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Direct *in vivo* interaction of the antibiotic primycin with the plasma membrane of *Candida albicans*: An EPR study

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ABSTRACT

The direct interaction of the antibiotic primycin with the plasma membrane was investigated by employing the well-characterized ergosterol-producing, amphotericin B-sensitive parental *Candida albicans* strain *33erg*$^+$ and its ergosterol-less amphotericin B-resistant plasma membrane mutant *erg-2*. The growth inhibition concentration in shaken liquid medium was 64 μg ml^{-1} for *33erg*$^+$ and 128 μg ml^{-1} for *erg-2*, suggesting that the plasma membrane composition influences the mode of action of primycin. To determine the primycin-induced changes in the plasma membrane dynamic, electron paramagnetic resonance (EPR) spectroscopy methods were used, the spin-labeled fatty acid 5-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid) being applied for the *in vivo* measurements. The phase transition temperatures of untreated strain *33erg*$^+$ and its mutant *erg-2* were 12.5 °C and 11 °C, respectively. After 128 μg ml^{-1} primycin treatment, these values increased to 17.5 °C and 16 °C, revealing a significant reduction in the phospholipid flexibility. Saturation transfer EPR measurements demonstrated that, the rotational correlation times of the spin label molecule for the control samples of *33erg*$^+$ and *erg-2* were 60 ns and 100 ns. These correlation times gradually decreased on the addition of increasing primycin concentrations, reaching 8 μs and 1 μs. The results indicate the plasma membrane "rigidizing" effect of primycin, a feature that may stem from its ability to undergo complex formation with membrane constituent fatty acid molecules, causing alterations in the structures of phospholipids in the hydrophobic surface near the fatty acid chain region.

1. Introduction

The important human pathogen *Candida albicans* is a diploid dimorphic unicellular yeast that has lost its sexual and parasexual cell cycles. More than 50% of *Candida* infections and deaths are caused by *C. albicans* [for reviews, see: [1–4]. Polyene antibiotics such as amphotericin B (AmB) or nystatin, and azole compounds such as miconazole or fluconazole are generally used for the treatment and prevention of systematic and superficial mycoses. The leakage of vital cell components and ions due to structural perturbation of the plasma membrane has been described as one of the effects of both types of antifungal drugs [5, 6]. In the case of AmB, this plasma membrane damage is a consequence of additional complex formation with fungal sterol molecules, the altered membrane permeability and ion leakage possibly involving the formation of aqueous pores in special experimental conditions consisting of an angulus of eight AmB molecules with the membrane sterols [7, 8]. The occurrence of strains resistant to both antibiotic families is very frequent, and in addition the possibility of serious side-effects of these antibiotics may arise, such as renal impairment [9, 10].

Primycin is a thermostable, absorptive, non-polyene macrolide lactone with a broad antimicrobial spectrum [11, 12]. In contrast with the above-mentioned antibiotics, its exact mode of action is not known so far. It is effective against human pathogen G-positive and G-negative bacteria, including polyresistant strains, and some yeasts and filamentous fungi. The primycin sulfate-containing Ebrimycin® gel has been successfully applied to prevent the bacterial infection of surface traumas and burned tissues. It is effective against various microbes present in destroyed skin, postoperative scabs and suppuration, bacterially infected trophic ulcers, necrotic-based open ulcers and superficial and deep suppuration [12–14]. The selective loss of alkali metal cations from primycin-treated erythrocytes was detected earlier [15]. The data suggested that the direct point of attack of primycin antibiotic is the plasma membrane. We have demonstrated an oleic acid–primycin

Abbreviations: AmB, amphotericin B; *C. albicans*, *Candida albicans*; 5-SASL, 5-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; EPR spectroscopy, electron paramagnetic resonance spectroscopy; G, Gauss; ST-EPR spectroscopy, saturation transfer electron paramagnetic resonance spectroscopy

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interaction *in vitro* [16] suggesting unsaturated fatty acids of the *C. albicans* plasma membrane as targets of primycin.

The high sensitivity of the spin-labeling electron paramagnetic resonance (EPR) spectroscopy allows analysis of the primycin–plasma membrane interaction. We utilized this technique to acquire information relating to the nature and extent of the primycin–plasma membrane interaction, using the ergosterol-producing strain *33erg*$^+$ of *C. albicans* (a clinical isolate) and its ergosterol-deficient plasma membrane mutant *erg*-2.

2. Materials and methods

2.1. Chemicals

A stock solution (5 mg ml^{-1}) of the spin probe 5-SASL [5-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid] was prepared in ethanol and stored at −18 °C until use. Primycin (MW = 1127.25, average mass) was provided by the manufacturer (PannonPharma Ltd., Pécsvárad, Hungary). The documentation indicated that, the content of active agent in the sample was 865.03 U mg^{-1}. Primycin consists of a mixture of more than 20 derivates; a detailed description of the components was reported by Virág et al. (2010). General structure of primycin and the functional groups at the terminal positions can be seen in Fig. 6 and Table 1. Primycin was dissolved in dimethyl sulphoxide and added to the cell suspensions in a final concentration of 1% in each case. All other chemicals were commercial products of analytical grade from Sigma-Aldrich Ltd.

