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The structuring effects of amphotericin B on pure and ergosterol- or cholesterol-containing dipalmitoylphosphatidylcholine bilayers: a differential scanning calorimetry study

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Abstract

Amphotericin B (AmB) is the most widely used polyene antibiotic to treat systemic fungal infections which affect an increasing number of immunocompromised patients. It is generally thought that AmB forms pores within the fungi membranes by interacting with ergosterol, the main sterol of fungi. However, it also interacts with the cholesterol contained in mammalian cells, hence its toxicity. In order to have a better understanding of the interactions prevailing between AmB and sterols, differential scanning calorimetry was used to study various mixtures incorporating from 6.5 to 25 mol% of AmB in pure dipalmitoylphosphatidylcholine (DPPC) vesicles and in ergosterol- or cholesterol-containing DPPC vesicles. The sterol concentration was kept constant at 12.5 mol% with respect to the phospholipid. Our results show that three phases coexist when AmB is dispersed in the pure phospholipid. One corresponds to the phospholipid phase alone. The two others are characterised by a broad transition at temperatures higher than the main transition temperature of the pure phospholipid, corresponding to the drug in interaction with the aliphatic chains of the lipid. The fact that the transition temperatures of these additional components are higher than that of the pure phospholipid suggests that AmB interacts strongly with the aliphatic chains of the lipid, consistent with the idea prevailing in the literature that AmB by itself may form pores in a lipid matrix. When AmB interacts with cholesterol-containing bilayers the thermograms also present three components. Upon increasing the concentration of AmB, though, an important broadening of these components is observed which is explained in terms of destabilisation of the organisation of the aliphatic chains. The situation is strikingly different if ergosterol is present in the lipid matrix. The thermograms remain unmodified as the concentration of AmB is increased and a broad transition, now involving only two components when the thermograms are decomposed, is observed. An analysis of the results shows that various interacting units, e.g. AmB+DPPC and (AmB+ergosterol)+DPPC, are present within the membrane. These units involve the phospholipid and hence contribute to its structurisation. The important differences between the thermograms obtained with the ergosterol- as compared to the cholesterol-containing bilayers, in spite of the structural similarity of these two sterols, provides strong evidence for the selectivity of interaction of AmB with ergosterol as compared to cholesterol. It is thus clear that the action of AmB on cholesterol- as compared to ergosterol-containing membranes results from different mechanisms. Finally, UV-visible spectra of AmB in pure as well as sterol-containing DPPC vesicles show the presence of absorption bands that give support to the interpretation derived from the calorimetric data.

Keywords: Amphotericin B; Ergosterol; Cholesterol; Lipid vesicles; Differential scanning calorimetry; UV-visible spectroscopy

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1. Introduction

Amphotericin B (AmB) is the most potent and effective polyene antibiotic clinically available. It has become the most widely used drug to treat serious systemic fungal infections, which have become an important clinical problem owing to the increasing number of immune-compromised patients [1,2]. Unfortunately, the classical formulation of AmB, Fungizone, has negative side effects (e.g. nephrotoxicity) that seriously impair its efficacy [3]. It has been shown that AmB acts at the membrane level where it increases the permeability of the cells to ions and small molecules. This activity depends on the presence of sterols in the membrane [1,4] and it has been proposed in the 1970s that the interaction between membrane sterols and AmB is responsible for the selectivity of the drug. It has therefore been assumed that the selective toxicity of AmB for fungi results from its capacity to bind more strongly to ergosterol, the principal fungal sterol, than to cholesterol, the principal sterol of mammalian cells [3,5].

Indeed, it has been suggested that AmB–sterol complexes would exist in the cell membrane environment, an idea originating from the fact that such complexes are present in aqueous solution [6–8]. It is now generally admitted that in phospholipid membranes, AmB associates with the sterols to form hydrophilic pores in which the AmB molecules are in a quasi-parallel orientation, their polar side pointing towards the inside of the pore and their lipophilic part interacting with the lipid environment [9,10]. The presence of such structures in a cellular membrane increases its permeability and causes damages resulting ultimately to the death of the cell [10–14]. It has been shown that ergosterol-containing membranes are more sensitive to the action of AmB than cholesterol-containing membranes [6,13,15–18] and as a consequence, the concentration of AmB necessary to obtain the same permeability is higher with cholesterol than with ergosterol [13].

