

ORIGINAL ARTICLE

Complex formation between primycin and ergosterol: entropy-driven initiation of modification of the fungal plasma membrane structure

Eszter Virág¹, Miklós Pesti¹ and Sándor Kunsági-Máté²

The interaction of the antibiotic primycin with the main fungal sterol, ergosterol, was investigated *in vitro* in order to monitor the effect of primycin on the fungal plasma membrane at the molecular level. The thermodynamic parameters of complex formation were determined by measuring Rayleigh scattering as a signal sensitive to particle size. The Benesi–Hildebrand method validated the 1 : 1 stoichiometry of the primycin–ergosterol complexes. A very low enthalpy change ($\Delta H = -1.14 \text{ kJ mol}^{-1}$) was measured during the complex formation, which itself cannot be responsible for the molecular association. However, the entropy production ($\Delta S = 29.78 \text{ J mol K}^{-1}$) observed during the complex formation can describe the molecular interaction. This effect is probably due to the partial destruction of the solvation shell of the interacting species before the interlinking of the molecules. The results highlight the importance of ergosterol as concerns the mode of effect of primycin in the treatment of fungal infections. As the entropy has a determinant role in the ergosterol–primycin interaction, this interaction exhibits a very high temperature dependence, with the important consequence that the effect exerted by primycin on the cell membranes increases with rising temperature, and the effect is therefore pronounced in febrile bodies.

The Journal of Antibiotics (2012) 65, 193–196; doi:10.1038/ja.2011.140; published online 25 January 2012

Keywords: antibiotic; ergosterol; interaction; membrane; primycin

INTRODUCTION

A knowledge of the point of attack and the mechanism of the action of an antimicrobial agent is an essential condition in approval processes and in the development of a drug or of its semi-synthetic variants. Primycin is a non-polyene macrolide lactone antibiotic complex that is strongly surface-active, thermostable and absorptive (Figure 1b); however, its exact mode of action is not yet known so far.^{1,2} It exhibits a broad antimicrobial spectrum and has been suggested to be a plasma membrane-destructive agent. It is effective against human pathogen gram-positive and gram-negative bacteria, including polyresistant strains, and some yeasts and filamentous fungi.^{3,4} For this reason, clarification of the mechanism of its action is an important challenge for pharmaceuticals. Primycin molecules have an amphiphilic structure that contain a lactone ring, a sugar component and a guanidine group.² It has been postulated that the dimeric form of primycin molecules is able to penetrate and form ion channels in an artificial lipid bilayer.⁵ The efflux of vital intracellular compounds, such as alkali metal cations, nucleotides, nucleosides and free bases has been described as a direct biological effect of primycin. Distortion of the shape of the *Candida albicans* cell has been demonstrated to be an indirect biological effect of the drug.^{5,6} The *in vitro* interaction of primycin with one of the main membrane components, oleic acid, was recently proved.⁷

The aim of our study was to investigate the interaction of primycin with ergosterol, the main sterol in the fungal plasma membrane (Figure 1a). The sterols are a particular class of membrane lipids with a polycyclic structure. The main sterol in the mammalian plasma membrane is cholesterol, whereas that in the fungal and protozoan membranes is ergosterol.⁸ Ergosterol comprises four fused cycles in *trans* configuration, a hydroxy group on C-3, C-7=C-8 and C-22=C-23 double bonds and an isononyl side-chain on C-17. The –OH group is responsible for the amphiphilic nature of ergosterol and consequently for its orientation in biological membranes.⁹ The shape of a membrane lipid depends on the effective area of its polar head group relative to the dimension of its hydrophobic moiety.^{10,11} Accordingly ergosterol has an essential role in yeast physiology, influencing membrane-associated processes such as stress tolerance or signal transduction.¹²

Detection of the ergosterol–primycin interaction in our study was based on the increase in particle size during complex formation. For this purpose, the molecular association was followed by means of Rayleigh scattering (RS). Quantum-chemical studies were applied to identify the bonds responsible for the ergosterol–primycin interaction. To characterize the molecular associations, the entropy and enthalpy changes are discussed in detail.

