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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

STUDY OF PHYSICAL AND CHEMICAL PROPERTIES OF ION CHANNELS CREATED BY POLYENE ANTIBIOTICS IN LIPID MEMBRANES

Speciality: 2406.01 – Biophysics

Field of science: Biology

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INTRODUCTION

The relevance of the topic and degree of processing. In addition to studying the cell's ultrastructure and physical and chemical properties, it is important to study the mechanisms of active and inactive transport in the cell, that is, the selective permeability for ions and organic compounds in cell membranes at the molecular level.

Amphotericin B (AmB) channels allow ions such as potassium and sodium to pass through the membrane, destabilizing the cell's internal environment and thus causing cell death. Many diseases caused by dysfunction of sodium, potassium, and other channels are already known to science - physiological disorders such as paralysis, myotonia, and Brugada syndrome and neurological diseases such as epilepsy, attacks, migraine, Lambert-Eaton syndrome, Alzheimer's disease, Parkinson's disease, schizophrenia. Currently, research on membranes is of great importance to treating such diseases more effectively in the future. Research of the ion channels created in the membrane of polyene antibiotics (PA), mainly used against bacterial, viral, and fungal infections, is the basis for medical science.

Since Amphotericin B is known as one of the antibiotics with high toxicity, one of the urgent issues is determining the non-toxic concentration for the human body. Analysis of literature data shows that AmB is toxic to cells only when the ratio of AmB present to sterol (cholesterol and ergosterol) is high. We propose three main methods to reduce the toxicity of AmB. The first method is to use a low dose of Amphotericin B to stay within the total membrane sterol to antibiotic ratio. A second method involves precomplexing Amphotericin B with a sterol (ergosterol or cholesterol) to reduce toxicity while retaining channel-forming activity. The third method uses alkyl derivatives obtained by modifying the amino and carboxyl groups of Amphotericin B and levorin. With these methods, it is possible to treat even severe diseases such as cystic fibrosis in the future.

Elucidating all the mechanisms mentioned above is of great importance both scientifically and practically, and research conducted in this direction will help open wide perspectives in treating diseases.

Purpose and tasks of the study. The main goal of the dissertation is to study the physical and chemical properties of ion channels formed due to the effect of polyene antibiotics taken in different concentrations in double lipid membranes and bacterial membranes with different methods and to conduct a comparative analysis of the results.

In order to achieve the goals set in the research process, it planned to carry out the following scientific research works:

- Different ultraviolet of the complex formed by polyene antibiotics separately and together with cholesterol do not swallow study with the spectrum.
- Antibiotics study of the properties of ion channels resulting from the effect.
- We are investigating the effects of Amphotericin B and levorin alkyl derivatives on membranes by perfusion and kinetic relaxation methods.
- The effect of antibiotics on bacteria's growth and the study of ion channels created by them in membranes.

Research methods. Bilipid lipid membranes (BLM) and bacterial membranes are used for research. The perfusion method determines the washing of antibiotics from the membranes, and the kinetic relaxation method reduces the membrane's integral permeability. Test-object and disk-diffusion methods were used when researching bacteria. Analyzes were performed using pClamp 8.2 software.

Amphotericin B, levorin, and dimethylsulfoxide were used for the study, and their ultraviolet spectra were obtained using a T92 + UV / VIS Spectrometer.

The main provisions of the dissertation presented for defence:

- Under the influence of polyene antibiotics, a polyene-cholesterol complex is formed in bacterial membranes, as in BLM, and causes the electrical resistance of the membrane to change.
- The ion selectivity, channel conductance, lifetime, and open time of AmB channels formed in bacteria and BLM vary depending on pH and temperature.

- AmB and levorin combine with the double bonds of cholesterol, causing a change in the ultraviolet absorption spectra.
- An increase in the length of AmB and levorin alkyl derivatives affects the change in the residence time of the molecules in the membrane.

The scientific novelty of the research. We investigated the channel formation activity of AmB polyene antibiotic in the membranes of *Escherichia coli (E.coli), Pseudomonas aeruginosa (Ps.aeruginosa), Staphylococcus aureus (St.aureus), Candida albicans (C.albicans).*

While studying the ion conductance of AmB channels in *Pseudomonas aeruginosa,* we found that the channel quickly switches to the fully open state. Depending on the time, we observed the formation of channels with low, medium, and high levels of permeability.

St. aureus appears predominantly anion-selective at neutral pH, and both channel conductance and anion selectivity increase at low pH. Increasing antibiotic concentration leads to linear asymmetry of the current-voltage characteristic and loss of selectivity. Higher temperature increases channel conductance in parallel with changes in electrical conductivity but does not change selectivity.

In *C.albicans* experiments, the application of 0.05 and 0.1 mM AmB decreases membrane resistance, which may be associated with a decrease in membrane density. AmB at different concentrations causes the loss of *C.albicans* reproductive capacity and several morphological and physiological disorders.

E.coli, we observed a well-defined conductance spectrum that allowed us to classify six types of AmB channels. Channels with the same conductance were rarely observed and strongly depended on the concentration of AmB. Channel lifetimes are slightly dependent on temperature, but there was a significant decrease in membrane conductance when cholesterol was added to both membranes for the three low-conductance channels. This is undoubtedly due to the increase in frequency. A channel with a large permeability could have been more-lived. The addition of cholesterol markedly reduced the formation of low-permeability channels. The addition of ergosterol increased duct

formation. The addition of cholesterol promoted the formation of channels but reduced their survival time. Decreasing the temperature produced a similar behavior, but this time, the channels remained open longer.

The parameters of bacteria's ion channels such as selectivity, permeability, and the lifetime of the channels in the open and closed state of the membranes are regulated by the structural modification of polyene molecules.

The theoretical and practical significance of the research. In addition to biology, the results obtained from the study of membranes are currently applied in medicine, physiology, immunology, xenobiology, and ecology.

AmB is an immunomodulator and can be offered as an immunostimulator in an appropriate dose range along with other antifungal drugs. AmB-containing drugs such as Fungizone and Ambisome are prepared and administered in the form of liposomes and micelles with lipid-containing phospholipids. AmB is used intrathecally in the treatment of coccidioidomycosis, mucoid leishmaniasis, invasive aspergillosis, blastomycosis, candidiasis, coccidioidomycosis, cryptococcal meningitis, cryptococcosis, severe central nervous system fungal infection in patients with HIV infection. It is used in the treatment of histoplasmosis in patients with HIV infection, pulmonary cryptococcosis in patients with HIV infection, Basidiobolus infection, mucormycosis, sporotrichosis and mycosis of the urinary tract, candidal vulvovaginitis, skin candidiasis, skin and mucous skin infection, and non-invasive gastrointestinal candidiasis. However, their relative toxicity, poor water solubility, and resistance of microflora limit the use of PA in medical practice.

