Department of Ophthalmology
Saarland University Medical Center, Homburg/Saar
Chairman: Prof. Dr. Berthold Seitz

The effect of antiamoebic agents on human corneal cells and the *Acanthamoeba castellanii* 1BU strain

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Submitted by Lei Shi
Born on July 15, 1978 in Anhui, P. R. China

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Dekan : Prof. Dr. med Michael D Menger

Erstberichterstatter: PD. Dr. Nora Szentmary

Zweitberichterstatter: Prof. Dr. Sören Becker

To my family

Declaration

I hereby declare that this thesis is my own original work and effort. All experiments, except for those specified, were exclusively performed by me. Except for the publications written by myself listed in the publication list, the data presented here have not been submitted anywhere else for any award. Where other sources of information and help have been used, they have been indicated and acknowledged.

Homburg/Saar, 21.9.2019

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Lei Shi

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AK	*Acanthamoeba* keratitis
aPA	Acathamoeba plasminogen activator
AUC	Area under curve
BrdU	Bromodeoxyuridine
CH	Chlorhexidine
CXL	Crosslinking
DD	Dibromopropamidine-diisethionat
DMEM/F12	Dulbecco's modified eagle medium: nutrient mixture F-12
E.coli	*Eshericia coli*
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GMS	Gömöri-methenamine silver
HCEC	Human corneal epithelial cell
HCEC-12	Human corneal endothelial cell
HD	Hexamidine-diisethionat
MF	Miltefosine
NM	Natamycin
PAS	Page’s amoebic saline
PAS1	Periodic acid Schiff
PCR	Polymerase-chain reaction
PD	Propamidin-isethionate
PDT	Photodynamic therapy
PVPI	Povidone iodine
PYG	Peptone-yeast extract-glucose
SD	Standard deviation

SUMMARY

The effect of antiamoebic agents on human corneal cells and the *Acanthamoeba castellanii* 1BU strain

Background: Acanthamoeba keratitis is a progressive, sight-threatening disease. The correct diagnosis is often delayed for the patients. In an early stage, ophthalmologists observe pseudodendritiformic epitheliopathy, “dirty epithelium”, spot-like multifocal stromal infiltrates and radial perineuritis. Later on, a ring infiltrate develops and in long-standing, recalcitrant *Acanthamoeba* keratitis, uveitis, retinal vasculitis and scleritis may occur and result in blindness. Since AK presents with heterogeneous clinical appearance and low incidence, there have been no clinical trials comparing different treatment modalities in this disease and no standardized treatment of AK has been established, yet. The lack of a standardized therapy against AK is also in part due to the lack of a generally agreed drug testing regime against *Acanthamoeba*.

Therefore, we had the following **purposes:**

- Histological analysis of two *Acanthamoeba* keratitis eyes with anterior and posterior segment inflammation and blindness.
- To analyze the effect of biguanides (polyhexamethylen biguanid (PHMB), chlorhexidine (CH)) and diamidines (hexamidine-diisethionat (HD), propamidin-isethionate (PD), dibromopropamidine-diisethionat (DD)) on human corneal epithelial cell, keratocyte and endothelial cell viability, proliferation and migration, in vitro.
- To compare LDH release assay, trypan blue and fluorescent stainings and non-nutrient *Escherichia coli* (*E. coli*) plate assay in determining treatment efficacy of antiamoebic-agents against *Acanthamoeba castellanii* trophozoites and cysts, *in-vitro*.
- To analyze the concentration dependent effect of biguanides (PHMB, CH); diamidines (HD, PD, DD); natamycin (NM); miltefosine (MF); povidone iodine (PVPI) and chlorin e6 photodynamic therapy (PDT) on *Acanthamoeba castellanii* trophozoites and cysts, in vitro.

Methods:

- Two eyes of 2 patients (age 45 and 51 years) with *Acanthamoeba* keratitis (PCR of

epithelial abrasion positive) were analysed. Patients underwent triple-topical therapy (polyhexamethilen-biguanide, propamidin-isethionat and neomycin) and with failed recovery, subsequent crosslinking, corneal cryotherapy, repeat penetrating keratoplasties, amniotic membrane transplantations and phacoemulsification with posterior chamber lens implantation. The patients developed ocular hypotony with central vein/artery occlusion and retinal/choroidal detachment and had no light perception; therefore, the inflamed eyes were enucleated. Histological analysis was performed using haematoxilin-eosin, periodic acid- Schiff and Gömöri-methenamine silver staining.

- For epithelial and endothelial cells a human cell line, and for keratocytes primary cultures were used (n=6 each). We used $7.8x10^{-5}$-0.02% PHMB or CH, $3.9x10^{-4}$-0.1% HD, PD or DD, $3.9x10^{-4}$-0.0125% PD concentration for 24 hours to determine viability (Cell Proliferation Kit XTT), proliferation (Cell Proliferation ELISA BrdU kit) and migration using wound healing assay. Viability/proliferation/migration values of each drug were summarized as "area under curve" (AUC) together with a Mann-Whitney test.
- *Acanthamoeba castellanii* strain 1BU-trophozoites/cysts were challenged with the antiamoebic-agents 0.02% PHMB, 0.1% PD and 0.0065% MF. Efficacies of the drugs were determined by LDH-release and trypan blue assays. The fluorescent dyes Hoechst 33343, calcein-AM, and ethidium-homodimer-1 were tested for their abilities to differentiate between viable and dead 1BU-trophozoites/cysts following treatment, a non-nutrient agar *Eshericia coli (E. coli)* plate assay was applied to monitor the outgrowth of 1BU-trophozoites/cysts challenged with antiamoebic-agents.
- *Acanthamoeba castellanii* 1BU strain was cultured in 712 peptone-yeast extract-glucose (PYG) medium. Thereafter, trophozoites or cysts were cultured in 0,005-0.02% PHMB, CH or 0.25-0.1% HD, PD, DD, or 5% NM or 0.001625-0.0065% MF or 0.25-1% PVPI containing PYG medium for 2 hours or underwent Chlorin e6-PDT. Then, the percentage of dead trophozoites was determined by CytoTox 96® Non-Radioactive Cytotoxicity assay and trypan blue staining and those of cysts using trypan blue staining. Treated cysts were also inoculated on non-nutrient agar *E. coli* plates and were observed for 5 weeks.

Results:

- We could not observe *Acanthamoeba* trophozoites or cysts neither in the cornea nor in other ocular tissues in the enucleated eyes. There were anterior synechiae in the chamber angle of both globes and lymphocytic infiltration around central retinal artery and vein, associated with fibrous metaplasia of the retinal pigment epithelium. We saw perivascular inflammatory cell infiltration (mainly lymphocytes) in the episclera and around ciliary nerves, analysing the first globe. This was associated with non-granulomatous uveitis,

cilioschisis and tractional retinal detachment. Cross sections of the optic nerve revealed gliosis and optic nerve atrophy. Histopathologic studies of the second globe revealed a multifocal, non-granulomatous choroiditis with lymphocytic infiltration.

- HCEC, keratocyte and HCEC-12 ***viability AUC,*** comparing PD and PHMB ($p \leq 0.014$ for all; PD better) or PD and HD ($p \leq 0.011$ for all; PD better) differed significantly. Keratocyte and HCEC-12 viability AUC comparing CH and HD ($p \leq 0.027$; CH better), HCEC-12 viability AUC comparing PD and HD ($p=0.005$; PD better) and HCEC viability AUC comparing CH and PHMB ($p=0.014$; CH better) differed significantly. HCEC ***proliferation AUC***, comparing PD with PHMB, CH, DD, HD ($p \leq 0.016$; PD worse for all) and keratocyte proliferation AUC, comparing PHMB with HD, PD ($p=0.004$; $p=0.002$; PHMB better for both), CH with HD, PD ($p \leq 0.001$; CH better for both) and DD with PD ($p=0.043$; DD better) differed significantly. Keratocyte ***migration AUC*** comparing PD with control, PHMB, CH, DD and HD differed significantly ($p \leq 0.012$; PD worse for all).
- All three antiamoebic-agents induced a significant LDH-release from trophozoites, when compared to controls ($p<0.0001$). There was a negligible fluorescent-dye staining in untreated 1BU-throphozoites/cysts, but after challenging with antiamoebic-agents, there was 59.3-100% trypan blue, 100% Hoechst 33342, 0-75.3% calcein-AM and 100% ethidium-homodimer-1 positivity. On non-nutrient agar *E. coli* plates, in controls and MF treated 1BU trophozoites/cysts, new trophozoites appeared within 24 hours, and encystment occured after 5 weeks. In PHMB and PD treated 1BU-throphozoites/cysts, irregularly shaped, smaller trophozoites appeared after 72 hours, which failed to form new cysts within 5 weeks.
- All concentrations of different antiamoebic agents had a significant cytotoxic effect on AK trophozoites and cysts ($p<0.05$), except 0.02% PHMB or CH, for trophozoites, and 0.005% CH or Ce6-PDT for cysts, using trypan blue assay. On agar plates, normal shaped trophozoites could not be observed using PHMB, CH, HD, PD, NM and PVPI treatment and cysts could not be formed again within 5 weeks. DD and MF treated cysts could excyst and after one week encyst again.

Conclusions:

- In long-standing, recalcitrant *Acanthamoeba* keratitis, uveititis, retinal vasculitis and scleritis may occur and result in blindness, even without further persistence of *Acanthamoeba* trophozoites or cysts. In this stage of *Acanthamoeba* keratitis, systemic immune suppression may be necessary for a longer time period.
- Chlorhexidin as biguanide and propamidin-isethionate as diamidine may be used clinically to reduce cytotoxicity of antiamoebic treatment on human corneal cells. Diamidines

reduce proliferation of human epithelial cells and keratocytes more than biguanides and propamidin-isethionate reduces migration of keratocytes. Therefore, in spite of lower cytotoxicity, the inhibitory effect on proliferation and migration indicates that extended use of propamidin-isethionate should be avoided in patients.

- None of the enzymatic- and dye-based viability assays tested here generated survival rates for trophozoites/cysts that were comparable with those yielded with the non-nutrient agar *E. coli* plate assay, suggesting that the culture-based assay is the best method to study the treatment efficacy of drugs against *Acanthamoeba*.
- PHMB, CH, HD, PD, NM and PVPI seem to be more effective against *Acanthamoeba castellani* 1BU strain, than DD and MF. In vitro analysis of treatment efficacy of different antiamoebic agents, especially the non-nutrient agar *Eschericia coli* plate assay may provide information on specific treatment of different *Acanthamoeba* strains in the future.

ZUSAMMENFASSUNG

Wirkung von Anti-Amöbika auf humane korneale Zellen und den *Akanthamöben castellanii* 1BU-Stamm

Hintergrund: Die *Akanthamöben*-Keratitis (AK) ist eine progressive, potentiell blindmachende Erkrankung. Die richtige Diagnose wird bei den Patienten oft erst verzögert gestellt. In einem frühen Stadium beobachten Augenärzte pseudodendritiforme Epitheliopathie, "schmutziges Epithel", punktförmige multifokale stromale Infiltrate und eine radiale Perineuritis. Später entwickelt sich ein Ringinfiltrat und bei langjähriger, hartnäckiger *Akanthamöben*-Keratitis, kann es zur Uveitis, retinale Vaskulitis und Skleritis und sogar zur Erblindung kommen. Da AK in der Regel mit einem heterogenem klinischen Erscheinungsbild und geringer Inzidenz auftritt, gab es keine klinischen Studien, die verschiedene Behandlungsmodalitäten bei dieser Krankheit verglichen hätten. Es wurde daher noch keine standardisierte Behandlung der AK etabliert. Das Fehlen einer standardisierten Therapie gegen die AK ist auch zum Teil auf das Fehlen einer allgemeinen Richtlinie zur Therapie einer *Akanthamöben* Infektion zurückzuführen.

Ausgehend davon hatten wir folgende Ziele für diese Arbeit:

- Histologische Analyse von zwei *Akanthamöben*-Keratitis-Augen mit Entzündungen im vorderen und hinteren Segment und Erblindung.
- Es sollte die Wirkung von Biguaniden (Polyhexamethylenbiguanid (PHMB), Chlorhexidin (CH)) und Diamidinen (Hexamidin-Diisethionat (HD), Propamidin-Isethionat (PD), Dibromopropamidin-Diisethionat (DD)) auf die Viabilität, Proliferation und Migration von humanen kornealen Epithelzellen, Keratozyten und Endothelzellen in vitro analysiert werden.
- Der Vergleich von LDH-Freisetzungstests, Trypanblau- und Fluoreszenzfärbungen und nährstofffreien-*Escherichia coli (E. coli)*-Platten-Tests zur Bestimmung der Effektivität von Anti-Amöbika gegen *Akanthamöben* castellanii Trophozoiten und Zysten in vitro.
- Es sollte die konzentrationsabhängige Wirkung von Biguaniden (PHMB, CH), Diamidinen (HD, PD, DD), NM, MF, PVPI und Chlorin e6 Photodynamische Therapie (PDT) bei *Akanthamöben* castellani Trophozoiten und Zysten in vitro analysiert werden.

Methoden:

- Zwei Augen von 2 Patienten (Alter 45 und 51 Jahren) mit *Akanthamöben*-Keratitis (Diagnose erfolgte durch PCR des epithelialen Abrasions positiv) wurden analysiert. Die Patienten wurden einer dreifach topischen Therapie (Polyhexamethilen-biguanid, Propamidin-Isethionat und Neomycin) und aufgrund fehlenden Therapieerfolges mit anschließendem Crosslinking, Hornhaut-Kryotherapie, wiederholten perforierenden Keratoplastiken, Amnionmembrantransplantationen und Phakoemulsifikation mit Kunstlinsenimplantation der hinteren Kammer unterzogen. Die Patienten entwickelten eine okuläre Hypotonie mit zentraler Venen/Arterien-Verschluss- und Netzhaut-Aderhautablösung und hatten keine Lichtwahrnehmung; daher wurden die entzündeten Augen enukleiert. Die histologische Analyse wurde mit Hämatoxilin-Eosin, Periodsäure-Schiff und Gömöri-Methenamin-Silberfärbung durchgeführt.
- Für die Zellkulturversuche wurde jeweils eine humane Epithelzell (HCEC)- und Endothelzelllinie (HCEC-12) und eine Keratozyten Primärkultur (n=6) verwendet. Wir verwendeten 7,8 x 10^{-5} - 0,02% PHMB oder CH, 3,9 x 10^{-4} - 0,1% HD, PD oder DD, 3,9 x 10^{-4} - 0,0125% PD-Konzentration für 24 Stunden, um die Viabilität (Cell Proliferation Kit XTT), die Proliferation (Cell Proliferation ELISA BrdU Kit) und die Migration mittels Wundheilungstest zu bestimmen. Die Ergebnisse der Viabilitäts-, Proliferations- und Migrationswerte wurden zusammen mit einem Mann-Whitney-Test berechnet und als "area under curve" (AUC) dargestellt.
- Der *Akanthamöben castellanii* Stamm 1BU (Trophozoiten und Zysten) wurde mit den Antiamöbika 0,02% Polyhexamethylen-biguanid (PHMB), 0,1% Propamidin-isethionat (PD) und 0,0065% Miltefosin (MF) inkubiert. Die Wirksamkeit der Medikamente wurde durch die LDH-Freisetzung und den Trypanblau-Assay bestimmt. Die Fluoreszenzfarbstoffe Hoechst 33343, Calcein-AM und Ethidium-Homodimer-1 wurden verwendet, um nach der Behandlung zwischen lebensfähigen und toten 1BU-Trophozoiten/Zysten zu unterscheiden. Ein nährstofffreier Agar-*Eschericia coli (E. coli*)-Plattentest wurde angewendet, um das Wachstum von 1BU-Trophozoiten und Zysten zu beurteilen, die mit den Anti-Acanhtamoeben-Medikamenten behandelt wurden.
- Die Anzucht des *Akanthamöben castellani* 1BU Stamm erfolgte in 712 Pepton-Hefeextrakt-Glukose (PYG) Medium. Anschließend wurden Trophozoiten oder Zysten in 0,005-0,02% PHMB, CH oder 0,25-0,1% HD, PD, DD oder 5% NM oder 0,001625-0,0065% MF oder 0,25-1% PVPI enthaltendes PYG-Medium für 2 Stunden inkubiert oder einer Chlorin e6-PDT unterzogen. Anschließend wurde der Anteil der toten Trophozoiten durch den CytoTox 96® Non-Radioactive Cytotoxicity

Assay und die Trypanblau-Färbung bestimmt, der Anteil der toten Zysten nur durch die Trypanblau-Färbung. Die behandelten Zysten wurden zusätzlich auf nährstofffreie Agar *E. coli*-Platten geimpft und 5 Wochen lang beobachtet.

Ergebnisse:

- *Akanthamöben* Trophozoiten oder Zysten konnten wir weder in der Hornhaut noch in anderen Augengeweben in den enukleierten Augen beobachten. Es gab anteriore Synechien im Kammerwinkel der beiden Augen und lymphozytäre Infiltration um die zentrale Netzhautarterie und Vene, verbunden mit fibröser Metaplasie des retinalen Pigmentepithels. Wir sahen eine perivaskuläre Infiltration inflammatorischer Zellen (hauptsächlich Lymphozyten) in der Episklera und um die Ziliarnerven herum. Die Analyse des ersten Auges zeigte eine nicht-granulomatöse Uveitis, Cilioschisis und traktive Netzhautablösung. Querschnitte des Sehnervs zeigten eine Gliose und eine Sehnervenatrophie. Die histopathologische Untersuchungen des zweiten Auges zeigten eine multifokale, nicht-granulomatöse Choroiditis mit lymphozytärer Infiltration.
- **AUC** der Viabilitätsassays von HCEC, Keratozyten und HCEC-12 unterschied sich signifikant im Vergleich von PD gegen PHMB ($p \leq 0.014$ für alle; PD besser) und im Vergleich von PD und HD ($p \leq 0.011$ für alle; PD besser). Die AUC des Viabilitätsassay unterschied sich signifikant bei Keratozyten und HCEC-12 im Vergleich von CH und HD ($p \leq 0.027$; CH besser), bei HCEC-12 im Vergleich von PD und HD ($p=0.005$; PD besser), und bei HCEC im Vergleich von CH und PHMB ($p=0.014$; CH besser). Bei den Proliferationstesten zeigte die AUC bei den HCEC im Vergleich von PD gegen PHMB, CH, DD und HD signifikante Unterschiede ($p \leq 0.016$; PD schlechter für alle). Die Ergebnisse der Proliferationsteste bei den Keratozyten zeigten Unterschiede bei der AUC im Verglich von PHMB mit HD und PD ($p=0.004$; $p=0.002$; PHMB besser für beide), CH mit HD und PD ($p \leq 0.001$; CH besser für beide), und im Vergleich von DD mit PD ($p=0.043$; DD besser). Bei den Migrationstesten zeigten sich signifikante Unterschiede bei den Keratozyten im Vergleich von PD mit der Kontrolle, PHMB, CH DD und HD ($p \leq 0.012$; PD schlechter für alle).
- Alle drei Anti-Amöbika induzierten im Vergleich zur Kontrolle eine signifikante LDH-Freisetzung bei den Trophozoiten ($p<0,0001$). Es gab eine vernachlässigbare Fluoreszenzfärbung bei unbehandelten 1BU-Throphozoiten/Zysten, aber nach der Inkubation mit den Anti-Amöbika zeigten sich in der Trypanblau Färbung 59,3-100%, nach der Inkubation mit Hoechst 33342 100%, bei Calcein-AM 0-75,3% und bei Ethidium-Homodimer-1 100% positive Zellen. Auf nährstofffreien Agar-*E.*

coli-Platten, traten bei der Kontrolle und den MF-behandelten 1BU-Trophozoiten/Zysten innerhalb von 24 Stunden neue Trophozoiten auf, nach 5 Wochen bildeten sich erneut Zysten. Bei den mit PHMB und PD behandelten 1BU-Throphozoiten/Zysten traten nach 72 Stunden unregelmäßig geformte, kleinere Trophozoiten auf, die innerhalb von 5 Wochen keine neuen Zysten bildeten.

- Bei allen Konzentrationen der verschiedenen amöbenhemmenden Mitteln zeigte sich eine signifikante zytotoxische Wirkung auf *Akanthamöben* Trophozoiten und Zysten ($p<0,05$), mit Ausnahme von 0,02% PHMB oder CH bei Trophozoiten und 0,005% CH oder Ce6-PDT bei Zysten unter Verwendung des Trypanblau-Tests. PHMB, CH, HD, PD, NM und PVPI Furhten zu morphologischen Veranderungen der *Akanthamöben*-Trophozoiten, die innerhalb von 5 Wochen keine Zysten mehr bilden Konnten. Die mit DD und MF behandelten Zysten konnten exzystisch und innerhalb von eine Woche wieder enzystisch werden.

Schlussfolgerungen:

- Bei langjähriger, andauernder *Akanthamöben*-Keratitis können Uveititis, retinale Vaskulitis und Skleritis auftreten und zur Erblindung führen, auch ohne weitere Persistenz von *Akanthamöben*-Trophozoiten oder Zysten. In diesem Stadium der *Akanthamöben*-Keratitis kann eine systemische Immunsuppression über einen längeren Zeitraum notwendig sein.
- Chlorhexidin als Biguanid und Propamidin-Isethionat als Diamidin können klinisch eingesetzt werden, um die Zytotoxizität der antiamöbischen Behandlung an humanen Hornhautzellen zu reduzieren. Diamidine reduzieren die Proliferation von humanen Epithelzellen und Keratozyten mehr als Biguanide. Propamidin-Isethionat senkt die Migrationaktivität von Keratozyten. Daher deutet die hemmende Wirkung auf Proliferation und Migration trotz geringerer Zytotoxizität darauf hin, dass eine längere Verwendung von Propamidin-Isethionat bei Patienten vermieden werden sollte.
- Da die enzymatischen und farbstoffbasierten Vitalitätsteste stark unterschiedliche Ergebnisse für Trophozoiten/Zysten aufwiesen, die mit denen des nährstofffreien Agar *E. coli-Platten-Assays* nicht vergleichbar waren, deutet es daraufhin, dass der kulturbasierte Assay die beste Methode ist, um die Behandlungseffektivität von Medikamenten gegen *Akanthamöben* zu untersuchen.
- PHMB, CH, HD, PD, NM und PVPI scheinen effektiver gegen den *Akanthamöben castellani* 1BU Stamm zu sein, als DD und MF. In vitro-Analysen, insbesondere die Analyse mit nährstofffreien Agars *Eschericia coli* Plattenassays können uns

Informationen über die Wirksamkeit der verschiedenen amöbenhemmenden Mittel bei verschiedenen *Akanthamöben*-Stämmen liefern.

1 INTRODUCTION

1.1 *Acanthamoeba*

Acanthamoeba species are free-living, ubiquitous protozoa in the nature. They can be isolated from soil, air, sea, swimming pool and bottled water. *Acanthamoeba* can even be isolated from nasopharyngeal mucosa of healthy individuals. [Niederkorn H; Siddiqui R 2012]

It exists in two life forms, active trophozoites and dormant cysts. Cysts are smaller, round, double-walled and are highly resistant to a stressful and extreme environment. *Acanthamoeba* cysts may remain dormant but viable for several years or even decades. Encystment occurs under stressful conditions such as pH changes or alterations of oxygen or food supply. [Mazur T; Aksozek A; Lorenzo-Morales J]

Trophozoites are the infectious form, resulting in human infection. They result in humans in two main types of infection; granulomatous amoebic encephalitis (GAE) - a rare but often fatal infection of the central nervous system [Geith S; Doan N], and *Acanthamoeba* keratitis (AK). Infection of the central nervous system predominantly occurs in immunocompromised patients and AK in immunocompetent subjects.

1.2 *Acanthamoeba* keratitis

1.2.1 Epidemiology

Acanthamoeba keratitis (AK) is a relatively rare, but progressive, sight-threatening corneal infection, occurring mostly in immunocompetent contact lens wearers. 68%-92.3% of AK patients wear contact lenses before development of a keratitis. [Radford CF; Butler TK; Chin J; Walochnik J 2015] With increasing use of contact lenses, the number of AK patients is likely to increase. [Mathers WD; Panjwani N]

In Germany, with about 80 million inhabitants, about 150 new AK cases have been reported in a 10-year-period [Daas L]. However, AK incidence have been described to increase worldwide [Stehr-Green JK; Chomicz L]. In 2002, its annual incidence was 17.53 to 21.14 per one million contact lens wearers in the UK [Radford CF]. In the US, an AK outbreak was associated to a contact lens solution [Verani JR]. Wearing cosmestic contact lenses have also shown a higher risk to develop AK [Lee SM].

1.2.2 Pathogenesis

Expression of mannosilated glycoproteins on corneal epithelial cell surface is upregulated in contact lens wearers [Huth S]. This plays an important role in AK pathogenesis. The *Acanthamoeba* throphozoite binds to these proteins though its mannose-binding site in order to release the so-called mannose-induced protease 133 (MIP-133) and *Acanthamoeba* plasminogen activator (aPA). MIP-133 and aPA give rise to lysis of epithelial cells, stromal cells and stromal matrix, leading to corneal erosions and ulceration [Alizadeh H 2007]. About 50-100% of the adult population posesses IgG serum antibody against *Acanthamoeba* antigens, however, mice experiments showed that the adaptive immune system cannot eradicate *Acanthamoeba* cysts [McClellan K]. Activation of the adaptive immune system through *Acanthamoeba* increases IL-17A protein expression, which leads to neutrophil activation and migration and, therefore, only moderate symptoms of AK, in mice [Suryawanshi A].

Although contact lens wear is considered as a risk of AK development, most interestingly, not

each contact lens wearer tends to develop AK, implying that the individual immune response may play a crucial role.

Through mucosal immune system, IgA is produced in human tears, which prohibits *Acanthamoeba* trophozoite binding to the ocular surface. Some contact lens wearers do not have this mucosal immune response and, therefore, have higher risk to develop AK [Neelam S; Alizadeh H 2001]. This suggests that *Acanthamoeba* patients may have an immunological "blind-spot", which prevents them from mounting an effective immune response to *Acanthamoeba* antigens.

In many aspects, the immunology of *Acanthamoeba* keratitis needs further research to better understand its pathogenesis and to find potential intervention points to prohibit its development and optimize the human immune response.

Presence of bacteria or fungi also supports *Acanthamoeba* growth, therefore, very often a co-infection is observed [Gupta N; Nunes TE; Raghavan A]. If the *Acanthamoeba* cysts reach deeper corneal layers, this may account for recurrences and a more severe AK in patients [Yokogawa H; AI Kharousi N].

1.2.3 Clinical signs

Acanthamoeba keratitis patients suffer at the early stage of the disease from tearing and ocular pain. At this time-point, the ophthalmologists observe a relative mild ophthalmological status, compared to the pronounced discomfort of the patient. Within the first 2 weeks in 50% of the patients there are chameleon-like epithelial changes ("dirty epithelium"). Grey epithelial opacities, pseudodendritiformic epitheliopathy, epithelial microerosions or microcyst can be observed at this stage (**figure 1A**). Multifocal stromal infiltrates may also be observed (**figure 1B)**, which are mostly central or paracentral. In the first month, 20% of the patients develop a ring infiltrate (Wessely immune ring) (**figure 1C**), formed from polymorphonuclear leukocytes, antigen-antibody-komplex and complement. Its incidence increases over time. In the first month of the disease 2.5-63% of the patients show perineural infiltrates (**figure 1D**), which are radially, from limbus to middle stroma oriented and may result in the long-term in neurotrophic keratopathy. Late symptoms (following months) include limbal stem cell deficiency (**figure 1E**), sterile anterior uveitis, scleritis, broad-based anterior synechiae,

secondary glaucoma, iris atrophy, mature cataract, chorioretinitis and retinal vasculitis. [Szentmáry N 2013; Szentmáry N 2017; Szentmáry N 2018; Daas L]

1.3 Treatment

Conservative treatment

AK is a serious, sight-threatening disease, in which patients need long-term treatment. However, until now, there is no standardized treatment of AK and there is no topical or systemic drug which could explicitly eliminate *Acanthamoeba* trophozoites and especially the highly resistant cysts from the human cornea.

The lack of a standardized therapy against AK is in part due to the lack of a generally agreed.

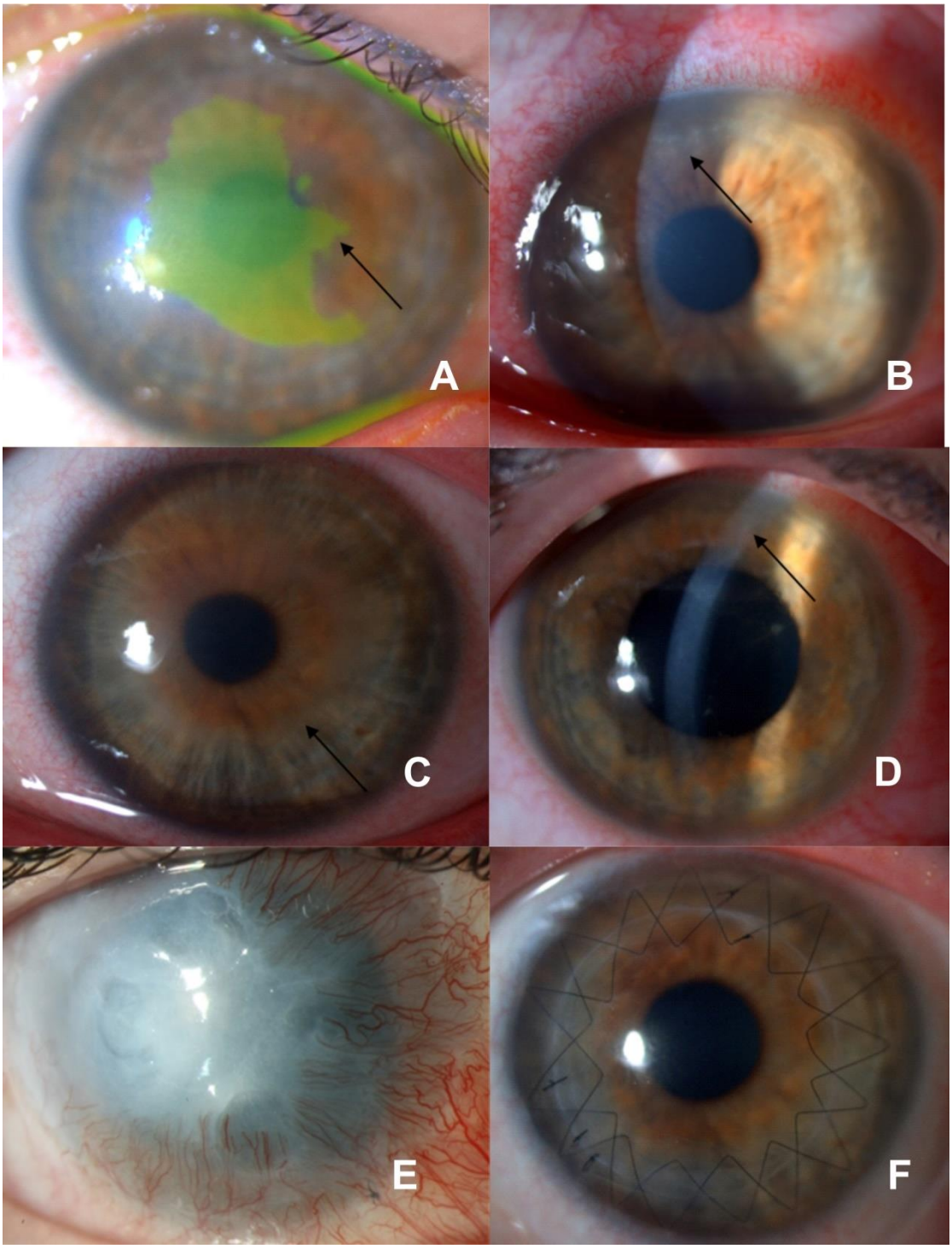


Figure 1. Nonhealing epithelial defect (fluorescein staining) (**A**), multifocal stromal infiltrates **(B)**, ring infiltrate **(C)**, perineuritis **(D)**, limbal stem cell deficiency and nonhealing epithelial defect with conjunctivalization of the cornea **(E)** and stage after curative penetrating keratoplasty **(F)** in AK. Images from the Department of Ophthalmology, Semmelweis University, Budapest, Hungary and from the Department of Ophthalmology, Saarland University Medical Center, Homburg/Saar, Germany.

drug testing regime against *Acanthamoeba*. Although a number of staining methods and

viability tests have been already used to test the efficacy of drugs against *Acanthamoeba* isolates in vitro [Lee X; Siddiqui R 2017; Mito T; Chen Z], a routine method, to enable a specific treatment in AK, against the isolated *Acanthamoeba,* is still not available.