¹Department of General and Environmental Microbiology, Faculty of Science, University of Pécs, Pécs, Hungary and ²Department of General and Physical Chemistry, Faculty of Science, University of Pécs, Pécs, Hungary
Correspondence: Professor S Kunsági-Máté, Department of General and Physical Chemistry, University of Pécs, Ifjúság 6, Pécs H-7624, Hungary.
E-mail: kunsagi@gamma.ttk.pte.hu

Received 23 October 2011; revised 15 December 2011; accepted 26 December 2011; published online 25 January 2012

RESULTS AND DISCUSSION

Complex-forming ability of primycin

Ergosterol is the main sterol in the fungal plasma membrane. We earlier demonstrated that the clinical isolate strain 33ery⁺ (ATCC 44829) of the most important human pathogenic yeast, *C. albicans*, contains 71.5% of this compound in the plasma membrane.¹³ As the weak fluorescence of the unsaturated ring in ergosterol can be detected, the increase in particle size during complex formation was used to determine the weak molecular interactions. Figure 2 depicts the intensities of RS at 366 nm of mixtures of ergosterol with various concentrations of primycin at the vital temperature of *C. albicans*, 303.16 K. It is well known that RS depends on the sixth power of the molecular radius, and it is obvious that the concentration of scattering particles is halved after complexes with 1 : 1 stoichiometry are formed. In view of the similar sizes of the ergosterol and primycin molecules, and assuming that both the associated and separated molecules are spherical, the RS scattering of the dissociated (I_r') and associated (I_r'') can be formulated as follows:

$$I_r' \sim r^6 \cdot c \quad (1)$$

$$I_r'' \sim (2r)^6 \cdot \frac{c}{2} = 32 \cdot I_r' \quad (2)$$

where r is the molecular radius and c is the concentration of the scattering particles. It is clear that the intensity of the RS would be 32 times higher if all of the primycin and ergosterol molecules interacted to form complexes. However, both the volume and the shape of the interacting species differ in these two molecules. To refine the above idea, quantum-chemical calculations were performed to determine the molecular volumes, and ellipsoids were applied to represent the

scattering particles. For calculation of the molecular volumes, the electron densities of the molecules were calculated at the DFT/BLYP/6-311G level, and the QSAR properties were determined by using the HyperChem 7.5 code (HyperChem, HyperCube, 2007). The molecular volumes of ergosterol, primycin and their complexes were found to be 1083 Å³, 1211 Å³ and 2412 Å³, respectively. The shapes of the representative ellipsoids, described in terms their $a/b/c$ axes, were determined to be 5.65/6.12/7.5 (ergosterol), 6.12/6.51/7.27 (primycin) and 9.11/8.41/7.51 (complexes). These results reveal the quasi-spherical shapes of both the interacting species and the complexes. Considering the ellipsoidal shapes of the interacted species and that of the complexes the calculated RS in this case would be 30.8 times higher if all the particles formed complexes. The reasonably good agreement with the above spherical approximation validates the RS method for determination of the association constants. Detailed information on the calculations is to be found in previous publications.^{14,15}

In accordance with previous studies on RS and weak host–guest interactions,^{14–16} the increase observed in the photoluminescence (PL) signal reflects the increase in concentration of ergosterol–primycin complexes. To obtain quantitative data on the thermodynamics of the chemical equilibrium in the system, the equilibrium constant was determined by the Benesi–Hildebrand method, utilizing the five series of data associated with the five different concentrations of primycin (0.002–0.006 M) (Figure 3). A detailed description of the method was

