Clinical & Experimental Ophthalmology RANZCO The Royal Australian and New Zealand College of Ophthalmologists

Check for updates

Clinical and Experimental Ophthalmology 2014; 42: 151–158 doi: 10.1111/ceo.12120

Original Article

Miltefosine and polyhexamethylene biguanide: a new drug combination for the treatment of *Acanthamoeba* keratitis

Zubeyde A Polat PhD,[1] Julia Walochnik PhD,[3] Andreas Obwaller PhD,[4] Ayse Vural MD,[2] Ayhan Dursun MD[2] and Mustafa K Arici MD[2]

Departments of [1]Medical Parasitology and [2]Ophthalmology, Cumhuriyet University School of Medicine, Sivas, Turkey; and [3]Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna, and [4]Orphanidis Pharma Research GmbH, Vienna, Austria

Abstract

Background: In this study, a series of compounds – miltefosine, polyhexamethylene biguanide, chlorhexidine and propamidine isethionate – and combinations of the latter three agents with miltefosine were prepared and used in a rat model for the topical treatment of *Acanthamoeba* keratitis.

Methods: The corneas of rats were infected with *Acanthamoeba hatchetti*. On the fifth day, all corneas were microscopically examined in order to determine the grade of infections. Nine groups were then prepared: miltefosine (65.12 μg/mL); chlorhexidine (0.02%); polyhexamethylene biguanide (0.02%), propamidine isethionate (0.1%), miltefosine plus chlorhexidine, miltefosine plus polyhexamethylene biguanide; miltefosine plus propamidine isethionate; infected control; and a non-infected control group. The treatment was continued for 28 days. After the treatment, the corneas were excised and used for *Acanthamoeba* culture to investigate the presence of *Acanthamoeba* growth. For the determination of cytotoxicity of the drugs on L929 cells, colorimetric assays were performed.

Results: The best treatment results were obtained from the polyhexamethylene biguanide plus miltefosine group; the ratio of fully recovered eyes was 28.4%. It was proven that the miltefosine–polyhexamethylene biguanide combination yielded the highest anti-acanthamoebal activity in that approximately 86% of the eyes were cleared from amoebae. The cytotoxicity values of the miltefosine and the control groups were compared with other groups and found to be statistically different ($P < 0.05$).

Conclusion: This *in vivo* study demonstrates that a miltefosine–polyhexamethylene biguanide combination is highly effective for the treatment of *Acanthamoeba* keratitis.

Key words: *Acanthamoeba*, keratitis, miltefosine, polyhexamethylene biguanide, rat keratitis model.

Introduction

Acanthamoebae are extracellular protozoan organisms that have been isolated from a variety of environments. *Acanthamoeba* keratitis (AK) is a severe and potentially vision-threatening corneal infection caused by free-living, pathogenic amoebae belonging to the genus *Acanthamoeba*.[1] It has been detected in almost all parts of the world.[2] Risk factors associated with contact lens wear are now well known: the combination of epithelial microtrauma, poor contact lens hygiene and the wearing of contact lenses in contaminated water environments such as swimming pools, spas and lakes. Insufficient or improper cleaning and disinfection of contact lenses, including

■ **Correspondence:** Assoc. Prof. Dr. Zubeyde Polat, Department of Medical Parasitology, University of Cumhuriyet School of Medicine, 58140 Sivas, Turkey. Email: zubeydeakin@yahoo.com

Received 4 January 2013; accepted 6 March 2013.

Competing/conflicts of interest: No stated conflict of interest.

Funding sources: This study was supported by Orphanidis Pharma Research GmbH, Vienna, Austria.

© 2013 Royal Australian and New Zealand College of Ophthalmologists

the use of tap water for cleaning and rinsing them, as well as improper storage, predispose for *Acanthamoeba* infection.[3,4] The earliest signs of AK are at the epithelial level, with patchy oedema, microcysts and epithelial haze. These symptoms cause an irregular dendritiform ulceration, which is often misdiagnosed as keratitis caused by herpes simplex virus. Stromal findings, which occur later, include single or multiple stromal infiltrates and nummular keratitis. Ring-shaped stromal infiltrates or satellite lesions usually suggest advanced disease.[5]

Effective medical treatment depends on early diagnosis.[2,5] Treatment regimens vary from the biguanides polyhexamethylene biguanide (PHMB) and chlorhexidine (CHX), which inhibit membrane function, to the diamidine propamidine isethionate (PI), which inhibit DNA synthesis. The drugs have been used in different combinations, with varying degrees of success.[3,6,7] Moreover, resistance against propamidine does occur,[8] and in a recent case, persistent amoebae were observed after 1 year of topical treatment with CHX.[9]

Miltefosine (MLT), an alkylphosphocholine, has been known to possess a diverse antiprotozoal activity; it is effective against visceral leishmaniasis,[10] *Trypanosoma* spp.,[11] *Trichomonas vaginalis*[12] and *Entamoeba histolytica*.[13] *In vitro* studies have also indicated a high therapeutic capacity of MLT against *Acanthamoeba* infections.[14–16] It has been reported that an immunocompromised patient with *Acanthamoeba* skin lesions and granulomatous amoebic encephalitis completely recovered after MLT treatment.[17] In a comparative study, we have applied the AK model to Syrian hamsters. *Acanthamoeba hatchetti* has been used for the infection of hamsters' eyes. Results obtained have shown the high therapeutic capacity of MLT.[18]

The aim of the current study was to compare AK treatment capacity of the compounds described earlier and their combinations with MLT, and to determine their cytotoxic potential.

