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The interactions of amphotericin B with various sterols in relation to its possible use in anticancer therapy

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Abstract

Amphotericin B (AmB) is still the most common anti-fungal agent used to treat systemic fungal infections. It is known that this antibiotic acts by forming pores with the ergosterol contained in the membranes of fungi, but it also interacts with the cholesterol contained in the membranes of eukaryotic cells, hence its toxicity. AmB may also interact with the most common oxidation products of cholesterol found in vivo, together with interacting with biosynthetic precursors of cholesterol, namely, lanosterol and 7-dehydrocholesterol (7-DHC). The purpose of the present work was to study the interactions in solution between AmB and these various sterols, the techniques used being UV-Vis spectroscopy and differential scanning calorimetry. The results are globally interpreted in terms of the structural differences between the sterols. We show that AmB selectively interacts with 7-DHC which, according to a recent hypothesis proposed in the literature, has been identified in connexion with a therapeutic strategy against hepatocellular carcinomas. We find that the affinity of AmB towards 7-DHC is even greater than the affinity of the antibiotic towards ergosterol. We also find that AmB selectively interacts with the principal oxidation product of cholesterol, 7-ketocholesterol, a situation that has to be taken into account when AmB is administered.

Keywords: Amphotericin B; Cholesterol; 7-DHC; Oxysterols; Hepatocellular carcinomas

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1. Introduction

Amphotericin B (AmB) has been extensively used in the last 40 years to treat systemic fungal infections that most often affect immune-compromised patients. In some cases, the drug is also administered as a preventive treatment in the case of leukemia, AIDS-affected individuals, or given to subjects who undergo organ transplantation [1–3]. Recently, AmB has also been used in preliminary studies as a potent agent against some viruses, parasites, and even in the case of prion infections [4,5]. It was also suggested quite a long time ago that AmB in the form of Fungizone, the only commercial form of AmB then available, could be used as an anti-cancer agent. However, the high nephrotoxicity of Fungizone soon appeared to be a major problem for such a use, but newer formulations of this antibiotic (e.g. the liposomal preparation known as Ambisome) which allows delivery of higher doses with fewer side effects, could revive the potential use of AmB in anticancer therapy.

In its use as an antibiotic, it is generally thought that AmB acts by interacting more specifically with the ergosterol contained in the fungi membranes, but it also interacts with the cholesterol of the mammalian cells, or that contained in the low density lipoproteins [6], hence its toxicity. These two sterols are very similar molecules, the main difference resulting from the presence in ergosterol of an additional methyl group and a double bond on the side chain, and the presence of an additional double bond on the steroid nucleus. In spite of these small differences in the structure of the two sterols, AmB has been shown, from UV-Vis studies in aqueous solutions, to interact more specifically with ergosterol than with cholesterol [7,8]. However, although the interactions of AmB with sterols occur through hydrophobic interactions, it is not yet clear which substructures on the sterols are predominantly involved in the hydrophobic interactions with AmB. The current explanations in the literature are given either with respect to the presence of the additional bond in the nucleus of ergosterol, which rigidifies this molecule, thus allowing stronger Van der Waals forces to be developed with the rigid heptaene moiety of AmB [9], or with respect to the double bond present in the side chain of ergosterol, which contributes to diminishing the conformational freedom of that chain, thus allowing closer contacts between AmB and this sterol [10,11].