However, the detailed molecular mechanism of the interaction of AmB with the membrane, as well as the formation of a transmembrane pore structure, are still imperfectly understood. In fact, in the light of recent observations, it appears that the mechanism of action of AmB on a cellular membrane is more complex than previously thought. For instance, interactions of AmB with the various components of the cell membrane (e.g. lipids and proteins), inducing lipid peroxidation [19], inhibition of membrane pumps [20,21], blockade of endocytosis and immune stimulation [22], have been invoked.

When studies are made with model membranes, the situation does not seem to be simpler and contradictory results are found in the literature. For example, Fujii et al. [23] have shown that sterols are not mandatory for the formation of AmB channels across the lipid membrane and thus that AmB may interact specifically with phospholipids, in contradiction with results previously published by Ockman [24]. On the other hand, Bolard et al. [25] have shown that when large unilamellar vesicles of egg phosphatidylcholine are exposed to the action of AmB, only the ergosterol- but not the cholesterol-containing membranes show the presence of pores, thereby implying that the action of AmB on cholesterol-containing membranes involves a different mechanism. This is in contradiction with the work done by Butyan and McPhie [26], who have shown that both cholesterol- and ergosterol-containing BLMs form channels of the same molecular structure, with the difference that the ergosterol–AmB channels have longer open-channel lifetimes.

In this context, in order to shed some light on the interactions between AmB, phospholipids and the sterols, we have performed differential scanning calorimetric studies on dipalmitoylphosphatidylcholine (DPPC) vesicles containing AmB or containing AmB and either cholesterol or ergosterol. The results show that AmB by itself induces a phase separation within the membrane, as clearly evidenced by the presence of new components in the thermogram at temperatures higher than that of the pure DPPC. In addition, when AmB is added to cholesterol- or ergosterol-containing DPPC vesicles, the thermograms of the two series of mixtures are completely different, although the effect of the sterols themselves on the thermogram of DPPC is the same. These results are interpreted in terms of the selectivity of interaction of AmB with the two sterols. We also show that the results are consistent with the presence of AmB–ergosterol units (or complexes) within the membrane matrix that have a structural ordering effect on their lipid environment.

2. Materials and methods

2.1. Materials

AmB, cholesterol, ergosterol and dimethyl sulphoxide (DMSO) were purchased from Sigma (St. Louis, MO). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, powder) was purchased from Avanti Polar Lipids (Alabaster, Al). Chloroform was obtained from Anachemia (spectroscopic grade, Montreal, Que.). The water used to prepare all the solutions was distilled and demineralised on a Sybron-Barnstead system (Fisher Scientific, Montreal, Que.). Phosphate buffer (PBS) 0.02 M at pH 7.0 was used for the liposome preparation.

2.2. Preparation of the liposome solutions

DPPC, ergosterol and cholesterol are first dissolved in chloroform while AmB is solubilised in the smallest volume of DMSO possible. From these batch solutions, a solution at the final concentration of the various components is prepared in a volumetric flask. The solvent is then evaporated (Rotovapor R 110, Buechi-Brinkman, Germany) to dryness and the lipid film is resuspended in the PBS buffer. It was verified by FTIR spectroscopy that at the concentrations used, DMSO was completely removed from the suspension during the evaporation. Large unilamellar vesicles are prepared from this lipid suspension through four freeze–thaw cycles, the high temperature of the cycle being at 50°C, i.e. higher than the main transition temperature of DPPC. In all cases, the final lipid concentration used was 4 mg/ml (5.45×10^{-3} M) while the concentration of the sterol was fixed at 12.5 mol% with respect to the phospholipid. Three different concentrations of AmB were used: 6.25, 12.5 and 25 mol% with respect to the lipid. The UV-visible spectra taken from the supernatant solution when the vesicles are centrifuged indicate that more than 98% of the AmB is indeed incorporated in the vesicles.

2.3. Differential scanning calorimetry

The thermograms were recorded on a Hart Scientific Differential Scanning 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. The decomposition of the thermograms was obtained by further processing the data using the software 'SPECTRA+' [27], based on algorithms developed by Barwicz et al. [28,29]. The software provided us with the parameters of the thermogram under study (magnitude, transition temperature, area under the peaks, half-height width, notably).

The molar enthalpy of the transition was calculated from the total area under the peak divided by the total number of moles of DPPC in the preparation. Each thermogram has been done at least three times to ensure reproducibility.

