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Effect of amphotericin B on dipalmitoylphosphatidylcholine membranes: calorimetry, ultrasound absorption and monolayer technique studies

Krzysztof Wójtowicz [a], Wiesław I. Gruszecki [b,*], Magdalena Walicka [b], Joanna Barwicz [c]

[a] *Department of Biophysics, Medical Academy, Lublin, Poland*
[b] *Department of Biophysics, Institute of Physics, Maria Curie-Skłodowska University, 20-031 Lublin, Poland*
[c] *Department de Chimie-Biologie, Universite du Quebec à Trois-Rivières Three Rivers, Que., Canada*

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Abstract

Amphotericin B (AmB) is a popular drug frequently applied in the treatment of mycosis. Differential scanning calorimetry (DSC), ultrasound absorption and monomolecular layer technique were applied to study the effect of AmB on organisation of dipalmitoylphosphatidylcholine (DPPC) membranes. DSC-determined enthalpy of the main phase transition of DPPC liposomes was found to be a sensitive parameter to monitor AmB–DPPC interaction. The enthalpy of the phase transition decreases with the increase in molar fraction of AmB incorporated to membranes. The exceptionally sharp decrease in the enthalpy of the transition was observed in the membranes containing 5–7 mol% AmB. Ultrasound absorption-monitored main phase transition of DPPC is very broad under the presence of 5 mol% AmB showing destabilisation and disorganisation of a membrane structure. These findings are discussed in comparison to monomolecular layer study of two-component DPPC–AmB system. Analysis of the surface pressure–molecular area isotherms of compressing DPPC–AmB films at the air–water interface shows pronounced increase in mean molecular area at AmB concentrations corresponding to those found to destabilise DPPC membranes of liposomes. Disorganisation of lipid bilayers due to the presence of AmB in concentrations below 10 mol% with respect to lipid is discussed in terms of toxicity and side effects of this drug.

Keywords: Amphotericin B; Liposome; Aggregation

1. Introduction

Amphotericin B (AmB) is the main antibiotic used in patients with deep-seated mycotic infections. The therapeutic usefulness of AmB and its clinical attraction is, however, limited because of severe side effects (see [1,2]). There are some hypotheses relating toxicity of AmB to its stereochemical structure (see Fig. 1) which is responsible for the formation of pores across lipid membranes, which are able to affect physiological ion transport considerably [3]. Several procedures have been elaborated to decrease the toxicity and harmful side effects of AmB. One of the approaches consists of preparation of pharmaceutics containing AmB admixed to surfactants [4,5]. A preparation of liposomal drug after admixture of AmB to lipids has also been proposed [6]. In that respect, the AmB–lipid interactions were recognised

* Corresponding author. Fax: +48 (81) 537-6191; E-mail: wieslaw@tytan.umcs.lublin.pl

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to be important and were the subject of several studies, including those based on calorimetry [6] and monomolecular layer technique [7,8]. It appeared that the composition of liposomes and concentration of AmB in a lipid phase had a significant effect on drug toxicity [9,10]. Hamilton et al. [6] proposed that these effects might be related to the existence of AmB oligomers forming phase-separated domains in the lipid phase. Such an interpretation is consistent with the findings that the presence of aggregated AmB in a preparation used for pharmacological treatment correlated with drug selective toxicity [11]. Aggregated structures of AmB in a form of hydrophobic pores in hydrated media may be formed spontaneously as was shown in our previous work [12]. In this report, we present some new results which seem to indicate an additional molecular mechanism operative at low molar fractions of AmB in a lipid phase, which may be responsible for the drug toxicity and its side effects. According to these results, the most pronounced effect of AmB in affecting molecular organisation of DPPC membranes is observable at low concentrations of AmB below those promoting massive formation of oligomeric structures of the drug in a lipid phase. Such findings led us to revise the current concepts concerning the molecular mechanism the of deleterious effect of AmB with respect to biomembranes.

