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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.

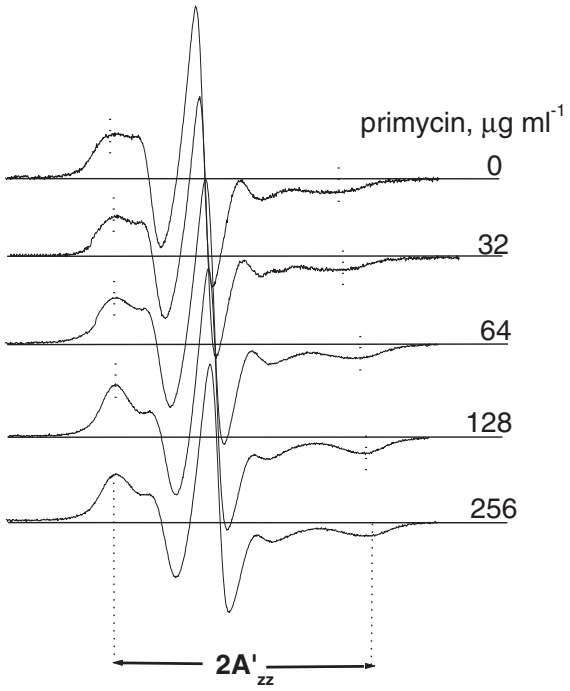

Fig. 2. Conventional EPR-spectra of 5-SASL. The concentration-dependence of the EPR spectra of 5-SASL incorporated in the plasma membrane of primycin-treated (0–256 μg ml^{-1}) strain *33erg*$^+$ of *C. albicans* at 20 °C is demonstrated. The spectral parameter $2A'_{zz}$ was defined as the distance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

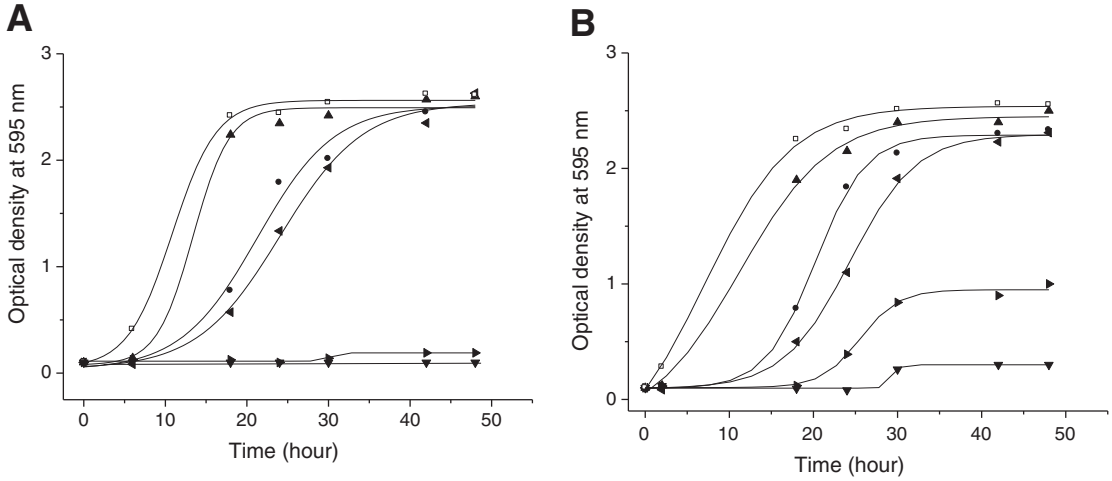

Fig. 1. Inhibition by primycin of the growth of the parental strain *33erg*$^+$ (A) and its plasma membrane mutant *erg-2* (B) of *C. albicans*. After cultivation for 40 h, the delayed growth curves achieved the normal stationary phase at slower primycin concentrations, whereas the multiplication of 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 (▼).

