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Small-angle neutron scattering studies of the effects of amphotericin B on phospholipid and phospholipid–sterol membrane structure

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ABSTRACT

Small-angle neutron scattering (SANS) studies have been performed to study the structural changes induced in the membranes of vesicles prepared (by thin film evaporation) from phospholipid and mixed phospholipid–sterol mixtures, in the presence of different concentrations and different aggregation states of the anti-fungal drug, amphotericin B (AmB). In the majority of the experiments reported, the lipid vesicles were prepared with the drug added directly to the lipid dispersions dissolved in solvents favouring either AmB monomers or aggregates, and the vesicles then sonicated to a mean size of ~100 nm. Experiments were also performed, however, in which micellar dispersions of the drug were added to pre-formed lipid and lipid–sterol vesicles. The vesicles were prepared using the phospholipid palmitoyloleoylphosphatidylcholine (POPC), or mixtures of this lipid with either 30 mol% cholesterol or 30 mol% ergosterol. Analyses of the SANS data show that irrespective of the AmB concentration or aggregation state, there is an increase in the membrane thickness of both the pure POPC and the mixed POPC-sterol vesicles—in all cases amounting to ~4 Å. The structural changes induced by the drug's insertion into the model fungal cell membranes (as mimicked by POPC-ergosterol vesicles) are thus the same as those resulting from its insertion into the model mammalian cell membranes (as mimicked by POPC-cholesterol vesicles). It is concluded that the specificity of AmB for fungal *versus* human cells does not arise because of (static) structural differences between lipid–cholesterol-AmB and lipid–ergosterol-AmB membranes, but more likely results from differences in the kinetics of their transmembrane pore formation and/or because of enthalpic differences between the two types of sterol-AmB complexes.

1. Introduction

Over the past decade there has been a dramatic rise in the frequency of diagnosed fungal infections [1,2]—encompassing both systemic infections (ascribed to increased numbers of patients immuno-compromised through chemotherapy or HIV infection [2,3]) and also transplant- and implant-related infections (attributable to mycotic biofilm development [4]). This sharp increase in fungal infections has been accompanied by an increased frequency with which these infections prove recalcitrant to standard anti-fungal therapy [5–8]. There is an emerging demand, therefore, for novel anti-fungal agents that can circumvent the pathogens' resistance. The successful development of such novel anti-mycotics will clearly require an appreciation of both the mechanism(s) of action of the failed compounds and the molecular mechanism(s) underlying the reduced susceptibility of the resistant pathogens.

Since its discovery in the mid-1950s, one of the mainstays of the anti-fungal armamentarium has been the polyene macrolide antibiotic, amphotericin B (AmB) (Fig. 1) [3]. Several recent reports, however, have attested to the emergence of AmB-resistant strains of a number of clinically problematic pathogenic yeasts including *Candida* spp [1–8]. Now, although it has long been held that AmB exerts its anti-fungal action through the generation of self-assembled ion channels within the fungal cell membranes, there is no *direct structural* evidence to support this hypothesis. It has been shown that the selectivity of AmB for fungal vs. human cell membranes is linked to the organisms' differing steroid content—with fungal cell membranes having ergosterol and their mammalian counterparts, cholesterol. It has been proposed (on the basis of ion and non-electrolyte permeability studies) that AmB - and related polyene macrolides such as nystatin - forms ion channels within bio-membranes [9]. Models of these ion channels have been derived based upon a consideration of the amphipathic structures of the drugs, together with an experimental demonstration of their cooperativity in development of the toxigenic membrane conductance (which leads to cell death through indiscriminate transfer of ions across the cell membranes) [10]. The selective toxicity of AmB

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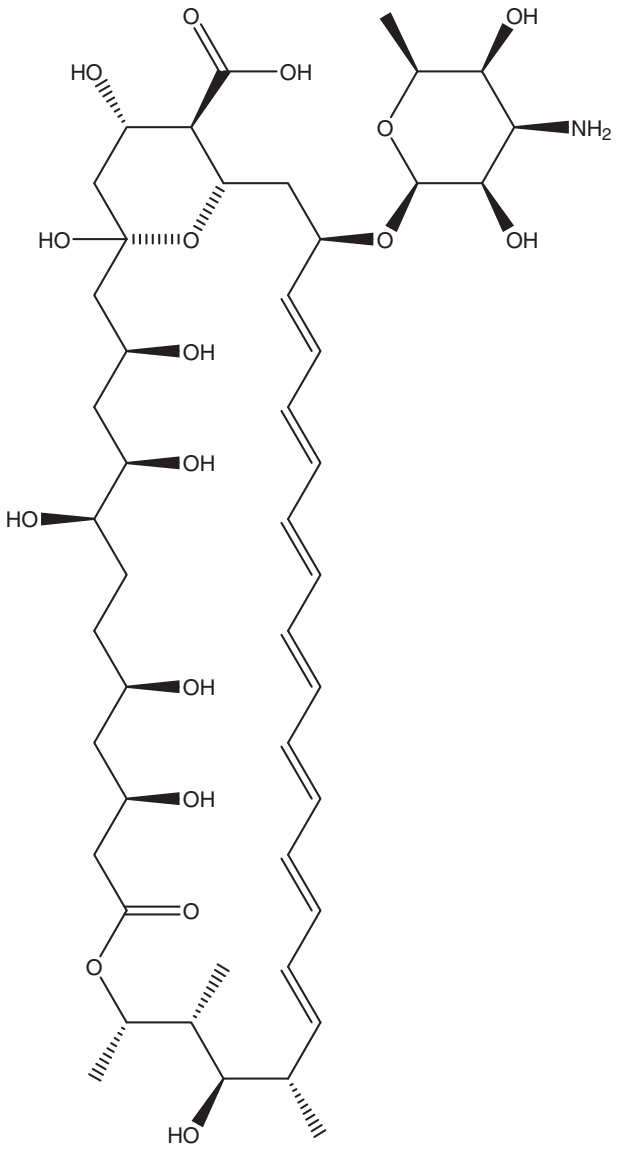

Fig. 1. Chemical structure of amphotericin B.

