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ORIGINAL ARTICLE

IMIDAZOLIUM IONIC LIQUID AS POTENTIAL CONTACT LENS DISINFECTANT INACTIVATING CYSTIC RESISTANCE FORMS FROM *Acanthamoeba* KERATITIS

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ABSTRACT

Keratitis caused by *Acanthamoeba* spp. is a rare disease, although increasingly common, especially among contact lens users. The occurrence and the devastating effect of this disease are associated with the lack of care in cleaning and disinfecting lenses and their storage cases, as well as ineffective drugs to mainly eliminate the parasite's cysts. This work evaluated the amoebicidal activity of the imidazolium salt 1-hexadecyl-3-methylimidazolium chloride (C_{16}MImCl) against cysts of two characterized isolates from *Acanthamoeba* keratitis cases (MZ404337 and MZ404332). The inactivation of 100% of the cysts was achieved at a concentration of 7.81 µg/mL for MZ404337 and of 1.95 µg/mL for MZ404332, both at 24 h and 48 h of exposure time. In contrast, a commercial formulation of chlorhexidine did not cause any reduction in the viability of the cysts. Related to the cytotoxicity to human HaCaT cells, C_{16}MImCl is biocompatible at the concentration required to inactivate cysts. This shows that C_{16}MImCl is a promising disinfectant for contact lenses and surfaces.

KEY WORDS: 1-hexadecyl-3-methylimidazolium chloride; *Acanthamoeba* keratitis; cysts; chlorhexidine; cytotoxicity.

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INTRODUCTION

Free-living amoebae (FLA) are ubiquitous organisms, widely dispersed in the environment, and they can be found in soils, cooling towers, rivers, lakes, sewers, swimming pools, and other water sources as well as in the air (Khan, 2006). Among the FLA, *Acanthamoeba* spp. stands out for its great prevalence in the environment. The genus *Acanthamoeba* has two forms of life: the trophozoite, which is the vegetative form and the cystic one, the latter being the form of resistance of the microorganism in the environment (Schuster & Visvesvara, 2004). These microorganisms are considered pathogens because they can cause disease in a healthy individual, or opportunistic, when they reach immunocompromised individuals (Visvesvara et al., 2007). *Acanthamoeba* spp. can cause granulomatous amoebic encephalitis (GAE) leading to very serious neurologic problems and amoebic keratitis (AK), which can culminate in blindness (Siddiqui & Khan, 2012; Lorenzo-Morales et al., 2015). *Acanthamoeba* spp. are excellent predators and they are considered the Trojan horse of the microbial world, being therefore involved in the dispersion and the persistence of microorganisms in the environment and in living beings (Horn & Wagner, 2004; Saberi at al., 2020; Chaúque & Rott, 2022). This parasite can encompass microorganisms such as bacteria, fungi, viruses and other protozoa that resist phagocytosis and live internalized. Some bacteria may increase their pathogenicity by coming into contact with the amoeba (Greub & Raoult, 2004; Khan & Siddiqui, 2014; Thewes et al., 2019).

Many cases of AK have been described primarily due to increased use of contact lenses (CL). Frequent reports reveal that CL users who contracted the disease had a history of recreation or diving in aquatic environments while using their CL, handling of the lens with wet hands and the use of non-sterile cleaning solutions, in addition to the lack of disinfection and maintenance of both lenses and their storage cases (Alves et al., 2018; dos Santos et al., 2018; Chaúque et al., 2022). It is important to note that there are no completely effective CL cleaning and preserving solutions against *Acanthamoeba*. This disease is aggravated by the possibility of the parasite producing cysts, hindering the action of cleaning and disinfecting products for storage cases and CL, as well as the solutions used for the treatment (Siddiqui & Khan, 2012; Lorenzo-Morales et al., 2013). Therefore, it is necessary to research new drugs that may combat the cysts. There is an effort to prospect drugs of biological and chemical origin that show activities against protozoa (Benitez et al., 2011; Faber et al., 2017; Fabres et al., 2020). A promising class of compounds for the development of new drugs is that of the imidazolium salts (IS).