Literature data show that AmB inhibits viral replication by inhibiting the synthesis and replication of viral proteins. Analysis of the data suggests that AmB, like other viruses, can stop the replication and protein synthesis of the COVID-19 virus.

Dissertation approval and application. The results of the dissertation work were heard at the following republican and international conferences and published in their materials: VI Biophysical Congress, (Russia, Sochi, 2019); Eurasian Scientific

Congress. First International Scientific and Practical Conference, (Barcelona, Spain, 2020); Medicinal Herbs: from Past Experience to New Technologies Proceedings of Ninth International Scientific and Practical Conference (Poltova, June 29-30, 2021); Symposium dedicated to the 120th anniversary of Academician V. I. Ulyanishshev of the Institute of Botany of ANAS and the Society of Botanists of Azerbaijan, (Baku, December 25, 2018); International Autumn school of young scientists (dedicated to the year of Heydar Aliyev), (Baku, 2023); Scientific-Practical Conference Modern Approaches in the study of the plant kingdom (dedicated to the year of Heydar Aliyev), (Baku, 2023).

The name of the institution where the dissertation work was performed. The dissertation work was performed at the Experimental botany department of the Botanical Institute of the Ministry of Science and Education of the Republic of Azerbaijan and at the Bacteriology section of the Microbiology department of the V.Y. Akhundov Scientific Research Institute of Medical Prophylaxis of the Ministry of Health of the Republic of Azerbaijan.

The structure of the dissertation and the total volume taken with the mark. The dissertation consists of 248825 characters (excluding spaces in the text and pictures, tables, graphs, appendices, and the bibliography-201730), 52 pictures, and ten tables. Dissertation introduction-10530, literature review-59701 (excluding gaps in the text and pictures, tables-55942), research methodology-35310 (excluding gaps in the text and pictures-34713), research results-109377 marks (gaps in the text and pictures, tables excluding-100545) and consists of a bibliography of 134 articles (28055 signs) and abbreviations - 1039 signs. More than 51% of references in the bibliography refer to the last ten years.

CHAPTER I. LITERATURE REVIEW

In this chapter of the thesis, a brief description of scientific research and literature data related to the general characteristics and chemical structure of polyene antibiotics, their mechanism of action on the membrane, and the structural-functional characteristics of ion channels is given in chronological order.

CHAPTER II. MATERIALS AND METHODS OF RESEARCH

Amphotericin B and levorin are research objects, and those antibiotics and their alkyl derivatives synthesized by Prof. Weinstein at St. Petersburg Institute of Technological Antibiotics and Enzymes. were used. Amino and carboxyl groups of levorin were modified and alkyl derivatives were obtained, and alkyl derivatives are indicated with the symbol R: R-CH_3: methyl-levorin; R-C_2H_5: ethyl-levorin; R-C_3H_7: propyl-levorin; R-C_4H_9: butel-levorin; R-C_5H_{11} : ammi-levorin. Levorin A_2 is one of the components selected for high membrane activity as the main component of levorin A. Alkyl derivatives of Amphotericin B and levorin were dissolved in DMSO at a concentration of 1 mg/ml and used.

Studies were conducted on BLM. A T92 + UV / VIS spectrometer was used to determine polyenes' biological activity.[1] A series of analyses was performed using Statistica 7.0 and pClamp 8.2 software. The studies on bacteria were conducted using the test-object and disk-diffusion methods.

CHAPTER III. RESULTS OF RESEARCH AND THEIR DISCUSSION

3.1. Studied ultraviolet spectra of polyene antibiotics in different concentrations

The main drawback of using AmB is its insolubility in water. Amphotericin B dissolves in organic solvents: 60-80 mg/ml in acidified dimethylformamide, 30-40 mg/ml in dimethylsulfoxide (DMSO), 2-5 mg/ml in propylene glycol, and 2-5 mg/ml in a weak alkaline solution or methanolic acid.[2] For our research work, we used AmB dissolved in a DMSO solution.

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[1]Вязьмин С.Ю. Электронная спектроскопия органических соединений / Рябухин Д.С., Васильев А.В. // - Санкт-Петербург, СПбГЛТА, - 2011, - с. 1-43.

[2] Cavassin F. B. Sixty years of Amphotericin B: An overview of the main antifungal agent used to treat invasive fungal infections. / Luiz Bau-Carneiro J., Vilas Boas R.R., Queiroz-Telles F. // Infect. Dis. Ther., – 2021, 10(1), – p. 115-147.

The DMSO molecule is amphiphilic and highly polar. Due to oxygen bonds, it has a chain structure. Examination of the absorption spectra of DMSO in the wavelength range from 350 nm to 2200 nm shows that DMSO is optically transparent in this range (Figure 3.1.1.). The absorption spectrum of dimethylsulfoxide molecules at the indicated wavelengths is due to the presence of the disulfide S=O group.[3]

Antibiotics are readily soluble in DMSO. When dissolved in DMSO, the biological activity of Amphotericin B and levorin increases dramatically. Antibiotics in DMSO solution are approximately 10-100 times more effective than the original water-soluble forms. DMSO has properties such as amphiphilicity, polarity, high resorption, molecular shape, and the ability to dissolve many organic compounds.

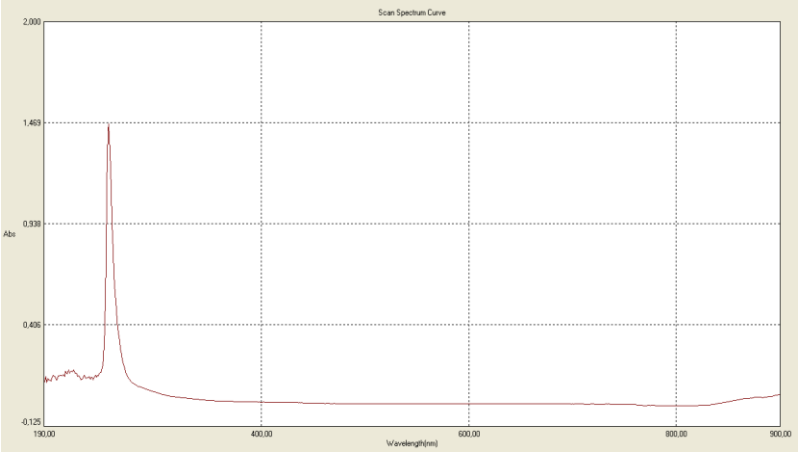

Figure 3.1.1. UV differential absorption spectrum of DMSO

DMSO molecules can cross tissue barriers, quickly entering cells and human skin. Their dielectric constant indicates that DMSO