(and related polyene macrolide antibiotics) towards ergosterol-containing fungal cell membranes compared to cholesterol-containing human cell membranes is believed to be a crucial factor in the specificity for fungi, with the commonly held view that the ion channels formed involve drug-sterol complexation, with complexes involving ergosterol being strongly preferred over those involving cholesterol [11]. Recent research, however, suggests that this "textbook" explanation of how AmB works is only partially correct and very oversimplified [12–16]. It has been shown, for example, that the nature of the interaction between AmB and cell membranes is influenced very significantly by the concentration of drug [15,17,21–23], the concentration of sterol [17–19,23], and the lipid composition [20] and physical state/properties of the membrane [15,17,18]. Moreover, there is now controversy regarding the role of sterols in the membrane—and some experiments in fact indicate that AmB may form pores in sterol-free membranes [20–22]. Suffice to say, therefore, that there is considerable research effort still required to determine the molecular basis for the anti-mycotic activity of AmB and its related polyene antibiotics such as nystatin. Without a detailed understanding of the drugs' mechanism(s) of action, through which the chemistry of the drugs can be quantitatively related to their biological efficacy, it will prove difficult to develop novel variants of the drug which exhibit the necessary specificity, whilst also being effective against AmB-resistant fungi.

The information required to furnish such understanding can only be secured by using biophysical analytical techniques to probe, in a systematic way, the interactions between the drug, sterols and lipids at the molecular level. The first such studies – employing a combination of small-angle neutron scattering (SANS), X-ray diffraction and Fourier transform infrared spectroscopy – were reported by Hereć et al. [17]. In these studies, however, the membrane interactions of AmB were investigated using vesicles prepared from egg lecithin alone—*without* incorporated cholesterol or ergosterol [17].

Here, we report SANS studies performed on both phospholipid and phospholipid–sterol vesicles, as a means to securing an understanding of how differences in a sterol's structure influence its effects on biomembranes, and as a means also then to explore how these differences influence the membrane interactions of the polyene macrolide antibiotic AmB.

2. Materials and methods

2.1. Sample preparation

All chemicals – other than those indicated below – were purchased from Sigma-Aldrich Ltd. (Gillingham, UK) and were used as received. Hydrogenated and deuterated phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The dispersions of the vesicles were prepared by means of thin film evaporation [24]. For the majority of the experiments detailed below, phospholipid and 2:1 phospholipid–sterol solutions were prepared in chloroform (5 mL, Fluka, UK, Ltd., Dorset; spectroscopic grade) at a concentration of 1.25 mg/mL (or the equivalent in the case of *d*-lipid samples), and were evaporated to dryness using a BUCHI 461 rotary evaporator. The resulting *h*-lipid films were then dispersed in 5 mL D_2O (Aldrich, UK, Ltd., Dorset; 99.7% D), and the corresponding *d*-lipid films were dispersed either in H_2O or D_2O. These aqueous lipid dispersions were vortexed for 5 min, placed in a water bath at ambient temperature for 10 min, and finally ultrasonicated for 5 min using a probe sonicator (Lucas Dawes Ultrasonicator Soniprobe). (The concentration of the lipid dispersions was ~1.25 mg/mL – with the precise concentration dependent on the particular lipid/lipid:sterol mixture used – and so the molar ratio of water:lipid was always in excess of 30:1.) Equivalent sets of samples were also prepared incorporating a final concentration of AmB in the lipid film of 0.1 μM, 1 μM, 10 μM or 100 μM. For these latter samples, the AmB was initially prepared as a 25 mg/mL stock solution in dimethyl sulfoxide (DMSO, Fluka, UK, Ltd., Dorset; spectroscopic grade), and the required volume of this stock then taken and added to the lipid or lipid:sterol solution dissolved in chloroform or chloroform:methanol 2:1 v/v. (The latter was employed in the case of the systems involving 10 μM and 100 μM AmB as a precaution against aggregation of the drug.) [25]. The level of DMSO in the final aqueous dispersions of these AmB-containing vesicles was always ≤0.04 vol%. Other samples were also prepared with an aqueous dispersion of AmB (containing ≤0.04 vol% DMSO) added to pre-formed lipid or lipid–sterol vesicles; for these experiments the AmB and lipid (or lipid–sterol) solutions (in either D_2O or H_2O) were prepared at twice the required final concentration (*viz*., ~2.5 mg/mL for the lipid, and 200 μM for the AmB), and the samples then prepared by mixing the drug and vesicle stocks in the ratio 1:1. For each vesicle suspension (diluted from the corresponding solution used for SANS studies by 1 in 16) photon correlation spectroscopic (PCS) measurements were recorded as a function of time (using a Brookhaven Zetaplus instrument), and indicated that the dispersions were stable (at ambient temperature) for up to 3 days (the time required to perform the SANS experiments), showing no significant change in particle size or polydispersity over this period (typically