In addition, since AK presents with heterogeneous clinical appearance and low incidence, there have been no clinical trials comparing different treatment modalities in this disease and no standardized treatment of AK has been established, yet [Szentmáry N 2013; Szentmáry N 2017; Szentmáry N 2018]. Therefore, several topical and systemic treatment modalities have been used in case series of AK patients.

Currently, biguanides such as polyhexamethylene biguanide (PHMB) and chlorhexidine (CH), diamidines such as hexamidine (HD), propamidin isethionate (PD) and dibromopropamidine-diisethionat (DD), are mainly used, as eye-drops (off-label medication) [Szentmáry N 2013; Szentmáry N 2017; Szentmáry N 2018; Seal D]. The antibiotic neomycin also showed promising antiamoebic effect as topical treatment. Biguanides, diamidines and neomycin together are used as a so called *triple-topical therapy* in clinical treatment of AK patients [Szentmáry N 2013; Szentmáry N 2017; Szentmáry N 2018; Seal D].

Miconazole and clotrimazole have also been previously successfully used as topical treatment against AK [D`Aversa G; Amoils SP].

Natamycin (NM) is known to inhibit fungal growth by binding to sterols and by impairment of membrane fusion via perturbation of ergosterol-dependent priming reactions that precede membrane fusion [te Welscher YM]. Some studies have shown topical NM successful in *Acanthamoeba* keratitis patients [Jackson TN; Sunada A].

Miltefosine (MF) is an intracellular targeting agent, which can denature essential cell proteins, and disrupt the cell membrane. MF was used successfully in amoebic encephalitis [Aichelburg AC] and in several *in vitro* experiments against *Acanthamoeba* [Walochnik J 2009; Mrva M; Polat ZA 2012; Polat ZA 2014].

PVPI is a broad spectrum microbicide that destroys microbial protein and DNA [Bigliardi PL]. 1% PVPI can cause ridges of the outer cyst wall and separation of the inner cyst wall from the outer one [Sunada A] and seemed to be effective against *Acanthamoeba in vitro* [Yamasaki K; Gatti S].

With increasing resistance of microorganisms to antibiotics, photodynamic therapy (PDT) may be one potential treatment option [Szentmáry N 2018]. Crosslinking as PDT seems not to be effective enough in AK [Hager T; del Buey MA; Makdoumi K]. PDT using Ce6 and red light seemed to be effective in treatment of Pseudomonas aeruginosa keratitis [Wu MF; Deichelbohrer M], therefore, could also be a potential antiamoebic agent.

In addition to topical antimycotic treatment, use of systemic voriconazole have also been reported in AK patients [D`Aversa G; Amoils SP]. Systemic antiamoebic therapy with oral itraconazole (100 mg daily) may probably be a useful adjuvant treatment to prevent progression of AK [Schuster FL 2004].

In the clinical practice, in addition to high recrudescence, AK patients often present with epithelial healing problems [Seal D; Dass L]. The main factors responsible for the cytopathic effect and cytolysis of the human cells in AK are the mannose-induced protein MIP-133 and the *Acanthamoeba* plasminogen activator (aPA), secreted through *Acanthamoeba* trophozoites [Niederkorn H]. However, a cytopathic effect through diamidines and biguanides has also been described [Johns KJ 1988a; Lee JE; Mafra CSP]. In addition, endothelial decompensation occurred following the use of 20% chlorhexidine [Shigeyasu C]. The role of diamidines/biguanides and the AK itself in these clinically observed non-healing epithelial defects is currently unknown.

Systemic antiinflammory treatment may additionally also be necessary in advanced cases of AK, with limbitis and scleritis, and scleral pain. These symptoms may be managed using oral nonsteroidal anti-inflammatory treatment (e.g. flurbiprofen 50 to 100 mg, 2 or 3 times a day), in moderate cases. In more severe cases, high-dose systemic steroid therapy (prednisolone 1 mg/kg/day) followed by systemic cyclosporine (3 to 7.5 mg/kg/day), can be used. Systemic immunosuppression is necessary in average for 7 months in such cases [Lee GA].

Surgical treatment

If conservative treatment is unsuccessful, surgical treatment methods are used to cure AK, or to decrease severity of the symptoms.

Using corneal cryotherapy, the infected corneal areas or the recipient corneal area before penetrating keratoplasty will be treated by “freeze-thaw-freeze" cycles 2–3 times in 360°

[Klüppel M, 1997]. The exact effect of this treatment method on limbal epithelial stem cells has not been clarified, yet.

An amniotic membrane transplantation (AMT) is often used in case of nonhealing epithelial defects, to support epithelial healing. AMT as “Graft” or “Sandwich” may also be applied to heal AK-related corneal ulcer and to reach a quiet stage of the eye (**figure 1F**) [Seitz B 2006].

In the case of progressive, therapy-resistant ulceration over weeks and months with peripheral reparative neovascularization, we suggest an early (<5 months disease course) à chaud penetrating keratoplasty [Laurik KL]. The aim is to remove the infected corneal tissue, before expansion of AK to the corneoscleral limbus.

Following penetrating keratoplasty, we generally continue with triple-topical-therapy over about a year [Hager T].

In the case of perforated corneal ulcers, a non-mechanical, excimer laser penetrating keratoplasty is advantageous [Küchle M; Seitz B 1999]. Using an elliptical excimer laser trephination with metal masks, we may remove the infected corneal area with a more homogeneous distance from the limbal vessels, in typically elliptical-shaped AK [Szentmáry N 2007].

Nevertheless, in some cases, removal of the eye though enucleation may also be necessary, due to loss of eye function and unbearable pain [Awwad ST; Laurik KL].

1.4 Thesis aims

In order to get more insight into an optimized clinical treatment of AK, our study ***purposes*** were as follows:

- Histological analysis of two *Acanthamoeba* keratitis eyes with anterior and posterior segment inflammation and blindness.
- To analyze the effect of biguanides (polyhexamethylen biguanid (PHMB), chlorhexidine (CH)) and diamidines (hexamidine-diisethionat (HD), propamidin-isethionate (PD), dibromopropamidine-diisethionat (DD)) on human corneal epithelial cell, keratocyte and endothelial cell viability, proliferation and migration, in vitro.
- To compare LDH release assay, trypan blue and fluorescent stainings and non-nutrient *Escherichia coli (E. coli)* plate assay in determining treatment efficacy of antiamoebic-agents against *Acanthamoeba castellanii* trophozoites and cysts, *in vitro*.
- To analyze the concentration dependent effect of biguanides (PHMB, CH); diamidines (HD, PD, DD); natamycin (NM); miltefosine (MF); povidone iodine (PVPI) and chlorin e6 photodynamic therapy (PDT) on *Acanthamoeba castellanii* 1BU trophozoites and cysts , *in vitro*.

2 MATERIALS AND METHODS

2.1 Histological analysis of *Acanthamoeba* keratitis eye globes

2.1.1 Patient history

We performed a retrospective record review between January 2006 and December 2017, at the Department of Ophthalmology of Saarland University Medical Center, Homburg/Saar searching for patients with the diagnosis of *Acanthamoeba* keratitis (polymerase-chain reaction (PCR) positive) and subsequent enucleation. During this time period, there were 30 PCR positive AK patients and 2 of them underwent enucleation.

These two patients were both contact lens wearers and their clinical history is described below. In these two eyes of 2 female patients (age 45 and 51 years) PCR of epithelial abrasion confirmed the clinical diagnosis of *Acanthamoeba* keratitis (time to diagnosis after first symptoms 2 weeks and 3 months). These cases had been treated previously as herpetic or herpetic/bacterial keratitis, in another hospital, respectively. There was no evidence of previous or subsequent systemic disease in any of the patients. Best corrected visual acuity at the time of diagnosis was 0.2 and 0.05 and clinical signs of AK were dirty epithelium and multifocal stromal infiltrates (**Figure 2A**) in the first and corneal ulcer, ring infiltrate, keratic precipitates, hypopyon, intrastromal bleeding and posterior synechiae in the second eye (**Figure 3A**).

The patients underwent triple-topical therapy (polyhexamethilen-biguanide, propamidin-isethionat and neomycin) and with failed recovery (2 and 5 months after first AK symptoms and with continuous triple-topical therapy), surgical therapies followed. Before surgery, during continuous triple therapy there were persisiting epithelial defects in both patients, with the size of about 4x5mms and 7x8mms. Repeat iatrogenic epithelial removals were not performed. Although persisting epithelial defects may also be related to the toxicity of the used triple-therapy itself, we interpreted their presence as lack of success with antiamoebic therapy and performed surgery.

The first patient received crosslinking (CXL) with amniotic membrane transplantation as patch, 2 months after first symptoms. All amniotic membranes have been prepared in our eye bank and were used following cold storage (-80°C), for both patients. Corneal cryotherapy with PKP was performed 3 months (8.0/8.1 mm excimer laser trephination) (**Figure 2B**) after initiation of keratitis. One month later, repeat PKP in combination with phacoemulsification and posterior chamber lens implantation and amniotic membrane transplantation as patch has

been performed (triple-procedure, 10.0/10.5 mm hand-held trephination, repeat PKP for AK recurrence and host calcification, along the interface) (**Figures 2C-D**). Histological analysis of both explanted corneal tissues (PKP and repeat PKP) revealed presence of trophozoites and cysts, veryfiing unsuccessfull previous triple-therapy.

The second patient underwent CXL, subsequent corneal cryotherapy with PKP and amniotic membrane transplantation as patch (7.5/7.6 mm excimer laser trephination) 5 months (**Figure 3B-C**) after first symptoms. Two months later, she had phacoemulsification with posterior chamber lens implantation and repeat PKP (8.0/8.1 mm excimer laser trephination, for non-healing epithelial defects). Histological analysis of both explanted corneal tissues (PKP and repeat PKP) revealed presence of trophozoites and cysts, also referring to failed previous triple-therapy. Thereafter, with non-healing epithelial defects, amniotic membrane transplantations as patch were performed 5 times.

Beside our "standard" systemic immune modulatory treatment after PKPs (250- 150- 150- 125- 125- 100- 100- 80- 80- 64- 64- 32- 32- 16- 16- 8 -8 mgs methylprednisolon), no additional immune suppression had been used after keratoplasties. We took this decision, as at this time-point, our patients did not show signs of severe anterior and posterior segment inflammation or corneal vascularization.

Following PKPs, best corrected visual acuity was hand movement and 0.1. Triple-topical therapy was continued 5x daily with additional prednisolone-acetate eye drops 5x daily. However, the epithelial defects further did not heal and the patients developed secondary glaucoma 3 and 6 months after presentation of first AK symptoms, which was successfully cured with conservative therapy. This was followed by central artery occlusion (CRAO) in the first patient 5 months and with central vein occlusion (CRVO) in the second patient 6 months after first AK symptoms. CRAO and CRVO were diagnosed through fundus examination. Fluorescein angiography could not give us additional information through the deepithelialized and oedematous transplanted corneas.

The first patient ended up with ciliary body, choroid and retinal detachment 11 months after first keratitis symptoms and, therefore, sclerotomy, pars plana vitrectomy with silicon oil implantation was performed. The second patient, with subsequent central retinal vein occlusion, received intravitreal Bevacizumab 9 and 10 months after first AK symptoms.

Ocular hypotony became obvious in both patients 11 and 9 months, respectively, after the first AK symptoms (**Figure 2E-F and Figure 3D**). A "filamentous, spider-net-like" inflammatory reaction was detected in the anterior chamber of the second patient simultaneously with ocular hypotony, 9 months after first AK symptoms **(Figure 3D**).

Three months after repeat PKP, both patients had no light perception (7 and 12 months

after the first symptoms of AK) and subsequently, also due to pain, the inflamed blind eyes were enucleated (13 months after the first symptoms of AK in both patients).

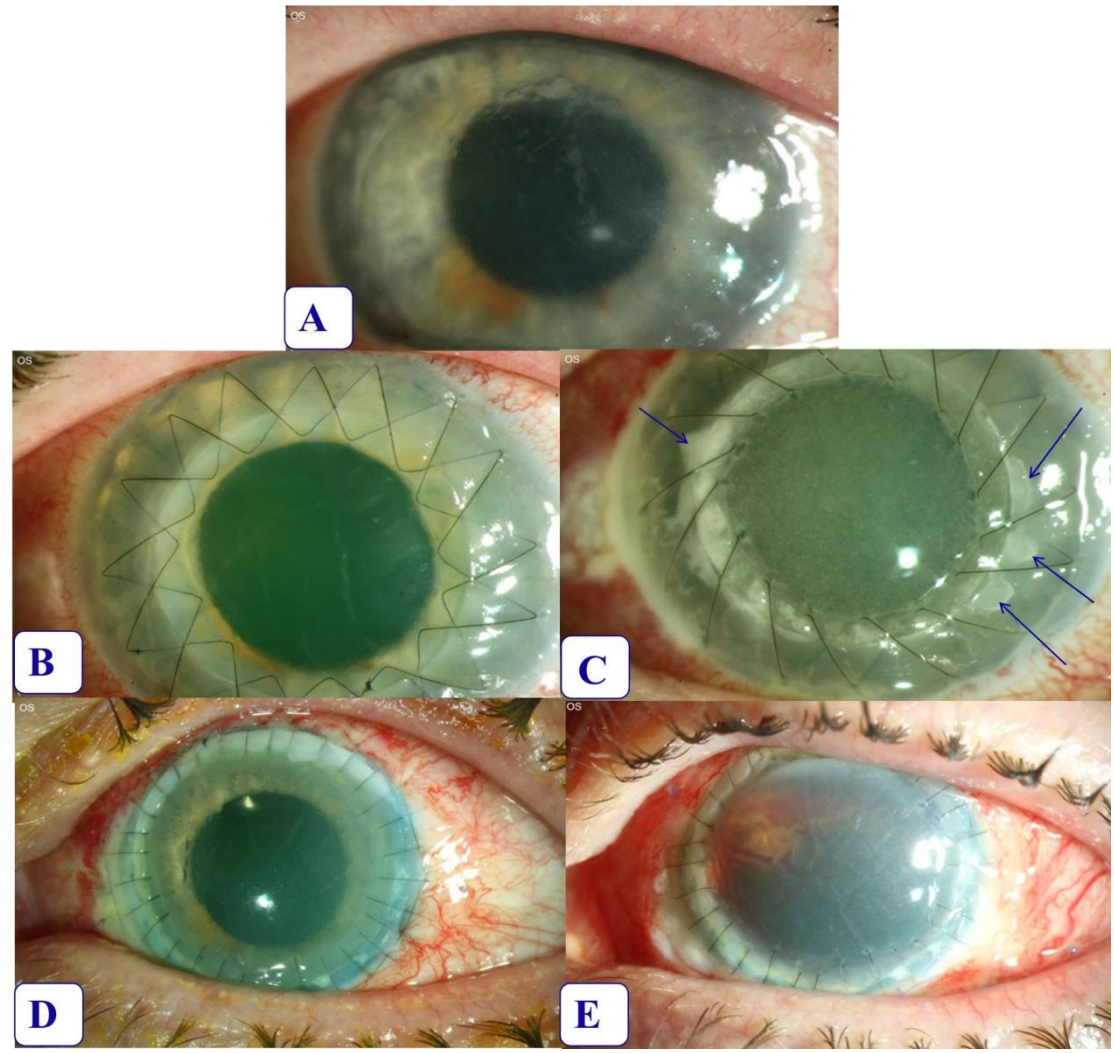


Figure 2. Images of the first case. *Acanthamoeba* keratitis with "dirty epithelium" and multifocal stromal infiltrates (A), after first PKP (B) and recurrence of AK and calcification in recipient along interface (arrows) (C), following repeat PKP with amniotic membrane transplantation as patch (D) and with ocular hypotony, retinal and choroidal detachment (E).

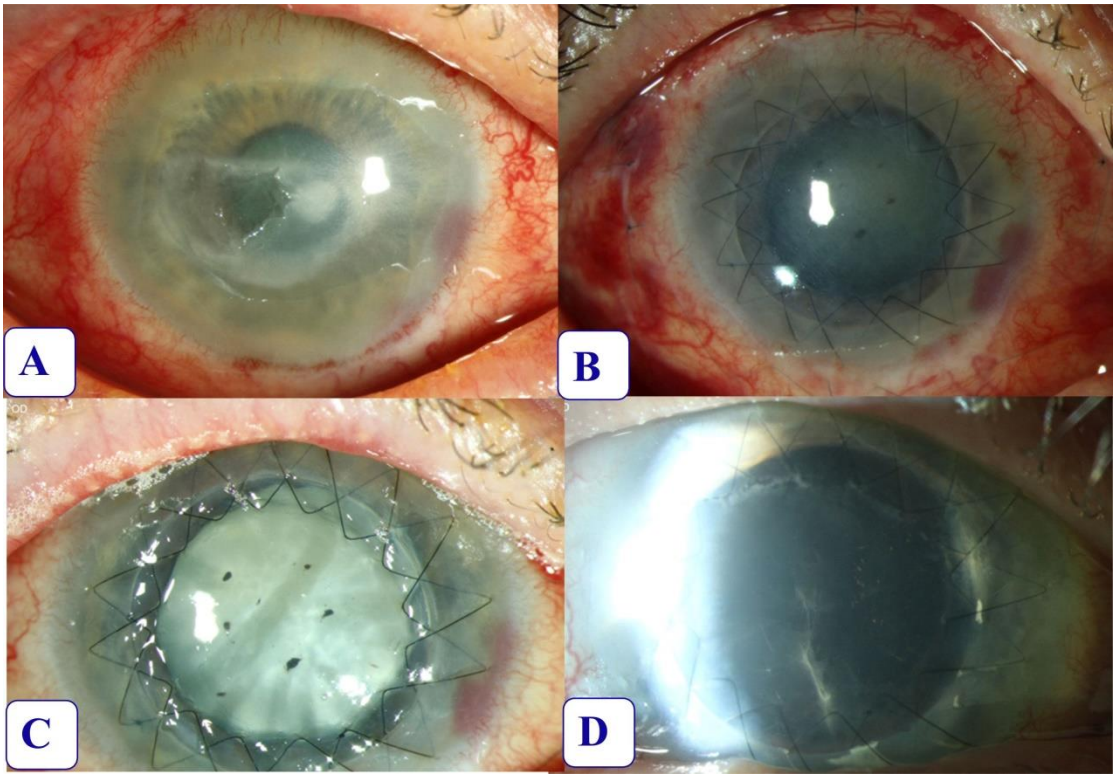


Figure 3. Images of the second case. *Acanthamoeba* keratitis with corneal ulcer, ring infiltrate, intrastromal bleeding and posterior synechiae (A), after first PKP and amniotic membrane transplantation as patch (B), with mature cataract (C) and with "filamentous, spider-net-like" inflammatory reaction in the anterior chamber (D).

2.1.2 Histological analysis

Histological analysis of the enucleated eyes was performed at the Department of Pathology of Saarland University, Homburg/Saar, Germany and at the Department of Ophthalmology of the Friedrich-Alexander University of Erlangen-Nürnberg, Erlangen, Germany.

After formaline-fixation and paraffin wax-embedding of the patients' enucleated eyes, 3 µm thickness sections were cut using a standard microtome and transferred onto microscope slides (SuperFrost, Menzel–Gläser, Braunschweig, Germany). We performed serial sections anteroposteriorly (parallel to the optical axis) and cross-sections of the optic nerves. The slides were dried at 37 °C overnight. Standard haematoxylin–eosin, periodic acid Schiff (PAS1) and Gömöri-methenamine silver (GMS) stainings were then performed. Using PAS1 and GMS stainings, we analyzed presence/absence of trophozoites or cysts in the enucleated eyes. With GMS, we also aimed to determine presence/abcence of a mycotic infection of the enucleated eyes. Further on we will name the first patient's eye globe as „first case", and the second patient's eye globe as „second case".

2.2 Human corneal cell cuture experiments

A part of this dissertation (chapters 2.2, 3.2 and 4.2 as much as Summary/Zusammenfassung on human cells) have been published in "*Shi L, Stachon T, Seitz B, Wagenpfeil S, Langenbucher A, Szentmáry N. The effect of antiamoebic agents on viability, proliferation and migration of human epithelial cells, keratocytes and endothelial cells. Curr Eye Res 2018; 43: 725-33.*". To use data of this publication for this doctoral dissertation was approved by *Current Eye Research.*

2.2.1 Reagents

The materials used included Culture Medium DMEM/F-12 (Life Technologies, Paisley, UK), Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, Steinheim, Germany), fetal bovine serum (FBS) (Life Technologies, Paisley, UK), penicillin-streptomycin (P/S) (Sigma-Aldrich, USA), dimethylsulfoxide (DMSO) (Central Chemical Storage of Saarland University, Saarbrücken, Germany), human epidermal growth factor (hEGF) (Biochrom GmbH, Berlin, Germany), insulin-transferrin-selenium (Life technologies, Paisley, UK), trypsin-EDTA solution (Sigma-Aldrich, USA), cell Proliferation Kit XTT (AppliChem, Darmstadt, Germany), cell proliferation ELISA BrdU (colorimetric) kit (Roche, Mannheim, Germany), sulfuric acid (Titrisol, Darmstadt, Germany), PHMB (Pharmacy of Saarland Medical University, Homburg/Saar, Germany) and CH (Sigma-Aldrich, USA), HD (European Pharmacopoeia, Strasbourg, France), DD (European Pharmacopoeia, Strasbourg, France), PD (Brolene, Patheon UK Ltd., Swindon, UK).

2.2.2 Cell culture

a) Human corneal epithelial cell line (HCECs), RCB2280/ HCE-T, SV40-large T (RIKEN BioResource Center, Ibaraki, Japan)

b) Primary normal human keratocyte cultures

c) Human corneal endothelial cell line (HCEC-12), ACC 646/HCEC-12m, SV40 large T-antigen and small t-antigen (RIKEN BioResource Center, Ibaraki, Japan)

For the following experiments, HCECs were cultured using DMEM/F12 culture medium with 5% FBS, 0.5% DMSO, 1% ITS, 1% P/S, and 10ng/ml hEGF (medium 1). Keratocytes and HCEC-12 were cultured using DMEM/F12 culture medium with 5% FBS (medium 2).

2.2.3 Isolation of primary human corneal keratocytes

Six normal human corneas were obtained from the LIONS Cornea Bank Saar-Lor-Lux,

Trier/Westpfalz, Germany. On the basis of endothelial cell density, these corneas did not meet the criteria for transplantation.

To isolate keratocytes, the human corneoscleral buttons were aseptically rinsed in phosphate-buffered saline (PBS) before removal of the endothelium including Descemet's membrane using a sterile surgical disposable scalpel. A central corneal button with epithelium was cut using a 8.0 mm hand-held trephine and thereafter incubated in culture medium containing 2.4 U/mL Dispase II for 4 h at 37 C. Thereafter, the corneal button was washed several times with PBS and the already loose corneal epithelium removed. The remaining corneal stroma was incubated in culture medium with 1.0 mg/mL collagenase A for 8–10 h at 37°C. The digested tissue and cells were pipetted three times and centrifuged at 800g for 7 minutes and finally resuspended in 1.0 mL culture medium, which consisted of basic medium (DMEM/F12) supplemented with 5% FCS and 1% P/S. The cell suspension was seeded in 6-well plates and the medium was changed 24 hours after seeding. The medium was changed every 2 to 3 days until keratocytes reached confluence. The cells were subcultured in 25 cm^2 culture flasks after 5 to 10 days following dispersal with 0.05% trypsin-EDTA for 3 to 5 min, and the passage four to eight of cells was used for experiments.

2.2.4 Drug preparation

We received HD, DD and CH in powder form, PHMB as 20% solution. Propamidine isethionate was available in 0.1% Brolene® eye drop form. Drugs were dissolved in different culture media, depending on cell type: for HCECs medium 1 and for HCEC-12 and keratocytes medium 2 was used.

The maximal concentrations used were 0.02% (w/v) for PHMB and CH, and 0.1% (weight/value (w/v)) for HD and DD (clinically used concentrations). The maximal concentration for PD was 0.0125% (w/v) (1/8 of 0.1% Brolene® eye drop concentration, as the eye drops had to be diluted with culture medium). Thereafter, all solutions underwent a serial two fold-dilution. The concentration gradients used were 1/256, 1/128, 1/64, 1/32, 1/16, 1/8, 1/4, 1/2 of the initial maximal concentration. Cell cultures without diamidine and biguanide were used as controls.

2.2.5 Determination of viability

Viability was determined using the Cell Proliferation Kit XTT. This measurement involves the activity of mitochondria in the living cells, causing reduction of the tetrazolium salt XTT to orange-colored compounds of formazan. The test was performed according to the manufacturer's protocol.

All cell types were seeded in 96-well plates. HCECs were seeded at 2.8 $x10^4$ HCECs/cm^2 (100µl/well) (n=6), keratocytes at 2.8 $x10^4$ keratocytes/cm^2 (100µl/well) (n=6) and HCEC-12

at $3.3x10^4$ HCEC-12/cm^2 (100µl/well) (n=6). Thereafter, all cell types were cultured for 24 hours until reaching 90% confluence. Once this stage was reached, the culture medium was replaced by drug containing media, using 9 different concentrations from $7.8x10^{-5}$% to 0.02% (from 1/256 to maximal concentration) for PHMB and CH and from $3.9x10^{-4}$% to 0.1% (from 1/256 to maximal concentration) for HD or DD, from $3.9x10^{-4}$% to 0.0125% (from 1/256 to 1/8 concentration) for PD (in a sequence of two fold-diluted concentrations, starting from the highest concentration). Cell cultures without drugs were used as controls.

After culturing the cells for another 24 h, 50 µl/well XTT-containing reaction solution was added and all cell types (with different concentrations) were incubated at 37°C for 2 h. Following this incubation period, the measurement was performed immediately using a 96-well microplate reader (Tecan Infinite Reader, TECAN Deutschland GmbH, Crailsheim, Germany) at 450 nm wavelength (reference wavelength: 690 nm).

2.2.6 Determination of proliferation

Cell proliferation was determined using the cell proliferation ELISA BrdU (colorimetric) kit, according to the manufacturer's protocol. In this assay, BrdU is incorporated instead of thymidine into the DNA of proliferating cells.

All cell types were seeded again in 96-well plates. HCECs were seeded at 2.0 $x10^4$ HCECs/cm^2 (100µl/well) (n=6), keratocytes at 2.0 $x10^4$ keratocytes/cm^2 (100µl/well (n=6) and HCEC-12 at 2.5 $x10^4$ HCEC-12/cm^2 (100µl/well) (n=6). Cells were cultured for 24 hours until reaching 70% confluence. Thereafter, the culture medium was replaced by drug containing media, using 3 different concentrations for all drugs: from $7.8x10^{-5}$% to $3.1x10^{-4}$% (from 1/256 to maximal concentration) for PHMB and CH (in between a series of two fold-diluted concentrations, starting from the highest concentration), and from $3.9x10^{-4}$% to 1.6 $x10^{-3}$% (from 1/256 to maximal concentration) for HD, PD and DD. Cell cultures without drugs were used as controls.

After culturing the cells in drug containing medium for another 24 h, 10µl/well BrdU labeling solution was added to the cultures and cells were incubated at 37°C for 2 h (BrdU incorporation). After removal of the drug containing medium, cells were fixed with FixDenat solution (provided with the test kit), followed by incubation with an anti-BrdU-POD (monoclonal antibody to the thymidine-analogue 5-bromo-2'-deoxyuridine Fab fragments conjugated with peroxidase) (100µl/well) for 90 min. This agent binds the incorporated DNA. Following removal of the solution, all 96-well plates were rinsed with 200µl/well washing buffer three times. Thereafter, 100µl/well tetramethyl-benzidine substrate solution was added, and then 50µl/well 1-N-sulfuric acid was added to stop the reaction after color development was sufficient for photometric detection (10-30 min). Plates were analyzed using a 96-well microplate reader (Tecan Infinite Reader, TECAN Deutschland GmbH, Crailsheim, Germany)

at 450 nm wavelength (reference wavelength: 690nm).

2.2.7 Wound healing assay

Cell migration was determined by the wound healing assay. For this assay, all cell types were seeded in 12-well plates again. HCECs were seeded at 3.8 x10^4 HCECs/cm^2 (n=6), keratocytes at 3.5 x10^4 keratocytes/cm^2 (n=6) and HCEC-12 at 4.5 x10^4 HCEC-12/cm^2 (n=6). Cells were cultured for 24 hours until reaching confluence. Thereafter, cell monolayers were scratched by 200µl yellow pipette tips (Eppendorf AG, Hamburg, Germany). Subsequently, culture medium was removed and cells were rinsed twice with PBS. Thereafter, for HCECs and for HCEC-12, a culture medium with the lowest drug concentration for all drug groups was added (3.9x10^{-4} % (1/256 of maximal concentration) for HD, PD and DD and 7.8x10^{-5} % (1/256 of maximal concentration) for PHMB and CH. For keratocytes, the concentrations used were 1.6x10^{-4} % (1/128 of maximal concentration) for PHMB and CH, and 7.8x10^{-4} % (1/128 of maximal concentration) for HD, PD and DD, due to the morphological and migration speed difference of these cells. Cell cultures without drugs were used as controls.