[3] Fei H. Ion transport through dimethyl sulfoxide (DMSO) induced transient water pores in cell membranes / Weirong L., Shengchao Z., Li Z. [et al.] // Molecular Membrane Biology, – 2012, 29(3-4) – p. 107-113.

lies between water and lipids and is characterized by a high degree of resorption into tissues. This suggests that DMSO can be used to transport various drug compounds into tissues. DMSO increases the permeability of many substances of low molecular weight through biomembranes and also increases the rate of entry into cells.
Figure 3.1.1. shows the absorption spectrum of DMSO. As can be seen from Figure 3.1.1., the absorption spectrum of DMSO is near the UV absorption region. The absorption spectrum of DMSO is between 222 and 224 nm**.**

The main feature of PA is that it produces an absorption spectrum with three maxima. The spectra of levorin and Amphotericin B also differ in three primary absorption spectra. The absorption spectra of antibiotics vary between 370 nm and 430 nm. UV absorption spectra reflect the characteristic spectrum of polyenes belonging to this class and their biological activity.

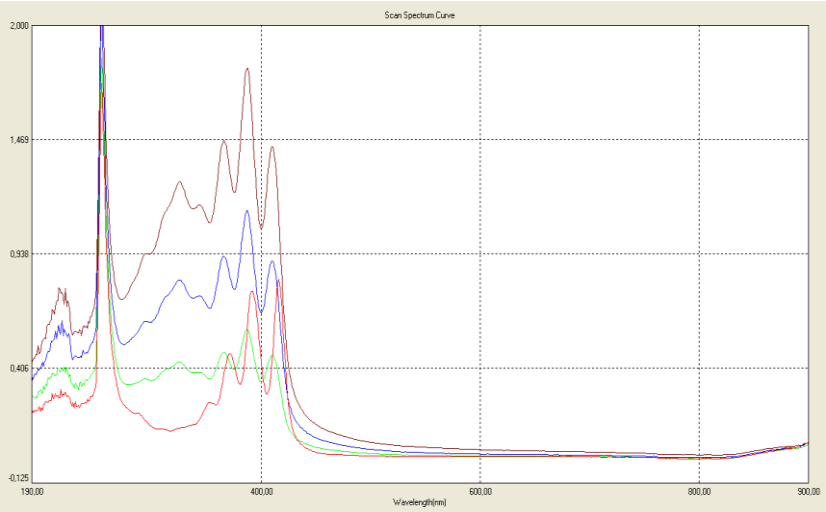

Figure 3.1.2. UV absorption spectra of levorin and Amphotericin B at different concentrations

The physicochemical properties of Amphotericin B and levorin in complex form and their dependence on their concentration

were studied in different proportions. The figure also shows the UV absorption spectrum of levorin and Amphotericin B at different concentrations in Figure 3.1.2. In Figure 3.1.2. the red curve was obtained with levorin at a concentration of 0.3 ml added to a cuvette in 3 ml of DMSO, blue curve was obtained from levorin at a concentration of 0.2 ml added to a cuvette in 3 ml of DMSO, the green curve was obtained from levorin at a concentration of 0.1 ml added to a cuvette in 3 ml of DMSO and the bright red line was obtained at a concentration of 0.01 ml of Amphotericin B in 3 ml of DMSO.

As can be seen from Figure 3.1.2, as the concentration of antibiotics increases, the maximum of the ultraviolet absorption spectrum also increases.

3.2. Investigation of the complex formed by cholesterol with polyenes by UV spectroscopy

The addition of cholesterol or other sterols to the aqueous solution of PA leads to a decrease in the antibiotic's UV absorption spectrum, which is the result of the formation of a complex of PA with cholesterol. The presence of sterols does not change the wavelength of polyenes' absorption maxima, only the absorption maxima. According to the effectiveness of the interaction of PA with cholesterol, they are in the following order: filipin > Amphotericin B > etruscomycin > pimaricin > nystatin.

In many cases, the structure of sterols determines the effectiveness of their interaction with polyenes. Thus, sterols containing the 3β-OH group are more effective against polyenes than those containing the 3α-OH or 3-keto groups. The interaction of polyenes with sterols is based on the C 17 carbon atom of the antibiotic forming a hydrogen bond with the 3β-OH group of the sterol molecule.[4]

Figure 3.2.1. shows the absorption spectra obtained during the interaction of Amphotericin B with cholesterol. Here:

[4] Pashazade T., The Properties and Clinical use of Polyene Antibiotics // Khazar Journal of Science and Technology, – 2023, v. 7, №1, – p. 5-13

the spectrum indicated by the dark red line by adding 0.03 ml of Amphotericin B dissolved in 1 mg/ml DMSO to 3 ml of DMSO in the first quartz cuvette;

the spectrum indicated by the blue line by adding 0.03 ml of Amphotericin B dissolved in 1 mg/ml DMSO and 0.5 mg cholesterol dissolved in ethanol to 3 ml DMSO in the first quartz cuvette;

and the spectrum indicated by the light red line with the addition of 0.03 ml of Amphotericin B solution dissolved in 1 mg/ml DMSO and 1 mg cholesterol dissolved in ethanol to 3 ml DMSO in the first quartz cuvette;

and the spectrum indicated by the green line was obtained by adding 0.03 ml of Amphotericin B solution dissolved in 1 mg/ml DMSO and 2 mg cholesterol dissolved in ethanol to 3 ml DMSO in the first quartz cuvette. A second quartz cuvette contained 3 ml of ethanol solution when each spectrum was taken.

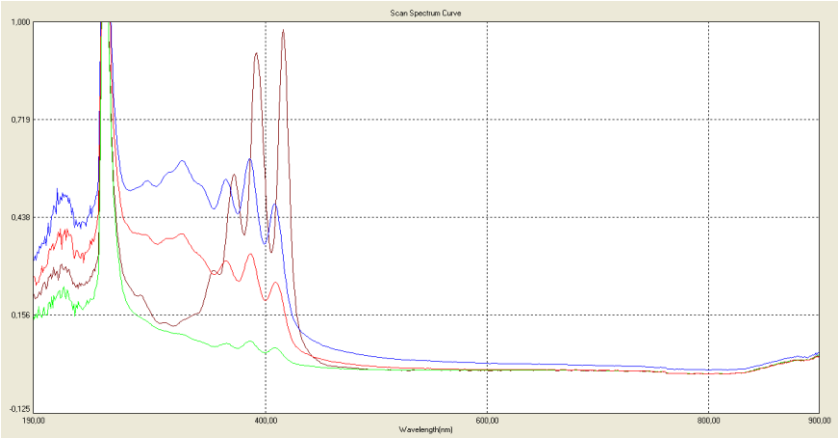

Figure 3.2.1. UV absorption spectrum of Amphotericin B obtained from its interaction with cholesterol