2.4. Absorption UV-visible spectroscopy

The UV-visible spectra were recorded against the pure PBS buffer on a Spectronic 3000 Array spectrophotometer (Milton Roy, Armonk, NY) using a 0.1-cm cell, the resolution being 0.35 nm.

3. Results and discussion

The thermograms of pure DPPC (4 mg/ml, curve A), DPPC plus cholesterol (curve B) or ergosterol (curve C) are presented in Fig. 1. The thermogram obtained here for the pure DPPC shows a first thermal transition at 33.5°C, corresponding to the pretransition of the lipid, and a second transition at 41.4°C, corresponding to the main gel to liquid–crystalline phase transition. Our thermogram is identical to those found in the literature for DPPC [30,31]. Fig. 1 also indicates that the effect of cholesterol or ergosterol (at the concentration used here, 12.5 mol%) on the thermogram of the phospholipid is almost identical: in both cases, the pretransition is abolished and the main transition peak is decreased, broadened and shifted to lower temperatures (40.5°C in both cases). Our results for the mixed cholesterol+DPPC vesicles are in close agreement with the literature data [31], while those with ergosterol have not been reported so far. It would therefore seem that the two sterols, considering the similarity of their molecular structure and the similarity of their thermograms when mixed with DPPC, would act in

the same way towards the lipid. Upon insertion of the sterol within the hydrophobic region of the phospholipid (it is generally admitted that the sterol is inserted paralleled to the aliphatic chains of the lipid), a disruption of the molecular interactions prevailing between the aliphatic chains results, thus yielding to a broadening of the main transition peak and a shift of this peak towards lower temperatures. These features correspond to what is generally expected when additives are mixed to a lipid.

A closer look at Fig. 1 also reveals that the transition peak of the lipid mixed with either sterol is not symmetrical. This feature is in agreement with the thermograms of DPPC mixed with cholesterol recently published by McMullen et al. [31] and Vist and Davis [32]. Indeed, the decomposition of the DSC endotherms (Fig. 1, dotted lines) shows the superimposition of a sharp and a broad component, the former being associated to the melting of sterol-poor regions within the bilayer while the latter is associated to the melting of sterol-rich DPPC domains. The striking similarity between the endotherms when ergosterol or cholesterol are mixed with DPPC suggests that for ergosterol also, poor- and rich-ergosterol regions would be found within the bilayer. However, a complete phase diagram of ergosterol mixed with DPPC would be necessary to clarify this situation, a task beyond the goal of the present work.

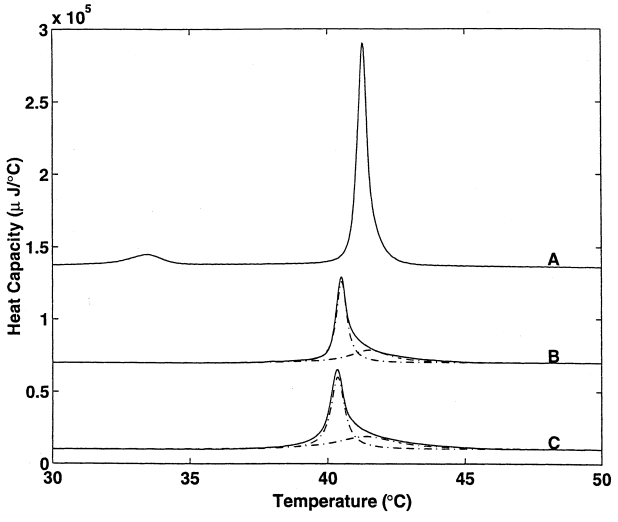

Fig. 1. The effect of cholesterol and ergosterol on the thermotropic phase behaviour of DPPC. A, DPPC; B, DPPC+12.5 mol% cholesterol; C, DPPC+12.5 mol% ergosterol. Dotted lines: the components of the decomposed thermograms. The thermograms are displaced vertically for more clarity.

