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How Do Sterols Determine the Antifungal Activity of Amphotericin B? Free Energy of Binding between the Drug and Its Membrane Targets

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Abstract: Amphotericin B (AmB) is a well-known polyene antibiotic used to treat systemic fungal infections. It is commonly accepted that the presence of sterols in the membrane is essential for the AmB biological activity, that is, for the formation of transmembrane ion channels. The selective toxicity of AmB for fungal cells is attributed to the fact that it is more potent against fungal cell membranes containing ergosterol than against the mammalian membranes with cholesterol. According to the "primary complex" hypothesis, AmB associates with sterols in a membrane to form binary complexes, which may subsequently assemble into a barrel-stave channel. To elucidate the molecular nature of the AmB selectivity for ergosterol-containing membranes, in the present work, we used computational methods to study the formation of the putative AmB/sterol complexes in a lipid bilayer. The free energy profiles for the AmB−sterol association in phospholipid bilayers containing 30 mol % of sterols were calculated and thoroughly analyzed. The results obtained confirm the formation of specific AmB/ergosterol complexes and are used to determine the energetic and structural origin of the enhanced affinity of AmB for ergosterol than for cholesterol. The significance of this affinity difference for the mechanism of action of AmB is discussed. The data obtained allowed us also to suggest a possible origin of the increased selectivity of a novel class of less toxic AmB derivatives.

Introduction

Sterols, essential components of eukaryotic cell membranes, are known to modulate the membrane function in two general ways.[1,2] First, they may act by modifying structural and thermodynamic properties of a lipid bilayer. Sterols such as cholesterol and ergosterol (Figure 1) have, for example, been shown to promote the separation of the so-called liquid-ordered (lo) phase, which is characterized by increased ordering of the lipid chains and, at the same time, retains translational mobility.[3−5] Thus, sterols can indirectly affect membrane proteins sensitive to changes in the lipid environment. In fact, it was demonstrated that sterol-rich lo domains (so-called "lipid rafts") sequester certain proteins and therefore may regulate such cellular functions as cell signaling and membrane trafficking.[6] Second, sterol molecules can directly bind to enzymes and other membrane components, thereby causing their activation or inactivation.[1,7]

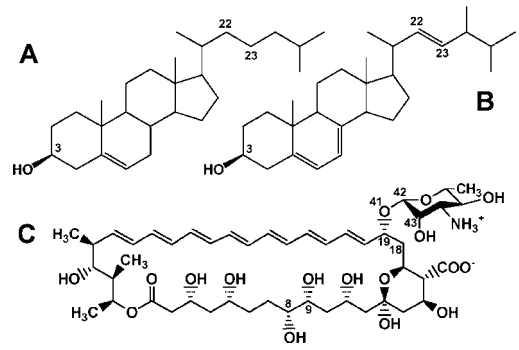

Figure 1. Chemical structures of the cholesterol (A), ergosterol (B), and AmB (C) molecules.

These two effects of sterols are also used to explain the mechanism of action of amphotericin B (AmB), an antifungal polyene antibiotic that is believed to act by forming barrel-stave channels in eukaryotic cell membranes (Figure 1).[8,9] It is believed that sterols determine pore-forming activity of AmB either by specific interaction with the antibiotic or, nonspecifically, by providing an appropriate environment (presumably,

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lo phase) for the formation of a functional pore. In the former case, the chemotherapeutic effect (selectivity of action) of AmB is due to its stronger binding to ergosterol (Erg), the principal membrane sterol of fungi, than to its mammalian counterpart, cholesterol (Cho). In this model, also called the "primary complexes" hypothesis, the more stable complexes with Erg more readily self-assemble to form transmembrane channels.[10,11] In the latter case, the selective targeting of fungal membranes by AmB is due to the stronger conformational ordering induced by Erg.[4,8,12,13] Direct interactions with sterols and disturbance of the lipid rafts are also considered when examining other biological effects of AmB, for example, its anti-HIV activity.[14,15] Recently, it has been proposed that specific binding to Erg may be responsible for antifungal activity of natamycin, another polyene antibiotic.[16]

Despite many unique advantages, evidenced by 50 years of its extensive use in clinical practice, AmB is not sufficiently selective and hence is highly toxic.[17,18] To rationally design less toxic derivatives, the mechanism of action of the antibiotic at the molecular level needs to be elucidated. Unfortunately, the experiments did not fully support nor eliminate any of the above two models.[8,9] NMR experiments showed that AmB slows the motions of Cho and that some form of loosely bound AmB/Cho associates is present in the membranes (given the current state of knowledge, this could be indicative of AmB incorporation into the sterol-rich lo domains).[19] The attempts to discern the differential affinity of AmB for both sterols yielded conflicting data, suggesting that either Cho[20] or Erg[21] is more prone to form complexes with the antibiotic in a lipid bilayer. No quantitative data as to the thermodynamics and kinetics of such complexes are, however, available.