2. Materials and methods

Dipalmitoylphosphatidylcholine (DPPC) and Amphotericin B (AmB) were purchased from Sigma. Liposome suspensions for the ultrasound absorption measurements were prepared according to the general procedure described briefly below. DPPC and AmB stock solutions were prepared in ethanol and 2-propanol, respectively. Ethanol was applied as a lipid solvent instead of the more frequently used chloroform in order to assure homogenous mixing of AmB and DPPC in a monomeric form of the drug. In the case of AmB, the stock solution was centrifuged at $15\,000\times g$ in order to remove drug which was not dissolved under such conditions and the final concentration was evaluated from light absorption measurements at 408 nm on the basis of the molar extinction coefficient in 2-propanol (1.3×10^5 $M^{-1}\,cm^{-1}$). The required amounts of lipid and AmB were mixed and evaporated under stream of dry nitrogen to form a thin homogeneous film on a glass tube. Residuals of organic solvents were removed in a vacuum and 10 ml of a phosphate buffer (10 mM, pH 8.0) was added to the tube containing a thin, dry and homogeneous film of DPPC–AmB. Small unilamellar liposomes were formed via sonication of the sample with ultrasonic disintegrator for 3 min at 45°C, above the main phase transition temperature of DPPC. After preparation, liposome suspension was cooled down to the temperature of 25°C and centrifuged at $15\,000\times g$ in order to remove large particles of AmB eventually not incorporated to liposomes. Not any residuals remaining as a pellet in the concentration range studied were detected by visual inspection and by light absorption measurements of 2-propanol added to centrifuge tubes after removing liposome suspension present as supernatant. On the other hand, the possibility may not be excluded that a certain small amount of AmB remained in a liposome suspension in an aggregated form not bound to the lipid phase. Spectral analysis of liposome suspensions showed that such AmB fraction may not exceed 10^{-6} M. Which was considered not to affect an interpretation of the results of this study. The final concentration of the lipid in water phase was 1.5×10^{-3} M. Liposomes for differential scanning calorimetric (DSC) measurements were prepared following the same protocol except that the sample volume was 100 μl, sonication was performed with the ultrasound bath and samples were not subjected to centrifugation after sonication. Liposomes were prepared directly before the measurements. Measurements of ultrasound absorption in the frequency range 1100–1400 kHz were carried out with the home-built interferometer similar to that described by Eggers and Funck [13]. The details concerning experimental set-up and conditions of measurements are described elsewhere [14]. Thermostatic conditions were kept with the accuracy of ±0.05°C. The measured excess of ultrasound absorption per wavelength ($\Delta\alpha\cdot\lambda$) is theoretically analysed and explained elsewhere [13,15] and is generally accepted to express the part of energy of ultrasound wave consumed in perturbation of equilibrium of the studied system. Calorimetric measurements were carried out with a DSC model UNIPAN 605M. Lip-

osome suspension was scanned versus pure buffer as a reference. The scan rate was 0.5°C per min. Monomolecular layers were formed at the air–water interface in a 300 cm^2 Teflon trough. Deionised double distilled water distilled the third time in glass set-up with the addition of $KMnO_4$ was applied as a subphase. DPPC and AmB to be deposited at the air–water interface were dissolved in benzene/ethanol (9:1, v/v) and 2-propanol/water (6:2, v/v), respectively. Drops of DPPC and AmB solutions were deposited very gently at the interface to form a monolayer. Such a procedure was extremely important for deposition of AmB in 2-propanol miscible with the subphase. To form mixed, two-component DPPC–AmB monolayers, DPPC was deposited as a first component and, after the 15 min required for evaporation of benzene AmB, was deposited at several different places at the air–water interface in order to obtain homogeneous monolayer. Such a method of a deposition of monolayer of AmB yields monomeric organisation of the drug at the air–water interface before film compression. In this respect, the initial molecular area in monolayers before compression was as large as 200 $Å^2$. Monolayers were compressed with a speed of 30 cm^2 per min. Surface pressure was monitored by NIMA-Wilhelmi

