Effect of antibiotic amphotericin B on structural and dynamic properties of lipid membranes formed with egg yolk phosphatidylcholine

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Abstract

Amphotericin B (AmB) is a popular antibiotic applied in treatment of deep-seated mycotic infections. The mode of action of AmB is based upon interactions with biomembranes but exact binding properties of the antibiotic to the lipid membranes still remain obscure. Effect of incorporation of AmB into egg yolk phosphatidylcholine membranes in the concentration range from 0.01 to 5 mol\% on structural and dynamic properties of lipid bilayers was studied with application of small-angle neutron scattering, X-ray diffractometry and Fourier-transform infrared spectroscopy (FTIR). The results of the experiments show that AmB is located predominantly in the headgroup region of the membranes at concentrations below 1 mol\%. The process of AmB aggregation, at concentrations above 1 mol\%, is associated with ordering effect within the acyl chain region and therefore indicates incorporation of AmB into the hydrophobic membrane core.

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1. Introduction

Antifungal polyene macrolide antibiotic amphotericin B (AmB; Fig. 1) is a representative of the group of chemotherapeutics which mechanism of action consists in change of membrane cell permeability (Aracava et al., 1981; Brajtburg et al., 1990; Gallis et al., 1990; Ellis, 2002; Baginski et al., 2006). Destroying natural selective membrane permeability is an effect of incorporation of amphiphilic molecules of amphotericin B into the lipid phase and their interaction with lipid molecules (Hartsel et al., 1991; Wolf and Hartsel, 1995). Rod-shaped structure of AmB molecule, with the polar head mycosamine, the hydroxyl groups on the one side of the macrolide ring and the polyene fragment on the opposite side, makes it possible to interact both with the polar part of the lipid membrane and acyl chains. The consequence of such a structure of AmB is also a formation of molecular aggregates (De Kruijff et al., 1974; De Kruijff and Demel, 1974; Bolard et al., 1991; Barwicz et al., 1993; Caillet et al., 1995; Fujii et al., 1997; Gruszecki et al., 2002)
that play an important role in AmB mode of therapeutic action.

It has been postulated that not only formation of porous molecular structures by AmB, but also modification of the physical properties of the lipid bilayers modulates membrane permeability to ions (Barwicz and Tancrede, 1997; Fournier et al., 1998; Wójtowicz et al., 1998; Charbonneau et al., 2001; Gagos et al., 2001; Milhaud et al., 2002; Paquet et al., 2002; Zumbuehl et al., 2004; Gagos et al., 2005; Baginski et al., 2006; Gabrielska et al., 2006). Such mechanisms can be particularly effective at low concentrations of AmB, at which formation of aggregated molecular structures of the drug within the lipid phase cannot be expected (Gagos et al., 2001; Gruszecki et al., 2003a,b). On the other hand, the recent findings show that AmB increases the barrier for transmembrane ion transport, while incorporated to the lipid membranes at low concentrations instead of acting as an ionophore (Herec et al., 2005). In the present work, we address the problem of molecular interactions between AmB and lipids in the membranes containing very low and relatively high concentrations of the drug, in order to explore further, the molecular mechanisms responsible for action of this antibiotic with respect to biomembranes.

2. Materials and methods

Amphotericin B was purchased from Sigma Chem. Co. (St. Louis, USA). AmB was dissolved in and recrystallized from 2-propanol–water (4:6, v/v) and purified by means of HPLC directly before use. A Supelco PKB-100 column was applied (length 25 cm, internal diameter 4.6 mm) and the solvent mixture 2-propanol–water (4:6, v/v) was used as a mobile phase. The final concentration of AmB was calculated from the absorption spectra. The molar extinction coefficient in the absorption maximum at 408 nm was $1.3 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$. Owing to the low concentration of AmB eluted from the column (absorbance level below 0.2 at the optical path-length 1 cm) and owing to the fact that absorbance level decreased linearly upon dilution we considered the solution as composed mostly of monomeric AmB and the determination based on absorbance measurement accurate. Egg yolk phosphatidylcholine (EYPC) was obtained from Sigma Chem. Co. and used without further purification.

The AmB-containing EYPC membranes were investigated in the form of either unilamellar liposomes (SANS, electronic absorption) or multibilayers (X-ray, FTIR).

The multibilayers composed of 40 bilayers were formed on glass support or on ZnSe crystal with EYPC and AmB at molar concentration from 0.01 to 5 mol% according to the procedure described in detail previously (Gruszecki and Sielewiesiuk, 1990, 1991). Briefly, the lipid multibilayers were deposited to a solid support by means of evaporation from ethanol. After deposition the lipid films were transferred to a vacuum, for 30 min, in order to remove possible residuals of organic solvent, and then exposed for 30 min to relative humidity 80% in order to hydrate the lipid multibilayers.