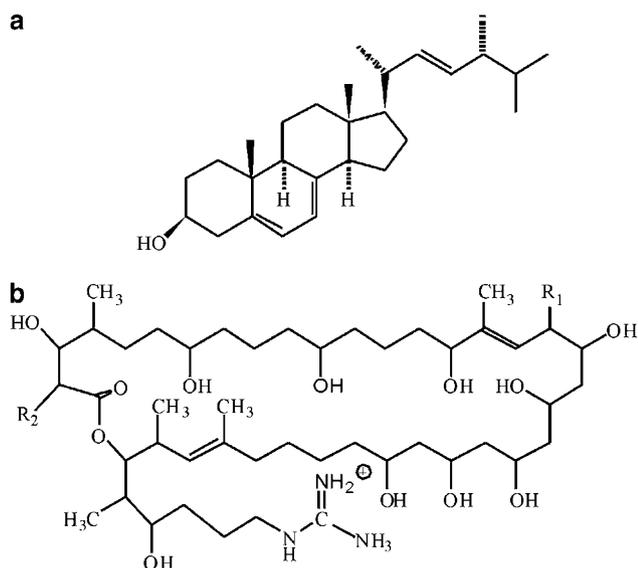


Figure 1 Structures of the compounds investigated in this work: ergosterol (a) and primycin (general structure) (b). The terminal functional groups of primycin are specified in Table 1.

Table 1 The functional groups at the terminal positions of primycin

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

The general structure of primycins has to be seen in Figure 1b.

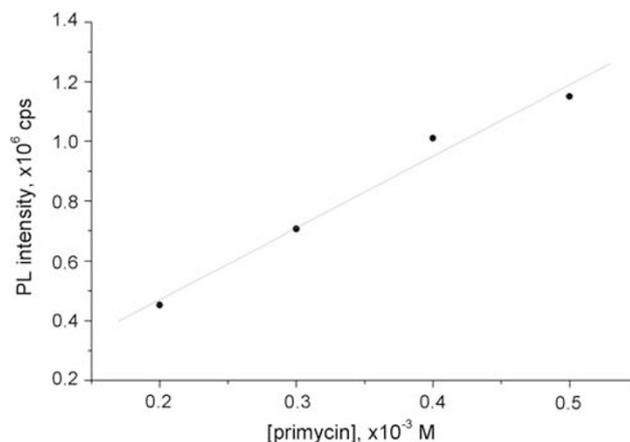


Figure 2 RS measured at 366 nm for 0.001 M ergosterol in the presence of different concentrations (0.002–0.005 M) of primycin at 303.16 K.

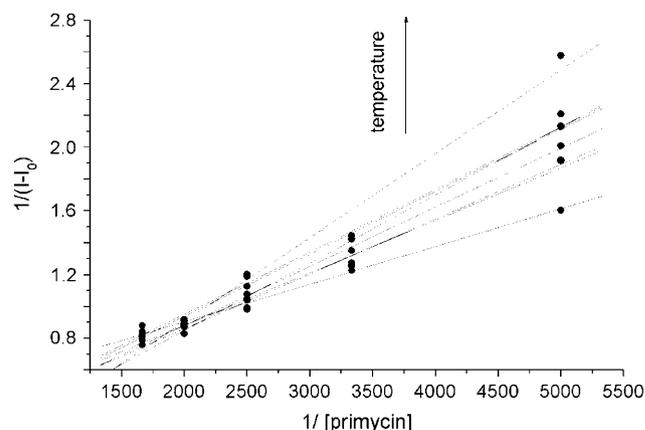


Figure 3 Benesi–Hildebrand plots of the PL signals of RS observed for the ergosterol–primycin complexes with 1 : 1 stoichiometry.

provided by Virág *et al.* (2010). Plots of the reciprocal RS change against the reciprocal primycin concentration were linear (Figure 3). The equilibrium constant could therefore be determined by using the following relationship:

$$\frac{1}{I - I_0} = \frac{1}{[e]} \cdot \frac{1}{[p]} \cdot \frac{1}{K} + \frac{1}{[e]} \quad (3)$$

where $[p]$ and $[e]$ are the analytical concentrations of primycin and ergosterol, respectively. I is the intensity of the samples containing both the reactants and I_0 is the intensity of ergosterol in the absence of primycin. Figure 3 shows the Benesi–Hildebrand plot for the PL RS at different temperatures.