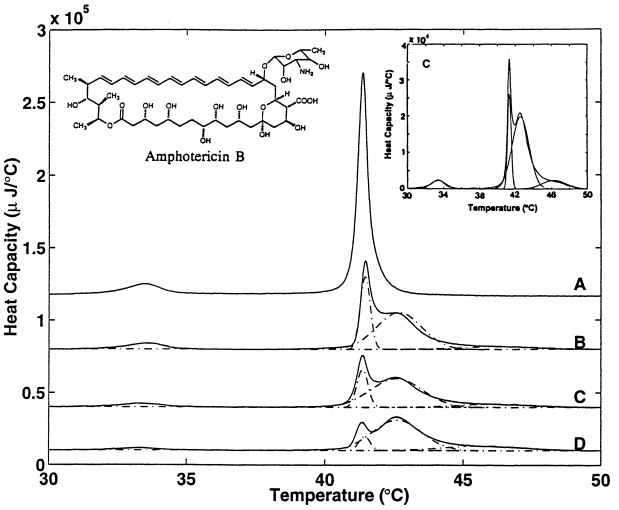

Fig. 2. Thermograms of DPPC vesicles incorporating AmB. A, DPPC; B, DPPC+AmB 6.25 mol%; C, DPPC+AmB 12.5 mol%; D, DPPC+AmB 25 mol%. Dotted lines: the components of the decomposed thermograms. The thermograms are displaced vertically for more clarity. Upper left: the molecular structure of AmB. Upper right: as curve C, but drawn to a different scale.

The effect of AmB itself on the thermogram of DPPC is shown in Fig. 2. In order to compare the results, the thermogram of the pure lipid is also reproduced in Fig. 2A while Fig. 2B–D present the results obtained for the mixtures at increasing concentrations of the drug (i.e. 6.25, 12.5 and 25 mol%). As the concentration of the drug increases, one observes that the pretransition and the main transition peaks of the lipid are decreased without affecting the transition temperatures or the cooperativity of the transition (this latter point will be discussed along with Fig. 6). The thermograms of the mixtures show that two new phase transitions are appearing at temperatures higher than that of the pure phospholipid. One is centred at 42.6°C, this phase taking more importance as the concentration of AmB increases. The second one is broad, centred at ~46°C. Its importance is also function of the amount of AmB present in the bilayer although Fig. 2B–D do not present this feature properly. To clarify this point, Fig. 2 (right upper corner) also presents one of the thermograms (thermogram C) drawn to a different scale which shows more clearly the presence of the component at ~46°C. It is thus

clear that three phases are co-existing when AmB is dispersed in the phospholipid, one corresponding exactly to the pure lipid phase alone (the main transition temperature of the pure DPPC is indeed observed in the thermograms even at 25 mol% of AmB), the others, characterised by broad transitions, corresponding to the lipid phase in interaction with the drug.

In order to rationalise the effect of AmB on the thermotropic transitions of the lipid, it might be important to recall that AmB is an amphiphilic molecule with a very special rigid structure: the hydrophobic side contains seven conjugated double bonds while the hydrophilic side contains several polar substituents (see insert, Fig. 2). It is generally admitted that in phospholipid membranes the AmB molecules are present as aggregates and form hydrophilic pores [9,10]. In this configuration, the lipophilic parts of AmB molecules are in contact with the aliphatic chains of the phospholipid molecules while the polar groups of AmB are located on the inner side of the pore, forming hydrogen bonds with water molecules. Such a molecular organisation provides ideal conditions for a rigidifying action of the acyl chains of the lipid by AmB through enhanced Van der Waals interactions. At the drug concentrations presented in this paper it is more than probable that the AmB molecules contained in the DPPC vesicles are indeed organised as aggregates as suggested by Fuji [23], these aggregates, in the light of the current literature on AmB, being most likely what is referred to pores.

Therefore, the fact that our mixtures present phase separations, together with the fact that the temperatures of the new transitions observed are higher than that of the pure phospholipid strongly suggest that AmB has a rigidifying effect on the hydrophobic part of the membrane which could directly result from the molecular arrangement of the drug within the bilayer, as discussed above.

The ability of AmB to rigidify its lipid environment has also been invoked by others in the literature. For instance Dufourc et al. [33], from ^{2}H-NMR studies on DMPC liposomes containing AmB, have noted a monotonic ordering effect of all the positions along the acyl chain of DMPC in contact with AmB. This ordering effect has also been suggested to explain the permeability results obtained with DPPC liposomes [34,35]. In addition, from an analysis of CD spectra of AmB in sterol-free vesicles, Balakrishnan et al. [36] have proposed the existence of an organised multimolecular structure within the bilayer, in which AmB interacts with the acyl chains, forming a 1:1 complex. This complex would form a non-aqueous pre-pore structure according to an hypothesis brought by Cohen [35]. In fact, the idea that AmB by itself, in the absence of sterols, would form pores within the membrane has been inferred by different authors in the literature [37–40]. In the light of the results presented here, however, it is clear that the presence of such units within the membrane would contribute to rigidify the lipid environment.