Here, we used a computational approach to study for the first time the formation of the putative AmB/sterol complexes directly at the molecular level. The free energy profiles for the AmB−sterol association in lipid bilayers containing 30 mol % of Erg or Cho were calculated and thoroughly analyzed. The results obtained confirm the formation of specific AmB/Erg complexes and are used to determine the energetic and structural origin of higher affinity of AmB for Erg than for Cho. The implications of these findings for AmB selectivity are discussed. The data obtained were also used to determine the molecular origin of the increased selectivity of the so-called second generation of AmB derivatives.

Methods

Molecular Dynamics Simulations. Molecular Model. The simulation model contained a single AmB/sterol complex (AmB/Erg or AmB/Cho) embedded in a DMPC membrane with ∼30 mol % of either ergosterol or cholesterol. These systems will be referred to as DMPC/Erg and DMPC/Cho, respectively. Note that DMPC/sterol model membranes are the simplest systems for which the AmB selectivity for Erg-containing bilayers is observed and which are, therefore, frequently used in experimental studies.[19,22,23] It was previously suggested that the presence of an ordered bilayer phase might be necessary for AmB to exert its action.[8,12] Thus, in choosing the DMPC:sterol molar ratio of 7:3, we were guided by the corresponding phase diagrams, which indicate that at 300 K the membrane of such a composition is entirely in the lo state.[24] The DMPC/sterol bilayers were prepared from the previously simulated models[25] by removing a certain number of DMPC and sterol molecules from the edge of the simulation box and by replacing an equal number of randomly picked lipid molecules with sterols to reach the desired sterol concentration (∼30%). Afterward, one DMPC molecule and one nearby sterol were replaced by an equilibrated AmB/sterol complex also taken from the previously simulated bilayer systems.[25] Each complex was placed in a manner consistent with the putative mechanism of action of AmB, that is, with the AmB's polar headgroup located at the bilayer/water interface and the lactone ring buried within the membrane hydrocarbon core. This orientation was indicated by experiments[26,27] and further verified by simulations.[13] The final models contained 31 DMPCs, 1 AmB, and 14 sterols in one leaflet, 32 DMPCs and 14 sterols in the other, and 2220 water molecules.

Molecular Dynamics Simulations. Molecular dynamics (MD) simulations were conducted with NAMD.[28] The CHARMM27 lipid force field was employed for the DMPC molecules.[29] The special parameter set taken from Cournia et al. and consistent with the CHARMM force field was used for the sterol molecules.[30] The antibiotic molecule was described using the previously validated CHARMM22-based set of parameters combined with the partial charges obtained by fitting to the quantum-mechanical electrostatic potential.[13,25,31,32] TIP3P model was used for water.

The standard CHARMM force field is known to underestimate values of the membrane area per lipid molecule when, the most natural for lipid bilayers in equilibrium, NPT conditions are applied.[30,33] The usage of a special set of parameters (reparametrized CHARMM27), which supposedly should solve this problem, led to highly disordered state of previously simulated sterol-

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containing bilayers.[25,34] Thus, we simulated our systems in the $NP_{zz}AT$ ensemble, which is a common practice when using CHARMM27. This means that the total number of particles, N, the normal component of pressure, P_{zz}, the membrane surface area, A, and the temperature, T, were kept constant during the simulations. The values of A were adjusted to reproduce the deuterium order parameter profiles as measured experimentally for membranes of identical composition (1891.5 Å^2 for DMPC/Erg and 1978.0 Å^2 for DMPC/Cho).[4] Importantly, the difference in packing of the Erg- and Cho-induced liquid-ordered phases was also reproduced (see Figure SI 1 in the Supporting Information). The temperature was kept at 300 K by means of the Langevin dynamics. The normal pressure was maintained at required value (1 bar) using the Langevin piston method.[35] Long-range electrostatic interactions were estimated using the Particle Mesh Ewald method.[36] The Lennard-Jones interactions were calculated using a smooth cutoff approach (8.0/10.0 Å). Covalent bonds between hydrogen atoms were constrained using the SHAKE method, except for water, for which the SETTLE algorithm was applied.[37] This permitted a time step of $\Delta t = 2$ fs to be used to integrate the equations of motion in the leapfrog Verlet algorithm.

Free Energy Calculations. To study the AmB/sterol complex formation in a lipid bilayer, we estimated the free energy profiles (potential of mean force, PMF) along the reaction coordinate, ξ, defined as the distance between the AmB's and sterol's centers of mass (COMs) projected on the *xy*-plane. To obtain such profiles, we used the adaptive biasing force (ABF) method.[38,39] This method allows for improved sampling of the phase-space by applying continuously updated biasing forces along the reaction coordinate. During the simulation, the average force acting along ξ is being accumulated. The free energy is then computed as an integral of this average force over ξ.

