

Influence of the lipid bilayer composition on interaction of polyanions with the alpha-toxin ion channel

Influência da composição da bicamada lipídica na interação de poliânions com o canal iônico da alfa-toxina

Influencia de la composición de la bicapa lipídica en la interacción de los polianiones con el canal iónico de la alfa-toxina

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Luciana Ramos Teixeira

ORCID: <https://orcid.org/0000-0002-2930-5962>

Universidade Federal de Pernambuco, Brazil

E-mail: lucianarateixeira@gmail.com

Gabriela Santos Pacheco Lima

ORCID: <https://orcid.org/0000-0003-2444-950X>

Centro Universitário Maurício de Nassau, Brazil

E-mail: gabispdl@gmail.com

Antônio Augusto Batista Neto

ORCID: <https://orcid.org/0000-0002-4870-2832>

Centro Universitário Maurício de Nassau, Brazil

E-mail: augusttobattista@gmail.com

Cláudio Gabriel Rodrigues

ORCID: <https://orcid.org/0000-0001-9015-5644>

Universidade Federal de Pernambuco, Brazil

E-mail: claudio.rodrigues@ufpe.br

Dijannah Cota Machado

ORCID: <https://orcid.org/0000-0002-5466-0229>

Universidade Federal de Pernambuco, Brazil

E-mail: dijannah.machado@ufpe.br

Abstract

The mechanism of interaction among polyanions and Staphylococcal alpha hemolysin (α HL) ion channel still was not elucidated completely. The initial interaction of polyanions with surface membrane of phospholipids is based in Ca^{2+} bridge formation. Such interaction increases polyanion concentration close to membrane surface that, in turn, increases the probability of polyanion enter into a channel and blocks it. Thus, this study proposed to investigate the role of the lipid membrane composition on interaction of polyanions, such as heparin, with α HL channel. It was found that the effectiveness of heparin to block α HL channels was significantly dependent on the lipid composition of the bilayers. The lipids on their ability to support heparin influence were ranked as follows: $\text{PC} \gg \text{PI} \approx \text{PS} > \text{DPhPS} \geq \text{PE} \geq \text{DPhPC} > \text{OChol}$. These results indicate that the interaction of Ca^{2+} with lipid membranes depends on the exposure and density of phosphate groups in phospholipids at membrane surface. On the other hand, the effectiveness of heparin to block α HL channel was more strongly correlated with the length of the hydrocarbons chain of fatty acids of the phospholipids. Thus, we demonstrate that the polar head group of phospholipids in the membranes affects their interaction with divalent ions by changing their surface potential, and therefore influences the effectiveness of heparin blockage in the formed channels. The results might be of interest for pharmacology, biomedicine, and research aiming to design mesoscopic pore blockers.

Keywords: Phospholipids; Heparin; Ion Channel; Alpha-Hemolysin.

Resumo

O mecanismo de interação entre os poliânions e o canal iônico formado pela alfa-hemolisina estafilocócica (α HL) ainda não foi completamente elucidado. A interação inicial dos poliânions com a membrana superficial dos fosfolípidios é baseada na formação de pontes de Ca^{2+} . Tal interação aumenta a concentração de poliânions próximo à superfície da membrana que, por sua vez, aumenta a probabilidade do poliânion entrar em um canal e bloqueá-lo. Assim, este estudo se propôs a investigar o papel da composição da membrana lipídica na interação de poliânions, como a heparina, com o canal iônico de α HL. Verificou-se que a eficácia da heparina para bloquear os canais de α HL era significativamente dependente da composição lipídica das bicamadas, os lípidios foram ranqueados da seguinte forma: $\text{PC} \gg \text{PI} \approx \text{PS} > \text{DPhPS} \geq \text{PE} \geq \text{DPhPC} > \text{OChol}$. Esses resultados indicam que a interação do Ca^{2+} com as membranas lipídicas depende

da exposição e densidade dos grupos fosfato nos fosfolipídios na superfície da membrana. Por outro lado, a eficácia da heparina em bloquear o canal de α HL foi mais fortemente correlacionada com o comprimento da cadeia de hidrocarbonetos dos ácidos graxos dos fosfolipídios. Assim, demonstramos que o grupo da cabeça polar dos fosfolipídios nas membranas afeta sua interação com íons divalentes alterando seu potencial de superfície e, portanto, influencia a eficácia do bloqueio da heparina nos canais de α HL. Os resultados podem ser de interesse para farmacologia, biomedicina e pesquisas com o objetivo de projetar bloqueadores de poros mesoscópicos.