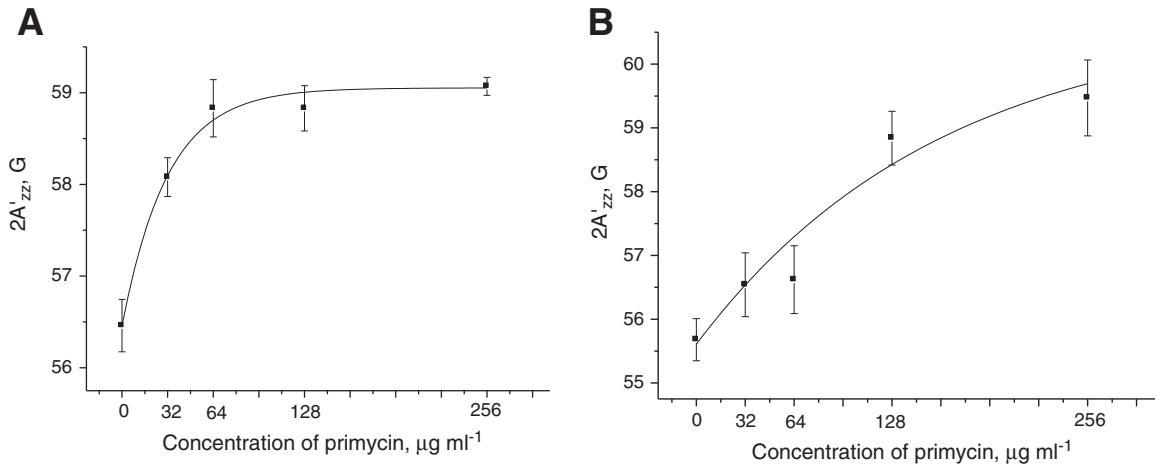

Fig. 3. Primycin concentration dependence of $2A'_{zz}$. Plots of $2A'_{zz}$ at 20 °C as a function of incremental primycin concentration for strain *33erg*$^{+}$ (A) and its mutant *erg-2* (B) of *C. albicans* are shown. Data are the means ± SD of three independent experiments.

multiplication was followed spectrophotometrically at 595 nm. The primycin–ergosterol interaction was measured as described by Virág et al. (2010).

2.4. Preparation of samples for spin labeling

Mid-exponential phase cells were collected by centrifugation (1071 *g* for 3 min) and washed twice with physiological salt solution. For protoplast formation, the suspension was incubated for 90 min with 2% lyophilized snail enzyme prepared from *Helix pomatia* and 0.2% lysing enzyme from *Trichoderma harzianum* in 0.6 M KCl as osmotic stabilizer at 37 °C. In order to perform the spin labeling of plasma membrane, cell-wall-free protoplasts were used. After incubation, the protoplasts were washed twice in stabilizer solution and diluted in 0.6 M KCl before the addition of spin probes [17]. Before spin-labeling, the protoplasts were treated with 32, 64, 128 or 256 μg ml^{-1} primycin for 15 min. Suspensions were washed twice by centrifugation at 3000 rpm for 5 min. A 3 μl aliquot of 5-SASL was added to a 500 μl of suspension containing 1×10^{8} protoplasts. The suspension was gently shaken for 3 min at room temperature to facilitate spin probe incorporation. The protoplasts were sedimented and resuspended in 100 μl of 0.6 M KCl. The pH was 5.4 in each experiment. The suspension was placed in a 100 μl capillary tube and centrifuged again at 3000 rpm at 4 °C for 5 min. The supernatant was removed. Under these conditions, no isotropic triplet arising from unincorporated spin probes was detected [18].

2.5. EPR measurements

2.5.1. Conventional EPR measurements

EPR spectra were recorded with an ESP 300E spectrometer (Bruker BioSpin, Germany) equipped with a diTC 2007 temperature regulator. The accuracy of temperature measurement was ± 0.2 °C. The EPR spectra of the membrane-incorporated fatty acid spin probe 5-SASL were registered in the temperature range 0 to 30 °C [17]. The conventional EPR spectra were obtained at a microwave power of 10 mW and a field modulation of 100 kHz with an amplitude of 2 G. The spectra were scaled to the same peak-to-peak amplitude or were normalized to an identical double integral. The spectra were characterized with the hyperfine splitting constant ($2A'_{zz}$), obtained from the conventional EPR spectra, as the distance between the outermost extremes (Fig. 2). The calculation was performed by a computer algorithm developed earlier, based on a non-linear least squares method for fitting [19-24]. Spectra were analyzed in terms of $2A'_{zz}$ against reciprocal absolute