Figure 3.2.2. shows the UV absorption spectra obtained as a result of Levorin's interaction with cholesterol. Here:

the spectrum indicated by the dark red line by adding levorin to 3 ml of DMSO, taken in a volume of 0.03 ml from a solution of levorin dissolved in 1 mg/ml DMSO in the first quartz cuvette;

blue-line spectrum with levorin taken in 0.03 ml of a solution of levorin dissolved in 1 mg/ml DMSO and 0.5 mg of cholesterol dissolved in ethanol added to 3 ml of DMSO in the first quartz cuvette;
green line - obtained by adding 0.03 ml of amphotericin B and 1 mg of cholesterol dissolved in ethanol to 3 ml of DMSO from a levorin solution dissolved in 1 mg/ml DMSO in the first quartz cuvette. During the acquisition of each spectrum, a second quartz cuvette contained 3 ml of ethanol solution.

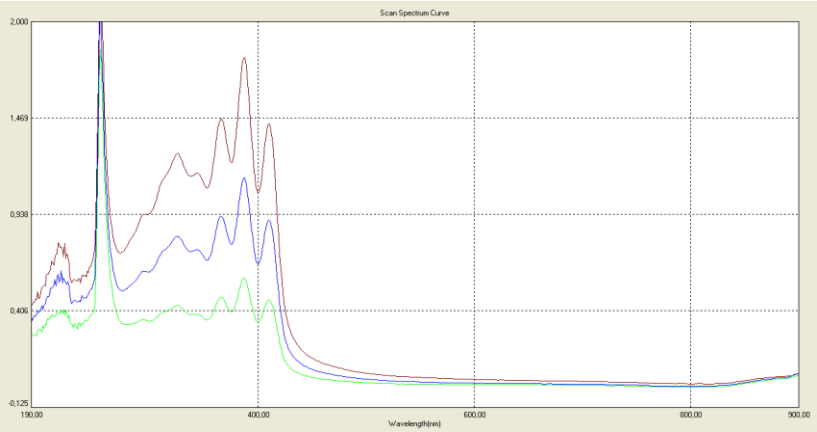

Figure 3.2.2. UV absorption spectra obtained as a result of the interaction of levorin with cholesterol

Amphotericin B and levorin form a complex with cholesterol and lower the maximum UV absorption spectra (Figure 3.2.1. and Figure 3.2.2.). An increased amount of cholesterol further lowers the maximum UV absorption spectra. Given the specificity of PA interactions with cholesterol, we hypothesized that polyene antibiotics can be used as fluorescence labels to quantify cholesterol in biological and lipid membranes.[5]

[5] Pashazade Turkan, Study of physical and chemical properties of ion channels created by polyene antibiotics in lipid membranes // Advances in Biology & Earth Sciences, – 2022, Vol.7, No.2, – p.128-134.

3.3. Effects of antibiotics on lipid membranes

In order to analyze the characteristics of ion channels depending on the structure of the lactone ring of polyene molecules, studies were conducted on lipid membranes obtained from a mixture of phospholipids with cholesterol, and the results were compared with the characteristics of channels obtained with Amphotericin B, nystatin and mycoheptin (Figure 3.3.1.).[6]

The molecules of these antibiotics differ only in the structure of the lactone ring. The heptaene chain of polyenes, e.g., is the same for Amphotericin B and mycoheptin. One double bond within the polyene chain in the nystatin molecule is hydrogenated (tetraene). The hydrophilic chain of the lactone ring is different for all three antibiotics. The support of hydrophilic groups is the same, but their locations are different. In mycoheptin, a hydroxyl group is replaced by a carbonyl group.

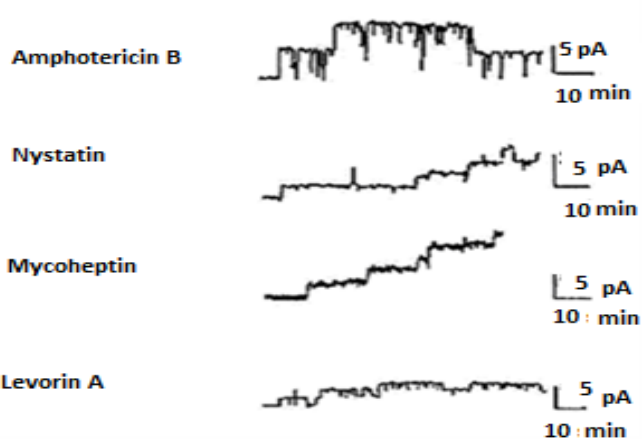

Figure 3.3.1. Recording of single ion channels in lipid membranes in the presence of PA

The concentration of antibiotics in taking single-ion channels is as follows: Amphotericin B ($2•10^{-8}$ M), nystatin ($1•10^{-7}$ M), mycoheptin ($2•10^{-8}$ M) and levorin ($5•10^{-9}$ M).

[6] Pashazade T.C. The action of nystatin and Amphotericin B on the thin lipid membranes. Действие нистатина и амфотерицина В на бислойные липидные мембраны. // VI Biophysical Congress,- Russia, Sochi, – 2019. v. 1, – p. 178-179.

Membranes were prepared from a liquid containing a 20:1 weight ratio of phospholipids and cholesterol dissolved in heptane. The liquid was available in aqueous solutions: 2 M KCl, pH 7.0, t = 22°C. The membrane potential was 200 mV.

The Amphotericin B channel has the highest permeability (6.5 pS), the nystatin channel has a lower conductance (2 pS), the mycoheptin channel exhibits minimal conductance (0.5 pS), and the levorin channel conductance is even lower (0.2-0.3 pS).

3.4. Determination of membrane residence time of Amphotericin B alkyl derivatives

One parameter characterizing PA's biological effectiveness is the level of induced antibiotic permeation and the constant time constant of permeation during antibiotic washout from BLM. Amphotericin B and its alkyl derivatives were modified on amino and carboxyl groups, and levorin and their alkyl derivatives were used in the research. Increasing the concentration of Amphotericin B alkyl derivatives by ten times makes it possible to observe a several-fold increase in membrane permeability.

The relaxation of the induced conductance varies from 100 to 1000. Thus, the concentration of PA can broadly regulate its biological effect.

