Cholesterol Reduces Partitioning of Antifungal Drug Itraconazole into Lipid Bilayers

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ABSTRACT: Cholesterol plays a crucial role in modulating the physicochemical properties of biomembranes, both increasing mechanical strength and decreasing permeability. Cholesterol is also a common component of vesicle-based delivery systems, including liposome-based drug delivery systems (LDSs). However, its effect on the partitioning of drug molecules to lipid membranes is very poorly recognized. Herein, we performed a combined experimental/computational study of the potential for the use of the LDS formulation for the delivery of the antifungal drug itraconazole (ITZ). We consider the addition of cholesterol to the lipid membrane. Since ITZ is only weakly soluble in water, its bioavailability is limited. Use of an LDS has thus been proposed. We studied lipid membranes composed of cholesterol, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), and ITZ using a combination of computational molecular dynamics (MD) simulations of lipid bilayers and Brewster angle microscopy (BAM) experiments of monolayers. Both experimental and computational results show separation of cholesterol and ITZ. Cholesterol has a strong preference to orient parallel to the bilayer normal. However, ITZ, a long and relatively rigid molecule with weakly hydrophilic groups along the backbone, predominantly locates below the interface between the hydrocarbon chain region and the polar region of the membrane, with its backbone oriented parallel to the membrane surface; the orthogonal orientation in the membrane could be the cause of the observed separation. In addition, fluorescence measurements demonstrated that the affinity of ITZ for the lipid membrane is decreased by the presence of cholesterol, which is thus probably not a suitable formulation component of an LDS designed for ITZ delivery.

INTRODUCTION

Pharmaceutical nanotechnology, also known as nanomedicine, is the development of nanoscale drug delivery vehicles, also known as nanoparticles. It can be seen as the development of mechanisms to both increase efficacy and reduce toxicity associated with a given drug by targeting the delivery to the desired tissue, through either active or passive means; a specific dose of the drug can thus have an increased efficacy with reduced side effects. The liposome-based delivery system (LDS) is, so far, the most successful form of the nanoparticle, representing more than half of all currently approved nanomedicine-based drug therapies. An LDS is composed of a phospholipid membrane formed into an enclosed sac; use of phospholipids and other biocompatible molecules for the membrane possesses the advantage of automatic biocompatibility. As a nanoparticle, the LDS is extremely versatile, it can carry hydrophobic drugs within the membrane or hydrophilic drugs within the internal pocket. Many aspects of the formulation can be altered to tune the properties of the LDS. A subset of the phospholipids can have polymers conjugated to their headgroup to create a protective polymer corona; poly(ethylene glycol) is currently the gold standard in this capacity. Other amphiphilic biocompatible molecules can be added to the membrane to tune its properties; the most commonly used of these is cholesterol (Chol).

A common component of the LDS formulation, Chol is present in 9 out of 15 clinically approved LDS-based drugs and an additional 12 products currently in clinical trials. As an important component that modulates the properties of

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biomembranes, Chol can play the same role in an LDS; it has the ability to modify the physical properties of a lipid membrane (for extensive reviews, see, e.g., refs 8–12). For example, the presence of Chol can increase the mechanical strength of the lipid bilayer13–16 leading to both increased stability and decreased passive permeability to water, ions, and small polar molecules, e.g., glucose and drugs.17–22 Liposomes with a high Chol concentration are also biocompatible since Chol is present in a high concentration in biomembranes; in particular, the cell membrane of erythrocytes has a Chol level as high as 50%23. There are, however, also disadvantages regarding the use of Chol; for example, Chol is prone to oxidation.24,25 Not surprising, oxidized derivatives of cholesterol have been found in a wide range of cosmetics,26 processed foods,27 and liposomal pharmaceutics.28

The drug itraconazole (ITZ) (Figure 1), used to treat mycotic infections, is an ideal candidate for delivery via LDSs.

