FUROSEMIDE BLOCKS THE ANION CHANNEL FORMED BY STAPHYLOCOCCUS AUREUS α-HEMOLYSIN

A furosemida bloqueia o canal aniônico causado por α-hemolisina de Staphylococcus aureus

Luciana R. Teixeira[1], Janilson J. S. Junior[2], Pedro H. S. Vieira[3], Anne G. Marciel de Figueirêdo[3], Marcus V. G. Canto[3], Dijanah C. Machado[4], Joelmir L.V. da Silva[1]

[1]Docente/Faculdade de Medicina de Olinda; [2]Docente/Centro Universitário Maurício de Nassau; [3]PRODIIC/ Faculdade de Medicina de Olinda; [4]Docente/Departamento de Biofísica e Radiobiologia/Universidade Federal de Pernambuco

Received in: 05/27/2022 | Approved in: 06/22/2022

ABSTRACT

This study aimed to investigate the effect of furosemide on the *Staphylococcus aureus* α-hemolysin (α-HL) channel in planar lipid bilayer membranes using electrophysiological characterization and molecular docking analyses. Planar lipid bilayer membranes were prepared, and α-HL (0.07 mg/mL) was added to the standard solution in the *cis* compartment of the experimental chamber. All experiments were conducted at room temperature using the Axopatch 200A amplifier in voltage--clamp mode. The α-HL channels typically exhibited high conductance (~4 nS) and rarely switched to low conductance states at pH 7.5. Furosemide was added to the solution in the *cis* compartment after the ion channel was incorporated into the planar lipid bilayer membrane. For docking analyses, the Protein Data Bank (ID: 7AHL) was consulted for the atomic coordinates of the α-HL heptameric channel, and the furosemide structure was obtained from PubChem. The coordinates were built and minimized using the Avogadro software, and molecular docking experiments were conducted through the DockThor online platform. Furosemide inhibited the conductance of the α-HL channel in a voltage-dependent manner ($p < 0.05$). The two best docking solutions for the α-HL channel showed that the highest interaction affinity involved multiple hydrogen bonds, and residues 113 and 147 formed part of the constriction region of the α-HL channel. In conclusion, furosemide blocked ion currents at the constriction region of the *Staphylococcus aureus* α-HL channel.

Keywords: furosemide; *Staphylococcus aureus*; ion channel; virulence factors; anti-bacterial agent.

RESUMO

Investigar o efeito de furosemida no canal α-hemolisina (α-HL) de *Staphylococcus aureus* em bicamadas lipídicas planares (BLP) por caracterização eletrofisiológica e estudos de *docking* molecular. As BLP foram preparadas e α-HL (0,07 mg/mL) foi adicionada à solução padrão no compartimento *cis* da câmara experimental. Todos os experimentos foram realizados em temperatura ambiente usando um amplificador Axopatch 200A no modo *voltage clamp*. Em pH 7,5, os canais α-HL estavam geralmente em uma alta condutância (~ 4 nS) e raramente mudavam para estados de baixa condutância. Após a incorporação do canal iônico na BLP, a furosemida também foi adicionada à solução padrão no compartimento cis. Para os estudos de *docking*, as coordenadas atômicas para o canal heptamérico α-HL foram recuperadas *do Protein Data Bank* (ID: 7AHL), a estrutura de furosemida foi obtida do PubChem e suas coordenadas foram elaboradas e minimizadas com o software Avogadro. Os experimentos de *docking* molecular foram realizados usando o Dockthor online. A furosemida inibiu ($p < 0,05$) a condutância do canal α-HL de maneira voltagem-dependente. Foram avaliadas as duas melhores soluções de *docking* e o canal α-HL, observando-se que o modo de conexão com maior afinidade de interação possuía maior número de ligações de hidrogênio. Os resíduos de ligação foram o 113 e o 147, que formam os remanescentes de constrição do canal α-HL. Em conclusão, a furosemida bloqueia as correntes iônicas na

constrição do canal causado pela α-HL de *Staphylococcus aureus.*

Palavras-chave: furosemida; *Staphylococcus aureus;* canal iônico; fator de virulência; agente antimicrobiano.