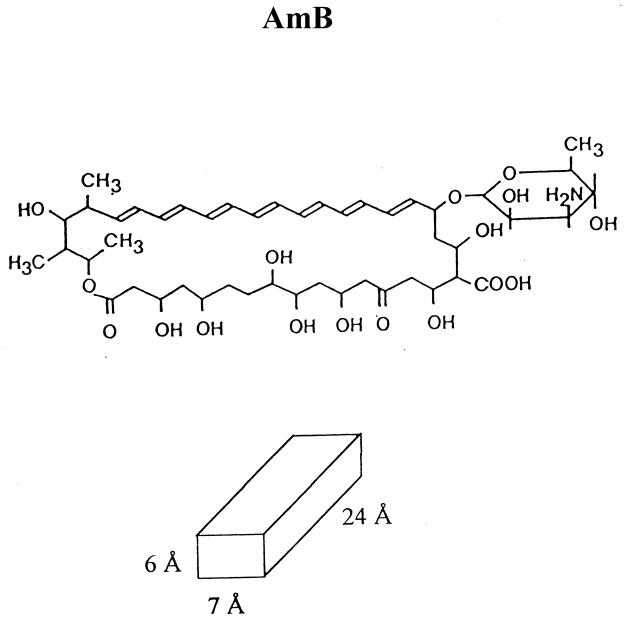

Fig. 1. Chemical structure of amphotericin B and schematic representation of molecular dimensions corresponding to this structure.

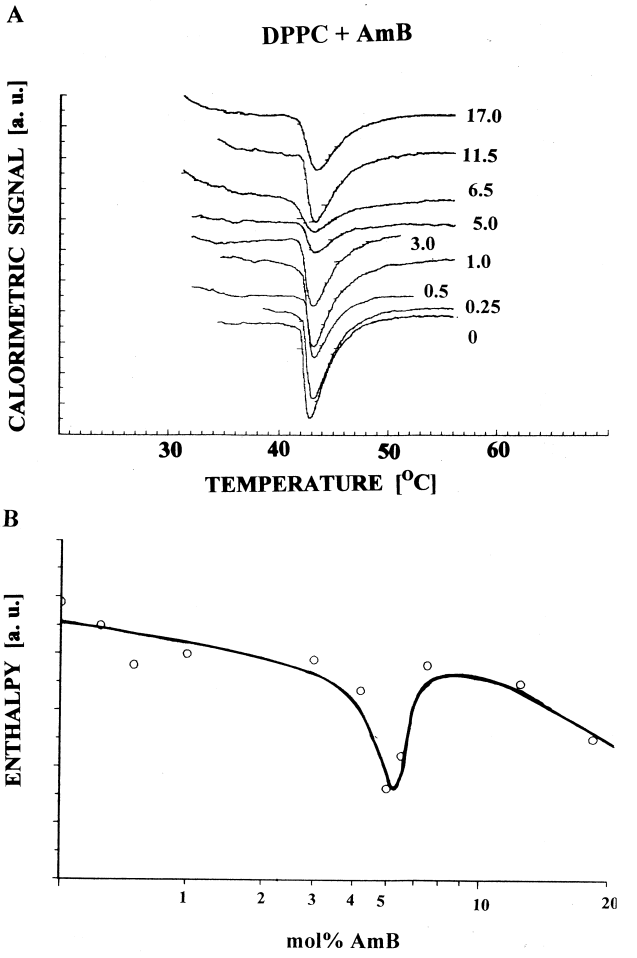

Fig. 2. (A) Original traces of DSC scanning of DPPC liposomes containing different molar percentage of AmB, indicated. (B) Enthalpy of the main phase transition of DPPC liposomes depicted versus concentration of AmB incorporated into the membranes. Experimental points were obtained by integration of DSC traces presented in (A).

plate tensiometer. Monolayer compression and data acquisition were controlled on-line by a personal computer. Monolayer formation and compression was carried out at 25°C.

3. Results and discussion

Several experimental techniques may be applied to detect the main thermotropic phase transition of phosphatidylcholines including NMR [16], EPR [17], DSC [18] and also ultrasound absorption [19]. The main phase transition of phosphatidylcholine membranes ($P_{\beta'}$–L_α) sometimes called 'melting' con-

