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Significance of Polyene Antibiotics in Increasing Membrane Permeability and in the Treatment of Animal and Plant Infections

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Using polyene antibiotics (PAs) in combination with dimethyl sulfoxide (DMSO) was shown to increase ion permeability of membranes and biological activity of antibiotics sharply. The comparative physical and chemical characteristics of dimethyl sulfoxide and PAs were determined. The effects of a complex relation between PAs and the bilayer lipid membranes (BLM) were studied. The parameters of biological activity of PAs and BLM were determined. It was shown that among all the studied PAs, amphotericin B and levorin were the most effective. Ion permeability of BLM was shown to depend on the concentrations of amphotericin B, levorin and cholesterol. On the basis of PAs, biological active preparations were developed against viral, bacterial and fungal diseases.

Keywords: *Polyene antibiotics, dimethyl sulfoxide, amphotericin B, levorin, lipid membranes, membrane permeability, animal and plant infections*

INTRODUCTION

Polyene antibiotics (PAs) are one of the most effective compounds in the fight against fungal infections (Borowski, 2000). The main representatives of PAs are amphotericin B, nystatin, mycoheptin and levorin. PA molecules contain a lactone ring, a conjugated system of double bonds and a hydrophilic chain consisting of hydroxyl and carbonyl groups. The biological activity of PAs depends on the presence of a certain structure in the membranes of sterol cells (Récamier et al., 2010). Polyenes are more sensitive to membranes containing ergosterol (Ciesielski et al., 2016). Due to this distinctive feature, polyenes are successfully used in medicine for therapeutic purposes. Currently, amphotericin B and nystatin are mainly used in the treatment of systemic fungal infections. A comparative analysis of the biological activity of amphotericin B and nystatin shows that amphotericin B is about 6 times more effective against most fungi than nystatin (Aszalos et al., 1985). The BLM method showed that the conductivity of the amphotericin channel is about 10 times higher than that of the nystatin channel (Gasymov, 2009). Amphotericin B and nystatin are very close to each other in their chemical structures, but membranes with cholesterol are more sensitive to amphotericin B than to nystatin. According to the studies, the presence of a certain number of double bonds in the chromophores of PAs is an important factor determining their biological activity (Samedova et al., 2018). Amphotericin B and nystatin differ in the number of double bonds in the chromophore structures of polyene molecules. Nystatin has less double bonds than amphotericin B, which is the reason of the markedly less biological activity of nystatin. PAs were not chosen randomly as objects of the study. The peculiarity of PAs is that they constitute the only class of compounds in nature that forms molecular-sized channels in the cell and lipid membranes selectively permeable to ions and organic compounds (Ibrahimova et al., 2006; Gasymov, 2009; Récamier et al., 2010; Cohen, 2010). The studies of the molecular mechanism of the interaction of PAs with membranes showed that polyenes in combination with sterols create channels with certain structures in the membranes (Gasymov, 2009). However, in spite of the presence of a large amount of PAs and their derivatives, none of them can prevail the effectiveness of the action of amphotericin B and levorin in the treatment of systemic fungal infections. In recent years, scientists aimed at obtaining new medicinal forms of PAs and the development of new ways of their delivery to the affected organs and tissues. Interest in antifungal preparations has increased due to the widespread HIV infection, which appeared to be sensitive to the presence of fungal infections in the organism (De Marie et al., 1994). About 90% of HIV-infected patients were found to be infected with a fungal infection, because of the weakening immune system of the organism (Mamidi et al., 2002). In addition, patients are administered immunosuppressive preparations in the transplantation of various organs and bone marrow. However, they create conditions

for the appearance of HIV and fungal infections in patients (Sepkowitz, 2002). Currently, researches focus on a more comprehensive study of the action mechanisms of PAs at the molecular level. Growing interest of scientists is largely due to the decoding chemical structures of PAs and the development of ways for the modification of the polyene molecule (Zotchev, 2008; Baginski, Czub, 2009). The use of antibiotics with a known molecular structure allows conducting researches at the molecular level. The main goal of the research was to determine the intensification degree of the biological activity of PAs by studying the physicochemical properties of PAs in combination with DMSO.