INTRODUCTION

In the last decade, the rational design of drugs by using known chemical compounds for new indications (i.e., new targets) has been the most effective innovation approach, recognized as a model to advance drug research and development.[1] The *Staphylococcus aureus* α-hemolysin (α-HL) channel has an important pathogenic role and is a virulence factor involved in infection and multidrug-resistant strains.[2] Thus, the interest in repurposing known chemical compounds as antibiotics has emerged to target different bacterial mechanisms.[2]

Previous studies have assessed the ability of several compounds to inhibit α-HL by hindering its assembly in the membrane or directly blocking the channel.[3-6] In this context, furosemide is a loop diuretic with a wide range of pharmacological properties that compete with chloride to bind to the sodium-potassium-chloride cotransporter, inhibiting the reabsorption of sodium and chloride.[7] The α-HL is an exotoxin that forms lytic pores in the host cell membrane, playing a role in invasive infections.[8] During the exponential growth of *Staphylococcus aureus*, α-HL is secreted as a monomer, which oligomerizes into a heptameric transmembrane pore, causing osmotic cytolysis.[9]

Molecular docking studies have provided atomic details on protein-ligand interactions, which may enhance the knowledge of how compounds interact with the heptameric pore. Considering that furosemide is suggested as a potential therapeutic adjuvant for treating infected patients, the present study assessed its antimicrobial effect through the inhibition of the α-HL channel, which is a major virulence factor from *Staphylococcus aureus*.

MATERIALS AND METHODS

α-HL protein and other chemicals

The α-HL from wild-type *Staphylococcus aureus* was obtained from List Biological Laboratories. Solvent-free planar bilayer lipid membranes (PLM) with a capacitance of 40 pF were formed using the lipid monolayer apposition technique with diphytanoylphosphatidylcholine (DPhPC) in hexane at 25 ± 1 °C. Furosemide (4-chloro-2-[furan-2-ylmethylamino]-5-sulfamoyl benzoic acid) was purchased from Sigma, and DPhPC was purchased from Avanti Polar Lipids.

Single channel reconstitution in PLM

PLM were formed as previously described[10] by using two DPhPC monolayers. After membrane stabilization, a stock solution (0.1 to 0.4 μL) containing *α*-HL (0.07 mg/ml) was added to the *cis* compartment of the experimental chamber with the standard solution (4 M potassium chloride [KCl], 5 mM tris(hydroxymethyl)aminomethane, pH 7.5), resulting in a final concentration of ~2 ng/mL. All experiments were performed at room temperature (25 ± 2 °C) using the Axopatch 200A amplifier in voltage-clamp mode. The α-HL channels typically exhibit high conductance (~4 nS) and rarely switch to low conductance states at pH 7.5. After the ion channel was incorporated into PLM, anion inhibitors were added to the standard solution. All steps (from current measurement to data analysis) were performed using the equipment and software described in previous studies.[5,11,12]

Multiple channel reconstitution in PLM

PLM for multiple channel experiments were formed using the painting technique with a phosphatidylcholine/cholesterol mixture (1:1, w/w) across a ~0.3 mm diameter hole in a 25 mm-thick Teflon partition in a Teflon cell. The current was converted to voltage, filtered through an eight-pole Butterworth low-pass filter, and digitized at a sampling frequency of 0.5 kHz (for multiple channel experiments) using the Whole Cell Electrophysiology Program (V1.7b) or the Electrophysiology software.

Channels were formed by adding several microfilters of the α-HL stock solution (5 to 50 mg/mL) to the *cis* compartment of the chamber. The mean single-channel insertion current was 0.3 pA in the presence of calcium chloride (50 mM) and 40 mV potential. The potential was defined as positive when it was higher on the side of

protein addition. The α-HL channels typically remain in a high conductance and rarely switch to low conductance states at pH 7.5. The *t*-test was used to compare conductance in the GraphPad Prism software, and the statistical significance of mean differences was set at $p < 0.05$.