The van't Hoff theory was used to determine the enthalpy and entropy changes associated with complex formation. As in the van't Hoff equation, the logarithm of the equilibrium constant was plotted against the reciprocal temperature (Figure 4):

$$\ln K = -\frac{\Delta H}{R} \cdot \frac{1}{T} + \frac{\Delta S}{R} \quad (4)$$

The enthalpy change can be determined from the slope, and the entropy change from the intercept of the line fitted to the experimental data. The results are summarized in Table 2.

The results revealed a very low enthalpy change during the formation of ergosterol–primycin complexes. At the same time, the entropy change was positive, and complex formation was therefore preferably based on entropy production. As a kinetic energy of about 4 kJ mol^{-1} can be assumed for the particles at around room temperature from the equipartition theorem, the enthalpy change itself cannot be responsible for the complex formation. Furthermore, the positive entropy change reflects the increased disorder of the system after the complexes are formed. This property cannot be associated with the increased freedom of the interacting species as the flexibility of the molecular skeleton of both interacting molecules is reduced in the complex relative to that before the association reaction. During the

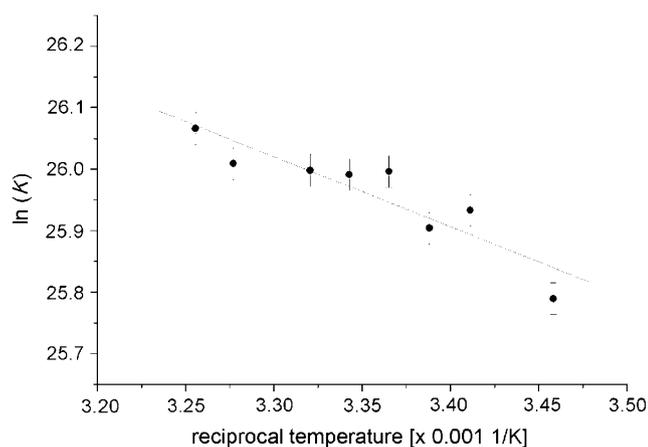


Figure 4 Van't Hoff plot of the equilibrium constants K determined by the Benesi–Hildebrand method. Thermodynamic parameters were determined from the slope and the intercept of the fitted line according to equation (4).

Table 2 Thermodynamic parameters calculated by applying the Van't Hoff theory to the reaction of the ergosterol–primycin association

ΔH (kJ mol ⁻¹)	-1.14 ± 0.22
ΔS (Jmol K ⁻¹)	29.78 ± 0.58
ΔG (30 °C) (kJ mol ⁻¹)	-10.17 ± 0.32

interaction of primycin with ergosterol, however, the association assumes at least partial destruction of the solvation shell a process that results in an increased number of free solvent molecules. This effect is similar to the well-known hydrophobic effect in the case of biological macromolecules, where the interaction is based on the releasing of water molecules from the surfaces of the interacting molecules, which are in contact during the interaction (Figure 5).

To investigate the mode of action of primycin in the plasma membrane, the minimal inhibitory concentrations on an ergosterol-producing and an ergosterol-deficient mutant of *C. albicans* were determined.⁶ The ergosterol-producing strain proved to be more sensitive than the mutant, the results support the occurrence of ergosterol–primycin complex formation *in vivo*. The interaction of oleic acid (accounting 35% of the fatty acids in *C. albicans*) with primycin was recently proved, based on the formation of molecular complexes stabilized by one or two H-bonds between the carboxyl group of oleic acid and one of the –OH groups of primycin.⁷ The terminal amidine group of primycin seems to be more reactive, and hence the ergosterol specificity of primycin has a major opportunity in the plasma membrane. It should be mentioned that an –OH group capable of complex formation is also present on other sterol molecules; for example, cholesterol, which could cause the decyptification effect of primycin on the human erythrocyte membrane.^{5,17,18}

