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Influence of amphotericin B on the physicochemical properties of model lipid membranes

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Amphotericin B (AmB) is a widely used antifungal antibiotic and has been extensively studied over the last decades due to its superior properties. However, the mechanism of action of AmB on the biological cell is still not completely understood. It is well known that the interaction of the antibiotic with the lipid membrane induces formation of anion-selective pores thus significantly increasing the permeability for ions through lipid cell membrane. The aim of the present work is to study the influence of AmB antibiotic on the phase behavior and the channel formation activity of the synthetic lipid membrane. The ability of AmB to form ion channels in lipid bilayers is studied by tip-dip patch clamp technique. We observed that AmB reduces the electrical resistance of the lipid membrane. The phase transition temperatures and corresponding enthalpies are obtained *via* differential scanning calorimetry. The obtained results show that the presence of AmB has a significant influence on the phase behavior of the lipid system.

Keywords: Amphotericin B, lipid membrane, phase behavior, enthalpy, ion channel.

INTRODUCTION

Amphotericin B (AmB) is one of the main polyene antibiotics widely used to treat deep-seated fungal infections [1]. The antibiotic is designated chemically as (1R,3S,5R,6R,9R,11R,15S,16R, 17R,18S,19E,21E,23E,25E,27E,29E,31E,33R,35S, 6R,37S)-33-[(3-amino-3,6-dideoxy-β-D-manno pyranosyl)oxy]-1,3,5,6,9,11,17,37-octahydroxy-15, 16, 18-trimethyl-13-oxo- 14,39-dioxabicyclo [33.3.1] nonatriaconta- 19,21,23,25,27,29,31-heptaene-36-carboxylic acid.

AmB was originally extracted in 1955 from *Streptomyces nodosus*, a filamentous bacterium, at the Squibb Institute for Medical Research from cultures of an undescribed streptomycete isolated from the soil collected in the Orinoco River region of Venezuela. Infections as a consequence of weakened immunity defense, such as from AIDS or from many cancer therapies, have renewed the scientific studies on the polyene antibiotic amphotericin B (AmB). The mechanism of biological action of AmB is most probably directly related to the ability of the drug to form hydrophilic pores in the hydrophobic membrane core, where it increases the permeability of the cells to ions and small molecules [2].

However, the detailed molecular mechanisms of the interaction of AmB with the membrane, as well as the formation of a transmembrane pore structure, are still imperfectly understood. The molecular structure of AmB used in the study is shown in Fig. 1.

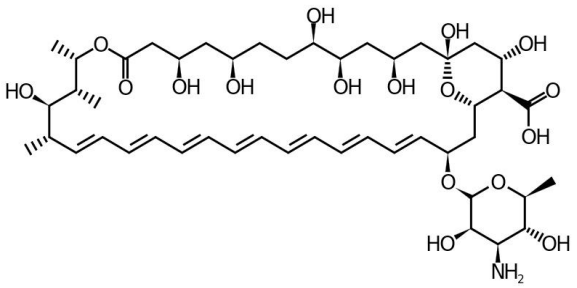

Fig. 1. Structure of the antibiotic amphotericin B

The molecule features a lactone ring containing conjugated double bonds, a chain of hydrophilic groups, and two ionizable groups - a carboxyl and a mycosamine.

The antibiotic forms channels in lipid membranes and has conducting and nonconducting states, with frequent transitions between them. One can induce a rise in conductance with amphotericin B on one or both sides of the planar bilayer membranes [3]. When the antibiotic is added to both sides, the electrical conductance is much higher and the permeability of univalent anions is larger than that of the cations [4]. When the antibiotic is added to only one side, it is selective to univalent cations.

Amphotericin B forms two types of channels that are structurally very similar but differ in length. It is assumed that the channels are formed by one or two "barrels" consisting of antibiotics and incorporated lipid molecules, with the long axes oriented perpendicularly to the membrane surface [5].

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The hydrophilic, charged, and hydrophobic sides of the antibiotic molecules are turned toward the water pore, water, and lipid phase, respectively. The one-sided action is attributable to channels having essentially the same structure as those formed by the two-sided action, except that only one "barrel" (half-pore) spanned the bilayer to form a functional channel. The two-sided channels are formed by two barrels bound end to end in the membranes [3, 6, 7].

The open channel does not undergo overall rearrangements, such as a change in the number of molecules in the pore or channel from the open to the closed state, it is probably not the result of such rearrangements, but may be caused by shrinking of the pores [8], those formed by the two-sided action, except that only one "barrel" (half-pore) spanned the bilayer to form a functional channel [5, 6].