METHODS

Animals

Sixty-three male Wistar rats weighing approximately 125 g were used for the present study (Table 1). The animals were fed with a standard laboratory diet, kept at 22 ± 2°C, 50–70% humidity, and a 12-h day-night cycle; drinking water was available ad libitum during the experiments. All corneas were examined before inoculation to exclude any abnormalities (Fig. 1). Experimental procedures involved in the study were approved by the Institutional Review and Animal Use Committee of the Cumhuriyet University and conducted by following accepted guidelines for the care and use of laboratory animals for research.

Cultivation of *Acanthamoeba*

Cornaeas were infected with *A. hatchetti* strain 11DS (American Type Culture Collection (ATCC) PRA-112), a human corneal isolate. Vegetative forms were

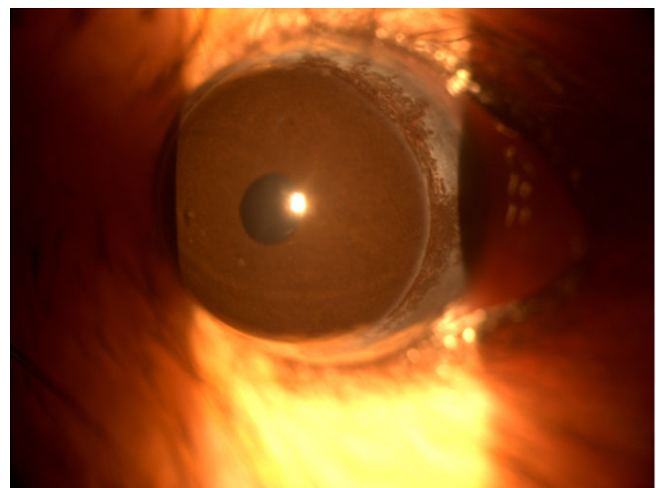

Figure 1. Appearance of a rat normal cornea (250×).

Table 1. Treatment groups

Group	Treatment	Formulation	No. of animals
1	Infected control	PBS/0.05% ethanol (vehicle)	7 (14 eyes)
2	MLT	miltefosine 65.12 μg/mL	7 (14 eyes)
3	CHX	0.02% cholorhexidine gluconate	7 (14 eyes)
4	PHMB	0.02% polyhexanide	7 (14 eyes)
5	PI	propamidine isethionate (Brolene)	7 (14 eyes)
6	MLT plus CHX	combination of 65.12 μg/mL miltefosine and 0.02% cholorhexidine	7 (14 eyes)
7	MLT plus PHMB	Combination of 65.12 μg/mL miltefosine and 0.02% polyhexanid	7 (14 eyes)
8	MLT plus PI	combination of 65.12 μg/mL miltefosine and Brolene	7 (14 eyes)
9	Non-infected control	PBS/0.05% ethanol (vehicle)	7 (14 eyes)

CHX, chlorhexidine; MLT, miltefosine; PHMB, polyhexamethylene biguanide; PBS, phosphate buffered saline; PI, propamidine isethionate.

© 2013 Royal Australian and New Zealand College of Ophthalmologists

14429071, 2014, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/ceo.12120 by Bezm-I Alem Vakif University, Wiley Online Library on [03/09/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

prepared in axenic cultures in 25-cm^2 flasks, containing 10-mL protease peptone, yeast extract and glucose (PYG) medium at 37°C. Trophozoites in the stage of exponential growth (72–96 h) were concentrated by centrifugation at 500 *g* for 10 min. The amoebae were washed twice in a sterile Neff's saline solution (1.2 g NaCl, 0.4 g $MgSO_4.H_2O$, 0.4 g $CaCl_2.2H_2O$, 1.42 g Na_2HPO_4, 1.36 g $KHPO_4$ in 100-mL distilled water), counted in a hemacytometer, adjusted to a final concentration in Neff's saline solution at a density of 1×10^6 amoebae/mL (95.0% trophozoites) and used immediately for infection.

Chemicals

MLT was provided by Orphanidis Pharma Research GmbH (Vienna, Austria) and prepared as a 2-mmol/L stock solution in 5% ethanol. CHX was provided by Sigma-Aldrich (C93949; St. Louis, MO), PHMB (Lavasept concentrate 20%) was purchased from Braun (Melsungen, Germany), and PI (Brolene) was purchased from Sanofi-Aventis (Surrey, UK). CHX and PHMB were diluted (to 0.02% final concentration) in artificial tear solution (Dacrolux, Alcon, Spain).