To increase the efficiency of the calculations, the AmB/sterol association pathway, the interval $3.0 \leq \xi \leq 19.0$ Å, was divided into eight equally sized windows. For each of these windows, a 250 ns long trajectory was generated. Instantaneous values of the force were stored in 0.1 Å wide bins. The standard error of the resulting free energies was estimated using the expression given by Rodriguez-Gomez et al.[40] The starting configurations for the ABF windows were obtained in the 40 ns steered MD simulations, carried out for the transition from the AmB/sterol complex to free AmB and sterol molecules. To eliminate the possible unphysical nature of the initial systems obtained, the 10 ns equilibration was performed, during which the AmB−sterol distance was harmonically restrained to its reference value.

The same starting configurations were also used in a set of umbrella sampling (US) simulations, which were performed to further evaluate the significance of the obtained free energy profiles. To ensure sufficient overlap of the consecutive probability distributions, this method required the division of the pathway ($3.0 \leq \xi \leq 19.0$ Å) into 16 equally spaced windows. The biasing potential of the form $U(\xi) = k/2(\xi - \xi_0)^2$ was used, where force constant k was set to 4.0 kcal/(mol × Å^2). Each window was simulated for 200 ns. The PMFs were determined using the standard weighted histogram analysis method (WHAM).[41] Error bars were estimated using Monte Carlo bootstrap analysis, taking into account the correlations in the time-series data.

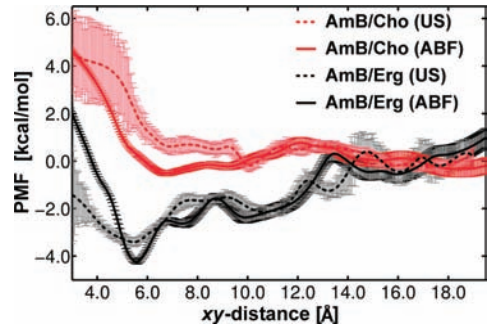

Figure 2. The free energy profiles (PMFs) along the reaction coordinate defined as the distance between the AmB's and sterol's centers of mass (COMs) projected on the *xy*-plane (*xy*-distance).

Results and Discussion

Free Energy of the AmB/Sterol Complex Formation. To compare the AmB molecule tendency to form binary complexes with sterols in Erg- and Cho-containing lipid bilayers, we calculated the free energy profiles along the reaction coordinate ξ, defined as the *xy*-distance between AmB and a single sterol molecule (Figure 2). Such a reaction coordinate is a natural choice for studying the association of two elongated molecules (e.g., AmB and sterol), whose equilibrium mobility is strongly restricted (by a lipid environment) to translations in the *xy*-plane and rotations around the *z*-axis.[42] The estimated statistical uncertainties indicate that, in our case, the ABF method ensures faster convergence of the PMFs than does the US approach. Regardless, the resulting profiles are, to a large extent, consistent, and some quantitative differences are mainly due to insufficient sampling of the barrier regions in the US approach (see, for example, large statistical errors for close contacts between AmB and sterol).

Clearly, both methods independently reveal that, relative to the unbound state (large ξ values), the AmB/sterol bound configurations are in equilibrium up to ∼4.0 kcal/mol more favorable for Erg than for Cho. In fact, the profiles for the AmB/Cho pair are roughly flat over the entire ξ range considered, indicating that there is no preferred distance between these molecules. For AmB/Erg, in turn, the free energy global minimum is found for the molecules held together at $\xi \approx 5.5$ Å (note also that Cho is not likely to approach AmB this closely). Additionally, for Erg, we can distinguish another well-pronounced minimum located at $\xi \approx 10.0$ Å. This corresponds to the weakly bound complex, mediated by a highly ordered DMPC molecule. The question that arises is whether the differences observed in the equilibrium distribution of Erg and Cho around AmB are of specific nature or merely reflect differences in the properties of the two lipid environments.

Indeed, the more irregular shapes of the AmB/Erg profiles might result from the tighter packing of the Erg-induced lo phase. To structurally characterize the local environment of the AmB molecule in the two considered bilayer systems, the radial distribution functions (RDFs) of DMPC acyl chain atoms around AmB were calculated and are shown in Figure 3. The plots clearly show that there are no substantial differences in the hydrophobic core packing around AmB between the two

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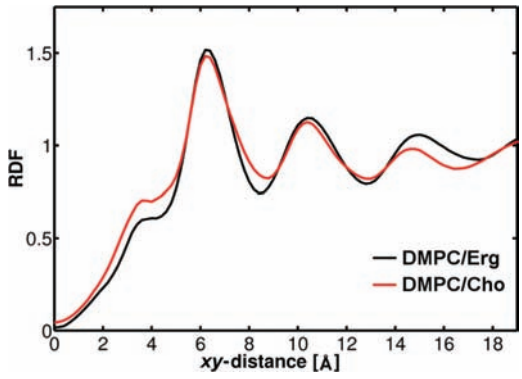

Figure 3. Two-dimensional radial distribution functions of the DMPC acyl chain carbon atoms around AmB COM for the DMPC/Erg and DMPC/Cho systems.