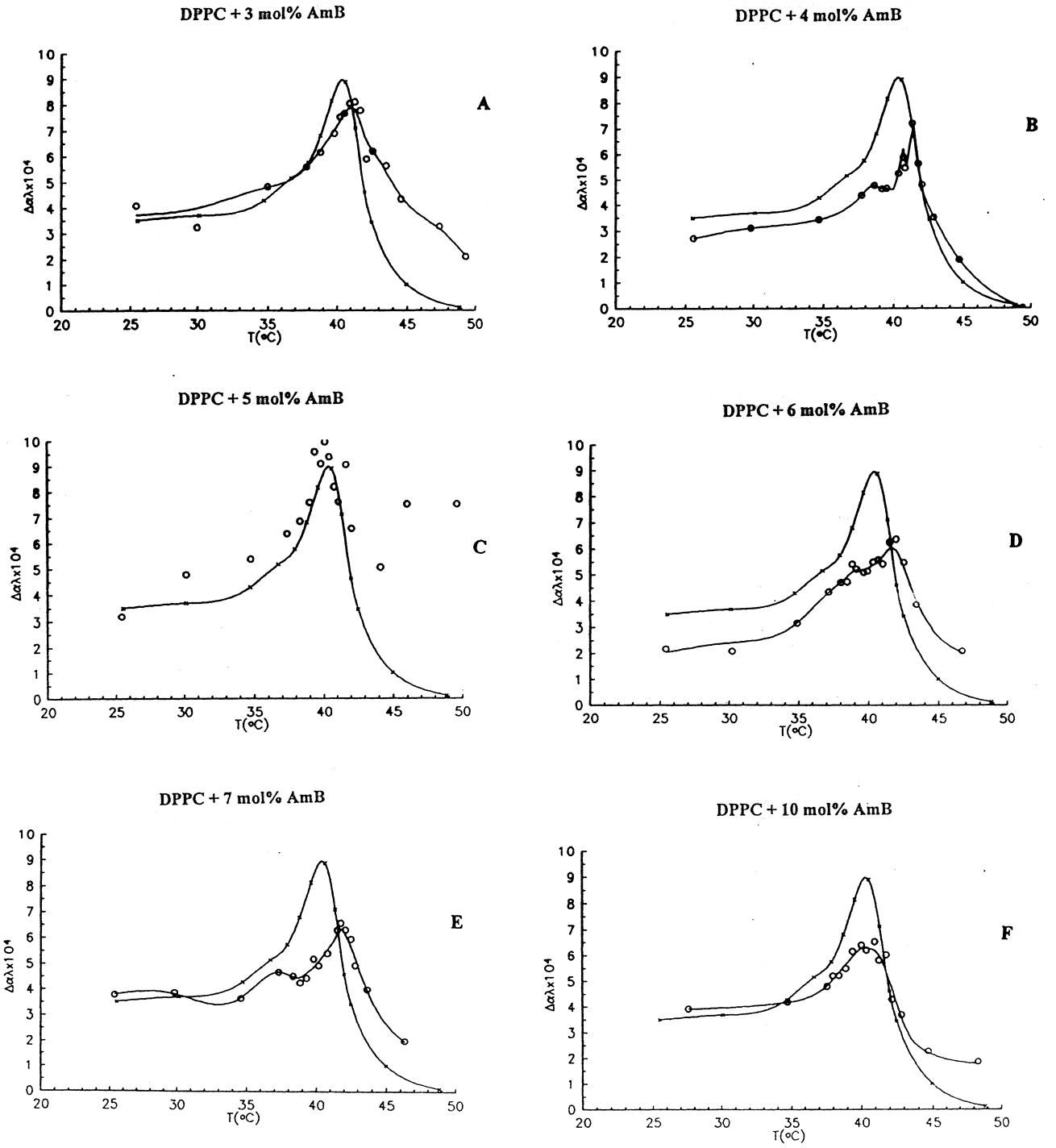

Fig. 3. Ultrasound absorption ($\Delta\alpha\cdot\lambda$) versus incubation temperature (T) of DPPC liposomes ($\times$) and DPPC liposomes containing different concentration of AmB ($\bigcirc$) indicated in each panel (A–F).

sists of a cooperative increase in the rate of *trans-gauche* isomerisation of acyl chains of lipid molecules forming hydrophobic core of a lipid bilayer. Going through the phase transition results in abrupt changes of several physical parameters of a membrane, like the membrane fluidity directly related to

the order of acyl chains. Fig. 2 presents the phase transition of DPPC membranes containing different amount of incorporated AmB, as analysed by means of DSC. The same transition monitored by means of ultrasound absorption technique is presented in Fig. 3. In most cases, additives to a membrane reduce the order within liquid crystalline lipid structures decreasing cooperativity and enthalpy of phase transition [17,20]. As may be predicted, the gradual increase in concentration of AmB in DPPC membranes results in a decrease in the enthalpy of the phase transition. This may be directly observed from the DSC traces (enthalpy of a phase transition being proportional to the surface of DSC peak). The dependence of enthalpy of DPPC phase transition on AmB content is depicted in Fig. 2B. As may be noticed from this dependency, a pronounced decrease