In this context, the purpose of the present work was to study the interactions in solution between AmB and various sterols and to discuss the results in terms of the structural differences in these sterols. For comparison purposes, cholesterol and ergosterol were studied. Also, AmB administered intravenously is known to provoke oxidative modifications of the cholesterol present in the blood [6,12] and, therefore, it is susceptible to interactions with these oxidation products. For these reasons, we also studied the affinity of AmB for the three oxysterols most commonly found in vivo [7-ketocholesterol (7-keto), 7-β-hydroxycholesterol (7-β-hydroxy) and 25-hydroxycholesterol (25-hydroxy)] [13] as compared to the affinity of AmB towards ergosterol and cholesterol. Finally, we also studied the interactions of AmB with two biosynthetic precursors of cholesterol, lanosterol and 7-dehydrocholesterol (7-DHC). These latter sterols were chosen in the context of an exciting new application for AmB in relation to cancer therapy recently suggested by Feigin [14]. This author has proposed a way to substitute cholesterol in hepatomas for its biosynthetic precursors, lanosterol or 7-DHC, without the accumulation of these precursors in healthy liver cells. This can be done through a rich cholesterol diet in addition to the administration of specific inhibitors of cholesterol biosynthesis. Owing to the fact that malignant hepatic cells have lost their regulation of cholesterol biosynthesis, its biosynthetic precursors would accumulate in these cells. Feigin supposes that since AmB has a higher affinity for these precursors as compared to cholesterol [15,16], it would preferably be found in higher amounts in the membranes of malignant cells, resulting in the destruction of these cells. We will, therefore, be in a position to compare the affinity of AmB towards these sterols to that of AmB to the other sterols studied.

2. Materials and methods

2.1. Materials

AmB, dimethyl sulfoxide (DMSO) and the various sterols were purchased from Sigma Co. (St. Louis, MO) while *n*-propanol was obtained from Aldrich Chem. Co. (Milwaukee, WI). 1,2-Dipalmitoylphosphatidylcholine (DPPC, powder) was purchased from Avanti Polar Lipids (Alabaster, AL). The water used to prepare all the solutions was doubly distilled and demineralized on a Sybron–Barnsted system (Fisher Scientific, Montreal, Quebec).

2.2. Preparation of the solutions

AmB dissolved in DMSO was diluted with water to obtain a 50-μM stock solution, always freshly prepared the same day as it was being used. The final concentration of DMSO in the stock solution was 1% (v/v). The sterols were solubilized in *n*-propanol, except 25-hydroxy which, for solubility reasons, was dissolved in DMSO. The samples used in our experiments were prepared from these stock solutions by adding to the AmB solution the required quantity of sterol. The final concentration of AmB was set at 6.5 μM and the solution contained 6.5% (v/v) of *n*-propanol. This specific concentration of AmB was chosen because it represents the concentration of AmB in circulation in the blood after administration [17]. The concentration of sterols was varied from a molar ratio of 1:1 to 20:1, with respect to AmB. The liposomes used in our study were prepared by the freeze–thaw technique from a suspension of DPPC, AmB and 7-DHC, as described elsewhere [18].

2.3. Apparatus used

The UV-Vis spectra were recorded within 30 min of their preparation against a 6.5% (v/v) solution of *n*-propanol in water on a Spectronic 3000 Array spectrophotometer (Milton Roy, Armonk, NY) using a 1-cm cell, the resolution being 0.35 nm. The thermograms were obtained on a Hart Scientific Differential Calorimeter (Calorimetry Sciences, Provo, UT) from 20 to 60°C at a scan rate of 10°C/h. The thermogram of the buffer used is subtracted from the thermogram obtained and then corrected for the thermal delay of the calorimeter. Finally, the critical micellar concentrations (CMC) data were obtained on a Shimadzu RF-540 spectrofluorimeter, the excitation and emission wavelengths being set at 450 nm.

3. Results and discussion

For a better understanding of the discussion to be held below, Fig. 1 presents the molecular structures of the sterols used in the present study. As noted above, the structural differences between cholesterol and ergosterol (the presence of an additional double bond at position 7 on the nucleus, and an additional methyl and double bond on the side chain) are such that this latter molecule would offer a more rigid structure susceptible to maximize hydrophobic interactions with AmB. 7-DHC is the metabolic intermediate immediately preceding cholesterol in the biosynthetic path yielding to this latter sterol, while lanosterol is higher in this biosynthetic chain. 7-DHC is similar in all aspects to cholesterol, except for the presence of a double bond, as in ergosterol, at position 7 on the nucleus. Lanosterol, on the other hand, has a much bulkier nucleus, notably due to the presence of two methyl groups at position 4 of the nucleus, and another methyl group at position 14. Finally, the oxysterols are characterized by the presence of polar substituents (carbonyl or hydroxy groups) at various positions on the sterol structure. Interestingly, 7-keto, the main oxidation product of cholesterol, also has conjugated double bonds at positions 5 and 7.