The unilamellar liposomes were prepared in 100 mM Tricine buffer (pH 7.6) by extrusion technique, with application of liposome extruder (Avestin Inc., Canada) using filters with 50 nm pores, as described in detail previously (Herec et al., 2005).

The small-angle neutron scattering (SANS) with twodetector system measurements were performed at the small-angle time-of-flight axially symmetric neutron scattering spectrometer MURN at the IBR-2 fast pulsed reactor of the Frank Laboratory of Neutron Physics, Joint Institute for Nuclear Research in Dubna. For complete description of technical details, see Kuklin et al. (2005). The samples were poured into quartz cells (Hellma, Müllheim, Germany) to provide the 2 mm sample thickness. The sample temperature was set and controlled electronically at $20.0 \pm 0.1 ^\circ\text{C}$. The sample in quartz cell
was equilibrated minimally for 1 h at this temperature before the measurements. The scattering patterns were corrected for background effect. The coherent scattering intensity was obtained by using a vanadium standard scatterer. Further details of measurements and data interpretation were described previously (Uhrikova et al., 2000, 2003).

The $\theta$–$2\theta$ X-ray diffractometric measurements were performed with Dron-HZG 4 apparatus equipped with a copper anode ($\lambda = 1.54 \text{ Å}$) and a nickel filter. The methodology of measurements and data interpretation were described previously (Klose et al., 1996).

Electronic absorption spectra of liposome suspension was recorded with a double-beam UV–Vis spectrophotometer Cary 300 Bio from Varian equipped with a thermostated cuvette holder.

Infrared absorption spectra were reordered with Fourier-transform infrared spectrometer (FTIR) from Bruker Optik, Germany, model Vector 33 supplied with an attenuated total reflection (ATR) accessory. Before (30 min) and during measurements the instrument was purged with argon. Lipid multilayers were deposited to a ZnSe crystal support as described above. Typically 100 interferograms were collected, Fourier transformed and averaged. Absorption spectra in the region between 4000 and 600 cm$^{-1}$, at a resolution of one data point every 0.6 cm$^{-1}$, were obtained using a clean crystal as the background. Spectral analysis was performed with Grams 32 software from Galactic Industries (USA). All measurements were done at 20°C.

3. Results and discussion

Fig. 2 presents the X-ray diffractograms of the EYPC multibilayers containing incorporated different amount of AmB. The multibilayer periodicity, representing the lipid bilayer thickness and the layer of tightly bound water between the lipid bilayers (Gruszecki et al., 1992), determined on the basis of the Bragg’s law for the samples containing different amount of AmB are presented in Fig. 3. As can be seen, incorporation of the drug does not influence the multibilayer periodicity parameter, in the concentration range between 0.1 and 3 mol%. Fig. 4 presents the electron density profiles across the EYPC bilayer and the bilayer containing incorporated AmB, calculated on the basis of the X-ray diffractograms. The thickness of the lipid bilayer, calculated as a distance between the maxima of the profile (minimum of the second derivative of the profile), was 39.3 Å in the case of pure EYPC, 39.2 Å in the case of 0.2 mol% AmB and...
values ± membrane thickness values are 37.6
some suspension (see Fig. 5). The calculated liposomal
neutron scattering technique applied to unilamellar lipo-
brane can be analysed with application of small-angle
antibiotic of thickness of a single bilayer lipid mem-
the periodicity parameter of the system. Effect of the
effect on the structural and dynamic properties of lipid
membranes owing to the inhomogeneous distribution.

Fig. 6 presents the IR absorption spectra in the “fin-
print” region of hydrated lipid multilayers formed
on ZnSe crystal with pure EYPC and multilayers con-
taining different amount of AmB. Several bands visible
in this spectral region represent vibrations of the groups
located in the polar headgroup region of the mem-
brane: antisymmetric N+–(CH3)3 stretching vibrations
of choline (969 cm−1), symmetric (1249 cm−1) and anti-
symmetric (1249 cm−1) stretching vibrations of PO2−
group, C–O stretching of the ester group (1169 cm−1) and anti-
symmetric (1249 cm−1) stretching vibrations of the C–O–P–O–C fragment (1066 cm−1) (Lewis and McElhaney, 1996). The fre-
Fig. 6. Infrared absorption spectra of multilayers formed with pure EYPC and EYPC containing different concentrations of amphotericin B (indicated) in 1300–950 cm$^{-1}$ spectral range. Multilayers were deposited on ZnSe crystal. Dashed lines indicate position of selected absorption bands in the lipid sample without AmB.