Table 1. Binding Free Energies (ΔG) and Association Constant ($K^*_{6.5}$) As Defined in the Text

	ABF				US			
	$\Delta G_{6.5}$	$\Delta G_{8.7}$	$\Delta G_{13.0}$	$K^*_{6.5}$	$\Delta G_{6.5}$	$\Delta G_{8.7}$	$\Delta G_{13.0}$	$K^*_{6.5}$
ERG	−0.53	−0.94	−2.44	95.7	−0.35	−0.65	−1.86	49.2
CHO	1.80	0.83	0.32	2.94	2.97	1.73	0.63	2.26

bilayers. First, this is in agreement with the previously reported ability of the rigid AmB molecule to increase the order of the acyl chains in DMPC/Cho to the level observed in DMPC/Erg.[13,43] Second, this finding suggests that the difference between the AmB/Erg and AmB/Cho PMFs (Figure 2) is due to specific AmB/sterol interactions rather than nonspecific environment-induced effects. Molecular determinants of the observed specificity will be analyzed in detail in the following sections. Note also that the global and local minima, identified above in the AmB/Erg PMF, indeed correspond to the first and second layers of the membrane hydrocarbon core around the AmB molecule as revealed in Figure 3.

To further analyze the association process on a quantitative level, we computed the free energy differences accompanying the binding of a single selected sterol molecule to the AmB monomer, both embedded in a bilayer in the sterol-induced lo phase. These "binding free energies" (Table 1) were obtained by integrating probability density functions, $\rho(\xi) = (2\pi\xi \exp(-\mathrm{PMF}(\xi)/k_\mathrm{B}T))$, over bound (b) and unbound (u) states:

$$\Delta G = -k_\mathrm{B}T \ln \frac{p^\mathrm{b}}{p^\mathrm{u}} = -k_\mathrm{B}T \ln \frac{\int_\mathrm{b} \rho(\xi)\,\mathrm{d}\xi}{\int_\mathrm{u} \rho(\xi)\,\mathrm{d}\xi}$$

where p^b and p^u are the probabilities that AmB and sterol are bound and not bound, respectively. Because the definition of intramolecular complex is arbitrary, ΔG values were calculated by setting the integration limit ξ^*, which divides the whole range of ξ and, therby, separates the b and u states, to three different values (6.5 Å for "tightly bound" complex, 8.7 Å for "bound" complex, and 13.0 Å for complex including also the "weakly bound" mode). All ΔG (from both ABF and US) values shown in Table 1 indicate that association of AmB with a selected sterol molecule is thermodynamically favorable only in the case of Erg. Positive values for AmB/Cho clearly show that in this case the entropic penalty for bringing the two molecules together (~1.5 kcal/mol, for $\xi^* = 6.5$) is not compensated by the other contributions to ΔG. It is worth noting that the Erg/Cho relative free energy differences, $\Delta\Delta G$, presumably important for the AmB antifungal activity, are also reasonably consistent between the two methods used. To enable experimental verification of our findings, we estimated a "macroscopic" association constant, $K^*_{6.5}$, defined as the ratio of the probability that AmB, when present in the DMPC bilayer containing 30 mol % of sterol, forms a tight binary complex with a sterol to the probability that it does not (or, equivalently, the respective concentration ratio). To obtain this ratio, ξ^* was set to 6.5 Å, and an upper limit to the u state was selected so as to correspond to the area of the bilayer per sterol molecule. The results (Table 1) indicate that the probability of finding AmB tightly bound to a sterol is ~32 (ABF) or ~22 (US) times higher for Erg than for Cho. Thus, we predict that within a lipid bilayer in the lo phase the specificity of AmB for Erg increases ~2−3 times as compared to the difference found experimentally in water solution.[44] The results here presented are in agreement with the ^{2}H NMR experiments, which demonstrate that AmB directly interacts with Erg and that Erg has stronger affinity for AmB than does Cho.[21] The qualitative character of the experimental data hinders more detailed comparison of the found differences.