Molecular docking analyses

Docking experiments were conducted to predict furosemide position and orientation on the surface of α-HL channels and understand its atomic-level action mechanisms. Atomics coordinates for the α-HL heptameric channel were retrieved from Protein Data Bank (ID 7AHL).[13] The furosemide structure was obtained from PubChem, and its coordinates were built and minimized using the Avogadro software.[14] Molecular docking experiments were performed using the DockThor online platform,[15] focusing on the constriction region of the α-HL channel, which is probably the main region interacting with small ligands.[16] The output conformers from the software were ranked based on their increasing affinity for the channel, and the two conformers with the highest affinity were selected for further analysis.

RESULTS AND DISCUSSION

The ability of furosemide to block ion conductance was assessed using pores in artificial membranes due to the large single-channel conductance of the α-HL (~4 nS in 4 mM KCl) (Figure 1).

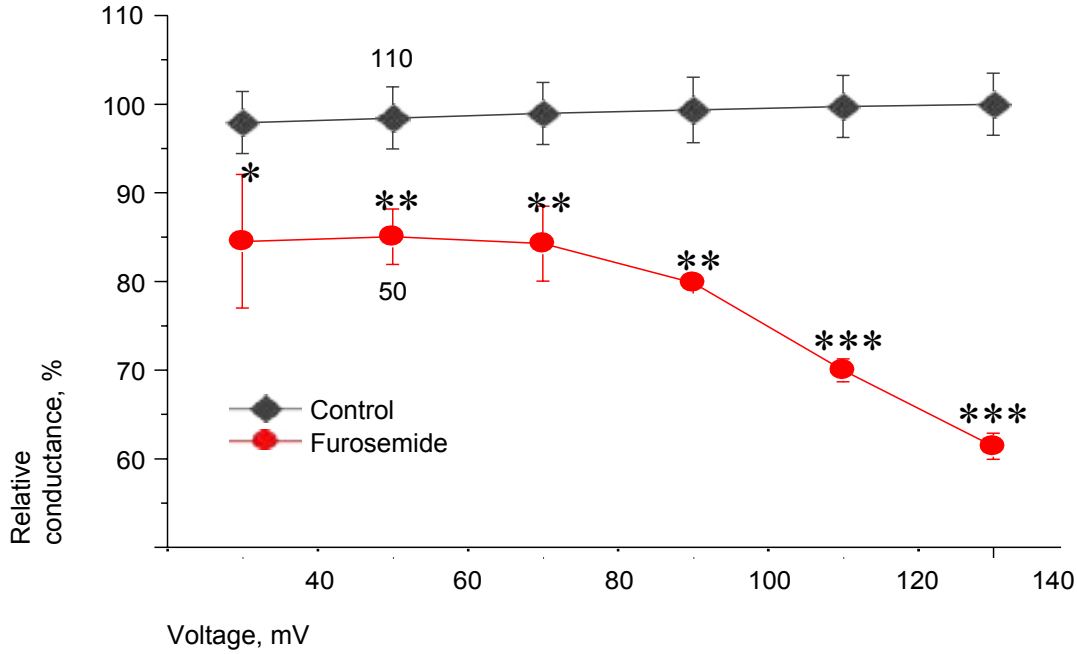

Figure 1. Voltage-dependent effect of inhibition from furosemide. The α-hemolysin channel conductance in the absence of anion inhibitors was considered 100% (control). The α-hemolysin channel conductance in the presence of 100 μM furosemide in the cis compartment was relativized to the control condition. Data presented as mean ± standard deviation for at least four experiments. *p < 0.05, **p < 0.01, ***p < 0.0001, t-test (control versus furosemide)

The relative conductance of the α-HL channel in the presence of furosemide was lower than in the standard solution and decreased with increasing transmembrane potential, indicating that its effect is voltage-dependent. The addition of 100 μM furosemide to the *cis* compartment of the membrane switched the channel to a closed state, similar to the "voltage-gated closed state" commonly observed for α-HL channels at ≥ 100 mV.