Anaesthesia

Ketamine HCl (ketamine, 10% 100 mg/kg body wt intraperitoneally; CP-Pharma, Burgdorf, Germany) and xylazine (Rompun, 10 mg/kg body wt intraperitoneally; Bayer, Leverkusen, Germany) were administered by an intramuscular injection, and one drop of preservative-free benoxinate was applied to each of the eyes.

In vivo corneal infection

The inoculation was performed under an operating microscope (Leica-M841; Leica Microsystems GmbH, Wetzlar, Germany). Initially, a half-thickness linear blade incision was made approximately 2 mm from the centre of the cornea. Using a 30-G needle attached to 1 μL-syringe, the needle was advanced from the point of incision through the lamella of the stroma to the centre of the cornea (Fig. 2a). One microlitre infection solution (1×10^6 amoebae/mL) was injected into the stroma (Fig. 2b). After 3 days, the infection was repeated. Control animals received a mock inoculum of 1-μL Neff's saline.

Clinical observation

Under general anaesthesia, the rats were examined under a slit-lamp microscope on day 5 after second inoculation. Infected corneas and irises were examined by retro-illumination, and by transillumination. The oblique slit beam was used to illuminate lesser degrees of opacity. The following grading scheme was used:[18] grade 0, normal; grade 1, corneal opacity visible only by oblique slit beam; grade 2, corneal opacity visible by retro-illumination but not sufficient to obscure iris vessels; and grade 3, corneal opacity visible by retro-illumination and obscure iris vessel details.

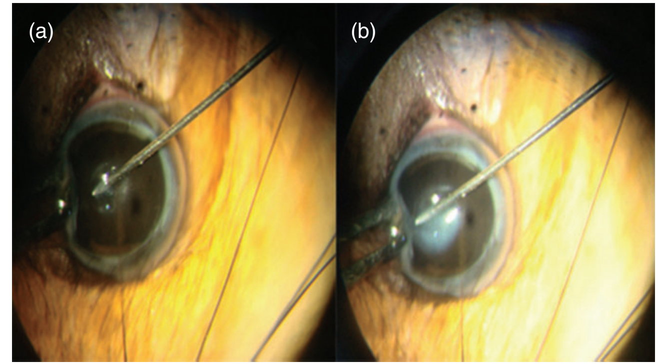

Figure 2. (a) Before injection of infection solution, (b) After injection of infection solution (×250).

Therapy

On the fifth day after inoculation, all the corneas were examined to grade infections as grade 0, grade 1, grade 2 or grade 3. Each grade included the same numbers of eyes to be able to use similar numerical parameters to better assess the effectiveness of the therapy. The therapy was performed for 1 month. During the first week, therapeutic agents were applied for eight times per day, and for the last 3 weeks, they were applied only three times per day.

Isolation of *Acanthamoeba* after the treatment

Non-nutrient agar was set in each well of six-well Petri dishes. A layer of *Escherichia coli* was added to the agar surface in each well. Corneas were excised and were then homogenized in 1-mL Page's amoeba saline in a glass grinder. Hundred microlitres of each of the homogenates were added onto *E. coli*-containing wells, and the dishes were incubated for 14 days at 30°C. Amoebic growth was inspected daily under the phase contrast microscopy (Eclipse TS 100; Nikon, Tokyo, Japan). Cultures containing no growth after the 14 days of incubation were discarded.

Cytotoxic potential of drugs

Cultivation of L929 mouse fibroblast cells

A mouse connective tissue fibroblast cell line, L929 (ATCC cell line, NCTC clone 929) was cultured in

© 2013 Royal Australian and New Zealand College of Ophthalmologists

14429071, 2014, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/ceo.12120 by Bezm-I Alem Vakif University, Wiley Online Library on [03/09/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Dulbecco's minimum Eagle medium (Sigma, St. Louis, MO, USA) supplemented with 10% foetal calf serum (Sigma) and 2 mmol/L/mL L-glutamine. No antibiotics were added to the cell culture medium. The cultures were cultivated in an incubator at 37°C and 5% CO_2, until the cell monolayer attained confluence, after approximately 7 days. Assays were always performed in the exponential growth phase of the cells.

Fibroblast cells were selected because they are the predominant tissue type in the body and are easy to cultivate and because of their favourable doubling time of 24 h. Moreover, these cells are recommended by many standard institutions.