![Chemical structure of molecules used in this study. Red arrows show long axes of the molecules. Vertical black-dotted lines show the fragments used for center of mass calculations (see Figure 9).](image)

With a solubility of only 1 ng/mL,29 ITZ bioavailability is a severe problem in terms of its efficacy. The incorporation of water insoluble drugs into an LDS has seen considerable success as a strategy to solve this problem.30 In fact, the incorporation of ITZ into multimamellar vesicles, a form of LDS, was shown to increase e

cacy in the treatment of

cf.13,14 The incorporation of

tions were performed for four systems containing hydrated

| system | no. of ITZ | no. of POPC | no. of Chol | no. of water | no. of Na ions | no. of Cl ions | no. of repeats | simulation time (ns) |
|--------|-----------|-------------|-------------|--------------|---------------|---------------|----------------|-------------------|
| S1     | 1         | 102         | 26          | 6940         | 37            | 37            | 3              | 3000              |
| S1*    | 1         | 102         | 26          | 3356         | 24            | 24            | 3              | 3000              |
| S2     | 9         | 918         | 234         | 62460        | 333           | 333           | 1              | 1000              |
| S3     | 9         | 918         | 234         | 34362        | 216           | 216           | 1              | 2000              |

**Table 1. Composition of Lipid Bilayers Used in This Study**

**Materials and Methods**

**Materials.** Synthetic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol (Chol), and itraconazole (ITZ) were received from Sigma-Aldrich. Methanol and chloroform were obtained from Sigma-Aldrich (HPLC grade, ≥99.9%). The ultrapure Milli-Q water, used in the experiments, had a surface tension of 72.6 mN/m (at 20 °C) and a resistivity of 18 MΩ cm.

**Langmuir Balance and Brewster Angle Microscopy (BAM) Measurements.** The measurements were performed using a KSV 2000 Langmuir trough (KSV Instruments Ltd., Helsinki, Finland) equipped with an ultraBAM (Accurion GmbH, Goettingen, Germany) microscope, as previously described.35–37 The BAM microscope was equipped with a 50 mW laser emitting p-polarized light at 658 nm, a 10× objective, and a CCD camera. The spatial resolution of the BAM images was 2 μm. To prepare stock solutions, POPC and Chol were dissolved in chloroform/methanol (4:1 v/v), and ITZ was dissolved in chloroform. Phosphate buffer (pH 7.4) was used as the subphase. All experiments were repeated at least twice to ensure consistent results. Surface pressure−area (π−A) isotherms were reproducible within an error of ±0.02 nm² molecules⁻¹.

**Liposome-Binding Constant Measurements.** POPC and POPC/Chol 4:1 liposomes were prepared by sonication using the modified procedure described previously.34 Briefly, POPC and Chol were dissolved in chloroform to form stock solutions. The appropriate volumes of the stock solutions were combined in a volumetric flask, and chloroform was evaporated under vacuum. Water was added to reach a lipid concentration of 2.5 mg/mL, and the sample was vortex mixed for several minutes. The lipid dispersion was subjected to five freeze−thaw cycles from liquid nitrogen temperature to 60 °C and sonication at ice temperature for 10 min using a titanium tip SONICS VC 130 sonicator. Binding constants (Kₙ)’s of the drug to liposomes were determined using a fluorescence titration technique.35 Fluorescence spectra were measured using a LS-55 PerkinElmer fluorimeter.