Palavras-chave: Fosfolipídios; Heparina; Canal Iônico; Alfa-Hemolisina.

Resumen

El mecanismo de interacción entre los polianiones y el canal iónico de la alfa-hemolisina estafilocócica (α HL) todavía no se elucidó por completo. La interacción inicial de los polianiones con la membrana superficial de los fosfolípidos se basa en la formación de puentes de Ca^{2+} . Tal interacción aumenta la concentración de polianión cerca de la superficie de la membrana que, a su vez, aumenta la probabilidad de que el polianión entre en un canal y lo bloquee. Por lo tanto, este estudio propuso investigar el papel de la composición de la membrana lipídica en la interacción de polianiones, como la heparina, con el canal α HL. Se encontró que la efectividad de la heparina para bloquear los canales α HL dependía significativamente de la composición lipídica de las bicapas, los lípidos se clasificaron de la siguiente manera: $\text{PC} \gg \text{PI} \approx \text{PS} > \text{DPhPS} \geq \text{PE} \geq \text{DPhPC} > \text{OChol}$. Estos resultados indican que la interacción de Ca^{2+} con las membranas lipídicas depende de la exposición y densidad de los grupos fosfato en los fosfolípidos en la superficie de la membrana. Por otro lado, la efectividad de la heparina para bloquear el canal α HL se correlacionó más fuertemente con la longitud de la cadena hidrocarbonada de los ácidos grasos de los fosfolípidos. Por lo tanto, demostramos que el grupo de cabeza polar de los fosfolípidos en las membranas afecta su interacción con los iones divalentes al cambiar su potencial de superficie y, por lo tanto, influye en la efectividad del bloqueo de la heparina en los canales formados. Los resultados pueden ser de interés para la farmacología, la biomedicina y la investigación con el objetivo de diseñar bloqueadores de poros mesoscópicos.

Palabras clave: Fosfolípidos; Heparina; Canal Iónico; Alfa-Hemolisina.

1. Introduction

Staphylococcus aureus is a gram-positive bacterium of the Micrococcaceae family. It is the main human pathogen causing several types of clinical infections from a simple infection, such as boils, to most severe diseases as endocarditis, bacteremia, sepsis and toxic shock syndrome (Lowy, 1998; Santos et al., 2007; Otto, 2014; Cohen et al., 2016; Ahmad-Mansour et al., 2021). This bacterium produces various exotoxins, such as alpha-hemolysin, that collaborate to its capacity to colonize mammalian hosts and to cause several diseases (Dinges et al., 2000; Cheung et al., 2021).

The alpha-hemolysin (α HL) is a water soluble 33 kD protein toxin (Bhakdi & Trantum-Jensen, 1991; Melo et al., 2016) that has the ability to incorporate into the cell membrane and, then, to form an ion channel. Besides that, the α HL is involved in inflammasome activation and induction of pro-inflammatory cytokines secretion implicated in tissue necrosis and inflammation processes (Craven et al., 2009).

The α HL represents one of the virulence factors of *S. aureus*, thus to use a drug that blocks the action of this toxin is a way to inhibit the staphylococcal infection progression (Santos et al., 2007; Tong et al., 2015; Teixeira et al., 2021). The great concern worldwide is the high incidence of hospital infection and resistance to antibiotics, mainly to the beta lactam class, presented by strains of this bacterium (Tong et al., 2015; Cohen et al., 2016). Therefore, is important to look for new compounds that inhibit the *S. aureus* toxins, particularly α HL, as well as the understanding of the mechanisms involved in the inhibition (Qiu et al., 2013; Rani et al., 2014).