During the antibiotic's leaching from the membrane, the effectiveness of the membrane's permeability to the antibiotic and the constant relaxation time is highly dependent on the membrane's sterol content. A significant decrease in the constant relaxation time decreases the toxicity of alkyl derivatives of amphotericin (Table 3.4.1.).[7]

The investigated derivatives are rapidly eluted from cholesterol-containing membranes, but antibiotics are slowly eluted from ergosterol-containing membranes. Metaphosin is comparatively

[7] Пашазаде Т.Д., Касумов Х.М. Свойства ионных каналов, образованных при одностороннем действии амфотерицина и N-метилпроизводного амфотерицина В в бислойных липидных мембранах // Ж. Биофизика, – 2021, т. 66, № 3. – с.504-510

ten times less toxic than natural amphotericin. This may be related to the rapid rate of metamphosin release from the membrane.

To estimate this rate, the release time of Amphotericin B alkyl derivatives from the membrane can be studied using the conductivity relaxation method. Extending the hydrocarbon chain by one CH_2 group decreases the relaxation time constant by about four times.

Extrapolation of this dependence to n=0, corresponding to the natural amphoteric, gives a measure of τ=160 seconds. By inducing membranes with alkyl derivatives of different lengths of the hydrocarbon chain-R = CH_3 - methyl; R = C_2H_5 - ethyl; R = C_3H_7 - propyl; R = C_4H_9 - butyl; R = C_5H_{11} - factor makes it possible to determine the life time of antibiotics in membranes. In Table 3.4.1., the constant relaxation time (τ_r) of BLM conductance in washing alkyl derivatives of amphotericin from one side of the membrane is determined.[8]

When cholesterol is replaced by ergosterol in membranes, the relaxation time constant (τ_r) increases dramatically. When there is a constant concentration of the antibiotic, it is impossible to observe the change in the permeability of cholesterol-containing membranes within 40-50 minutes.[9]

The described data show that the alkyl derivatives of Amphotericin B and levorin have a high and practical effect on ergosterol-containing fungal cells.

[8] Пашазаде Т.С. Поиск мембраноактивных препаратов на основе алкильных производных амфотерицина В для профилактики растительных инфекций. Лекарственное растениеводство: от опыта прошлого к современным технологиям. Материалы девятой Международной научно-практической конференции -Полтава. – 2021., – с. 139-141

[9] Пашазаде Т.Д., Касумов Х.М. Свойства ионных каналов, образованных при одностороннем действии амфотерицина и N-метилпроизводного амфотерицина В в бислойных липидных мембранах // Ж. Биофизика, – 2021, т. 66, № 3. – с.504-510

Table 3.4.1.

Determination of the constant relaxation time (τ_r) of the BLM conductance in washing alkyl derivatives of amphotericin from one side of the membrane

Antibiotic	The composition of the membrane	Concentration, M	pH	time , min .	Registration
Metaphosin	Etgosterin : phospholipid = 0.05	$3 \cdot 10^{-7}$	7.0	30	Conductivity change in 30 minutes is not registered .
Ethamfosine	«----------»	$1.5 \cdot 10^{-7}$	7.0	-	was recorded within 30 minutes .
Propamphosin	«----------»	$3 \cdot 10^{-7}$	7.0	-	was recorded within 30 minutes .
Butamphosin	«----------»	$3 \cdot 10^{-7}$	7.0	-	was recorded within 50 minutes .
Amphotericin B	Cholesterol phospholipid = 0.5	$5 \cdot 10^{-7}$	7.0	160	conductivity change in 30 minutes was not recorded.
Metaphosin	«----------»	$5 \cdot 10^{-7}$	7.0	38	
Ethamfosine	«----------»	$5 \cdot 10^{-7}$	7.0	84	
Propamphosin	«----------»	$5 \cdot 10^{-7}$	7.0	0.46	
Amphotericin B*	«----------»	$3 \cdot 10^{-6}$	3.0	-	was recorded within 30 minutes .
Metaphosin *	«----------»	10^{-6}	3.0	10.6	
Butamphosine *	«----------»	10^{-5}	3.0	5.1	$19\ ^0C$
Butamphosine * -	«----------»	10^{-5}	3.0	2.6	$27\ ^0C$

3.5. Antibiotics unilateral study of the properties of ion channels resulting from the effect

Amphotericin B was at a concentration of 10^{-6} M, and a sharp increase in the permeability of the membranes (up to the conductivity of the electrodes) was observed when inserting it into

both sides of the membrane. Studies have shown that when Amphotericin B was added to one side of membranes containing cholesterol at a concentration of $5 \cdot 10^{-4}$ M, the membrane's permeability remained unchanged for a long time (1 hour). However, when Amphotericin B is on one side of cholesterol-containing membranes, and the membranes have a phospholipid content of 10 mg/ml and a ph as low as 3.0, the activity of ion channels has been studied, and the high permeability of the membranes has been shown in studies. When Amphotericin B was inserted into one side of the membrane at a concentration of $2 \cdot 10^{-8}$ M, it formed ion channels in the membrane. The image shows a recording of single ion channels formed when Amphotericin B is present on one side of the membrane. Figure 3.5.1. shows a histogram of the conductance size distribution of single unitary ion channels.

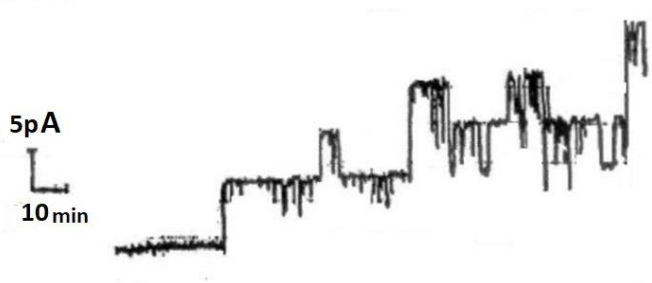

Figure 3.5.1. Dynamics of operation of single ion channels formed on one side as a result of the action of Amphotericin B on the membrane at a concentration of $2 \cdot 10^{-8}$ M

This experiment was conducted in 2 M KCl aqueous solution, pH 3.0, t=22 ^{o} C, membrane potential +200 mV, and composition of the membrane consist of 10 mg of phospholipids and 4 mg of cholesterol in 1 mg of heptane. A large dispersion of single channel conductances is seen from 2 pS to 20 pS. Channels from 7 pS to 10 pS appear with the highest probability. The time of accumulation of channels does not depend on the size and direction of the membrane's electric field. During the unilateral study of Amphotericin B, the selective permeability of the membranes was

determined, and it was shown that the membrane's selective permeability is mainly for anions and does not depend on the concentration of cholesterol in the membrane.

During the one-way modification of membrane permeability pH=3 from the concentration of Amphotericin B and concentration of methamphosin at neutral pH, the membrane permeability coefficient shows the same n=4 degree. During unilateral modification of metafosine, the selective permeability of membranes is mainly for anions and does not depend on the concentration of cholesterol in the membrane.