Although, as discussed, what is known about AmB dispersed within membranes does suggest that the drug could have a rigidifying effect on the lipid environment, it is not possible to completely rule out the fact that the increase in the transition temperature we observe might also be interpreted in terms of mismatch effect between AmB and its lipid environment. Indeed, according to Zhang et al. [41] the hydrophobic thickness of the bilayer formed by DPPC in the gel state is 39.4 Å. The length of the hydrophobic part of the AmB molecule is close to 20 Å [42], thus corresponding almost exactly to the hydrophobic thickness of one layer of the DPPC membrane in the gel state. On the other hand, during the main transition, the mismatch between the hydrophobic length of an AmB molecule and the hydrophobic thickness of the DPPC bilayer (32.9 Å on average in the phase transition [41]) cannot be excluded. However, the effect of mismatch is suggested here as an additional, but minor effect and cannot replace, in our opinion, the important rigidifying effect of AmB. In this sense, both effects could contribute to the increase of the transition temperature observed.

The effect of AmB on the DPPC bilayer containing 12.5 mol% cholesterol is presented in Fig. 3, which also presents the decomposition of the endotherms. To ensure an easier comparison of the results, Fig. 3A reproduces the thermogram of the lipid containing 12.5 mol% of cholesterol (i.e. Fig. 1B) while Fig. 3B–D represent the thermograms of the cholesterol-containing DPPC bilayers in the presence of increasing concentrations of AmB (6.25, 12.5 and 25 mol%, Fig. 3B–D, respectively). At 6.25 mol% of AmB (Fig. 3B), the decomposed thermogram also

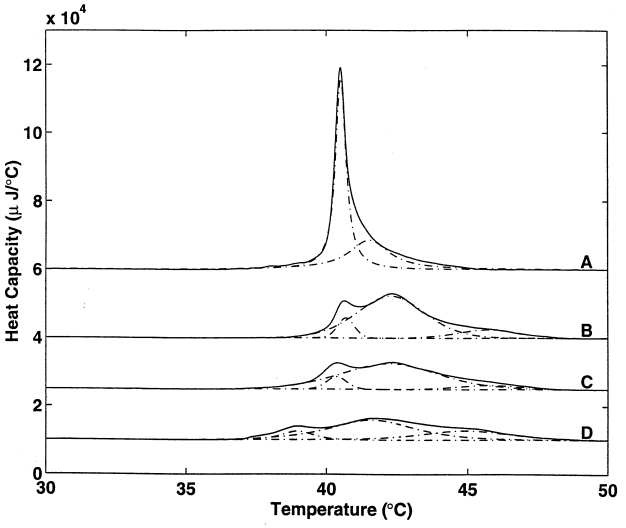

Fig. 3. The effect of AmB on the thermotropic phase behaviour of DPPC vesicles containing 12.5 mol% cholesterol. A, DPPC+12.5 mol% cholesterol. From B to D, the bilayer contains in addition 6.25, 12.5 and 25 mol% of AmB. Dotted lines: the components of the decomposed thermograms. The thermograms are displaced vertically for more clarity.

shows that a phase separation is occurring, one of the phases being DPPC in interaction with the sterol (the endotherm at 40.5°C in Fig. 3B corresponds to the endotherm in Fig. 3A), the other phases presenting transition temperatures at 42.3 and ~46°C. These phases may correspond either to DPPC in interaction with AmB (the peaks in Fig. 3B are found at the same temperatures as those observed in Fig. 2B–D for the binary AmB+DPPC mixtures) or may also correspond to DPPC+cholesterol in interaction with AmB.