These IS are organic compounds made up of an imidazolium-based cation and organic anion. Among the IS, those that are in the molten state at 100 °C are classified as ionic liquids (Riduan & Zhang, 2013; Biczak et al., 2014). The IS have very versatile physicochemical properties, including

negligible volatility, non-flammable, high thermal and chemical stability, electrical conductivity and ability to dissolve organic and inorganic compounds (Biczak et al., 2014). Several biological activities of IS have been reported: antibacterial, anti-inflammatory, anti-yeast, antifungal and antiparasitic (Fang et al., 2010; Schrekker et al., 2013; Dalla Lana et al., 2015; Ghashghaei et al., 2018). IS were recently tested for the first time in *Acanthamoeba* spp. trophozoites showing promising activities (Fabres et al., 2020).

This present study evaluated the amoebicidal activity of the IS (1-n-hexadecyl-3methylimidazolium chloride (C_{16}MImCl) (Figure 1) against *Acanthamoeba* spp. cysts. The amoebicidal effect was tested against cysts of MZ404337 and MZ404332 isolates, obtained from the corneal scraping of patients with AK, belonging to the T4 genotype, already characterized and deposited in GenBank (dos Santos et al., 2022). The MZ404332 isolate has an endosymbiont called *Candidatus Paracaedibacter acanthamoebae* (Horn et al., 1999; Schmitz-Esser et al., 2008) and it was obtained from a patient with AK who had undergone a corneal transplant in both eyes. Several authors (Heinz et al., 2007; Rayamajhee et al., 2021) claim that endosymbionts can potentiate the virulence of the amoeba, exacerbating the diseases, reinforcing the importance of research on clinical samples of amoebas containing endosymbionts.

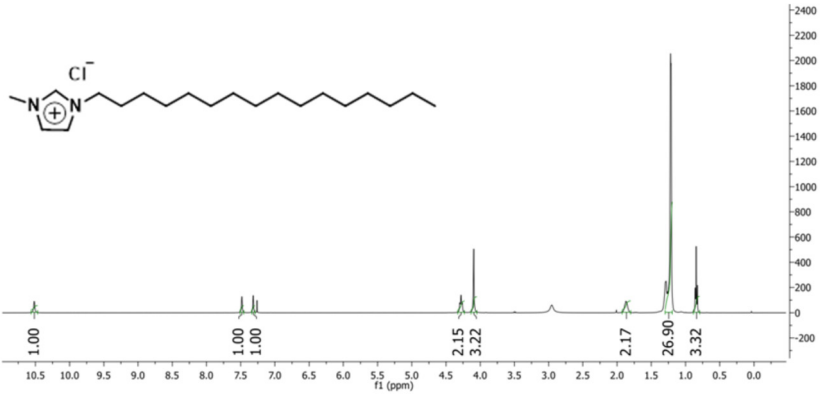

Figure 1. Chemical structure of 1-n-hexadecyl-3-methylimidazolium chloride (C16MImCl), and 1H NMR spectrum of C16MImCl (400 MHz, CDCl3).

MATERIAL AND METHODS

Preparation of the imidazolium salt

C_{16}MImCl was prepared as previously described in the literature (Zhou & Antonietti, 2004; Shen et al., 2019) using the following adapted method: 1-chlorohexadecane (39.79 mmol) was added to 1-methylimidazole (39.79 mol) in a 50 mL round-bottom flask, mixed at 90 °C for 24 h, and then it was cooled down to room temperature. The product was purified by recrystallization in tetrahydrofuran. After being washed three times with tetrahydrofuran and two times with diethyl ether, the white crystalline powder was collected by filtration and dried at room temperature in a vacuum oven. The spectral data were in accordance with those reported (see the Nuclear Magnetic Resonance (^{1}H NMR) spectrum in the Figure 1). Before use the IS was diluted in Milli-Q® water to the desired concentration.

