UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Disciplina de Trabalho de Conclusão de Curso

Activity of 1-*n*-hexadecyl-3-methylimidazolium methanesulfonate salt against *Candida tropicalis* biofilm

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Eduardo Álvares Balbueno

Prof. Dr. Alexandre Meneghello Fuentefria

Orientador

MSc. Bruna Pippi

Co-orientadora

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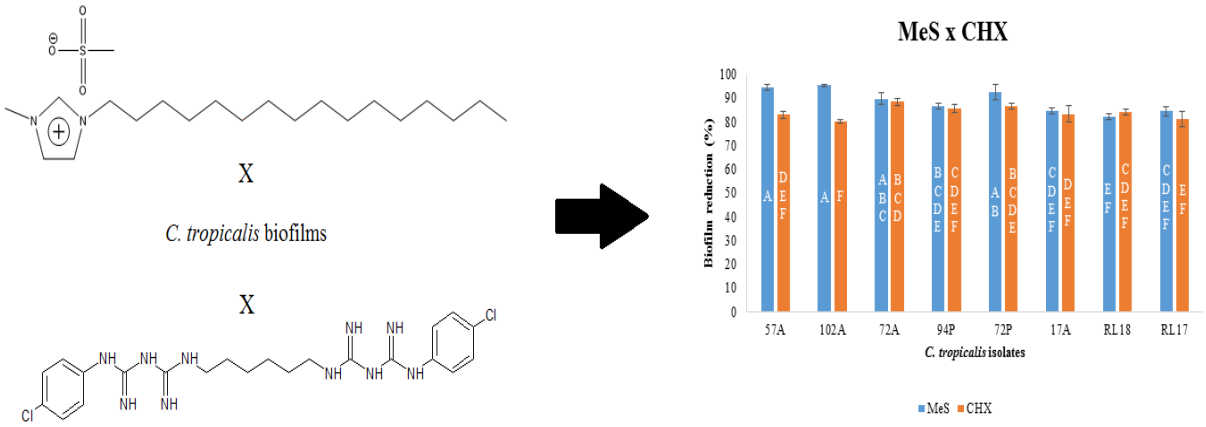

Biofilm reduction percentage between C16MImMeS and CHX, against biofilms formed by different isolates of *C. tropicalis*. Different letters represent a statistically significant difference ($p < 0.05$).

Activity of 1-*n*-hexadecyl-3-methylimidazolium methanesulfonate salt against *Candida tropicalis* biofilm

Eduardo Álvares Balbueno[a]; Camila Hatwig[a]; Bruna Pippi[a]; Ricardo Donato[b]; Henri S. Schrekker[b]; Alexandre Meneghello Fuentefria[*a,c];

[a]Laboratório de Micologia Aplicada and [c]Departamento de Análises Clínicas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul. Avenida Ipiranga, 2752 Porto Alegre-RS, Brazil.

[b]Laboratório de Processos Tecnológicos e Catalises, Instituto de Química, Universidade Federal do Rio Grande do Sul. Avenida Bento Gonçalves, 9500 Porto Alegre-RS, Brazil.

*Corresponding Author: alexandre.fuentefria@ufrgs.br

RESUMO: O uso de cateteres em pacientes críticos é essencial. Embora esses dispositivos sejam necessários para o tratamento, eles apresentam vários riscos à saúde do paciente. *Candida tropicalis* é uma levedura patogênica altamente prevalente que tem a capacidade de desenvolver uma extensa matriz polimérica, dificultando a penetração de fármacos. Esse estudo tem como objetivo avaliar a capacidade do sal metanosulfonato de 1-*n*-hexadecil-3-metilimidazol (C_{16}MImMeS) em erradicar biofilme dessa espécie e comparar a sua eficácia com a clorexidina (CHX), a qual é um desinfetante de primeira escolha em hospitais. As concentrações mínimas necessárias de C_{16}MImMeS e de CHX para erradicar o biofilme foram avaliadas por meio da exposição do biofilme formado em microplaca a diferentes concentrações dessas substâncias. C16MImMeS foi capaz de remover o biofilme em uma concentração oitenta vezes menor que a CHX, onde foram necessários 15,625 μg/mL de sal imidazólico e 1250 μg/mL de CHX para a erradicação do biofilme, demonstrando o grande potencial desta substância para o controle de infecções hospitalares.

