

ORIGINAL ARTICLE

Antifungal activity of the primycin complex and its main components A1, A2 and C1 on a *Candida albicans* clinical isolate, and their effects on the dynamic plasma membrane changes

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The *in vitro* antifungal activities of the macrolide lactone antibiotic complex primycin (PC) and its main components, A1 (50%), A2 (7.3%) and C1 (13%), against the opportunistic pathogenic fungus *Candida albicans* 33erg⁺ were determined by microdilution testing. The MIC₁₀₀ (the minimal concentration required for 100% growth inhibition) values found, A2 (2 µg ml⁻¹), PC (32 µg ml⁻¹), A1 (32 µg ml⁻¹) and C1 (64 µg ml⁻¹), suggested that the biological activity of PC is highly dependent on the proportions of its constituents. *In vivo* measurements of the biophysical properties of plasma membranes were carried out by electron paramagnetic resonance (EPR) spectroscopic methods, using the spin probe 5-(4,4-dimethylloxazolidine-*N*-oxyl)stearic acid. Conventional EPR measurements demonstrated altered phase transition temperatures (T_m) of the plasma membrane of strain 33erg⁺ as a consequence of treatment with PC or its constituents: for cells treated with 128 µg ml⁻¹ PC, A1, A2 or C1 for 15 min, T_m was 17, 21, 14.5 and 15 °C, respectively; that is significantly higher than the T_m of untreated cells, 12 °C. The molecular motions of the near-surface hydrophobic region of the plasma membrane, estimated by saturation transfer EPR spectroscopy, reflected changes in the membrane phases after the treatment. Two physiological membrane phases were detected in control samples: liquid-ordered and liquid-disordered, characterized by molecular movements ~10⁻⁶–10⁻⁸ s and ≥10⁻⁹ s. The cells treated with the investigated compounds showed the strong presence of a non-physiological gel phase additional to the above phases, characterized by movements ≤10⁻⁵ s.

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INTRODUCTION

The incidence of human fungal infections caused by the known 13 pathogenic *Candida* species has increased dramatically over the past two decades, among them, *C. albicans* being responsible for 45–60% of the high rate of mortality.¹ These yeast species are diploid without sexual and parasexual cell cycles. Dimorphism in the cell morphology is one of the pathogenicity factors: hyphae and pseudohyphae cell types can form from the vegetative blastospore cells as a consequence of altered environmental factors.² In most cases of fungal infections after antifungal treatment, resistant strains can be detected with high frequency.³ However, resistance to the broad-spectrum antibiotic primycin (PC) is still not found under hospital and laboratory conditions. PC is effective against Gram-positive, Gram-negative bacteria and all investigated fungi in the concentration ranges of 0.02, 10 and 5 µg ml⁻¹, respectively, including multiresistant human pathogenic bacterial strains.^{4–6} PC has been reported to increased permeability of erythrocytes as concerns alkali metal cations and the

leakage of Mg²⁺.⁷ It has been applied effectively in superficial infections and burn injuries as the active agent of *Ebrimycin* gel.^{8–10} Synergistic and additive effects of PC with various statins against human pathogenic yeasts and mould species were recently proved *in vitro*.¹¹ In our previous work, the mode of action of PC was studied. Investigations of the plasma membrane of living cells of the ergosterol-producing strain *C. albicans* 33erg⁺ and its ergosterol-deficient mutant *erg-2* have been reported. An increased phase-transition temperature (T_m) of the plasma membrane was detected by electron paramagnetic resonance (EPR) measurements for both strains. The T_m values of untreated 33erg⁺ and *erg-2* cells, 12.5 °C and 11 °C, respectively increased to 17.5 °C and 16 °C on treatment with 128 µg ml⁻¹ PC for 15 min. Saturation transfer (ST) EPR measurements demonstrated that the rotational correlation times of 60 ns and 100 ns of the spin label 5-SASL (5-(4,4-dimethylloxazolidine-*N*-oxyl)stearic acid) for the control samples of 33erg⁺ and *erg-2* gradually decreased on the addition of increasing