As implied by the positive ΔG for the association of AmB with a selected Cho molecule, it is due to the high concentration of sterol in our "standard state" that $K^*_{6.5}$ for Cho is larger than 1. By comparing the activation barriers $\Delta G^\ddagger$, we conclude that the formed AmB/Erg complex is >100 times more kinetically stable than its Cho counterpart. Using a simple expression for the rate constant $k_\mathrm{off} = \nu \exp(-\Delta G^\ddagger/k_\mathrm{B}T)$, where the frequency prefactor ν was estimated on the basis of equilibrium simulations to be ~1.0 ns^{-1},[25] we obtained $k_\mathrm{off} \approx 5.3 \times 10^{-3}$ ns^{-1} as the dissociation constant in 300 K for the AmB/Erg binary complex. In contrast, the dissociation of AmB/Cho is diffusion-controlled.

Energetic and Entropic Contributions to $\Delta\Delta G$. To determine the origin of the difference in binding free energies found between AmB/Erg and AmB/Cho, we analyzed energy and entropy contributions to ΔG. The following analysis is intended to highlight the main differences between the two types of complexes, that is, the terms contributing the most to $\Delta\Delta G$. Note also that the contributions presented do not sum up to ΔG mainly due to inconsistency of the methods used to obtain them.

Energies of association $\Delta E = E_\mathrm{b} - E_\mathrm{u}$, shown in Table 2, were calculated from the ABF- and US-generated ensembles and can be treated as binding enthalpies if one assumes $\Delta pV = 0$. The ABF energies were first averaged in ξ-bins of a width of the PMF grid spacing. Subsequently, two energy values were computed as weighted averages: E_b for a bound state and E_u for an unbound state (defined as above), using $\xi\exp(-\mathrm{PMF}(\xi)/k_\mathrm{B}T)$ as weights. Corresponding energies for US were obtained as averages over the biased ensemble with weights of the form $\exp((U(\xi) - F_i)/k_\mathrm{B}T)$, where U is the US biasing potential and F_i is a free energy constant obtained in WHAM for ith US window. In both cases, uncertainties were calculated as the standard errors of the mean corrected for time-series correlation. The two methods yielded comparable results, at least for well-converged values, and thus the selected data presented in Table 2 have been averaged over the ABF and US results. The complete set of the energetic contributions obtained, separately

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Table 2. Selected Energetic Contributions to ΔG[a]

		$\Delta E_{AmB-ster}$ [kcal/mol]		$\Delta E_{ster-membr}$ [kcal/mol]	
	ξ^* [Å]	vdW	el	vdW	el
ERG	6.5	-9.22 ± 0.40	-1.28 ± 0.22	5.26 ± 0.57	-0.18 ± 0.50
	8.7	-11.93 ± 0.25	-1.27 ± 0.19	8.94 ± 0.46	1.50 ± 0.47
	13.0	-11.15 ± 0.21	-1.59 ± 0.12	8.27 ± 0.54	1.50 ± 0.46
CHO	6.5	-9.75 ± 0.39	0.17 ± 0.18	6.28 ± 0.44	1.07 ± 0.46
	8.7	-8.63 ± 0.37	0.26 ± 0.12	5.99 ± 0.39	0.41 ± 0.46
	13.0	-5.10 ± 0.23	0.03 ± 0.08	5.04 ± 0.45	-1.28 ± 0.47

[a] The contributions were calculated as the respective energy differences between the ξ^*-separated bound and unbound states ($\Delta E_{AmB-ster}$ and $\Delta E_{ster-membr}$ denote the AmB−sterol and the sterol−membrane interaction energy changes, respectively). van der Waals (vdW) and electrostatic (el) contributions are shown separately. The results were averaged over the ABF and US data. The uncertainties were calculated as standard errors of the mean corrected for the correlations in the time series.

for ABF and US, is presented in the Supporting Information (Table SI 1).

Table 2 presents the energetic terms that appear to contribute the most to $\Delta\Delta G$. Note that all these terms are fully converged and that they correspond to the change of the local environment the sterol molecule experiences upon association. In general, it is more energetically favorable for AmB to form tightly bound ($\xi^* = 6.5$) or bound ($\xi^* = 8.7$) complexes with Erg than with Cho. This is mainly due to the AmB−sterol interactions, which, in the case of Erg, compensate more effectively for the unfavorable decrease in sterol−lipid contact area as reflected in positive $\Delta E_{ster-membr}$. With its side chain stabilized by an extra C22−C23 double bond and practically parallel to the planar ring system, the Erg molecule benefits from better van der Waals (vdW) interactions with the planar AmB macrolactone ring as compared to the more flexible Cho molecule.[25,45] The larger electrostatic energy (el) gain observed for Erg is in turn due to the interactions between its 3β-OH group and the AmB polar head (see below).