However, as the concentration of AmB is increased from 6.25 to 25 mol%, the transitions at 40.5, 42.3 and ~46°C are broadened and shifted towards lower temperatures, a point that will be presented further (Fig. 6). This indicates an important destabilisation of the organisation of the aliphatic chains of the phospholipid. Since neither cholesterol alone at the concentration used here (Fig. 1B) or at higher concentrations (up to 25 mol%, [31,32]), nor AmB (Fig. 2B–D) present such an effect when mixed with DPPC, one must conclude that the destabilizing effect observed in Fig. 3C,D results from the presence of the two components, AmB and cholesterol, simultaneously within the bilayer. The fact that we observe separated phases when AmB is incorporated in cholesterol-containing lipid vesicles is consistent with the results reported by Balakrishnan and Easwaran [36] and Fujii et al. [23]. Thus, the general behaviour of the various peaks as the concentration of AmB is increased in presence of cholesterol, is different from what was observed when AmB itself was mixed with DPPC (Fig. 2). This shows how cholesterol may perturb the interactions between AmB and the phospholipid.

Strikingly different results are obtained if cholesterol is substituted for ergosterol in the membrane. Fig. 4A presents the thermogram of DPPC containing 12.5 mol% ergosterol while Fig. 4B–D show the thermograms of the ergosterol-containing DPPC bilayers in the presence of increasing concentrations of AmB (6.25, 12.5 and 25 mol%). One notes that the thermograms for the ternary mixtures are very similar for the various AmB concentrations used. A broad transition, centred at about 42.7°C, is recorded which remains unmodified when the concentration of AmB is increased by a factor of 4. The transition temperature observed here for the ternary mixtures is similar to the one found in Fig. 2B–D when AmB alone was mixed with the lipid. In addition, the decomposition of the thermograms shows a small, broad transition at about 40.5°C which corre-

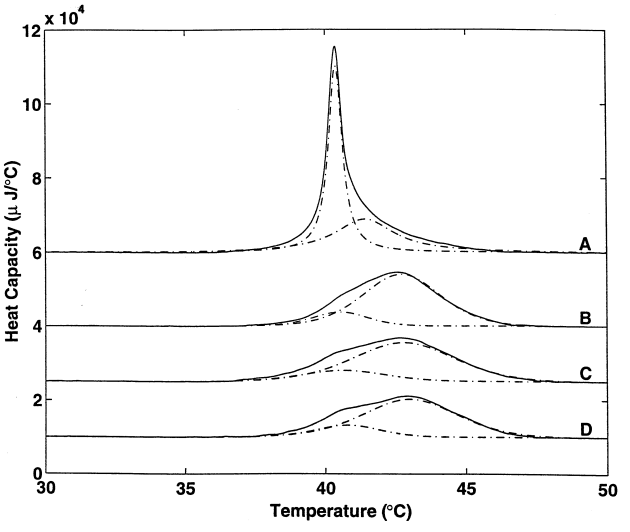

Fig. 4. The effect of AmB on the thermotropic phase behaviour of DPPC vesicles containing 12.5 mol% ergosterol. A, DPPC+12.5 mol% ergosterol. From B to D, the bilayer contains in addition 6.25, 12.5 and 25 mol% of AmB. Dotted lines: the components of the decomposed thermograms. The thermograms are displaced vertically for more clarity.

sponds to the transition temperature observed for the binary ergosterol+DPPC mixture. The important differences between the thermograms obtained with the ergosterol- as compared to the cholesterol-containing bilayers, in spite of the structural similarity of these two sterols, bring a strong evidence for the selectivity of interaction of AmB with ergosterol- as compared to cholesterol-containing lipid bilayers. Such a selectivity of interaction with sterol-containing systems has also been observed by us in a previous work using monolayers [43].

The fact that the thermograms of ergosterol and DPPC incorporating various amounts of AmB remain almost unmodified upon increasing the concentration of AmB by a factor of 4 is an important result. It is certainly not accidental either that the broad transition recorded for the ternary mixtures studied here (Fig. 4B–D) is centred around the same temperature (42.7°C) as the phase transition recorded when AmB by itself is dispersed in DPPC (Fig. 2B–D). In this latter case, as discussed above, the phase transition was associated to the presence of regions enriched in AmB within the bilayer in which the lipid is organised by its interactions with AmB.