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PC concentrations, reaching 8 and 1 μs , respectively. The results indicated the plasma membrane 'rigidizing' effect of PC and its ability to undergo complex formation with membrane constituent molecules.¹² The interactions between PC and the main *C. albicans* plasma membrane components, such as oleic acid and ergosterol, were therefore investigated in *in vitro* fluorescence experiments. At room temperature, oleic acid–PC complex formation stabilized by single or double H-bonds with interaction energies of -12.46 and -33.16 kJ mol^{-1} was described.¹³ However, ergosterol–PC complex formation was detected only at elevated temperature ($>37^\circ\text{C}$), the interaction comprising an entropy-driven addition process involving the release of a water molecule.¹⁴ The biological consequences of PC treatment on *C. albicans* strains *33erg*⁺ and *erg-2* were: (i) an increased efflux (~ 62 – 76%) of the intracellular substances absorbing at 260 nm (nucleotides, nucleosides and free bases) after treatment for 2 h with $64 \mu\text{g ml}^{-1}$ PC in hypotonic phosphate buffer, suggesting a disturbance of the barrier function for both strains, while (ii) the scanning electron microscopy records indicated that the dimorphic *C. albicans 33erg*⁺ cells sustained a morphological change as a consequence of 32 or $64 \mu\text{g ml}^{-1}$ PC treatment, suggesting a modification in the regulation of the cell morphology. Some cells displayed deep depressions in the cell wall, whereas most were deformed and thread-like formations were visible among them. Instead of blastospores, the cells were mostly pseudohyphae after $64 \mu\text{g ml}^{-1}$ PC treatment.¹⁵

PC is a mixture of mainly three components in which the molecular skeleton is a lactone ring bearing different functional groups R1 and R2 (Figure 1). The components have been shown to exert synergistic activity in various combinations.¹⁶ PC is a water-insoluble, lipid-soluble material, similar to the polyene antibiotics. As water solubility is important in medicine, it is necessary to consider the properties of each of the individual PC components. A knowledge of the biological and plasma membrane effects of the PC in comparison with those of the individual components would have an essential role in pharmaceutical development. The structure of an antimicrobial agent influences its biophysical and biological effects,

such as it has been proved in the case of semisynthetic derivatives of amphotericin B, which has a similar structure to that of PC.¹⁷

In this study, the effects of the main components of PC A1 (50%), A2 (7.3%) and C1 (13%), on the dynamical changes of plasma membrane of *C. albicans* strain *33erg*⁺ were investigated in comparison with the data on PC itself.

MATERIALS AND METHODS

Chemicals

PC (average MW = 1127.25) and the investigated components A1 (MW = 1137.55), A2 (MW = 1151.57) and C1 (MW = 1005.43) were dissolved in dimethyl sulfoxide. The general structure of PC and the terminal functional groups of the components are to be seen in Figure 1. Detailed description of the structure of PC was given in the study by Virág *et al.*¹³ PC and its partially purified components were provided by the manufacturer of *Ebrimycin* gel, PannonPharma Ltd., Pécsvárad, Hungary. The HPLC purities at 210 nm of the samples were as follows: The A1 sample contained 87.18% A1 and 7.77% C1. The A2 sample contained 67.66% A2 and 11.93% A1. The C1 sample contained 86.51% C1 and 5.28% A1. The compounds accounting for the remaining percentages of the samples were not identified. Stock solutions of the samples (A1: 66.8 mg ml^{-1} , A2: 71.3 mg ml^{-1} , C1: 67.3 mg ml^{-1}) were stored at $+4^\circ\text{C}$. A 5 mg ml^{-1} stock solution of the spin probe 5-SASL was prepared in ethanol and stored at -18°C until use. All other chemicals were commercial products of analytical grade from Sigma-Aldrich Ltd (Budapest, Hungary).