MATERIALS AND METHODS

PAs dissolve well in DMSO. Polyenes have amphoteric properties and when ionized, they form a cation in an acidic medium, and an anion in an alkaline medium (Yu, Quinn, 1994). In combination with DMSO, polyenes are a liquid with a dark yellow color, bitter taste and a specific smell.

When preparing active forms of PAs, it is necessary first to convert the antibiotic from the powdery form (crystalline) to the molecular form. Wherein the conversion of the antibiotic into the most effective form occurs. After thoroughly mixing PA with DMSO, the composition is kept for 24 hours at room temperature. Then the liquid is filtered and stored in a dark, cool place. The obtained stock solution is ready for using. The use of PA in this combination of components is highly effective. The biological activity of PAs is determined by the method of bilayer lipid membranes (BLM) (Ibrahimova et al., 2006). BLM was obtained from total phospholipids isolated from cells by applying a drop of phospholipids to a hole in the teflon cell. Total phospholipids were purified from cholesterol and other neutral lipids by acetone washing and stored at 0°C at a concentration of 20 mg/ml in a chloroform-methanol solution in a volume ratio of 2:1. The integrated conductivity of the membranes was studied depending on the concentration of the antibiotic in the mode of fixation of the potential. At a certain concentration of the antibiotic, maximum membrane conductivity is achieved, which is considered as the active component of the PAs. The main information about the mechanism of PAs functioning in membranes was obtained using the BLM method. The method is based on the ability of PAs to dramatically increase the permeability of lipid membranes for the corresponding ions by registering changes in the electrical conductivity of the membranes. Studies of the integrated conductivity and measurement of the membrane potential were carried out in the mode of fixation of the potential and current using an electrometric DC amplifier Keithley-301 (USA).

RESULTS AND DISCUSSION

Dimethyl sulfoxide $(CH_3)_2SO$ is obtained by oxidizing dimethyl sulfide $(CH_3)_2S$ with nitric acid (Vaisman, Berkowitz, 1992). At present, for this purpose hydrogen peroxide H_2O_2 is used as an oxidizing agent. Dimethyl sulfoxide is the first member of the homologous series of sulfoxides R_2SO. As a result of their further oxidation, R_2SO_2 sulfones are formed. The chemical structure of DMSO contains two methyl groups, sulfur and oxygen (Fig. 3). DMSO is a clear, colorless, slightly bitter-tasting liquid, with a specific smell and it is highly water soluble (Yu, Quinn, 1994). Organic sulfoxides have a pyramidal structure with a sulfur atom at the top. In sulfoxides (RR′SO), the radicals R and R ′ differ from each other and exist in two optically active forms. The DMSO molecule is amphiphilic and highly polar. The negative pole of the dipole is on the oxygen atom. DMSO has an ordered structure, because of the temperature dependence of the refractive index, density, viscosity and other characteristics. DMSO is a protophilic solvent, and therefore, its associates are easily destroyed by the addition of substances that are proton donors. DMSO absorption spectrum in the wavelength range of 350 nm - 220 nm appeared to be optically transparent. A high degree of solubility of a number of organic compounds in DMSO is usually used to study their physicochemical characteristics and molecular structure (Yu, Quinn, 1994). Table 1 shows some physical characteristics of DMSO and water. The relatively high boiling point and large latent heat of vaporization (53 J/M at 25 ° C) indicate that DMSO molecules are strongly associated (Vaisman, Berkowitz, 1992). DMSO has properties such as amphiphilicity, polarity, high resorption.