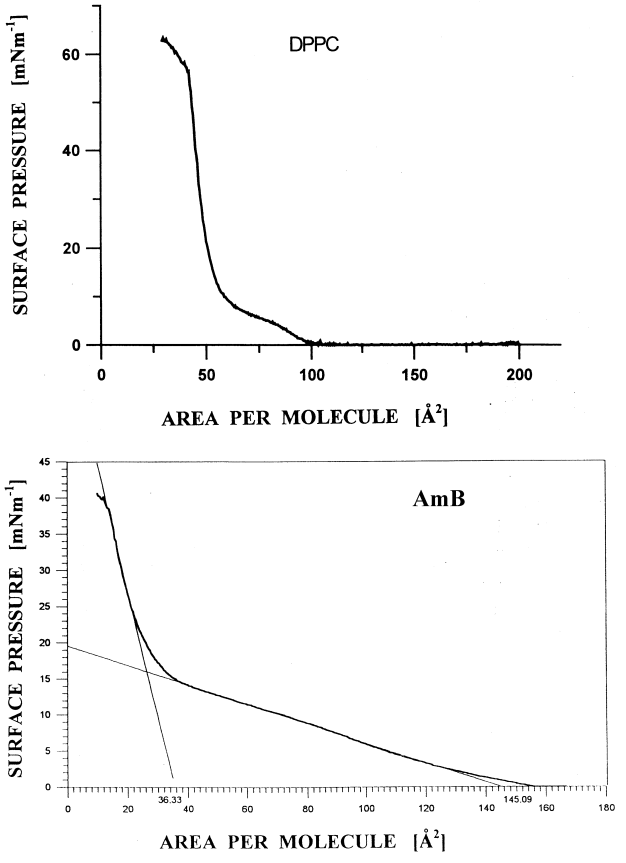

Fig. 4. Surface pressure–molecular area isotherms of compression of monocomponent monomolecular layers formed with DPPC (A) and AmB (B). The straight lines interpolated to the linear parts of isotherms are indicated along with the corresponding molecular areas.

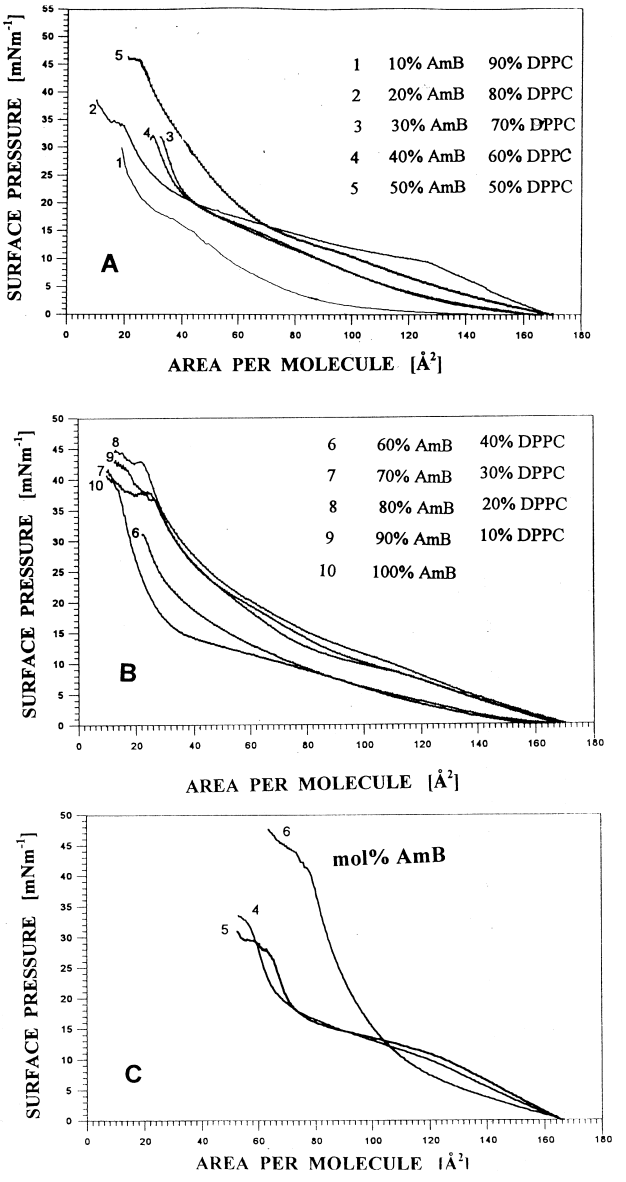

Fig. 5. Surface pressure–molecular area isotherms of the compression of two-component monomolecular layers formed with DPPC and AmB. Composition of monolayers is presented.

