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Amphotericin B as a Potential Probe of the Physical State of Vesicle Membranes

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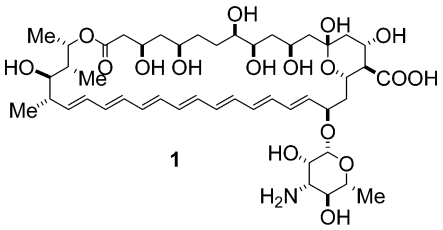
ABSTRACT



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The investigations described introduce a new role for a natural product such as amphotericin B as a potential biophysical reporter group to probe the physical state of a membrane. Specifically, we demonstrated that the K^+ efflux pattern reveals an interesting sterol dependence. This is suggested to be correlated to the physical state of the membrane showing high efflux in a vesicle membrane of intermediate fluidity.

Over the last 50 years, the antimycotic agent amphotericin B (**1**) has been the subject of thousands of research papers.[1] Although many findings remain contradictory, it is generally agreed that amphotericin B induces leakage of small electrolytes from yeast cells. This may not be the only cause of cell death, and, in fact, there may be several parallel mechanisms of action for amphotericin B.[2] It is generally accepted that the molecule differentiates between mammalian and fungal cells. Kotler−Brajtburg et al. suggested that this is due to the different sterols present in the two membranes: ergosterol in yeast and cholesterol in mammalian cells.[3] However, it is not clear if this effect is caused by the preferential formation of a complex of amphotericin B with ergosterol over cholesterol or if the observed effects result from differential preorganization of membranes by the two sterols a priori.[4]

We have recently reported an improved assay allowing fast on-line measurements of K^+-efflux from 100 nm large unilamellar POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) vesicles prepared by the extrusion technique ($LUVET_{100}$).[5] In this communication we fully exploit this vesicle assay and report on the interaction of amphotericin

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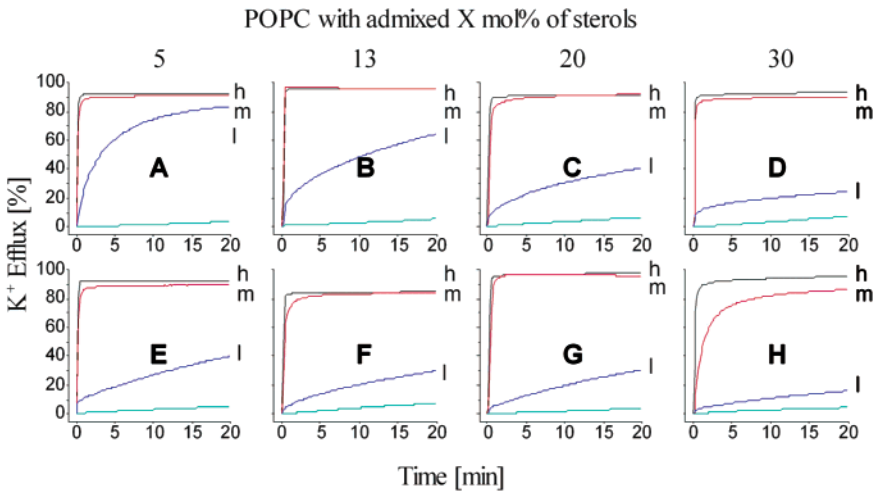
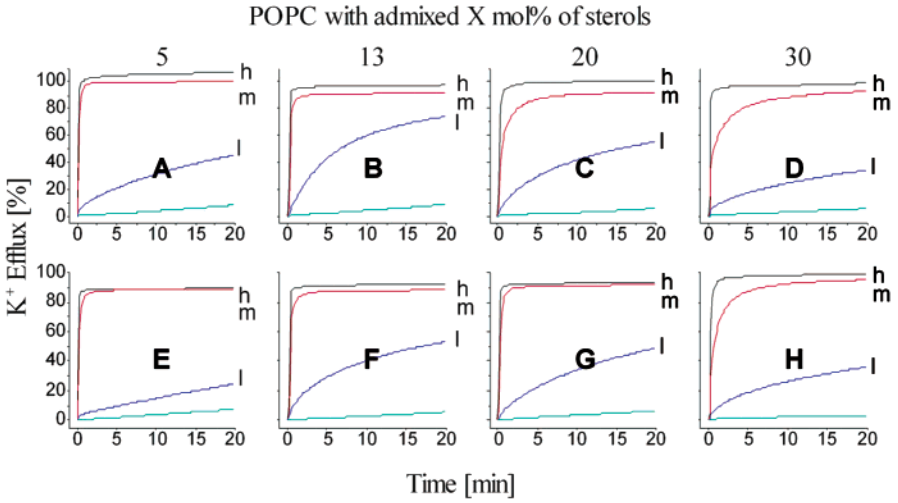


The vesicles were prepared from POPC with admixed ergosterol (**2**) A-D and cholesterol (**4**) E-H (5, 13, 20, 30 mol% from left to right). The concentrations of amphotericin B after external addition (as DMSO solution) to the stirred vesicle solution were: high concentration (h): 10 μM (black), medium concentration (m): 1 μM (red), low concentration (l): 0.1 μM (blue). The green line depicts the lack of K+-release after addition of 32 μL of pure DMSO (the amount in which amphotericin B was added). The total concentration of lipids (POPC + sterol) was 1 mM in 5 mM HEPES buffer at pH = 7.4.