Cell proliferation assay

The proliferation assay analyses the number of viable cells by the cleavage of tetrazolium salts added to the culture medium, using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) labelling reagent (Roche, Mannheim, Germany). During the assay, tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which correlates directly with the number of metabolically active cells in the culture. The formazan dye produced by the metabolically active cells was quantified by a scanning multiwell spectrophotometer by measuring the absorbance of the dye solution at 450 nm. Cells were seeded into 96-well microtitre plates at a concentration of 1×10^5 cells/mL in a final volume of 100 μL per well. For evaluating the cytotoxicities of the respective compounds and combinations, concentrations of 65.12 μg/mL MLT, 0.02% CHX, 0.02% PHMB, 0.01% PI, 65.12 μg/mL MLT plus 0.02% CHX, 65.12 μg/mL MLT plus 0.02% PHMB, and 65.12 μg/mL MLT plus 0.01% PI were used. Cells were then incubated for 24 h in a humidified atmosphere (37°C, 5% CO_2). After this, 10 μL of the XTT labelling reagent were added to 10 μL of culture medium into each well, and the absorbance of the samples was measured at 450 nm against the control (the same cells without any treatment) using a microtitre plate reader (Thermo Scientific Microplate Photometer, Multiskan FC, Canada). The same volume of culture medium and XTT labelling reagent (10 μL of XTT labelling reagent/100 μL of culture medium) was added to one well as a background control (absorbance of culture medium plus XTT in the absence of cells) as a blank position for the microplate reader. The absorbance was measured after 2 h from the start of the tetrazolium reaction. The experiments were conducted in quadrubles. The optical densities (ODs) of the samples were compared with that of the negative control to obtain the percentage viability, as follows: cell viability (%) = ([OD450 {sample}/OD450 {negative control}] × 100).

The results, expressed as mean ± standard deviation from four replicates, were analysed statistically by using one-way analysis of variance at 95% confidence levels for multiple comparisons and Student's *t*-test for two-group comparisons.

RESULTS

The treatment of AK

Five days after the inoculation with *Acanthamoeba* trophozoites, all of the eyes (100%) developed keratitis. They were grouped with respect to the degree of opacity observed as follows: 35 of the eyes (31.25%), G1; 28 (25%), G2; and 49 (43.75%), G3. After this, eight different groups of eyes were formed, and into each of the groups, six G3, four G2 and four G1 eyes were included (Fig. 3).

The best treatment results were obtained from the PHMB plus MLT group: on day 0, there were six G3, four G2 and four G1 opacity eyes; on day 28, these numbers were reduced to one G2 and nine G1 eyes. The ratio of fully recovered eyes was 28.4%. In the group treated with PHMB alone, there were two G3, five G2, six G1 and one G0 eyes on the 28th day. And in the MLT group, the distribution of opacity was

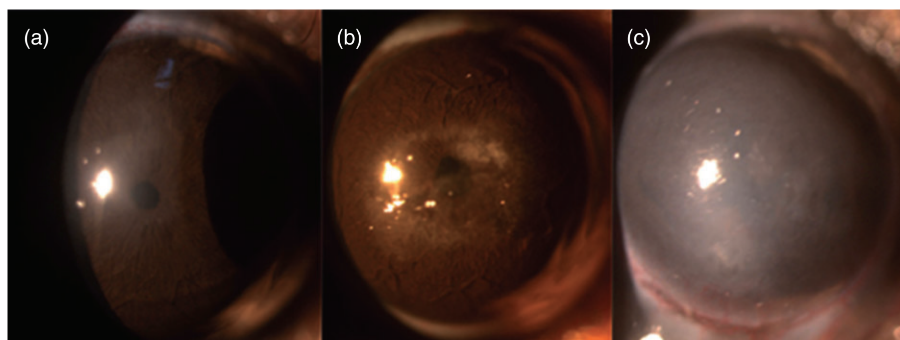

Figure 3. Grades 1 (a), 2 (b), and 3 (c) corneal opacity (250×).

© 2013 Royal Australian and New Zealand College of Ophthalmologists

14429071, 2014, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/ceo.12120 by Bezm-I Alem Vakif University, Wiley Online Library on [03/09/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

as follows: five G2, seven G1 and two G0. In the infected control group, infection appeared to have progressed until the 28th day, and six G3, five G2 and 3 G1 eyes were obtained. It also was observed on day 28 that opacity areas reduced vascularization, and veins appeared to become thinner and shortened in the MLT, CHX, PHMB, MLT plus CHX and MLT plus PHMB groups. In addition, development of ghost veins was detected in the MLT, CHX, PHMB, MLT plus CHX and MLT plus PHMB groups at ratios of 50%, 28.4%, 42.8%, 35.5% and 71.4%, respectively. No changes were observed in the uninfected control group's eyes during the 28 days of the experiment (Table 2).

Isolation of *Acanthamoeba* after the treatment

After completion of the treatment, there was an attempt to recover *Acanthamoeba* from the excised eyes, eye tissue were inoculated onto non-nutrient agar medium (NNA) solid media. After 14 days of incubation at 30°C, four eyes in each of the MLT, CHX and PI groups (28.5%); three eyes in each of the PHMB, MLT plus CHX, and MLT plus PI groups (21.4%); and two eyes in the MLT plus PHMB group (14.2%) revealed amoebal growth. Amoebal growth was obtained from 10 eyes (71.42%) of the infected control inoculations; no growth was observed for uninfected control eyes (Fig. 4).