Figure 2 illustrates typical recordings of an ion current through a single α-HL channel. Even before the addition of compound 1, the conductance level of the single α-HL channels showed significant noise (Figure 2A, top track), which is characteristic of pores channels, exhibiting rapid flickering between open and fully closed conformations. The addition of furosemide to the *cis* compartment of the membrane (i.e., the toxin side) caused additional stepwise closures with an average duration of 6 ms (Figure 2B, middle).

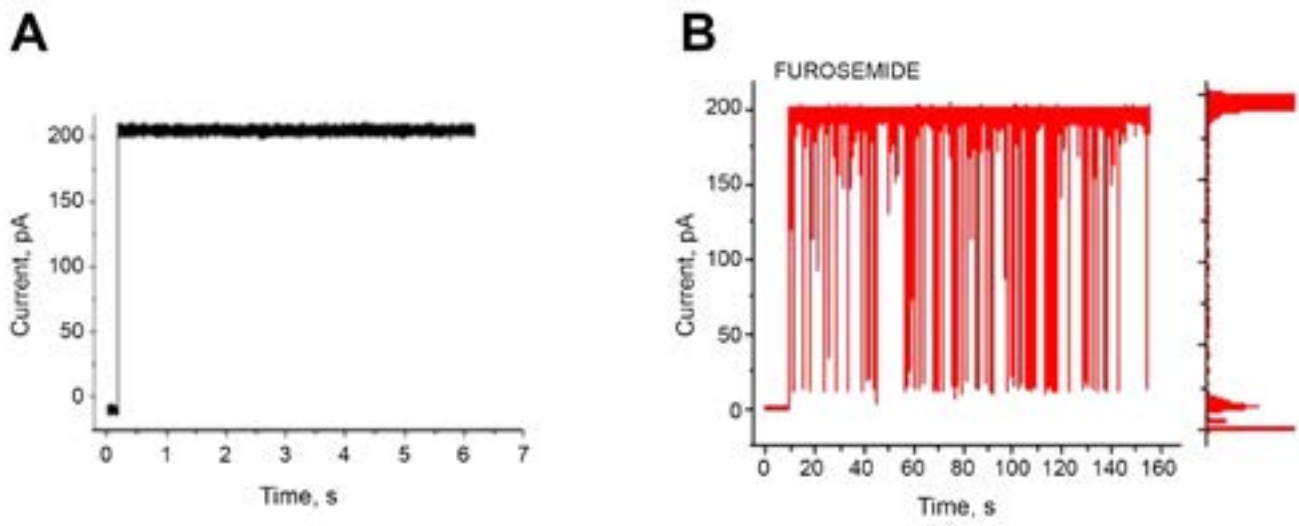

Figure 2. Effect of anion channel inhibitors on the α-hemolysin channel in planar lipid bilayer membrane. Ionic current through a single α-hemolysin channel in the absence of furosemide (A) and representative current recordings and all point current amplitude histograms illustrating the behavior of the α-hemolysin channel in the presence of 100 μM furosemide (B, red records) in the cis compartment. Voltage, +50 (upper traces) mV; filtering, 0.5 KHz; digitizing, 2.5 KHz.

These fluctuations were rapid transitions between the fully open and non-conducting states of the channel, and the blockade became frequent with 100 μM furosemide, similar to the voltage-gated closed state observed for α-HL channels at ≥ 50 mV (Figure 2B, bottom). The residual conductance in this closed state ranged between 1% and 15% of the total channel conductance. The current through a single α-HL channel remained stable, with no gating events observed at ≥ 100 mV.