UV-Vis spectroscopy has been extensively used in the context of understanding the action of AmB as an anti-fungal agent [19–21]. It has thus become well-known that the UV-Vis spectra of AmB in the region of 300–450 nm are very sensitive to the environment around the antibiotic molecule [22,23]. Indeed, the AmB spectra present specific characteristics related either to the

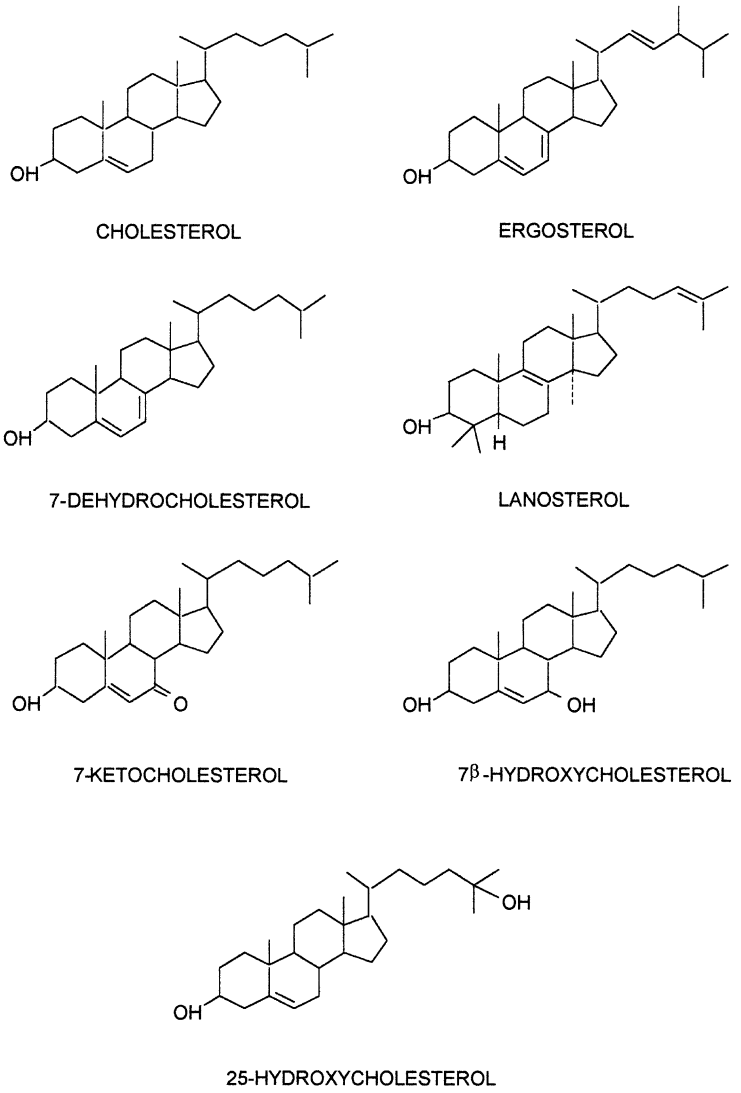

Fig. 1. The structure of the various sterols used.

aggregation state of the antibiotic or to its interactions with sterols, namely [7,18,22,24]. This is apparent in Fig. 2, which presents the spectra of either AmB alone, or mixed with some of the sterols used here, in a 6.5% solution of *n*-propanol in water. This solvent was used to ensure proper solubility of the sterols and, at such a concentration, it has been shown that *n*-propanol has no effect on the interactions of the antibiotic with the sterols [7]. The results presented in Fig. 2 correspond to a sterol to AmB molar ratio of 5:1. The spectrum of AmB alone (Fig. 2a) shows, among others, two characteristic peaks: one located at 409 nm, which has been attributed to the monomeric form of AmB; and one at 328 nm, characteristic of the aggregated form of the antibiotic [7,22,23]. Fig. 2a also shows the spectrum of AmB mixed with lanosterol. It is observed