**Molecular Dynamics (MD) Simulations.** MD simulations were performed for four systems containing hydrated lipid bilayers composed of POPC and Chol (20 mol %) (the POPC/Chol bilayer) and ITZ, at a concentration of one simulation of the LDS membrane and Brewster angle microscopy (BAM) of monolayers in a Langmuir balance (LB). Both computational and experimental results are in agreement and present a surprising result: ITZ and cholesterol do not coexist in the membrane; rather, they separate within the membrane. The separation of ITZ and Chol is the reason for the lower affinity of the drug for the lipid membrane containing Chol, as shown by fluorescence measurements. We thus propose that our results indicate that inclusion of Chol into the lipid membrane is probably not beneficial for the case of ITZ delivery.
molecule per 128 lipids. Detailed compositions of all systems are given in Table 1. In system S1, a single ITZ molecule was inserted into the water phase and allowed to spontaneously insert into the lipid bilayer. System S2 was constructed by replicating the frame of system S1 three times over periodic boundary conditions in the bilayer plane (XY) to create a lipid bilayer nine times larger than that of S1. System S1* was constructed from model S1 by decreasing the number of water molecules. A physiological salt concentration (140 mM of NaCl) was used. The results were averaged over simulated replicates and molecules present in the bilayer.

To parametrize all molecules and ions, we used the OPLS-AA force field. We used lipid models derived in our prior studies (molecular topologies of POPC and Chol are provided in Supporting Materials of ref 40). Partial charges for the ITZ molecule were derived in the previously published work. To model water we used the TIP3 parameter set. All simulations were performed using the GROMACS software.

Figure 2. Snapshots of simulated systems at several time windows: ITZ molecules are shown in standard colors, cholesterol is shown in red, POPC phosphate groups are shown as violet spheres, and POPC acyl tails are shown in gray. The figure was prepared using VMD software.

Figure 3. Snapshot of the one leaflet containing ITZ (A), and close views of an ITZ molecule in system S2 from top (B) and side (C). Cholesterol is shown in red, ITZ in blue with red oxygens and white hydrogens, POPC in gray, and POPC phosphorus atoms in dark blue.
The LINCS algorithm was used to maintain covalent bond lengths between hydrogens and heavy atoms, allowing for a 2 fs time step. Simulations were performed at constant temperature (300 K) and pressure (1 atm). Temperature and pressure were controlled using the Nose–Hoover and Parinello–Rahman algorithms, respectively. The temperature of solute and solvent were controlled independently, and semi-isotropic pressure coupling was used. The long-range electrostatic interactions were calculated using Particle-Mesh-Ewald algorithm with a real space cutoff of 1 nm. The neighbor lists were updated every 10 steps.

**RESULTS AND DISCUSSION**

**MD Simulations.** Figure 2 shows snapshots of the systems taken at various simulation times. For the case of system S1, the ITZ molecule, placed initially in the water phase, entered the membrane after approximately 350 ns of the simulation. Insertion was observed in two out of the six replicas, and partial insertion was observed in only one replica. For comparison, in our previous studies using a pure POPC bilayer, all ITZ molecules entered the bilayer after less than 450 ns. This observation is consistent with the known reduction in permeability of the bilayer containing Chol. On the other hand, the insertion process is similar for both POPC and POPC/Chol bilayers. During insertion, ITZ is oriented perpendicular to the bilayer surface. The difference lies in the ITZ orientation after insertion: in the POPC bilayer, the drug molecules orient their long axis parallel to the bilayer surface, while in the POPC/Chol membrane, ITZ molecules remain perpendicular to the membrane surface.

For the case of system S2, nine ITZ molecules were partially inserted into the bilayer at the beginning of the simulation and separated from each other (Figure 2). During the simulation, all ITZ molecules fully entered the bilayer core. Although the drug/lipid ratio of systems S1 and S2 is identical, the behavior of ITZ in these two systems is significantly different. In system S2, the drug molecules adopt an orientation parallel to the bilayer surface, similar to the behavior of ITZ in the pure POPC bilayer (Figure 3). In addition, the ITZ molecules form aggregates of three molecules, and the drug tends to accumulate in Chol-depleted regions (Figure 3).