Model organism and culturing conditions

The adenine-requiring, ergosterol-producing eukaryotic yeast strain *C. albicans 33erg*⁺ (ATCC 44829, American Type Culture Collection, Rockville, MD, USA) was used in all experiments. The strain was cultured at 30°C in YPD liquid medium (1% yeast extract, 2% peptone, 2% glucose and $50 \mu\text{g ml}^{-1}$ adenine at pH 6.5) or maintained on YPD medium supplemented with 2% agar. For each experiment, mid-exponential phase 8-h cultures were used to ensure the same physiological state of the cells. For the EPR measurements, cell-wall-free protoplasts from vegetative cells were prepared according to the procedure described by Virág *et al.*¹²

Antifungal susceptibility testing with PC and its constituents

The *in vitro* antifungal activities of PC and its three partially purified constituents A1, A2 and C1 were determined against the strain *C. albicans 33erg*⁺ by using the broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines.¹⁸ MIC₁₀₀ (the minimal concentration required for 100% growth inhibition) and IC₅₀ (the concentration required for 50% growth inhibition) values were determined in 96-well flat-bottomed microtitre plates (Costar 3599, Corning, NY, USA) by measuring the optical densities of the yeast cultures at 620 nm. A series of two-fold dilutions was prepared from the drug stock solutions in the appropriate solvent to yield a solution that had 100 times the final concentration required for the tests in the broth microdilution assays. Each intermediate solution was further diluted in RPMI 1640 to twice the final required concentration. *C. albicans* cell inocula were prepared from 1-day-old cultures grown on adenine-complemented YPD agar slants. The cell suspension was diluted in adenine-complemented RPMI 1640, pH = 7.0. Aliquots of 100 μl of various concentrations of the tested materials diluted in RPMI 1640 were mixed with 100 μl of cell suspension in the microtitre plates. The final concentrations of PC and its investigated components in the wells ranged from 0.25 to 64 mg ml^{-1} . The initial cell concentration was $1 \times 10^3 \text{ cells ml}^{-1}$. The microplates were incubated for 72 h at 35°C , and the OD was measured at 620 nm with a microtitre plate reader (Jupiter HD; ASYS Hitech, Salzburg, Austria). The uninoculated medium was used as background for the spectrophotometric calibration; the growth control wells contained cell suspension in drug-free medium. For calculation of the extent of inhibition, the OD₆₂₀ of the drug-free control cultures was set at 100% growth in each case. The MIC₁₀₀ value was defined as the lowest concentration causing a

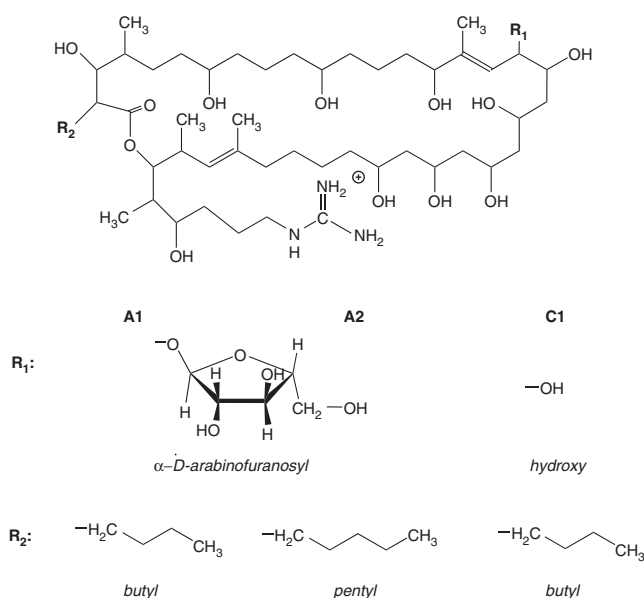


Figure 1 General structure of PC and terminal functional groups of investigated PC complex components.

