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Tamoxifen inhibits the anion channel induced by *Staphylococcus aureus* α-hemolysin: electrophysiological and docking analysis

Tamoxifeno inibe o canal aniônico induzido por α-hemolisina de *Staphylococcus aureus*: análise eletrofisiológica e de docking

El tamoxifeno inhibe el canal aniónico inducido por la α-hemolisina de *Staphylococcus aureus*: análisis electrofisiológico y de acoplamento

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Abstract
To investigate the effects of tamoxifen on *Staphylococcus aureus* α-hemolysin channel (α-HL) in planar lipid bilayers with electrophysiological characterization and docking studies. Planar lipid bilayer membranes were prepared and *α*-HL (0.07 mg/mL) was added to the standard solution in *cis* compartment of the experimental chamber. All experiments were performed at room temperature using an Axopatch 200A amplifier in the voltage clamp mode. At pH 7.5, α-HL channels were usually in a high conductance ~4 nS and rarely switch to low conductance states. After the ion channel was incorporated in bilayer membrane, the tamoxifen was also added to the standard solution to the *cis* compartment. To docking studies, atomics coordinates for the α-HL heptameric channel was retrieved from PDB ID (7AHL) and the structure of tamoxifen was removed from the Pubchem, their coordinates were built and minimized with Avogadro software. The molecular docking experiments were performed using the Dockthor online portal. The tamoxifen inhibited ($P < 0.05$) α-HL channel conductance and it was a voltage-dependent manner. The three best docking solutions and the α-HL channel were evaluated, it was observed the connection mode with the highest affinity of interaction has a greater number of types of polar interaction. The residues present interactions of greater energy were 111 and 147 that form the remainders of the constriction in the channel of α-HL. The other conformers were accommodated in a region with more hydrophobic characteristics (valine 149). The mechanism of *Staphylococcus aureus* α-hemolysin inhibition by tamoxifen was blockade over the constriction of channel.
Keywords: Tamoxifen; *Staphylococcus aureus*; Ion channel; Virulence factors; Anti-bacterial agent.

Resumo
Investigar os efeitos do tamoxifeno sobre o canal de α-hemolisina obtido de *Staphylococcus aureus* (α-HL) em bicamadas lipídicas planas com caracterização eletrofisiológica e estudos de docking. As membranas planares 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 alta condutância ~ 4 nS e raramente mudam para estados de baixa condutância. Depois que o canal iônico foi incorporado na membrana, o tamoxifeno também foi adicionado à solução padrão no compartimento *cis*. Para estudos de docking, as coordenadas atômicas do canal heptamérico α-HL foram obtidas do PDB ID (7AHL) e a estrutura do tamoxifeno do Pubchem, suas coordenadas foram construídas e minimizadas com o programa Avogadro. Os experimentos de docking molecular foram realizados usando o portal online Dockthor. O tamoxifeno inibiu (P < 0,05) a condutância do canal α-HL de maneira de dependente de voltagem. Foram avaliadas as três melhores soluções de docking com o canal, observou-se que o modo de conexão com maior afinidade possui um maior número de tipos de ligações polares. Os resíduos que apresentam interações de maior energia foram o 111 e o 147, que formam os resíduos da constrição do canal α-HL. As outras conformações foram acomodadas em uma região com características mais hidrofóbicas (valina 149). O mecanismo pelo qual o tamoxifeno inibiu o canal α-hemolisina de *Staphylococcus aureus* foi por bloqueio da constrição deste canal.
Palavras-chave: Tamoxifeno; *Staphylococcus aureus*; Canal iônico; Fatores de virulência; Agente anti-bacteriano.