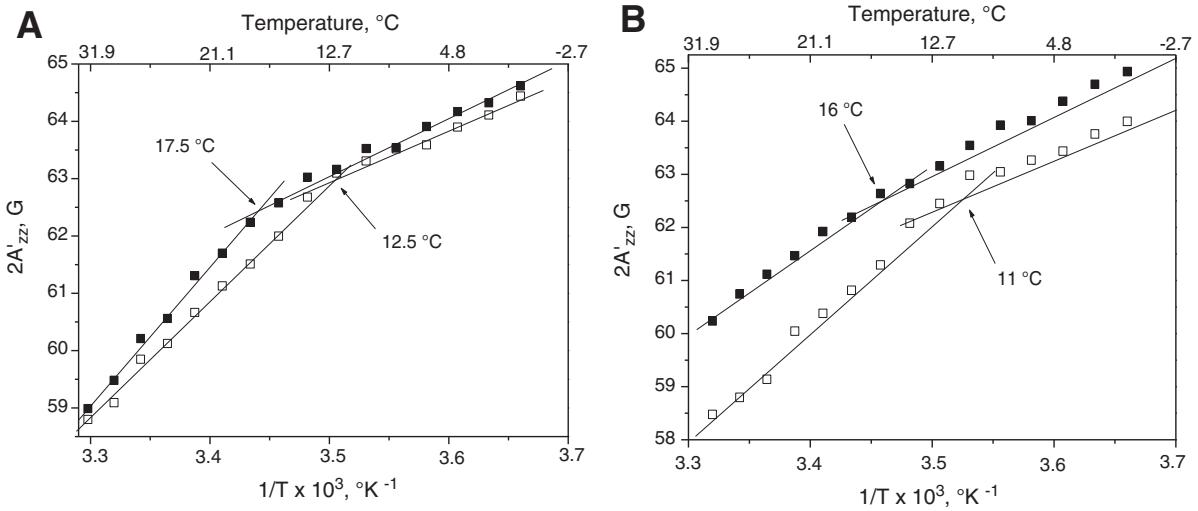

Fig. 4. Temperature dependence of $2A'_{zz}$. Plots of $2A'_{zz}$ of the 5-SASL spin-labeled protoplasts of strain *33erg*$^{+}$ (A) and its mutant *erg-2* (B) as a function of reciprocal temperature are shown. The phase transition breakpoints (°C) are indicated. Confidence interval of phase transition temperature: ± 1 °C. Symbols: control (□), treated with 128 μg ml^{-1} primycin (■).

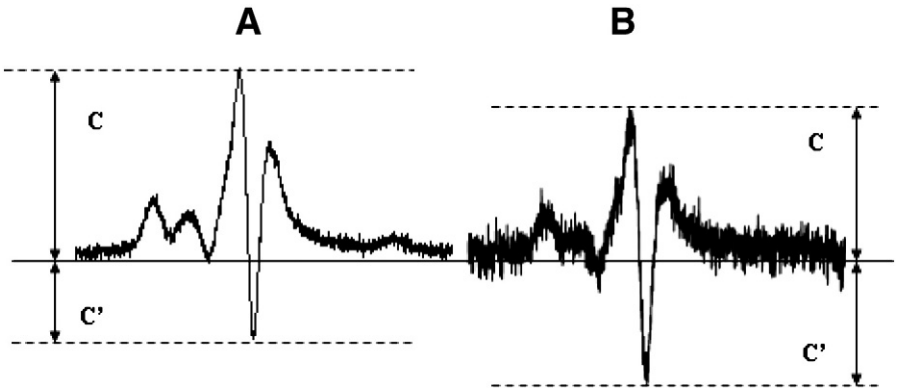

Fig. 5. ST-EPR spectra of 5-SASL. Representative ST-EPR spectra of the 5-SASL-labeled plasma membrane of the *C. albicans 33erg*$^+$ protoplasts treated with 128 μg ml^{-1} (A) or 32 μg ml^{-1} (B) of primycin at 20 °C are demonstrated. To calculate the rotational correlation time of the probe molecules, the ratio of the peaks C′/C was determined.

temperature ($1/T \times 10^3$, °K^{-1}). The experimental data could not be satisfactorily fitted to a straight line. When an otherwise linear Arrhenius plot exhibits a break, it suggests the existence of a phase change [25]. The problem of finding the possible breakpoint of two straight lines requires the application of statistical methods [26]. A computer-assisted procedure was used to derive the breakpoint and to calculate the statistical significance of the difference between the slopes of the straight lines. For all samples, our fitting resulted in a better approximation to two straight lines. This supports the suggestion of a phase change in the membrane with increasing temperature.