Over the years, studies have reported protective properties of polyanions against bacterial infections (Remington & Merigan, 1970; Zaretzky et al., 1995; Guo et al., 2017). Our group demonstrated that the polyanion heparin blocks ion channels formed by α HL in a concentration and molecular weight-dependent manner, besides that, we also emphasized the importance of the presence of divalent cations in the action of the polyanions (Teixeira et al., 2009). In presence of Ca^{2+} , the sulfate groups of the heparin bind to negatively charged phosphate groups of the phospholipids by divalent cation bridges (Huster & Arnold, 1998; Krasilnikov et al., 1999). Such interaction increases polyanion concentration close to membrane surface that, in turn, increases the probability of polyanion enter into a channel and blocks it (Sinn et al., 2006; Teixeira et al., 2009).

It is believed that the length and degree of unsaturation of the hydrocarbon chains of phospholipids may modify the local environment and orientation of the phosphate groups (Rolland et al., 1996; Meshkov et al., 1998) responsible for interaction with ions and other charged molecules. In this context, we decided to investigate the influence of lipid composition of planar lipid bilayers (PLM) on the effectiveness of heparin for blocking α HL channels. The results are discussed in terms of the influence of the bilayer composition on Ca^{2+} /heparin interaction and its repercussion on the blockade of the α HL channel.

2. Methodology

2.1 Chemicals

The wild type of *Staphylococcus aureus* α -hemolysin was purchased from Calbiochem (Madison, WI). The oxidized cholesterol (OChol) and all phospholipids: phosphatidylcholine (PC); phosphatidylethanolamine (PE); phosphatidylinositol (PI); phosphatidylserine (PS); 1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine (DPhPC) and 1,2-diphytanoyl-sn-glycero-3-phosphatidylserine (DPhPS) were purchased from Avanti Polar Lipids (Alabaster, AL). Heparin 6000 g/mol (H-5284, Hep6000), CaCl_2 , EDTA, 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS), and HCl were purchased from Sigma (St. Louis, MO). All reagents were used as received. Milli-Q plus water (Millipore, Bedford, MA) with resistivity of 18 M Ω cm was used to prepare all aqueous buffer. The bath solution was composed of 50 mM CaCl_2 , 1 mM EDTA, 10 mM TRIS-HCl, at pH 7.5.

2.2 Electrophysiological measurements

Multichannel experiments were carried out at bilayer lipid membranes formed by painting technique (Mueller et al., 1963) from PC/Chol; PE/Chol; PS/Chol; PI/Chol mixture (ratio 2:1, M/M) and OChol across a hole (~0.3 mm diameter) in a 25-mm-thick Teflon[®] partition in a Teflon[®] chamber. The bilayers formed from synthetic lipids as 1,2-Diphytanoyl-sn-glycero-3-phosphatidylcholine (DPhPC) and 1,2-Diphytanoyl-sn-glycero-3-phosphatidylserine (DPhPS) were performed by the solvent-free method. The phospholipids were dissolved at 5 mg/ml in hexane and spread at the air-water interface of the aqueous buffered solutions on both sides of the partition. Membranes were formed on the orifice by sequentially raising the level of the aqueous electrolyte solutions (Montal & Mueller, 1972).

After the formation of the membrane, ± 100 mV voltage pulses were applied to verify the basal conductance of the membrane, which was inferior to 6pS for bilayers formed from PC/Chol; PE/Chol; PS/Chol mixture, and inferior to 16pS for membranes formed from OChol.

Channels were formed by adding several microliters of the α HL stock solution (5 ng/ml) to one side of the chamber (here in defined as Cis side). The mean value of single-channel current was 5 pA for 50 mM de CaCl_2 , 1 mM de EDTA, 10 mM de TRIS-HCl, pH 7.5 and 40 mV applied potential.

The ionic current was measured at room temperature (25 ± 2 °C) using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) in the voltage-clamp mode. Membrane potential was maintained using Ag/AgCl electrodes in 3 M KCl 2% agarose bridges assembled within standard 200 μ L pipette tips. Currents were filtered by a low-pass eight-pole Bessel filter (Model 9002, Frequency Devices, Haverhill, MA), digitized with a sampling frequency of 0.5 kHz (for multichannel experiments) or 50 kHz (for single channel recording), stored on a computer and analyzed by electrophysiology software developed by Dr. M. A. Pustovoi (Petersburg Nuclear Physics Institute, Gatchina, Russia).