Resumen
Investigar los efectos del tamoxifeno sobre el canal de α-hemolisina obtenido de *Staphylococcus aureus* (α-HL) en bicapas lipídicas planas con caracterización electrofisiológica y estudios de acoplamiento. Se prepararon membranas planas y se añadió α-HL (0,07mg/ml) a la solución estándar en la cámara *cis* de la cámara experimental. Todos los experimentos se llevaron a cabo a temperatura ambiente usando un amplificador Axopatch 200A en el modo de "sujeción de voltaje". A pH 7,5, los canales de α-HL estaban generalmente en alta conductancia ~4nS y rara vez cambian a estados de baja conductancia. Después de que el canal de iones se incorporó a la membrana, también se añadió tamoxifeno a la solución estándar en el compartimento cis. Para los estudios de acoplamiento, las coordenadas atómicas del canal heptamérico se obtuvieron del PDBID (7AHL) y la estructura del tamoxifeno de Pubchem, sus coordenadas se construyeron y minimizaron con el programa Avogadro. Los experimentos de acoplamiento molecular se llevaron a cabo utilizando el portal en línea Dockthor. El tamoxifeno inhibió (P<0,05) la conductancia del canal α-HL de forma dependiente del voltaje. Se evaluaron las tres mejores soluciones de atraque con el canal, se observó que el modo de conexión con mayor afinidad tiene mayor número de tipos de enlaces polares. Los residuos con las interacciones energéticas más altas fueron 111 y 147, que forman los residuos de la constricción del canal. Las otras conformaciones se acomodaron en una región con características más hidrofóbicas (valina 149). El mecanismo por el cual el tamoxifeno inhibió el canal de α-hemolisina de *Staphylococcus aureus* fue bloqueando la constricción de este canal.
Palabras clave: Tamoxifeno; *Staphylococcus aureus*; Canal iónico; Factores virulentos; Agente antimicrobiano.

1. Introduction

The *Staphylococcus aureus* α-hemolysin channel (α-HL), importance in pathogenesis, is regarded as a virulence factor playing a role in infection (Bryant et al., 2019). The α-HLs are exotoxins that create lytic pores in the host cell membrane. They are recognized as being mainly for the development of invasive infections and are thus potential targets for antivirulence treatments (Liu et al., 2020). It is one of the first important bacterial and viral virulence factors which mechanism of action was disclosed (Fussle et al., 1981; Krasilnikov, et al., 1988) and is based on pore formation in lipid bilayer membranes.

α-HL is a monomer secreted during *Staphylococcus aureus* exponential growth, and oligomerizes into the host membranes as an heptameric transmembrane pore, causing osmotic cytolysis (Gouaux et al., 1994). Previous studies have evaluated the activity of several compounds to inhibit α-HL action, by hindering its membrane assembly (Qiu et al. 2013; Rani et al., 2014) or by direct blocking α-HL ion channel (Melo et al, 2015; Karginov et al., 2007; Teixeira et al., 2009).

Thus, this study proposed to investigate the tamoxifen, the first selective estrogen receptor (ER) modulators, is a triphenylethylene derivative used for the treatment of ERα-positive breast cancer (Gómez-Coronado et al., 2020). Tamoxifen have been related inhibiting the inward rectifier potassium current in cardiac myocytes (Ponce-Balbuena et al., 2009) and the calcium-activated chloride currents in epithelial cells (Imberti et al., 2018).

Moreover, molecular docking studies provided atomic level details on protein–ligand interactions. These are expected to expand the knowledge on compounds molecular recognition by the heptameric pore, while tamoxifen is suggested as possible cotherapeutical agents for the treatment of infected patients. Thus, this work proposes to verify the effects of

tamoxifen on *Staphylococcus aureus* α-hemolysin (α-HL) currents and its molecular mechanism by docking studies.

2. Methodology

This research is quantitative and experimental (Pereira et al., 2018).