Interestingly, the introduction of positive charges in the furosemide molecule enhanced its ability to block the α -HL channel on the *cis* compartment of the membrane. Unmodified drugs weakly bind to the heptameric α-HL channel when added to the *trans* (i.e., intracellular) compartment of the membrane. Also, furosemide presented similar effects to tamoxifen.[6]

The two furosemide conformers with the highest binding affinity to the α-HL channel presented binding energies of -6.766 and -6.634 kcal/mol, respectively. This binding showed the conformers were positioned in the same constriction region, occupying a small volume (Figure 3A). Furosemide binds to residues 113 and 147 of the α-HL channel, and four hydrogen bonds were observed with residue 147 near the constriction region of the channel (Figure 3B and 3C). Although these bonds stabilize furosemide within the nanopore lumen,[17] its polarity helps the solubilization within the solvent, justifying the rapid binding events observed in the experiments (Figure 2B).

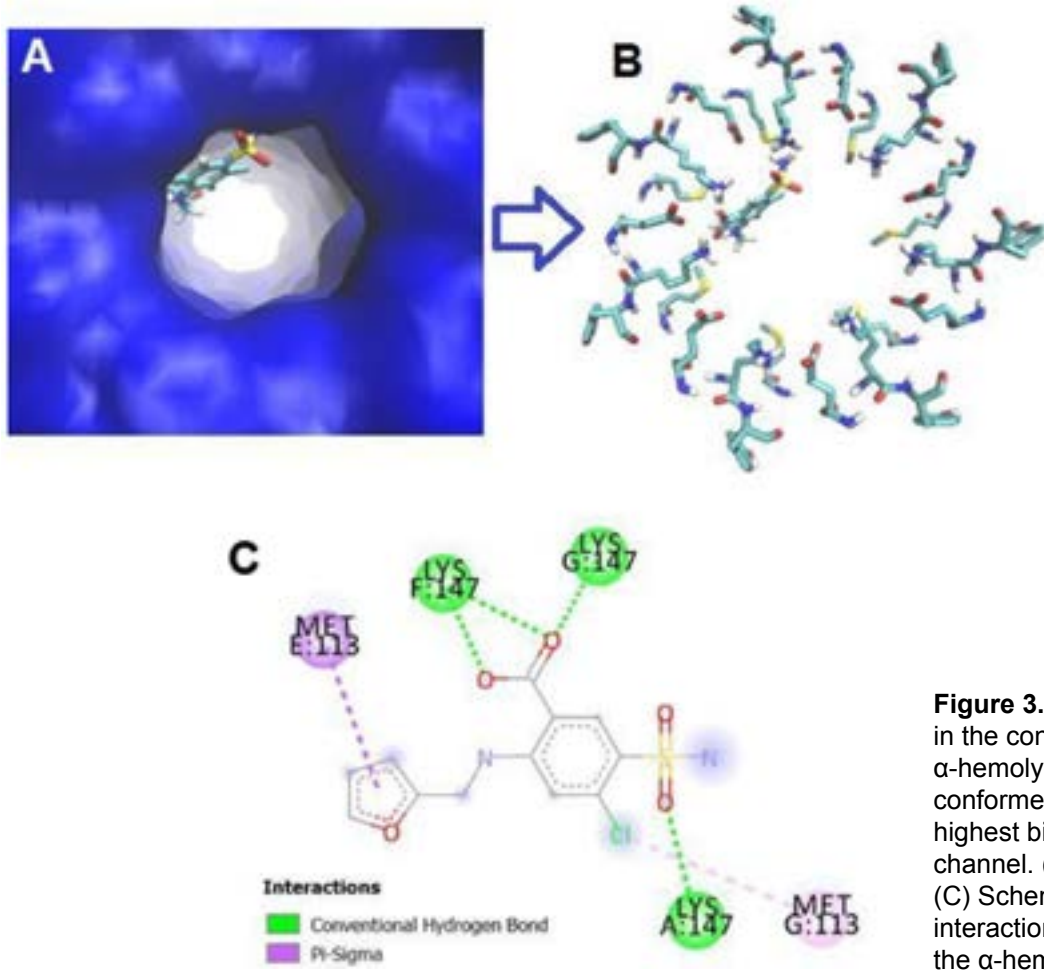

Figure 3. Illustration of furosemide in the constriction region of the α-hemolysin channel. (A) Docked conformer of furosemide with the highest binding energy coupled to the channel. (B) Licorice representation. (C) Schematic representation of interactions involving furosemide and the α-hemolysin channel.