The qualitative differences in the behavior of the ITZ molecules in the two systems (S1 and S2) can be attributed either to the limited size of the bilayer in system S1 or to the need for the collective action of ITZ molecules to form local clusters oriented parallel to the bilayer surface. Thus, the
behavior of ITZ molecules in system S1 would be representative of the highly diluted system where isolated ITZ molecules adopt the orientation of cholesterol molecules. On the other hand, the simulations of system S2 clearly demonstrate a tendency of ITZ molecules to aggregate even at low concentrations in Chol-containing lipid bilayers. The cause of ITZ aggregation in the lipid bilayer is probably the drug–Chol separation, which significantly reduces the volume available for the drug in cholesterol-containing membranes. As a result, the drug concentration increases locally.

Figures 4 and 5 provide quantitative results regarding the location and orientation of ITZ in the lipid bilayers. Figure 4 shows the density profiles of ITZ and selected POPC atoms. In system S2, ITZ locates preferentially between the headgroup and the double bonds in the sn2 chain of POPC with the maximum at 1.4 nm from the bilayer center. In system S1, ITZ spreads over the entire leaflet, reflecting its orientation parallel to the membrane normal. Figure 5 shows time development and the distribution of the angles of the long molecular axes of Chol and ITZ. The long axis of Chol molecules makes an average angle of 17.2 ± 0.8° with the bilayer normal. Thus, Chol adopts an orientation approximately parallel to the bilayer normal. The POPC acyl tails have a similar orientation (21.6 ± 0.6°), considering the vector connecting the first and last atom in the sn1 chain. In contrast, ITZ molecules predominantly adopt a perpendicular orientation (72 ± 3°), thus parallel to the membrane plane. However, in system S1, in which only one ITZ molecule was inserted into the bilayer, the ITZ orientation is more similar to the Chol orientation (the ITZ long axis makes an average angle of 33 ± 5° with the bilayer normal).

To characterize the interactions between ITZ and Chol in the lipid membrane, we calculated the number of contacts between heavy atoms of both molecules. We assumed that a contact occurred when the distance between two (non-hydrogen) atoms was smaller than 0.6 nm. Figure 6 shows the time development of the number of contacts during the simulation. In the first 400 ns of the simulation, the ITZ–Chol contacts were insignificant. After this time, the number of contacts increased quickly due to the change in the ITZ orientation from parallel to the bilayer normal (initial arrangement) to parallel to the membrane surface. With this arrangement of ITZ, the increase in contact is expected, since ITZ is a long molecule, thus interacting with many lipids while adopting an orientation parallel to the membrane surface. In the next part of the simulation, we observed a decrease in the number of contacts with Chol; this can be interpreted as a sign of separation. This process is, however, not completed within the simulation time.

To further evaluate the ITZ–Chol interactions, we calculated the radial distribution functions (RDFs) for heavy atoms of ITZ and lipids according to the equation

\[
RDF = \frac{V}{N} \frac{n(r)}{4\pi r^2 dr}
\]

where \(n(r)\) is the number of atoms \(\beta\) in the spherical ring with radius \(r\) and width \(dr\) around the atom, \(4\pi r^2 dr\) is the ring volume, \(V\) is the volume of the system, \(N\) is the number of atoms, and \(\langle \rangle\) denotes averaging over time and ensemble. The RDF function (Figure 7) for the ITZ-POPC pair shows a narrow maximum located at 0.5 nm, which indicates ITZ preference for interacting with POPC. In the case of a ITZ–Chol pair, the RDF function has a broad maximum centered at 2.8 nm, demonstrating that Chol tends to be located away from the drug. A small maximum can be noticed at approximately 0.6 nm, which indicates that Chol–ITZ interactions are also possible.