2.1 Chemicals

The wild type of *S. aureus* α-hemolysin was purchased from (List Biological Laboratories, Campbell, CA). Solvent-free planar bilayer lipid membranes (PLM), with capacitance of 40 pF, were formed by the lipid monolayer apposition technique, using DPhPC in hexane (J. T. Baker, Phillipsburg, NJ) at 25 ± 1 °C. Tamoxifen (*Z*)-2-[4-(1,2-difenilbut-1-enil)fenoxi]-*N*,*N*-dimetil etanamina) was purchased from Sigma (St. Louis, MO). Diphytanoylphosphatidylcholine (DPhPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2 Single channel reconstitution in planar lipid bilayer

Planar lipid bilayer membranes were formed as previously described (Montal & Mueller, 1972), where the bilayers were composed of two DiPhyPC monolayers. After membrane stabilization, a 0.1–0.4 μl of the stock solution containing α-HL (0.07 mg/ml) was added to the standard solution (4 M KCl, 5 mM Tris- OH, pH 7.5) in *cis* compartment of the experimental chamber, to provide final concentrations of ~2 ngml−1. All experiments were performed at room temperature (25 ± 2º C) using an Axopatch 200A amplifier in the voltage clamp mode. All steps from measuring current to analyzing data were performed with equipment and software that are described in previous studies (Krasilnikov et al., 2000; Teixeira et al., 2009; Rodrigues et al., 2011). At pH 7.5, α-HL channels are usually in a high conductance ~4 nS and rarely switch to low conductance states. After the ion channel was incorporated in bilayer membrane, the tamoxifen (100 µM) was also added to the standard solution to the *cis* compartment. All steps from measuring current to analyzing data were performed with equipment and software that are described in previous studies (Krasilnikov et al., 1999, 2000; Teixeira et al. 2009; Rodrigues et al., 2011). Data were analyzed statistically by GraphPad Prism, that media difference was calculated using t-Test and significant when value $P < 0.05$.

2.3 Molecular docking studies

In order to obtain atomic-level insights into the Tamoxifen mechanism of action, docking experiments were carried out to predict the position and orientation of tamoxifen on the surface of α-HL. To docking studies, atomics coordinates for the α-HL heptameric channel was retrieved from PDB ID 7AHL (Song et al., 1996). The structure of tamoxifen was removed from the Pubchem and their coordinates were built and minimized with Avogadro software (Hanwell et al., 2012). The molecular docking experiments were performed using the Dockthor online portal (Magalhães et al., 2014). Molecular docking was performed in the constriction region, as it is the region most likely to have interactions between α-HL and little ligands (Melo et al., 2016).

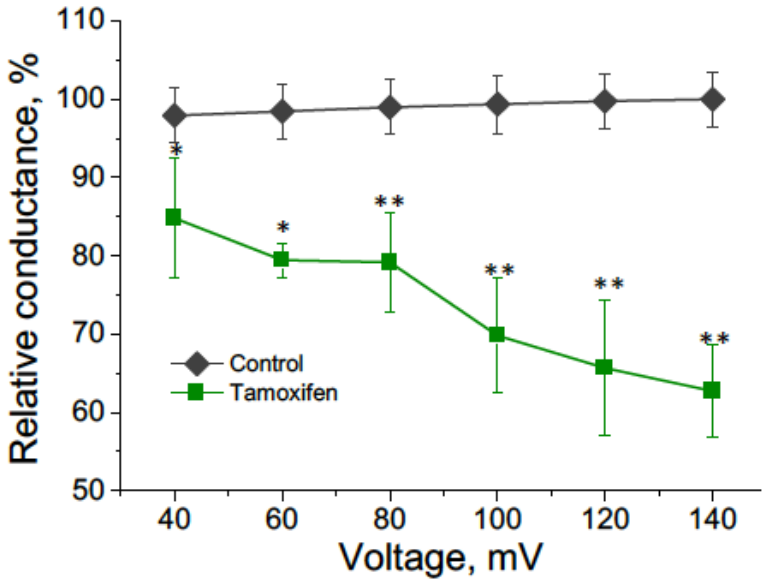
The output conformers from program are ranked in order of increasing affinity with the protein. The three conformations the drug with biggest affinity docked were retrieved for further analysis.