Biological consequence of primycin–ergosterol complex formation

The various membrane phases contain different amounts of ergosterol.¹⁹ When ergosterol comes into contact with the plasma membrane, it binds the primycin molecules on the membrane surface, whereas the ergosterol-less phases reach in fatty acids (for example, oleic acid) interact with the –OH groups of primycin and consequently the drug is localized between the phospholipids. Both formations caused a decreased flexibility in the plasma membrane, with different molecular motions. For these reasons primycin rearranges the membrane phases, leading to alterations in the conformation of membrane proteins, including channel proteins and proteins having a role in the signal transduction,²⁰ with the consequence of loss of the plasma membrane barrier function.⁶

EXPERIMENTAL PROCEDURE

Chemicals

Primycin (molecular weight=1127.25, average mass) was provided by the manufacturer, PannonPharma Ltd., Pécsvárad, Hungary. All other chemicals were commercial products of analytical grade from Sigma-Aldrich (St Louis, MO, USA). The documentation indicated that the content of active agent in the primycin sample was 865.03 U mg^{-1} (Figure 1b). The primycin was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 6.4 mg ml^{-1} , and the stock solution was stored at 4 °C. A detailed description of the composition of primycin was reported by Virág *et al.* (2010). The functional groups at the terminal positions of primycin can be seen in Table 1. Ergosterol (molecular weight=396.66) was dissolved in chloroform and stored in the dark at –20 °C (Figure 1a). The purity of both species was >95%.

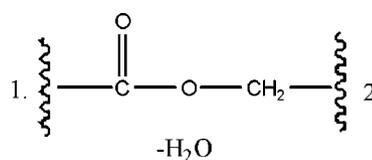


Figure 5 A schematic outline of the possible addition reaction and the formation of a dative bond between primycin (1) and ergosterol (2).

Sample preparation

The experiments were carried out by means of PL studies and data were evaluated by the Benesi–Hildebrand method.²¹ For the measurements, stock solutions of 0.002 M of ergosterol and 0.0024 M of primycin were prepared in chloroform and DMSO, respectively. The stock solutions of ergosterol and primycin were mixed in five different molar ratios, the concentration of ergosterol being kept constant at 0.001 M, while the concentration of primycin was varied from 0.002 to 0.005 M.

Instruments

The RS spectra were recorded on a Fluorolog τ 3 spectrofluorometer (Jobin-Yvon/SPEX, Longjumeau, France). For data collection, the photon-counting method with an integration time of 0.2 s was used. Excitation and emission bandwidths were set to 5 nm. Front-face detection was used to eliminate the inner-filter effect. The spectral region examined was from 200 nm to 700 nm. The measurements were taken at 11 different temperatures (in 2 K steps from 289.16 to 311.16 K). The average of 10 scans was used for data evaluation with DataMax 2.20 software (Jobin-Yvon, Longjumeau, France).

CONCLUSIONS

The antibiotic primycin shows considerable interaction with ergosterol. The thermodynamic parameters derived from PL studies reveal weak, but stable complexes at around the vital-temperature of pathogenic *C. albicans*, demonstrating that complex formation process with the most significant fungal membrane component, ergosterol, and can occur *in vivo*. Furthermore, the entropy-driven interaction is pronounced at elevated temperature, highlighting the enhanced effect of primycin in febrile bodies. This behavior of primycin is of pharmaceutical significance in the possible application of the drug in the specific therapy of fungal infections. In order to understand the exact mode of action of primycin, further investigations are planned with phospholipids as the main membrane structure components and with artificial membranes.

ACKNOWLEDGEMENTS

This research was supported by grants RET-08/2005 and INNO-08-DA-PRIMYCIN, by PannonPharma, Ltd., Pécsvárad, Hungary, and by Developing Competitiveness of Universities in the South Transdanubian Region (SROP-4.2.1.B-10/2/KONV-2010-0002).