Another experiment was related to studying the effect of Amphotericin B antibiotic on the properties of the double lipid membrane depending on the pH and the properties of the formed single ion channels (Figure 3.5.2.).[10] The pH on the cis and trans side of the lipid bilayer membranes was equated, and pH seven was taken. The pH was adjusted by adding small amounts of concentrated solutions of HCl or KOH. At 40 and 20 volts, the single-channel current amplitude decreased after the pH was lowered to 6 on the cis side of the membrane, whereas the single-channel current amplitude did not change when the pH was increased from 7 to 8 on the cis side of the membrane. The pH 7 and 8 conductance was 500 and 560 pS, respectively. On the opposite side, the single channel conductance dropped to 250 pS as the pH decreased to 6. The potential on the trans side was 35, 36, and 40 mV at pH 6, 7, and 8, respectively. The probability of the channel opening on the trans side increased at potentials above 40 mV at pH 7 and 8; on the opposite side, lowering the pH to 6 decreased the channel open probability at all voltages, and we explain this by the mechanical narrowing of the pore in the AmB channel.

[10] T.C. Pashazade. Study of physical and chemical properties of ion channels created by polyene antibiotics in lipid membranes, Advances in Biology & Earth Sciences, – 2022, v. 7, No.2, – p.128-134.

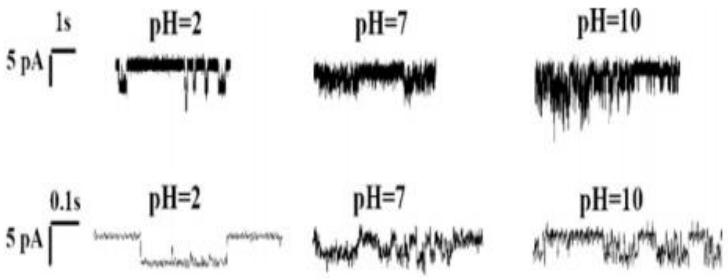

Figure 3.5.2. Typical current recordings of single ion channels induced by Amphotericin B in bilipid membranes, potentials at different pH values in buffer solutions on the cis side of the membrane

3.6. Experiments on bacteria and bacterial membrane

The bacterial membrane is biologically compatible with the living cell model and can better aid in testing antibiotics.[11]

E. coli, while Amphotericin B 1 mg/ml had no effect at 30 and 60 minutes of exposure but showed an antimicrobial effect at 120 and 180 minutes. When exposed to dark-colored substances, Amphotericin B 1 mg/ml has an antimicrobial effect at all exposure times. Exposure to 10 mg/ml of amphotericin shows an antimicrobial effect during the entire exposure period. Levorin C 10 mg/ml is antimicrobial at all exposure times. Carbolevorin and isolevorin showed antimicrobial effects at all exposure times.

Ps.aeroginosa - levorin did not show an antimicrobial effect at exposure times of 30 and 60 minutes when exposed to 1 mg/ml and showed an antimicrobial effect at exposure times of 120 and 180 minutes. Amphotericin B 1 mg/ml did not show an antimicrobial effect at 30 and 60 minutes of exposure, but it did at 120 and 180 minutes. Amphotericin B 1 mg/ml dark-colored solution,

[11] Pashazade T.C., Sultanova G.H., Effect of Polyene Antibiotics on a bacteria and bacterial membrane // International Autumn school of young scientist dedicated to the year of Heydar Aliyev, – Baku -2023, – p.104-105.

Amphotericin B 10mg/ml, Levorin 10 mg/ml, Carbolevorin, and Isolevorin showed an antimicrobial effect in all exposure periods.

St.aureus - levorin 1 mg/ml has no antimicrobial effect for 30 minutes. It had a bacteriostatic effect at 60 and 120 minutes of exposure time. In 180 minutes, the microbial cells developed again and ended. Amphotericin B 1 mg/ml had no effect for 30, 60, or 120 minutes but showed an antimicrobial effect for 180 minutes of exposure. Amphotericin B 1 mg/ml dark-colored solution, Levorin 10 mg/ml, and Amphotericin B 10 mg/ml were affected at all exposure times. However, carbolevarine and isolevarine had no effect.

C.albicans - levorin 1 mg/ml, Amphotericin B 1 mg/ml, Amphotericin B 1 mg/ml dark-colored, levorin 10 mg/ml, and isolevorin were effective at all exposure times. During exposure to carbolevarine, there was no effect for 30 minutes, but it had an effect for 60, 120, and 180 minutes.

The experiments show that Amphotericin B 10 mg/ml, levorin 10 mg/ml, and Amphotericin B 1 mg/ml are among the best antimicrobial agents. The DMSO taken for the control test culture itself has antimicrobial properties.

Let us first review the experiments on *Ps.aeroginosa* more broadly. AmB has an antimicrobial effect on *Ps.aeruginosa,* and its efficacy, i.e., the lethal effect, increases in a dose- and time-dependent manner (Table 3.6.1.).

We investigated the channel-forming activity of the polyene antibiotic AmB in *Ps.aeruginosa* using the method of ion channel reconstruction. In the unilateral insert, we failed to detect the formation of any channels with well-defined conductance levels. We only rarely saw channel formation. However, we observed that channels with reproducible conductance were formed when we included AmB at different concentrations. When studying the ion conductance of single AmB channels, we found that the channel briefly switches to the fully open state. We observed the formation of channels with different levels, medium, and high permeability depending on the time (Figure 3.6.1.).

Table 3.6.1.
Percent survival *of Ps.aeroginosa* as a function of Amphotericin B dose

Amphotericin B used dose, mg/ml	Survivors in %
20	39.4±24.3
5	59.1±20.1
1	86.7±19.3
0.2	90.6±19.1
Control	100±16.2

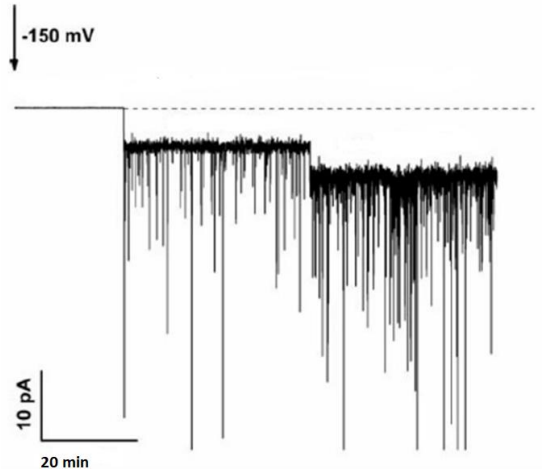

Figure 3.6.1. Typical ionic current obtained from the membrane after addition of 50 mg AmB to 1.5 ml DMSO solution

The AmB channel formed in *St.aureus* appears to be predominantly anion-selective at neutral pH, and both channel conductance and anion selectivity increase at low pH. Increasing antibiotic concentration leads to a linear asymmetry of the current-voltage characteristic and loss of selectivity. Higher temperature and pH increase channel conductance in parallel with changes in electrical conductivity but do not change selectivity (Figure 3.6.2.).