2.3 Time constant measurements of α -HL in bilayer membranes

To quantify the change in the current reduction of the channel, as a result of the presence of Hep6000, it was calculated the characteristic time constant of the current decay (τ). This parameter was also used to analyze the influence of the Hep6000

for multichannel experiments in bilayers constructed with different phospholipids. The data had been adjusted perfectly to the described equation:

$$I(t) = I_0 \exp\left(\frac{-t}{\tau}\right) + I_{ss} \quad [1]$$

where, I_0 is the ionic current amplitude in reply to the voltage pulse applied at multichannel bilayer; t is the time of the process; τ is the characteristic time and I_{ss} is the steady-state current value, after the phase of decline in reply to the voltage pulse.

Value of IC_{50} , that represents a heparin concentration that causes 50% of the blocking effect on α HL channels at multichannel bilayers, was determined by fitting of the experimental dependence of τ on heparin concentration as follows:

$$\tau = \tau_{\max} \times \frac{\text{Hep}^n}{IC_{50} + \text{Hep}^n} \quad [2]$$

where, τ is the characteristic time of the α HL channels; n is a parameter that determines a number of possible binding or cooperative sites in the interaction of heparin (or other polyanions) with the α HL channel; and IC_{50} is the responsible concentration for 50% of the the modulation effect in the α HL channels.

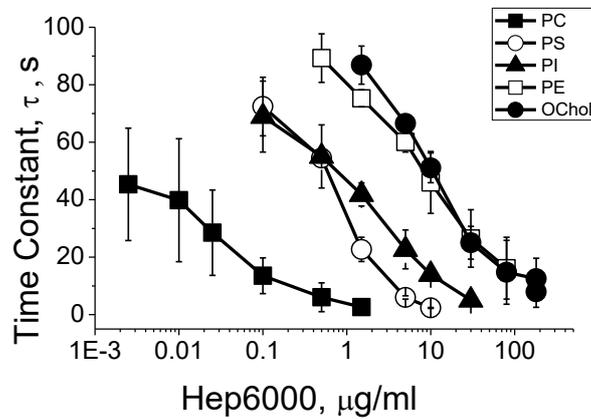
For adjustment of the curve with the experimental data, the equations were adjusted using the statistical software Origin 7.5. Data are reported as the mean \pm SD obtained in 3–7 independent experiments.

3. Results and Discussion

To evaluate the influence of bilayer lipid composition on effectiveness of heparin in blocking the α HL channel, we performed multichannels experiments with bilayer membrane formed by OChol and phospholipids from different polar heads: phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE).

It was observed an influence of the type of lipid in membrane compositions on the α HL ion channel sensitivity to Hep6000. The results analysis had demonstrated that the influence of composition of the lipid bilayer on the sensitivity of the α HL channel to Heparin 6000 is ranked as follows: PC \gg PI \approx PS $>$ PE \geq OChol (Figure 1).

Figure 1. Influence of phospholipids from different polar heads on α HL channel time constants (τ). The PLM-lipid composition induced reduction on α HL channel time constant as a function of heparin concentration. The lipid bilayers had been formed with different lipids + cholesterol (in the ratio of 2:1, M/M). Hep6000 was added in the compartment *TRANS*, which was submitted the pulses of voltage of 100 mV.



Source: Authors (2022).

The results demonstrate that the reduction of the characteristic time constant values under effect of the Hep6000 was bigger in channels incorporated in bilayers formed by neutral phospholipid phosphatidylcholine (PC).

The half-maximal inhibitory Hep6000 concentration was defined as IC_{50} . The reduction of the values of the characteristic times under effect of Hep6000 was always bigger in bilayers formed by egg-derived phosphatidylcholine (PC) (neutral lipid, $IC_{50} = 0.03 \pm 0.01 \mu\text{g/mL}$), than in experiments that used lipid bilayers composed of any other type of lipids, as phosphatidylserine (PS) (negative lipid, $IC_{50} = 0.91 \pm 0.05 \mu\text{g/mL}$), phosphatidylinositol (PI) (negative lipid, $IC_{50} = 0.88 \pm 0.05 \text{ mg/mL}$) and phosphatidylethanolamine (PE) (neutral lipid, $IC_{50} = 5.17 \pm 1.08 \mu\text{g/ml}$).