Cytotoxic potential of drugs

Cytotoxicity of MLT, CHX, PHMB, PI, MLT plus CHX, MLT plus PHMB and MLT plus PI was tested for 24 h by quantitative analysis using the XTT test. The cytotoxicity values of MLT and then control were compared with that of CHX, PHMB, PI, MLT plus CHX, MLT plus PHMB and MLT plus PI, and found to be statistically different (Fig. 5, $P < 0.05$). As can be seen, MLT 65.12 μg/mL had no negative effect on cell viability.

Discussion

It has been recognized in almost all parts of the world that *Acanthamoeba* species are an important

Table 2. Degrees of opacity after 28 days of treatment (G-Grade)

Brolene					Brolene and miltefosine				
Day	G0 (%)	G1 (%)	G2 (%)	G3 (%)	Day	G0 (%)	G1 (%)	G2 (%)	G3 (%)
0	0	35.5	21.4	42.8	0	0	28.4	21.4	50
7	0	35.5	21.4	42.8	7	0	28.4	28.4	42.8
14	0	35.5	21.4	42.8	14	0	28.4	42.8	28.4
21	0	42.8	21.4	35.5	21	0	42.8	35.5	21.4
28	7,1	35.5	21.4	35.5	28	7.1	35.5	42.8	14.2
Chlorhexidine					**Chlorhexidine and miltefosine**				
Day	G0 (%)	G1 (%)	G2 (%)	G3 (%)	Day	G0 (%)	G1 (%)	G2 (%)	G3 (%)
0	0	35.5	21.4	42.8	0	0	28.4	28.4	42.8
7	0	35.5	28.4	35.5	7	0	35.5	35.5	28.4
14	0	35.5	35.5	28.4	14	0	50	42.8	7.14
21	0	42.8	35.5	21.4	21	0	57.1	42.8	0
28	7,1	50	28.4	14.2	28	14.2	57.1	28.4	0
PHMB					**PHMB and miltefosine**				
Day	G0 (%)	G1 (%)	G2 (%)	G3 (%)	Day	G0 (%)	G1 (%)	G2 (%)	G3 (%)
0	0	28.4	28.4	42.8	0	0	28.4	28.4	42.8
7	0	35.5	28.4	35.5	7	0	35.5	42.8	21.4
14	0	35.5	35.5	28.4	14	7.1	50	35.5	7.1
21	0	42.8	35.5	21.4	21	14.2	64.2	21.4	0
28	7,1	42.8	35.5	14.2	28	28.4	64.2	7.1	0
Miltefosine					**Infected control**				
Day	G0 (%)	G1 (%)	G2 (%)	G3 (%)	Day	G0 (%)	G1 (%)	G2 (%)	G3 (%)
0	0	35.5	21.4	42.8	0	0	28.4	28.4	42.8
7	0	35.5	28.4	35.5	7	0	14.2	35.5	50
14	0	42.8	35.5	21.4	14	0	7.1	42.8	50
21	7,1	50	42.8	0	21	0	14.2	42.8	42.8
28	14,2	50	35.5	0	28	0	21.4	35.5	42.8

PHMB, polyhexamethylene biguanide.

© 2013 Royal Australian and New Zealand College of Ophthalmologists

14429071, 2014, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/ceo.12120 by Bezm-I Alem Vakif University, Wiley Online Library on [03/09/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

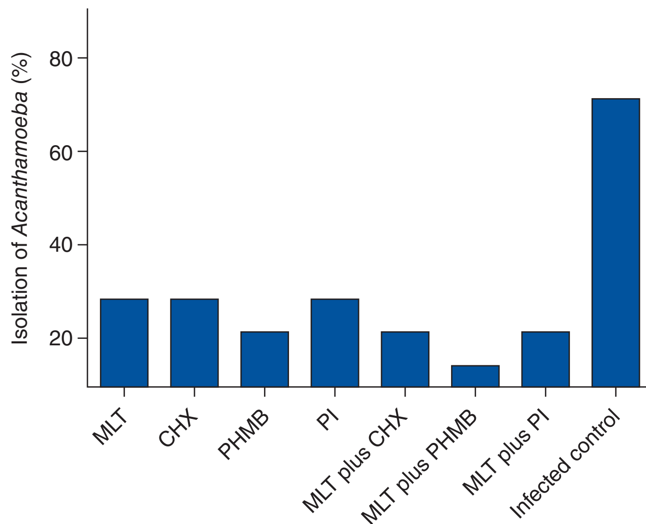

Figure 4. Pecentage of isolation of *Acanthamoeba* after the treatment in the groups. CHX, chlorhexidine; MLT, miltefosine; PHMB, polyhexamethylene biguanide; PI, propamidine isethionate.