Table 1. Physical properties of DMSO and water

Physical properties	DMSO	Water
Molecular weight	78.13	18.02
Density at 20◦C	1.1014	18.02
Melting point 20◦C	18.4	0.00
Boiling point	189.0	100.00
Surface tension at 20◦C (10^{-3}x Pa x c)	46.2	72.75
Viscosity at 20◦C(10^{-3}x Pa x c)	2.20	1.002
Dielectric constant at 20◦C	48.9	80.20

Studies of the biological activity of PAs showed that these compounds specifically interact with sterols of antibiotic-sensitive organisms, such as fungi and protozoa (Grayetal., 2012). Studies of the molecular mechanism of interaction of PAs with

membranes showed that polyenes create channels in the membranes through which ions and intracellular components can diffuse from the cells into the external medium, leading to cell lysis (Cohen, 2010).The biological activity of PAs is assumed to depend on the nature of the intermolecular interactions between the charged groups of antibiotic molecules and phospholipids. Probably, the incorporation of antibiotics into the membrane occurs as a result of the formation of a hydrogen bond between PAs and the phosphate groups of phospholipid molecules. A comparative analysis of the biological activity of amphotericin B and nystatin shows that amphotericin B is more effective against most fungi than nystatin (Aszalosetal., 1985). It was found that the polyene chains in nystatin A1 and amphotericin A are the same and antifungal activities of these two antibiotics are identical (Aszalosetal, 1985). According to these data, the presence of a certain number of double bonds in the chromophores of PAs is an important factor determining their sensitivity to membranes. There is a direct relationship between the number of double bonds in the chromophore and the biological activity of antibiotics – high biological activity corresponds to the high number of double bonds in the chromophores of PAs (Gasymov, 2009). Levorin has a higher selectivity of action on the membrane and differs from other PAs by its high solubility in water. The structure of the lipid bilayer, as well as the structure of the penetrating molecules, is an important factor determining the permeability of water-soluble compounds. A high degree of resorption of DMSO molecules is attributed to the fact that the value of the dielectric constant of DMSO is between that of water and fats (Table 1). This indicates that DMSO enhances the permeability of a large number of medicinal compounds through biological membranes, and also contributes to their quite deep penetration into the cell (Ibrahimova et. al. 2002).

For the first time, the physicochemical properties of amphotericin B and levorin in combination with DMSO and their mixed solutions in various ratios were studied. The dependence of the conductivity of bimolecular membranes on the concentration of amphotericin B and levorin was studied. Amphotericin B dramatically increases membrane permeability to ions, water, non-electrolytes and organic compounds. The dependence of the membrane conductivity on the concentration of amphotericin B increases in proportion to the 8-10th degree and this degree depends on the structure of the PA molecules. The sharp dependence of the conductivity of the membranes on the concentration of amphotericin B allows us to suggest that ion permeability is associated with the formation of oligomeric structure in the membranes of the polyene channels. Probably, the system responsible for the selective permeability of membranes is localized in the hydrophilic chain of the amphotericin B molecule. With the increasing concentration of DMSO in an aqueous solution, the efficiency of assembly of polyene channels and the stability of the channels in the conducting state increase. At a concentration of $1 \cdot 10^{-6}$ mamphotericin B reduces (10^5-10^6 times) the initial specific resistance of membranes ($1\text{-}5 \cdot 10^{-8} Om \cdot cm^2$), prepared from common phospholipids. The dependence of the conductivity of bimolecular membranes on the concentration of amphotericin B was studied at various concentrations of cholesterol in the membranes. The addition of cholesterol to phospholipids increases the effectiveness of the antibiotic. Membranes in the presence of amphotericin B are selectively permeable to monovalent anions. However, in the study of aromatic antibiotics, it was found that, unlike amphotericin B, levorin causes selective permeability not for anions, but for alkali metal cations. This antibiotic differs from nystatin, amphotericin B and mycoheptinum by the presence of an additional aromatic group- ρ-aminoacetophenone, which contains positively charged nitrogen. In the presence of levorin, with increasing cholesterol concentration, membrane conductivity increases. Studies of the dependence of the conductivity of membranes on the concentration of levorin led to the assumption that there are molecular-sized channels in the membranes that induce ion permeability. Selective permeability for cations is thought to be associated with the formation of negatively charged pores in the membranes. Probably, the transfer of cations through membranes occurs in the hydrophilic parts of the channel. Essential information on the mechanism of membrane permeability in the presence of aromatic antibiotics can be obtained from data on the transfer of small ions through the membrane, such as guanidine and hydrazine. In the presence of levorin, these ions penetrate the membrane much better than K^+ and Na^+ ions. The presence of the same number of double bonds in the chromophores of amphotericin B and levorin is an important factor determining their high sensitivity to membranes. The most effective of the studied PAs appeared to be amphotericin B and levorin. Dimethyl sulfoxide (DMSO) plays a special role in the formation of the amphotericin and levorin channels inside the membrane. DMSO has the ability to dramatically enhance the biological activity of PAs and induce selective permeability for ions and organic compounds in membranes.