Trophozoites culture and cyst stock preparation

Trophozoites of *Acanthamoeba* spp. from AK (MZ404337 and MZ404332) were isolated and cultivated in peptone yeast glucose (PYG) medium (2% proteose peptone, 0.2% yeast extract and 1.8% glucose), supplemented with antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL), and incubated at 30 °C. For the experiments, 1 mL of each culture was centrifuged for 5 min at 2,500 rpm. The supernatant was discarded, and the precipitate washed twice with phosphate buffered saline (PBS) and then it was diluted in PYG medium to obtain a final concentration of 10^4 cells/mL (Schuster, 2002).

For the cyst stock the trophozoites were suspended in Neff encystment solution (0.1 M KCl, 0.02 M trisamine, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$) supplemented with antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL) and incubated for 72 h at 30 ºC. Dilution was performed in encystment solution to obtain a final concentration of 10^4 cysts/ mL (Schuster, 2002).

Assessment of amoebicidal activity

The amoebicidal activity assessment of C_{16}MImCl on cysts was performed by serial dilution (100 µL of Milli-Q® water and 100 µL of IS) and the final concentrations tested were: 250, 125, 62.5, 31.25, 15.62, 7.81, 3.91, 1.95, 0.97 and 0.49 µg/ mL. The minimum inhibitory concentration (MIC) values of C_{16}MImCl were determined using the concentration of 10^4 cysts/ mL in 96-well plates (100 µL). The plates were sealed and incubated at 30 °C for 48 h. These experiments were performed in triplicate and with three repetitions. Cysts were counted using a Fuchs-Rosenthal chamber and viability was checked using the trypan blue exclusion test (0.4%). Chlorhexidine (0.02%) and untreated cysts

were used as positive and negative controls, respectively. MIC values were defined as the lowest concentration of compound capable of inhibiting visible amoebic viability. The cysts, after the amoebicidal evaluation, were plated on non-nutrient agar (NNA) with a layer of heat inactivated *Escherichia coli* (gold standard to evaluate the viability) (Visvesvara, 2013), to be monitored for seven days and to confirm its viability, using a concentration below and above the MIC (Bernardes et al., 2020; Fabres et al., 2020).

Cell culture and cytotoxicity assay

The cell line 3T3 (embryo fibroblasts from mice NIH-3T3), SH-SY5Y (derived from a human neuroblastoma) and HaCaT (human keratinocytes cells) were originally obtained from Cell Bank of Rio de Janeiro (BCRJ/ Brazil). The 3T3 and HaCaT cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Sigma®) supplemented with 10% fetal bovine serum (FBS) (Cultilab®). The SH-SY5Y cells were cultivated in DMEM Low+Ham F12 (1:1, v/v) medium supplemented with 10% FBS. The cultures were kept in a semi-open system (standard condition) under a humid atmosphere with 5% CO_2, 37 ºC, using the trypsinization process for their maintenance. For the assays, 100,000 cells/ well of SH-SY5Y and 15,000 cells/ well of 3T3 or HaCaT were plated on 96-well polystyrene microplates and they were kept under usual conditions until reaching sub confluence (Capes-Davis & Freshney, 2015).

For exposure to C_{16}MImCl, the cell culture medium was replaced by another media containing 1% FBS and C_{16}MImCl at concentrations of 0.001, 0.01, 0.1, 1, 10 and 100 µM, in quadruplicates, and maintained for 96 h. As negative control, cultures kept in culture medium with 1% FBS were used, and as positive control were used cultures incubated with 1% hydrogen peroxide (Synth®) for 60 min.