The configurational entropy change on AmB/sterol association can be approximately expressed as a sum of complex- ($\Delta S_{AmB,ster}$) and environment-related (ΔS_{rest}) terms. The computation of these individual contributions from MD simulations is still a challenging task. Because AmB is a rigid molecule,[13] conformational contribution to $\Delta S_{AmB,ster}$ is dominated by the change in sterol side-chain flexibility due to the AmB−sterol binding. To obtain this contribution, we calculated the weighted covariance matrices for all atoms of the associating sterol, separately for the bound and unbound states, using the US data only, where weights were of the form $\exp(U(\xi) - F_i/k_BT)$. These matrices were then diagonalized and used to obtain entropy estimates within the quasi-harmonic approximation.[46] As expected, the sterol conformational entropy counteracts the association with AmB. For Cho, the tighter is the associate, the more entropically unfavorable it becomes, with $-T\Delta S \approx 1.6$, 3.5, and 5.1 kcal/mol for $\xi^* = 8.7$, 6.5, and 5.5 Å, respectively. For Erg, in contrast, no such trend is observed, with $-T\Delta S \approx 1.5$ kcal/mol obtained for all three ξ^* (see also Table SI 2 in the Supporting Information). This is due to the fact that even though both sterols are being ordered by AmB, the loss in flexibility of the sterol side chain is greater for less rigid Cho. The decrease in the conformational entropy, in the case of Cho, seems to compensate for the energy gain ΔE, whereas it does not fully counterbalance ΔE for more favorably interacting Erg (Table 2). The large entropic penalty might be the reason why Cho cannot approach as close to the AmB molecule as can Erg.

Overall, our analysis indicates that the specificity of AmB for Erg is due to the intrinsic properties of the sterol molecule that, especially in the side-chain region, differ markedly from Cho. Other contributions, apparently less significant in terms of the association energy or entropy, are discussed in the Supporting Information.

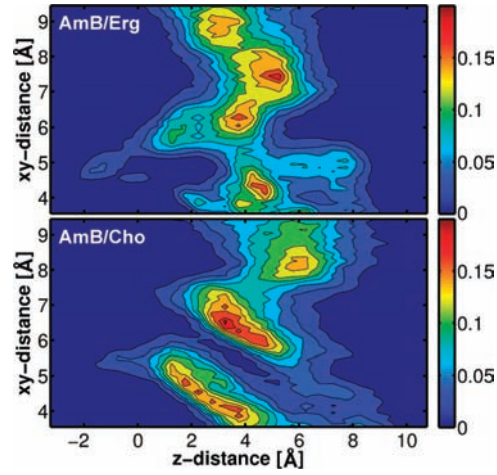

Figure 4. The distributions of distances between AmB and sterol COMs projected onto the *z*-axis (i.e., *z*-distances) calculated separately for each AmB−sterol *xy*-distance. The negative *z*-distances indicate that the sterol COM lies farther away from the aqueous phase than does the AmB COM.

Structure of AmB/Sterol Associates versus AmB Selectivity. To find out how the relative transverse position of the AmB and sterol molecules varies with the distance between them, we calculated the distributions of the *z*-component of the vector connecting their COMs (where the *z*-axis is perpendicular to the membrane surface). These distributions, averaged over ABF and US data, are presented in Figure 4 as a function of the *xy*-distance. It is clear that for both AmB/Erg and AmB/Cho, the energetically optimal *xy*-distance ($4.0 \le \xi \le 6.0$ Å) allows for the greatest relative mobility along the *z*-axis. This is due to the interactions between the sterol 3β-OH group and the AmB poliol chain (the "OH-ladder"), which enable the former group to move deeper into the membrane hydrocarbon core from its typical position at the level of the DMPC carbonyl groups. Figure 4 indicates that Cho generally gets closer to the membrane center and stays there longer, while Erg prefers to interact with the AmB polar head located at the bilayer surface.

The analysis of hydrogen bonds (HBs) between AmB and sterol confirms the above findings. The geometric criterion for hydrogen-bonding presence was used (donor−acceptor distance <3.5 Å and donor−H−acceptor angle >120°). We found that in AmB/Erg complexes, HBs are mainly formed between the sterol 3β-OH group and the AmB polar head moiety, in particular, the OH-43 hydroxyl group (with 13% probability) or the glycosidic linkage O atoms (3%). In contrast, Cho does