Palavras chave: *Candida tropicalis,* biofilme, sais imidazólicos, clorexidina

ABSTRACT: The use of catheters in critically ill patients is essential. Although these devices are needed for treatment, they present several risks to patients' health. *Candida tropicalis* is highly prevalent pathogenic yeast, which has the ability to develop an extensive polymeric matrix hindering the drugs' penetration. This study is an attempt to demonstrate the ability of the 1-*n*-hexadecyl-3-methylimidazolium methanesulfonate salt (C_{16}MImMeS) for eradicating biofilm of this species and to compare these results with chlorhexidine (CHX), which is the standard disinfectant in hospitals. The minimum concentration required of C_{16}MImMeS and CHX for eradicating biofilm was evaluated through biofilm exposure to

different concentrations of these substances on microtiter plates. C_{16}MImMeS was able to remove biofilm with eighty times lower concentration than with CHX, i.e. for biofilm eradication were required 15.625 μg/mL of the imidazolium salt and 1250 μg/mL of CHX, demonstrating the great potential of this substance for nosocomial infections control.

Key words: *Candida tropicalis*, biofilm, imidazolium salt, chlorhexidine.

Introduction

The use of central venous catheters in critically ill patients; such as those with cancer, total parenteral nutrition and hemodialysis therapies, is essential.[1] Although their importance for treatment, they present several risks and their use has been related with a variety of complications. Bacteria and fungi can colonize the catheters and form biofilms, thus these medical devices are constantly associated with bloodstream infections, especially candidemia. The skin and mucous membranes changes caused by the use of catheters are predisposing factors for invasive candidiasis, e.g. in patients admitted to intensive care units.[2] Furthermore, the resistance of *Candida* spp., as well as other species of fungi, to antifungal agents is enhanced by biofilm formation in medical implants.[1,3] *Candida* biofilms are matrix-enclosed microcolonies of sessile yeast cells, which are effectively shielded from the antibiotics and more virulent than planktonic cells.[3,4]

Candida species usually coexist with humans, as commensals, without causing diseases to healthy individuals. They can become, however, opportunistic pathogens, especially in immunocompromised patients.[6] *Candida tropicalis* is one of the highly prevalent pathogenic yeasts in *Candida* non-*albicans* species and is frequently reported in immunocompromised patients with bloodstream infections, urinary tract infections and also have been associated with cancer, especially in patients with leukemia or neutropenia.[3,5] The use of antimicrobial substances in the external and internal surfaces of the catheters has helped reducing the risk of biofilm formation related candidemia episodes, becoming a standard practice in hospitals and medical centers.[1-3]

Chlorhexidine gluconate (CHX) is a broad spectrum antimicrobial agent widely used

in hospital routine. Mainly, it is applied for *in loco* catheter insertion asepsis, but is also has been used as hands disinfector and in mouthwashes. Several reports in the literature show that CHX is effective against both Gram-negative and Gram-positive bacteria, also various species of fungi, including *C. tropicalis.*[5] The increased microbial evolution against antibiotics is a matter of concern throughout the medical community. Susceptibility of *Candida* biofilms to CHX has been significantly reduced when compared with non-adhered organisms.[7] The development of innovative approaches to avoid this microbial tolerance, e.g. the search for new compounds which can act on fungal biofilm, is necessary and the use of imidazolium salts (IMS) can be an alternative confront such issues.[8]

The IMS are ionic compounds constituted of a cationic imidazolium unity. The neutral (uncharged) version of this study is found in known antifungal agents, such as ketoconazol and miconazole, and is responsible for the biological activities presented by these drugs[9]. IMS exhibit various specific and interesting properties, such as; neglectable volatility and flammability, low melting points, high thermal and chemical stability and tunable viscosity[10]. Furthermore, recent studies show that these salts exhibit antifungal activity against *Candida* species at extremely low concentrations.[9,10]

Considering these properties, this study aims demonstrating the ability of IMS 1-n-hexadecyl-3-methylimidazolium methanesulfonate (C_{16}MImMeS) to eradicate *C. tropicalis* at very low concentrations. In order to evaluate the applicability of IMS for the asepsis of catheters, its antimicrobial antiseptic properties were compared to CHX in biofilm from eight clinical isolates of *C. tropicalis*.