Figure 1. K^+-release from $LUVET_{100}$ after external addition of amphotericin B (**1**).

B with liposomes containing different sterols (cholesterol (**4**), ergosterol (**2**), 7-dehydrocholesterol (**3**), dihydrocholesterol (**5**)) at different concentrations (5, 13, 20, 30 mol %) (Chart 1). We find that at amphotericin B concentrations above the critical association constant (1 μM),[6] immediate full K^+ release is induced regardless of the sterol composition of the POPC vesicle. The K^+ efflux pattern of monomeric amphotericin B, however, reveals an interesting sterol dependence, which we suggest is correlated to the physical state of the membrane, showing high efflux in a membrane of intermediate fluidity. The investigations introduce a new role for a natural product such as amphotericin B as a biophysical reporter group to probe the physical state of a vesicle membrane.

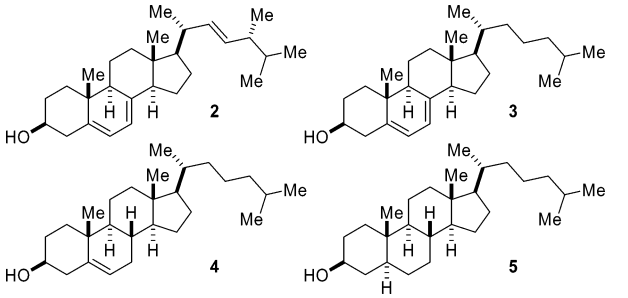
A series of POPC $LUVET_{100}$ was prepared containing different amounts of various sterols (Figures 1 and 2). Ergosterol (**2**), 7-dehydrocholesterol (**3**), cholesterol (**4**), and dihydrocholesterol (**5**) (Chart 1) were chosen, as they retain



The vesicles were prepared from POPC with admixed 7-dehydrocholesterol (**3**) A-D and dihydrocholesterol (**5**) E-H (5, 13, 20, 30 mol% from left to right). The concentrations of amphotericin B after external addition (as DMSO solution) to the stirred vesicle suspensions were: high concentration (h): 10 μM (black), medium concentration (m): 1 μM (red), low concentration (l): 0.1 μM (blue). The green line depicts the lack of K+-release after addition of 32 μL of pure DMSO (the amount in which amphotericin B was added). The total concentration of lipids (POPC + sterol) was 1 mM in 5 mM HEPES buffer at pH = 7.4.

Figure 2. K^+-release from $LUVET_{100}$ after external addition of amphotericin B (**1**).

Chart 1



the 3β-hydroxy group and side-chain at C-17[7], albeit displaying various degrees of unsaturation. The vesicles were prepared from a thin film of the lipids in the presence of KCl.[5] Following dialysis against a NaCl solution, an ion-gradient was established with K^+ ions inside and Na^+ ions outside of the vesicles. A K^+ selective electrode (valinomycin) was immersed into the vesicle suspension. Addition of amphotericin B induced a K^+ efflux from the vesicles, which could be conveniently measured via potentiometry at room temperature.[8] After destruction of the vesicles by adding the detergent sodium cholate, the total amount of the initially trapped K^+ was calculated. Each measurement was repeated at least two times.[9]

We started our investigations on a system containing ergosterol (**2**) or cholesterol (**4**) as a consequence of their putative biological relevance (see Figure 1).[3] The amounts of added amphotericin B were chosen such as to be above (h, 10 μM), at (m, 1 μM), and below (l, 0.1 μM) the critical self-association concentration of 1 μM in water.[6] Thus, at 10 and 1 μM concentrations, amphotericin is found in various degrees of aggregation, while at 0.1 μM, it exists as a monomer.[10]

Analysis of the efflux data in Figure 1 reveals that at and above a threshold value of 1 μM, amphotericin B leads to very rapid release of K^+ from vesicles independent of the type of sterol in the vesicle membrane (Figure 1Ah−Hh, Am−Hm). Indeed, it has been suggested that aggregates of amphotericin B are responsible for the severe side-effects of the drug in humans,[1h] a feature that is reflected in the nonselective interactions of the drug with either ergosterol- or cholesterol-containing membranes.

A different picture emerges when monomeric amphotericin B is added to the vesicle suspension (Figure 1Al−Hl). In this case, the kinetics of K^+-release depends on the type of sterol and the sterol concentration in the vesicle membrane. At low sterol content (5 mol %, Figure 1Al, El) the K^+-release from the vesicles containing ergosterol was much faster than from cholesterol-containing liposomes. This observation highlights that amphotericin B is able to differentiate between ergosterol- and cholesterol-containing membrane vesicles only as its monomer and is consistent with previous reports concerning differentiation between mammalian and fungal cells.