2.5.2. Saturation transfer (ST)-EPR measurements

Second harmonic, 90° out-of-phase absorption spectra were recorded at a microwave power of 63 mW and a field modulation of 50 kHz, with an amplitude of 5 G, the signals being detected at 100 kHz out-of-phase. The microwave power of 63 mW corresponds to an average microwave field amplitude of 0.25 G in the central region of a standard cavity. The values were obtained by using the standard protocol of Fajer and Marsh (1982). The ratio of the central-field diagnostic peaks C′/C was used to obtain an estimate of the rotational correlation time τ_c in the range 10^{-7} to 10^{-3} s of the incorporated probe molecules [27].

3. Results and discussion

3.1. Primycin-induced growth inhibition and viability of strains

In all experiments, mid-exponential phase cultures of vegetative cells or protoplasts of the *C. albicans* AmB-sensitive parental strain *33erg*$^+$ and its AmB-resistant strain *erg*-2 were used. In contrast with the situation with AmB, neither lysis nor shrinking of primycin-treated protoplasts was observed (data not presented). The absence of ergosterol in the mutant *erg*-2 resulted in an increased accumulation of Δ^8 sterols, a decreased fatty acid chain length and a lower proportion of unsaturated fatty acids in the plasma membrane in comparison with the strain *33erg*$^+$ [5, 28]. The inhibition of the growth of the two strains at the various primycin concentrations in shaken liquid media is illustrated in Fig. 1A and B. A given primycin concentration exerted significantly less inhibition on the mutant *erg*-2 than on its parental strain *33erg*$^+$. The dynamics of cell multiplication of both strains was slowed by primycin in processes that proved to be concentration-dependent. After cultivation for 40 h, the delayed growth curves achieved the normal stationary phase at primycin concentrations of 16, 24 and 32 μg ml^{-1}, 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. No resistant mutants of human pathogenic *Candida* spp. or clinical isolates of *C. albicans* have been found [14, 29].

The viability data reports about the retardation effect of primycin on the cell multiplication, however the viability of survival colonies remains the same during 1 week. Data reflects a concentration dependent adaptation to primycin at a very low ratio (Table 2).

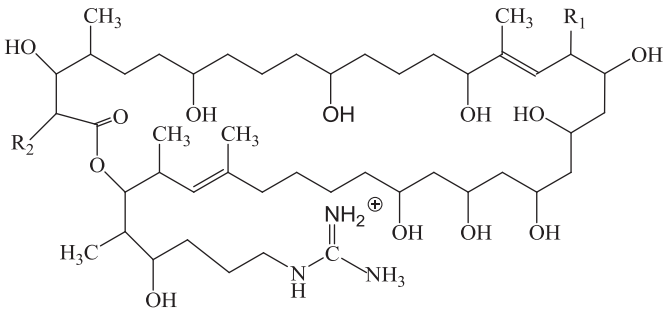

Fig. 6. General structure of primycin. The possible combinations of R1 and R2 terminal functional groups can be seen in Table 1.

3.2. Interaction of primycin with plasma membrane: conventional EPR measurements

The conventional EPR and the ST-EPR techniques cover the whole range of interesting molecular motions in lipid and membrane systems [27]. For the EPR measurements a spin label, the 5-SASL molecule, was employed to monitor the near-surface hydrophobic region of the plasma membrane. The hyperfine splitting constant $2A'_{zz}$ is one of the parameters most commonly used to evaluate changes in membranes; it can be derived from the EPR spectra. From the $2A'_{zz}$ values, the correlation time of the probe molecules can be estimated in the ns order of magnitude. It characterizes the tumbling motion of the incorporated molecules, and thereby reflects the "fluidity" of the analyzed region. In our experiments, the increase in $2A'_{zz}$ as a function of increasing primycin concentration indicated that the probe mobility was decreasing. The concentration-dependence of the hyperfine splitting constants at 20 °C is demonstrated in Fig. 2. The plots of $2A'_{zz}$ for strains *33erg*$^+$ and *erg-2*

Table 1
The functional groups at the terminal positions of primycin.

	A1	A2	A3	B1	B2	B3	C1	C2	C3
R1	a-D-arabinofuranosyl			H	H	H	OH	OH	OH
R2	butyl	pentyl	hexyl	butyl	pentyl	hexyl	butyl	pentyl	hexyl

The general structure of primycin can be seen in Fig. 6.

Table 2
Effectiveness of primycin on the viability of strain *33erg*$^+$.