The results of the experiments suggest that the

mechanism of selective action is based on the specific interaction of antibiotic molecules with membranes. The chromophores of PA molecules, interacting with phospholipids, form a channel in a 1:1 stoichiometric ratio. The stoichiometric coefficient of assembly of single channels for various PAs can be different and range from 3 to 17 (Ibrahimova et al., 2006). It should be noted that the molecular structure of the hydrophilic part of the channel has not yet been established due to the lack of appropriate methods for determining the exact localization of the molecular groups in the internal cavity of the channel. According to the research, the internal diameter of the channel is 7-10 Å (Ibrahimova et al., 2006). A computer analysis showed that during the formation of the ion channel in the presence of an amide derivative of amphotericin B, the ionizing groups of molecules can be turned both inward and outward, i.e. polar groups can be in two conformational forms, due to the rotation of mycosamine around the glycosidic bond. There is an assumption that the biological activity of PAs may depend on the nature of the intermolecular interactions between the charged groups of antibiotics and phospholipids. The incorporation of antibiotics into the membrane occurs as a result of the formation of a hydrogen bond between PAs and the phosphate groups of phospholipid molecules.

THE PRACTICAL SIGNIFICANCE OF THE RESEARCH

Antibiotics in animal and crop husbandry became widespread after the adverse effects of using some preparations had been established. Because, suppressing phytopathogenic microflora, they poison useful species of birds and animals that feed on pollinated plants. Compared with other substances, antibiotics have a number of valuable advantages in the fight against phytopathogenic microorganisms. Antibiotics act selectively and, suppressing the development of phytopathogenic bacteria and fungi, they are practically harmless to plants and animals (Lewis, Papavizas, 1987; Ibrahimova et al., 2014). The absence of toxicity is required when choosing antibiotics. For example, PAs used in low concentrations (10^{-6}-10^{-4}M), are non-toxic for plants and animals. Most antibiotics penetrate into the tissues of animals and plants and are well absorbed. The concentration of antibiotics necessary to suppress pathogenic microflora in animal and plant tissues depends on the properties of the antibiotic and external conditions. PAs were used as the basis for the development of effective antiviral, antibacterial and antifungal preparations. Based on the data obtained, the minimum concentration of the antibiotic corresponding to its maximum biological activity was calculated. New active compounds, which have the ability to effectively and selectively suppress pathogenic infections, were revealed in the group of PAs. The preparations were found to suppress viral and fungal infections of plants. Spraying a solution of the preparation on plants affected by a viral and fungal infections leads to the effective destruction of plant infections. Laboratory analysis of a soil sample on which vegetables were grown showed that soil contains a small amount of nitrogen, a large amount of phosphorus and a small amount of potassium, and pH of the soil sample was slightly alkaline. Table 2 shows mineral elements in the soil composition, based on soil gradation. Despite the missing mineral elements in the soil, where vegetables were grown, studies have shown the high efficiency of the preparations against pathogenic microorganisms of vegetable cultures. It should be noted that the preparations have the ability to completely inhibit the growth of the tobacco mosaic virus (*Tobacco mosaic virus*) (Ibrahimova et. al., 2014). Infected plants after the treatment are not only cured, regeneration of plants faded from infection also occurs. The antiviral and antifungal effects of infanvir are attributed to the binding of the antibiotic with the membranes with the subsequent formation of a complex in them. This complex is a molecular-sized channel formation, which is reflected in the inhibitory effect of the preparation on the reproduction of viruses and fungal cells. The proposed preparation is non-toxic and harmless, which contributes to its rational use in agriculture for growing vegetable and fruit crops.