In this study, it was used bath solution contained Ca^{2+} ions, divalent cations interact with phosphate groups of phospholipids and introduce a positive charge at the membrane surface (Papahadjopoulos, 1968; Alsop et al., 2016). This action can provide Ca^{2+} bridge formation between phosphate groups of phospholipids in the membranes and the sulfate groups on the polyanions molecules (Arnold et al., 1990) as well as an increase in concentration of negatively charged polyanions at membrane-solution interfaces. In turn, this negatively charged polyanions concentration-increase should increase the probability of the channel block by polyanions.

Our results indicate that PC membranes bind to Ca^{2+} ions with high affinity, which determines a significant electrostatic attraction with Hep6000 and consequently a more effective channel blocking effect. For the other neutral phospholipid, phosphatidylethanolamine (PE), the effectiveness of the Hep6000 in blocking the α HL channel was eight-fold lower than PC membrane. This indicates that addition of divalent cations did not produce detectable surface potential, i.e., little or no Ca^{2+} binding occurs to the polar head of PE phospholipid.

The influence of the phospholipids charge used in our studies demonstrated that the effectiveness of Hep6000 on the channel α HL decreased when these channels were incorporated in lipid bilayers constituted by phospholipids which present negatively charge polar heads, such as phosphatidylserine (PS) and phosphatidylinositol (PI). In comparison to lipid bilayer formed by PC, the channels were less sensitive to the action of Hep6000 on membranes composed of PS and PI because of repulsion electrostatic dust interaction with the negatively of heparin 6000.

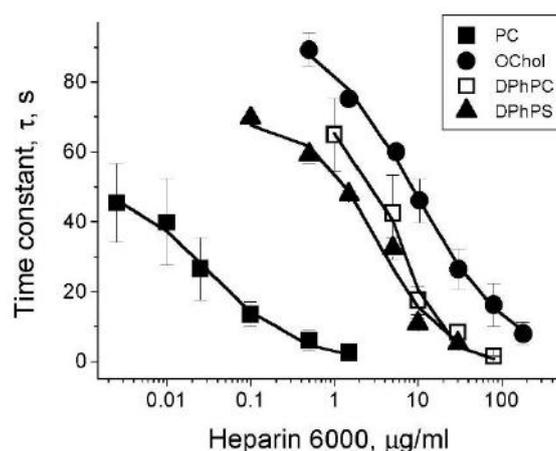
As Ca^{2+} -phospholipids interaction is a result of electrostatic interaction between the phosphate group of phospholipids with Ca^{2+} ions, which is directly related to the exposure of the phosphate, the charged polar head of phospholipids can influence the effectiveness of Hep6000 on the channel formed by αHL from *Staphylococcus aureus* by decreasing this exposure phosphate groups, and therefore reducing Ca^{2+} binding.

Studies on model membranes have demonstrated that when the bilayers were formed from a neutral lipid, phosphatidylethanolamine, the addition of cations produced no detectable surface potential, indicating that little or no binding occurs to the polar head group with these ions. Ions interact with charged phospholipids via Coulombic forces. The apparent association of cations with lipid membranes is distinctly more intense for anionic lipids than for neutral (zwitterionic) ones. This behavior can be explained taking into account that the net negative surface charges of membranes of lipids increases cation concentration near the lipid–water interface, according to the Gouy–Chapman theory of the electrical double layer (Valeva et al., 2006). When the bilayers were formed from a negatively charged lipid, phosphatidylserine, the surface potential decreased 27 mv for a 10-fold increase in Ca^{2+} concentration in the millimolar range (McLaughlin et al., 1971). Ethanolamine head groups result in phospholipid bilayers that are more tightly packed decreasing the exposure and the density of phosphate groups at the membrane interphase.

These findings indicate that the interaction of Ca^{2+} with lipid membranes depends on the exposure and density of phosphate groups in phospholipids at membrane surface. Thus, we demonstrate that the polar head group of phospholipids in the membranes affects their interaction with divalent ions by changing their surface potential, and therefore influences the effectiveness of heparin in blocking αHL channels.

Still evaluating the sensitivity of the channel to the action of the hep6000 in different PLM-lipid composition, also it was observed a great difference when αHL channels were incorporated in bilayers formed with phospholipids that have the same polar head, however, with different composition of acid fatty (Figure 2).