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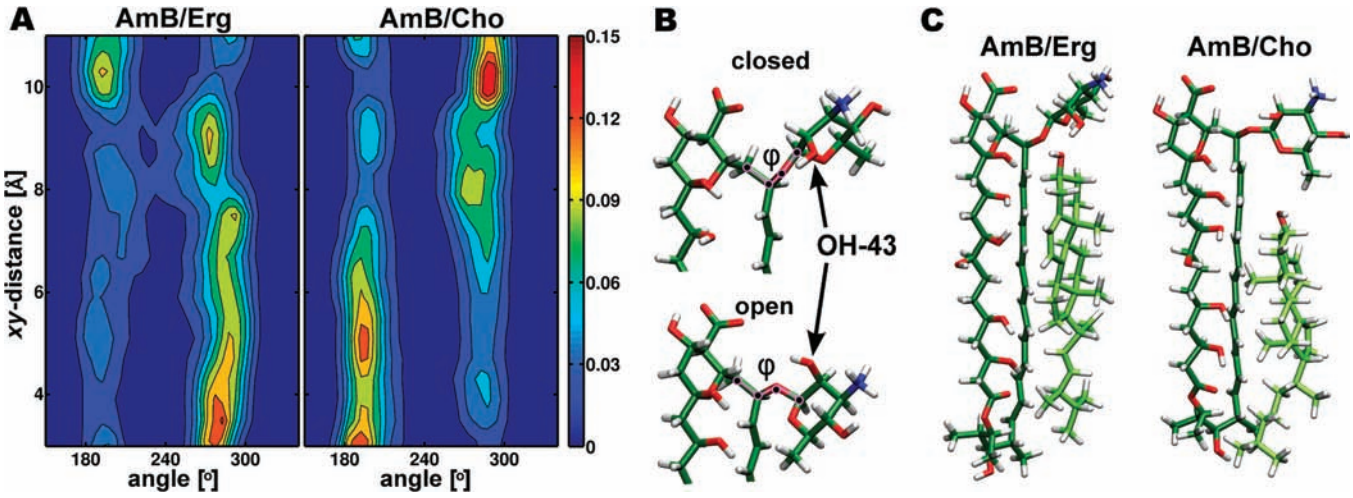

Figure 5. (A) Distributions of mutual orientations of the aminosugar moiety and the macrolide ring described by the φ angle, where φ is C18−C19−O41−C42, and calculated separately for each AmB−sterol *xy*-distance. (B) Zoom-in on the AmB polar head conformations. (C) Comparison of the typical structures of the 1:1 associates formed by AmB with Erg and Cho. The closed conformation (AmB/Erg) leads to a more tightly bound complex than does the open one (AmB/Cho).

not actually participate in HBs with AmB. The only noteworthy HBs are located closer to the membrane center where the 3β-OH group interacts with the AmB poliol chain (OH-9 and OH-8 with 2% and 1% probability, respectively). The above equilibrium probabilities correspond to the tightly bound state and were obtained as PMF-weighted averages of HB probability over the respective *xy*-distance range.

The contrasting HB patterns found for AmB/Erg and AmB/Cho may be explained by the different molecular geometries adopted by the AmB polar head, depending on the type of sterol bound to it. As previously shown, the AmB molecule, when in the membrane, can assume two main conformations. These conformations, that is, the "closed" one defined by the dihedral angle φ (the only flexible dihedral angle C18−C19−O41−C42) of ∼280°, and the "open" one for φ of ∼200°, differ in the orientation of the aminosugar moiety with respect to the planar macrolactone ring.[23,32,47] The current study confirms this observation (Figure 5A,B) and further suggests that the complex formation affects the AmB's conformational behavior in a sterol-dependent manner. In an AmB/Erg complex, conformational equilibrium is shifted toward the closed conformer, whereas the opposite tendency is observed for AmB/Cho. As illustrated in Figure 5C, the closed conformation allows for HB formation between the sterol 3β-OH and AmB OH-43 groups. The open conformation, in turn, prevents sterol from forming close contacts with the AmB polar head and causes it to move deeper into the membrane hydrocarbon core. It was demonstrated above that the Erg molecule and the AmB macrolactone are held together by vdW forces between their relatively rigid and smooth surfaces. These favorable interactions keep the Erg 3β-OH close to the AmB polar head and thus enforce its transition toward the HB-forming closed conformation. For the more flexible Cho molecule, which was found to interact with AmB less intimately, this effect is not observed.

According to the commonly accepted hypothesis, the selectivity of AmB for fungal, as opposed to mammalian, cell membranes is due to its higher affinity for Erg than for Cho. This idea, originally based on the affinity difference found in aqueous solutions, is further supported by our data showing an increase of this difference in the lipid bilayer. The above analysis of the structural properties indicates that the AmB head conformation may influence the complex stability, and thus, according to the hypothesis, may be crucial for the AmB selective toxicity. This is especially since some more selective (less toxic) AmB derivatives, in which the two ionizable groups of the AmB polar head were modified, were previously shown to assume only the closed conformation when in a membrane.[32,48] Therefore, to determine what effect the aminosugar moiety orientation may have on the AmB/sterol association process, we obtained two separate association PMFs for AmB either in the open or in the closed conformation. The profiles shown in Figure 6 were computed by integrating the average force acting along ξ in the same way as the overall ABF-derived profiles, but the force averages were calculated separately over all configurations with $\varphi < 230°$ (open) and $\varphi \geq 230°$ (closed). Note that the presented profiles are only a rough approximation of actual partial PMFs because for both sterols only one of the two conformers is appreciably populated. Figure 6 reveals that