Images were taken from the original scratch gap and after 6 h, 9 h, 12 h and 24 h of incubation. For each well, two images were taken from different parts of the gap and the mean of these two measurements was used for the subsequent statistical analysis. The area of the scratch gap (for each image, and for each time-point) was measured using the Image J Program MRI-wound-healing-tool (National Institute of Health, USA).

2.2.8 Statistical analysis

Statistical analysis was performed using SPSS 20.0 (IBM, Armonk, NY: IBM Corp, USA).

A Kruskal-Wallis test was performed to check for the overall effect of drugs on human corneal cell viability, proliferation and migration (separate for HCEC, keratocytes and HCEC-12).

To test for the overall efficacy of the drugs, viability, proliferation and migration values for each drug and for each sample (cornea 1 to 6) were summarized for all concentrations as a measure of area under the curve (AUC) (separate for HCEC, keratocytes and HCEC-12).

If the Kruskal-Wallis test indicated an effect of the drug on human corneal cell viability, proliferation and migration, a non-parametric Mann-Whitney U-test was used to compare viability, proliferation and migration AUC values between pairs of drugs (separate for HCEC, keratocytes and HCEC-12). Bonferroni correction was performed for multiple testing. P values <0.05 were considered statistically significant.

2.3 Comparison of assays to study the effectiveness of antiparasitics against *Acanthamoeba castellanii* trophozoites and cysts

2.3.1 Medium and non-nutrient agar preparation

Peptone-yeast-glucose (PYG) medium: 20 g of proteose-peptone (Sigma-Aldrich, St. Louis, USA), 1 g of yeast extract (Sigma-Aldrich), 8 ml of a 0.05M $CaCl_2$ (Gruessing GmbH, Filsum, Germany) solution, 10 ml of 0.4 M $MgSO_4 \times 7H_2O$ (Gruessing GmbH) solution, 10 ml of 0.25 M $Na_2HPO_4 \times 7H_2O$ (Sigma-Aldrich), 10 ml of 0.25M KH_2PO_4 (Gruessing GmbH) solution, 1 g of Na Citrate$\times 2H_2O$ (Gruessing GmbH), and 10 ml of 0.005 M $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$ (Carl Roth GmbH, Karlsruhe, Germany) solution were added to 0.95 L distilled water. The pH of the medium was adjusted to 6.5, and the solution was subsequently autoclaved at 121°C for 15 min. 18 g glucose (Sigma-Aldrich) was added to 50 ml distilled water. Thereafter it was filter sterilized and added to the above solution.

Neff's constant-pH encystment medium: 7.46 g of KCL (Gruessing GmbH), 1.97 g of $MgSO_4 * 7H_2O$ (Gruessing GmbH), 44.40 mg of $CaCL_2 * 2H_2O$ (Gruessing GmbH), 84.01 mg of $NaHCO_3$ (Gruessing GmbH), and 2.42 g of Tris-HCL (Gruessing GmbH) were dissolved in 1 L distilled water. The pH of the solution was adjusted to 9.0, and the medium autoclaved at 121°C for 15 minutes. As these cooled to room temperature, solution A and B were mixed.

Page's amoeba saline (PAS): 0.14 g of Na_2HPO_4 (Sigma-Aldrich) and 0.14 g of KH_2PO_4 (Sigma-Aldrich) were dissolved in 500 ml of distilled water as soltion A, 0.12 g of NaCl (Gruessing GmbH), 4 mg of $MgSO_4 \times 7H_2O$ (Gruessing GmbH) and 4 mg of $CaCL_2 * 2H_2O$ (Gruessing GmbH) were dissolved in 500 ml of distilled water as solution B, then both solutuions were autoclaved at 121°C for 15 minutes. Following reaching room temperature, solutions A and B were mixed.

Non-nutrient agar: 15 g of agar (Sigma-Aldrich) was mixed with 100 ml PAS and 900 m1 distilled water, then was autoclaved at 121°C for 15 minutes.

2.3.2 *Acanthamoeba* isolate

The *Acanthamoeba castellanii* strain 1BU which was isolated from an AK patient, was received from the Unit for Mycotic, Parasitic and Mycobacterial Infections (FG16) of the Robert Koch Institute, Berlin, Germany.

2.3.3 *Acanthamoeba* cultures

1BU trophozoites were grown in tissue culture flasks containing 5 ml of PYG broth medium

at 30°C, in an airtight container. Encystment was induced by adding Neff's constant-pH encystment medium to trophozoites, instead of PYG broth medium, after trophozoites had reached confluence.

Following incubation of the 1BU trophozoites in Neff`s constant-pH encystment medium for 1 week at 30°C, cysts were harvested through 3-times repeated washing (PBS, Sigma-Aldrich) and centrifugation (800 x g) steps, then were resuspended in 5ml PAS with a concentration of 3.30 x 10^6 cysts/ml and were stored at 4°C until usage.

2.3.4 Antiamoebic agents and their preparation

We used as antiamoebic agents polyhexamethylen biguanid (PHMB, Pharmacy of Saarland Medical University, Homburg/Saar, Germany), propamidin isethionate (PD, Brolene, Patheon UK Ltd., Swindon, UK) and miltefosine (MF, Sigma-Aldrich).

PHMB was received as a 20% solution, PD as 0.1% Brolene® eye drops, and MF in powder form. These agents were dissolved or diluted in PYG medium for treatment of 1BU trophozoites, and in PBS for treatment of 1BU cysts (final concentrations were 0.1% PHMB, 0.02% PD and 0.0065% MF). The above concentrations were set analog the clinically used concentrations in human AK treatment (eye drops) (**Table 1**). As controls, 1BU trophozoites were cultivated in PYG medium and 1BU cysts in PBS

Table 1. Antiamoebic agent concentrations in experiments to test *Acanthamoeba* viability.

Agents	Concentrations
PHMB	1079 µM (0.02%)
PD	1771 µM (0.1%)
MF	160 µM (0.0065%）

2.3.5 LDH release assay (cytotoxicity)

Lactate dehydrogenase (LDH) activity in the supernatants of untreated and drug-challenged *Acanthamoeba* was determined with the CytoTox 96® Non-Radioactive kit (Promega Corporation, Madison, USA). For the determination of the cytotoxic effects of antiamoebic

drugs on *A. castellanii* trophozoites, $1x10^4$ 1BU trophozoites were incubated in 96-well plates with 100 µl PYG medium/well at 30°C for 24 hours. During this time, 1BU trophozoites attached to the plate bottom and reached confluence. After 24 hours, PYG medium was replaced by 100 µl of the antiameobic agent-containing medium (preparation described above) and the throphozoites were incubated for 2 hours at 30°C.

To determine the cytotoxic effect of antiamoebic drugs on *A. castellanii* cysts, 2 x 10^4 1BU cysts were incubated in 96-well plates with 100 µl antiamoebic agent containing medium at 30°C for 2 hours. Negative controls received at the same time point 100 µl fresh PYG medium, and positive controls were incubated with 100 µl fresh PYG, supplemented with 10 µl lysis solution (provided by the kit). Controls were also incubated at 30°C for 2 hours. Thereafter, 96-well plates were centrifuged at 200 x g (trophozoites) and 400 x g (cysts) for 5 minutes, respectively, and 50 µl of the supernatants were removed from each well and transferred into the wells of a fresh 96-well plate. 50 µl of substrate reagent was added to each well, the solution was carefully mixed and was incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 µl stop solution to each well. The LDH catalyzed formazan formation was determined spectroscopically with a multimode microplate reader (PerkinElmer EnsightTM, USA) at 490 nm, that was blanked with medium and antiamoebic agent containing medium, respectively.

Cytotoxicity (%) was determined as follows:

Cytotoxicity (%) = 100 × (Experimental LDH Release OD_{490}-Blank control OD_{490}) / (Maximum LDH Release OD_{490} -Blank control OD_{490})

2.3.6 Trypan blue assay

2 x 10^4 1BU trophozoites or cysts were suspended in 100 µl of PYG or antiamoebic agent containing medium (preparation described above for each agent) in 1.5 ml reaction tubes (Sarstedt, Nümbrecht, Germany) and incubated in a Thermomixer (Eppendorf AG, Hamburg, Germany) with a shaking rate of 650 rpm at 30°C for 2 hours. Thereafter, all reaction tubes were centrifuged at 200 g for 5 minutes and 80 µl supernatant was removed from each tube. As a next step, 20 µl of a 0.4% trypan blue solution (Sigma-Aldrich) was added to each tube and throphozoites or cysts were incubated at room temperature for 5 minutes. 1BU trophozoites and cysts without trypan blue staining served as negative controls. Thereafter, 10 µl of the trypan blue treated cells was pipetted to a hemocytometer (C-Chip, NanoEnTek, Waltham, USA) and bright field images were taken with a Leica DMI4000 B microscope (Leica Microsystems, Wetzlar, Germany) at 10-fold magnification, using Leica Application Software (LAS) v3.7.

Using Image J (National Institute of Health, USA), we analyzed grey value for each blue stained cell. Unstained cells in control group and other treated groups were not included. Then,

we calculated the mean grey value for all blue stained cells in each group separately and defined the grey value for each blue-stained cell, compared to the mean value as percentage. With this method, we summarized the cell amount in grey value ranges 85%-90%, 90%-95%, >95%.

2.3.7 Fluorescent staining

1BU trophozoites or cysts (2 x 10^4 cells/well) were resuspended in 100 µl PYG (trophozoites) or PBS (cysts) in absence or presence of the antiamoebic agents, and were pipetted into a 96-well plate, which was subsequently incubated for 2 hours at 30°C. As a positive control, primary human corneal keratocytes (10^4 cells/well) were resuspended in DMEM/F-12 medium (Thermofisher, Paisley, UK), pipetted into 96-well plates, and were incubated for 2 hours at 37°C.

1BU containing 96-well plates were subsequently centrifuged at 200 x g (trophozoites) or 400 x g (cysts) for 5 minutes, to remove the antiamoebic agent containing media.

As a next step, cells were resuspended in 100 µl of fluorescent stain solution (Hoechst 33342 [10 µg/ml] or a mixture of calcein-AM [4 µM] and ethidium homodimer-1 [1 µM]) and were incubated in the well plates for 30 minutes at room temperature in the dark. Thereafter, wells were washed 3-times with PBS to remove unbound fluorescent dyes, and cells were resuspended in PBS.

Cell images were taken with an inverted fluorescence microscope (Leica Microsystems) using the following settings: Hoechst 33342 fluorescence was excited at 340 nm and the light emission was determined at 510 nm, calcein-AM was excited at 490 nm and light emission was determined at 515 nm, and ethidium homodimer-1 was excited at 493 nm and light emission was determined at 617 nm.

Hoechst 33343 stains nuclei of dead and viable cells to blue colour, calcein-AM stains cytoplasm of viable cells to green color and ethidium homodimer-1 stains nuclei of dead cells to red color.

2.3.8 Non-nutrient agar *Escherichia coli* plate assay

The non-nutrient agar *Escherichia coli* plate assay was carried out essentially as described by Narasimhan [Narasimhan S] and Kowalski [Kowalski RP]. First, *E. coli* strain IM08B [Monk IR] was grown overnight on sheep blood agar plates (Becton Dickinson, Heidelberg, Germany) at 35°C. Fresh grown IM08B colonies were picked with a cotton swab and suspended in PAS, equivalent to a McFarland of 4. 50 µl of this suspension was pipetted onto the surfaces of non-nutrient agar plates, and spread out using an L-shaped spreader.

In a second step, 2 x10^4 trophozoites or cysts were mixed with 100 µl medium (PYG for trophozoites and PBS for cysts) in absence and presence of the antiamoebic agents, and were

incubated at 30°C for 2 hours in a Thermomixer (Eppendorf AG) with a shaking rate of 650 rpm. 10 µl of the cell suspensions was transferred into fresh tubes, mixed with 10 µl of a trypan blue solution (0.4%) and was incubated for 5 minutes at room temperature. 980 µl PBS was subsequently added to each tube (the drugs were diluted 100 folds) and the cells were carefully suspended by pipetting gently up and down. 50 µl of the stained cell suspensions (100 cells) were next pipetted into the middle of the non-nutrient agar *E. coli* plates, and the suspension desiccated for around 10 minutes. Next, circles were drawn on the bottom of the plates to confine the borders of the *Acanthamoeba* cell solution, and bright field images of these regions were taken. *A. castellanii* inoculated plates were incubated upside up for 24 hours at 30°C. Thereafter, plates were sealed by parafilm (Pechiney, Menasha, USA) and were incubated upside down for up to 5 weeks at 30°C.

Bright field images were taken with a Leica DMI4000 B microscope every 24 hours until 72 hours, then every week until the end of the 5th week, using a 10-fold magnification. These experiments were repeated three times on different days.

2.3.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.02. Results of the LDH release (cytotoxicity) and trypan blue assays were statistically analyzed using one-way ANOVA followed by Turkey's multiple comparisons test (to compare the effect of each antiamoebic agent).

2.4 Efficacy of antiamoebic agents and Ce6-PDT against *Acanthamoeba castellanii* 1-BU

2.4.1 Medium and non-nutrient agar preparation

PYG medium, Neff's constant-pH encystment medium, Page's amoeba saline (PAS) and ***Non-nutrient agar*** preparation was performed as described at capter **2.3.1**

2.4.2 *Acanthamoeba* isolate

For these experiments, the same *Acanthamoeba castellanii* 1BU strain has been used, as described under chapter **2.3.2**

2.4.3 *Acanthamoeba* cultures

Culturing conditions of *Acanthamoeba castellanii* 1BU strain were also equivalent to the ones described under chapter **2.3.3**

2.4.4 Antiamoebic agents and their preparation

For the experiments, the antiameobic agents polyhexamethylen biguanid (PHMB, Pharmacy of Saarland Medical University, Homburg/Saar, Germany), chlorhexidine (CH, Sigma-Aldrich, USA), dibromopropamidine diisethionat (DD, European Pharmacopoeia, Strasbourg, France), hexamidine diisethionat （HD, European Pharmacopoeia, Strasbourg, France), propamidin isethionate (PD, Brolene, Patheon UK Ltd., Swindon, UK), natamycin (5% natamycin ophthalmic suspension, Alocon laboratories, Fort Worth, USA), povidon-iod (PVPI, B. Braun, Melsungen, Germany), miltefosine (MF, Sigma-Aldrich, Switzerland) and Chlorin e6 (Ce6, ORPEGEN Pharma, Heidelberg, Germany) were used.

We received HD, DD, CH, MF and Ce6 in powder form, PHMB as 20% solution and PVPI as 7.5% solution. Propamidine isethionate was available in 0.1% Brolene® eye drops, natamycin in 5% Natamet® eye drop form. These agents were dissolved or diluted in PYG medium for treatment of 1BU trophozoites and in PBS for treatment of 1BU cysts (final concentrations were the clinically used concentrations and ½ and ¼ of the clinically used concentrations) (**Table. 2**) Trophozoites cultured with PYG medium and cysts cultured with PBS were set as controls.

Table 2. Antiamoebic agent concentrations to test antiamoebic agent efficacy against *Acanthamoeba castellanii* 1BU strain. In LDH and trypan blue assays the below 3 different concentrations were used. However, for non-nutrient *E.coli* agar plate assay, the maximum

concentration alone used.

agents	maximum	Half of maximum	Quarter of maximum
PHMB	0.02% (1079µM)	0.01% (539.5µM)	0.005% (269.75µM)
CH	0.02% (396µM)	0.01% (198µM)	0.005% (99µM)
HD	0.1% (1648µM)	0.05% (824µM)	0.025% (412µM)
PD	0.1% (1771µM)	0.05% (885.5µM)	0.025% (442.75µM)
DD	0.1% (1384µM)	0.05% (692µM)	0.025% (346µM)
NM	5% (75106µM)	2.5% (37553µM)	1.25% (18776.5µM)
MF	0.0065% (160µM)	0.00325% (80µM)	0.001625% (40µM)
PVPI	1% (27401µM)	0.5% (13700.5µM)	0.25% (6850.25µM)
Ce6	0.0152% (256µM)	0.0076% (128µM)	0.0038% (64µM)

2.4.5 Photodynamic therapy (PDT) using Chlorin e6 (Ce6), *trophozoites and cysts*

We received Chlorin e6 stock solution (ORPEGEN Pharma, Heidelberg, Germany) in PBS (30mM). For the experiments, fresh Ce6 with 64µM, 128µM and 256µM concentrations was prepared, using PYG medium or PBS.

1 x10^4 *trophozoites* were seeded in 96-well plates and were allowed to grow for 24 hours with 100µl PYG medium before photodynamic treatment. After 24 hours, trophozoites attached to plate bottom and reached confluence. Then, we replaced PYG medium by the above 3 different Ce6 containing PYG medium and incubated the cultures at 37 °C for 60 minutes in the dark. As a negative control, a culture with fresh PYG medium was used. Thereafter, cultures were washed by 100 µl PBS 3 times to remove the Ce6 containig PYG medium and 100 µl fresh PYG medium was added.

2 x10*4 cysts* were seeded in 96-well plates. Thereafter, the above 3 different Ce6 containing PBS medium were added and we incubated the cultures at 37 °C for 60 minutes in the dark. As a negative control, a culture with PBS was used. Thereafter, the plate was centrifuged at 400 x g for 5 minutes and was washed by 100 µl PBS 3 times to remove the Ce6 containig PBS and 100 µl fresh PBS was added.

Then, we exposed the cultures (*trophozoites* or *cysts*) to red (670 nm) light for 13 minutes (24 J/cm^2). As a negative control without illumination, we kept the culture after removal of the Ce6 containing PYG or PBS medium in dark for the illumiation time of the other cultures. The used illumination box was developed previously through the Department of Physics of the University of Kaiserslautern (Kaiserslautern, Germany) [Wang J; Wu MF; Winkler K].

2.4.6 LDH release assay (cytotoxicity), *trophozoites*

To determinate the cytotoxic effect of antiamoebic drugs on *A. castellanii trophozoites,* $1x10^4$ 1BU trophozoites were incubated in 96-well plates with 100 µl PYG medium/well at 30°C for 24 hours. During this time 1BU trophozoites attached to the plate bottom and reached confluence. After 24 hours, PYG medium was replaced by 100 µl of the antiameobic agent-containing medium (preparation described above) and the throphozoites were incubated for 2 hours at 30°C. Negative controls received at the same time point 100 µl fresh PYG medium, and positive controls were incubated with 100 µl fresh PYG supplemented with 10 µl lysis solution (provided by the kit). Blank controls were incubated with medium or antiamoebic agent-containing medium, but without trophozoites. All controls were also incubated at 30°C for 2 hours. As lysis buffer in this kit could not lyse cysts, these were not analysed using this assay [Shi L, 2019 in press].

Thereafter, 96-well plates were centrifuged at 200 x g for 5 minutes, then 50 µl of the supernatant was removed from each well and was transferred into wells of a fresh 96-well plate. 50 µl of substrate reagent was added to each well, the solution was carefully mixed and was incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 µl stop solution to each well. The LDH catalyzed formazan formation was determined spectroscopically with a multimode microplate reader (PerkinElmer EnsightTM, USA) at 490 nm, that was blanked with medium and antiamoebic agent containing medium, respectively. Cytotoxicity (%) was determined as follows:

Cytotoxicity (%) = 100 × (Experimental LDH Release OD_{490} - Blank control OD_{490}) /(Maximum LDH Release OD_{490} - Blank control OD_{490})

These experiments were repeated 5 times on different days.

As the dark brown color of PVPI was similar to the color of the reaction, the effect of PVPI on *A. castellanii* could not be tested using this assay.

2.4.7 Trypan blue assay, *trophozoites and cysts*

Trypan blue assay was performed as we described previously under chapter **2.3.6**

2.4.8 Non-nutrient agar *Escherichia coli* plate assay, *cysts*

The non-nutrient agar *Escherichia coli* plate assay was carried out essentially as described by Narasimhan [Narasimhan S] and Kowalski [Kowalski RP].

First, *E. coli* strain IM08B17 was grown overnight on sheep blood agar plates (Becton Dickinson, Heidelberg, Germany) at 35°C. Fresh grown IM08B colonies were picked with a cotton swab and were suspended in PAS (equivalent to a 4.5 McFarland turbidity standard). This suspension was pipetted onto the surface of non-nutrient agar plates, and was spread out

using an L-shaped spreader.

In a second step, 2 x10^4 cysts were mixed with 100 µl PBS in absence and presence of one of the antiamoebic agents (0.02% PHMB, 0.02% CH, 0.1% HD, 0.1% PD, 0.1% DD, 5% NM, 0.0065% MF, 1% PVPI), and were incubated at 30°C for 2 hours in a Thermomixer (Eppendorf AG) with a shaking rate of 650 rpm. Thereafter, 10 µl of the cell suspensions was transferred into fresh tubes, was mixed with 10 µl trypan blue solution (0.4%) and was incubated for 5 minutes at room temperature. 980 µl PBS was subsequently added to each tube (the drugs were diluted 100 folds) and the cells were carefully suspended by pipetting gently up and down. Then, 50 µl of the stained cell suspensions (100 cells) was pipetted into the middle of the non-nutrient agar *E. coli* plates, and the suspension was desiccated for around 10 minutes. As a next step, circles were drawn on the bottom of the plates to confine the borders of the *Acanthamoeba* cyst solution, and bright field images of these regions were taken. *A. castellanii* inoculated plates were incubated upside up for 24 hours at 30°C.

Thereafter, plates were sealed by parafilm (Pechiney, Menasha, USA) and were incubated upside down for up to 5 weeks at 30°C.

As a negative control, cysts were incubated in 100 µl PBS (without antiamoebic agents) for 2 hours. As a positive control, cysts were incubated in lysoform (Rossmann GmbH, Berlin, Germany) for 2 hours.

Bright field images were taken with a Leica DMI4000 B microscope every 24 hours until 72 hours, then every week until the end of the 5th week, using a 10-fold magnification. These experiments (with each antiamoebic agent) were repeated five times on different days.

2.4.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.02, by one- or two-way ANOVA tests, followed by Dunnett's or Sidak's multiple comparisons tests. $P \leq 0.05$ was considered as statistically significant.

One-way ANOVA test was used to make a comparison between groups with different antiamoeabic agents. Then, the Dunnett's multiple comparison test was used to compare each group (3 different antiamoebic agent concentrations for each drug) to controls. For Ce6-PDT, the two-way ANOVA test was used, to compare groups with and without illumination, then the Didak's multiple comparisons test was applied to compare groups with 3 different concentrations.

3 RESULTS

3.1 Histological analysis of *Acanthamoeba* keratitis eye globes

Images of the histological analysis are shown at **figures 4**.

There was no central corneal epithelium on the analysed globes. We could not observe *Acanthamoeba* trophozoites or cysts neither in the cornea (**Figures 4 A and B**) nor in other ocular tissues. There was one corneal endothelial cell per field of view (original magnification 40x) analyzing the first and no corneal endothelial cells examining the second case.

There were anterior synechiae in the chamber angle of both cases and lymphocytic infiltration around the central retinal artery and vein, associated with fibrous metaplasia of the retinal pigment epithelium (**Figures 4 C-E**).

Additionally, we observed perivascular inflammatory cell infiltration (mainly lymphocytes) in the episclera and around ciliary nerves, analysing the first case (**Figure 4A**). This was associated with non-granulomatous uveitis, cilioschisis and tractional retinal detachment. Cross sections of the optic nerve revealed gliosis and optic nerve atrophy.

Histopathologic studies of the second case revealed a multifocal, non-granulomatous choroiditis with lymphocytic infiltration (**Figure 4E**).

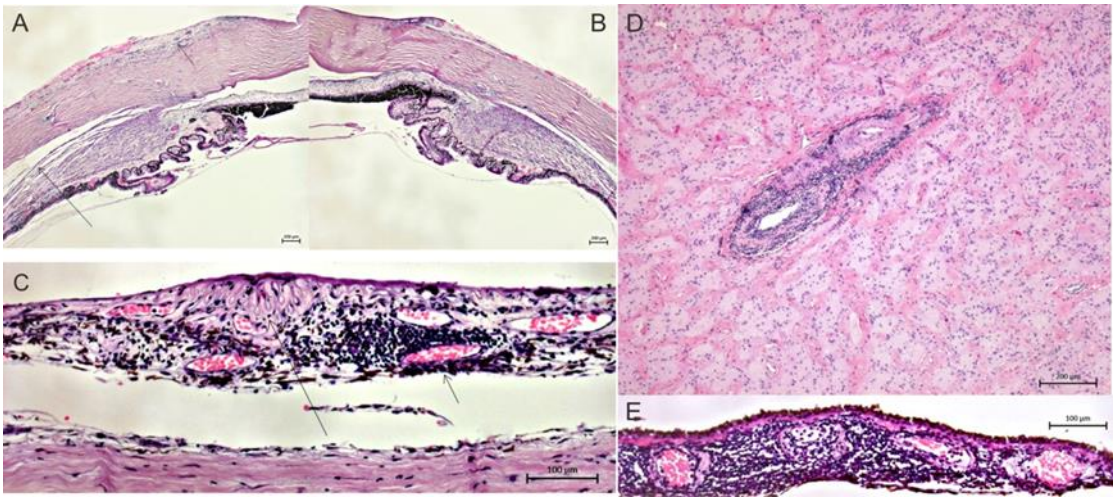


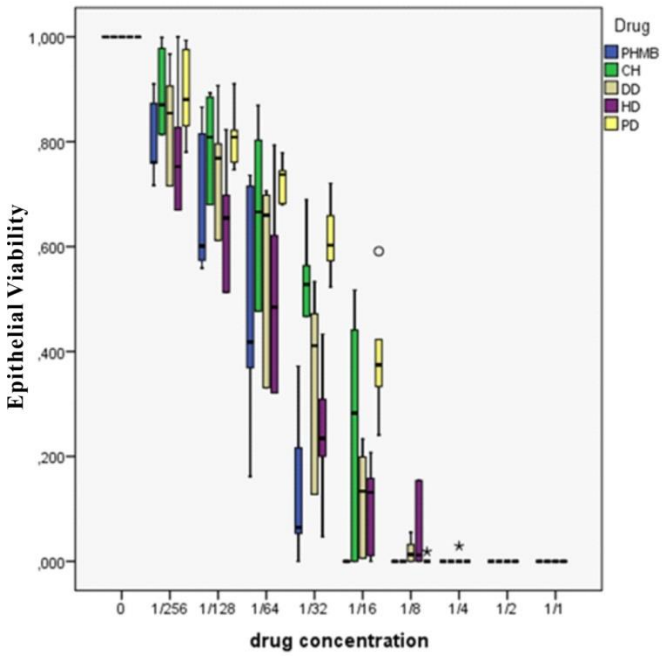
Figure 4. Histological images of both cases. In the first case (A and B, haematoxylin-eosin), *Acanthamoeba* trophozoites or cysts were not detectable in the corneal or other ocular tissues, but lymphocytic infiltration of the episclera (arrow) and choroidal detachment (long arrow) were shown. In the first case (C, haematoxylin-eosin), perivascular lymphocytic infiltration (arrow) and retinal atrophy (long-arrow) and perivascular lympocytic infiltration around central retinal artery

was detectable (D, haematoxylin-eosin). In the second case, there was lymphocytic infiltration of the choroid (E, haematoxylin-eosin).

3.2 Human corneal cell cuture experiments

A part of this dissertation (chapters 2.2, 3.2 and 4.2 as much as Summary/Zusammenfassung on human cells) have been published in "*Shi L, Stachon T, Seitz B, Wagenpfeil S, Langenbucher A, Szentmáry N. The effect of antiamoebic agents on viability, proliferation and migration of human epithelial cells, keratocytes and endothelial cells. Curr Eye Res 2018; 43: 725-33.*". To use data of this publication for this doctoral dissertation was approved by *Current Eye Research.*

Epithelial cell, *keratocyte* and *endothelial* cell viability, proliferation and migration using different concentrations of PHMB, CH, DD, HD and PD are displayed at **figures 5-7**. Viability and proliferation data are displayed using boxplots (including median, 25% and 75% quartile as well as 95% confidence interval) grouped by concentration and drug, and migration data are displayed grouped by time interval and drug.