100% decrease in turbidity as compared with the growth in the control well. All experiments were repeated three times.

EPR measurements

The preparation of samples for the spin labeling and, the conventional EPR and ST-EPR measurements were carried out under the same conditions and with the same settings as described by Virág et al. (2012).¹² Correlation times were obtained by using the calibrations of the ratios in the diagnostic regions of the low-field ratio L''/L and the central ratio C'/C of the ST-EPR spectra described by Horváth and Marsh.¹⁹ Representative conventional and ST-EPR spectra are to be seen in Figure 2. The rotation correlation times of the spin label from the spectral parameter $2A'_{zz}$ were calculated according to the Goldman equation^{20,21} applied in the *in vivo* membrane system of *C. albicans*.²²

RESULTS AND DISCUSSION

Sensitivities of *C. albicans* 33erg⁺ to PC and its constituents A1, A2 and C1

The sensitivities to PC of 13 human pathogenic *Candida* species and 74 *C. albicans* clinical isolates were earlier determined by an internationally accepted method: the MIC₁₀₀ ranges for the two groups were 4–64 and 16–32 µg ml⁻¹, respectively. Resistant strains were not found¹⁵ (see Introduction). The MIC₁₀₀ values for *C. albicans* strain 33erg⁺ with the same methods were: A2 (2 µg ml⁻¹), PC (32 µg ml⁻¹), A1 (32 µg ml⁻¹) and C1 (64 µg ml⁻¹), and IC₅₀ values were: A2 (0.5 µg ml⁻¹), PC (4 µg ml⁻¹), A1 (16 µg ml⁻¹) and C1 (32 µg ml⁻¹).

Both IC₅₀ and MIC₁₀₀ demonstrated well-detectable differences in biological activity (growth inhibition) between PC and its components (Table 1).

Effects of PC and its components on the fungal plasma membrane: an EPR study

The question arose: as to whether the antifungal efficacy of these compounds is associated with alterations in membrane-dynamic features such as T_m and molecular motions of the plasma membrane. Previous data indicated decreased fluorescence anisotropy in the TMA-DPH-labeled plasma membrane of strain 33erg⁺ after treatment with 128 µg ml⁻¹ PC for 15 min.¹⁵ EPR analyses of the 5-SASL-labeled plasma membrane of the same strain revealed an increased T_m and a longer rotational correlation time after the same PC treatment. The PC concentration dependence of these parameters was proved.¹² With an *erg-2* ergosterol-deficient mutant strain of ergosterol-producing strain 33erg⁺, the PC dependence of the biophysical

parameters of the plasma membrane composition was confirmed.¹² In the present study, the effects of PC and three of its components on the dynamics of the 5-SASL-labeled plasma membrane of strain 33erg⁺ were investigated at a concentration of 128 µg ml⁻¹ for 15 min.

Conventional EPR measurements

T_m, which depends strongly on the structure of the hydrophobic region of lipids,²³ was determined by conventional EPR measurements by plotting $2A'_{zz}$ against reciprocal temperature (Figure 3). For monitoring the near-surface hydrophobic region of the fungal plasma membrane, the 5-SASL molecule was employed. In Table 2, the data are compared with earlier findings¹² on PC. The conventional spectra of protoplasts treated with the PC components gave similar $2A'_{zz}$ values at 20 °C, confirming the presence of molecular motions in the ns range in the investigated region of the lipids (Table 2). However, significant differences in T_m were observed on the action of the individual components (Figure 3). Thus, the T_m for the untreated sample, 12 °C, increased on treatment with PC or its three main components: 21 °C (A1), 17 °C (PC), 15 °C (C1) and 14.5 °C (A2). These data indicate that A1 diminished the fluidity of the analyzed region of the plasma membrane most strongly. A2 and C1 had similar effects; and both seemed to less effective as 'rigidizing' agents than PC or A1. Considering the accuracy (± 0.03 mT) of the determination for $2A'_{zz}$, the \pm s.d. of the rotational correlation time was 0.045 ns. The calculated rotational correlation time for the untreated sample, 0.33 ns at 20 °C, increased to 0.34, 0.40, 0.44 and 0.52 ns on treatments with A2, C1, PC or A1, respectively (Table 3). Consequently, the treatment caused a decrease in membrane flexibility, which was most pronounced for A1.