Figure 2. Influence of phospholipids with different composition of acid fatty on αHL channel time constants (τ). The lipid bilayers with been formed with PC/Chol (in the ratio of 2:1, M/M), OChol and synthetic lipids (DPhPC and DPhPS). Hep6000 was added in the compartment *TRANS*, which was submitted the pulses of voltage of 100 mV.



Source: Authors (2022).

This can be seen in the comparison between bilayers formed of phosphatidylcholine (PC) of egg ($\text{IC}_{50} = 0.03 \pm 0.01 \mu\text{g/mL}$) and 1,2-Diphytanoyl-sn-glycero- 3-phosphatidylcholine (DPhPC) ($\text{IC}_{50} = 6.26 \pm 1.04 \mu\text{g/mL}$). The channels in

bilayers composed by oxidized cholesterol (OChol) had been less sensible ($IC_{50} = 9.18 \pm 1.37 \mu\text{g/mL}$) to the action of Hep6000 than the other bilayers investigated in this study.

In order to clarify which membrane factor determines the sensitivity of the channel, we performed a correlation analysis among the type of phospholipids used with the fatty acid composition, length of hydrocarbon chain and IC_{50} for Hep6000 (Table 1).

Table 1. Average order parameter* of the natural phospholipids in saturated and unsaturated PC/PE/PS and their correlations with IC_{50} .

phospholipids	unsaturated %	Saturated/unsaturated	hydrocarbon chain 20:4(3) %	hydrocarbon chain ≥ 20 , %	hydrocarbon chain < 20 , %	IC_{50} $\mu\text{g/ml}$
<i>phosphatidylcholine (PC). Eggs</i>	53.5	0.87	3	3	97	0.03
<i>phosphatidylserine (PS). Brain</i>	50.5	0.98	2	12	77	0.913
<i>phosphatidylethanolamine (PE). Brain</i>	63.3	0.58	20	33	57	5.165
<i>phosphatidylinositol (PI). Liver</i>	47.4	1.11	22.6	22.6	74.4	1.956
Correlation Coefficient.	0.746	-0.691	0.728	0.954	-0.931	

*Parameters provided by the manufacturer, Polar Avanti Lipids. Source: Authors (2022).

We found a significant positive correlation among the IC_{50} and the amount of unsaturated fatty acid and a negative correlation among the IC_{50} and the ration of saturated and unsaturated fatty acid. The highest correlation coefficient established was ~ 0.9 with the length of hydrocarbon chain ≥ 20 , in this manner the degree of saturation and the increase of the hydrocarbon chain had influenced in the effect of the heparin on the ion channel. These two factors seem to influence through the properties of the membrane.

The main variable considered here to interpret the results of affinity of Ca^{2+} to the different surfaces is the exposure of the phosphate groups. There is another important variable related to the same group that is the proximity of one group to another, meaning the density of the phosphate groups in each surface. In the case of PCs, the differences in the area per lipid molecule could be related to the differences in the calcium affinity. As it is known from the bibliography (Li et al., 1996; Nagle & Tristram-Nagle, 2000), PCs with unsaturated acyl chains present a greater area per lipid molecule than PCs with saturated acyl chains, caused by the spacer effects produced by the wobbling of the acyl chains with unsaturations. The smaller area per lipid molecule of PCs with saturated acyl chains determines a higher density of the phosphate groups in the surface (Nagle & Tristram-Nagle, 2000) which it determines a significant electrostatic force in the interaction polyanion and ion channel, increasing the probability of the polyanion is blocking the αHL channel, as demonstrated in this study.

4. Conclusion

Our results show that the interaction of Ca^{2+} to lipid membranes depends on the exposure and the density of phosphate groups at the membrane interphase and these ones are influenced by the degree of lipid saturation and length of hydrocarbon chain. The longer the hydrocarbon chain, the lower the exposure of phosphate groups, decreasing the Ca^{2+} bridges between phospholipid groups negatively charged and Hep6000 sulfate groups, thereby decreasing the effective ability to block the αHL channel. These findings suggest that the lipid constitution of membranes in different cells can influence heparin activity, thus decreasing the effective ability to block the alpha hemolysin ion channel secreted by *Staphylococcus aureus*. These results may

be of interest for pharmacology and future investigations with the objective of prospecting new drugs that have the ability to block the α HL ion channel through the influence of the lipid constitution of membranes in different cells.

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