3. Results and Discussion

Importantly, the large α-HL single-channel conductance ~4 nS in 4 M KCl, tamoxifen was assayed for its ability to block ion conductance through the pores formed in artificial membranes α-HL. The tamoxifen induced inhibition ($P < 0.05$) of

channel conductance (Figure 1). In addition, the relative conductance of the channel on the presence of the compound is smaller than in the standard solution and decrease the transmembrane potential, so the effect is voltage-dependent. Differently, tamoxifen inhibited the inward rectifier potassium current in cardiac myocytes in a manner voltage-independent (Ponce-Balbuena et al., 2009). Addition of about 100μM to the cis-side of the membrane switched the channel to a closed state similar to the 'voltage-gated closed state' seen commonly for α-HL at 100 mV and higher voltages.

Figure 1. Effects of tamoxifen on α-HL conductance.

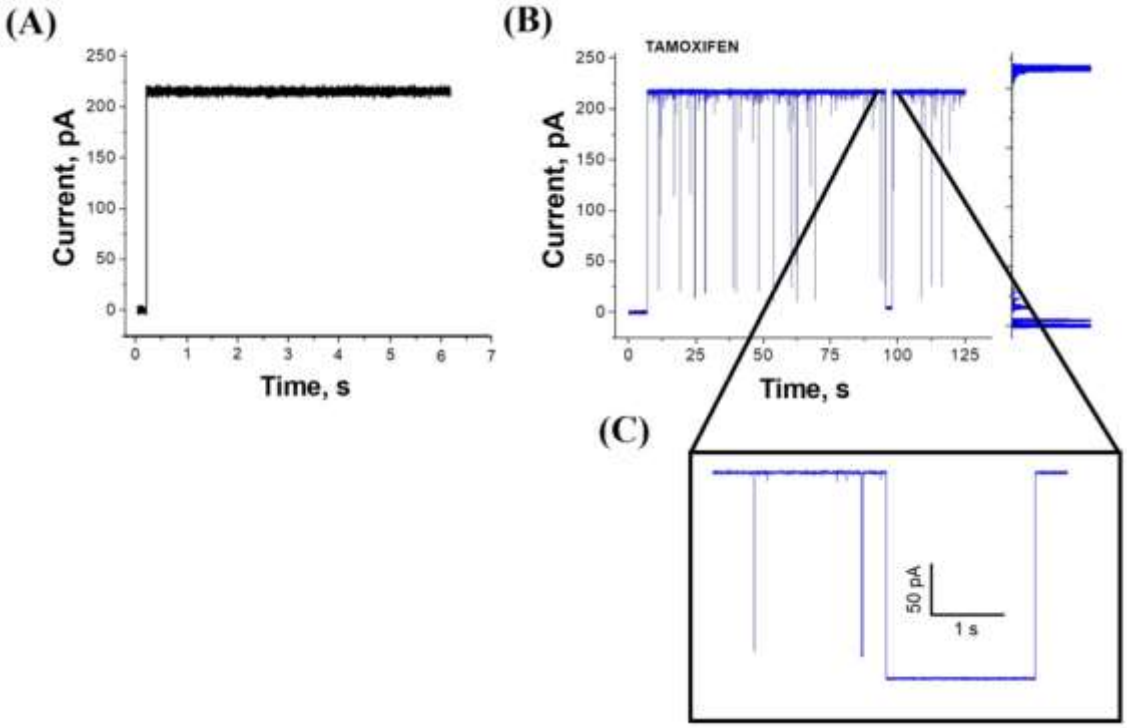


Being: Symbols represent relative conductance (%) at different voltages (mV). The channel conductance in the absence (◆, control) and presence of 100 μM tamoxifen (■, Tamoxifen) in the cis compartment was relativized as the control condition. *P < 0.05, **P < 0.001, t-Test (control x Tamoxifen). Data are as mean ± SD for at least four experiments.
Source: Authors (2021).