Artificially created lipid vesicles mimic the cell membrane. They present a simple model for investigation of membrane properties and can be formed from various lipids with controlled composition in controlled environment. With a better understanding of AmB mechanism of action on model lipid membranes we will have a clearer image on how this antibiotic interacts with living cells.

The aim of the present study is to investigate the effect of amphotericin B (AmB) on the physicochemical properties of model lipid membranes formed from synthetic lipids.

MATERIALS AND METHODS

L-α-lecithin from soybean (choline content 20%), and amphotericin B were obtained from Sigma, HEPES. KCl (p.a.), n-propanol (p.a.) and n-hexane (p.a.) were purchased from Valerus Co. The synthetic lipid 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) 18:0/18:1 was purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). The solvents, chloroform (purity 99%) and methanol (purity 99:8%), were purchased from Sigma-Aldrich Corporation, part of Merck & Co., Inc. (Kenilworth, USA). All the chemicals were used without any further purification.

Patch-clamp technique

Lipid bilayers were self-assembled at the tips of patch pipettes, using the tip-dip patch clamp technique, from monolayers formed by spreading L-α-lecithin from soybean in n-hexane (10 mg ml^{-1}) onto the surface of electrolyte solutions contained in Petri dishes (Fig. 2).

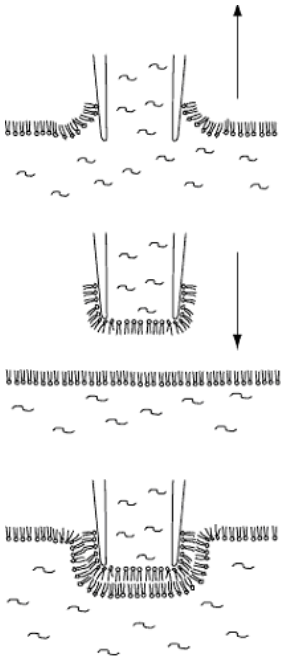

Fig. 2. Scheme of lipid bilayer formation at the tip of a patch pipette

Patch pipettes (tip diameter 1-2 μm) and Petri dishes (10 cm^2 area) were filled with aqueous solutions of KCl (0.1M) buffered with HEPES (0.01M) at pH 7. Only bilayers with seal resistances >1 GΩ were used. AmB dissolved in n-propanol:distilled water (4:6, v/v), was added to the Petri dish after bilayer formation to a final concentration of 5×10^{-6} M and 8×10^{-4}M.

Single AmB channel currents were monitored using a patch clamp amplifier Model 2400 (A-M Systems, Inc.) and patch clamp software. The currents were stored on a PC hard disc with 1 ms time resolution. All measurements were performed at room temperature (~20°C).

Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to study the influence of amphotericin B on the phase behavior of the SOPC lipid system. DSC is an experimental method that determines the difference in heat required to increase the temperature of an examined sample and a reference as a function of temperature. When the sample under consideration experiences a phase transition, a certain amount of heat flows to the reference sample in order to maintain both samples at the same temperature [10, 11]. Comparing the heat flows during a phase transformation identifies the process as being exothermic (heat is generated) or endothermic (heat is absorbed). The output from the DSC measurements consists of the heat required to maintain both cells containing the samples, at the

same temperature converted into heat flow, or equivalently heat capacity. The values of the changes in the thermodynamic quantities, such as enthalpy, entropy, and heat capacity at any transition might be deduced from the ensuing calorimetric data.

SOPC was dissolved in chloroform, while AmB antibiotic was dissolved in methanol. The final lipid–cholesterol solution was prepared by mixing SOPC and AmB in the desired molar proportion for every AmB concentration. The investigated quantity of the final lipid/antibiotic solution was placed in a flask that was places under vacuum for about 4 – 5 h until the entire solvent evaporation. Then, the appropriate amount of double-distilled water was added to the dry lipid/AmB film and the samples were positioned in a warm (40 – 45ºC) ultrasonic bath for at least 2 h to hydrate the lipid film and achieve homogeneity of the lipid-water mixture. For all AmB concentrations, the samples were prepared to contain 20 wt.% of water (80 wt.% of the lipid/AmB mixture).

RESULTS AND DISCUSSION

Patch clamp technique

Lecithin bilayers were used to study the effect of AmB channels formed by several molecules self-assembled into a "bundle" structure which traverses the lipid bilayer. Incorporation of AmB molecules into the hydrophobic core of the membrane results in the formation of molecular aggregates, which probably take the form of hydrophilic pores composed of six to nine molecules.