Table 1 Growth inhibition exerted by PC, A1, A2 and C1 on *C. albicans* strain 33erg⁺, expressed in IC₅₀ and MIC₁₀₀

Material	IC ₅₀ (µg ml ⁻¹)	MIC ₁₀₀ (µg ml ⁻¹)
PC	4	32
A1	16	32
A2	0.5	2
C1	32	64

Abbreviation: PC, primycin. 50% (IC₅₀) and 100% (MIC₁₀₀) growth inhibitions were determined in the same 72-h cultures at 35 °C.

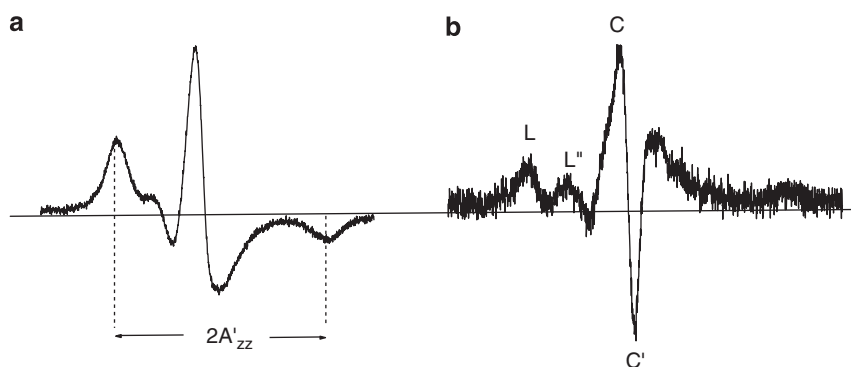


Figure 2 Representative conventional EPR and ST-spectra of 5-SASL-labelled plasma membrane of the *C. albicans* 33erg⁺ protoplasts. Symbols: conventional EPR spectra (a), ST-EPR spectra (b). In the EPR procedures, the hyperfine splitting constant ($2A'_{zz}$), the low-field ratio L''/L and the central-field ratio C'/C were determined.