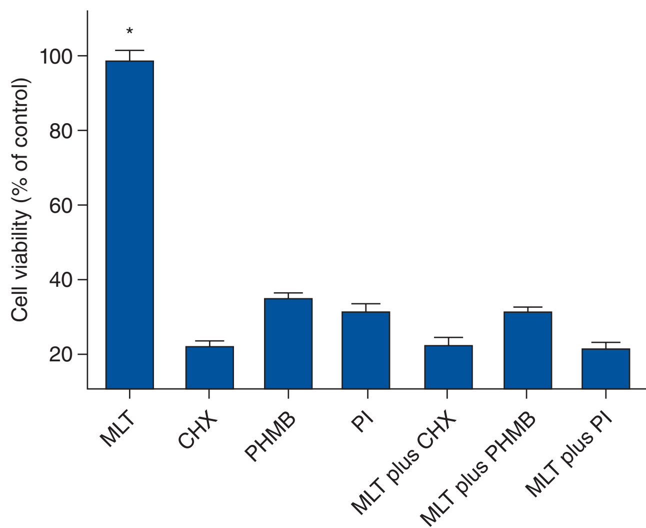

Figure 5. *In vitro* cytotoxic effect of 65.12 μg/mL miltefosine (MLT), 0.02% chlorhexidine (CHX), 0.02% polyhexamethylene biguanide (PHMB), 0.01% propamidine isethionate (PI), 65.12 μg/mL MLT plus 0.02% CHX, 65.12 μg/mL MLT plus 0.02% PHMB and 65.12 μg/mL MLT plus 0.01% PI on L929 fibroblast cells measured by XTT assay. *$P < 0.05$ *versus* CHX, PHMB, PI, MLT plus CHX, MLT plus PHMB and MLT plus PI. MLT 65.12 μg/mL had no negative effect on cell viability.

cause of microbial keratitis that may lead to severe ocular inflammation and visual loss. Evidence from numerous studies has suggested that soft contact lens wearers are at the greatest risk for AK; in a recent study, contact lens wear was reported in 80–86% of cases.[1,4,6,19] As active trophozoites and also cysts attach to the surface of contact lenses, *Acanthamoeba* can easily be transmitted from the storage case onto the eye.[5]

AK symptoms are non-specific and can be misdiagnosed as a viral, bacterial or fungal keratitis. Thus, an early diagnosis is required to achieve a successful therapeutic outcome. Satisfactory treatment for amoebic keratitis is lacking. Providing adequate medical therapy is difficult for several reasons. Amoebae are resistant to most ocular antibiotics.[6,19] Further, different species, as well as different strains of the same species, demonstrate variable drug susceptibilities.[20] Cysts are even more resistant than trophozoites and persist in the cornea indefinitely requiring prolonged topical medical treatment to ensure eradication before keratoplasty.[21]

In AK, a biguanide (0.02% PHMB or 0.02% CHX digluconate) in combination with a diamidine (0.1% PI, also known as Brolene, or 0.1% hexamidine, also known as Desomedine), is the first-line treatment.[1,9] Hourly drops may be tapered down after 48 h to alleviate the epithelial toxicity caused by both of these compounds. Topical therapy for AK needs to be continued much longer than antibacterial therapy regimes because of the encystment of the amoebae, which is much harder for the drugs to penetrate. Typical regimes will taper over around 6 months.[22] Biguanides are used most often because of their relatively good efficacy against cysts and are frequently combined with a diamidine because of a presumed additive anti-amoebic effect.[19] Moreover, CHX and PHMB as monotherapy agents at normal concentrations have been proven to be insufficiently effective against several clinical or environmental strains of acanthamoebae, and this has highlighted the importance of multiple-strain testing of drugs against acanthamoebae.[9,23] It also is important to mention that drugs to be used for the treatment of AK should be both amoebicidal and cysticidal, as there is a risk of relapse because of the re-emergence of amoebae from the more resistant cysts.[3,5] CHX is generally active against both *Acanthamoeba* trophozoites and cysts. It acts by the binding of its highly charged positive molecules to the mucopolysaccharide plug of the ostiole, resulting in penetration through it to the internalized amoeba, where they bind to the phospholipid bilayer of the cell membrane of the internalized amoeba. This results in membrane damage with irreversible loss of calcium and cell electrolytes from the cytoplasm causing cell lysis and death.[22] However, it is difficult to believe that a chemical destroying the membrane of the amoeba should not at the same time affect the plasma membranes of the ocular cells, where the epithelium ulcerates, the keratocytes disappear and the endothelium does not function properly. With continued medical treatment, the iris cells die, the lens cells die, and cataract

© 2013 Royal Australian and New Zealand College of Ophthalmologists

14429071, 2014, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/ceo.12120 by Bezm-I Alem Vakif University, Wiley Online Library on [03/09/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

develops.[24] In this study, in an AK model in rats, we investigated both single and combined treatments with MLT with PHMB, CHX and PI, all of which alone had been shown to be effective against acanthamoebae in previous studies.[14–18] In conclusion, compared with the infected control group, infections regressed in all treatment groups to different degrees, but the best results were obtained from the PHMB plus MLT group. In this group, opacity regressed in all eyes, particularly also in G3 grade corneal opacity. In this group, 28.4% of the eyes completely recovered. Vascularization substantially decreased in the PI and MLT plus PI groups. In addition, development of ghost vessels following treatment was observed in the MLT plus PHMB, the MLT and the PHMB groups.