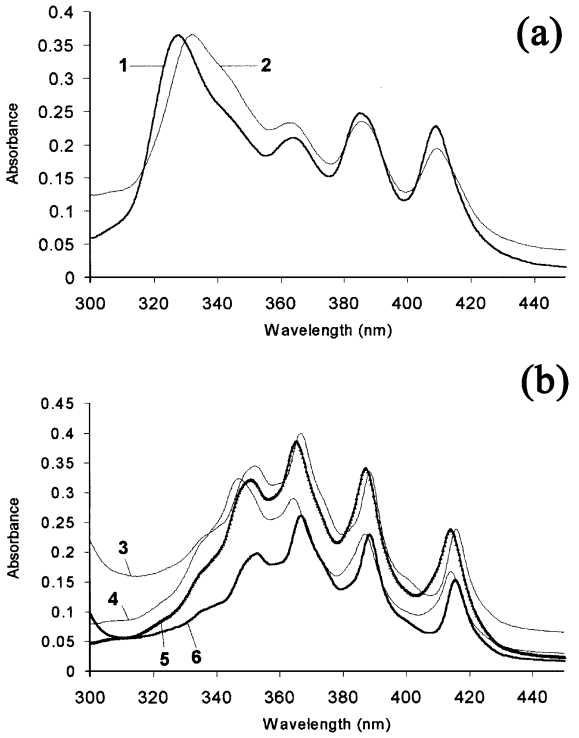

Fig. 2. The UV-Vis spectra of AmB recorded in a 6.5% *n*-propanol solution in water. (1): AmB alone. The other spectra are for AmB mixed at a 5:1 sterol/AmB molar ratio with (2): lanosterol; (3): ergosterol; (4): cholesterol; (5): 7-keto; and (6): 7-DHC.

that the spectrum recorded in this case is almost the same as the spectrum recorded for AmB alone, thus suggesting that lanosterol does not interact with AmB in such conditions. In fact, even when the concentration of lanosterol is increased up to a molar ratio of 20:1, the spectrum of AmB superimposes to that of Fig. 2a, thereby indicating that, indeed, even under such conditions, this sterol does not interact noticeably with AmB. This may not be completely surprising owing, as noted above, to the steric hindrance brought by the methyl groups present at positions 4 and 14 on the nucleus of lanosterol which prevents the close proximity necessary for hydrophobic interactions with AmB to take place.

On the other hand, the spectra recorded for AmB in the presence of all the other sterols are strikingly different. These spectra are shown in Fig. 2b. They manifest the overall shape typical of AmB in interaction with either cholesterol or ergosterol [18]. Fig. 2b also presents, as reference spectra, those recorded for AmB mixed with these two sterols and the spectra of AmB mixed with 7-DHC, the intermediate of cholesterol which, according to Feigin [15], should interact favorably with the antibiotic, or mixed with 7-keto, the main oxidation product of cholesterol found in vivo. The spectra recorded for the two other oxysterols implied in our study bear the same general characteristics as those of Fig. 2b, but, for the sake of clarity, these spectra are not shown here. However, the data obtained with these latter sterols have been incorporated in the summary appearing in Table 1, to be discussed below.

Table 1
The ratio of the absorbance at 415 nm to that at 350 nm for the UV-Vis spectra of AmB in interaction with the sterols under study[a]

AmB	Band I		Band IV		A_{415}/A_{350}
	λ (nm)	*A*	λ (nm)	*A*	
+ Chol.	414.2	0.126	347.2	0.262	0.481
+ Ergo.	416.0	0.149	352.2	0.210	0.709
+ 7-DHC	415.6	0.125	352.6	0.152	0.822
+ 7-keto	414.2	0.210	351.5	0.286	0.734
+ 7β-hydroxy	414.9	0.157	351.5	0.268	0.584
+ 25-hydroxy	414.9	0.186	351.9	0.273	0.681

[a] The results shown are for a 5:1 sterol/AmB molar ratio. The spectral data have been corrected for light scattering, when necessary.