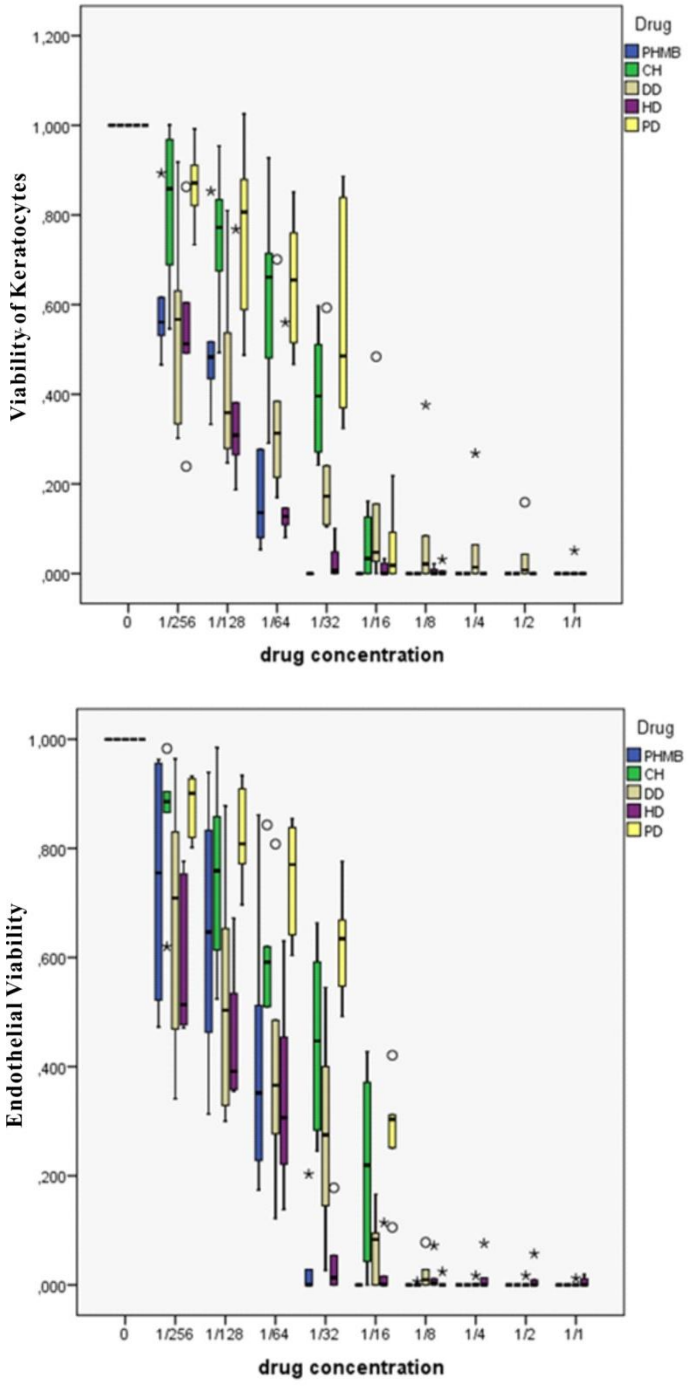
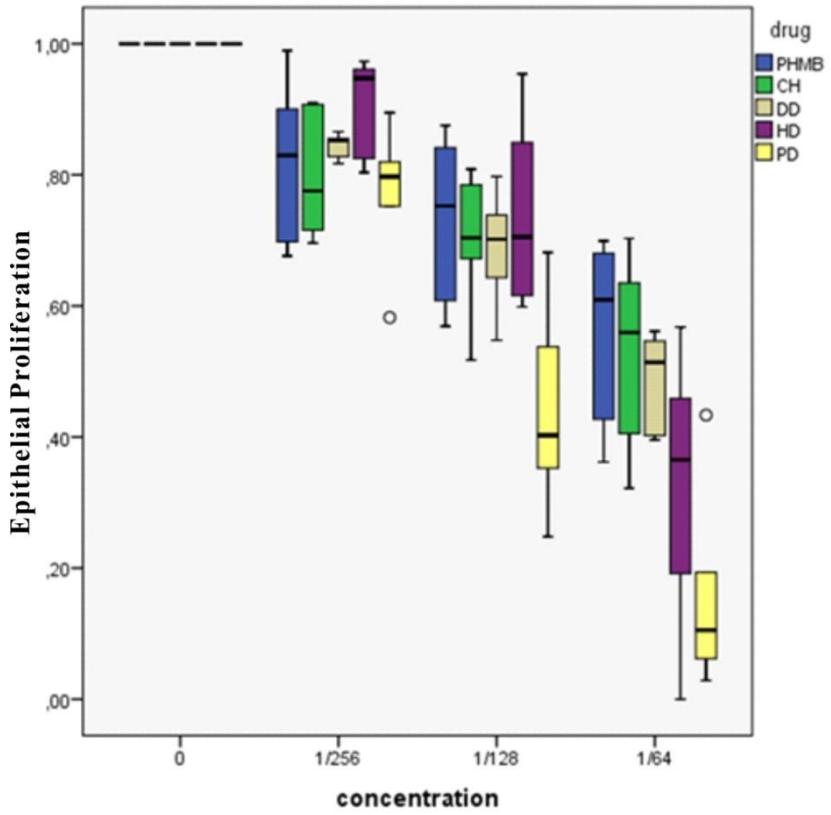
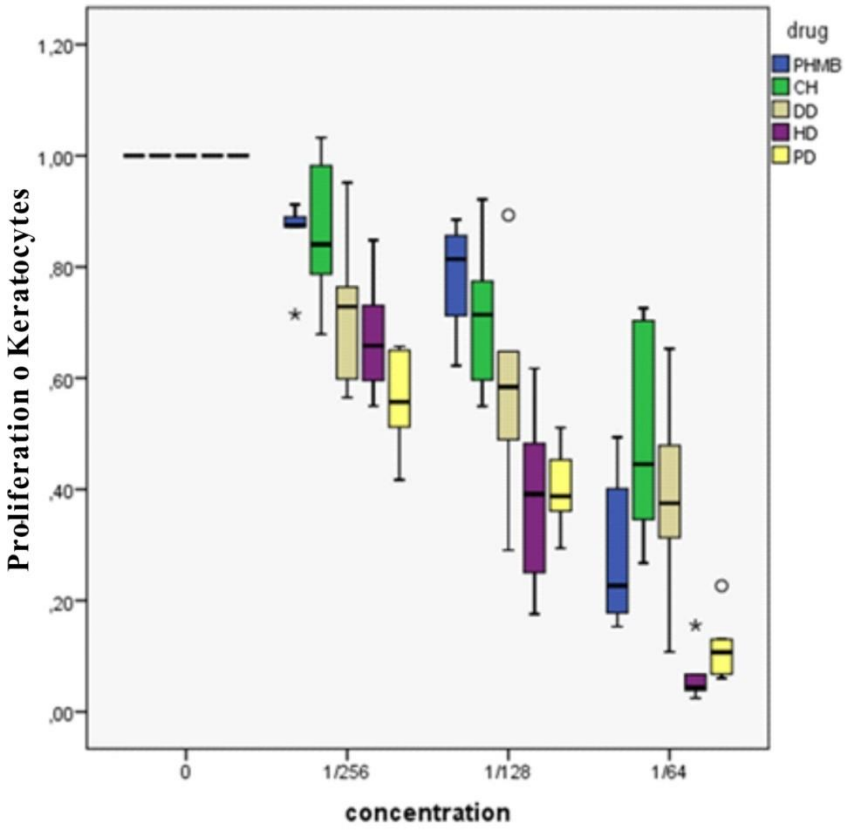


Figure 5. *Human corneal epithelial cell (HCEC), keratocyte and human corneal endothelial cell (HCEC-12)* **viability**, using different dibromopropamidine-isethionat (DD), propamidin isethionat (PD), hexamidine-diisethionat (HD), polyhexamethylen biguanid (PHMB) and chlorhexidine (CH) concentrations. 100% represents viability of controls. Data are displayed with boxplots (including median, 25% and 75% quartile as well as 95% confidence interval) grouped by concentration and drug. The maximal concentrations used were 0.1% for HD and DD, and 0.02% for PHMB and CH. The maximal concentration for PD was 0.0125% (1/8 of 0.1% Brolene® eye drop concentration, as the eye drops had to be diluted with culture medium). Thereafter, all solutions underwent a serial two fold-dilution.


drug
PHMB
CH
DD
HD
PD
Epithelial Proliferation
1,00
,80
,60
,40
,20
,00
0
1/256
1/128
1/64
concentration


drug
PHMB
CH
DD
HD
PD
Proliferation o Keratocytes
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1,00
,80
,60
,40
,20
,00
0
1/256
1/128
1/64
concentration

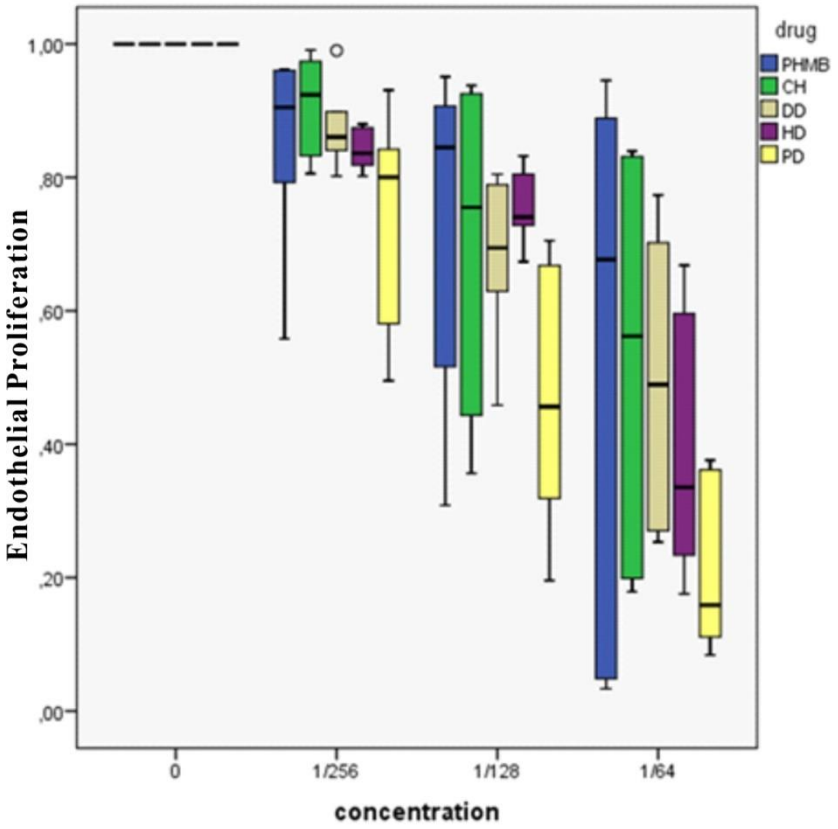
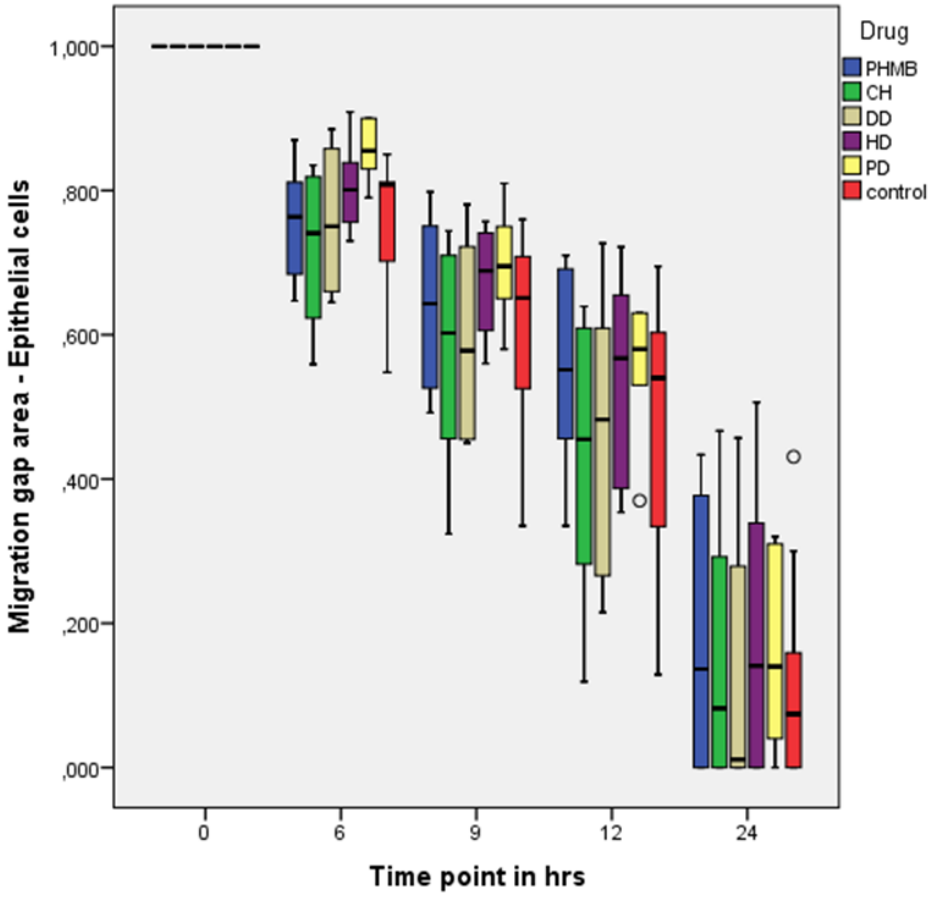
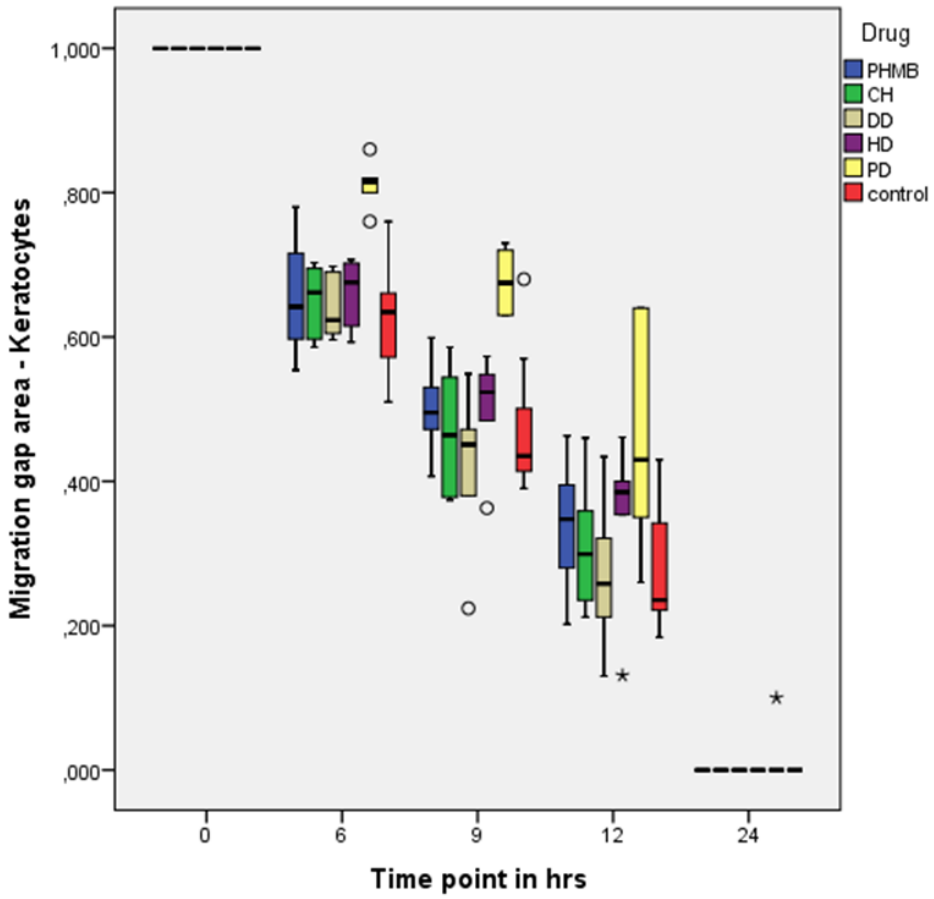


Figure 6. *Human corneal epithelial cell (HCEC), keratocyte and human corneal endothelial cell (HCEC-12)* **proliferation**, using different dibromopropamidine-isethionat (DD), propamidin isethionat (PD), hexamidine-diisethionat (HD), polyhexamethylen biguanid (PHMB) and chlorhexidine (CH) concentrations. 100% represents proliferation of controls. Data are displayed with boxplots (including median, 25% and 75% quartile as well as 95% confidence interval) grouped by concentration and drug. The maximal concentrations used were 0.1% for HD and DD, and 0.02% for PHMB and CH. The maximal concentration for PD was 0.0125% (1/8 of 0.1% Brolene® eye drop concentration, as the eye drops had to be diluted with culture medium). Thereafter, all solutions underwent a serial two fold-dilution.


Drug
PHMB
CH
DD
HD
PD
control
Migration gap area - Epithelial cells
1,000
,800
,600
,400
,200
,000
0
6
9
12
24
Time point in hrs


Drug
PHMB
CH
DD
HD
PD
control
Migration gap area - Keratocytes
1,000
,800
,600
,400
,200
,000
0
6
9
12
24
Time point in hrs

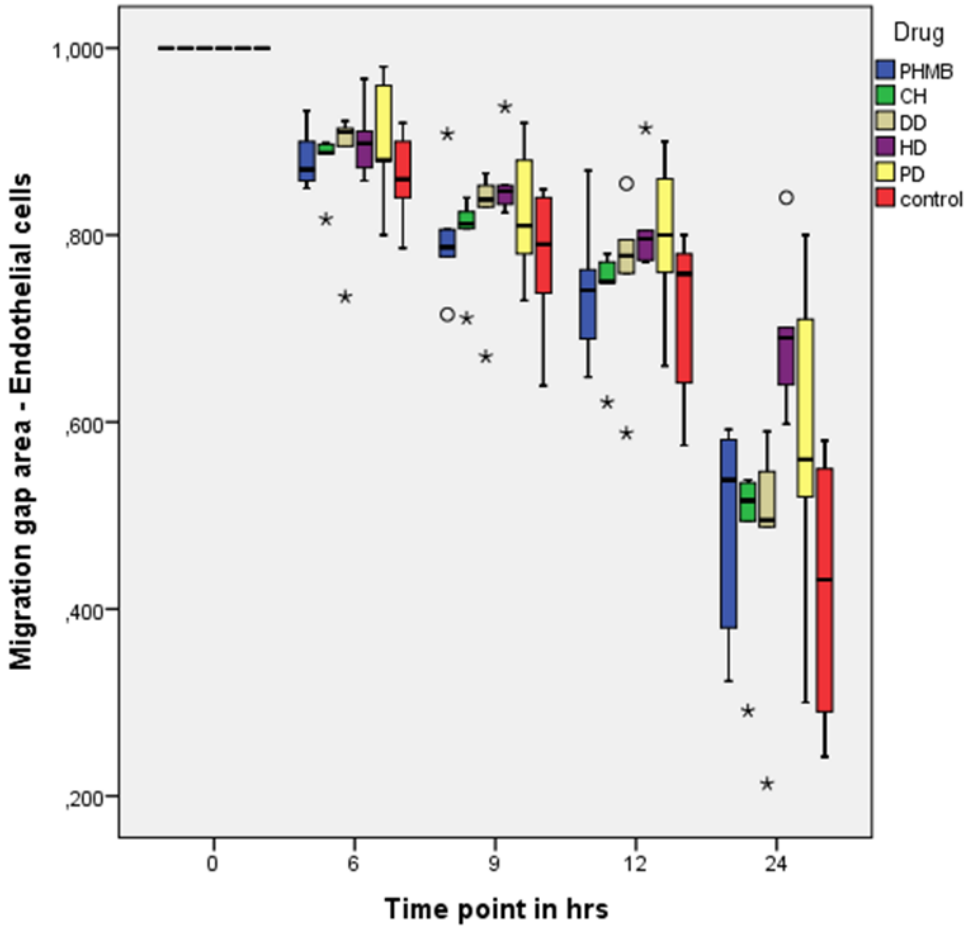


Figure 7. Migration of *epithelial cells (HCEC) and endothelial cells* (HCEC-12) in 24 hours using $3.9x10^{-4}$ % (1/256) dibromopropamidine-isethionat (DD), $3.9x10^{-4}$ % (1/256) propamidin isethionat (PD), $3.9x10^{-4}$ % (1/256) hexamidine-diisethionat (HD), $7.8x10^{-5}$ % (1/256) polyhexamethylen biguanid (PHMB) and $7.8x10^{-5}$ % (1/256) chlorhexidine (CH) concentrations and migration of *keratocytes* in 24 hours using $7.8x10^{-4}$ % (1/128) DD, $7.8x10^{-4}$ % (1/128) PD, $7.8x10^{-4}$ % (1/128) HD, $1.6x10^{-4}$ % (1/128) PHMB and $1.6x10^{-4}$ % (1/128) CH concentrations. The area of the gap is displayed. Data are displayed with boxplots (including median, 25% and 75% quartile as well as 95% confidence interval) grouped by time interval and drug. Cell cultures without drugs were used as controls.

Images of the gap (migration assay) using HCEC, keratocytes and HCEC-12 at different timepoints (0, 6, 9, 12 and 24 hours following scratch by yellow pipette tips) in control cell cultures are shown in **figure 8**.

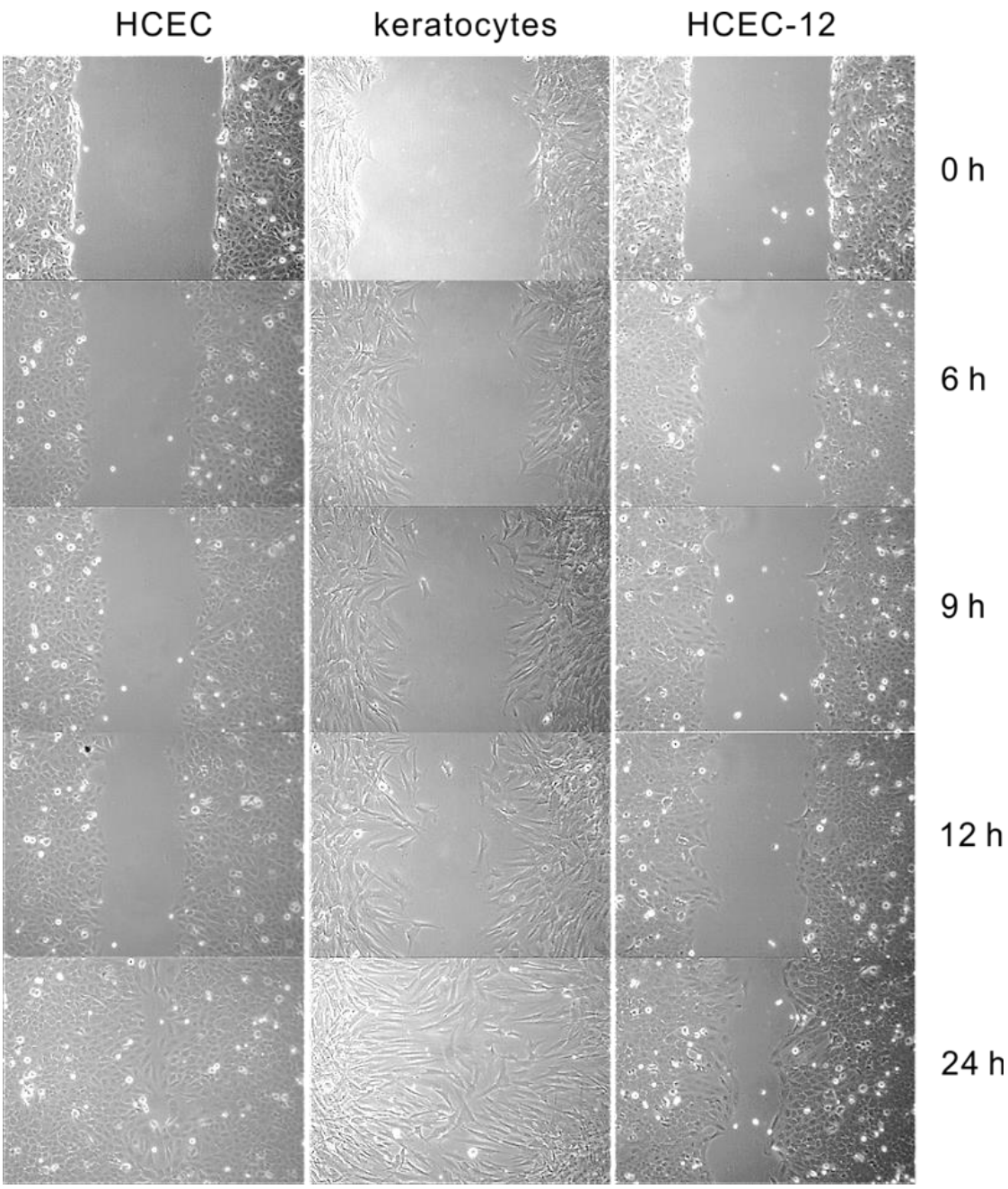


Figure 8. Images of the gap are shown at different timepoints (0, 6, 9, 12 and 24 hours following scratching by yellow pipette tips) in control cell cultures. The area of the gap was calculated as a percentage, compared to the size of the area directly after scratching. Different cell types have shown different cell growth speed.

Using the Kruskal-Wallis test, all diamidines and biguanides had a significant overall effect on HCEC, keratocyte and HCEC-12 ***viability*** (p=0.001; p=0.001; p=0.003).

The "area under the curve" (AUC) using different anti-*Acanthamoeba* drugs is displayed in **figure 9.**

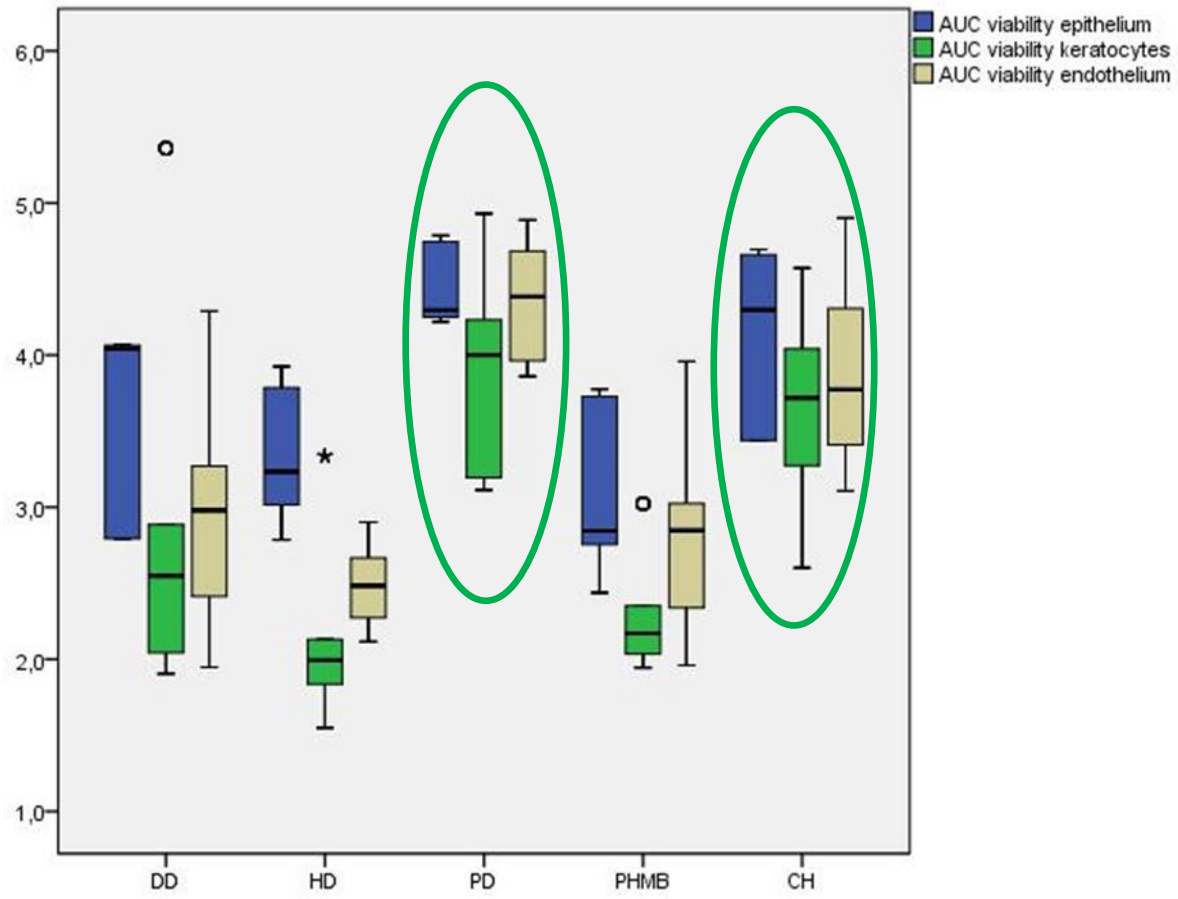


Figure 9. Epithelial cell (HCEC), keratocyte and endothelial cell (HCEC-12) ***viability*** "area under curve" (AUC) for hexamidine diisethionat (HD), propamidin isethionate (PD), dibromopropamidine diisethionat (DD), polyhexamethylen biguanid (PHMB) and chlorhexidine (CH). CH and PD, with the highest viability AUC for all cell types are marked with green circle.

Using a Mann-Whitney test, there was a significant difference in viability AUC of HCEC, keratocytes and HCEC-12 comparing PD and PHMB ($p \leq 0.014$ for all; PD better) or comparing PD and HD ($p \leq 0.011$ for all; PD better). There was a significant difference in viability AUC of keratocytes and HCEC-12 comparing CH and HD ($p \leq 0.027$; CH better), and in viability AUC of HCEC-12 comparing PD and HD ($p = 0.005$; PD better) and viability AUC of HCEC differed significantly comparing CH and PHMB ($p = 0.014$; CH better) (**Table 3.**)

		PHMB	CH	DD	HD
CH	HCEC	***0.014***			
	Keratocyte	0.057			
	HCEC-12	0.054			
DD	HCEC	0.686	1.0		
	Keratocyte	1.0	1.0		
	HCEC-12	1.00	0.151		
HD	HCEC	1.0	0.123	1.0	
	Keratocyte	1.0	***0.027***	1.0	
	HCEC-12	1.00	***0.004***	1.0	
PD	HCEC	***0.001***	1.0	0.131	***0.011***
	Keratocyte	***0.014***	1.0	0.322	***0.006***
	HCEC-12	***0.002***	1.0	***0.005***	***<0.001***

Table 3. Analysis of epithelial cell (HCEC), keratocyte and endothelial cell (HCEC-12) ***viability*** „area under curve" (AUC) for hexamidine diisethionat (HD), propamidin isethionate (PD), dibromopropamidine diisethionat (DD), polyhexamethylen biguanid (PHMB), chlorhexidine (CH) with Mann-Whitney test. Significant p values are bold. In case of statistically significant difference in viability AUC, the drug with better (higher) viability AUC is marked in green.

Using the Kruskal-Wallis test, all diamidines and biguanides had a significant overall effect on HCEC and keratocyte ***proliferation*** (p=0.022; p=0.001), but they had no significant overall effect on HCEC-12 proliferation (p=0.167). Therefore, a non-parametric Mann-Whitney U-test was used to compare proliferation AUC values between pairs of drugs for HCEC and keratocytes, but not for HCEC-12.

The "area under the curve" (AUC) for HCEC and keratocytes using different anti-*Acanthamoeba* drugs is displayed in **figure 10.** Using a Mann-Whitney test, there was a significant difference in proliferation AUC of HCEC, comparing PD with PHMB, CH, DD, HD (mean 2.36 vs 3.11, 3.02, 3.02, 2.97; PD worse for all) ($p \leq 0.016$ for all).

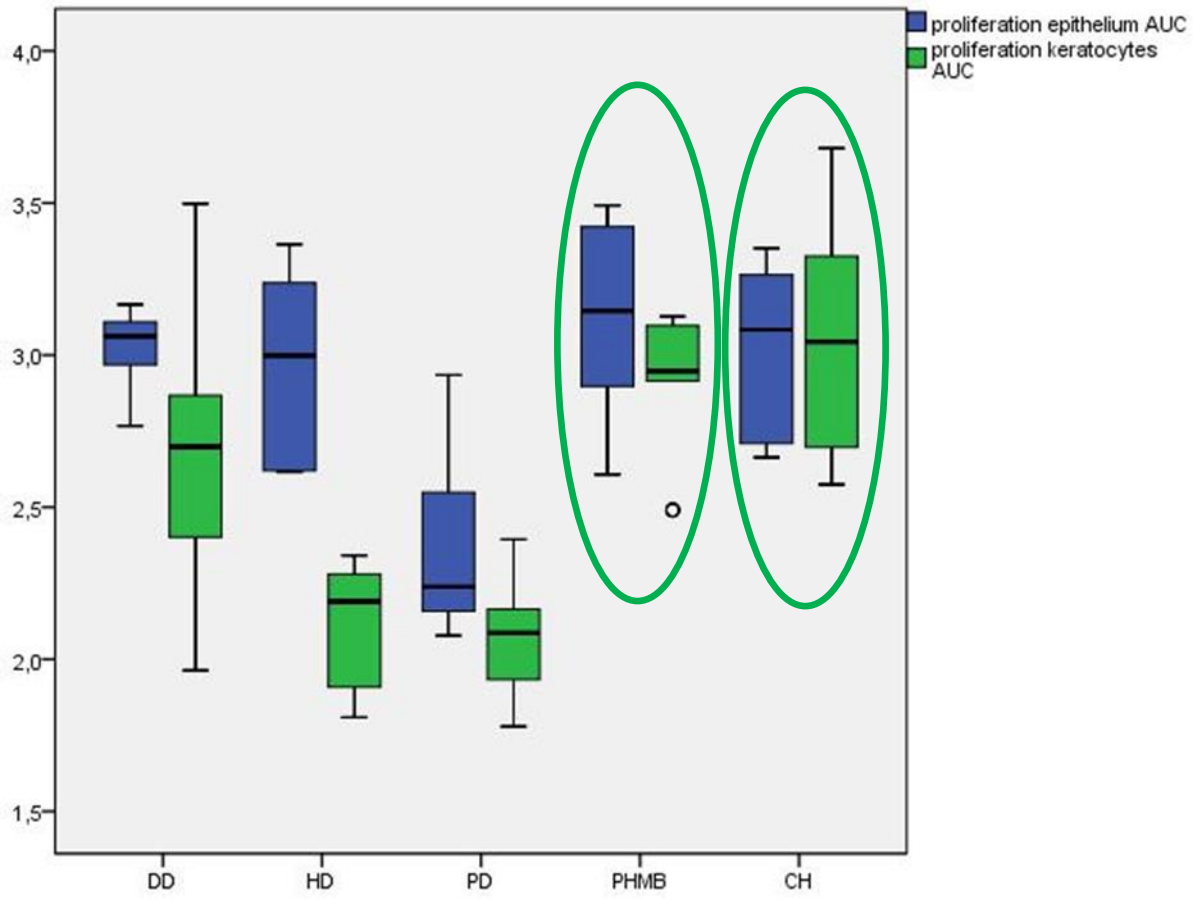


Figure 10. Epithelial cell (HCEC) and keratocyte ***proliferation*** "area under curve" (AUC) for hexamidine diisethionat (HD), propamidin isethionate (PD), dibromopropamidine diisethionat (DD), polyhexamethylen biguanid (PHMB) and chlorhexidine (CH). PHMB and CH with the highest proliferation AUC for both cell types are marked with green circle.