Experimental

Fungal strains

Eight clinical isolates of *C. tropicalis* (72A, 72P, 94P, 102A, 17A, 57A, RL17 and RL18) were used in this study. The isolates 72A, 72P and 94P are resistant to Fluconazole (Cristália®), Amphotericin B (Sigma), Voriconazole (Sigma) and Anidulafungin (Pfizer®). All microbial strains are deposited in the Mycology Collection of the Universidade Federal do Rio Grande do Sul-UFRGS, Porto Alegre, Brazil.

Chemical compound

The 1-*n*-hexadecyl-3-methylimidazolium methanesulfonate salt [C_{16}MImMeS] was synthesized as previously reported in the literature.[11-13]

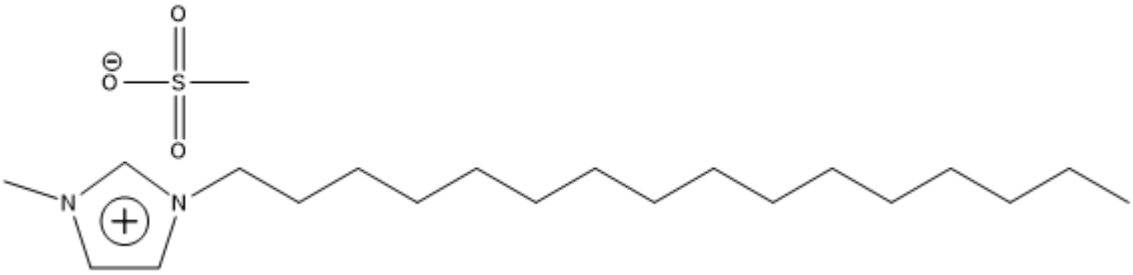

Figure 1: Chemical structure of 1-*n*-hexadecyl-3-methylimidazolium methanesulfonate.

Working solutions to conduct the experiments were prepared in sterile Roswell Park Memorial Institute 1640 broth medium (RPMI 1640; Gibco), using a commercially acquired CHX 10% *solution*.

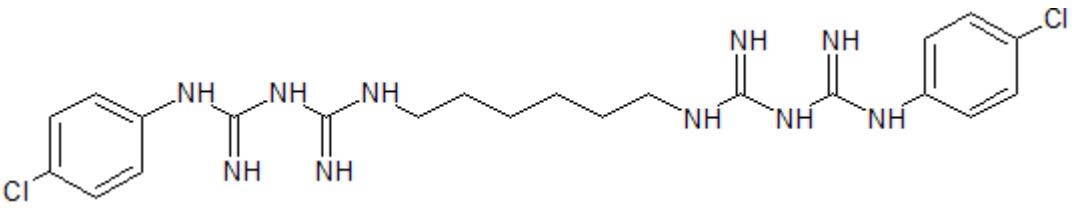

Figure 2: Chemical structure of chlorhexidine.

Minimal biofilm eradication concentration (MBEC)

For testing the susceptibility of biofilm cells to C_{16}MImMeS and CHX, the method of Ramage *et al.*,[14] was used with some modifications. The isolates were cultivated in Sabouraud Agar for 24 h at 35 °C and then the inoculum of fungal suspensions of each isolate was prepared (0.5 McFarland). A 20 µL of inoculum were added to each well of a pre-sterile commercial polystyrene flat-bottom 96-well microtiter plate, which was then filled with 180 µL per well of RPMI 1640 medium. The plates were incubated for 48 h at 35 °C to favor the formation of the fungal biofilm and, then, non-adherent cells were removed by washing the wells with 150 µL of sterile saline solution. The remaining attached fungi were covered with 100 µL of RPMI 1640 medium and exposed to different concentrations of C_{16}MImMeS (125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.625 µg/mL and 7.813 µg/mL) and CHX (10000 µg/mL, 5000 µg/mL, 2500 µg/mL and 1250 µg/mL). Untreated biofilm wells were used as biofilm formation controls, where only inoculum and RPMI were added. The plates were incubated for 48 h at 35 °C. The solutions of C_{16}MImMeS and CHX were removed and the wells were rinsed three times with sterile saline solution. The effect of the substances was determined by addition of the colorimetric reagent MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) and isopropyl alcohol (Vetec) was used to extract the MTT formazan crystals, which were responsible for the coloration of viable cells of the biofilm. The absorbance was measured in a microtiter plate reader SpectraMax at a dual wavelength of 570 and 690 nm. Absorbance is adjusted by subtracting computed values at 570 nm from absorbance at 690 nm.