To gain insight into the local ITZ impact on POPC properties, we calculated the order parameter, \(S_{CD}\), for the sn1 chains of POPC molecules located at the distances up to 1 nm, between 1 and 2 nm, and above 2 nm. The distance was calculated between the center of mass of the acyl tails and the center of mass of three ITZ fragments (see Figure 1) in the plane of the bilayer (only lipids in the same leaflet were included into the calculation). The \(S_{CD}\) order parameter is defined as follows:

\[
S_{CD} = \frac{3}{2} (\cos^2 \theta) - \frac{1}{2}
\]

where \(\theta\) is the angle between the C–H bond of the ith carbon atom and the bilayer normal. The angle brackets mean averaging over time and over appropriate C–H bonds in the bilayer. The \(S_{CD}\) parameter profiles along the PA sn1 chains (Figure 8) show a decrease in the order of hydrocarbon tails of the lipids in the vicinity of the drug molecules. Therefore, the presence of ITZ in the membrane should increase its fluidity.
Monolayer Experiments. The $\pi$–$A$ isotherms recorded during compression of the POPC/Chol films and their mixtures with ITZ are presented in Figure 9. To determine if the ITZ effect depends on the Chol content in the model membrane, POPC and Chol were mixed in two different proportions (POPC/Chol = 4:1 and 1:1). The physical state of both POPC/Chol films was determined on the basis of the compression modulus calculated on the basis of the isotherms (Figure S1, the Supporting Information). The calculated values indicate that both monolayers are in the liquid-condensed (LC) phase at larger surface pressures. We introduced a variety of concentrations of ITZ (5, 10, and 15 mol %) into these films.

The addition of ITZ in the investigated concentration range does not drastically affect the position of the isotherms; it however significantly alters their slopes. This indicates that the incorporation of the drug, even at low concentrations, into the POPC/Chol membranes disturbs their structures. The fact that the isotherm slope for ITZ-containing monolayers is less steep than that for the POPC/Chol films indicates that the addition of itraconazole increases the fluidity of the model membranes. This is confirmed by the compression modulus values calculated for the ITZ-containing monolayers (Figure S1, Supporting Information).

The BAM images obtained during the compression of the films are shown in Figure 10. BAM images taken for both two-component (POPC/Chol) monolayers are similar. At low surface pressure ($\pi = 1$ mN/m), brighter oval domains of the liquid expanded (LC) phase that coexist with the gaseous (G) phase (darker areas in the images) are visible. When compressing films, the LC domains merge and the LC phase covers the whole interface up to the collapse point. This is reflected in the homogeneous BAM images that confirm the miscibility of POPC and Chol. Our results are consistent with previous studies showing that the excess Gibbs energy of mixing ($\Delta G_{mix}$) for the POPC/Chol binary system is negative for the entire range of monolayer compositions. The only difference that can be noticed between POPC/Chol films is that the LE domains observed for the POPC/Chol 4:1 monolayer are smaller than that for the POPC/Chol (1:1) monolayer. This indicates a higher condensation of the latter monolayer. The observed effect is due to the higher Chol concentration, whose condensing property on phospholipids is well-known.

For the case of ternary POPC/Chol/ITZ monolayers, in which the ratio of POPC to Chol was 4:1, their morphology does not change up to 10 mol % of ITZ in the mixed film (BAM images are practically identical to those for the POPC/Chol monolayer, data not shown). The higher content of ITZ (15 mol %), however, causes the condensed phase domains observed at lower surface pressure ($\pi = 1$ mN/m) to be smaller than those observed for the two-component POPC/Chol (4:1) monolayer. This confirms that the POPC/Chol/ITZ monolayers have a more liquid character than the POPC/Chol films. In addition, at higher surface pressures ($\pi \geq 10$ mN/m), the monolayers are heterogeneous, and small condensed domains can be observed in the BAM images, suggesting phase separation. ITZ exerts a similar effect on the POPC/Chol 1:1 film; however, the morphology of the monolayer changes at the lower itraconazole content (10 mol %), and monolayers are inhomogeneous over the entire range of surface pressures. Furthermore, at a higher concentration of ITZ (15 mol %), the domains observed at higher surface pressures are very bright. This suggests that multilayer (3-D) structures are present, indicating that, at higher surface pressures, the film-forming molecules (most probably ITZ) are squeezed out from the monolayer. This is
strongly supported by the formation of ITZ clusters observed in the simulations of system S2, in which the concentration of the drug was much smaller. The obtained results show that the effect of ITZ on the model membranes depends on the Chol concentration. In our previous studies with the pure POPC bilayer, we showed that the POPC/ITZ monolayers were homogeneous at ITZ concentration levels up to 15 mol % over the entire range of surface pressures.32 It can therefore be concluded that the higher the level of Chol in the lipid monolayer is, the lower the concentration of ITZ is at which the membrane morphology starts to be disturbed.