Days	Control	225 μg ml^{-1}	200 μg ml^{-1}	175 μg ml^{-1}	150 μg ml^{-1}
1	448	0	0	2	0
2	453	0	1	2	0
3	453	0	1	2	110
4	453	0	1	26	118
5	453	0	1	66	118
6	453	1	2	72	127
7	453	2	3	72	127

Number of colonies that rose after streaking 500 cells on primycin containing media.

as a function of primycin concentration are given in Fig. 3A and B. The data are the means of the results of three independent measurements, with the standard deviations. Differences were detected in the angular offset of the curves in the exponential phase for the two strains. Saturation of the curves of *33erg*$^+$ and *erg-2* started at primycin concentrations of 64 μg ml^{-1} and 256 μg ml^{-1}, respectively, at which concentrations the growth of these strains was fully blocked (Fig. 3A and B). The increase in $2A'_{zz}$ indicates that the mobility of the incorporated spin label molecules decreased in the monitored region of the plasma membrane. This "rigidizing" effect of the primycin proved to be concentration-dependent. The plot of $2A'_{zz}$ against reciprocal temperature exhibited a non-linear dependence; accordingly, a well-defined phase-transition temperature could be deduced (Fig. 4). The phase-transition breakpoints of the untreated protoplasts of *33erg*$^+$ and *erg*-2 were at 12.5 °C and 11 °C, respectively. Treatment of the protoplasts with 128 μg ml^{-1} primycin resulted in significant increases of the breakpoints of *33erg*$^+$ and *erg-2*, from 12.5 °C and 11 °C to 17.5 °C and 16 °C, respectively (Fig. 4A and B). These results showed that primycin molecules are able to interact with the components of the plasma membrane, and thereby to decrease the flexibility of the lipid chains. Increased membrane rigidity was experienced in both strains, but the phenomenon was more pronounced for the mutant *erg-2*. It is noteworthy that, up to 28 °C, the distance between hyperfine splitting extremes could be estimated without any difficulty with changing temperature. At higher temperature, the intensities of both the inner and the outer hyperfine splitting constants were no longer resolved, and the spectra tended to become isotropic three-line EPR spectra. The measured differences in the plasma membrane parameters of the two strains might be consequences of the adaptation induced by the altered sterol composition. The strain *33erg*$^+$ accumulates ergosterol (71.5%) and ergosta-7-en-3β-ol (14.3%), while the mutant *erg-2* membrane contains fecosterol (55.6%) and zymosterol (42.7%) as the main sterols, indicating a block of Δ8-Δ7 isomerase [28]. The ergosterol level in the plasma membrane of yeast influences the membrane dynamics. For example, higher ergosterol levels led to a higher order parameter in the EPR spectra when the 5-SASL spin probe was used [30], indicating a more ordered structure of the membrane [31]. The absence of ergosterol in *erg-2* suggested that ergosterol is not the main target of primycin [28]. In contrast to AmB, no detectable interaction was measured between ergosterol and primycin using the methods described earlier for the primycin-fatty acid interaction (data not presented; [16]). In comparison with *33erg*$^+$, *erg-2* contained lower amounts of phosphatidylcholine and phosphatidylserine (described as fluidizing lipids) and higher amounts of phosphatidylinositol, phosphatidylethanolamine and phosphatidic acid (considered to be rigidifying lipids) in the membrane [28, 32]. However, the mutant *erg-2* overcompensated the absence of ergosterol, since the order parameter measured by EPR for the total lipid extracts detected in lipid-water dispersions proved to be higher for *erg-2* than for *33erg*$^+$ (0.640 ± 0.005 vs. 0.611 ± 0.003) as a consequence of the altered lipid composition of the mutant *erg-2* [28]. The above data allowed the conclusion that the target molecules of primycin are located in the cell membrane.

3.3. Interaction of primycin with plasma membrane: ST-EPR measurements

The ST-EPR spectra were analyzed in order to characterize the effects of primycin treatment on the very slow molecular motions in the phospholipid bilayer. The ST-EPR technique has proved very useful for the determination of rotational correlation times (τ_c) between 10^{-7} and 10^{-3} s in physical and biological systems [22, 27, 33, 34]. In the ST-EPR spectra of 5-SASL, the ratio C′/C was analyzed at 20 °C so as to estimate the value of τ_c [35]. Representative ST-EPR spectra of the plasma membrane are to be seen in Fig. 5. The estimated τ_c values of the lipid motions in the plasma membrane of strains *33erg*$^+$ and *erg-2* are presented in Table 3. The slower correlation time indicates that the probe mobility decreased after the primycin treatment, as a consequence of the primycin–lipid interactions in the investigated region of the plasma membrane. The correlation time of the incorporated 5-SASL molecule exhibited a non-linear dependence above a primycin concentration of 64 μg ml^{-1}. At higher primycin concentrations, τ_c revealed significantly less retardation, indicating saturation of the bulk of the primycin molecules in the plasma membrane.