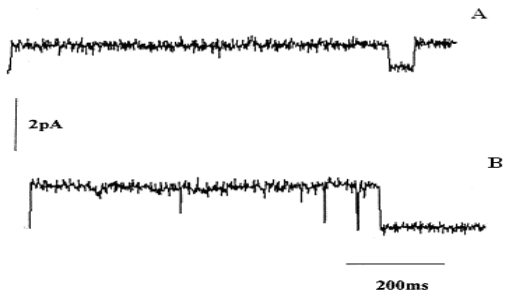

Fig. 3. Record showing two types (A, B) of amphotericin B channels in a lecithin bilayer. Transmembrane potential 100 mV. A - AmB concentration 5×10^{-6} M; B - AmB concentration 8×10^{-4}M.

Differential scanning calorimetry

For the investigation of the influence of amphotericin B antibiotic on the thermal behavior of the synthetic SOPC lipid system DSC measurements at three different molar concentrations of AmB in the lipid system were performed - 10^{-5} M, 10^{-3} M and 10^{-1} M, respectively. Special attention was paid to the heating rate and incubation procedure. The amount of AmB in the membrane was chosen to match the conditions of the channel formation experiments, performed by patch-clamp technique. The influence of water content on the thermograms of pure SOPC was studied in detail [12]. It was shown that the optimal water content in the lipid-water samples is from 10 to 20 wt.%. We used 20 wt.% of water in all the experiments with all three molar concentrations of AmB. The used DSC equipment was Discovery 250 (TA Instruments, USA). The optimal quantity of the investigated sample, typically 20-30 mg, was inserted in the special DSC pan. The pan containing SOPC sample was closed with a hermetic cover and matched with the reference pan. The incubation procedure for all the performed experiments consisted of inserting the sample at room temperature in the DSC oven and heating from 30°C to 50°C then the sample was cooled at a rate of 5 ºC min^{-1} from 50 to −10° C. The sample was further heated up at a rate of 5 ºC min^{-1} from −10 °C to 70 ºC.

On Fig. 4 the obtained thermograms at heating for 10^{-5} M, 10^{-3} M and 10^{-1} M AmB in the lipid matrix, respectively, are shown in the temperature range where the phase transition peaks are seen (from 3 to 40 °C).

On Fig. 5 the thermogram for pure SOPC lipid system, obtained at identical conditions (water content, heating rate, etc.) as those in presence of AmB, is shown for comparison [12]. As it can be seen from the obtained thermogram, even at the least amount of amphotericin B in the lipid matrix, (10^{-5} M AmB in the SOPC system, down left picture on Fig. 4) the phase behavior of the system substantially changes. The sharp shape of the transition observed for pure SOPC (Fig. 5) is smeared out and split into several less pronounced peaks.

As the concentration of AmB within the lipid membrane increases, the behavior of the phase transition curve does not change significantly. The peaks remain 2-3 with a slight change in the locations of their maxima and their relative intensities. In Table 1 the obtained main transition temperatures of the most pronounced peaks for all studied systems and the corresponding enthalpies are presented.

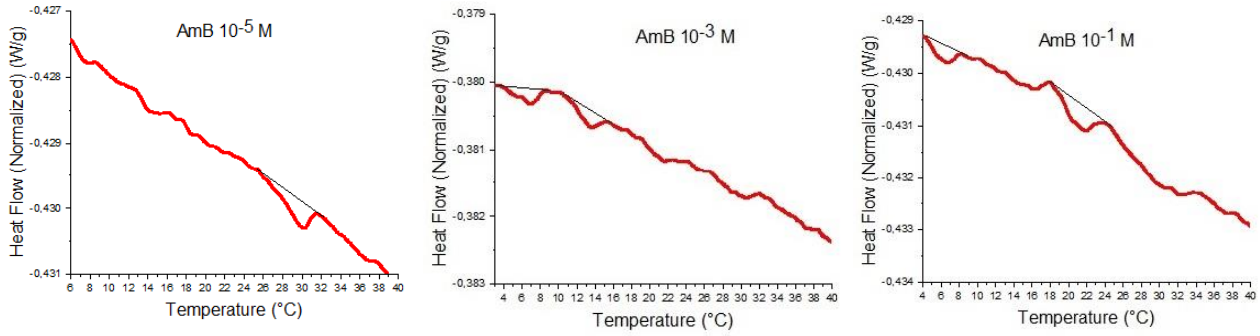

Fig. 4. DSC thermograms for SOPC with various molar concentrations of AmB at heating.

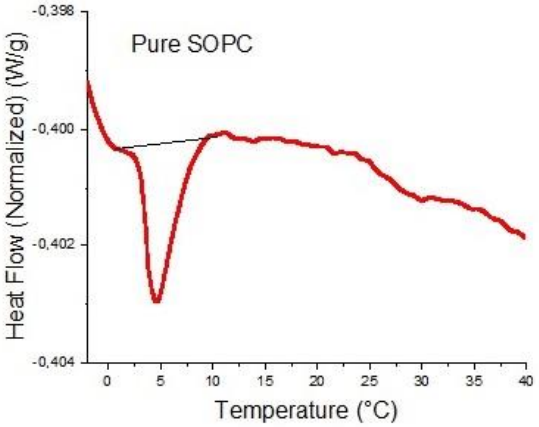

Fig. 5. DSC thermogram for pure SOPC at heating for comparison.