The results of the present study suggested that furosemide blocked ion currents in the constriction region of the α-HL channel formed by *Staphylococcus aureus*. Thus, furosemide may be proposed as a potential therapeutic adjuvant for treating infected patients.

REFERENCES

1. Santoro FM. Innovación y sendero evolutivo en la industria farmacéutica: los casos de Argentina y España. Cadernos de Gestão Tecnológica 2000;48.
2. Bryant, A. E., Gomi, S., Katahira, E., Huang, D. B., & Stevens, D. L. The effects of iclaprim on exotoxin production in methicillinresistant and vancomycin- intermediate Staphylococcus aureus. Journal of Medical Microbiology 2019;684:56–466.
3. Qiu J, Wang D, Zhang Y, et al. Molecular modeling reveals the novel inhibition mechanism and binding mode of three naturalcompounds to Staphylococcal alpha-hemolysisn. PLOS One 2013;8:e80197.
4. Rani N, Saravanan V, Lakshmi PTV, et al. Inhibition of pore formation by blocking the assembly of Staphylococcus aureus alpha-hemolysin through a novel peptide inhibitor: an in silco approach. Int J Pept Res Ther 2014;20:575– 83.
5. Teixeira LR, Merzlyak PG, Valeva A, et al. Interaction of heparins and dextran sulfates with a mesoscopic protein nanopore. Biophys J 2009;97:2894–903.
6. Teixeira LR, Silva Júnior JJ, Vieira PHS, Canto MVG, Figueirêdo AGM, Silva JLV. Tamoxifen inhibits the anion channel induced by Staphylococcus aureus α-hemolysin: electrophysiological and docking analysis. RSD [Internet]. 2021;10(2):e13010212326.
7. Ellison DH, Felker GM. Diuretic treatment in heart failure. N Engl. J Med.2017;377(20):1964-1975.
8. Liu J, Kozhaya L, Torres VJ, Unutmaz D, Lu M. Structure-based discovery of a small-molecule inhibitor of methicillin-resistant Staphylococcus aureus virulence. J Biol Chem. 2020;295(18):5944-5959.
9. Gouaux JE, Braha O, Hobaugh MR, et al. Subunit stoichiometry of staphylococcal alpha-hemolysin in crystals and on membranes: a heptameric transmembrane pore. Proc Natl A Sci USA 1994;91:12828–31.
10. Montal M, Mueller P. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. Proc Natl A Sci USA 1972;69:3561–6.

11. Krasilnikov OV, Capistrano MP, Yuldasheva LN, et al. Influenceof Cys-130 S. aureus alpha-toxin on planar lipid bilayer and erythrocyte membranes. J Membrane Biol 1997;156:157–72.
12. Rodrigues CG, Machado DC, Silva AM et al. Hofmeister effect in confined spaces: halogen ions and single molecule detection. Biophys J 2011;100:2929–35.
13. Song L, Hobaugh MR, Shustak C, et al. Structure of staphylococcal alpha- hemolysin, a heptameric transmembrane pore. Science 1996;274:1859–66.
14. Hanwell MD, Curtis DE, Lonie DC, et al. Avogadro: An advanced semantic chemical editor, visualization, and analysis platform. Journal of Cheminformatics 2012; 4:17.
15. Magalhães CS, Marinho-Almeida D, Barbosa HJC, et al. A dynamics niching genetic algorithm strategy for docking of highly flexible ligands. Inform Sciences 2014;289:206–24.
16. Melo M. C. A., Teixeira. L. R., Pol-Fachin. L, Rodrigues C. G. Inhibition of the hemolytic activity caused by Staphylococcus aureus alpha-hemolysin through isatin-Schiff copper(II) complexes. FEMS Microbiology Letters, 2016,363(1):1-5.
17. Yunta MJR. It Is Important to Compute Intramolecular Hydrogen Bonding in Drug Design? American Journal of Modeling and Optimization. 2017,5(1):24-57.