**Fluorescence Measurements.** To assess the effect of Chol on ITZ partitioning between the liposomal and aqueous phases, we determined the so-called binding constant, \( K_b \), defined as:

\[
K_b = \frac{c_L}{c_w[L]}
\]

where \( c_L \) and \( c_w \) are ITZ concentrations in the liposomal and aqueous phases, respectively, \([L]\) is the lipid concentration in the system. Two sets of samples containing constant ITZ concentration and increasing content of the POPC or POPC/Chol 4:1 liposomes were prepared, and emission spectra were measured. We observed an increase in ITZ fluorescence intensity after the addition of the liposomes. Figure 11 presents a typical dependence of fluorescence intensity \( F \) and \([L]\) for the ITZ solution titrated with the POPC and POPC/Chol 4:1 vesicles. \( K_b \) was determined by fitting the experimental data to the formula:

\[
F = F_{\text{init}} + \frac{F_{\text{comp}}K_b[L]}{1 + K_b[L]}
\]

where \( F_{\text{init}} \), \( F \), and \( F_{\text{comp}} \) are the fluorescence intensity of the drug measured without lipid, after adding lipid to the concentration \([L]\), and the asymptotic intensity achieved at complete binding, respectively; the fitted line is shown in
Figure 11. Changes in ITZ fluorescence intensity ($\varepsilon_{ITZ} = 1 \mu M, \lambda_{ex} = 270 \text{ nm}$) upon titration with the POPC liposomes (○) and the POPC/Chol 4:1 liposomes (□). The red lines show the best fits to eq 2.

**CONCLUSIONS**

Our results clearly demonstrate that Chol and ITZ do not mix in lipid bilayers but rather separate into different domains, thus reducing membrane stability. The orientation of the ITZ molecules in the bilayer results from the shape and distribution of the polar groups in the molecule, and this orientation clashes with that of Chol. Cholesterol is evolutionarily optimized to increase order of the lipids in biological membranes and adopts a slightly tilted orientation toward the normal to the bilayer. This orientation is maintained by its (1) hydroxyl group that locates to the interface between polar and hydrophobic regions, (2) rigid steroid ring that neighbors the ordered section of the lipid tails, and (3) isooctyl tail spanning the most disordered section of the bilayer. While also a rigid molecule, ITZ is, however, longer than the cholesterol molecule or POPC acyl tails; thus, ITZ, in orientation parallel to the bilayer normal, can span the disordered region of the bilayer or even protrude into the opposite leaflet. These two situations are entropically unfavorable due to the ordering effects that result from the presence of a rigid molecule in the highly disordered region of the bilayer. In addition, polar groups are distributed along the entire length of the molecule; thus, in orientation parallel to the bilayer normal, some of them would be buried in the hydrophobic core of the membrane. These two factors lead to the strong preferences of ITZ to locate to the interface between the hydrocarbon chain region and the polar region and then orient parallel to the membrane surface. The drug—Chol separation in the lipid bilayer strongly affects ITZ accumulation in Chol-containing liposomes. The presence of Chol causes the membrane volume available for the drug to be significantly reduced. As a result, at higher concentrations the drug molecules either aggregate within the membrane or are expelled from it entirely.

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