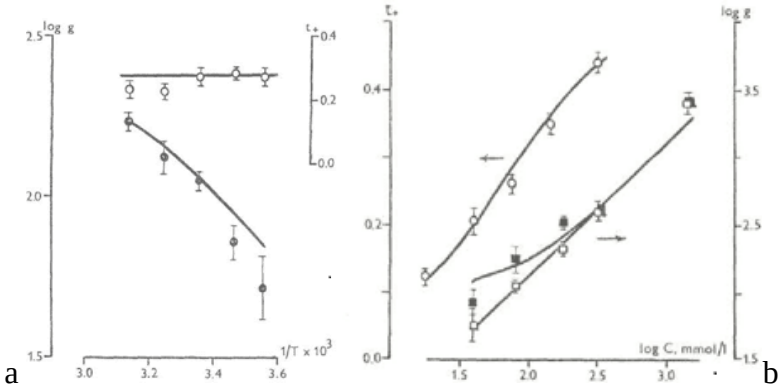

Figure 3.6.2. Effect of temperature (a) and activity of AmB (b) on conductance and cation-anion selectivity of AmB channels in *St. aureus* , pH=7.5

The *Candida albicans* bacterial membrane is reflected in the graphs below.

C.albicans showed that the application of 0.05 and 0.1 mM AmB decreased resistance, which may be associated with a decrease in membrane density. AmB applied at a concentration of 0.05 mM also decreased the number of channels but did not significantly change their open probability. At different concentrations, AmB did not rapidly decrease the number of *C.albicans* cells. However, significant loss of proliferative capacity and numerous morphological and physiological disturbances, including cytoplasmic shrinkage and oxidative stress, occurred (Figure 3.6.3.).

C.albicans culture with increasing concentration of Amphotericin B in the medium, a strain resistant to the effect of Amphotericin B, was isolated. It also became clear that instead of ergosterol, which is characteristic of bacterial membranes, the cells of the resistant strain contain a heterogeneous sterol component that absorbs in the 212-242 nm region and does not interact with PA. The strain resists 50 μg/ml Amphotericin B and 5 μg/ml levorin. However, *C.albicans* had low resistance against PA at low concentrations of antibiotics: Amphotericin B (0.35 μg/ml) and

levorin (0.15 μg/ml). The resistant strain cells do not differ from the original culture in terms of morphological characteristics and produce a small amount of biomass in an antibiotic-free medium (Figure 3.6.4.).

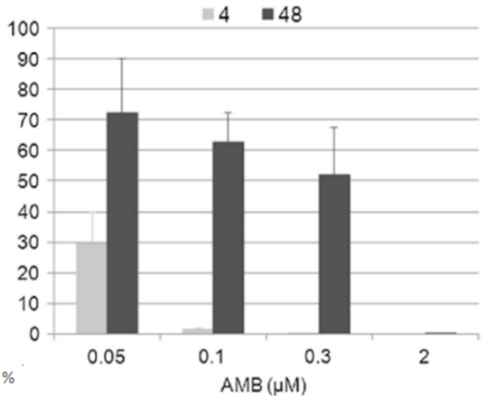

Figure 3.6.3. Proliferative capacity of control culture treated with AmB

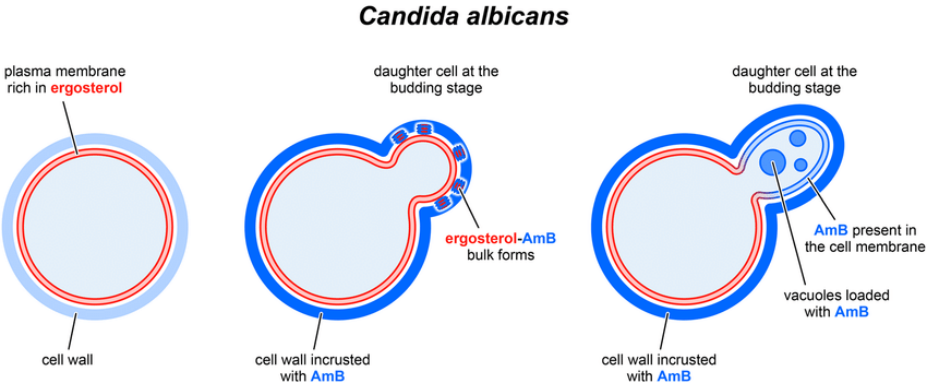

Figure 3.6.4. Model of the effect of Amphotericin B *on Candida albicans*

In addition, several parameters of single ion channels in the membranes of *E.coli* were studied.

A well-defined conductance spectrum was observed, allowing the classification of six types of AmB channels. These experiments were conducted in 2 M KCl and 1 mM CaCl2 buffer solution, pH 8.0, and potential 200 mV. These different conductances cannot be assigned as substates of a single AmB channel. This is because channels with overlapping conductances were rarely observed, which strongly depended on the concentration of AmB, indicating that the events were independent. Conductivity for channel I is between 1.5 and 4.2 pS; from 4.3 to 12.5 pS for channel II; 12.6 to 25.0 pS for channel III; 25.1 - 47.5 pS for channel IV; 45.6 to 58.0 pS for channel V; and varies from 58.1 to 75.0 pS for channel VI (Figure 3.6.5.).

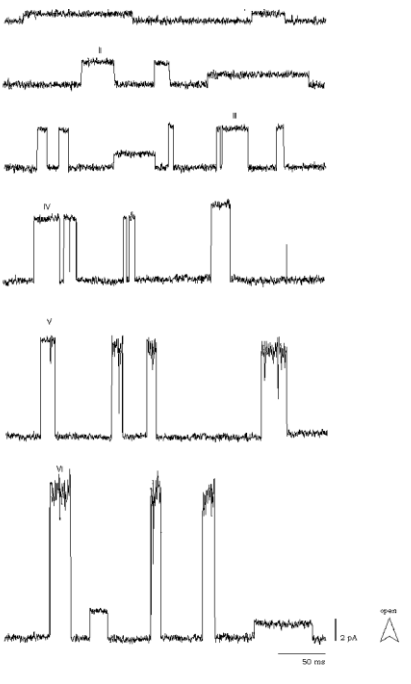

Figure 3.6.5. Typical ion recordings of six types of AmB channels in the membranes of *E.coli*

Channel lifetimes are slightly dependent on temperature, but there was a significant decrease in membrane conductance when

cholesterol was added to both membranes for the three low-conductance channels. This is undoubtedly due to the increase in frequency. A channel with a large permeability could have been more-lived. The addition of cholesterol markedly reduced the formation of low-permeability channels. The addition of ergosterol increased formation of the channels. The addition of cholesterol promoted the formation of channels but reduced their survival time. Decreasing the temperature produced a similar behavior, but this time, the channels remained open longer.