Using a Mann-Whitney test, there was a significant difference in proliferation AUC of keratocytes, comparing PHMB with HD, PD (mean 2.92 vs 2.12, 2.07; PHMB better for both) (p=0.004; p=0.002), comparing CH with HD, PD (mean 3.06 vs 2.12, 2.07; CH better for both) (p≤0.001 for both) and comparing DD with PD (mean 2.68 vs 2.07; DD better) (p=0.043) (**Table 4**).

		PHMB	CH	DD	HD
CH	HCEC	1.0			
	Keratocyte	1.0			
DD	HCEC	1.0	1.0		
	Keratocyte	1.0	0.684		
HD	HCEC	1.0	1.0	1.0	
	Keratocyte	***0.004***	***0.001***	0.076	
PD	HCEC	***0.002***	***0.007***	***0.008***	***0.016***
	Keratocyte	***0.002***	***<0.001***	***0.043***	1.0

Table 4. Analysis of epithelial cell (HCEC) and keratocyte ***proliferation*** „area under curve" (AUC) for hexamidine diisethionat (HD), propamidin isethionate (PD), dibromopropamidine diisethionat (DD), polyhexamethylen biguanid (PHMB), chlorhexidine (CH) with Mann-Whitney test. Significant p values are bold. In case of statistically significant difference in proliferation AUC, the drug with better (higher) proliferation AUC is marked in green.

Using the Kruskal-Wallis test, all diamidines and biguanides had a significant overall effect on keratocyte ***migration*** (p=0.006), but they had no significant overall effect on HCEC and HCEC-12 migration (p=0.657; p=0.178). Therefore, a non-parametric Mann-Whitney U-test was used to compare migration AUC values between pairs of drugs for keratocytes, but not for HCEC and HCEC-12.

The "area under the curve" (AUC) using different antiamoebic drugs is displayed in **figure 11.**

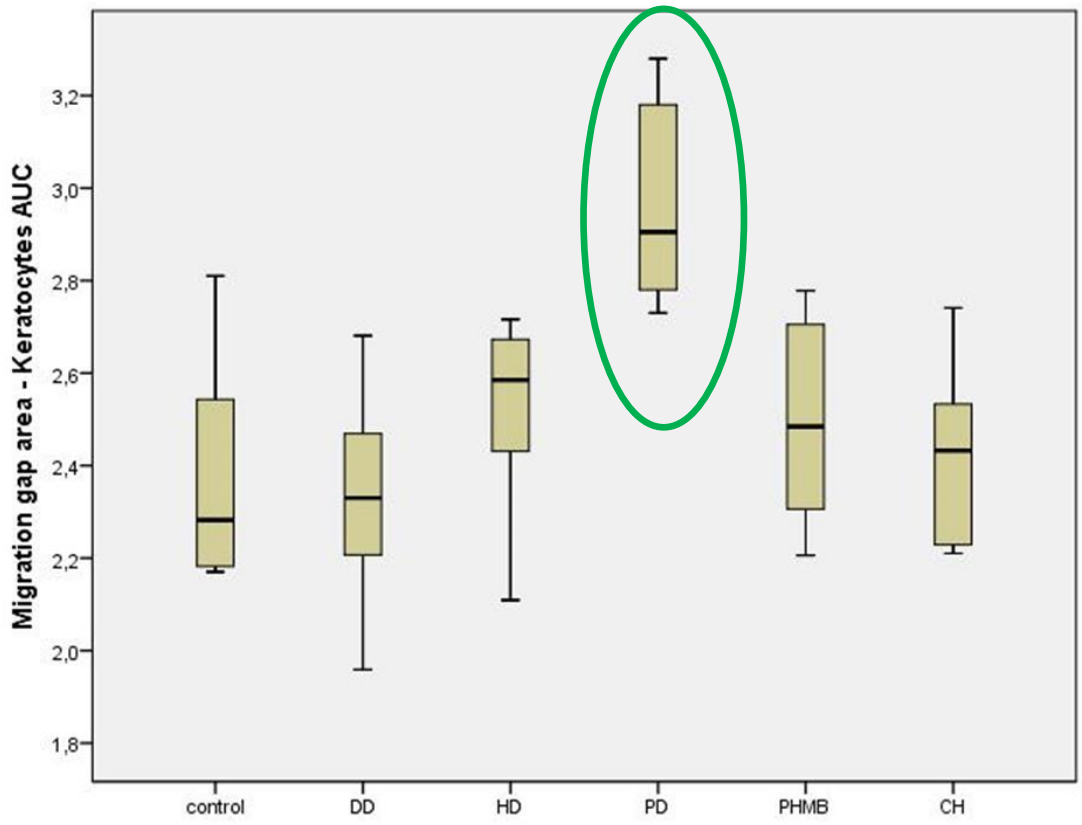


Figure 11. Keratocyte ***migration*** "area under curve" (AUC) for controls, hexamidine diisethionat (HD), propamidin isethionate (PD), dibromopropamidine diisethionat (DD), polyhexamethylen biguanid (PHMB) and chlorhexidine (CH). PD, the highest migration AUC (slower migration) is marked with green circle.

Using a Mann-Whitney test, there was a significant difference in migration AUC of keratocytes comparing PD with control, PHMB, CH, DD and HD (mean 2.93 vs 2.37, 2.49, 2.43, 2.32, 2.51; PD worse for all) ($p \leq 0.012$). However, no significant difference was noted when comparing keratocyte migration AUC of other pairs of drugs ($p=1.0$ for all) (**Table 5**).

		PHMB	CH	DD	HD	PD
CH	Keratocyte	1.0				
DD	Keratocyte	1.0	1.0			
HD	Keratocyte	1.0	1.0	1.0		
PD	Keratocyte	***0.012***	***0.003***	***<0.001***	***0.020***	
Control	Keratocytes	1.0	1.0	1.0	1.0	***<0.001***

Table 5. Analysis of keratocyte ***migration*** „area under curve" (AUC) for control, hexamidine diisethionat (HD), propamidin isethionate (PD), dibromopropamidine diisethionat (DD), polyhexamethylen biguanid (PHMB), chlorhexidine (CH) with Mann-Whitney test. Significant p values are bold. In case of statistically significant difference in migration AUC, the drug with better (lower) migration AUC is marked in green.

3.3 Comparison of assays to study the effectiveness of antiparasitics against *Acanthamoeba castellanii* trophozoites and cysts

3.3.1 LDH release assay

In order to confirm the antiamoebic potentials of the drugs PHMB (0.02%), PD (0.1%), and MF (0.0065%) on *Acanthamoeba* trophozoites [Narasimhan S; Kowalski RP; Sunada A], a series of LDH release assays were carried out (**Figure 12)**. All antiamoebic agents induced a significant release of LDH into the supernatants of the drug-challenged trophozoite cultures when compared to the unchallenged control group ($p<0.0001$), in line with previous studies reporting that all three drugs exhibited a significant cytotoxicity on *Acanthamoeba* trophozoites [Siddiqui R 2016]. Based on the LDH release values, MF was identified as the most effective drug of this test panel causing a significantly higher LDH release than PD ($p=0.0011$) and PHMB ($p<0.0001$). PD was significantly more effective in releasing LDH than PHMB ($p=0.0004$).

As the lysis buffer supplied by the CytoTox 96® Non-Radioactive kit could not lyse 1BU cysts, we could not set a maximum LDH release control for these and we could only perform LDH assay for 1 BU trophozoites.

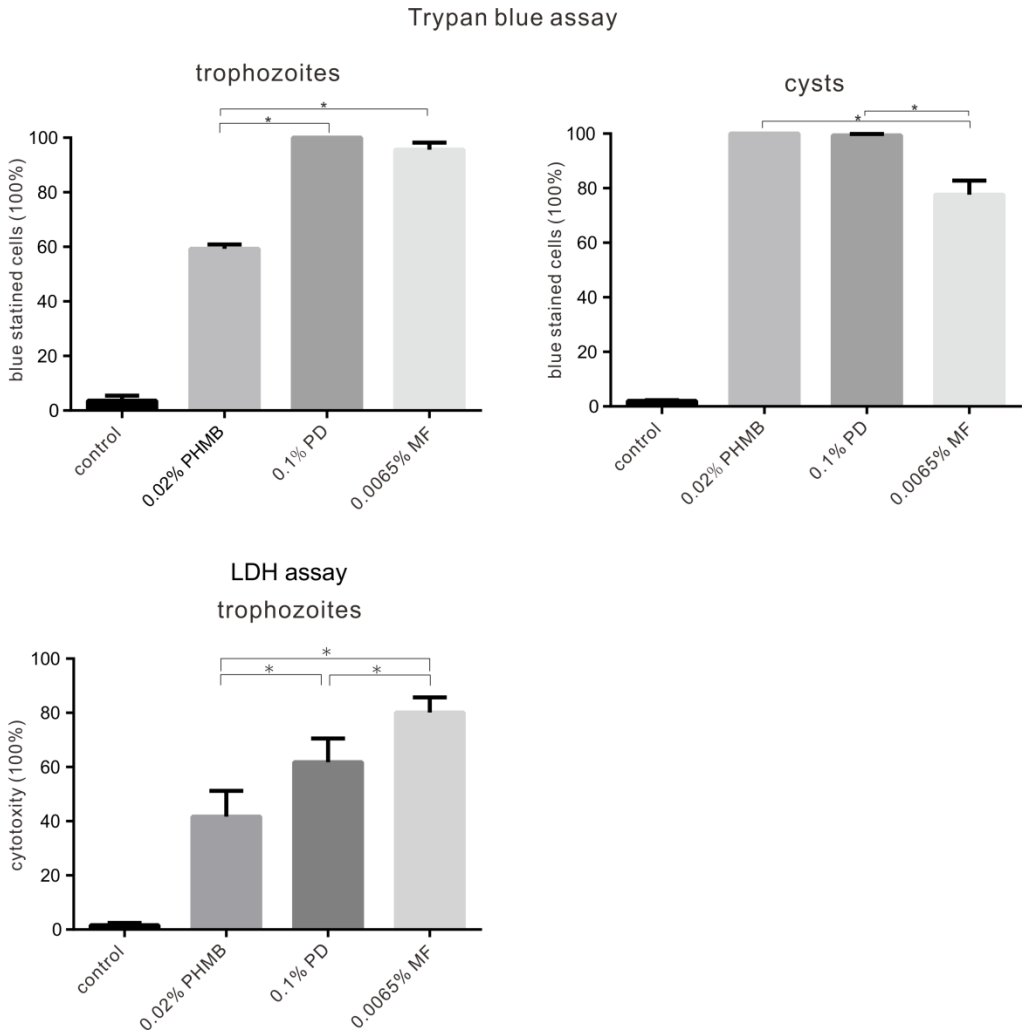


Figure 12. Cytotoxic effect of 0.02% PHMB, 0.1% PD and 0.0065% MF, on 1BU trophozoites and cysts, using ***trypan blue*** assay (n=3) and on 1BU trophozoites, using ***LDH assay*** (n=5).

Trypan blue assay: 0.02% PHMB, 0.1% PD and 0.0065% MF had a significant cytotoxic effect on 1BU trophozoites and cysts ($p<0.0001$). 0.1% PD and 0.0065% MF had a significantly higher cytotoxic effect on 1BU trophozoites, than 0.02% PHMB ($p<0.0001$). There was no significant difference between 0.1% PD and 0.0065% MF cytotoxicity for 1 BU trophozoites ($P=0.0608$).

The cytoxic effect on 1BU cysts was significantly less using 0.0065% MF, than 0.02% PHMB or 0.1% PD ($P<0.0001$). However, there was no significant difference between cytotoxic effect of 0.02% PHMB and 0.1% PD, observing 1BU cysts ($P=0.9883$)

LDH assay: 0.0065% MF was significantly more cytotoxic than 0.1% PD ($p=0.0011$) and 0.02% PHMB ($p<0.0001$), and 0.1% PD was significantly more cytotoxic than 0.02% PHMB ($p=0.0004$), observing 1BU trophozoites.

3.3.2 Trypan blue assay

The effects of PHMB, PD and MF on the viability of 1BU cells were next determined with a trypan blue exclusion assay [Strober W], which monitors the integrity of cell membranes (**Figure 12**, and the corresponding phase-contrast images of **Figures 13 and 14**). In untreated

1BU trophozoites and cysts (controls) we observed only a small proportion of cells that displayed a relevant blue staining. Following trypan blue staining, about 3.7 ± 1.8% of the drug-free trophozoites and 2.1 ± 0.2% of the cysts displayed a clear blue signal. Challenging 1BU cells with PHMB clearly increased the ratios of blue stained cells to 59.3 ± 1.5% (trophozoites) and 100% (cysts), respectively. This effect was even stronger when 1BU cells were challenged with PD and MF, respectively. The latter drug increased the ratios of blue stained cells to 100% (trophozoites) and 77.7 ±5.1% (cysts), while almost all PD-treated 1BU trophozoites/cysts became trypan blue positive (**Table 6**). When drug-challenged cysts were screened for blue staining intensities, some differences between drugs were noticed. Challenging cysts with PD yielded a highly homogeneous trypan blue staining pattern with all cysts displaying mean grey values >95%. However, when the cysts were treated with MF and PHMB, respectively, a more heterogeneous staining pattern was observed. MF and PHMB produced small proportions of blue stained cysts that showed lower grey values (3.45% with 85%-90%, 6.90% with 90%-95%, and 89.65% with >95% mean grey value for PHMB, and 9.1% with 90-95%, and 90.9% with >95% mean grey value for MF).

Table 6. Percentage of ***trypan blue, Hoechest 33342, calcein-AM and ethidium homodimer-1*** positive 1BU trophozoites and cysts, with and without antiamoebic agent treatment. Hoechest 33342, calcein-AM and ethidium homodimer did not stain untreated trophozoites and cysts

	Trophozoites				**Cysts**			
	control	**0.02% PHMB**	**0.1% PD**	**0.0065% MF**	**control**	**0.02% PHMB**	**0.1% PD**	**0.0065% MF**
Trypan blue	3.66 ± 1.83%	59.33 ± 1.53%	100%	95.67 ± 2.52%	2.06 ± 0.24%	100%	99.33 ± 0.58%	77.67 ± 5.13%
Hoechest 33342	0%	100%	100%	100%	0%	100%	100%	100%
Calcein-AM	0%	75.33 ± 4.04%	9.67 ± 2.52%	0%	0%	60.67 ± 3.51%	36.67 ± 4.51%	0%
Ethidium homodimer	0%	100%	100%	100%	0%	100%	100%	100%

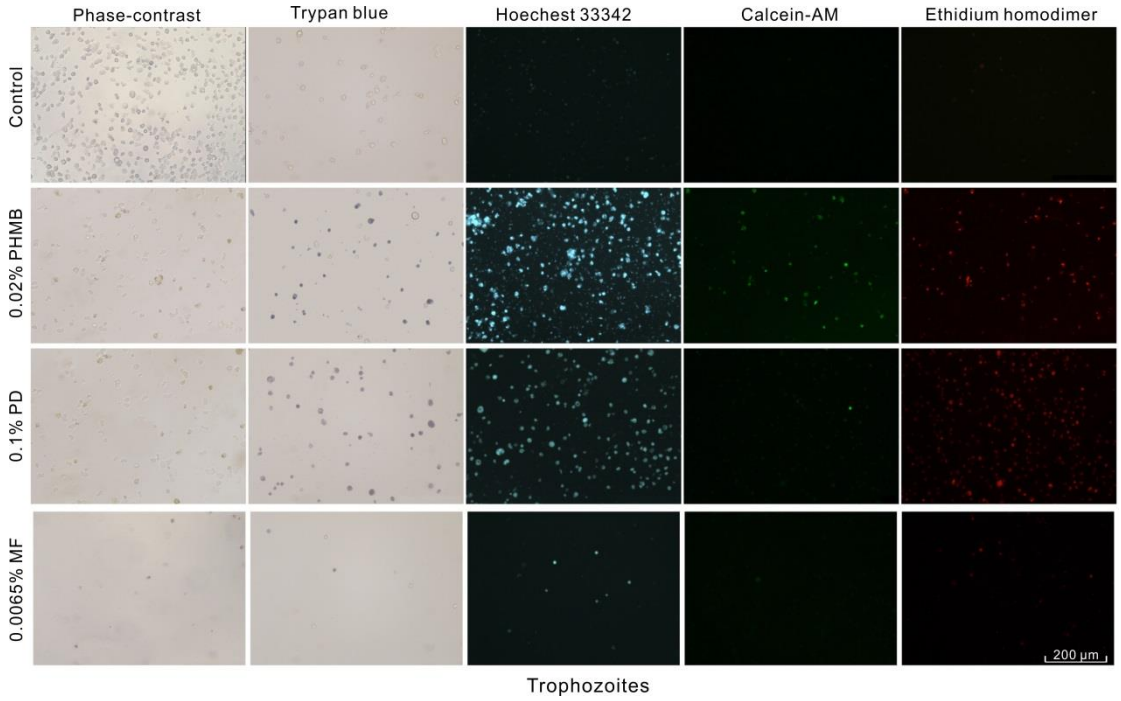


Figure 13. ***Trypan blue, Hoechest 33342, calcein-AM and ethidium homodimer-1 staining*** of 1BU trophozoites, with and without antiamoebic agent treatment. Hoechest 33342, calcein-AM and ethidium homodimer-1 did not stain untreated trophozoites.

Trypan blue stained 3.6%, Hoechest 33342 100%, ethidium homodimer-1 100%, calcein-AM 75.33% of 0.02% PHMB treated trophozoites. Following 0.1% PD treatment, Hoechst 33342 and ethidium homodimer-1 were 100%, trypan blue 59.33%, calcein-AM 9.67% positive in trophozoites. 0.0065% MF exposure resulted in 100% Hoechst 33342 and 100% ethidium homodimer-1, 95.67% trypan blue and in 0% calcein-AM positivity of trophozoites.

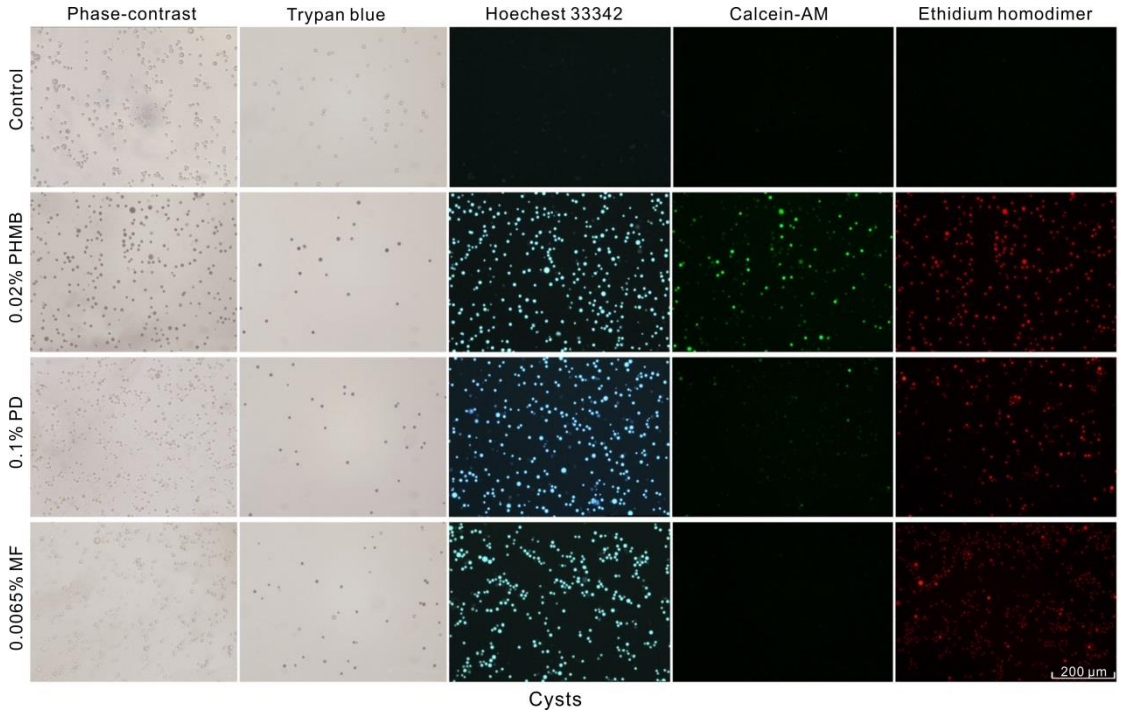


Figure 14. ***Trypan blue, Hoechest 33342, calcein-AM and ethidium homodimer-1 staining*** of

1BU cysts, with and without antiamoebic agent tretament. Hoechest 33342, calcein-AM and ethidium homodimer-1 did not stain untreated cysts.

Trypan blue stained 100%, Hoechest 33342 100%, ethidium homodimer-1 100%, calcein-AM 60.67% of 0.02% PHMB treated cysts. Following 0.1% PD treatment, Hoechst 33342 and ethidium homodimer-1 were 100%, trypan blue 99.33%, calcein-AM 36.67% positive in cysts. 0.0065% MF exposure resulted in 77.67% trypan blue, 100% Hoechst 33342 and 100% ethidium homodimer-1 and in 0% calcein-AM positivity of cysts.

3.3.3 Fluorescent staining

Drug treated trophozoites and cysts and healthy controls were mixed either with Hoechst 33342 or a combination of calcein-AM and ethidium homodimer-1, and fluorescence images were taken as outlined in Materials and Methods. Representative fluorescence images of Hoechst 33342 and calcein-AM/ethidium homodimer-1 treated cells are displayed in Figures 12 and 13, respectively, and percentages of Hoechst 33342, calcein-AM and ethidium homodimer-1 positive trophozoites/cysts are given in (**Table 6)**. Notably, neither Hoechst 33342 nor the calcein-AM/ethidium homodimer-1 mixture stained drug-free trophozoites and cysts. However, this changed when 1BU cells were challenged with the antiamoebic drugs. Hoechst 33342 and ethidium homodimer-1 stained 100%/100% of the PHMB treated 1BU cells blue and red, respectively, and calcein-AM stained 75.3±4.0%/60.7±3.5% of the PHMB challenged trophozoites/cysts green. Following PD treatment, Hoechst 33342 and ethidium homodimer-1 were 100%/100% positive in trophozoites/cysts, and calcein-AM stained 9.7±2.5%/36.7±4.5% of the PD treated trophozoites/cysts green. Similarly, MF exposure yielded in 100%/100% Hoechst 33342 and ethidium homodimer-1 stained trophozoites/cysts. However, none of the MF treated 1BU trophozoites/cysts emitted relevant amounts of green fluorescence upon exposure to light of the wavelength 490 nm. 75.3±4.0%/60.7±3.5% of the PHMB challenged trophozoites/cysts were positive for both ethidium homodimer-1 and calcein. Similar results were observed with PD treated 1BU cells, which were in 9.7±2.5% (trophozoites) and 36.7±4.5% (cysts) positive for both calcein and ethidium homodimer-1.

3.3.4 Non-nutrient agar *E.coli* plate assay

In drug free controls, 1BU trophozoites appeared within 24 hours including central and peripheral areas of the agar plate. After 72 hours, a considerable amount of trophozoites could be seen (**Figure 15**). After 5 weeks of cultivation, a number of cysts appeared in central and peripheral parts of the agar plate in controls. Notably, a similar kinetic was observed with MF treated trophozoites (**Figure 15**). Although MF can diminish the trophozoite amount (**Figure 13**) and result in the highest amount of LDH release (**Figure 12**), the surviving cells are still viable.

However, when PHMB and PD treated 1BU *trophozoites* were spotted onto the non-nutrient agar *E. coli* plates, respectively, and monitored for growth and encystment, a more cohesive picture to those seen with the afore mentioned assays emerged (**Figure 15**). Small, strange shaped structures appeared on central and peripheral parts of the agar plate after 72 hours of cultivation (**Figure 16**). Nevertheless, after 5 weeks of cultivation, no fresh cysts were observed at central or peripheral parts of the agar plate.

When PHMB and PD treated *trophozoites* were monitored after 5 weeks of growth on non-nutrient agar *E. coli* plates, no fresh cysts were found at the central or peripheral parts of the agar plates (**Figure 15**).

When drug treated *cysts* were tested with the non-nutrient agar *E. coli* assay, the following observations were made (**Figure 16**): in the control and the MF treated 1BU cyst groups, trophozoites became visible on central and peripheral parts of the agar plate after 24 hours of cultivation (**Figure 16**).

A different picture emerged, when PHMB and PD treated *cysts* were tested with this assay (**Figure 16**). In both drug treated groups, irregularly shaped, smaller trophozoites with a thinner cell wall appeared after 72 hours in the central and peripheral parts of the agar plates, which did not form cysts again within the 5 weeks of monitoring.

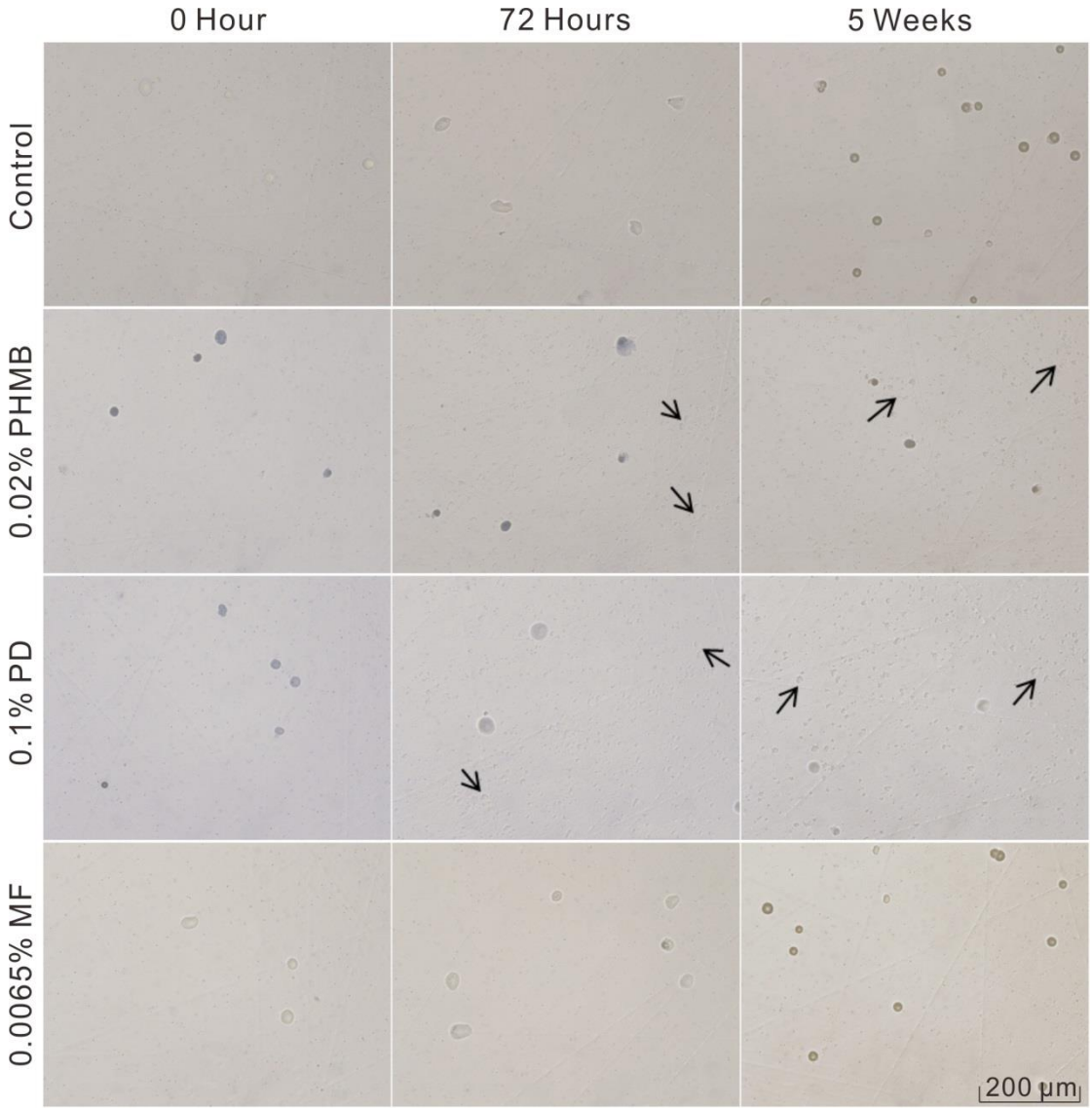


Figure 15. ***Phase-contrast images of 1BU <u>trophozoites</u> on non-nutrient agar E. coli plates***, 0 and 72 hours and 5 weeks following 0.02% PHMB, 0.1% PD and 0.0065% MF treatment and trypan blue staining. In controls and in 0.0065% MF treated 1BU trophozoites groups, new trophozoites appeared outside than marked circle after 72 hours, and encystment could be observed following 5 weeks. In 0.02% PHMB and 0.1% PD treated 1BU trophozoites groups, strange shaped trophozoites appeared after 72 hours (arrows), which did not form cysts following 5 weeks. The strange trophozoites were smaller and thinner compared to the normal trophozoites

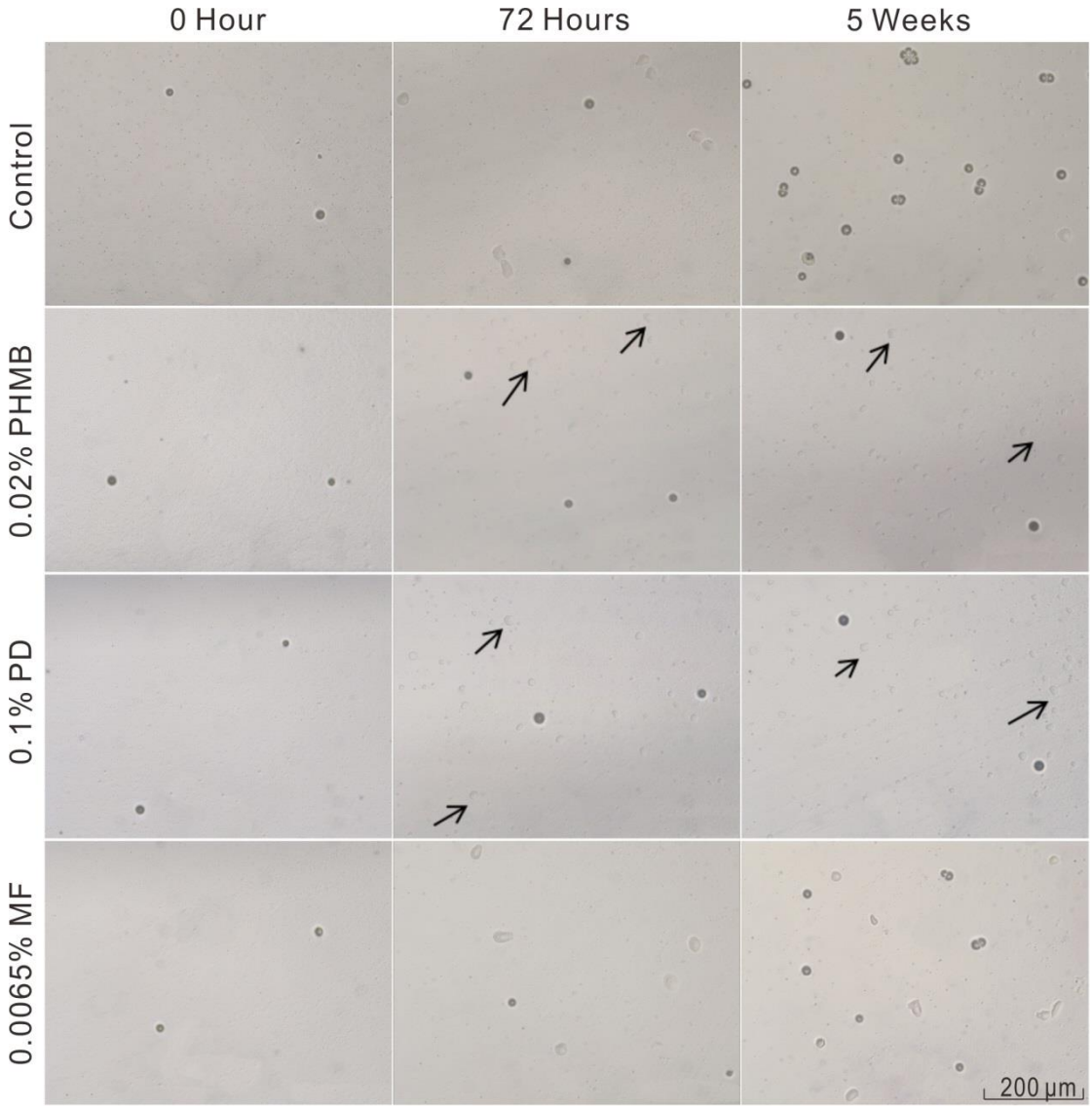


Figure 16. ***Phase-contrast images of 1BU cysts on non-nutrient agar E. coli plates***, 0 and 72 hours and 5 weeks following 0.02% PHMB, 0.1% PD and 0.0065% MF treatment and trypan blue staining. In controls and in 0.0065% MF treated 1BU cyst groups, trophozoites appeared after 72 hours, and encystment could be observed following 5 weeks. In 0.02% PHMB and 0.1% PD treated 1BU cyst groups, strange shaped trophozoites appeared after 72 hours (arrows, see also **Figure 17**), which did not form cysts following 5 weeks. The strange trophozoites were smaller and thinner compared to the normal trophozoites.