Illustrate typical recordings of an ion current through single α-HL pores (Figure 2). It is seen that even before the addition of 1, the single α-HL pores conductance level was rather noisy (Figure 2 A, topmost track). This noisiness represents the well-known pore channels which appears as fast flickering between the open and completely closed conformations. The addition of about μM these drugs to the cis-side of the membrane (the side of toxin addition) caused additional step-wise closures of 6 ms average duration (Figure 2B, middle). Just as gating, these fluctuations are fast transients between the fully open and non-conducting channel. Even though the amplitude of these events coincides with the amplitude of the regular α-HL channel (complete channel closure), the difference in the residence times allowed us to sort all observed events into the two different processes. The tamoxifen concentrations 33.60 μM channel blockades are more frequent (Figure 2B, bottom). It was observed that the current through the single α-HL pore was stable; no gating events were seen under applied + 60 mV. Even though the amplitude of these events coincides with the amplitude of the regular α-HL channel (complete channel closure), the difference in the residence times allowed us to sort all observed events into the two different processes (Figure 2A). Rapid events (with an average duration of 6ms) are observed, as well as some lasting events (with an average duration of 2s) (Figure 2B and 2C). Even though the amplitude of these events coincides with the amplitude of the regular α-HL channel (complete channel closure), the difference in the residence times allowed us to sort all observed events into the two different processes: events of shorter duration and greater frequency and events of longer duration and less frequency (Figure 2B and 2C). It was observed that the current through the single α-HL pore was stable; no gating events were seen under applied 60 mV. Addition

of about 100μM to the cis-side of the membrane switched the channel to a closed state similar to the 'voltage-gated closed state' seen commonly for α-HL at +60 mV and higher voltages. The residual conductance of the closed state was between 1% and 15% of the total channel conductance. It is remarkable that the introduction of positive charges to the drugs molecule leads to its ability to block the α-HL channel from the cis-side. It is known that unmodified drugs binds only weakly to the heptameric α-HL channel when added from the trans (intracellular) side of the membrane.

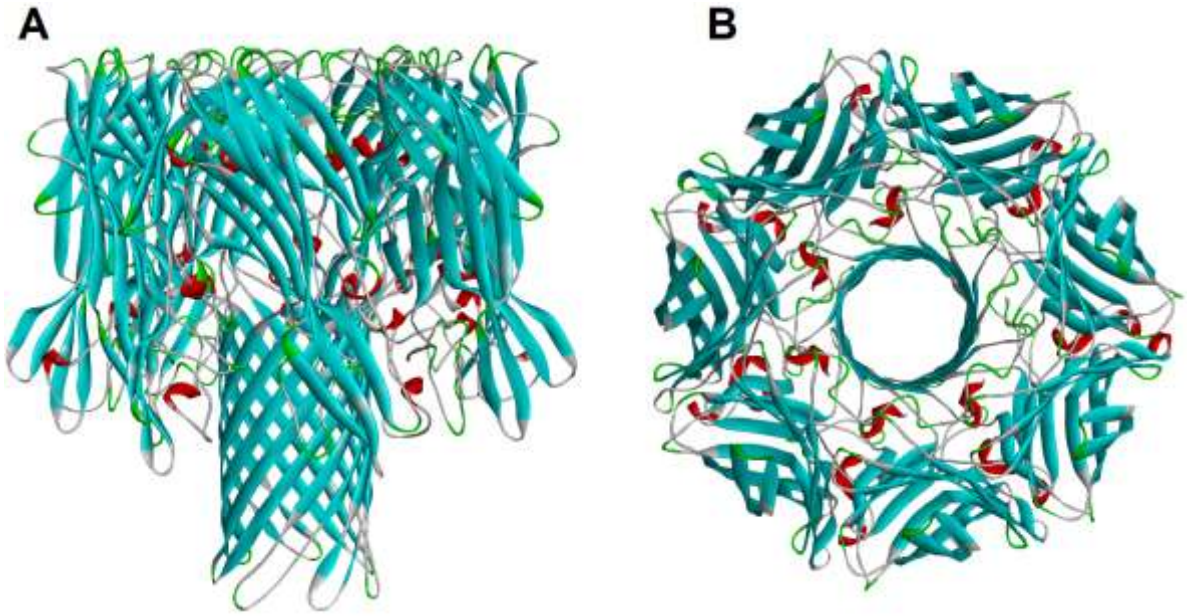
Figure 2. Effect of anion channel inhibitors on the α-HL channel in planar lipid bilayers.