Determination of the ST-EPR spectra allowed calculation of the probe molecule mobility in the order of magnitude of 10^{-6} s [35]. As a result of primycin treatment, the anisotropic rotation of the spin label was changed. In the control plasma membrane, ST-EPR spectroscopy indicated angular motion ($\tau_c \approx 10^{-6}$–10^{-8} s) and long axis rotation ($\tau_c \approx 10^{-8}$–10^{-9} s) of the spin label. Primycin addition eliminated this motion and the molecule displayed much slower motion. The data demonstrated the interesting phenomenon that the correlation time for the liquid crystalline phase at room temperature was of the order of magnitude of 10^{-9} s, as compared with 10^{-5} s for the gel phase of the investigated plasma membrane systems. The retardation of τ_c by approximately 1–1.5 orders of magnitude appears to indicate the disorganization of the structure of the plasma membrane. The fatty acid composition plays an important role in the membrane fluidization: a plasma membrane containing a higher level of unsaturated fatty acids has a lower fluidity [36]. Thus the differences in sensitivity of the two strains to primycin might be based on their significantly different compositions of polyunsaturated fatty acids 16:2, 16:3 and 18:3 in the plasma membrane in the strains *33erg*$^+$ and *erg-2* (0.9 *vs*. 1.7, 2.2 *vs* 3.7,. 2.3 and *vs* 4.2%, respectively) [28]. The interaction of oleic acid (comprising 35% of the fatty acids in *C. albicans*) with primycin exhibited a complex character, based on the formation of molecular complexes stabilized by one or two hydrogen-bonds). In agreement with this, it was recently reported that the interaction energies associated with these sites for one and two hydrogen-bonds were −12.46 and −33.16 kJ mol^{-1}, respectively [16]. New spatial structures may be created by primycin-fatty acid complexes stabilized by one or two hydrogen-bonds, the plasma membrane integrity being destroyed in a primycin concentration-dependent manner, leading to the loss of its barrier function [29].

Table 3
Values of C′/C and rotational correlation time of the 5-SASL of strains *33erg*$^+$ and *erg-2* after treatment with various primycin concentrations (0–256 μg ml^{-1}).

Primycin concentration (μg ml^{-1})					
Strains	0	32	64	128	256
Ratio of the peaks, C′/C					
33erg$^+$	−1.30 (±0,1)	−0.64 (±0,03)	−0.23 (±0,03)	−0.17 (±0,01)	0.14 (±0,01)
erg-2	−0.91 (±0,03)	−0.7 (±0,02)	−0.5 (±0,02)	−0.6 (±0,03)	−0.6 (±0,04)
Rotational correlation time, τ_c 10^{-6} *s*					
33erg$^+$	0.06	0.5	3	4	8
erg-2	0.04	0.5	1	1	1

Data of C′/C are the means ± SD of three independent experiments.

4. Conclusions

4.1. Conclusion of EPR measurements

Primycin is a water-insoluble and, lipid-soluble compound. Due to these features primycin was able to get into the plasma membrane. In the course of treatment the residual primycin molecules localized in the extracellular space were cleared away by washing. It has been proved that a part of the amount of primycin molecules integrated in the plasma membrane has formed stable bounds with certain membrane component compounds, likely with the fatty acid molecules [16]. Furthermore we presumed that a certain amount of primycin molecules was fixated in the plasma membrane without creating chemical bounds with membrane components and did not left into the aqueous phase during the washing.

The following processes in the plasma membrane were taking place influenced by primycin treatment: During the conventional EPR measurements the characteristic spectra were of the spin-labeled lipids received, giving information about the movement of spin label with magnitude of ns correlation time that was ordered to an anisotropic movement (the $2A'_{zz}$ values were between 50 and 60 G). This motion suggested that in the plasma membrane a phase, where the label molecule had a free rotational diffusion existed (presumably it was a layer closer to the cell surface). A saturation curve of $2A'_{zz}$ values was detected caused by enhancement of primycin concentration so the correlation time of the membrane probe decreased for a while, and then it was jointed a constant value, referring to a flexibility developed in his phase of the membrane that was not become reduced further by addition of many great primycin molecules.