Cytotoxicity was evaluated at the end of the incubation period through the reduction of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) that determines mitochondrial functionality; and neutral red uptake (NRU), used to assess lysosomal viability. Briefly, in the MTT assay, the cells were washed with buffered saline solution (calcium and magnesium free-CMF), culture medium containing 200 µg/mL of MTT was added and the cultures were kept in an incubator for 2 h (Mosman, 1983). After discarding this medium, the formazan crystals were dissolved with DMSO (Nuclear®), followed by reading in a microplate spectrophotometer (Spectramax M3, Molecular Devices®) at 570 nm. For the NRU assay, after washing with CMF, medium containing 50 µg/ mL of NRU was added and incubated for 3 h. After washings with CMF and fixation with 1% $CaCl_2$ solution in 0.5% formaldehyde, the incorporated NR was eluted with 1% acetic acid in 50% ethanol, followed by the reading at 540 nm (Borenfreund & Puerner, 1985).

Data analysis

Data normality was assessed using the Shapiro-Wilk test. The two-tailed paired *t*-test, as well as analysis of variance followed by Tukey's test were used to assess the significance of the difference in the data. The Kruskal-Wallis test was used in cases of non-normal data distribution. A value of $p < 0.05$ was considered significant. The BioEstat 5.0 software was used to perform the analysis and the GraphPad prism 8.02 program to plot the graphs.

The selectivity index (SI) of the IS was calculated as the ratio of cytotoxicity to biological activity (SI= CC_{50} cells/IC_{50}cysts). The CC_{50} was estimated by the minimum square method and IC_{50} corresponds to the dose capable of killing 50% of the parasites.

RESULTS

Assessment of amoebicidal activity against cysts

Total reduction in cyst viability was obtained with a concentration of 7.81 and 1.95 µg/mL of C_{16}MImCl at both exposure times (24 and 48 h) for MZ404337 and MZ404332, respectively (Figure 2). The half-maximal inhibitory concentration (IC_{50}) values of 6.43 µg/mL and 1.70 µg/mL were calculated for C_{16}MImCl against cysts of the MZ404337 and MZ404332 isolates respectively. The cysts that remained viable at concentrations below 7.81 and 1.95 µg/mL of C_{16}MImCl (Figure 3) for MZ404337 and MZ404332, respectively, did not differ significantly from the corresponding controls, either for 24 h or for 48 h of exposure time ($p < 0.05$). In contrast, to C_{16}MImCl no reduction in cyst viability was observed when the cysts were exposed to chlorhexidine (200 µg/mL) for 24 and 48 h.

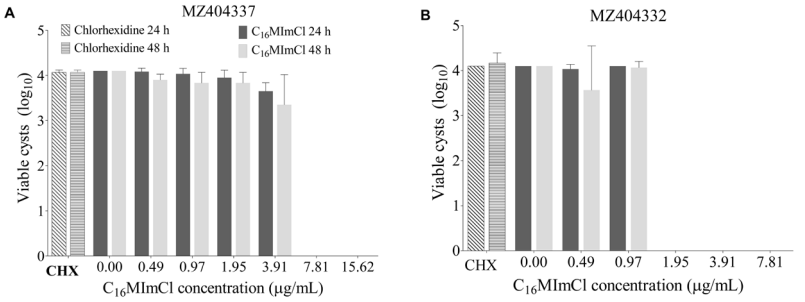

Figure 2. Cyst inactivation profile of MZ404337 (A) and MZ404332 (B) isolates exposed to C_{16}MImCl for 24h and 48h.

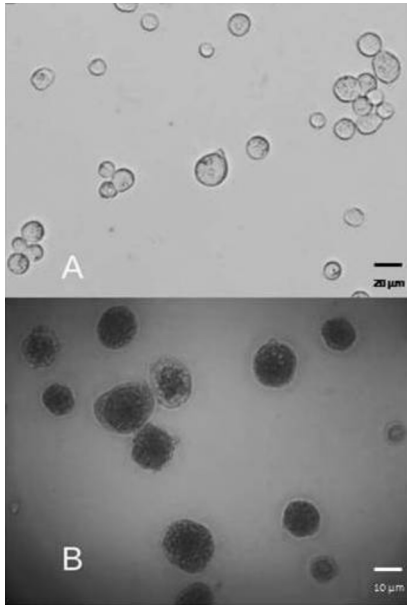

Figure 3. Optical micrographs (×1000) of A - viable cysts (control) and B - unviable cysts exposed to C_{16}MImCl for 48 h and stained with trypan blue.