Being: The ionic current through a single *α*-HL channel in the absence Tamoxifen (**A**) and representative current recordings and all point current amplitude histograms illustrating the behavior of α-HL channel in in the presence 33.50 μM Tamoxifen (**B** and **C** blue records) in the *cis* compartment. Voltage, +60 (upper traces) or mV. Filtering, 0.5 KHz; digitizing, 2.5 KHz. (F–J): amplitude histograms obtained from longer segments of the recordings shown to their left.
Source: Authors (2021).

In order to predict the position and orientation of tamoxifen on the surface of α-HL and obtain atomic-level insights into these drugs mechanism of action, docking experiments were carried out to drug on the surface of α-HL (Figure 3). The copal region of alpha hemolysin is represented, where tamoxifen was added (Figure 3B), as well as the stem region that is inserted in the membrane (Figure 3B).

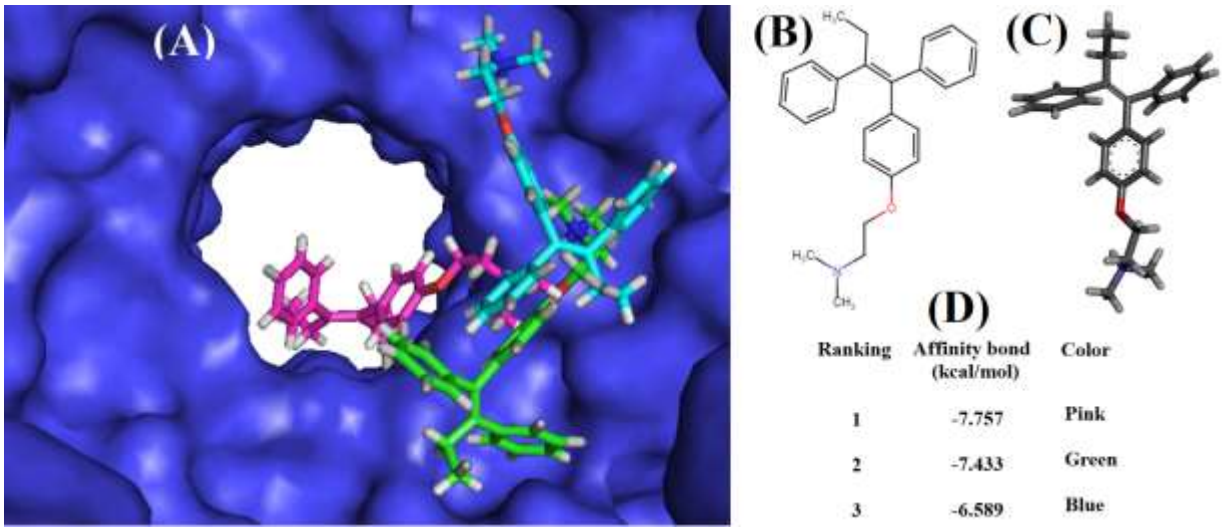
Figure 3. Structure of α-HL, a heptameric transmembrane pore.



Being: α-HL shown from the side (**A**) and cytoplasmic (**B**) views.
Source: Authors (2021).

Figure 4A represents the three conformers that showed greater binding affinity with α-HL for tamoxifen. Conformers of tamoxifen has little atomic overlap over the constriction of α-HL. It is possible to observe that tamoxifen conformers with the three major binding affinities can occupy different positions of the constriction (Figure 4A). The tamoxifen conformer with the highest binding affinity occupies the most central region of the constriction, occupying a larger volume and justifying the events of greater amplitude of α-HL block and greater reduction in channel conductance (Figure 2C) observed in the experimental results.