We proceeded to employ amphotericin as a potential probe for the physical state of the membrane. Amphotericin B monomers appear to interact more readily with ordered membrane phases.[4a] Since cholesterol is more effective at increasing order and restricting the mobility of POPC than ergosterol, one would expect a higher degree of K^+-release from cholesterol-containing vesicles.[11] However, the data show a decrease in K^+-efflux when compared to the case of ergosterol (Figure 1Al vs 1El; 1Bl vs 1Fl; 1Cl vs 1Gl; 1Dl vs 1Hl). The observed diminution in K^+-efflux strongly implicates an upper limit in membrane ordering beyond which amphotericin B is no longer capable of inserting into the membrane. Specifically, this suggests that amphotericin B may instead prefer an intermediate membrane phase between the liquid-disordered state (l_d) and the liquid-ordered state (l_o).

Recent biophysical studies have refined the picture of the physical states of biomembranes. In particular, the phase diagram for DPPC-cholesterol[12] mixtures at temperatures above the gel-to-liquid transition (T_m) predicts such a coexisting phase between the liquid-disordered state and the liquid-ordered state.[13] Such a phase coexistence is absent in POPC-cholesterol bilayers at room temperature, which remains in a liquid-ordered state up to 50 mol % cholesterol.[14] Our findings of monotonic K^+-efflux decrease with increasing sterol content (Figure 1Al−Dl,El−Hl) are consistent with this.

We next examined vesicles incorporating 7-dehydrocholesterol (**3**) and dihydrocholesterol (**5**). At 10 and 1 μM amphotericin B concentration, an immediate total K^+-efflux from vesicles is recorded (Figure 2Ah−Hh, Am−Hm) depicting the lack of selectivity of amphotericin B also for these types of membrane sterols as observed for cholesterol and ergosterol. By contrast, the investigations of 7-dehydrocholesterol (**3**) and dihydrocholesterol (**5**) afforded some interesting contrasting results at the low concentration where amphotericin is added in monomeric form. At low amphotericin B concentrations (Figure 2Al−Dl, El−Hl), with both 7-dehydrocholesterol (**3**) and dihydrocholesterol (**5**),

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(8) Small changes in temperature may have an effect on the phase boundaries; however, it has been shown that phase boundaries do not necessarily shift with temperatures; see discussion in: Virtanen, J.; Ruonala, M.; Vaukonen, M.; Somerhau, P. *Biochemistry* **1995**, *34*, 11568−11581.

(9) We have performed dynamic light-scattering measurements with POPC liposomes in the presence of added amphotericin B/DMSO solutions; no change in vesicle diameter was noted.

(10) For the dispersion in this study using 10, 1, and 0.1 μM amphotericin, the nominal loadings are 300, 30, and 3 amphotericins per vesicle. The calculation is based on the following assumptions: mean headgroup area of one POPC molecule of 72 Å^2, bilayer thickness of 37 Å, and a mean sectional area of 33 Å^2 for both cholesterol and ergosterol. It has been proposed that as little as 2 amphotericin B molecules per vesicle are sufficient to cause total leakage, see: van Hoogevest, P.; de Kruijff, B. *Biochim. Biophys. Acta* **1978**, *511*, 397−407.

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the K^+-efflux goes through a maximum at 13 mol % and thus does not decrease monotonically as observed for cholesterol- and ergosterol-containing membranes (Figure 1Al−Dl, El−Hl). We suggest that this implicates that 7-dehydrocholesterol (**3**) and dihydrocholesterol (**5**) induce a coexisting liquid-disordered/liquid-ordered phase at medium sterol concentrations (Figure 2Bl−Cl, Fl−Gl) with which amphotericin B preferentially interacts. This would necessitate that part of the membrane is more ordered (rich in sterol) and partly less so (poorer in sterol);[15] amphotericin B is then able to interact with the less ordered part leading to K^+-release.

In conclusion, we have shown that at concentrations below the aggregation threshold, amphotericin B induces remarkably different K^+-efflux from vesicles containing various sterols at different concentrations. We have thus employed amphotericin B as a probe to report on the phase diagram of the different membranes at room temperature. This has led to the proposal that amphotericin B shows a preference for a coexisting $l_d + l_o$ (liquid-disordered/liquid-ordered) phase. More generally, the observations are consistent with a model for amphotericin B in which membrane selectivity arises from differential preorganization of membranes caused by the different sterols.

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Supporting Information Available: Experimental procedures and spectroscopic data of the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) This conclusion is analogous with the superlattice model that has been proposed for cholesterol. For a review, see: Chong, P. L.-G.; Sugar, I. P. *Chem. Phys. Lipids* **2002**, *116*, 153−175.