Cytotoxicity assay

For the cytotoxicity of C_{16}MImCl, the CC_{50} (50% cytotoxic concentration), calculations provided the following results: 16.42 µg/mL (3T3), 17.57 µg/mL (SH-SY5Y) and 171.50 µg/mL (HaCaT).

DISCUSSION

Our findings show that C_{16}MImCl has strong biocidal properties against *Acanthamoeba* spp. cysts. A drastic reduction in cyst viability (100% inactivation 4 $\log_{10}$) of MZ404337 was obtained after the exposure to this IS at a concentration of 7.81 µg/mL for 24 h. On the other hand, no reduction in cyst viability was obtained in treatments with exposure to chlorhexidine (200 µg/mL) both for 24 h and 48 h. As far as we know, this is the first study to report on amoebicidal activity of IS against cysts of *Acanthamoeba* spp. The high efficacy of C_{16}MImCl against *Acanthamoeba* cysts suggests that this IS has a high capacity to the double layered cyst's wall layer, which usually makes them highly resistant to several biocidal factors, including UV radiation, salinity, chlorine-based disinfectants (Chaúque & Rott, 2021), as well as several drugs (Anwar et al., 2018; Wekerle et al., 2020). On the other hand, it is known

that chlorhexidine also leads to bacterial cell membrane disruption, causing the microorganism's death (Karpinski & Szkaradkiewicz, 2015) thus, at low concentrations it is bacteriostatic and bactericidal at higher concentrations (Steinsapir & Woodward, 2017). Importantly, the inability of chlorhexidine to inactivate *Acanthamoeba* cysts suggests that this drug could not cross the barrier established by the rigid cyst wall.

The possible explanation for the best result (MIC of 1.95 µg/mL) obtained for C_{16}MImCl against MZ404332 cysts may be related to the presence of the endosymbiont. Similar cases have been reported in literature. For instance, the study on the relationship between the *Wolbachia* endosymbiont (obligate intracellular bacterial) and filarial worm revealed that the death of *Wolbachia* occurred at low concentrations of antibiotics that may not initially cause damage to the worm's cells. Nevertheless, these may have been sufficient for the antibiotics to infiltrate in the bacteria before signaling and triggering host defense mechanisms (Bulman et al., 2021). Another study (da Luz Becker et al., 2015) revealed a high incidence of *Mycoplasma hominis* infected *Trichomonas vaginalis* susceptible to metronidazole. Interestingly, studies with microbiota present in *Aedes aegypti* larvae suggested that bacterial communities interacted with the IS, enhancing its larvicidal action (Pilz-Júnior et al., 2019). When comparing the cysts of the two isolates, most likely the IS may have made the obligatory endosymbiont unfeasible, which contributed to the death of the amoeba.

The cytotoxicity assays revealed that C_{16}MImCl is cytotoxic to 3T3 and SH-SY5Y cells at the concentrations tested. This agrees with the cytotoxicity reported for C_{16}MImCl to macrophages and brain cells (Fabres et al., 2020). Although, these findings suggest that this IS has little potential to be applied in the production of eye drops, complementary cytotoxicity studies with human corneal cells are necessary to verify the effect of direct contact with human eyes. On the other hand, C_{16}MImCl was less cytotoxic to HaCaT cells revealing that it is possible to manipulate the product as a disinfectant without damage or injury to the skin.

In the context of *Acanthamoeba* spp., C_{16}MImCl was identified as a highly effective cysticide, outperforming the gold standard chlorhexidine. As this compound is biocompatible for contact with skin, it is possible for the preparation of a disinfectant for surfaces and for the contact lens cases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest to disclose.

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