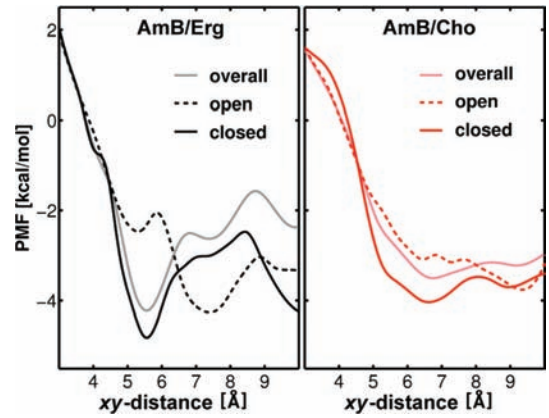

Figure 6. The PMFs describing the AmB/Erg and AmB/Cho association for AmB either in the open or in the closed conformation. The overall ABF free energy profiles are shown for comparison. Note that the PMF values are given to within an additive constant.

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AmB, when in the closed conformation, allows Erg to approach it more closely (preferred distance at $\xi \approx 5.5$ Å), and thus it promotes more stable and tightly bound complexes. The open conformer of AmB, in turn, is responsible for the second minimum (at $\xi \approx 7.5$ Å in the overall PMF) corresponding to the more loose type of complex. The AmB conformation does not appear to have such a strong discriminative effect on binding with Cho. Thus, to sum up, the difference in the affinity of Erg and Cho for AmB in the closed conformation appears to be even more pronounced. Assuming the relation between sterol-binding specificity and selectivity of action, we can suggest that the chemical modifications of the AmB polar head, which lead to stabilization of the closed conformation in a lipid bilayer, should possibly increase selectivity of such a derivative for Erg-containing membranes. This hypothesis is currently being investigated in ongoing research.

Conclusions

Here, we investigated the possibility of the formation of the specific 1:1 AmB/sterol complexes in sterol-containing lipid bilayers. To this end, the free energy profiles along the reaction coordinate defined as the *xy*-distance between AmB and a single sterol molecule were calculated using two independent methods. We found that AmB, when embedded in a lipid bilayer, has a significantly higher affinity for the fungal sterol, Erg, than for its human counterpart, Cho (in a membrane environment, this affinity difference is actually 2−3 times greater than that observed experimentally in aqueous solution). The here demonstrated binding specificity of AmB for the two sterols correlates with the known permeabilizing effect of the antibiotic on the respective membranes, and, therefore, it may be expected to be responsible for the selectivity of AmB action. Indeed, according to the "primary complexes" hypothesis, AmB is more active against fungal membranes, because the more stable AmB/Erg complexes can more effectively assemble into functional transmembrane channels.

We also found that the specific AmB/Cho complexes are not kinetically stable in the lipid bilayer. Because AmB is fairly effective against Cho-containing membranes,[49,50] this instability may, on the one hand, support the experimentally motivated modification of the above model in which the channels formed in the two types of membranes differ in terms of their building blocks.[51] It was suggested that, in the Cho-containing membrane, the channels are formed only at higher AmB concentrations and consist of AmB dimers or higher associates. On the other hand, one may also argue that the actual mechanism by which the affinity difference induces the AmB's selectivity of action is different from that postulated by the "primary complexes" hypothesis. The higher affinity for Erg may, for example, result in a higher capacity of AmB to bind to fungal cell membranes or to Erg-enriched membrane microdomains, which would translate into an increased susceptibility to the drug. This supposition is also consistent with certain experimental findings.[52,53]

According to our data, the difference observed in the AmB affinity for Erg and Cho is mainly of energetic origin. The more rigid and elongated molecular geometry of Erg facilitates favorable interactions with the AmB molecule, both by maximizing the contact area with the macrolactone moiety (vdW interactions) and by inducing the formation of hydrogen bonds with the AmB polar head (el interactions). In the case of Cho, not only is the complexation energy less favorable, but it is also, to a larger extent, compensated by the entropic loss associated with the decrease of the side-chain conformational flexibility. It was also demonstrated that the discussed affinity difference is mainly due to the different chemical structure of the sterols, and, thus, one may speculate that the generalizability of our results is not limited to the chosen simulation model but that they are also valid for other membranes in the lo phase.

The relation between the polar head conformation and the ability of AmB to bind a sterol molecule allows us to suggest that the reduced toxicity of the so-called second generation of AmB derivatives results from the previously observed shift toward the closed conformation.

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Supporting Information Available: Order parameters, complete energetic and entropic contributions, and structural properties. This material is available free of charge via the Internet at http://pubs.acs.org.

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