Early clinical signs of AK include epithelial irregularities, opacities, microerosions, microcystic oedema and patchy anterior stromal infiltrates. As the infection progresses, many eyes develop an annular central or paracentral stromal infiltrate, which tends to be most dense at its peripheral margins, giving the appearance of the classic ring infiltrate, usually with overlying epithelial defects. The ring infiltrate is considered to be the representative sign of AK.[2] In the present study, corneal stromal oedema and epithelial defect from staining with fluorescein were seen in all the rats' eyes inoculated with *Acanthamoeba* in the early period. In the chronic stage of experimental keratitis, linear ridge-like lesions of the cornea developed in the infected control group by day 28. None of the treatment groups developed a ring infiltrate. Acanthamoebae are ubiquitous protozoa that exist in two forms. Under unfavourable conditions, trophozoites transform into cysts that are resistant to extremes of temperature, pH and desiccation.[25] Cysts are difficult to kill, which is one reason why this infection is so hard to eradicate. In a series of *in vitro* studies using corneal organ cultures, Clarke and Niederkorn[26] demonstrated that *Acanthamoeba* trophozoites can penetrate the corneal endothelium and the underlying Descemet's membrane, suggesting that the trophozoites can, enters the anterior chamber. One of the major causes of repeated infection following the treatment is the amoebae transforming to cysts within the tissue and failure or low efficacy of the treatment against cysts. The cysts remaining in the tissue after treatment may be reactivated resulting in recurrent infection. In this study, the eyes of the rats were removed after treatment, and the eye tissues were monitored in NNA plates coated with *E. coli* for 14 days. Among the treatment groups, *amoebae* were most frequently recovered from the eyes of the MLT, CHX and PI groups (28.5%). Thus, recurrence of infection is most likely when MLT, CHX or PI are implemented alone. In the MLT plus PHMB group, amoebae could be recovered in only 14.2% of the treated eyes, thus suggesting a much lower probability hence for recurrence of the infection. PHMB has been shown to be able to kill both the trophozoite and cyst, and was shown to have no toxic effects to the healing epithelium in a previous study.[19] In this study, we comparatively investigated the cytotoxic potentials of all compounds used in the treatment groups by the XTT method. When comparing 0.02% CHX, 0.02% PHMB and 0.01% PI, PHMB showed the lowest cytotoxic potential. MLT at a concentration of 65.12 $\mu g \cdot mL^{-1}$ did not show any cytotoxic impact on the cells, whereas of the combinations, the lowest cytotoxicity was found for MLT combined with PHMB, CHX and PI, respectively.

The severity, chronicity and difficulty in the treatment of AK as well as the increased use of contact lenses make this infection serious. A number of drugs have been tested *in vitro* and *in vivo* with varying degrees of efficacy, sometimes leading to recovery,[2,3] but sometimes not.[1] Present therapeutic regimens for AK rely mainly on topical applications of antimicrobial agents, but the emergence of drug-resistant strains and the recurrence of dormant infectious forms underscore the need for more effective treatments. This *in vivo* study demonstrated that a combination of MLT-PHMB is highly effective for the treatment of AK in the rat model used and that further investigations employing animal models with larger sample size and volunteer patient groups are warranted.

References

1. Mattana A, Biancu G, Alberti L *et al*. *In vitro* evaluation of the effectiveness of the macrolide rokitamycin and chlorpromazine against *Acanthamoeba castellanii*. *Antimicrob Agents Chemother* 2004; **48**: 4520–7.
2. Trabelsi H, Dendana F, Sellami A *et al*. Pathogenic free-living amoebae: epidemiology and clinical review. *Pathol Biol* 2012; **60**: 399–405.
3. Hammersmith KM. Diagnosis and management of *Acanthamoeba* keratitis. *Curr Opin Ophthalmol* 2006; **17**: 327–31.
4. Stapleton F, Carnt N. Contact lens-related microbial keratitis: how have epidemiology and genetics helped us with pathogenesis and prophylaxis. *Eye* 2012; **26**: 185–93.
5. Panjwani N. Pathogenesis of *Acanthamoeba* keratitis. *Ocul Surf* 2010; **8**: 70–9.
6. Dart JK, Saw VP, Kilvington S. *Acanthamoeba* keratitis: diagnosis and treatment update. *Am J Ophthalmol* 2009; **148**: 487–99.
7. Lin HC, Hsiao CH, Ma DH *et al*. Medical treatment for combined *Fusarium* and *Acanthamoeba* keratitis. *Acta Ophthalmol* 2009; **87**: 199–203.
8. Ficker L, Seal D, Warhurst D, Wright P. *Acanthamoeba* keratitis-resistance to medical therapy. *Eye* 1990; **4**: 835–8.