Table 2. Mineral composition of soil based on soil gradation.

Sample name	pH	The degree to which soil is provided with mineral elements on the basis of soil gradation			Conductivity on particle size distribution of the soil (mS)	NaCl (mg/kg) Standart 150-300	KCl (mg/kg) Standart 350-700
		nitrogen, 40-120 mg/kg	phosphorous, 15-60 mg/kg	potassium, 300-600 mg/kg			
		Index of mineral sample provision					
		nitrogen, N/NH_3 mg/kg	phosphorous, P_2O_5 mg/kg	potassium, K_2O mg/kg			
Soil	7.55	7.76	133.32	212.08	1.18	520	516

For the first time the effect of preparations against viral, staphylococcal and fungal infections has been studied. The effect of the preparation formed by Streptomyces microorganisms on a number of pathogens - Staphylococcus, Escherichiosis, Candida, opportunistic bacteria and Coxsacke A, ECHO virus and type I and II herpes simplex virus has been investigated. Antimicrobial activity of preparations has been studied in various test systems. It was found that in low doses (10^{-7}-10^{-6} M) the preparations had antibacterial and antifungal effects on the cultures *Salmonella typhimuium, Pseudomonas aeruginosa*, *Proteus vulgaris, Escherichia coli,* gram-positive cultures *Staphylococcus aureus* and cells of the fungus *Candida albicans.* They also have an antiviral effect on Coxsacke A 20, ECHO9 virus and type I and II herpes simplex virus. Eurasian patents were obtained for both preparations.

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Membran Keçiriciliyinin Artırılmasında və Heyvan və Bitki İnfeksiyaların Müalicəsində Polien Antibiotiklərin Rolu

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Göstərilmişdir ki, polien antibiotiklərin (PA) dimetilsulfoksidlə (DMSO) kompleks istifadəsi nəticəsində membranların ion keçiriciliyini və antibiotiklərin bioloji aktivliyini kəskin sürətdə artırır. PA və DMSO- in müqayisəli fiziki-kimyəvi xüsusiyyətləri göstərilmişdir. DMSO və PA–nın bimolekulyar lipid membranları BLM-lə kompleks əlaqəsinin təsir effekti tədqiq edilmişdir. BLM-lə PA-nın bioloji aktivliyinin parametrləri təyin olunmuşdur. Göstərilmişdir ki, bütün öyrənilmiş PA-dən ən effektivi amfoterisin B və levorindir. BLM-nın ion keçiriciliyinin amfoterisinin B və levorinin membranlarda mövcud olan xolesterinin konsentrasiyasından asılılığının nəticələri verilmişdir. PA-nın əsasında heyvan və bitkilərdə olan virus, bakteriya, göbələk xəstəliklərinə qarşı bioloji aktiv preparatlar yaradılmışdır.

Açar sözlər: *Polien antibiotiklər, dimetilsulfoksid, amfoterisin B, levorin, lipid membranları, membran keçiriciliyi, heyvan və bitki infeksiyaları*

Значение Полиеновых Антибиотиков в Увеличении Мембранной Проводимости и при Лечении Животных и Растительных Инфекций

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Показано, что при комплексном использовании полиеновых антибиотиков (ПА) с диметилсульфоксидом (ДМСО) резко усиливается ионная проницаемость мембран и биологическая активность антибиотиков. Представлены сравнительные физико-химические характеристики ДМСО и ПА. Рассмотрены эффекты комплексного взаимодействия ДМСО и ПА с бислойными липидными мембранами (БЛМ). Методом БЛМ определены параметры биологической активности ПА. Показано, что из всех изученных ПА самыми эффективными оказались амфотерицин В и леворин. Изложены результаты зависимости ионной проводимости БЛМ от концентрации амфотерицина В и леворина и от концентрации холестерина в мембранах. На основе ПА разработаны эффективные мембраноактивные препараты против вирусных, бактериальных и грибковых заболеваний животных и растений.

Ключевые слова: *Полиеновые антибиотики, диметилсульфоксид, амфотерицин В, леворин, липидные мембраны, проводимость мембран, животные и растительные инфекции.*