After 5 weeks of incubation, the appearance of roundish but flat cells with a discontinuous wall (sometimes double-wall) was noticed in the PD and PHMB treated cyst groups (**Figure 17**). The amount of these irregularly shaped structures increased over time and was also observed in the central and peripheral parts of the agar plates.

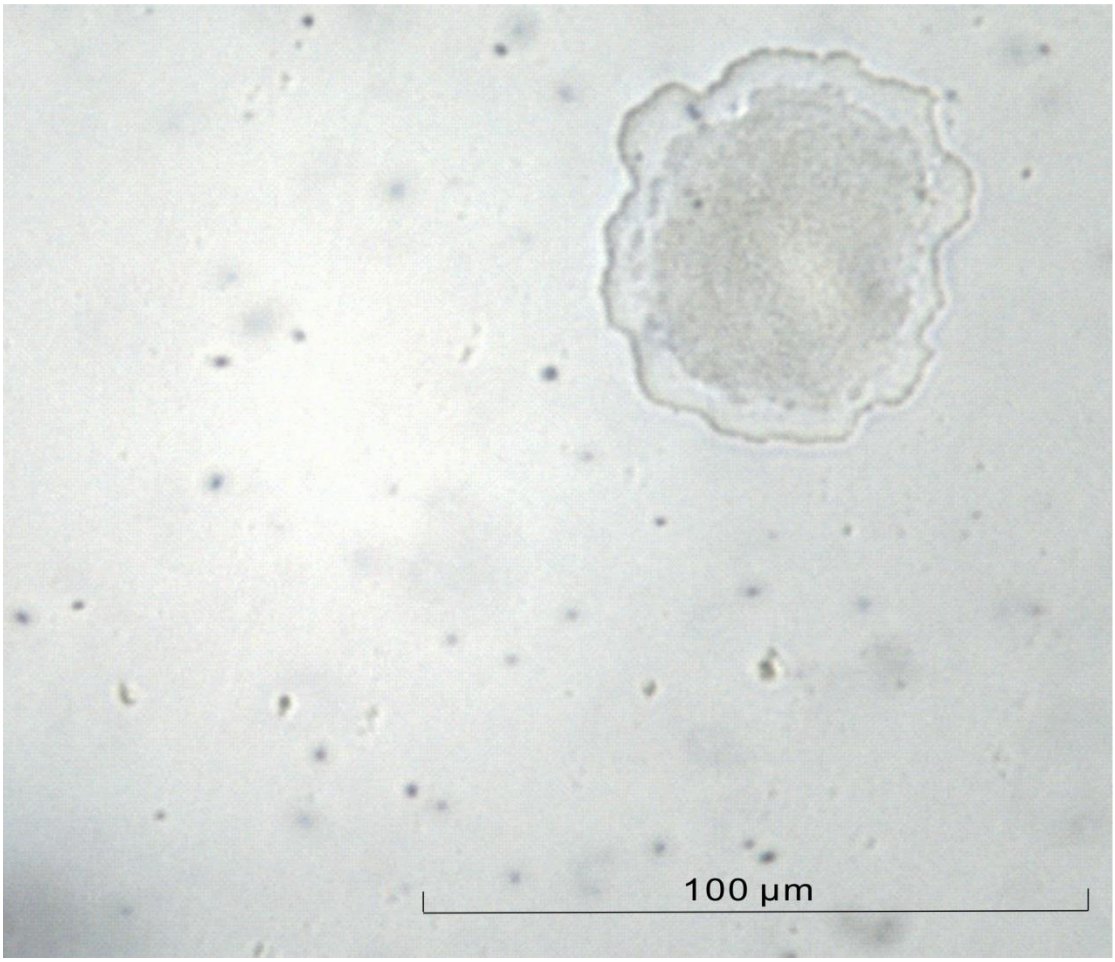


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Figure 17. In 0.02% PHMB and 0.1% PD treated 1BU cyst groups, strange shaped structures appeared after 72 hours, which did not form cysts again following 5 weeks. The strange trophozoites were smaller and thinner compared to the normal trophozoites.

3.4 Efficacy of antiamoebic agents and Ce6-PDT against *Acanthamoeba castellanii* 1-BU

3.4.1 LDH assay

Results of the LDH assay for *trophozoites* (n=5) are displayed in **figure 18.**

PHMB, CH, HD, PD, DD, NM, MF, and chlorin e6-PDT increased LDH activity significantly in trophozoite cultures ($P<0.01$), compared to controls.

The use of Ce6 without illumination did not change LDH activity ($P=0.96$), compared to Ce6 with illumination. There was no LDH activity difference between groups with different Ce6 concentration, but without illumination ($P=0.09$). Illumination alone did not increase LDH activity significantly ($P=0.96$), compared to controls.

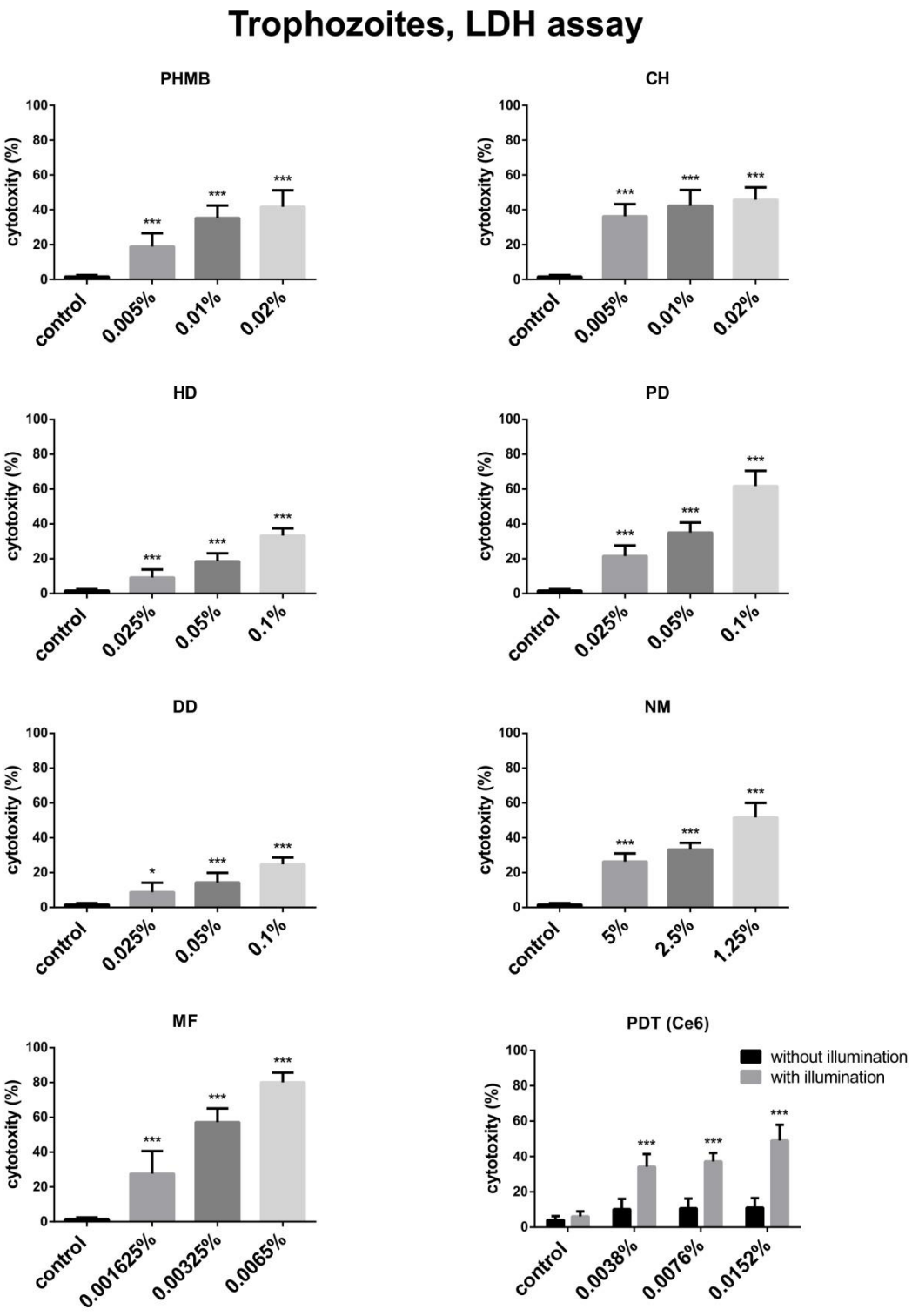

Figure 18. Cytotoxic effect (mean ± standard error) of polyhexamethylen biguanid (PHMB), chlorhexidine (CH), hexamidine-diisethionat (HD), propamidin-isethionate (PD), dibromopropamidine-diisethionat (DD), natamycin (NM), miltefosine (MF), and chlorin e6 photodynamic therapy (PDT) on 1BU trophozoites (***LDH assay;*** n=5).

PHMB, CH, HD, PD, DD, NM, MF, and chlorin e6-PDT increased LDH activity significantly in

trophozoite cultures (P<0.01), compared to controls. The use of Ce6 without illumination did not change LDH activity (P=0.96), compared to Ce6 with illumination. („*”: P≤0.05; „**”: P≤0.011; „***”: P≤0.005)

3.4.2 Trypan blue assay

Cytotoxicy of different agents, using trypan blue assay is shown in **figures 19 and 20.**

All used drugs increased percentage of trypan blue stained 1BU *trophozoites* significantly (P<0.01), compared to controls. Concerning different drug concentrations, all increased cytotoxicity on 1BU trophozoites significantly (P<0.01) compared to controls, except 0.005 % PHMB (P=0.59), 0.005 % CH (P= 0.10) and Ce6 without illuminaton (P=0.99). Illumination alone (P=0.99) or the use of different Ce6 concentrations without illumination (P=0.09) did not increase trypan blue positivity, compared to controls.

All used drugs increased percentage of trypan blue stained 1BU *cysts* significantly (P<0.01), compared to controls. Concerning different drug concentrations, all increased cytotoxicity on 1BU cysts significantly (P<0.01) compared to controls, except 0.005 % CH (P=0.29), Ce6 without illumination (P=0.94), 0.00095% or 0.0038% Ce6 with illumination (P=0.14; P=0.06) and illumination alone (P=0.94).

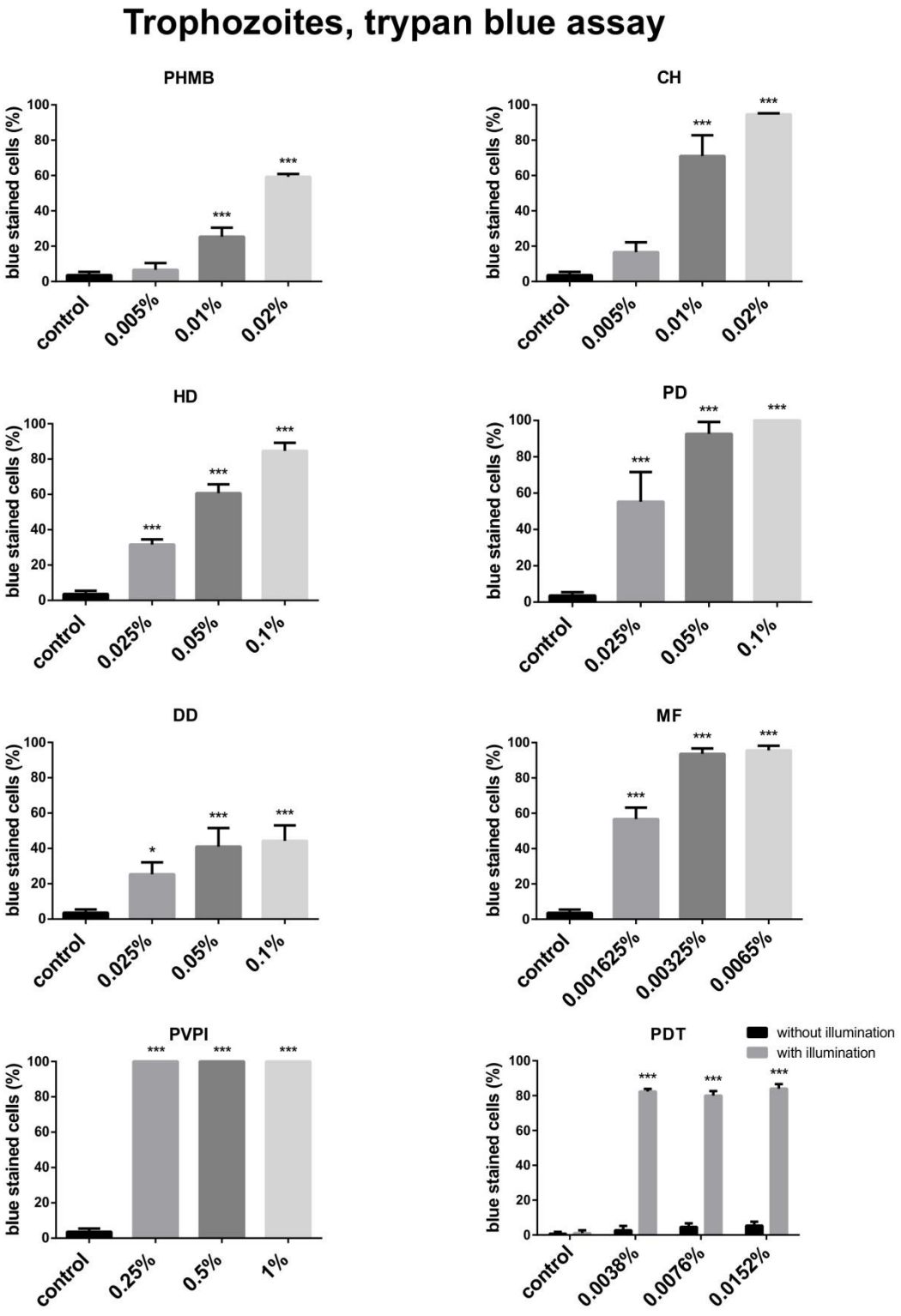

Figure 19. Cytotoxic effect (mean ± standard error) of polyhexamethylen biguanid (PHMB), chlorhexidine (CH), hexamidine-diisethionat (HD), propamidin-isethionate (PD), dibromopropamidine-diisethionat (DD), miltefosine (MF), povidone iodine (PVPI) and chlorin e6 photodynamic therapy (PDT) on 1BU trophozoites (***trypan blue*** assay; n=3).

All used drugs increased percentage of trypan blue stained 1BU *trophozoites* significantly

(P<0.01), compared to controls. Concerning different drug concentrations, all increased cytotoxicity on 1BU trophozoites significantly (P≤0.02) compared to controls, except 0.005 % PHMB (P=0.59), 0.005 % CH (P= 0.10) and Ce6 without illuminaton (P=0.99). („*": P≤0.05; „**": P≤0.011; „***": P≤0.005)

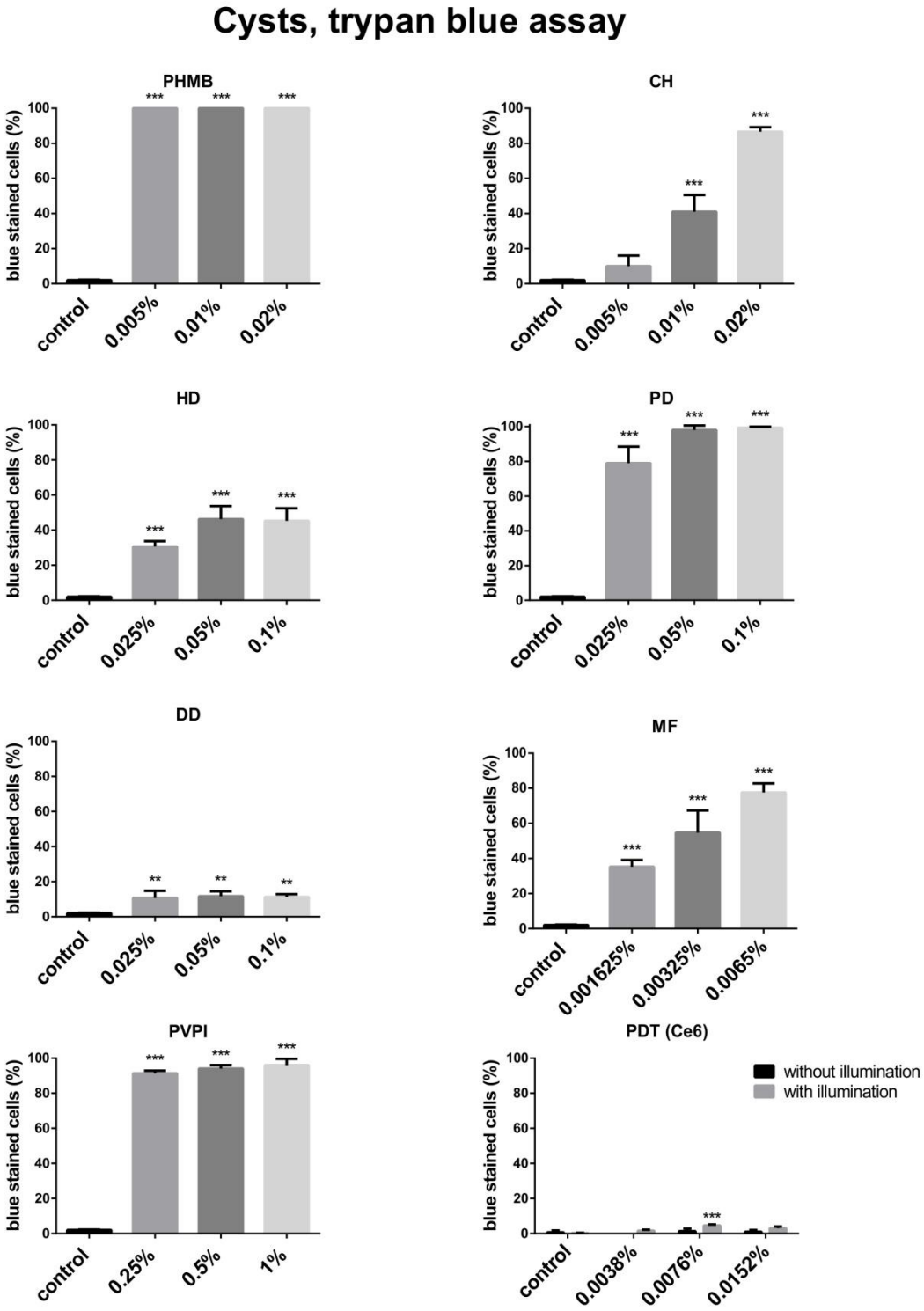

Figure 20. Cytotoxic effect (mean ± standard error) of polyhexamethylen biguanid (PHMB),

chlorhexidine (CH), hexamidine-diisethionat (HD), propamidin-isethionate (PD), dibromopropamidine-diisethionat (DD), miltefosine (MF), povidone iodine (PVPI) and chlorin e6 photodynamic therapy (PDT) on 1BU cysts (***trypan blue*** assay; n=3).

All used drugs increased percentage of trypan blue stained 1BU *cysts* significantly (P<0.01), compared to controls. Concerning different drug concentrations, all increased cytotoxicity on 1BU cysts significantly (P<0.01) compared to controls, except 0.005 % CH (P=0.29), Ce6 without illumination (P=0.94) and 0.00095% or 0.0038% Ce6 with illumination (P=0.14; P=0.06). („*” : P≤0.05; „**” : P≤0.011; „***” : P≤0.005).

3.4.3. Non-nutrient agar *E.coli* plate assay

Images of the non-nutrient agar *E. coli* plate at the center and periphery of the plates at three different time-points are displayed at **figure 21.**

Following excystment, in all plates, remnants of the original cysts remained observable at the same place during the complete follow-up.

In lysoform treated control group, no trophozoites or fresh cysts emerged during follow-up.

In control, 0,1% DD and 5% MF groups, fresh, normal-shaped trophozoites appeared after 24-72 hours and the trophozoites could move out from the center to the peripheral area of the plate. In these groups, encystment happened again after 1 week.

In 0.02% PHMB, 0.1% PD, and 1% PVPI groups, normal shaped trophozoites could not be observed after 24-72 hours (for all 5 repeat experiments). No fresh cysts appeared during follow-up in these groups.

In the 5% NM groups, NM was deposited on the plates like powder, which made differentiation of NM powder and acanthamoeba difficult under light-microscopy. However, in 0.1% HD and 5% NM groups, we observed the same phenomenon as for control, 0,1% DD and 5% MF groups (see above) in 1 of 5 repeat experiments. In the other 4 of 5 experiments, the same phenomenon as for 0.02% PHMB, 0.1% PD or 1% PVPI groups was observed (see above).

In 0.02% CH group, treated cysts behaved similarly to control, 0,1% DD and 5% MF groups (see above) in 2 of the 5 repeat experiments. In the other 3 of 5 experiments, the same phenomenon as for 0.02% PHMB, 0.1% PD or 1% PVPI groups (see above) was observed.

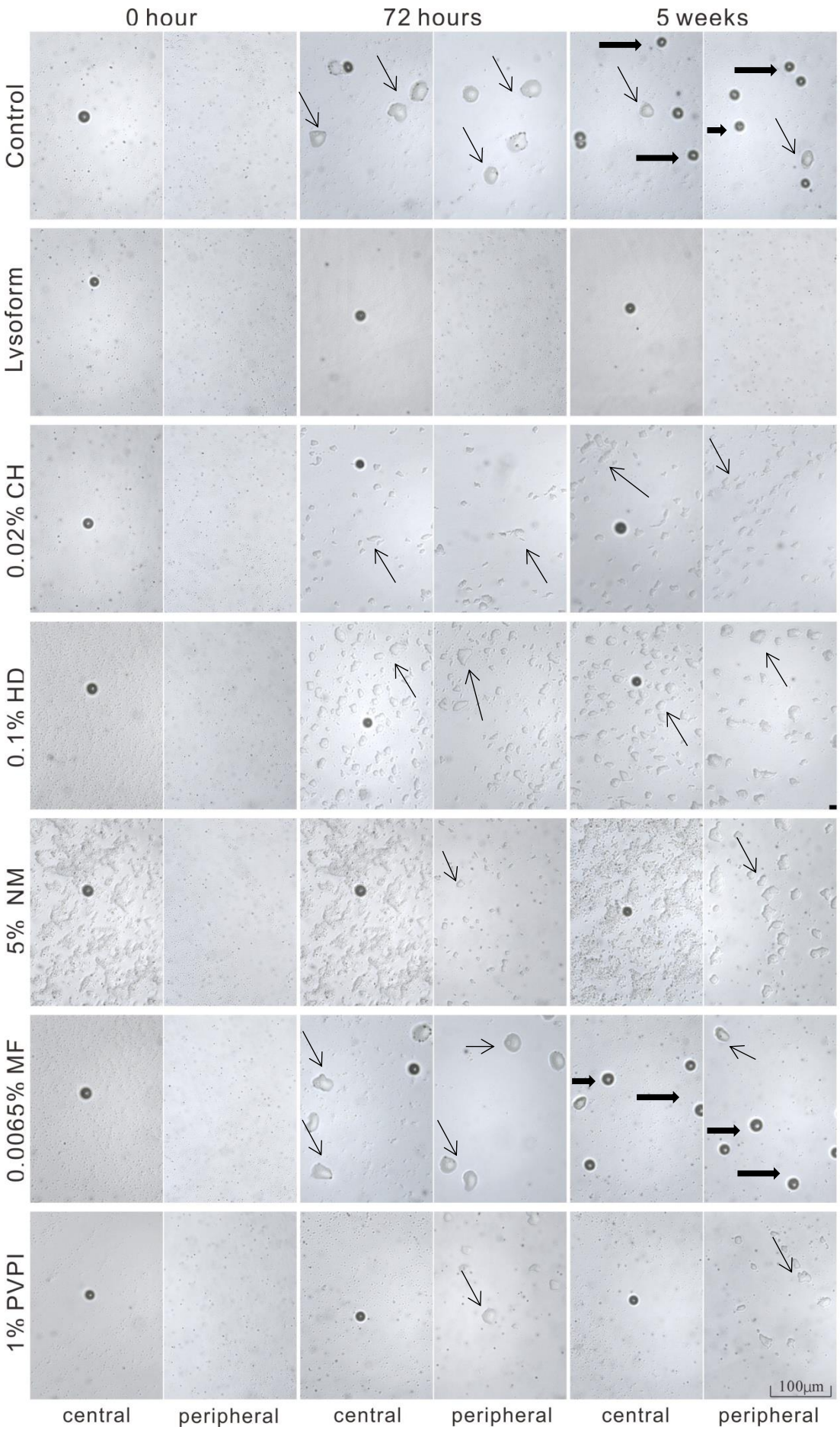
0 hour
72 hours
5 weeks
Control
Lvsoform
0.02% CH
0.1% HD
5% NM
0.0065% MF
1% PVPI
100μm
central
peripheral
central
peripheral
central
peripheral

Figure 21. Images of the non-nutrient agar *E. coli* plate assay (following inocuation of cysts), at the center and periphery of the plates at three different time-points.

In lysoform treated positive control group, no trophozoites or fresh cysts emerged during follow-up.

In control and 0.0065% MF groups, normal-shaphed fresh trophozoites appeared from 24 hours (see central and periphreal areas at 72 hours and 5 weeks, arrows) and the trophozoites could move out from the central area of the plate (see peripheral images at 72 hours and 5 weeks, arrows). In these groups, encystment happened again from 1 week (see central and peripheral images at 5 weeks, bold arrows).

In 0.02% CH, 0.1% HD and 1% PVPI groups, we observed strange-shaped structures after 24-72 hours**.** No fresh round cysts appeared during follow-up.

At the 5% NM images we also see the „natamycin-suspension", which made differentiation of NM and *acanthamoeba* difficult. There were no new cysts during 5 weeks (see peripheral images at 72 hours and 5 weeks). However, some spherical structures could be observed (arrows).

4 DISCUSSION

4.1 Histological analysis of *Acanthamoeba* keratitis eye globes

In 2007, Awwad et al. reported chronic chorioretinal inflammation with perivascular lymphocytic infiltration and diffuse neuroretinal ischemia as a new potentially blinding syndrome in 4 of 5 enucleated eyes after AK [Awwad ST]. In 4 of these patients, there were *Acanthamoeba* cysts in the cornea, nevertheless, the posterior segment of the eye failed to demonstrate *Acanthamoeba* cysts or throphozoites. Burke et al. had reported similar results in one patient in 1992 [Burke JP].

Most interestingly, we observed episcleritis, non-granulomatous uveitis with choroidal and central retinal artery/vein lymphocytic infiltration (vasculitis) and neuroretinal degeneration, without presence of *Acanthamoeba* throphozoites or cysts in the cornea or other ocular tissues, in two enucleated eyes of two patients.