Figure 4. Docked α-HL constriction region of the channel in (**A**). Docked conformers of tamoxifen of greater binding energy coupled in the channel of α-HL.

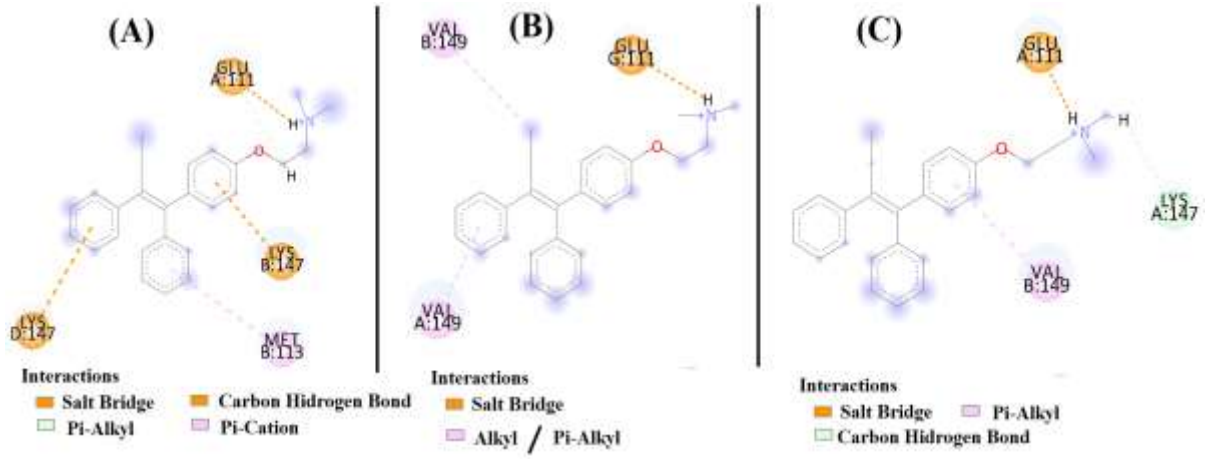


Ranking	Affinity bond (kcal/mol)	Color
1	-7.757	Pink
2	-7.433	Green
3	-6.589	Blue

Being: (**A**) The conformers are represented in the context of the entire protein. (**B**) 2D structure of tamoxifen. (**C**) 3D structure of tamoxifen. (**D**) Three higher values of link affinity generated by the Dockthor platform, in order of ranking.
Source: Authors (2021).

When evaluating the types of interaction involved in the connection between the three best docking solutions and the α-HL channel, it is possible to observe that the connection mode with the highest affinity of interaction has a greater number of types of polar interaction (Figure 5A). It is observed that this conformer interacts with a greater amount of polar residues in the α-HL, which provides greater stability and longer residence time inside the channel of α-HL. The residues present interactions of greater energy are 111 and 147 (Figure 5) that form the remainders of the constriction in the channel of α-HL. The other conformers are accommodated in a region with more hydrophobic characteristics, mainly due to the presence of valine 149 (Figure 5B e C).

Figure 5. Schematical presentation of the interactions from three conformers the best position at docking and α-HL channel.



Being: Ranking first (**A**), second (**B**) and third (**C**) position, respectively.
Source: Authors (2021).

Thus, the mechanism of action of this complex is similar to heparin, cyclodextrins derivatives (Karginov et al., 2007; Teixeira et al., 2009; Melo et al., 2015) and microcystins (Júnior et al., 2019). Those finding show tamoxifen as a putative drug to control multi-resistant bacteria in hospital environment (Silva et al., 2020).

4. Conclusion

The mechanism of *Staphylococcus aureus* α-hemolysin inhibition by tamoxifen has been identified. Here, we demonstrate the ability of tamoxifen to block α-HL channels correlates it in a potential dependent manner and this blockade is over the constriction of α-HL.

In the future, the effects of tamoxifen in the microbiological assays on culture of *Staphylococcus aureus* are will performed for corroborate those findings.

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