Fig. 2 shows that upon addition of sterols to AmB, the spectrum of the antibiotic tends to present the general characteristics found in the spectrum of AmB in its monomeric form. Indeed, upon interaction with AmB at the concentrations used here, all the sterols, except lanosterol, tend to break the AmB aggregates normally found at the concentration of the antibiotic used here (6.5 μM, final concentration). The band at 328 nm characteristic of such aggregates is indeed much weaker (compare Fig. 2a to Fig. 2b). Two bands of the spectrum will be of particular interest here. One is located at approximately 415 nm. This band has been identified in the literature as characteristic of AmB in interaction with the sterols

[18]. The other band is located at approximately 350 nm. It has been found that upon increasing the sterol/AmB molar ratio, the band at approximately 415 nm is increased while the band at approximately 350 nm is decreased. It has been suggested by Gruda et al. [25] that the interactions between AmB and the sterols could be followed through the ratio of the absorbances at these two wavelengths.

On this basis, Table 1 (column 6) displays the values of the ratio of the absorbance at 415 nm to that at 350 nm for all the sterols under study, with the exception of lanosterol, which does not present a band at 415 nm, indicating that it does not interact with AmB. The spectra that showed an important difference in absorbance between 300 and 400 nm, due to light scattering, were corrected accordingly. The results shown are for a 5:1 sterol/AmB molar ratio. The values observed here, as well as those obtained for all the molar ratios studied, represent the average of at least three different experiments using different batch solutions each time. In this table, band 1 is referred to as the band at approximately 415 nm and band 4 is referred to as the band at approximately 350 nm. One notes that the highest ratio among the sterols used is observed when AmB is mixed with 7-DHC, thereby suggesting that this sterol has the highest affinity for AmB. 7-Keto presents the second highest affinity towards AmB while the other two oxysterols have affinities in between those of ergosterol and cholesterol. This latter sterol has the lowest affinity towards AmB.

The same trend in the results is observed for all the sterols used as shown in Fig. 3, which displays the ratio of the absorbances discussed so far as a function of the various sterol to AmB ratios covered. It is observed that in the sterol/AmB molar ratios range of 1:1–5:1, the selectivity of interaction of AmB with some of the sterols increases rapidly. This is even more so for 7-DHC, 7-keto, ergosterol and 25-hydroxy. For higher sterol/AmB molar ratios, 7-DHC still shows the highest affinity for AmB, while cholesterol shows the lowest. One has to recall that the only structural difference between 7-DHC and cholesterol is the

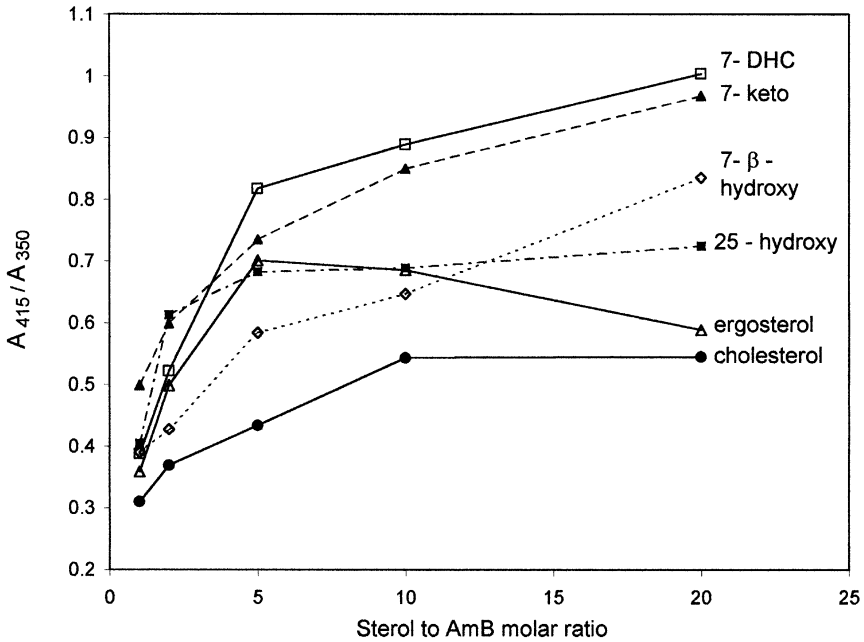

Fig. 3. The ratio of the absorbance at 415 nm to the absorbance at 350 nm for AmB mixed with the various sterols as a function of the sterol to AmB molar ratio. The spectral data used have been corrected for light scattering, when necessary.

