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Membrane activity of the pentaene macrolide didehydroroflamycoin in model lipid bilayers

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ABSTRACT

Didehydroroflamycoin (DDHR), a recently isolated member of the polyene macrolide family, was shown to have antibacterial and antifungal activity. However, its mechanism of action has not been investigated. Antibiotics from this family are amphiphilic; thus, they have membrane activity, their biological action is localized in the membrane, and the membrane composition and physical properties facilitate the recognition of a particular compound by the target organism. In this work, we use model lipid membranes comprised of giant unilamellar vesicles (GUVs) for a systematic study of the action of DDHR. In parallel, experiments are conducted using filipin III and amphotericin B, other members of the family, and the behavior observed for DDHR is described in the context of that of these two heavily studied compounds. The study shows that DDHR disrupts membranes via two different mechanisms and that the involvement of these mechanisms depends on the presence of cholesterol. The leakage assays performed in GUVs and the conductance measurements using black lipid membranes (BLM) reveal that the pores that develop in the absence of cholesterol are transient and their size is dependent on the DDHR concentration. In contrast, cholesterol promotes the formation of more defined structures that are temporally stable.

1. Introduction

Polyene macrolides are biologically active metabolites isolated from *Streptomyces* [1]. Due to their antifungal activity, some of them, e.g., amphotericin B (AmB) or nystatin, have been used in human medicine to treat fungal infections for several decades [2]. Their mode of action is assumed to heavily involve biological membranes [3]. Although polyene antibiotics share a similar structure, the mechanism of the interaction with the membrane can substantially differ and cannot be easily predicted. For instance, AmB and nystatin form ion channel pores [3], but the pentaene filipin III acts as a general disruptor through membrane protrusions that arise from altered phase behavior [4–6]. The action of most polyenes strongly depends on the presence of sterols in membranes [7–10]. Furthermore, a target organism can be identified by the sterol composition of its membrane [11], and despite the importance of sterols, the involvement of sterols is not thoroughly understood. Pore formation occurs even in sterol-free bilayers, indicating that sterols merely facilitate the incorporation of antibiotics into the membrane via modulation of the membrane mechanical properties [6,12]. In contrast, specific interactions of the mycosamine moiety of AmB have been proposed to be crucial for the interaction with ergosterol [13].

In this manuscript, we investigate the membrane interactions of a recently isolated polyene macrolide, 32,33-didehydroroflamycoin (DDHR, Fig. 1) [14]. To date, the only known aspect of its mechanism of action is associated with the dose-dependent hemolysis of red blood cells [15].

In this study, leakage assays were used to study the creation of pores, as well as other membrane formations (buds, non-spherical shape), in well-defined, free-standing model membranes of giant unilamellar vesicles (GUVs). In particular, we focus on the role of cholesterol and its participation in the enhancement/attenuation of membrane disruption. By combining these assays with conductance measurements of black lipids membranes (BLMs), we demonstrate that the pores formed in cholesterol-containing bilayers are defined in size and temporally stable. In contrast, the pores formed in the absence of cholesterol resemble general membrane disruptions, which are transient and whose size depends on the concentration.

Furthermore, we study membranes consisting of coexisting fluid phases and the ability of DDHR to promote these phases in a homogeneous bilayer. Additionally, experiments are also performed using filipin III and AmB (Fig. 1), other members of the polyene macrolide

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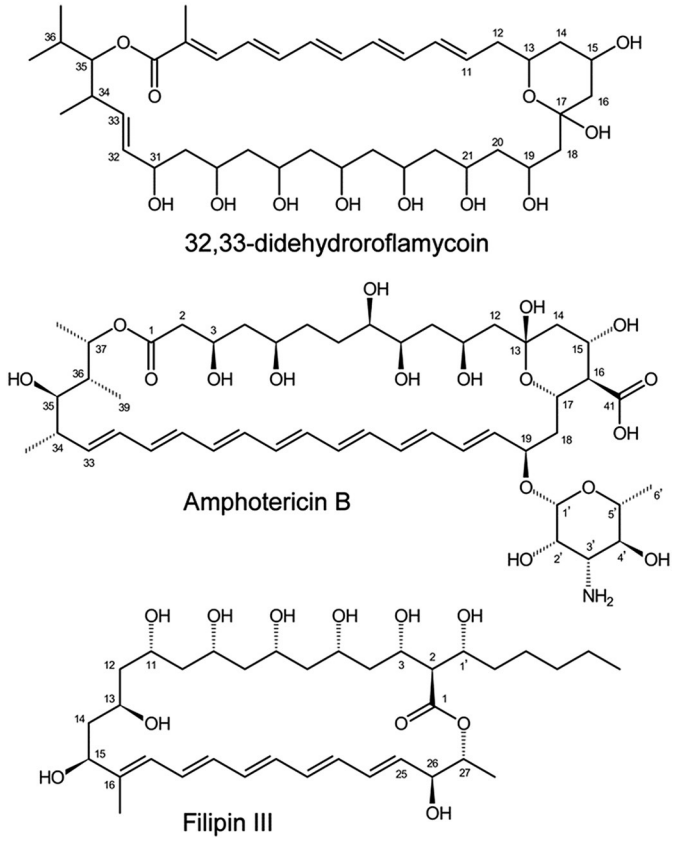

Fig. 1. Structures of the polyene macrolides 32,33-didehydroroflamycoin, amphotericin B and filipin III.

family. The similarities and differences in their action are demonstrated, showing that although cholesterol substantially participates in the action of all the presented antibiotics, its final effect on the fate of a membrane and potentially on the fate of a cell may be very different.