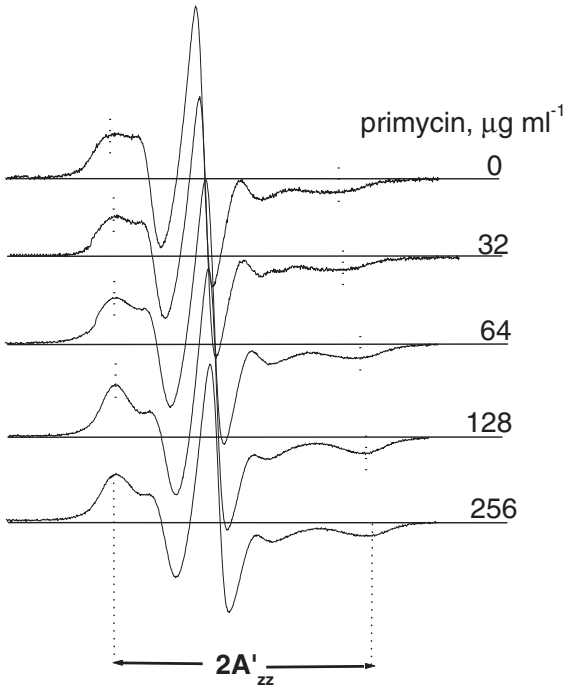

Fig. 2. Conventional EPR-spectra of 5-SASL. The concentration-dependence of the EPR spectra of 5-SASL incorporated in the plasma membrane of primycin-treated (0–256 μg ml^{-1}) strain *33erg*$^+$ of *C. albicans* at 20 °C is demonstrated. The spectral parameter $2A'_{zz}$ was defined as the distance of the low-field maximum and the crossover point of the 5-SASL signal. The field scan was 100 G.

2.2. Strains and culturing conditions

Two eukaryotic model organisms were investigated: an adenine-requiring ergosterol-producing *C. albicans 33erg*$^+$ strain (ATCC 44829) and its ergosterol-deficient mutant *erg-2* strain (ATCC 44831) [5,28]. The strains were cultured in YPD liquid medium (yeast extract 1%, peptone 2%, glucose 2% and 50 μg ml^{-1} adenine at pH 6.5) or maintained on YPD medium supplemented with 2% agar. Mid-exponential phase cultures were used. The growth inhibitory effect of primycin was measured in liquid YPD cultures for 48 h at 30 °C on a shaker operating at 150 rpm. The starting cell number was 10^6 cells ml^{-1}; cell numbers were determined spectrophotometrically (a Spectronic® Genesys^{TM}2 instrument) via the optical density at 595 nm. The viability of parental strain was analyzed by streaking stationer-phase cells on primycin containing minimal media (glucose 1%, agar 2%, $(NH_4)_2SO_4$ 0.5%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, vitamin solution 0.1%, 50 μg ml^{-1} adenine at pH 5.6).

2.3. Investigation of the viability and growth inhibition by primycin and the primycin–ergosterol interaction

The viability of strain *33erg*$^+$ was investigated by determining the number of colonies that rose after plating 500 cells on minimal media contained 0, 150, 175, 200, 225 μl ml^{-1} primycin. Incubation of plates carried out at 30 °C and the colonies were counted and controlled day by day for 1 week. Growth inhibition by primycin was measured at 30 °C in shaken liquid media containing 0, 16, 24, 32, 64 or 128 μg ml^{-1} primycin, with initially 1×10^6 cells ml^{-1} Cells

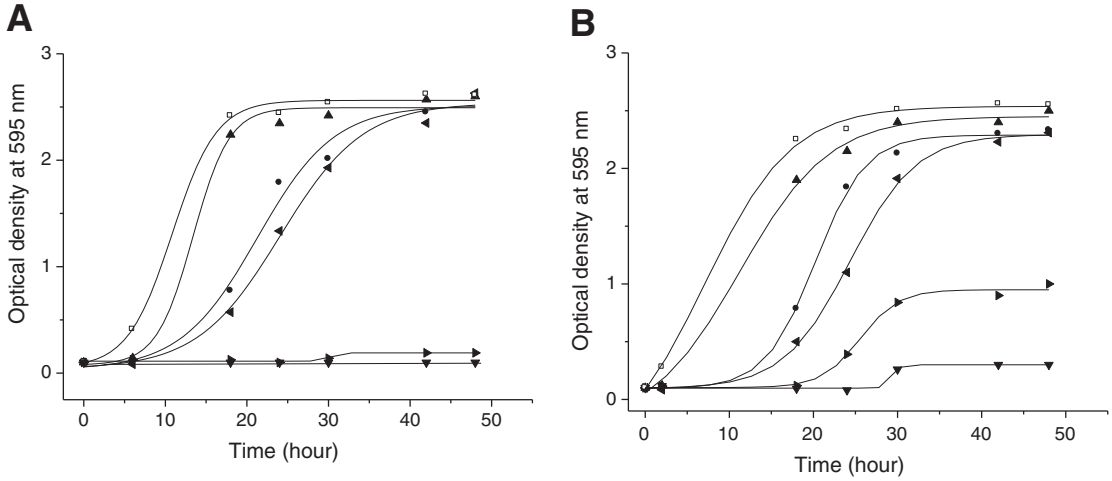

Fig. 1. Inhibition by primycin of the growth of the parental strain *33erg*$^+$ (A) and its plasma membrane mutant *erg-2* (B) of *C. albicans*. After cultivation for 40 h, the delayed growth curves achieved the normal stationary phase at slower primycin concentrations, whereas the multiplication of the cells of strains *33erg*$^+$ and *erg*-2 was blocked completely by 64 μg ml^{-1} and 128 μg ml^{-1} primycin, respectively. Primycin concentrations (μg ml^{-1}): control (□), 16 (▲), 24 (●), 32 (◄), 64 (►), 128 (▼).