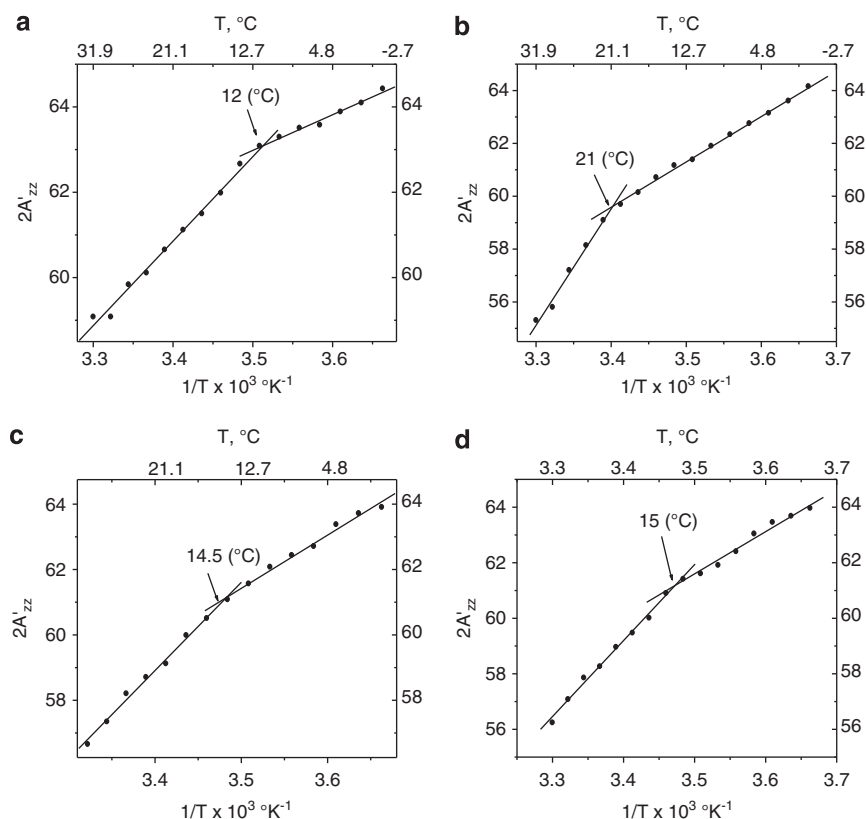


Figure 3 T_m of plasma membrane of strain *33erg*⁺. Symbols: control cells (a) and cells treated with PC components A1 (b), A2 (c) and C1 (d). Means of data of three independent experiments are plotted; confidence interval of $T_m \pm 1.5$ °C.

Table 2 Correlation times of the 5-SASL spin probe incorporated in the plasma membrane of control and PC, A1, A2 and C1-treated cells of *C. albicans* strain *33erg*⁺

	Compound of treatment				
	Untreated	PC	A1	A2	C1
<i>Phase-transition temperature</i>					
T_m (°C)	12.5	17.5	21	14.5	15
<i>Ratio of peaks</i>					
C'/C	-1.2 (± 0.05)	-0.17 (± 0.01)	-1.1 (± 0.01)	-0.7 (± 0.05)	-0.5 (± 0.05)
L''/L	0.4 (± 0.05)	0.9 (± 0.05)	0.4 (± 0.05)	1 (± 0.1)	0.9 (± 0.1)
<i>Correlation times</i>					
τ_c (s)	1×10^{-8}	4×10^{-6}	5×10^{-8}	5×10^{-7}	1×10^{-6}
τ_l (s)	8×10^{-6}	6×10^{-5}	1×10^{-5}	1×10^{-4}	8×10^{-5}

Abbreviations: PC, primycin; T_m , phase-transition temperature.

Data are calculated from the ST-EPR parameter ratios C'/C and L''/L at 20 °C. The correlation times of PC presented in the Table were from the study of Virág *et al.* (2012)¹². The correlation times τ_c were estimated via the ratios C'/C, and τ_l via L''/L. Data are the means of the results of three independent experiments \pm s.d.

ST-EPR measurements

The changes in the 'very slow' molecular motions after the treatment with the investigated compounds were analyzed by ST-EPR. Molecular motions of the phospholipid bilayer in the μ s range were estimated from the ratios of diagnostic peaks L''/L and C'/C at 20 °C.^{24,19} From the ratio L''/L in the ST-EPR spectra, the anisotropic angular motion (correlation times τ_l) of the spin label can be estimated to a good approximation²⁵ in view of the composition of

the *in vivo* membrane system analyzed in our study, this kind of movement was presumable. The approximate estimated values of τ_l in the investigated region of the plasma membrane were: A2 (1×10^{-4} s), C1 (8×10^{-5} s), PC (6×10^{-5} s), A1 (1×10^{-5} s) and untreated (8×10^{-6} s), reflecting the high degree of molecular blocking exerted by A2. The axial rotation movement (correlation times τ_c) of the spin probe estimated via the ratio C'/C revealed significant differences. The compounds blocked this anisotropic

Table 3 The detected phases with diagnostic correlation times in the plasma membrane of protoplasts of *C. albicans* 33erg⁺

Phases of plasma membrane with characteristic correlation times			
Compound of treatment	Gel (10 ⁻⁴ s)	Liquid-ordered (10 ⁻⁷ s)	Liquid-disordered (10 ⁻⁹ s)
Control	Not detected	0.1	0.33
PC	0.6	40	0.44
A1	0.1	0.5	0.52
A2	1	5	0.34
C1	0.8	10	0.40

For the comparison, the correlation times listed in Table 2 and the correlation times calculated from 2A_{zz} are presented in the Table.