All biofilm experiments were performed in triplicate and the MBEC was defined as the minimum concentration of the substance required eradicating the biofilm. It is considered

as a positive result the compound concentrations with ability to remove more than 80% of the microplate formed biofilm. The biofilm removal percentage results described below were obtained using the eq. 1.

$$100.00\% - \left(\frac{Abst}{Abspc} x\ 100\right)$$

Abs_t – Subtract of computed values at 570 nm and 690 nm of substances CHX and C_{16}MImMeS.

Abs_{pc} – Subtract of computed values at 570 nm and 690 nm of untreated biofilm wells.

Statistical analysis

Results were statistically analyzed by one-way ANOVA, followed by a comparison of means by Tukey. $P < 0.05$ was considered statistically significant.

Results and Discussions

The biofilm removal results obtained for the different concentrations of C_{16}MImMeS are shown in Table 1. It could be seen that a solution with 15.625 µg/mL of this substance was able to eradicate more than 80% of the biofilm for all isolates tested. This concentration was considered the MBEC of C_{16}MImMeS against biofilms formed of *C. tropicalis* species. Furthermore, diluting the previous solution to 7.813 µg/mL allowed observing that this IMS is still effective to eradicate the biofilm formed by 50% of the isolates (57A, 102A, 72P and 17A). Another study, also conducted by our research group, evaluated the MBEC of the chloride analog of C_{16}MImMeS, 1-*n*-hexadecyl-3-methylimidazolium chloride (C_{16}MImCl),

against biofilms of *C. tropicalis*. The results of this assay showed that low concentrations of C_{16}MImCl have the ability to eradicate biofilms of *C. tropicalis* formed on polystyrene microtiter plates, which demonstrate the potential use of this class of substances for such purposes.[15]

Table 1: Percentage of biofilm removal of *C. tropicalis* after exposure to C_{16}MImMeS for 48 h

	Concentration of C_{16}MImMeS (μg/mL)				
Strain	125	62.5	31.25	15.625	7.813
57A	97.65%	93.56%	93.56%	94.50%	94.94%
102A	95.31%	95.35%	96.92%	95.35%	93.52%
72A	93.46%	91.75%	90.69%	89.79%	73.94%
94P	90.35%	88.46%	87.56%	86.55%	72.84%
72P	100.00%	95.40%	93.37%	92.34%	87.86%
17A	98.77%	94.11%	90.23%	84.74%	82.97%
RL18	100.00%	90.88%	86.86%	82.16%	74.38%
RL17	95.95%	90.47%	86.42%	84.50%	78.12%

The results for CHX are shown in Table 2. This compound presents biofilm eradication above eighty percent with a concentration of 1250 μg/mL. The main difference between CHX and C_{16}MImMeS is the concentration required for this elimination occur. While CHX needs 1250 μg/mL to achieve only a satisfactory result for biofilm removal, C_{16}MImMeS acquire even higher removal yields with application of only 15.625 μg/mL. IMS demonstrated an equivalent biofilm removal action in concentration eighty times smaller than CHX against the 72A, 94P, 72P, 17A, RL18 and RL17 *C. tropicalis* isolates. For the biofilms formed by 57A and 102A, a 15.625 μg/mL concentration of C_{16}MImMeS

was more effective than 1250 μg/mL of CHX ($p < 0.05$). The biofilms formed by these two isolates, together with the 72P isolate, showed a reduction greater than 90% when exposed to 15.625 μg/mL of C_{16}MImMeS. Differently, when applied a 1250 μg/mL concentration of CHX, the biofilm removals values observed for all *C. tropicalis* isolates were below 90%, from which isolates 72A, 72P and 94P were the most affected.

