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Molecular organization of antifungal antibiotic amphotericin B in lipid monolayers studied by means of Fluorescence Lifetime Imaging Microscopy

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ABSTRACT

Amphotericin B (AmB) is a life-saving polyene antibiotic used to treat deep-seated mycotic infections. Both the mode of therapeutic action as well as toxic side effects are directly dependent on molecular organization of the drug. Binding of AmB to lipid monolayers formed with dipalmitoylphosphatidylcholine, pure and containing 40 mol% cholesterol or ergosterol, the sterols of human and fungi respectively, has been examined by means of Fluorescence Lifetime Imaging Microscopy. AmB emits fluorescence with the characteristic lifetimes dependent on actual molecular organization: $\tau_{M2} \leq 10$ ps and $\tau_{M1} = 0.35$ ns in the monomeric state, the emission from the S_2 and the S_1 states respectively and $\tau_D = 14$ ns and $\tau_A = 3.5$ ns in the form of a dimer and associated dimers respectively. Analysis of the Langmuir–Blodgett films reveals that AmB binds to the lipid membranes and to the cholesterol-containing lipid membranes preferentially in the form of associated dimers. The same form of AmB appears in the membranes containing ergosterol but additionally the monomers and dimers of the drug. can be observed, which can severely affect molecular organization of the lipid membrane. The results are discussed in terms of selectivity of AmB towards the ergosterol-containing biomembranes of fungi.

1. Introduction

Amphotericin B (AmB, Fig. 1) is a life-saving polyene antibiotic used to treat deep-seated mycotic infections [1,2]. Despite very strong side effects and toxicity to patients AmB is constantly being applied owing to its pharmaceutical effectiveness [3]. Severe mycotic infections accompanying AIDS are frequently treated with AmB formulations. Both the pharmacologic as well as toxic side effects of AmB are associated with specific molecular organization of the drug. Efforts of multiple research laboratories in the world are directed toward elaboration of a formulation of the drug characterized by minimized toxic side effects but still preserving the antibiotic activity toward fungi. In this respect, understanding of molecular mechanisms of biological action of AmB seems to be a primary target [1,2].

According to a popular conviction AmB present in biomembranes associates into molecular aggregates in the form of transmembrane pores that affect physiological ion transport [4–8]. The concept that such structures are formed more efficiently in the presence of ergosterol, the sterol present in the membranes of fungi, than in the presence of cholesterol is a key paradigm of selectivity of AmB. It has been also proposed that selectivity toward cells of fungi is based upon a difference of the radii of porous structures of AmB binding ergosterol and cholesterol [6,9]. On the other hand, very recent reports show that alternatively both the biological action of the drug as well as toxic side effects may be directly related to the effect of AmB on physical properties of the membranes [10–13]. The ^{1}H NMR technique studies demonstrated that the polar headgroup region of the membranes, rather than the hydrophobic core, is a predominant site of binding of AmB from the water phase [12]. Analysis of the effect of AmB on structural and dynamic properties of lipid membranes carried out with application of SANS (small angle neutron scattering), ^{1}H NMR and FTIR techniques showed that AmB molecules, even bound to the lipid polar groups, influence motional freedom of acyl lipid chains [11–13]. This effect is particularly pronounced in the case of the lipid membranes containing ergosterol but less in the membranes containing cholesterol. Linear dichroism studies carried out with UV–Vis and FTIR absorption spectroscopy techniques revealed also that the membrane-bound AmB molecules are distributed between two pools: one oriented parallel and the other one perpendicular with respect

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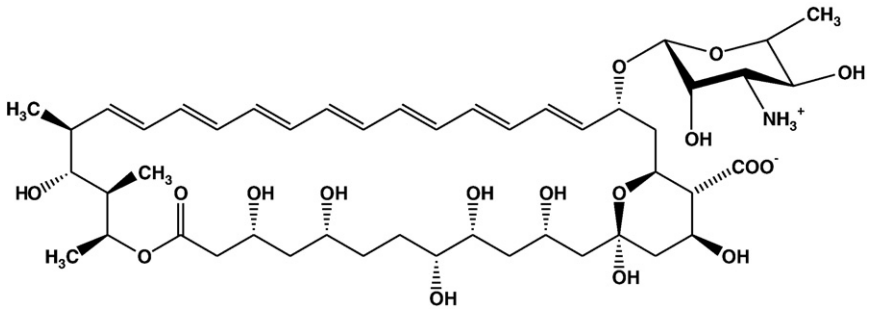

Fig. 1. Chemical structure of amphotericin B.

to the plane of the membrane [13–15]. It appeared that a laterally-oriented pool was particularly large in the membranes containing ergosterol and that such orientation of the drug had strongest effect on the membrane organization [13]. According to our earlier studies, AmB is able to form hydrophilic pores which can disturb transmembrane ion transport [16], indeed, but the results of the most recent studies, outlined above, seem to suggest that molecular mode of action of AmB with respect to biomembranes is primarily based on interference with the structural and dynamic properties of the lipid bilayers. This hypothesis requires further verification. In particular the finding that the effect of AmB on membranes is totally dependent on molecular organization of the drug (monomers and dimers display the opposite effect as compared to the larger aggregates) awaits interpretation and explanation at the molecular level. AmB at low concentrations in the lipid phase restricts the transmembrane proton transfer but acts in the opposite direction at higher concentrations (molar fractions above 3 mol%) [17].