in enthalpy accompanies the AmB concentrations close to 5 mol%. The DSC-monitored energy uptake by the DPPC membranes containing 5 mol% AmB is very weak and broad compared to the typical DPPC calorimetric signal characteristic of a phase transition. Ultrasound absorption measurements of the sample of the same composition (Fig. 3C) clearly show affected temperature profile of ultrasound energy uptake representing cooperative relaxation processes related directly to a molecular motion of lipid

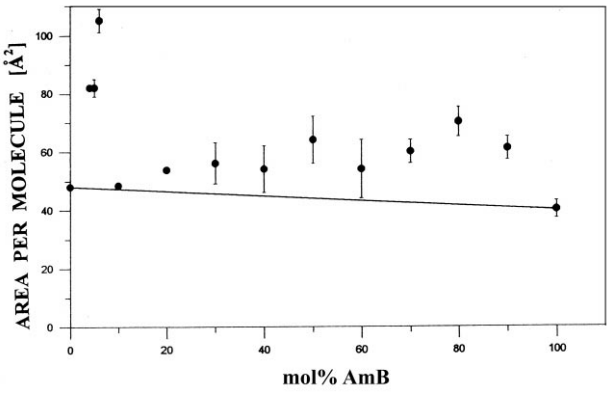

Fig. 6. Molecular area in a two-component monolayers versus molar percentage of AmB. Each point represents mean from three to five experiments. Error bars correspond to standard deviation. Molecular area values were evaluated on the basis of extrapolation to the zero surface pressure lines fitted to the final linear part of an isotherm corresponding to monolayer compression preceding collapse (see Fig. 4).

molecules. Such a result is a further indication of disorganisation of the DPPC membrane structure brought about by low-concentrated AmB. This effect was further studied by means of monomolecular layer technique. Fig. 4 presents surface pressure–molecular area isotherms of compressing pure DPPC and AmB monomolecular films. The specific molecular area of DPPC, evaluated by the method of extrapolation of the linear part of the isotherm directly preceding the monolayer collapse to the zero surface pressure (47 Å^2, see straight lines in Fig. 4), well corresponds to the molecular area of phosphatidylcholines at temperatures below the phase transition [21]. Two distinct phases of the AmB monomolecular layer compression are clearly visible from the isotherm presented in Fig. 4. Such a shape of the isotherm is very close to those reported previously for AmB in a single component monolayer [7] and indicates monomeric organisation of AmB at the air–water interface before compressing. The isotherm reflects two different orientations of AmB at the air–water interface in which the drug occupies a mean molecular area 145 and 36 Å^2 (as deduced by the isotherm extrapolation to the zero surface pressure as in the case of DPPC explained above). These mean molecular areas directly correspond to such orientations of AmB at the air–water interface where the molecule contacts subphase by its hydrophilic side determined by the OH groups (6×24 Å) or by the sugar ring and the COOH groups forming a specific polar head (6×7 Å). Fig. 5 presents exemplary isotherms of compressing two-component DPPC–AmB monolayers at the air–water interface. Mean molecular areas in two-component monolayers are presented in Fig. 6 versus molar percentage of AmB. As can be seen, the mean molecular areas in two-component monolayers are always higher than the corresponding areas of pure components. This effect is exceptionally high in the low AmB concentration region (below 10 mol%). However, such an effect was not observed by Lance et al. [8] under similar conditions, most probably due to the differences in solvents and in the technique of a monolayer deposition. On the other hand, a similar effect of a decrease in enthalpy of the main phase transition corresponding to 25 mol% AmB was observed in multilamellar small vesicles formed with another lipid (DMPC) [6]. The molecular areas, higher than expected on the basis of the additivity rule (represented by the straight line in Fig. 6), may be explained in terms of an empty space in the monolayer due to AmB pores. This over-additivity is observed in the region of high concentrations of AmB, in which formation of pore-like aggregated structures is very likely. Pores formed in a hydrophobic environment are organised in such a way that they cover inside polar OH groups and in consequence occupy a higher area than all molecules involved in the formation of such a structure. A rough estimate for 90 mol% AmB at which the mean molecular area is larger by ca. 49 Å^2 leads to a model of AmB pore formed by six molecules and a pore internal radius of 6.1 Å. Such an estimate is close to a model of hydrophobic pore formed by 6 AmB molecules (a radius of 6.8 Å, [12]). The most striking effect is, however, the exceptionally high over-additivity corresponding to the low concentrations of AmB in a lipid phase. Such an effect may be explained in terms of a very strong repulsive hydrophobic interactions between polar groups of AmB located at one side of the molecule (see Fig. 1) and acyl chains of DPPC. Most probably, such an effect, distinctly demonstrated in the mixed AmB–DPPC monolayers, is responsible for the disorganisation of the structure of lipid bilayer pronounced in thermotropic phenomena.