Table 1. Thermodynamic quantities (phase transition temperatures and associated enthalpies) derived from DSC measurements for SOPC lipid–AmB antibiotic samples. All the samples contain 20 wt. % double distilled water.

Sample	Temperature [˚C]	Enthalpy [J.g^{-1}]
Pure lipid	4.6	0.147
10^{-5} AmB	30	0.012
10^{-3} AmB	6.9; 13.5	0.007; 0.03
10^{-1} AmB	6.6; 21.5	0.01; 0.017

The values for pure SOPC lipid are given for comparison [12]. Analyzing the obtained values for the peak positions and enthalpies it is seen that with the addition of AmB in the lipid matrix the typical peak of the phase transition from L_β to L_α phase at around 5°C for SOPC lipid is not only split into several peaks, but also slightly shifted towards higher temperatures, resulting with at least one order of magnitude lower enthalpies. Similar phase transition temperature shift, but in the opposite direction is observed with addition of cholesterol in the lipid membrane [13]. It was reported in the literature [14] that the addition of hydrophobic gold nanoparticles in the lipid membrane results in a comparable smearing of the transition thermograms.

CONCLUSIONS

Patch-clamp technique measurements showed that amphotericin B reduces the electrical resistance of the lipid membrane. AmB forms ion channels in the lipid membrane with millisecond dwell times in the lipid membrane. The obtained volt-ampere characteristics were linear over a range of ± 100 mV.

The rate of rise of the membrane conductance for small times is proportional to the rise in the antibiotic concentration. The addition of a higher concentration of AmB drastically reduced the membrane resistance.

The experiment provided further evidence in support of the widely held view that at high concentrations of the antibiotic AmB will drive the membranes into a state of instability. It is possible that at these large concentrations, large pores are formed, perhaps by confluence of the small pores.

By means of differential scanning calorimetry aiming to investigate the influence of amphotericin B on the thermal properties of SOPC phospholipid, we have found that even a very small amount of AmB, inserted in the phospholipid SOPC, is enough to substantially modify the structural and dynamical properties of the phosphatidylcholine bilayer system. It was shown that AmB antibiotic, when added to the SOPC lipid system, smears out the phase transition curve and splits it into several less pronounced peaks, slightly shifts the main phase transition temperature and significantly reduces the transition enthalpy.

REFERENCES

1. J. Brajtburg, W. G. Powderly, G. S. Kobayashi, G. Medoff, *Antimicrobial Agents and Chemotherapy*, **34**,183 (1990).

2. L. N. Ermishkin, K. M. Kasumov, V. M. Potseluyev, *Biochim. Biophys. Acta*, **470**, 357 (1977).
3. J. Bolard, *Biochim. Biophys. Acta*, **864** 257 (1986).
4. M. P. Borisova, R. A. Brutyan, L. N. Ermishkin, *J. Membrane Biol.*, **90**, 13 (1986).
5. A. Finkelstein, R. Holz, in: Membranes: Lipid Bilayers and Antibiotics, G. Eisenman (ed.), vol. 2, New York, Marcel Dekker, Inc., 1973. p. 377.
6. R. A. Brutyan, P. McPhie, *J. Gen. Physiol.*, **107**, 69 (1996).
7. B. E. Cohen, *J. Membrane Biol.*, **238,** 1 (2010).
8. V. Khutorsky, *Biophys. J.*, **71**, 2984 (1996).
9. G. Fujii, J.-En Chang, T. Coley, B. Steere, *Biochemistry*, **36**, 4959 (1997).
10. T. M. Koyama, C. R. Stevens, E. J. Borda, K. J. Grobe, D. A. Cleary, *Chem. Educator*, **4**, 12 (1999).
11. S. M. Ohline, M. L. Campbell, M. T. Turnbull, S. J. Kohler, *J. Chem. Ed.*, **78(9)**, 1251 (2001).
12. J. Genova, Z. Slavkova, H. Chamati, M. Petrov, *Phase Transit.*, **92 (4),** 323 (2019).
13. J. Genova, Z. Slavkova, H. Chamati, M. Petrov, *Journal of Surfactants and Detergents*, **22**, 1229 (2019).
14. J. Genova, Z. Slavkova, H. Chamati, *Coll. Surf. A*, **603,** 125090 (2020).