3.7. Proposing polyene antibiotics as antiviral drugs in the treatment of COVID-19

PA can affect the penetration of viruses into the cell and inhibit their reproductive activity. The effect of Amphotericin B and levorin on fungi and bacteria has long been known to science. Recently, AmB and levorin have been used in research as antiviral agents. According to the experiments carried out in our laboratory, levorin in small concentrations (10^{-7} -10^{-6} M) along with fungi and bacteria such as *Salmonella typhimurium (I), Salmonella typhimurium (II), Proteus vulgaris, Pseudomonas aeruginosa, E.coli, Staphylococcus aureus, Candida albicans* also has antiviral effect like *Coxsackie A20 and ECHO 9.* The effect of Levorin on viruses was studied in fibroblasts of human embryos. In the primary cell culture, the virus growth is stopped by coming into contact with the lipid components, and the yellow color reaction of the medium indicates the stop of the virus development. Levorin can also destroy viral and fungal infections so that it can be used therapeutically as a biologically active substance.

Like levorin, AmB is effective against bacteria and fungi in laboratory conditions. However, many studies are currently focused on the different mechanisms of action and potential therapeutic effects of AmB derivatives against viruses, including human immunodeficiency virus (HIV), herpes virus (HSV), Japanese encephalitis virus, rubella virus, hepatitis B, enterovirus 71.

Literature data show that AmB inhibits viral replication by inhibiting the synthesis and replication of viral proteins. Data

analysis suggests that, like other viruses, AmB can stop the replication and protein synthesis of the COVID-19 virus. While studies and clinical trials of AmB may require time, based on the results of previous studies on the unique properties and efficacy of AmB against various viruses, the administration of AmB alone or in combination regimens can reduce the side effects of drugs and improve the health of patients with COVID-19. It can be effective.

3.8. The practical significance of the work done

At a relatively low concentration (0.2-3.7 mcg/ml), Amphotericin B inhibits the growth of many fungi that cause disease in humans, animals, and plants. Its concentration of 0.03-1 mcg/ml is an example of activity against Cryptococcus, Blastomyces, and Histoplasma, which cannot be affected by high doses of other antifungal antibiotics. Actinomyces, Nocardia, bacteria, and protozoa are also resistant to amphotericin.[12]

Combining Amphotericin B with rifampicin and tetracycline greatly enhances its antifungal effect. The application of Amphotericin B combined with rifampicin against Aspergillus, Candida, Coccidiodes, Immitis, Blastomyces, Dermatitidis, and others, combined with tetracycline against Histoplasma and Capsulatium is synergistically important. The therapeutic concentration of Amphotericin B has a fungistatic effect on fungi. The fungicidal effect is revealed when the pH 4.0 concentration of the medium in vitro is taken slightly above the amount of 3 μg/ml, and the effect lasts for more than 12 hours.

Resistance to Amphotericin B in the causative agent develops slowly. When applied together with nystatin and other antifungal antibiotics, cross-resistance to it is observed in the causative agent. The optimal effect is revealed when the environment's pH reaches 5.5-7.0. This antibiotic's activity is weakened when cysteine, magnesium, and calcium ions are introduced into the medium. Like

[12] Pasha-zade T.C. Pharmacology of Amphotericin B // Eurasian Scientific Congress. I-st International Scientific and Practical Conference, – Barcelona, Spain, – 2020, – p. 33-38

other polyene antibiotics, Amphotericin B binds to sterol in the cell, causing its permeability to the fungal cell membrane.

The body poorly absorbs oral Amphotericin B. At the high dose used (3-7 g), the blood serum level is 0.3-1.9 μg/ml. The concentration of the drug administered intravenously at the rate of 0.5-1 μg/kg is 2-3 μg/ml, and it can remain in the blood for only 6-8 hours. Amphotericin B is very late in the kidneys, i.e., 5% of the administered dose is excreted within the first 24 hours and 20-40% within seven days.

The methods we propose in our research work help reduce AmB's toxicity, which creates great opportunities for the effective treatment of the above-mentioned diseases and the creation of new drugs.

RESULTS

1. Since the polyene-cholesterol complex is formed in bacterial membranes, as in bimolecular lipid membrane, the membrane's electrical resistance decreases sharply under the influence of polyene antibiotics.
2. AmB channels formed in bimolecular lipid membrane and bacteria have selective permeability for anions at neutral pH, but levorin channels are cation transporters depending on the number of carbonyl and carboxyl groups. At low pH, both channel and ion selectivity increase, but while channel permeability increases at high temperatures, ion selectivity does not change.
3. The open time of Amphotericin B channels increased at pH 7 and 8 in both bimolecular lipid membrane and bacterial membranes and decreased with a decrease in pH to 6, with a lifetime of 3-5 milliseconds. Although the concentration of polyene antibiotics does not affect the opening time of the channels, temperature changes do.
4. Amphotericin B and levorin combine with the double bonds of cholesterol, lowering the maximum ultraviolet absorption spectra.
5. As the length of Amphotericin B and levorin alkyl derivatives increases, the length of stay of molecules in the membrane decreases.
6. Membrane channels have a selective permeability for anions and do not depend on the cholesterol concentration in the membrane during exposure to metamphosin. Metamphosin increases membrane permeability at neutral pH.
7. Carbolevorin and isolevorin at 1 mg/ml concentrations and 10 mg/ml do not show an antimicrobial effect on *St.aureus* for 30-120 minutes.

LIST OF PUBLISHED SCIENTIFIC PUBLICATIONS RELATED TO THE DISSERTATION TOPIC

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6. **Pashazade T.C.** The action of nystatin and Amphotericin B on the thin lipid membranes, Действие нистатина и амфотерицина В на бислойные липидные мембраны // VI Biophysical Congress, – Russia, Sochi, – 2019, v. 1, – p. 178-179.
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16. **Pashazade T.C.** Analysis of spectral characteristics of certain polyene antibiotics in the presence of DMSO and cholesterol (Анализ спектральных характеристик некоторых полиеновых антибиотиков в присутствии дмсо и холестерина) / Sultanova G.G., Kasumov Kh.M. // Антибиотики и Химиотерапия, - Россия: – 2023. т. 68, № 11-12, – с. 10-18.

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