© 2013 Royal Australian and New Zealand College of Ophthalmologists

14429071, 2014, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/ceo.12120 by Bezm-I Alem Vakif University, Wiley Online Library on [03/09/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

9. Gooi P, Lee-Wing M, Brownstein S, El-Defrawy S, Jackson WB, Mintsioulis G. *Acanthamoeba* keratitis: persistent organisms without inflammation after 1 year of topical chlorhexidine. *Cornea* 2008; **27**: 246–8.
10. Cojean S, Houzé S, Haouchine D *et al*. *Leishmania* resistance to miltefosine associated with genetic marker. *Emerg Infect Dis* 2012; **18**: 704–6.
11. Santa-Rita RM, Santos Barbosa H, Meirelles MN, de Castro SL. Effect of the alkyl-lysophospholipids on the proliferation and differentiation of *Trypanosoma cruzi*. *Acta Trop* 2000; **75**: 219–28.
12. Blaha C, Duchêne M, Aspöck H, Walochnik J. *In vitro* activity of hexadecylphosphocholine (miltefosine) against metronidazoleresistant and -susceptible strains of *Trichomonas vaginalis*. *J Antimicrob Chemother* 2006; **57**: 273–8.
13. Seifert K, Duchêne M, Wernsdorfer WH *et al*. Effects of miltefosine and other alkylphosphocholines on human intestinal parasite *Entamoeba histolytica*. *Antimicrob Agents Chemother* 2001; **45**: 1505–10.
14. McBride J, Ingram PR, Henriquez FL, Roberts CW. Development of colorimetric microtiter plate assay for assessment of antimicrobials against *Acanthamoeba*. *J Clin Microbiol* 2005; **43**: 629–34.
15. Walochnik J, Duchêne M, Seifert K *et al*. Cytotoxic activities of alkylphosphocholines against clinical isolates of *Acanthamoeba* spp. *Antimicrob Agents Chemother* 2002; **46**: 695–701.
16. Walochnik J, Obwaller A, Gruber F *et al*. Anti-acanthamoeba efficacy and toxicity of miltefosine in an organotypic skin equivalent. *J Antimicrob Chemother* 2009; **64**: 539–45.
17. Aichelburg AC, Walochnik J, Assadian O *et al*. Successful treatment of disseminated *Acanthamoeba* sp. infection with miltefosine. *Emerg Infect Dis* 2008; **14**: 1743–6.
18. Polat ZA, Obwaller A, Vural A, Walochnik J. Efficacy of miltefosine for topical treatment of *Acanthamoeba* keratitis in Syrian hamsters. *Parasitol Res* 2012; **110**: 515–20.
19. Visvesvara GS. Amebic meningoencephalitides and keratitis: challenges in diagnosis and treatment. *Curr Opin Infect Dis* 2010; **23**: 590–4.
20. Akin Polat Z, Vural A. Effect of combined chlorhexidine gluconate and Neosporin on experimental keratitis with two pathogenic strains of *Acanthamoeba*. *Parasitol Res* 2012; **110**: 1945–50.
21. Sharma N, Sachdev R, Jhanji V, Titiyal JS, Vajpayee RB. Therapeutic keratoplasty for microbial keratitis. *Curr Opin Ophthalmol* 2010; **2**: 293–300.
22. Clarke B, Sinha A, Parmar DN, Sykakis E. Advances in the diagnosis and treatment of *Acanthamoeba* keratitis. *J Ophthalmol* 2012; **2012**: 484892.
23. Lim N, Goh D, Bunce C *et al*. Comparison of polyhexamethylene biguanide and chlorhexidine as monotherapy agents in the treatment of *Acanthamoeba* keratitis. *Am J Ophthalmol* 2008; **145**: 130–5.
24. Ehlers N, Hjortdal J. Are cataract and iris atrophy toxic complications of medical treatment of *Acanthamoeba* keratitis? *Acta Ophthalmol Scand* 2004; **82**: 228–31.
25. Aksozek A, McClellan K, Howard K, Niederkorn JY, Alizadeh H. Resistance of *Acanthamoeba castellanii* cysts to physical, chemical, and radiological conditions. *J Parasitol* 2002; **88**: 621–3.
26. Clarke DW, Niederkorn JY. The pathophysiology of *Acanthamoeba* keratitis. *Trends Parasitol* 2006; **22**: 175–80.

© 2013 Royal Australian and New Zealand College of Ophthalmologists

14429071, 2014, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/ceo.12120 by Bezm-I Alem Vakif University, Wiley Online Library on [03/09/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License