The ST-EPR signal detected in the sample suggested that in the plasma membrane another phase, where the correlation time of the probe moved in microsecond scale, exists. This phase was in a region where the bound with certain membrane compounds could be created by primycin in the lipid bilayer (probably in the fatty acid region). If the number of entered primycin molecules into the membrane-system was increased, simultaneously the number of primycin molecules being able to form chemical bounds also was enhanced, until free binding sites for them was findable. The saturation was detected also in this phase after a certain concentration. Presumably the primycin added in "excess" was penetrated into the deeper region of plasma membrane and passed into the cell. (Since the applied spin label provided information about the hydrophobic region of lipid bilayer near surface, the effect evolved in the deeper region was not detectable.)

The phase transition temperature, investigated at a selected, intermediate concentration, showed that the plasma membrane switched off the gel phase to the fluid phase at higher temperature, meaning the membrane was stiffened by primycin that phenomenon was related to the above-mentioned chemical bonds formation of primycin and membrane compounds.

Striking difference were between the saturation curves of parental and mutant strains due to different membrane structure. The saturation of the "membrane phase of nanosecond" was found at higher concentrations by the mutant strain indicating that more primycin specific compounds was contained in the membrane at the "phase of microsecond". Previous literature data reports about that, the membrane of *erg*-2 strain contained more polyunsaturated fatty acids that were the assumed primycin binding compounds.

4.2. Conclusion of the mode of action of the antibiotic primycin

The direct interactions of the antibiotic primycin with the plasma membrane of the pathogenic yeast *C. albicans* strain *33erg*$^{+}$ and its plasma membrane mutant strain *erg-2* (with a different membrane composition) have been demonstrated for the first time. These interactions involve concentration-dependent complex "rigidizing" processes as primycin treatment (i) increases the phase transition temperatures of both 5-SASL-labeled strains (this study), and (ii) decreases the rotational correlation times measured by ST-EPR. The absence of ergosterol–primycin interactions and the existence of fatty acid–primycin interactions [this study; 16] strongly suggested that steric alterations in the interacting molecules induced the earlier-mentioned membrane perturbations rather than formation of pores or channels as suggested in the case of AmB [8]. This assumption may explain the fact that primycin interacts with all types of microbial and animal cells [37].

The presented experimental items reflect a structural change in the plasma membrane that investigation was designed knowing preliminary studies on the action of primycin. The efflux of compounds absorbing at 260 nm (nucleotides, nucleosides and free bases), appearing to originate in the free intracellular pool of cells was recently proved [29] and selective increases in the permeability of erythrocytes to alkali cations according to the sequence: $Cs^{+} > Rb^{+} = K^{+} \gg Na^{+}$, and leakage of Mg^{2+} were described [37, 38]. Furthermore the other fallowing effects on membrane-associated processes were defined as action of primycin in bacteria and host cells: an increasing conductance derived by selective efflux of ions was measured in *Bacillus subtilis*, red-blood cells and mitochondria [39, 40] In the case of neuromuscular neurons an increasing efflux of acetylcholine was demonstrated, however there were a lacking data about the effect of primycin directly on the ion channels [15, 37, 40–42]. Studies of *Escherichia coli* reported about changes in the signal transduction system such as an enhanced effect of primycin on activity of tyrosine aminotransferase and blocking effect on RNA polymerase I. Primycin had inhibitory effect on tryptophan pyrrolase and it stimulated the activity of tyrosine aminotransferase in the liver cells of rats [11, 12, 43]. It should be mentioned that the presented primycin-induced enzymatic changes could come into existence as a pleiotropic physical effect via perturbation of plasma membrane. Interaction of primycin molecules with compounds of plasma membrane leads to the structural alteration of membrane phases that consequence may be the destruction of signal transduction systems and loss of function of membrane proteins [44] - such as ion channels — rather than pore formation feature of primycin in plasma membrane.

The pharmaceutical significance of our results is based on the certification of non-intracellular target of the drug with low potential genetic mutation of pathogenic species. Hence application of primycin in diseases associated opportunistic infections will have a big advantage in the future because the adaptation to the drug showed a very low ratio and no resistant strains was described.

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