2. Materials and methods

2.1. Extraction of DDHR and other macrolides

Dry DDHR powder was kindly gifted by the Laboratory of Fungal Genetics and Metabolism (Institute of Microbiology, Academy of Sciences of the Czech Republic v.v.i., Prague, Czech Republic). The extraction procedure has been described elsewhere [14]. The dry DDHR powder was kept in the dark at −20 °C. A stock solution of 5 mM DDHR was prepared by dissolving the DDHR powder in methanol. This solution was stored as aliquots at −80 °C and was protected from light. Pure methanol was used as a solvent due to the poor solubility of DDHR in water. Additionally, 5 mM stock solutions of AmB and filipin III were prepared in methanol.

2.2. Solvent and reagents

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), porcine brain sphingomyelin (Sph) and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). All lipids were used without purification after the phospholipid purity was confirmed using thin-layer chromatography. Stock solutions were prepared in chloroform using standard quantitative techniques. Atto488 was purchased from ATTO-Tec (Siegen, Germany) and prepared as a stock solution in 105 mOsm glucose buffer. DiIC18(5) (DiD), AlexaFluor®488-labeled dextran 3000 and dextran 10 000 were purchased from Life Technologies Corporation (Carlsbad, CA), and the dextrans were dissolved in 105 mOsm glucose buffer. Filipin III and AmB were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. GUV formation

GUVs were prepared using a modified electroformation method originally developed by Angelova [16]. Lipid mixtures were prepared from stock solutions in chloroform. The total amount of all lipids (100 nmol in approximately 200 μL of chloroform) together with DiD (0.1 mol%) was spread onto two hollowed titanium plates, which were placed on a heater plate at approximately 50 °C to facilitate solvent evaporation, and the mixture was subsequently placed in high vacuum for at least 1 h for evaporation of the remaining solvent traces. The lipid-coated plates were assembled using one layer of Parafilm for insulation [17]. The electroswelling chamber was filled with 1 mL of preheated sucrose solution (100 mM sucrose, osmolarity of 103 mOsm/kg) and sealed with Parafilm. An alternating electrical field of 10 Hz that increased from 0.02 V to 1.1 V (peak-to-peak voltage) during the first 45 min was applied and was then maintained at 1.1 V for an additional 2.5 h at 55 °C; this field was followed by 30 min of 4 Hz and 1.3 V to detach the formed liposomes. Finally, approximately 40 μL of the GUV suspension was placed in a microscopy chamber containing 360 μL of glucose buffer (~80 mM glucose, 10 mM HEPES and 10 mM NaCl, pH 7.2) with an osmolarity of 103 mOsm/kg. The presence of glucose in the final solution allowed the liposomes to sediment and decreased the vesicle movement.

For all the experiments, DDHR, filipin III and AmB were added to the glucose buffer prior the addition of GUVs. For the leakage assays, the glucose buffer also contained Atto488, labeled dextrans or methanol in the desired concentration. The leaking vesicles were counted after 1 h of incubation. All the measurements were performed at room temperature.

Simultaneously with the leakage experiments, control experiments were conducted. Instead of DDHR or the other investigated polyenes, methanol in the same volume as the volume of the polyene solution was added. The maximum methanol volume fraction was 1%. The control GUVs were stable, as shown in Table 1.

2.4. LUV formation

For LUV formation, an appropriate mixture containing 10^{-6} mol of lipids was prepared in chloroform. Chloroform was evaporated using a rotary evaporator, and the lipid film was rehydrated using 1 mL of buffer solution (10 mM HEPES, 150 mM NaCl and 2 mM EDTA, pH 7). A turbid solution containing the multilamellar vesicles was extruded 10 times using 100 nm filters in a LIPEX extruder (Northern Lipids Inc., Canada) [18].

2.5. Absorption/emission spectra

Absorption spectra were measured on a UV2600 UV–VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Emission spectra were monitored using a FluoroLog 3 steady-state fluorescence spectrometer (model FL3-11; Horiba Jobin Yvon Inc., Edison, NJ). DDHR was excited by 370 nm light.

2.6. Confocal microscopy

Confocal microscopy imaging was performed on an FV1000 (Olympus, Hamburg, Germany), and the microscope was equipped with a UPLSAPO 60× W N.A. 1.20 objective lens. Atto488, AlexaFluor®488 and DiD were excited using the 488 and 632 nm laser lines, respectively. DDHR and filipin III was excited by a Coherent Chameleon Vision II titanium:sapphire laser (Coherent, Santa Clara, CA) using multiphoton excitation at 800 and 750 nm, respectively.