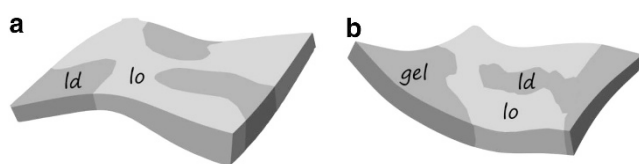


Figure 4 Concept of phase separation in the plasma membrane. Symbols: control membrane (a), PC-treated membrane (b), liquid-disordered (ld), liquid-ordered (lo) and gel (gel) phases. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

rotation in the sequence: PC (4×10^{-6} s), C₁ (1×10^{-6} s), A₂ (5×10^{-7} s), A₁ (5×10^{-7} s) and untreated (1×10^{-8} s) (Table 3). PC and its components decreased both estimated correlation times of the 5-SASL molecule in the near-surface hydrophobic region of the plasma membrane.

Altered phase separation of plasma membrane

The molecular motions estimated by conventional EPR and ST-EPR measurements clearly revealed two membrane phases in the plasma membrane of the untreated cells: the liquid-disordered (or fluid) phase displayed molecular motions at around 10^{-10} s, and the liquid-ordered phase exhibited motions in the range 10^{-6} – 10^{-8} s, reflecting the state of plasma membrane under physiological conditions. However, a third phase similar to the gel state was detected by ST-EPR in the PC, A1, A2 and C1-treated samples, with spin probe correlation times of 10^{-4} – 10^{-5} s, reflecting motions not present in the natural biological membranes.²⁶ These phenomena relate to the interactions of PC molecules with unsaturated fatty acids in the near-surface hydrophobic region of the lipid bilayer, leading to alterations in the lipid structures and phase separation in the membrane. The strongly ordered gel phase is presumably organized by saturated alkyl chains, the liquid-ordered phase is structured by phospholipids and sterols in interaction with PC molecules, and the liquid-disordered phase reflects the unperturbed plasma membrane regions (Figure 4). The detected phases with the diagnostic correlation times are compared in Table 3.

CONCLUSIONS

Treatment with PC or its three main components induced dynamic changes in the fungal plasma membrane affecting T_m, the molecular motion and the phase separation. The biological activities of the investigated compounds did not correlate with the plasma membrane flexibility.

The investigation of the three main components of PC facilitates the understanding of the mode of action of PC: (i) PC integrates directly into the plasma membrane;^{12,15} (ii) in the plasma membrane it interacts with the fatty acids and at elevated temperature with ergosterol, giving rise to complex formation;^{13,14} (iii) consequently, changes in biophysical parameters occur, for example an increase in T_m, rearrangement of the membrane phases and formation of the gel phase in the plasma membrane (Virág *et al.*,¹² this study); (iv) the rearrangement of the membrane phases leads to alterations in conformations of the membrane proteins (for example, ion-channels, transporters and signal transduction-participating proteins); (v) hence, there is a loss in barrier function of the plasma membrane and a change in the internal ion-milieu of the cells;¹⁵ (vi) in consequence, the cell morphology is changed;¹⁵ (vii) cell death occurs depending on the concentration of PC.^{12,15}

The pharmaceutical significance of this behavior of PC could be its possible application in the therapy of non-intracellular targets, preventing cell toxicity in the treated organism and the knowledge of the biological effects of individual components may be utilized in the pharmaceutical development of this antibiotic.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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