presence in the former of an additional double bond at position 7 on the nucleus. This tends to rigidify the sterol backbone, thus enabling the adequate proximity of the sterol with AmB necessary to maximize the hydrophobic interactions between the sterol and the rigid apolar heptaene moiety of the antibiotic. This important difference of affinity of 7-DHC as compared to cholesterol towards AmB is at the heart of the hypothesis proposed by Feigin [14], that AmB would interact specifically with 7-DHC, with the consequence that the antibiotic would accumulate in membranes containing this sterol. Our experimental finding is, thus, most important since it represents the first experimental confirmation that, indeed, 7-DHC can interact favorably with AmB.

Interestingly, ergosterol and 7-keto are the other two sterols that present the highest affinity for AmB. They are also the two sterols that present conjugated double bonds at positions 5 and 7 on the nucleus. According to these results, therefore, one may infer, according to Langlet and collaborators [9], that the presence of an additional conjugated double bond on the nucleus seems to represent the main structural aspect of the sterol, contributing to maximizing its interactions with AmB. This conclusion is reinforced by the fact that lanosterol, which bears additional methyl groups on its nucleus, does not interact with AmB (Fig. 2a) owing to the steric hindrance brought about by these additional substituents. Finally, the other two oxysterols present intermediate affinities, probably due to the presence of additional hydroxy substituents that could interact with the polar constituents also present on AmB.

Inasmuch as ergosterol is concerned, Fig. 3 shows that it presents an atypical behavior as compared to the other sterols. While at the lower sterol/AmB ratios ergosterol shows an affinity for AmB that is comparable to the affinity observed with 7-DHC and 7-keto, its affinity towards AmB decreases at molar ratios higher than 5:1. Visual observations of the samples used in this case led us to conclude that aggregation of the components was taking place. To confirm this, we measured the critical micellar concentration (CMC) of ergosterol in the presence of AmB in the solvent used, i.e. a 6.5% *n*-propanol solution in water. The CMC was determined by light scattering measurements, as discussed elsewhere [24]. A value of 4.4×10^{-5} M was obtained, i.e. lower than the concentrations of ergosterol used to prepare the 10:1 and 20:1 sterol/AmB solutions. It is thus clear that the decrease observed in Fig. 3 for ergosterol is due to the fact that part of the sterol is lost into micelles and precipitates; thus, the sterol is not available to interact with AmB.

In the context of our finding that 7-DHC, owing to its molecular structure, interacts favorably with AmB, together with the fact that this sterol could be implied in a new therapy against hepatocellular carcinomas [14], we were led to explore the behavior of 7-DHC towards AmB in a more biological system. We, therefore, undertook a preliminary study of the thermotropic properties of DPPC bilayers incorporating these two components. The goal was to compare the results obtained with these constituents to those involving AmB, ergosterol and cholesterol that we have published elsewhere [18]. Fig. 4 shows the thermograms obtained for DPPC mixed with 12.5

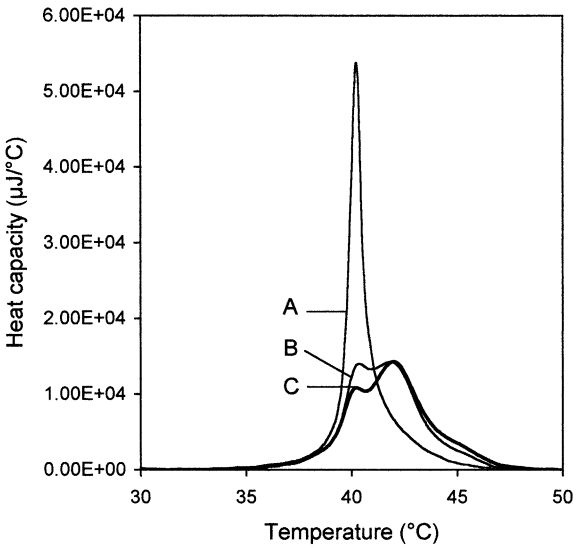

Fig. 4. The thermograms of DPPC + 12.5 mol.% 7-DHC (A); the other thermograms are for this same lipid matrix containing in addition 6.25% or 12.5% AmB (curves B and C, respectively).