Table 2: Percentage of biofilm removal of *C. tropicalis* strains after exposure to CHX for 48 h

	Concentration of CHX (μg/mL)			
Strain	10000	5000	2500	1250
57A	100.00%	96.00%	90.41%	83.06%
102A	96.93%	91.72%	85.55%	80.31%
72A	100.00%	95.05%	93.94%	88.48%
94P	98.82%	95.32%	94.10%	85.49%
72P	96.45%	95.02%	93.63%	86.52%
17A	100.00%	91.72%	86.30%	83.35%
RL18	97.48%	94.20%	88.47%	83.96%
RL17	98.01%	95.32%	87.33%	81.31%

The treatment after the adjusting and subsequently forming the biofilm has been accomplished with antibacterial, antifungal and antiseptics agents. A small number of antifungal drugs able to preventing candidiasis associated with implanted and infected medical devices, although, they usually need to be further removed.[16] *Candida* biofilms over polyvinyl chloride materials was reported to be more than 30 to 2000 times more resistant to antimicrobial agents than their planktonic form.[17-19] A study on *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* biofilm formation and their antifungal

susceptibility demonstrated that *C. tropicalis* species was the strongest biofilm former.[20] Moreover, all biofilms of the tested species are resistant to fluconazole. Among the evaluated *C. tropicalis* strains, a fluconazole concentration of 2048 μg/mL was not sufficient to eradicate the biofilm formed. *C. tropicalis* is able developing a more extensive polymer matrix than *C. albicans* and, therefore, allowing a smaller penetration of this drug into the formed biofilm. Beside these authors' observations on *C. tropicalis* biofilms resistance to fluconazole, it is also worth noting their high resistance to amphotericin B.[21] *C. tropicalis* biofilms resistance to echinocandins has also been reported.[22] There is no effective and non-invasive technique to treat biofilms associated contaminations on medical implants. Efforts to develop a non-invasive and effective treatment for such contaminations include the use of electrical and ultrasonic booster, also the use of antibiotics and antimicrobials applied on biomaterials.[23] *Candida tropicalis* is a frequent cause of candidemia in Latin American hospitals and is among the main Candida species isolated in Brazilian hospitals. In southern Brazilian states, which presents sub-tropical to temperate climate, *C. tropicalis* was the most frequent agent of fungal infections, representing 13.3 to 15% of infections causes.[24-26] The biofilms' resistance to currently available antifungal agents and their ability to form large polymer matrices lead to the search for new therapies. The seek for synthetic compounds with the capacity to remove of yeast biofilm is a current topic of interest. Among those, IMS has been shown promising results; different IMS were tested against *C. tropicalis* biofilm on polystyrene pegs, and demonstrated efficacy in a concentrations of 66 μg/mL,[27] and 62.5 μg/mL.[28] Interestingly, our tested IMS presenting a MeS anion presented the ability to remove fungal biofilms in a four times smaller concentration (15.625 μg/mL), showing the importance of the anion role for the antifungal and antibiofilm activity. Based on the data presented in this work, the IMS C16MImMeS may become an effective alternative for

controlling medical device associated *C. tropicalis* infections, since it presents similar biofilm removing capacity to CHX, but in extremely lower concentrations.

Conclusions

The results presented in this study are promising, considering the high persistence of multiresistant *C. tropicalis* biofilms to antifungal agents. The IMS C_{16}MImMeS demonstrated ability to effectively remove biofilm at very low concentrations, i.e. it demonstrated the same effectiveness of CHX (an efficient antibiofilm agent for catheter asepsis) at eighty times lower concentration. IMS has the potential to become a powerful alternative to CHX in the nosocomial infections control. However, more tests are still necessary to further elucidate the mechanisms of biofilm eradication, as well as the detailed toxicity studies are still been conducted.

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