Frequency of antisymmetric stretching ($\nu_a$) of PO$_2^-$ is exceptionally sensitive to hydrogen bonding (Barth and Zscherp, 2002). As can be seen, the position of this band is sensitive to AmB presence in the system. Fig. 7 presents the AmB concentration dependence of the $\nu_a$ PO$_2^-$ band position. The band becomes shifted towards lower frequencies upon incorporation of AmB to the membranes. This effect can be readily interpreted in terms of hydrogen bonding to PO$_2^-$ group, either AmB or water molecules, influenced by the presence of the drug in the system. The dependency presented in Fig. 7 is clearly biphasic: a strong concentration dependence below 1 mol% AmB and practically unchanged band position in the concentration range above 1 mol%. Similar, biphasic concentration dependency can be observed in the case of the C–O–P–O–C stretching vibrations (Fig. 8), that can be also interpreted in terms of hydrogen bonding to this group. Interestingly, the shape of the dependencies corresponds well to the concentration-dependent aggregation of AmB in the system (Fig. 9). This can be seen from the analysis of the electronic absorption spectra of the EYPC liposome suspension containing different amount of AmB (Fig. 9). The absorption spectra of AmB in liposomes display relatively complex structure owing to the fact that they are

Fig. 7. Dependence of the position of the absorption band corresponding to the $\nu_a$ PO$_2^-$ vibrations in IR absorption spectra of EYPC multilayers on AmB concentration.

Fig. 8. Dependence of the position of the absorption band corresponding to the stretching vibrations of the C–O–P–O–C fragment in IR absorption spectra of EYPC multilayers on AmB concentration.
composed of the spectra of various organization forms of the drug, including monomers (absorption maximum at 408 nm), dimers (absorption maximum at 350 nm) and larger aggregates (absorption maximum at 325 nm) (Barwicz et al., 1993; Gaboriau et al., 1997; Gagos et al., 2001; Gruszecki et al., 2003a,b; Baginski et al., 2006). The dependency of the absorbance ratio recorded at 408 and 325 nm, presented in the inset to Fig. 9, shows that AmB remains largely in the aggregated form at concentrations above 1 mol% with respect to lipid. This particular concentration can be referred to as the aggregation threshold of AmB in the lipid system applied. The symmetric stretching vibrations of the N+(CH₃)₃ groups are also sensitive to the AmB presence in the membranes (Fig. 10). Interestingly, the corresponding spectral band, that peaks at 926 cm⁻¹, becomes shifted to 910 cm⁻¹ upon AmB incorporation but the maximum of this band can be seen again at 926 cm⁻¹ at the relatively high concentration of the drug (5 mol%). According to the analysis presented in Fig. 9, AmB remains largely aggregated at such a high concentration. Apparently, aggregated drug has limited influence on the membrane region characterized by location of the choline groups. This effect is most probably associated with inhomogeneous distribution of aggregated molecules of AmB with respect to the lipid phase. Aggregation of AmB in the lipid membrane system is considered as a principal molecular mechanism that enables penetration of the drug into the hydrophobic core of the lipid bilayer owing to the amphiphilic character of AmB molecule.

Fig. 9. Electronic absorption spectra recorded from EYPC liposomes containing incorporated different amount of AmB (indicated). The inset shows AmB concentration dependency of the absorbance ratio recorded at 408 and 325 nm, corresponding to the absorption maxima that represent monomeric and aggregated form of AmB, respectively.
nostic of alkyl chain ordering (Lewis and McElhaney, 1996) and therefore reflects binding of AmB molecules into the hydrophobic core of the membrane. Such a binding results in van der Waals interactions between alkyl chains of the lipid molecules and rigid polyene chains of AmB, that restrict gauche-trans isomerization of alkyl chains. An ordering effect of AmB with respect to lipid membranes has been also demonstrated with application of differential scanning calorimetry (Fournier et al., 1998) and \(^{2}H\) NMR technique (Paquet et al., 2002). Another aspect of such a binding is formation of porous aggregated structures, characterized by the internal diameter of 0.6 nm (Gruszecki et al., 2002).

The results of the experiments presented in this work show that the polyene antibiotic amphotericin B in a monomeric form binds preferentially to the phospho-

Fig. 11. Infrared absorption spectra of multilayers formed with pure lipid EYPC and EYPC containing different concentrations of AmB, in 2870–2830 cm\(^{-1}\) spectral range. Multilayers were deposited on ZnSe crystal. Dashed lines indicate position of the absorption band assigned to the \(v_s\) CH\(_2\) vibrations.

lipid membrane polar headgroup region. This fraction of the antibiotic molecules is most probably responsible for the “sealing” effect manifested by an increase of the barrier for transmembrane ion transport (Herec et al., 2005). At concentrations exceeding the aggregation threshold (1 mol% in EYPC membranes at room temperature) AmB forms molecular structures that penetrate the hydrophobic core of the lipid bilayer.

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