Extracorneal invasion of *Acanthamoeba* had in the literature only been described in 8 patients between 1975 and 2013. In three of these cases, scleral invasion and in 5 others *Acanthamoeba* sclerokeratitis have been described. Iovieno et al. reported 18.5% occurrence of sclerokeratitis in their case series with presence of degraded necrotic cysts in scleral nodule biopsy of these patients [Iovieno A 2014]. They considered sclerokeratitis as a T-cell-mediated immune response, which requires systemic immunosuppression [McClellan K; Lee GA]. *Acanthamoeba* antigens elicit an immune response that leads to generation of T cell clones. These T cell clones then cross-react with antigens expressed in the normal eye, which may lead to the generation of additional T cell clones by a process called “epitope spreading” [Cornaby C].

We hypothesize that lymphocytes are more efficient than neutrophils and macrophages to chemo attract *Acanthamoeba*. But on the other hand, it can induce an immune response, which may also destroy other structures of the eye.

Lee has reported, that corneal antigen presenting cells can reside in the central cornea, migrate to the cervical lymph nodes and activate T-cells [Lee HS]. These T-cells then trigger an inflammatory reaction in the vascularized ocular tissues, such as uvea and retina. Interestingly, Johns at al. reported on chorioretinitis without vitritis in the contralateral eye of an immunocompetent AK patient, which might have been a regional immune-related inflammation, induced by local tissue infection through *Acanthamoeba* [Johns KJ 1988b].

There is another hypothesis that *Acanthamoeba* may induce a state of autoimmunity through

molecular mimicry via corneal antigen presenting cells or a type III immune reaction, which may target vascular receptors leading to vasculitis and thrombosis.

In our cases, there was retinal artery occlusion in one patient and retinal vein occlusion in the second patient, before enucleation. Histopathological examination found lymphocytic infiltration of these vessels. This may indicate a possible local immune-mediated vasculitis with secondary thrombosis and occlusion. We hypothesize that the peripheral vasculitis might be rather related to reactive inflammation than to the *Acanthamoeba* itself. This could have happened similarly in three patients reported by Awwad et al. [Awwad ST] and Burke at al. [Burke JP].

Interestingly, necrotizing vasculitis, leukocytoclastic vasculitis, thrombosis of small vessels and thrombo-occlusive vasculitis have also been described in systemic *Acanthamoeba*-related diseases, such as cutaneous *Acanthamoeba* infections and *Acanthamoeba* encephalitis.

There are only 4 case reports on *Acanthamoeba* in the posterior part of the eye. Jones et al. described a case in a 7-year-old boy with meningoencephalitis, with throphozoites in the ciliary body [Jones DB]. Heffler et al. reported on *Acanthamoeba* cysts in the aqueous humor and in the vitreous in a patient with acquired immune deficiency syndrome [Heffler KF]. In both patients, chrorioiditis and retinal vasculitis were present. Moshari et al. found *Acanthamoeba* cysts and throphozoites in the human retina, without chronic choroidal and retinal perivascular inflammation [Moshari A]. Mammo et al. report a recurrent *Acanthamoeba* infection presenting initially as keratitis, followed by retinitis and histolopathology-confirmed endophthalmitis [Mammo Z].

Interestingly, Clarke et al. showed that the clearance of the anterior chamber happens within 15 days following injection of *Acanthamoeba* trophozoites to the anterior chamber of hamster eyes [Clarke DW]. This was supported through a robust neutrophilic reaction in these eyes. This also supports the hypothesis, that choroid and retinal inflammation is rather immune-mediated and not related to the presence of *Acanthamoeba*. However, there might be a difference in human an animal immune response.

Previous studies have shown that polyhexamethilen-biguanide and propamidin-isethionat may be cytotoxic for human corneal cells in clinically relevant concentrations. [Shi L] It has also been suggested, that posterior segment inflammation may be related to toxicity of topical treatment used in AK. However, previous studies also reported AK patients with long-lasting topical treatment and absence of posterior pole inflammation, which contradicts this theory. Nevertheless, mature cataract formation in both patients could be related to toxicity of biguanides. These can then disrupt the lens surface, provoke lenticular oxidative or osmotic stress, and contribute to cataract formation by altering lipid membranes, damaging lens fibers, and inducing electrolyte imbalance [Ehlers N; Herz NL].

In our study, enucleation was performed at the end of patient histories with repeat (intraocular)

surgeries. The most conspicious finding of the histological analysis is that there were no trophozoites or cysts in both enucleated eyes. Although there were *Acanthamoeba* trophozoites and cysts in the explanted corneal buttons of PKPs and repeat PKPs previously, these were not persisting in corneal and ocular tissues subsequently. However, intraocular inflammation with central retinal artery/vein occlusion developed. Therefore, we hypothesize that *Acanthamoeba* or the long lasting triple-therapy trigged an immune response, which was persisting without microorganisms.

In long-standing, recalcitrant *Acanthamoeba* keratitis, uveitis, retinal vasculitis and scleritis may occur and result in blindness, even without further persistence of *Acanthamoeba* trophozoites or cysts. The etiology of these inflammatory complications is unclear, but may be explained with molecular mimicry or type III immune-reaction. Therefore, in late stage of *Acanthamoeba* keratitis, systemic immune suppression may be necessary for a longer period of time.

4.2 Human corneal cell cuture experiments

A part of this dissertation (chapters 2.2, 3.2 and 4.2 as much as Summary/Zusammenfassung on human cells) have been published in "*Shi L, Stachon T, Seitz B, Wagenpfeil S, Langenbucher A, Szentmáry N. The effect of antiamoebic agents on viability, proliferation and migration of human epithelial cells, keratocytes and endothelial cells. Curr Eye Res 2018; 43: 725-33.*". To use data of this publication for this doctoral dissertation was approved by *Current Eye Research.*

To the best of our knowledge, this is the first comparative study on the effect of diamidines and biguanides on human epithelial cells, keratocytes and endothelial cells. In *AK*, the cysts can reach close to the endothelium [Hager T]. Therefore, an effective drug must penetrate all corneal layers. To obtain information on the effect of diamidines and biguanides on corneal cells, cells of all corneal layers were analyzed in these experiments.

In our present study we have demonstrated that PD decreases human cell viability less than DD and HD, whereas CH decreases viability less than PHMB. Diamidines reduce proliferation of human epithelial cells and keratocytes more than biguanides, and propamidin isethionate reduces migration of keratocytes.

The genus *Acanthamoeba* is classified into 19 different genotypes including T1 to T19 based on rRNA gene sequence analysis. T4 genotypes are typically associated with human *AK*, but non-T4 genotypes have also been identified as pathogenic strains of AK [Iovieno A 2010; Arnalich-Montiel F]. However, correlation between agent effectiveness and *Acanthamoeba* genotype has not been verified until now [Sunada A; Wright P; Brasseur G]. Therefore, based on current literature, clinicians have no means to decide for or against the use of a specific diamidine or biguanide depending on *Acanthamoeba* genotype.

From the diamidine group, propamidine (Brolene®) was considered not to have an excellent cystcidal effect [Sunada A] and hexamidine (Hexacyl®) has higher amebicidal activity than propamidine [Perrine D]. However, as the percentage of trophozoites and cysts in the cornea is currently not objectively measurable, clinicians also cannot base their decision (preferred topical therapy) on an objectibe measure.

PHMB (Lavasept ®) is known to interact with membrane phospholipids, affecting membrane fluidity and conformation thus leading to ionic leakage and cell death. CH (Curasept®) is positively charged and ionizing with the negatively charged plasma membrane of the *Acanthamoeba*, resulting in structural and permeability changes, ionic leakage, and cytoplasmic disruption causing cellular damage and cell death of *Acanthamoeba*.

Comparing biguanides and diamidines clinically, literature on the *in vitro* cysticidal effect of biguanides and diamidines is controversial [Lee JE; Arnalich-Montiel F; Khunkitti W;

Kowalski RP; Lim N]. Some authors describe biguanides as having the most promising effect against *Acanthamoeba in vitro*, particularly against the highly resistant cyst form of the organism [Lee JE; Dart JK]. The use of PHMB and CH has been described as effective cysticidal monotherapy or combination therapy against different *Acanthamoeba* strains [Lee JE; Mafra CSP; Moon EK; Mathers W]. In contrast, Sunada *et al.* observed only a weak cysticidal effect of PHMB [Sunada A]. Some other reports have considered PHMB equivalent or more effective than CH against trophozoites and cysts of different *Acanthamoeba* species. Conversely, some studies report CH as more effective. [Narasimhan S; Turner NA]

Turner studied biocide susceptibility during the differentiation process of *Acanthamoeba castellanii* and observed that the time of emerging resistance during encystment varies for different biocides [Turner NA]. Resistance to minimal amoebicidal concentrations of PHMB and PD occurred 24 hours after initiation of the encystment process. Resistance to CH developed during 36 hours. The smaller molecular size of CH compared with PHMB may contribute to the prolonged uptake of the biguanide derivative in immature cysts.

After a systematic database review summarizing all of the available clinical data in 2015, Alkharasi *et al*. concluded that there is insufficient evidence to evaluate the relative effectiveness and safety of medical therapy for the treatment of AK [Alkharasi M]. Results yielded no difference with respect to outcomes reported (resolution of disease, visual acuity, need for keratoplasty, adverse events) between chlorhexidine and PHMB. However, the sample size was inadequate to detect clinically meaningful differences between both.

Which diamidine or biguanide is more effective *in vitro* or in the clinical practice against *Acanthamoeba* trophozoites and cysts, is not yet proven. Therefore, it is important to evaluate the safety and potential toxic effect on human corneal cells. To date there is only limited information in the literature on the effect of diamidines and biguanides on human corneal cells.

Moon *et al*. could not verify a cytopathic effect on HCEC using 0.02% PHMB for 30 minutes, *in vitro* [Moon EK]. In contrast, using the same PHMB concentration for 24 hours, no viable HCEC were seen in our experiments. Lee *et al*. determined similar to our findings, that PHMB damages keratocytes more than CH [Lee JE]. In contrast, and interestingly, CH used alone tended to be more cytotoxic than PHMB analyzing human umbilical vein endothelial cells (HUVEC), since CH induced cell necrosis and PHMB cell apoptosis. [Mafra CSP]

Fernández-Ferreiro *et al*. described PD to be more cytotoxic on primary keratocytes than CH [Fernández-Ferreiro A]. In contrast, there was no significant difference in viability AUC comparing PD vs CH for HCEC, keratocytes and HCEC-12 in our study, but PHMB resulted in significantly worse viability AUC than PD for all human corneal cell types.

There is also limited information in the literature on *in vivo* effects of biguanides on the

cornea. Vontobel *et al*. tested the effect of 0.02% PHMB and CH on rabbit corneas and found that none of them penetrated through the cornea to the anterior chamber [Vontobel SF]. In contrast, Murthy *et al*. observed absence of epithelial cells, loss of keratocytes with apparent apoptosis and loss of endothelial cells after 0.02% chlorhexidine use [Murthy S]. Shigeyasu described limbal stem cell deficiency, corneal neovascularization, cataract formation and endothelial cell loss following exposure of the ocular surface to 20% chlorhexidine [Shigeyasu C] . Other case reports linked corneal necrosis, iris atrophy, cataract formation and ischemic ocular inflammation to 0.02% chlorhexidine use [Murthy S; Ehlers N; Awwad ST]. Concerning diamidines, some authors describe mild epithelial keratopathy in the form of epithelial microcysts or superficial punctate keratitis [Johns KJ 1988a; Duguid IG]. following the use of propamidine. On the other hand, Herz *et al*. and Ehlers & Hjordtal reported on rapidly progressive cataract and iris atrophy during propamidine isethionate or hexamidine diisethionate treatment [Herz NL; Ehlers N]. However, these clinical changes could also be related to the *AK* itself or to the effect of topical corticosteroids used.

In summary, PD decreases human cell viability less than DD and HD, whereas CH decreases viability less than PHMB. Our results suggest that propamidin-isethionate as diamidine and chlorhexidin as biguanide should be used in order to reduce the cytotoxicity of antiacanthaemoeba treatment on human epithelial cells, keratocytes and endothelial cells.

Diamidines reduce proliferation of human epithelial cells and keratocytes more than biguanides and propamidin isethionate reduces migration of keratocytes. Therefore, although propamidin isethionate is less cytotoxic than other diamidine, its negative effect on cell proliferation and migration indicates that its application should not be extended in patients.

A limitation of our study is that cell cultures were analyzed, and, therefore, our experience might not completely correspond to results of an *in vivo* analysis. A multicenter, randomized clinical trial could further clarify advantages and disadvantages of the use of diamidines and biguanides in the treatment of *AK*.

4.3 Comparison of in vitro assays to study the effectiveness of antiparasitics against *Acanthamoeba castellanii* trophozoites and cysts

In order to compare the efficacy of different antiamoebic agents, survival of *Acanthamoeba* trophozoites and cysts have to be observed. Up-to date the use of trypan blue assay [Lee X; Siddiqui R 2017], calcein-AM-ethidium homodimer staining [Chen Z], 5-cyano-2,3-tetrazolium chloride (CTC) staining [Mito T] and observation of treated *Acanthamoeba* on agar plate [Narasimhan S; Kowalski RP] or in fresh medium [Kobayashi T 2011; Kobayashi T 2012, Mito T; Baig AM] have been described in the literature. Nevertheless, all these examination methods have pitfalls, especially with difficulties in using an appropriate negative control**.**

As a first step, in our present work, the XTT assay was used to determine the efficacy of antiamoebic agents (data not shown). This assay measures the activity of mitochondria in the living cells to reduce the tetrazolium salt XTT to orange-colored compounds of formazan. Previously, Moss [Moss BJ] and Loures [Loures FV] used the XTT assay to estimate metabolic activity of fungi. Although it has recently been described, that NAD(P)H dehydrogenase has a similar function in plants, fungi and *Acanthamoeba castellanii* mitochondria [Antos-Krzeminska N], our trophozoite cultures did not show a positive XTT assay, even after the use of antiamoebic agents (data not shown).

As a next step, we used the LDH assay, to evaluate percentage of dead *Acanthamoeba*. LDH is an enzyme found in nearly all living cells, with a half-life time of approximately 9 hours. LDH is released during tissue damage. Most interestingly, during our measurement series, LDH release was the highest using 2 hours incubation time for all antiamoebic agents, but there was no LDH release after 24 hours of incubation. Therefore, we suggest that the LDH release peak of 1BU trophozoites is after 2 hours incubation time and subsequently, LDH is degraded.

Using the LDH assay, all antiamoebic agents had a significant cytotoxic effect on 1BU trophozoites, but 0.0065% MF shown a significantly higher effect as both other drugs, and 1BU count through 0.0065% MF was relatively reduced. This might be explained through a lytic effect of miltefosine on trophozoites with more LDH release, but has to be proven in further experiments.

The effects of PHMB, PD and MF on the viability of 1BU cells were next determined with a trypan blue exclusion assay [Strober W], which monitors the integrity of cell membranes (**Figure 12**, and the corresponding phase-contrast images of **Figures 13** and **14**). Living cells possess intact cell membranes that exclude penetration of certain dyes, such as trypan blue,

eosin, propidium, whereas dead cells do not. This is true for human cells, funghi [McMahon TA] and plant cells [van Wees S].

The results in our experiments also suggested in untreated 1BU trophozoites and cysts (controls) that these cells featured compromised cell membranes/cyst walls. However, challenging 1BU cells with the antiamoebic drugs clearly increased the number of trypan blue positive cells for all three drugs tested (**Figure 12 and Table 6**). Challenging 1BU cells with PHMB clearly increased the ratios of blue stained cells to 59.3 ± 1.5% (trophozoites) and 100% (cysts), respectively, suggesting that the membranes/cyst walls of the majority of PHMB treated cells became permeable for trypan blue by this drug. This effect was even stronger when 1BU cells were challenged with PD and MF, respectively. These findings are basically in line with the LDH release assay results (**Figure 12**), suggesting that all tested drugs exerted highly significant cytotoxic effects on 1BU cells, with PD and MF displaying very strong effects on trophozoites, while PHMB was significantly less effective in destabilizing trophozoites membranes (**Figure 12**). PHMB and PD exerted the strongest cytotoxic effect on cysts, while MF was significantly less effective against cysts (**Figure 12**).

The impact of the antiamoebic drugs on cell viability of *A. castellanii* trophozoites and cysts were next tested with a small set of fluorescent dyes. Hoechst 33342 is a cell permeable blue fluorescent dye that is specific for DNA and can be used to stain the nuclei of viable and fixed cells [Crowley LC; Eddaoudi A]. The cell permeable vital stain calcein-AM is a non-fluorescent derivative of the green fluorescent dye calcein, which can be converted to calcein due to the activity of cytosolic esterases [Romano V]. The vital stain is often used in combination with ethidium homodimer-1, a cell impermeable DNA-specific red-fluorescent dye that stains nuclei of dead cells [Chan LL].

Chen et al. determined the proportions of live/dead *Acanthamoeba* following photodynamic therapy, but their publication does not display the control group [Chen Z]. Notably, in our experiments, neither Hoechst 33342 nor the calcein-AM/ethidium homodimer-1 mixture stained drug-free trophozoites and cysts, indicating that both cell permeable dyes are not readily taken up and/or converted to a fluorescent dye by both *A. castellanii* growth stages.

However, this changed when 1BU cells were challenged with the antiamoebic drugs. The fact suggested that the membranes or cyst walls of the PHMB and PD challenged 1BU cells were sufficiently compromised to allow the diffusion of ethidium homodimer-1 into the cells but were viable enough to convert calcein-AM to calcein. *A. castellanii* cysts contain sufficient amounts of active non-specific esterases to cleave the acetoxymethyl esters from the non-fluorescent calcein derivative to generate a fluorescent probe. Concerning MF, the results indicate that MF treated *A. castellanii* trophozoites and cysts cannot convert the non-fluorescent calcein derivative calcein-AM to the green fluorescent calcein. We suggest that it might be because MF affects *Acanthamoeba* cystoplasm.

The afore mentioned enzyme- and cell permeability based viability assays suggested that the antiamoebic drugs PHMB, PD, and MF can kill significant amounts of, if not all, *A. castellanii* trophozoites and cysts under in vitro conditions. In order to confirm that these high killing rates suggested by the afore mentioned assays are real, a non-nutrient agar *E. coli* plate assay was next carried out, in which trophozoites were fed on an *E. coli* lawn. This assay also allows to monitor whether drug challenged cysts can still germinate in a growth favoring environment, and if cysts can be formed once the *E. coli* lawn was consumed by viable trophozoites. In this assay, untreated and drug challenged trophozoites/cysts were stained by trypan blue and subsequently placed in the center of a non-nutrient agar plate inoculated with a layer of viable *E. coli* cells, and the germination of cysts and the growth of trophozoites was monitored over time by light microscopy. Representative phase-contrast images of 1BU trophozoites and cysts that were cultivated for 0, 3, and 35 days on non-nutrient agar *E. coli* plates are shown in **Figures 15 and 16**. For both 1BU trophozoites and cysts, the trypan blue staining disappeared within 24 hours and, therefore, a follow-up of the fate of trypan blue positive *Acanthamoeba*e beyond this time frame was not possible.

The results in MF challenged trophozoites were the same as in the drug free control group, indicating that MF neither substantially affected the ability of trophozoites to migrate on the agar plate, nor was encystment markedly affected (**Figure 15**). Although MF can diminish the trophozoite amount (**Figure 13**) and result in the highest amount of LDH release (**Figure 12**), the surviving cells were still viable. *A. castellanii* 1BU cysts can also germinate on non-nutrient agar *E. coli* plates and migrate on the agar, and that these abilities were not markedly affected by the MF treatment. Chambers & Thompson reported that empty cysts will be left after excystment, which can be distinguished by scanning electron microscope [Chambers JA]. Unfortunately, we were unable to determine the percentage rates of 1BU excystment, as empty cysts were not clearly distinguishable from filled cysts by our light microscopy approach. After 5 weeks of cultivation, cysts could be observed in the central and peripheral parts of the agar plates, showing that MF treated cysts did not lose their excystment and encystment abilities (**Figure 16**).

When put into relation with the trypan blue assay results and the calcein-AM/ethidium homodimer-1 stainings (**Figures 12 and 13**), one can furthermore come to the conclusion that a positive staining of 1BU trophozoites for trypan blue or ethidium homodimer-1 does not allow to state that all these cells are dead. This conclusion is also strengthened by the findings that considerable amounts of PD and PHMB treated 1BU trophozoites were also positive for calcein, indicating that these cells were intact enough to allow the conversion of calcein-AM to calcein. In addition, PHMB and PD treated trophozoites released a lower LDH amount. Nevertheless, the high percentage of trypan blue positive PHMB and PD treated trophozoizes

may refer to a damage of the cell membrane, which may also be the reason of their inability to form normal shaped trophozoites and encyst again.

4.4 Efficacy of antiamoebic agents and Ce6-PDT against *Acanthamoeba castellanii* 1-BU

There are controversial literature data on effectivity of different drugs against *Acanthamoeba* isolates *in vitro* and *in vivo.* We aimed to analyze the effect of nine potential antiamoebic agents on *Acanthamoeba castellanii* trophozoites and cysts *in vitro*, using LDH assay, trypan blue staining and non-nutrient *E. coli* agar plate assay. With these methods we planned to get a better insight into the potential specific treatment against the 1BU *Acantahmoeba castellanii* isolate.

PHMB and CH belong to biguanides and are membrane-acting agents. Literature on their *in vitro* cysticidal effect is controversial. Some authors describe them as the most promising agents against different *Acanthamoeba* strains *in vitro*, particularly against the highly resistant cyst form of the organism [Mathers W; Lee JE, Moon EK]. In contrast, Sunada et al. observed weak cysticidal effect of PHMB [Sunada A]. Some other reports have considered PHMB equivalent or more effective than CH against trophozoites and cysts of different *Acanthamoeba* species. Some other studies, however, have reported CH as more effective [Narasimhan S; Turner NA].

Diamidines are nucleic acid-acting drugs. PD (Brolene®) was considered not to have an excellent cysticidal effect [Sunada A] and HD (Hexacyl®) was described to have higher amoebicidal activity than PD. [Perrine D] Also the efficacy of PD was considered lower than that of CH or MF against *Acanthamoeba* [Yamazoe K; Polat ZA 2012].

5% natamycin is known to inhibit fungal growth by binding to sterols and by impairment of membrane fusion via perturbation of ergosterol-dependent priming reactions that precede membrane fusion [te Welscher YM]. A case report described complete healing of AK within two months, using 2% natamycin [Jackson TN]. Sunada determined an excellent cysticidal effect of 5% natamycin [Sunada A].

MF is an intracellular targeting agent, which can denature essential cell proteins, and disrupt the cell membrane. MF was used successfully in amoebic encephalitis [Aichelburg AC] and in several *in vitro* experiments against *Acanthamoeba* [Walochnik J 2009; Polat ZA 2012; Polat ZA 2014].

PVPI is a broad spectrum microbicide that destroys microbial protein and DNA [Bigliardi PL, 2017]. 1% PVPI can cause ridges of the outer cyst wall and separation of the inner cysts wall from the outer one [Sunada A] and seemed to be effective against *Acanthamoeba in vitro* [Yamasaki K; Gatti S].

With increasing resistance of microorganisms to antibiotics, photodynamic therapy (PDT) may be one potential treatment option [Szentmáry N 2018]. Crosslinking as PDT alone

seemed not to be effective enough in AK [Hager T; del Buey MA; Makdoumi K]. Nevertheless, PDT using Ce6 and red light seemed to be effective in the treatment of Pseudomonas aeruginosa keratitis [Wu MF, Deichelbohrer M]. Obviously, the photosensitizer uptake through the microorganism and availibilty of oxygen seems to be decisive in treatment efficacy and this is extremely difficult in case of double-walled *Acanthamoeba* cysts [Winkler K; Chen Z].

Concerning the overall effect of the drugs, in our experiments, PHMB, CH, HD, PD, DD, NM, MF, and chlorin e6-PDT increased LDH activity and trypan blue positivity significantly in trophozoites ($P<0.01$), compared to controls. All used drugs also increased percentage of trypan blue stained 1BU *cysts* significantly ($P<0.01$), compared to controls, all clinically used concentrations of the drugs were significantly increasing trypan blue positivity of trophozoites and cyst ($P<0.01$). Concerning different drug concentrations, the effect of 0.005% PHMB ($P=0.59$) and 0.005% CH ($P= 0.10$) on *trophozoites* was weaker compared to other concentrations and the effect of 0.005% CH ($P=0.29$), 0.00095% or 0.0038% Ce6 with illumination ($P=0.14$; $P=0.06$) on *cysts* was lower than other applied concentrations, using the trypan blue assay. These results proof the potential of the clinically used drug concentrations but the ineffectivity of Ce6-PDT against *acanthamoeba* cysts.

It is well known, that *Acanthamoeba* cysts have a double cellulose wall, which is extremely resistant to enviromental damage. Using trypan blue assay, all drugs had significant cytotoxicity on *Acanthamoeba* trophozoites and cysts. To better understand the behaviour of trophozoites and cysts, we inoculated them after treatment to non-nutrient agar *E.coli* plates, which served as nutrition for the Acanthamoeba. In our opinion, the most striking observations could be made analyzing these plates.

In DD and MF groups, similar to controls, normal-shaped trophozoites with migratory potential appeared after 24-72 hours and encystment could happen again from 1 week. In PHMB, CH, HD, PD, NM and PVPI groups, there were no normal-shaped trophozoites after 24-72 hours and cysts could not be formed again during 5 weeks (**figure 21**). PHMB, PD and PVPI seemed to be even more effective in repeat experiments than CH, HD and NM against the *Acanthamoeba castellanii* 1-BU strain, as PHMB, PD and PVPI were effective in all 5 experiments against the 1BU strain, however, CH, HD and NM only in 3 or 4 of 5 repeat experiments.

Our experiments show, that 1BU *Acanthamoeba castellanii* was more vulnerable to PHMB, CH, HD, PD, NM and PVPI treatment than to DD and MF. However, none of these could completely eradicate trophozoites and cysts.

In summary, PHMB, CH, HD, PD, NM and PVPI seem to be more effective against *Acanthamoeba* castellani 1BU strain, than DD and MF. In vitro analysis of treatment efficacy of different antiamoebic agents, especially the non-nutrient agar *Eschericia coli* plate assay

may provide us information on specific treatment of different *Acanthamoeba* strains in the future.

4.5 Conclusions and outlook to the future

Acanthamoeba keratitis is a severe, sight threatening infection.

Acanthamoeba keratitis patients at the early stage of the disease suffer from tearing and ocular pain. At this time-point, the ophthalmologists observe a relative mild ophthalmological status, compared to the pronounced discomfort of the patient. A pseudodendritiformic epitheliopathy, “dirty epithelium”, typically spot-like multifocal stromal infiltrates and radial perineuritis can be observed at this stage. Later on, a Wessely immune ring (ring infiltrate) develops. In long-standing, recalcitrant *Acanthamoeba* keratitis, uveitis, retinal vasculitis and scleritis may occur and result in blindness, even without further persistence of *Acanthamoeba* trophozoites or cysts. The etiology of these inflammatory complications is unclear, but may be explained with molecular mimicry or type III immune reaction. Therefore, in late stage of *Acanthamoeba* keratitis, systemic immune suppression may be necessary for a longer period of time.

Since AK presents with heterogeneous clinical appearance and low incidence, there have been no clinical trials comparing different treatment modalities in this disease and no standardized treatment of AK has been established, yet.

The lack of a standardized therapy against AK is also in part due to the lack of a generally agreed drug testing regime against *Acanthamoeba*. Although a number of staining methods and viability tests have been already used to test the efficacy of drugs against *Acanthamoeba* isolates in vitro, a routine method, to enable a specific treatment in AK, against the isolated *Acanthamoeba,* is still not available.

Analysing human corneal cells, PD decreases human cell viability less than DD and HD, whereas CH decreases viability less than PHMB. Considering these effects of antiamoebic agents on human corneal cells, we suggest the use of propamidin-isethionate as diamidine and chlorhexidin as biguanide in order to reduce the cytotoxicity of antiamoebic treatment on human epithelial cells, keratocytes and endothelial cells. Diamidines reduce proliferation of human epithelial cells and keratocytes more than biguanides and propamidin isethionate reduces migration of keratocytes. Therefore, although propamidin isethionate is less cytotoxic than other diamidines, its negative effect on cell proliferation and migration indicates that its application should not be extended in patients. A multicenter, randomized clinical trial could further clarify advantages and disadvantages of the use of diamidines and biguanides in the treatment of AK.

Acanthamoeba species can develop two life-forms, trophozoites and cysts. To be effective

against these protozoan pathogens a drug should also attack the dormant cyst state of the pathogen, and as a consequence, efficacy of an antiamoebic drug should be evaluated with both life-forms. We tested a number of common enzymatic- and dye-based viability assays to study the killing efficacies of antiamoebic drugs against *A. castellanii* 1BU trophozoites and cysts. When compared with the non-nutrient agar *E. coli* plate assay, which served as gold-standard in this comparison, none of the enzymatic- and dye-based assays tested here yielded results with both life-forms that were comparable with the results obtained with the culture-based method. Discrepancies were especially evident with the drug MF, which yielded almost no effect on the viability of 1BU trophozoites and cysts when assayed with the culture-based method, while all enzymatic- and dye-based assays tested here suggested a good to excellent killing efficacy of this drug to at least one of the life-forms of this pathogen. These findings suggest that commonly used viability assays such as trypan-blue staining and life-dead staining with calcein-AM and ethidium homodimer-1 may - depending on the drug - overestimate the killing efficacy of the antiamoebic agent, while the culture-based non-nutrient agar *E. coli* plate assay proofed to be a reliable method to study the drug efficacy of antiamoebic agents against both life-forms of this protozoan pathogen.

Based on the culture-based non-nutrient agar *E. coli* plate assay, we have shown, that *Acanthamoeba castellanii* 1BU strain was more vulnerable to PHMB, CH, HD, PD, NM and PVPI treatment than to DD and MF. However, none of these could completely eradicate trophozoites and cysts. The non-nutrient agar *Eschericia coli* plate assay may provide us information on specific treatment of different *Acanthamoeba* strains.

The limitation of our study is that we included only one *Acanthamoeba* isolate for our experiments. In subsequent studies, different *Acanthamoeba* isolates - especially clinical isolates from AK patients - should be tested, in order to enable a more specific treatment for AK patients, in the long-term.