According to our earlier report [15], confirmed recently by Stoodley et al. [18], AmB displays measurable fluoresce which can be analyzed, providing that fluorescing contaminations are separated [19]. Despite relatively low quantum yield fluorescence can be applied to distinguish different organization forms of molecules of the drug in different environments (even in not transparent natural samples). In particular, the presence of AmB dimers can be detected by means of fluorescence spectroscopy, owing to distinguished spectral signature. Detection of AmB dimers by means of other spectroscopic techniques is extremely difficult, due to spectral overlap with large aggregated structures. It appeared only possible, so far, in a very simple and well defined model system such as a monomolecular film [20]. Interestingly, very recent reports show that, most probably, more complex molecular structures of the drug are formed out of dimers [21].

In the present work we apply the fluorescence lifetime measurements and FLIM (Fluorescence Lifetime Imaging Microscopy) in order to understand more deeply molecular mechanisms responsible for the effect of AmB on biomembranes as well as toxic side effects and selectivity toward fungi.

2. Materials and methods

2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), cholesterol, ergosterol and crystalline amphotericin B (AmB) were purchased from Sigma Chem. Co. (USA). AmB was dissolved in 40% 2-propanol and then centrifuged for 15 min at 15000 ×g in order to remove micro crystals of the drug that may still remain in the sample. AmB was further purified by means of HPLC on YMC C-30 coated phase reversed column (length 250 mm, internal diameter 4.6 mm) with 40% 2-propanol in H_2O as a mobile phase. The final concentration of AmB was calculated from the absorption spectra on the basis of the molar extinction coefficient $1.3\times10^5\ M^{-1}\ cm^{-1}$ in the 0–0 absorption maximum at 408 nm.

2.2. Monomolecular layers

AmB was dissolved in water alkalized to pH 12 with KOH and then centrifuged for 15 min at 15000 ×g in order to remove microcrystals of the drug, still remained in the sample. A stock solution of AmB was adjusted to 1 mg/ml. Lipids (DPPC, cholesterol and ergosterol) were dissolved in chloroform. Monomolecular layers of pure DPPC and two-component monolayers composed of 60 mol% DPPC and 40 mol% of sterols were formed at the air–water interface. Monolayers were formed at the air–water interface and the water subphase was buffered with 10 mM Hepes, pH 7.2. Monomolecular layers were formed in computer-controlled Minitrough2 system from KSV Helsinki (Finland) composed of a Wilhelmy type tensiometer and of a Teflon trough (364 mm × 75 mm). Monolayers were compressed along the long side with a speed of 15 mm/min. After compression of the lipid monolayers to the surface pressure of 22 mN/m the solution of monomeric AmB in water alkalized to pH 12 was injected into the water subphase, beneath the monolayer. Final concentration of AmB in the subphase was 2.2 μM. Monomolecular layers were deposited to non-fluorescent quartz glass slides (10 mm × 20 mm) by means of the Langmuir–Blodgett technique (L–B films), with a speed of the lift 5 mm/min at a constant, computer-controlled surface pressure which

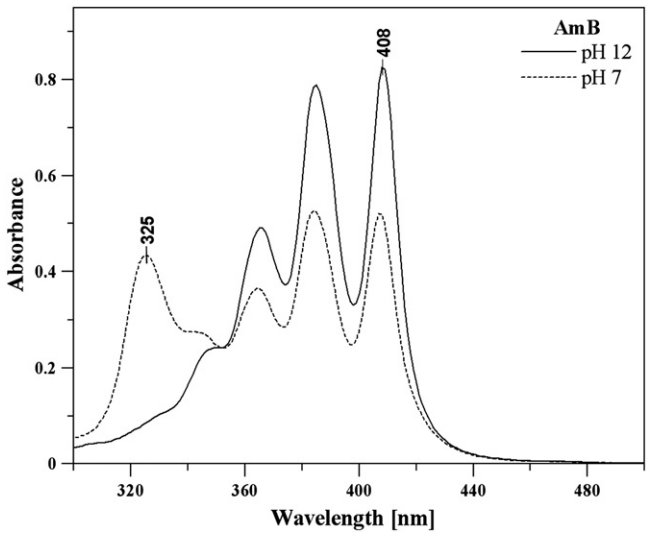

Fig. 2. Electronic absorption spectra of amphotericin B in water medium alkalized with KOH to pH 12 and 7, indicated. Sample concentration 6.2 μM.