As it follows from the experiments analysed above, AmB present in lipid membranes at low concentra-

tions, below those promoting its aggregation in lipid phase, strongly affects membrane properties. It is very probable that just this effect is responsible for very strong toxicity and severe side effects of this drug. In this respect, it is worth mentioning that 5 mol% AmB in 7:3 DMPC/DMPG sonicated liposomes was found to be exceptionally toxic to mice [9].

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References

[1] G.B.J. Mackanes, J. Liposome Res. 1 (1990) 521–528.
[2] J. Brajtburg, I. Gruda, G.S. Kobayashi, in: H. Yamaguchi, G. Kobayashi, H. Takahashi (Eds.), Recent Progress in Antifungal Chemotherapy, Dekker, New York, 1992, p. 65.
[3] I. Gruda, N. Dussault, Biochem. Cell. Biol. 66 (1988) 177–183.
[4] I. Gruda, E. Gauthier, S. Elberg, J. Braitburg, G. Medoff, Biochem. Biophys. Res. Commun. 154 (1988) 954–958.
[5] C. Tasset, V. Preat, M. Roland, J. Pharm. Pharmacol. 43 (1991) 297–302.
[6] K.S. Hamilton, K.R. Barber, J.H. Davis, K. Neil, C.W.M. Grant, Biochim. Biophys. Acta 1062 (1991) 220–226.
[7] M. Saint-Pierre-Chazalet, C. Thomas, M. Dupeyrat, C.M. Gary-Bobo, Biochim. Biophys. Acta 944 (1988) 477–486.
[8] M.R. Lance, C. Washington, S.S. Davis, Pharmaceut. Res. 13 (1996) 1008–1014.
[9] A.S. Janoff, L.T. Boni, M.C. Popescu, S.R. Minchey, P.R. Cullis, T.D. Madden, T. Taraschi, S.M. Gruner, E. Shyamsunder, M.W. Tate, R. Mendelsohn, D. Bonner, Proc. Natl. Acad. Sci. USA 85 (1988) 6122–6126.
[10] G. Lopez-Berestein, R.L. Juliano, in: M.J. Ostro (Ed.), Liposomes from Biophysics to Therapeutics, Marcel Dekker, New York, 1987, pp. 195–218.
[11] J. Barwicz, S. Christian, I. Gruda, Antymicrob. Agents Chemother. 36 (1992) 2310–2315.
[12] J. Barwicz, W.I. Gruszecki, I. Gruda, J. Colloid. Interf. Sci. 158 (1993) 71–76.
[13] F. Eggers, Th. Funck, Naturwissenschaften 63 (1976) 280–285.
[14] K. Wójtowicz, W.I. Gruszecki, J. Biol. Phys. 21 (1995) 73–80.
[15] L.D. Ma, R.L. Magin, F. Dunn, Biochim. Biophys. Acta 929 (1987) 183–192.
[16] A. Watts, P.J.R. Spooner, Chem. Phys. Lipids 57 (1991) 195–211.
[17] W.K. Subczyński, E. Markowska, W.I. Gruszecki, J. Sielewiesiuk, Biochim. Biophys. Acta 1105 (1992) 97–108.
[18] H. Lin, Z. Wang, C. Huang, Biochim. Biophys. Acta 1067 (1991) 17–28.
[19] J.E. Harkness, R.D. White, Biochim. Biophys. Acta 552 (1979) 450–456.
[20] L. Cruzeiro-Hansson, J.H. Ipsen, O.G. Mouritsen, Biochim. Biophys. Acta 979 (1989) 166–176.
[21] D. Chapman, Q. Rev. Biophys. 8 (1975) 185–235.