- 1 Vályi-Nagy, T., Uri, J. & Szilágyi, I. Primycin, a new antibiotic. *Nature* **174**, 1005–1006 (1954).
- 2 Aberhart, J., Fehr, T., Jain, R. C., de Mayo, P. & Motl, O. J. Primycin. *Am. Chem. Soc.* **92**, 5816–5817 (1970).
- 3 Nógrádi, M. Primycin (Ebrimycin)–A new topical antibiotic. *Drugs of Today* **24**, 563–566 (1988).
- 4 Papp, T., Ménesi, L. & Szalai, I. Experiences in the ebrimycin gel treatment of burns. *Ther. Hung.* **38**, 125–128 (1990).
- 5 Blaskó, K., Shagina, L. V., Györgyi, S. & Lev, A. A. The mode of action of some antibiotics on red blood cell membranes. *Gen. Physiol. Biophys.* **5**, 625–635 (1986).
- 6 Virág, E. *et al.* *In vivo* interaction of the antibiotic primycin on a *Candida albicans* clinical isolate and its ergosterol-less mutant. *Acta Biol. Hung.* **63**, 2012 (2011)(in press).
- 7 Virág, E., Pesti, M. & Kunsági-Máté, S. Competitive hydrogen bonds associated with the effect of primycin antibiotic on oleic acid as a building block of plasma membranes. *J. Antibiot.* **63**, 113–117 (2010).
- 8 Brennan, P. J., Griffin, P. F. S., Losel, D. M. & Tyrrell, D. The lipids of fungi. *Prog. Chem. Fats Other Lipids* **14**, 49–89 (1979).
- 9 Tanford, C. *The hydrophobic effect: formation of micelles and biological membranes* (John Wiley & Sons: New York, 1980).
- 10 Cullis, P. R. & de Kruijff, B. Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochem. Biophys. Acta* **559**, 399–420 (1979).
- 11 Chernomordik, L. Non-bilayer lipids and biological fusion intermediates. *Chem. Phys. Lipids* **81**, 203–213 (1996).
- 12 Swann, T. M. & Watson, K. Stress tolerance in a yeast sterol auxotroph: role of ergosterol, heat shock proteins and trehalose. *FEMS Microbiol. Lett.* **169**, 191–197 (1998).
- 13 Pesti, M., Horváth, L., Vigh, L. & Farkas, T. ESR determination of plasma membrane order parameter, lipid content and phase transition point in *Candida albicans* sterol mutants. *Acta Microbiol. Hung.* **32**, 305–313 (1985).
- 14 Kunsági-Máté, S., Csók, Zs., Iwata, K., Szász, E. & Kollár, L. Role of the Conformational Freedom of the Skeleton in the Complex Formation Ability of Resorcinarene Derivatives toward a Neutral Phenol Guest. *J. Phys. Chem. B* **115**, 3339–3343 (2011).
- 15 Li, H., Nie, J. C. & Kunsági-Máté, S. Modified dispersion of functionalized multi-walled carbon nanotubes in acetonitrile. *Chem. Phys. Lett.* **492**, 258–262 (2010).
- 16 Kunsági-Máté, S., Kumar, A., Sharma, P., Kollár, L. & Nikfardjam, M. P. Effect of molecular environment on the formation kinetics of complexes of malvidin-3-O-glucoside with caffeic acid and catechin. *J. Phys. Chem. B* **113**, 7468–7473 (2009).
- 17 Blaskó, K. & Györgyi, S. Alkali ion transport of primycin modified erythrocytes. *Acta Biol. Med. Germ.* **40**, 465–469 (1981).
- 18 Blaskó, K., Györgyi, S. & Horváth, I. Effect of primycin on monovalent cation transport of erythrocyte membrane and lipid bilayer. *J. Antibiot.* **32**, 408–413 (1979).
- 19 Brown, D. A. & London, E. Structure and origin of ordered lipid domains in biological membranes. *J. Membr. Biol.* **164**, 103–114 (1998).
- 20 Wisniewska, A., Draus, J. & Subczynsky, W. K. Is a fluid mosaic model or biological membranes fully relevant? Studies on lipid organization in model and biological membranes. *Cell Mol. Biol. Lett.* **8**, 147–159 (2003).
- 21 Benesi, H. & Hildebrand, J. A. Spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. *J. Am. Chem. Soc.* **71**, 2703–2707 (1949).