mol.% 7-DHC only (curve A). Curves B and C are associated with the bilayer containing in addition 6.25 and 12.5 mol.% of AmB with respect to DPPC. The latter two mixtures thus correspond to a 2:1 and 1:1 sterol/AmB molar ratio, respectively.

Curve A shows a transition centered at 40.2°C, typical of what was observed when cholesterol or ergosterol were present in the membrane, for which the transition temperature was centered at 40.5°C in both cases [18,26]. The effect of adding 7-DHC to a DPPC bilayer, therefore, contributes to the slight decrease of the transition temperature of the pure lipid normally found at 41.4°C, together with decreasing the enthalpy of the transition. When AmB is added to the DPPC + 7-DHC mixture, two phase transitions are clearly observed. The first one is centered at 40.2°C, corresponding to the sterol in interaction with the phospholipid. The relative amount of the phase corresponding to this transition decreases as the concentration of AmB within the membrane is increased. The second phase, centered at 42.1°C, appears at higher temperatures and its relative importance does not seem to change much as the concentration of AmB is increased. As observed with cholesterol and ergosterol, the fact that a new phase transition appears at temperatures higher than that recorded for DPPC itself or for DPPC mixed with 7-DHC implies that the presence of both the sterol and AmB have a structuring effect on the phospholipid. In comparison, in our previous study, when both cholesterol and AmB were present in the membrane, a destabilizing effect was observed, the transitions being broadened to a large extent as the amount of sterol was added. It is clear that such an effect is not observed here with 7-DHC. On the other hand, similar to what was observed in our previous study when ergosterol and AmB were present in the bilayer [18], the position of the high temperature peak when both 7-DHC and AmB are present does not change. However, contrary to what was observed with ergosterol, the thermograms of Fig. 4 clearly show the presence of the transition at 40.2°C, characteristic of the interactions between the lipid and 7-DHC. One must, therefore, conclude that the affinity of 7-DHC for DPPC is greater than the affinity of ergosterol for this lipid. It is clear, though, that the general behavior of 7-DHC in a DPPC matrix containing AmB more closely resembles that of ergosterol contained in an AmB-DPPC matrix than that of cholesterol under the same conditions. By this technique, therefore, through comparisons with the systems previously studied [18], one can also conclude that the affinity of 7-DHC towards AmB in a lipid matrix is higher than the affinity of cholesterol towards the antibiotic.

In conclusion, the present work has shown that the interactions of sterols with AmB are correlated to the presence of conjugated double bonds on the sterol nucleus. These conjugated double bonds contribute to rigidifying the sterol backbone, thus allowing the molecular proximity necessary to maximize the hydrophobic interactions with AmB. In this sense, our results tend to support the idea prevailing in the literature [9] that this structural feature, more than the presence of an additional double bond on the side chain [11], is the determinant factor in understanding the interactions of sterols with AmB. Our finding that AmB has strong affinities with 7-DHC also gives support to the idea that AmB could be used in a therapeutic strategy against some forms of cancer [14], provided that this sterol, through diet and adequate medication, can indeed accumulate in the membranes of malignant cells. However, beyond the biophysical studies presented here, further work using AmB against tumor cells will be needed to ascertain this point. Finally, our results have also shown that AmB interacts favorably with 7-ketocholesterol, the main oxidation product of cholesterol found in vivo, a situation that has to be taken into account for the possible use of this antibiotic either as an anti-tumor agent or in the newer therapies that are being proposed against viral or prion infections.

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