a dilution in PBS for a prolonged period of time (≥12 months) without any loss of activity. This makes it not only suitable as a potential topical drug in Acanthamoeba infections, but also as an additive, e.g. in contact lens solutions. Previous studies have shown that miltefosine is effective against clinical and environmental isolates of Acanthamoeba, and also against Balamuthia mandrillaris and Naegleria fowleri, starting at concentrations of ~5 μM.\(^{11–13}\) Storage without loss of activity is essential for any possible application, either as topical ointment or solution (or even as a disinfectant). Another advantage is the rapid mode of action. Anti-Acanthamoeba efficacy is reached within 1 h, leading to a complete destruction of trophozoites and to a 3 log reduction of cysts within 24 h.\(^{11}\) The exact mode of action of miltefosine is still unknown; however, it has been shown that the point of attack is the cell membrane.\(^{18}\) Miltefosine accumulates in the cell membranes and perturbs cell metabolism.\(^{19}\) Acanthamoeba skin lesions and Acanthamoeba pneumonitis, both potential portals of entry for amoebae into the blood stream and thus eventually leading to Acanthamoeba-GAE,\(^{20}\) occur almost exclusively in the immunodeficient host, who of course is also highly susceptible for other common opportunistic pathogens, such as S. aureus and C. albicans. Moreover, acanthamoebae are known to function as vehicles for other microorganisms and even to promote their virulence.\(^{21,22}\) Thus, it is desirable that a substance used for anti-Acanthamoeba treatment also shows activity against other opportunists. Although the required residence time for S. aureus and C. albicans was considerably longer than that for Acanthamoeba, they were completely eradicated by 75 μM miltefosine within 6 and 18 h, respectively. Broad-spectrum antifungal activity has already been demonstrated for miltefosine in vitro and in vivo.\(^{23}\) The activity against S. aureus and C. albicans might also be interesting with respect to eye infections, as both are also major causes of corneal ulcers.\(^{24}\) Additionally, by irritating the eye or by functioning as a food source for acanthamoebae, they can pave the way for AK. S. aureus and, particularly, C. albicans are common contaminants of contact lens cases if contact lens hygiene is inadequate, and

![Figure 1. EZ4U test: treatment of keratinocytes with 10, 50, 250 and 1000 μM miltefosine, and 0.02% and 0.1% chlorhexidine. Ctrl, control; Milf, miltefosine; CIHex, chlorhexidine. Absorbance was measured at 450 nm with a reference wavelength of 620 nm. Effects of treatment were recorded after 1, 2 and 4 h, means were calculated from triplicates. The horizontal line shows the toxicity cut-off.](image-url)
they can even enhance binding of the amoebae to the lenses. In the USA the prevalence of AK is increasing and most cases occur in contact lens wearers, which is also true for Europe. Contaminated contact-lens care systems usually are the first step in AK pathogenesis, tap water being the most important source of amoebae. However, under normal conditions amoeba density in tap water is very low and amoebae must first multiply within the lens case, thriving on syntopically occurring bacteria before they reach infective doses. Acanthamoebae, particularly the cysts, are resistant against a variety of disinfectants, including many of those commonly used in contact lens solutions.

The in vitro toxicity of miltefosine as evaluated in the EZ4U test was rather high in long-term incubation; however, it was still significantly lower than the toxicity of chlorhexidine, which is the least toxic of the currently used standard drugs for AK treatment. Moreover, the in vivo tolerability usually is considerably higher than the in vitro tolerability, because in vivo the cells are aggregated in a united cell structure exhibiting better

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**Figure 2.** Acanthamoebae penetrating skin equivalent. (a–d) HE stain. (a and b) Untreated skin equivalent after 48 h [(a) without and (b) with inoculation of 20 μL of PBS]. (c and d) Penetration of amoebae within 48 h [(c) control and (d) amoebae (10⁴) applied in 20 μL of PBS to keratinocytes]. Arrows indicate cell layers of the stratum corneum (KC, keratinocytes; FB, fibroblasts embedded in collagen lattice). (e–g) Immunofluorescence staining for amoebae. (e–g) Penetration of amoebae after (e) 6 h, (f) 24 h and (g) 48 h, stained for amoebae (green) and Hoechst counter stain (blue). (h) Detailed view of the penetration of the amoebae after 24 h, indicating the cell layers of the skin equivalent (SC, stratum corneum; D KC, differentiating keratinocytes; B KC, basal keratinocytes; Col, collagen lattice with embedded dermal fibroblasts). (i–l) Immunofluorescence staining for amoebae after miltefosine treatment. (i) Skin equivalent treated with 50 μM miltefosine (M) without inoculation of amoebae. (j) Untreated control (C). (k and l) Skin equivalent 24 h post-inoculation of amoebae [(k) without treatment and (l) treated with 50 μM miltefosine 1 h post-inoculation of amoebae].

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protection. This is corroborated by our results demonstrating that 50 μM miltefosine was well tolerated by the organotypic skin model. In vivo, even a 6% (~150 mM) solution of miltefosine is well tolerated by human skin.30

The virulence of Acanthamoeba strains is usually assessed in animal models or on human cell monolayers. While the mouse model, in which acanthamoebae are usually injected into the nostrils and reisolated from the brain, seems to be a good model for GAE and a good indicator for the virulence of a particular strain, there is no model for Acanthamoeba skin lesions. Moreover, even in the case of AK the situation is complicated, because mice are not susceptible.31 Nevertheless, a pig and a rat model for AK have been developed.32–34 There also have been many attempts to avoid animal experiments, and Cursons and Brown35 compared the time needed for the development of cytopathic effects in cell cultures with that needed to cause death in mice, and established the use of cell cultures as an indicator of the pathogenicity of free-living amoebae. Although monolayers are an excellent tool to determine the cytopathic capability of Acanthamoeba strains, they cannot serve as a model for evaluating the usability of a drug candidate. The organotypic skin equivalent adapted as a model for Acanthamoeba infection seems to be a good alternative as it demonstrates the penetration of the amoebae into the cell layer, on the one hand, and the tolerance of the skin equivalent to the drug candidate, on the other. The strain used in the current study, originally isolated from the eye, penetrated the artificial skin in our model within 24 h. This further corroborates the assumption that virulence is not ‘organ-specific’ in Acanthamoeba, but is dependent on a combination of certain prerequisites, e.g. temperature tolerance, ability to bind to human cells and protease activity.1,15,36,37 Taken together, it was shown that acanthamoebae can penetrate into the skin within 24 h and that miltefosine treatment prevents the penetration of Acanthamoeba. The toxicity of anti-Acanthamoeba-effective concentrations of miltefosine lies below those of standard topically applied anti-Acanthamoeba drugs and miltefosine also shows at least partial effect against Staphylococcus aureus and Candida albicans. Miltefosine has been approved for the oral and topical treatment of leishmaniasis, and has already been successfully used to treat Acanthamoeba skin lesions in a case of disseminated Acanthamoeba infection.14 It may thus be a promising candidate for the topical treatment of Acanthamoeba infections. As B. mandrillaris, the second causative agent of amoebic skin lesions, is also susceptible to miltefosine in vitro,13 it is even conceivable that miltefosine might be a candidate to treat Balamuthia infections.

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Transparency declarations
None to declare.

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