REFERENCES

1. AI Kharousi N, Wali UK (2012) Confoscan: an ideal therapeutic aid and screening tool in *Acanthamoeba* keratitis. Middle East Afr J Ophthalmol 19: 422–425.
2. Aichelburg AC, Walochnik J, Assadian O, Prosch H, Steuer A, Perneczky G, Visvesvara GS, Aspöck H, Vetter N (2008) Successful treatment of disseminated *Acanthamoeba* sp. infection with miltefosine. Emerg Infect Dis 14: 1743-1746.
3. Aksozek A, McClellan K, Howard K, Niederkorn JY, Alizadeh H (2002) Resistance of *Acanthamoeba castellanii* cysts to physical, chemical, and radiological conditions. J Parasitol 88: 621-623.
4. Alizadeh H, Apte S, El-Agha MS, Li L, Hurt M, Howard K, Cavanagh HD, McCulley JP, Niederkorn JY (2001) Tear IgA and serum IgG antibodies against *Acanthamoeba* in patients with *Acanthamoeba* keratitis. Cornea 20: 622-627.
5. Alizadeh H, Neelam S, Niederkorn JY (2007) Effect of immunization with the mannose-induced *Acanthamoeba* protein and *Acanthamoeba* plasminogen activator in mitigating *Acanthamoeba* keratitis. Invest Ophthalmol Vis Sci 48: 5597-5604.
6. Alkharashi M, Lindsley K, Law HA, Sikder S (2015) Medical interventions for acanthamoeba keratitis. Cochrane Database Syst Rev 24:CD010792.
7. Amoils SP, Heney C (1999) Acanthamoeba keratitis with live isolates treated with cryosurgery and fluconazole. Am J Ophthalmol 127:718-720.
8. Antos-Krzeminska N, Jarmuszkiewicz W (2014) External NAD(P)H dehydrogenases in *Acanthamoeba castellanii* mitochondria. Protist 165: 580-593.
9. Arnalich-Montiel F, Lumbreras-Fernández B, Martín-Navarro CM, Valladares B, Lopez-Velez R, Morcillo-Laiz R, Lorenzo-Morales J (2014) Influence of *Acanthamoeba* genotype on clinical course and outcomes for patients with *Acanthamoeba* keratitis in Spain. J Clin Microbiol 52: 1213–1216.
10. Awwad ST, Heilman M, Hogan N, Parmar DN, Petroll WM, McCulley JP, Cavanagh HD (2007) Severe reactive ischemic posterior segment inflammation in *Acanthamoeba* keratitis: a new potentially blinding syndrome. Ophthalmology 114: 313-320.

11. Baig AM, Iqbal J, Khan NA (2013) In vitro efficacies of clinically available drugs against growth and viability of an *Acanthamoeba castellanii* keratitis isolate belonging to the T4 genotype. Antimicrob Agents Chemother 57: 3561-3567.
12. Bigliardi PL, Alsagoff SAL, El-Kafrawi HY, Pyon JK, Wa CTC, Villa MA (2017) Povidone iodine in wound healing: A review of current concepts and practices. Int J Surg 44: 260-268.
13. Brasseur G, Favennec L, Perrine D, Chenu JP, Brasseur P (1994) Successful treatment of *Acanthamoeba* keratitis by hexamidine. Cornea 13: 459-462.
14. Burke JP, Webber SK, Kerr-Muir MG, Talbot JF, Parsons MA (1992) Acanthamoeba polyphaga panophthalmitis. Cornea 11: 274-275.
15. Butler TK, Males JJ, Robinson LP, Wechsler AW, Sutton GL, Cheng J, Taylor P, McClellan K (2005) Six-year review of *Acanthamoeba* keratitis in New South Wales, Australia: 1997-2002. Clin Exp Ophthalmol 33: 41-46.
16. Chambers JA, Thompson JE (1972) A scanning electron microscopic study of the excystment process of *Acanthamoeba castellanii*. Exp Cell Res 73: 415-421.
17. Chan LL, McCulley KJ, Kessel SL (2017) Assessment of cell viability with single-, dual-, and multi-staining methods using image cytometry. Methods Mol Biol 1601: 27-41.
18. Chen Z, Xuguang S, Zhiqun W, Ran L (2008) In vitro amoebacidal activity of photodynamic therapy on *Acanthamoeba*. Br J Ophthalmol 92: 1283-1286.
19. Chin J, Young AL, Hui M, Jhanji V (2015) Acanthamoeba keratitis: 10-year study at a tertiary eye care center in Hong Kong. Cont Lens Anterior Eye 38: 99-103.
20. Chomicz L, Conn DB, Padzik M, Szaflik JP, Walochnik J, Zawadzki PJ, Pawłowski W, Dybicz M (2015) Emerging treats for human health in Poland: Pathogenic Isolates from drug resistant *Acanthamoeba* keratitis monitored in terms of their in vitro dynamics and temperature adaptability. Biomed Res Int 2015: 231285.
21. Clarke DW, Alizaden H, Niederkorn JY (2005) Failure of *Acanthamoeba castellanii* to produce intraocular infections. Invest Ophthalmol Vis Sci 46: 2472-2478.
22. Cornaby C, Gibbons L, Mayhew V, Sloan CS, Welling A, Poole BD (2015) B cell epitope spreading: mechanisms and contribution to autoimmune disease. Immunol Lett 163: 56-68.
23. Crowley LC, Marfell BJ, Waterhouse NJ (2016) Analyzing cell death by nuclear

staining with Hoechst 33342. Cold Spring Harb Protoc 1: 2016(9).

24. Daas L, Szentmáry N, Eppig T, Langenbucher A, Hasenfus A, Roth M, Saeger M, Nölle B, Lippmann B, Böhringer D, Reinhard T, Kelbsch C, Messmer E, Pleyer U10, Roters S, Zhivov A, Engelmann K, Schrecker J, Zumhagen L, Thieme H, Darawsha R, Meyer-Ter-Vehn T, Dick B, Görsch I, Hermel M, Kohlhaas M, Seitz B (2015) Das Deutsche Akanthamöbenkeratitis-Register - Erste Ergebnisse einer multizentrischen Erhebung. Ophthalmologe 112: 752-763.
25. Dart JK, Saw VP, Kilvington S (2009) *Acanthamoeba* keratitis: diagnosis and treatment update 2009. Am J Ophthalmol 148: 487-499.
26. D'Aversa G, Stern GA, Driebe WT Jr (1995) Diagnosis and successful medical treatment of Acanthamoeba keratitis. Arch Ophthalmol 113: 1120-1123.
27. Deichelbohrer M, Wu MF, Seitz B, Hüttenberger D, Laschke MW, Foth HJ, Wonnenberg B, Wagenpfeil S, Meier C, Bischoff M, Tschernig T (2017) Bacterial keratitis: Photodynamic inactivation reduced experimental inflammation. Exp Ther Med 14: 4509–4514.
28. del Buey MA, Cristóbal JA, Casas P, Goñi P, Clavel A, Mínguez E, Lanchares E, García A, Calvo B (2012) Evaluation of in vitro efficacy of combined riboflavin and ultraviolet a for *Acanthamoeba* isolates. Am J Ophthalmol 153: 399-404.
29. Doan N, Rozansky G, Nguyen HS, Gelsomino M, Shabani S, Mueller W, Johnson V (2015) Granulomatous amebic encephalitis following hematopoietic stem cell transplantation. Surg Neurol Int 6: S459-462.
30. Duguid IG, Dart JK, Morlet N, Allan BD, Matheson M, Ficker L, Tuft S (1997) Outcome of *Acanthamoeba* keratitis treated with polyhexamethyl biguainide and propamidine. Ophthalmology 104: 1587-1592.
31. Eddaoudi A, Canning SL, Kato I (2018) Flow Cytometric Detection of G0 in Live Cells by Hoechst 33342 and Pyronin Y Staining. Methods Mol Biol. 1686: 49-57.
32. Ehlers N, Hjortdal J (2004) Are cataract and iris atrophy toxic complications of medical treatment of *Acanthamoeba* keratitis? Acta Ophthalmol Scand 82: 228-231.
33. Fernández-Ferreiro A, Santiago-Varela M, Gil-Martínez M (2017) *In vitro* evaluation of the ophthalmic toxicity profile of chlorhexidine and propamidine isethionate eye drops. J Ocul Pharmacol Ther 33: 202-209.

34. Gatti S, Cevini C, Bruno A, Penso G, Rama P, Scaglia M (1998) In vitro effectiveness of povidone-iodine on *Acanthamoeba* isolates from human cornea. Antimicrob Agents Chemother 42: 2232-2234.
35. Geith S, Walochnik J, Prantl F, Sack S, Eyer F (2018) Lethal outcome of granulomatous acanthamoebic encephalitis in a man who was human immunodeficiency virus-positive: a case report. J Med Case Rep 12: 201.
36. Gupta N, Samantaray JC, Duggal S, Srivastava V, Dhull CS, Chaudhary U (2010) Acanthamoeba keratitis with Curvularia co-infection. Indian J Med Microbiol 28: 67-71.
37. Hager T, Hasenfus A, Stachon T, Seitz B, Szentmáry N (2016) Crosslinking and corneal cryotherapy in *Acanthamoeba* keratitis -- a histological study. Graefes Arch Clin Exp Ophthalmol 254: 149-153.
38. Heffler KF, Eckhardt TJ, Reboli AC, Stieritz D (1996) Acanthamoeba endophthalmitis in acquired immunodeficiency syndrome. Am J Ophthalmol 122: 584-586.
39. Herz NL, Matoba AY, Wilhelmus KR (2008) Rapidly progressive cataract and iris atrophy during treatment of *Acanthamoeba* keratitis. Ophthalmology 115: 866-869.
40. Huth S, Reverey JF, Leippe M, Selhuber-Unkel C (2017) Adhesion forces and mechanics in mannose-mediated acanthamoeba interactions. PLoS One 12: e0176207.
41. Iovieno A, Oechsler RA, Ledee DR, Miller D, Alfonso EC (2010) Drug-resistant severe *Acanthamoeba* keratitis caused by rare T5 *Acanthamoeba* genotype. Eye Contact Lens 36: 183-184.
42. Iovieno A, Gore DM, Carnt N, Dart JK (2014) Acanthamoeba sclerokeratitis: epidemiology, clinical features, and treatment outcomes. Ophthalmology 121: 2340-2347.
43. Jackson TN, Heinze JB, Tuxen J, Weiner JM (1986) Successful medical treatment of a corneal ulcer due to *Acanthamoeba* polyphaga. Aust N Z J Ophthalmol 14: 139-142.
44. Johns KJ, Head WS, O'Day DM (1988a) Corneal toxicity of propamidine. Arch Ophthalmol 106: 68-69.
45. Johns KJ, O'Day DM, Feman SS (1988b) Chorioretinitis in the contralateral eye of a patient with *Acanthamoeba* keratitis. Ophthalmology 95: 635-639.

46. Jones DB, Visvesvara GS, Robinson NM (1975) *Acanthamoeba* polyphaga keratitis and *Acanthamoeba* uveitis associated with fatal meningoencephalitis. Trans Ophthalmol Soc UK 95: 221-232.
47. Khunkitti W, Avery SV, Lloyd D, Furr JR, Russell AD (1997) Effects of biocides on *Acanthamoeba castellanii* as measured by flow cytometry and plaque assay. J Antimicrob Chemother 40: 227-233.
48. Klüppel M1, Reinhard T, Sundmacher R, Daicker B (1997) Therapy of advanced amoeba keratitis with keratoplasty à chaud and adjuvant cryotherapy. Ophthalmologe 94: 99-103.
49. Kobayashi T, Gibbon L, Mito T, Shiraishi A, Uno T, Ohashi Y (2011) Efficacy of commercial soft contact lens disinfectant solutions against *Acanthamoeba*. Jpn J Ophthalmol 55: 547-557.
50. Kobayashi T, Mito T, Watanabe N, Suzuki T, Shiraishi A, Ohash Y (2012) Use of 5-Cyano-2,3-Ditolyl-Tetrazolium Chloride staining as an indicator of biocidal activity in a rapid assay for anti-*Acanthamoeba* agents. J Clin Microbiol 50: 1606–1612.
51. Kowalski RP, Abdel Aziz S, Romanowski EG, Shanks RM, Nau AC, Raju LV (2013) Development of a practical complete-kill assay to evaluate anti-*Acanthamoeba* drugs. JAMA Ophthalmol 131: 1459-1462.
52. Küchle M, Seitz B, Langenbucher A, Naumann GO (1999) Nonmechanical excimer laser penetrating keratoplasty for perforated or predescemetal corneal ulcers. Ophthalmology 106: 2203-2209.
53. Laurik KL, Szentmáry N, Daas L, Langenbucher A, Seitz B (2019) Early penetrating keratoplasty à chaud may improve outcome in therapy-resistant *Acanthamoeba* keratitis. Adv Ther 36: 2528-2540.
54. Lee GA, Gray TB, Dart JK, Pavesio CE, Ficker LA, Larkin DF, Matheson MM (2002) Acanthamoeba sclerokeratitis. Treatment with systemic immunosuppression. Ophthalmology 109: 1178-1182.
55. Lee HS, Amouzegar A, Dana R (2017) Kinetics of Corneal Antigen Presenting Cells in Experimental Dry Eye Disease. BMJ Open Ophthalmol 1: e000078.
56. Lee JE, Oum BS, Choe HY, Yu HS, Lee JS (2007) Cysticidal effect on Acanthamoeba and toxicity on human keratocytes by polyhexamethylene biguanide and chlorhexidine. Cornea 26: 736-741.

57. Lee SM, Lee JE, Lee DI, Yu HS (2018) Adhesion of *Acanthamoeba* on cosmetic contact lenses. J Korean Med Sci 33: e26.
58. Lee X, Reimmann C, Greub G, Sufrin J, Croxatto A (2012) The Pseudomonas aeruginosa toxin L-2-amino-4-methoxy-trans-3-butenoic acid inhibits growth and induces encystment in *Acanthamoeba castellanii*. Microbes Infect 14: 268-272.
59. Lim N, Goh D, Bunce C, Xing W, Fraenkel G, Poole TR Ficker L (2008) Comparison of polyhexamethylene biguanide and chlorhexidine as monotherapy agents in the treatment of *Acanthamoeba* keratitis. Am J Ophthalmol 145: 130-135.
60. Lorenzo-Morales J, Khan NA, Walochnik J (2015) An update on Acanthamoeba keratitis: diagnosis, pathogenesis and treatment. Parasite 22: 10.
61. Loures FV, Levitz SM (2015) XTT Assay of Antifungal Activity. Bio Protoc 5. pii: e1543.
62. Mafra CSP, Carrjio-Carvalho LC, Chudzinski-Tavassi AM, Taguchi FM, Foronda AS, Carvalho FR, de Freitas D (2013) Antimicrobial action of biguanides on the viability of Acanthamoeba cysts and assessment of cell toxicity. IOVS 54: 6363-6372.
63. Makdoumi K, Bäckman A, Mortensen J, Magnuson A, Crafoord S (2013) Comparison of UVA- and UVA/riboflavin-induced growth inhibition of *Acanthamoeba castellanii*. Graefes Arch Clin Exp Ophthalmol 251: 509-514.
64. Mammo Z, Almeida DR, Cunningham MA, Chin EK, Mahajan VB (2017) *Acanthamoeba* endophthalmitis after recurrent keratitis and nodular scleritis. Retin Cases Brief Rep 11: 180-182.
65. Mathers W (2006) Use of higher medication concentrations in the treatment of Acanthamoeba keratitis. Arch Ophthalmol 124: 923.
66. Mathers WD (2004) Acanthamoeba: a difficult pathogen to evaluate and treat. Cornea 23: 325.
67. Mazur T, Hadaś E, Iwanicka I (1995) The duration of the cyst stage and the viability and virulence of Acanthamoeba isolates. Trop Med Parasitol 46: 106-108.
68. McClellan K, Howard K, Mayhew E, Niederkorn J, Alizadeh H (2002) Adaptive immune responses to Acanthamoeba cysts. Exp Eye Res 75:285-293.
69. McMahon TA, Rohr JR (2014) Trypan blue dye is an effective and inexpensive way to determine the viability of Batrachochytrium dendrobatidis zoospores.

Ecohealth 11: 164-167.

70. Mito T, Suzuki T, Kobayashi T, Zheng X, Hayashi Y, Shiraishi A, Ohashi Y (2012) Effect of photodynamic therapy with methylene blue on Acanthamoeba in vitro. Invest Ophthalmol Vis Sci 19; 53: 6305-6313.
71. Monk IR, Tree JJ, Howden BP, Stinear TP, Foster TJ (2015) Complete bypass of restriction systems for major staphylococcus aureus lineages. MBio 6: e00308-15.
72. Moon EK, Hong Y, Chung DI, Goo YK, Kong HH (2015) Potential value of cellulose synthesis inhibitors combined with PHMB in the treatment of Acanthamoeba keratitis. Cornea 34: 1593-1598.
73. Moshari A, McLean IW, Dodds MT, Damiano RE, McEvoy PL (2001) Chorioretinitis after keratitis caused by acanthamoeba: case report and review of the literature. Ophthalmology 108: 2232-2236.
74. Moss BJ, Kim Y, Nandakumar MP, Marten MR (2008) Quantifying metabolic activity of filamentous fungi using a colorimetric XTT assay. Biotechnol Prog 24: 780-783.
75. Mrva M, Garajová M, Lukáč M, Ondriska F (2011) Weak cytotoxic activity of miltefosine against clinical isolates of *Acanthamoeba* spp. J Parasitol 97: 538-540.
76. Murthy S, Hawksworth NR, Cree I (2002) Progessive ulcerative keratitis related to the use of topical chlorhexidine gluconate (0.02%). Cornea 21: 237-239.
77. Narasimhan S, Madhavan HN, K LT (2002) Development and application of an in vitro susceptibility test for Acanthamoeba species isolated from keratitis to polyhexamethylene biguanide and chlorhexidine. Cornea 21: 203-205.
78. Neelam S, Niederkorn JY (2017) Pathobiology and Immunobiology of Acanthamoeba Keratitis: Insights from Animal Models. Yale J Biol Med 90: 261-268.
79. Niederkorn H, Alizadeh H, Leher JP (1999) The pathogenesis of Acanthamoeba keratitis. Microbes Infect 1: 437-443.
80. Nunes TE, Brazil NT, Fuentefria AM, Rott M (2016) Acanthamoeba and Fusarium interactions: A possible problem in keratitis. Acta Trop 157: 102-107
81. Panjwani N (2010) Pathogenesis of Acanthamoeba keratitis Ocul Surf 8: 70-79.
82. Perrine D, Chenu JP, Georges P, Lancelot JC, Saturnino C, Robba M (1995) Amoebicidal efficiencies of various diamidines against two strains of Acanthamoeba polyphaga. Antimicrob Agents Chemother 39: 339-342.

83. Polat ZA, Obwaller A, Vural A, Walochnik J (2012) Efficacy of miltefosine for topical treatment of Acanthamoeba keratitis in Syrian hamsters. Parasitol Res 110: 515-520.
84. Polat ZA, Walochnik J, Obwaller A, Vural A, Dursun A, Arici MK (2014) Miltefosine and polyhexamethylene biguanide: a new drug combination for the treatment of *Acanthamoeba* keratitis. Clin Exp Ophthalmol 42: 151-158.
85. Radford CF, Minassian DC, Dart JK (2002) *Acanthamoeba* keratitis in England and Wales: incidence, outcome, and risk factors. Br J Ophthalmol 86: 536-542.
86. Raghavan A, Baidwal S, Venkatapathy N, Rammohan R (2019) The Acanthamoeba-Fungal Keratitis Study. Am J Ophthalmol 201: 31-36.
87. Romano V, Parekh M, Ruzza A, Willoughby CE, Ferrari S, Ponzin D, Kaye SB, Levis HJ (2018) Comparison of preservation and transportation protocols for preloaded Descemet membrane endothelial keratoplasty. Br J Ophthalmol 102: 549-555.
88. Schuster FL, Visvesvara GS (2004) Opportunistic amoebae: challenges in prophylaxis and treatment. Drug Resist Updat 7:41-51.
89. Seal D, Hay J, Kirkness C, Morrell A, Booth A, Tullo A Ridgway A, Armstrong M (1996) Successful medical therapy of *Acanthamoeba* keratitis with topical chlorhexidine and propamidine. Eye 10: 413-421.
90. Seitz B, Langenbucher A, Kus MM, Küchle M, Naumann GO (1999) Nonmechanical corneal trephination with the excimer laser improves outcome after penetrating keratoplasty. Ophthalmology 106: 1156-1164.
91. Seitz B, Resch MD, Schlötzer-Schrehardt U, Hofmann-Rummelt C, Sauer R, Kruse FE (2006) Histopathology and ultrastructure of human corneas after amniotic membrane transplantation. Arch Ophthalmol 124: 1487–1490.
92. Shi L, Stachon T, Seitz B, Wagenpfeil S, Langenbucher A, Szentmáry N (2018) The effect of antiamoebic agents on viability, proliferation and migration of human epithelial cells, keratocytes and endothelial cells, in vitro. Curr Eye Res 43: 725-733.
93. Shigeyasu C, Shimazaki J (2012) Ocular surface reconstruction after exposure to high concentrations of antiseptic solutions. Cornea 31: 59-65.
94. Siddiqui R, Khan NA (2012) Biology and pathogenesis of Acanthamoeba. Parasit Vectors 5:6.

95. Siddiqui R, Aqeel Y, Khan NA (2016) The Development of Drugs against Acanthamoeba Infections. Antimicrob Agents Chemother 60: 6441-6450.
96. Siddiqui R, Abjani F, Yeo CI, Tiekink ER, Khan NA3 (2017) The effects of phosphanegold (I) thiolates on the biological properties of *Acanthamoeba castellanii* belonging to the T4 genotype. J Negat Results Biomed 16: 6.
97. Stehr-Green JK, Bailey TM, Visvesvara GS (1989) The epidemiology of Acanthamoeba keratitis in the United States. Am J Ophthalmol 107: 331-336.
98. Strober W (2001) Trypan blue exclusion test of cell viability. Curr Protoc Immunol Appendix 3: Appendix 3B. doi: 10.1002/0471142735.ima03bs21.
99. Sunada A, Kimura K, Nishi I, Toyokawa M, Ueda A, Sakata T, Suzuki T, Inoue Y, Ohashi Y, Asari S, Iwatani Y (2014) In vitro evaluations of topical agent to treat Acanthamoeba keratitis. Ophthalmology 121: 2059-2065.
100. Suryawanshi A, Cao Z, Sampson JF, Panjwani N (2015) IL-17A-mediated protection against Acanthamoeba keratitis. J Immunol 94: 650-663.
101. Szentmáry N, Langenbucher A, Kus MM, Naumann GO, Seitz B. (2007) Elliptical nonmechanical corneal trephination: intraoperative complications and long-term outcome of 42 consecutive excimer laser penetrating keratoplasties. Cornea 26: 414-420.
102. Szentmáry N, Daas L, Matoula P, Goebels S, Seitz B (2013) Akanthamöbenkeratitis. Ophthalmologe 110: 1203-1210.
103. Szentmáry N, Daas L, Shi L, Lenke Laurik K, Seitz B (2017) SOP Akanthamöbenkeratitis – klinische Zeichen, Diagnose, Therapie. Klin Monbl Augenheilkd 7: 281-287.
104. Szentmáry N, Daas L, Shi L, Laurik KL, Lepper S, Milioti G, Seitz B (2018) Acanthamoeba keratitis - Clinical signs, differential diagnosis and treatment. J Curr Ophthalmol 31: 16-23.
105. te Welscher YM, Jones L, van Leeuwen MR, Dijksterhuis J, de Kruijff B, Eitzen G, Breukink E (2010) Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. Antimicrob Agents Chemother 54: 2618-2625.
106. Turner NA, Russell AD, Furr JR, Lloyd D (2000) Emergence of resistance to biocides during differentiation of Acanthamoeba castellanii. J Antimicrob Chemother 46: 27-34.

107. van Wees S (2008) Phenotypic analysis of Arabidopsis mutants: trypan blue stain for fungi, oomycetes, and dead plant cells. CSH Protoc 3: pdb.prot4982.

108. Verani JR, Lorick SA, Yoder JS, Beach MJ, Braden CR, Roberts JM, Conover CS, Chen S, McConnell KA, Chang DC, Park BJ, Jones DB, Visvesvara GS, Roy SL (2009) National outbreak of Acanthamoeba keratitis associated with use of a contact lens solution, United States. Emerg Infect Dis 15: 1236–1242.

109. Vontobel SF, Abad-Villar EM, Kaufmann C, Zinkernagel AS, Hauser PC, Thiel MA (2015) Corneal penetration of polyhexamethylene biguanide and chlorhexidine digluconate. Clin Exp Ophthalmol 6: 1000430.

110. Walochnik J, Obwaller A, Gruber F, Mildner M, Tschachler E, Suchomel M, Duchêne M, Auer H (2009) Anti-Acanthamoeba efficacy and toxicity of miltefosine in an organotypic skin equivalent. J Antimicrob Chemother 64: 539-545.

111. Walochnik J, Scheikl U, Haller-Schober EM (2015) Twenty years of Acanthamoeba diagnostics in Austria. J Eukaryot Microbiol 62: 3-11.

112. Wang J, Stachon T, Eppig T, Langenbucher A, Seitz B, Szentmáry N (2013) Impact of photodynamic inactivation (PDI) using the photosensitizer chlorin e6 on viability, apoptosis, and proliferation of human keratocytes in vitro. Graefes Arch Clin Exp Ophthalmol 251: 2725-2731.

113. Winkler K, Simon C, Finke M, Bleses K, Birke M, Szentmáry N, Hüttenberger D, Eppig T, Stachon T, Langenbucher A, Foth HJ, Herrmann M, Seitz B, Bischoff M (2016) Photodynamic inactivation of multidrug-resistant Staphylococcus aureus by chlorin e6 and red light (λ=670nm). J Photochem Photobiol B 162: 340-347.

114. Wright P, Warhurst D, Jones BR (1985) Acanthamoeba keratitis successfully treated medically. Br J Ophthalmol 69: 778-782.

115. Wu MF, Deichelbohrer M, Tschernig T, Laschke MW, Szentmáry N, Hüttenberger D, Foth HJ, Seitz B, Bischoff M (2017) Chlorin e6 mediated photodynamic inactivation for multidrug resistant Pseudomonas aeruginosa keratitis in mice in vivo. Sci Rep 7: 44537.

116. Yamasaki K, Saito F, Ota R, Kilvington S (2018) Antimicrobial efficacy of a novel povidone iodine contact lens disinfection system. Cont Lens Anterior Eye 41: 277-281.

117. Yamazoe K, Yamamoto Y, Shimazaki-Den S, Shimazaki J (2012) Visual outcome in Japanese patients with Acanthamoeba keratitis. Eye (Lond) 26: 517-522.

118. Yokogawa H, Kobayashi A, Yamazaki N, Ishibashi Y, Oikawa Y, Tokoro M, Sugiyama K (2012) Bowman's layer encystment in cases of persistent Acanthamoeba keratitis. Clin Ophthalmol 6: 1245–1251.

LIST OF PUBLICATIONS

Original articles

[1]. Shi L, Stachon T, Seitz B, Wagenpfeil S, Langenbucher A, Szentmáry (2018) The effect of antiamoebic agents on viability, proliferation and migration of human epithelial cells, keratocytes and endothelial cells, in vitro.Curr Eye Res 43: 725-733.

[2]. Orosz E, Kriskó D, Shi L, Sándor GL, Kiss HJ, Seitz B, Nagy ZZ, Szentmáry N (2019) Clinical course of *Acanthamoeba* keratitis by genotypes T4 and T8 in Hungary. Acta Microbiol Immunol Hung 5: 1-12.

[3]. Shi L, Stachon T, Latta L, Elhawy MI, Gunaratnam G, Orosz E, Kiderlen AF, Seitz B, Bischoff M, Szentmáry N. Comparison of in vitro assays to study the effectiveness of antiparasitics against *Acanthamoeba castellanii* trophozoites and cysts. Acta Microbiol Immunol Hung. [2019 in press]

[4] Shi L, Hager T, Fries FN, Daas L, Holbach L, Hofmann-Rummelt C, Zemova E, Seitz B, Szentmáry N. Reactive uveitis, retinal vasculitis and scleritis as ocular end-stage of *Acanthamoeba* keratitis – a histological study. Int J Ophthalmol. [Revision submitted]

[5] Shi L, Stachon T, Latta L, Elhawy MI, Gunaratnam G, Orosz E, Kiderlen AF, Seitz B, Bischoff M, Szentmáry N. The effect of antiamoebic agents and Ce6-PDT on *Acanthamoeba castellanii* trophozoites and cysts, in vitro. [Submitted to Graefe's Arch Clin Exp Ophthalmol].

Review articles

[1]. Szentmáry N, Daas L, Shi L, Lenke Laurik K, Seitz B (2017) SOP Akanthamöbenkeratitis – klinische Zeichen, Diagnose, Therapie. Klin Monbl Augenheilkd 7: 281-287.

[2]. Szentmáry N, Daas L, Shi L, Laurik KL, Lepper S, Milioti G, Seitz B (2018) Acanthamoeba keratitis - Clinical signs, differential diagnosis and treatment. J Curr Ophthalmol 31: 16-23.

Lectures and posters

[1]. Shi L, Stachon T, Seitz B, Wagenpfeil S, Langenbucher A, Szentmáry N (2017) The effect of antiamoebic agents on viability, proliferation and migration of human epithelial cells, keratocytes and endothelial cells, in vitro. DOG Congress; Berlin, Germany; Poster.

[2]. Shi L, Stachon T, Latta L, Seitz B, Wagenpfeil S, Langenbucher A, Szentmáry N (2017) The Effect of Antiamoebic Agents on the Viability of Human Epithelial Cells, Keratocytes and Endothelial cells, *in vitro.* Congress of Rhein-Mainischer Augenärzte; Frankfurt, Germany; Poster.

[3]. Shi L, Stachon T, Seitz B, Wagenpfeil S, Langenbucher A, Szentmáry N (2018) The effect of antiamoebic agents on viability, proliferation and migration of human epithelial cells, keratocytes and endothelial cells, in vitro. ARVO Congress; Hawaii, USA; Poster.

[4]. Szentmáry N, Shi L, Stachon T, Latta L, Wagenpfeil S, Langenbucher A, Seitz B (2018) The effect of antiamoebic agents on viability, proliferation and migration of human epithelial cells, keratocytes and endothelial cells, in vitro. EuCornea Conference; Wien, Austria; Poster.

[5]. Szentmáry N, Shi L, Hager T, Holbach L, Hofmann-Rummelt C, Zemova E, Seitz B (2018) Reactive uveitis, retinal vasculitis and scleritis as ocular end-stage of acanthamoeba keratitis –a histological study. Congress of the Hungarian Ophthalmological Society; Szeged, Hungary; Lecture.

[6]. Szentmáry N, Shi L, Stachon T, Latta L, Wagenpfeil S, Langenbucher A, Seitz B (2018) The effect of antiamoebic agents on viability, proliferation and migration of human epithelial cells, keratocytes and endothelial cells, in vitro. European Cornea Conference; Potsdam, Germany; Lecture.

[7]. Shi L, Hager T, Fries FN, Daas L, Holbach L, Hofmann-Rummelt C, Zemova E, Seitz B, Szentmáry N (2018) Reactive uveitis, retinal vasculitis and scleritis as ocular end-stage of *Acanthamoeba* keratitis – a histological study. DOG Congress; Bonn, Germany; Poster.

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