In addition, the observation that the magnitude of the peak at 40.5°C associated to the ergosterol–lipid interactions is so much decreased even for the lowest concentration of AmB used (Fig. 4B) strongly suggests that AmB interacts favourably with ergosterol thus diminishing the amount of ergosterol molecules free to interact with the lipid. The situation was indeed completely different when cholesterol was present in the bilayer. In this latter case, the peak at 40.5°C corresponding to the lipid in interaction with the sterol was always clearly present (Fig. 3B) and contributed significantly to the overall transition recorded in this case. On the other hand, the corresponding peak when ergosterol is used almost disappears and becomes included in a broad transition centred at 42.7°C. Also, the fact that this latter peak is much broader than the peak observed at the same temperature when AmB itself interacts with DPPC (compare Fig. 2D to Fig. 4D) indicates that the cooperativity of the transition is much lower. This shows that various interacting units, e.g. AmB+DPPC and (AmB+ergosterol)+DPPC, might be present within the membrane which rigidify their lipid environment, the transition being again observed at temperatures higher than the main transition temperature of DPPC. This is also a strong indication that the lipid is involved in the interactions with AmB and ergosterol, an idea which is reinforced when one realises that, as the concentration of AmB is increased up to two molecules of AmB per molecule of ergosterol (Fig. 4D), one still observes that the broad transition is not modified.

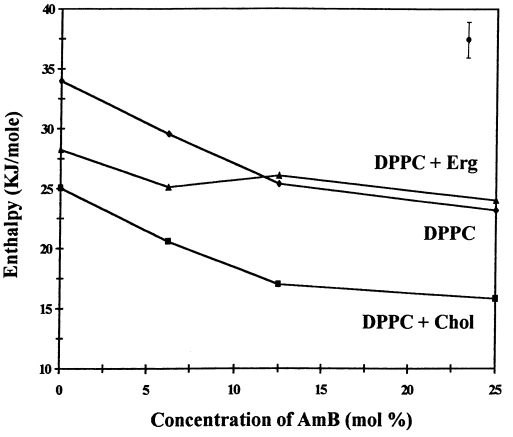

Fig. 5. The effect of AmB on the total molar enthalpy.

The analysis of our results can be pushed further by calculating the overall transition enthalpy for all the systems studied, as presented in Fig. 5. The total molar enthalpy obtained for the pure DPPC is about 34.0 kJ/mol, a value in agreement with the data obtained by McElhaney et al. (32.4 kJ/mol, [31]) and Finegold et al. (36.4 kJ/mol, [44]). In the presence of 12.5 mol% of either sterol alone, the molar enthalpy of the lipid decreases to 25.0 kJ/mol for the cholesterol-containing DPPC vesicles, a value in agreement with the literature, ~24 kJ/mol [31]. For the ergosterol-containing DPPC vesicles, on the other hand, the value obtained is 28.2 kJ/mol (data not available in the literature). The different enthalpy values for these two systems suggest that, in similar conditions, the perturbing action of cholesterol on the phospholipid chains is more important than that of ergosterol even though the temperature of the main transition in both cases is almost the same (Fig. 1).

When AmB is introduced either in the lipid alone or in the sterol-containing vesicles, the general tendency observed is that the molar enthalpy of the overall transition is decreased, although the situation is not that clear for the ergosterol-containing vesicles. Indeed, the decrease in the molar enthalpy observed

when the concentration of AmB is increased up to 25 mol% is about 10 kJ/mol for either the pure DPPC or the cholesterol containing vesicles while the decrease is only 4 kJ/mol for the ergosterol-containing vesicles. One also notes that at 12.5 mol% AmB or higher the molar enthalpy for the pure DPPC or the ergosterol-containing vesicles is about the same. So, what is observed here is the effect of the ergosterol–AmB complexes on the overall thermotropic transition of the phospholipid, a situation which is strikingly different from what is observed when cholesterol instead is present in the bilayer (compare the results of Fig. 5 for the ergosterol and cholesterol-containing bilayers). In the latter case, the results obtained can be interpreted as representing the sum of both the effects of AmB and cholesterol on the lipid.

The decomposition of our thermograms allows us to investigate the detailed effect of the incorporation of AmB on either the main transition temperatures or the cooperativity of the transitions (half-height width), as shown in Fig. 6A and B, respectively. In Fig. 6, the various peaks resulting from the decomposition of the thermograms of Figs. 2–4 are identified by a number referring to the position of the peaks and a code identifying the system under study. Thus 1-Chol refers to the peak centred at 40.5°C for the cholesterol-containing lipid vesicles while 3-DPPC, for example, refers to the peak centred at ~46°C for the sterol-free DPPC. The positions of the various peaks are thus plotted as a function of the AmB concentration.

Fig. 6A shows that the transition temperatures of the various peaks of the decomposed thermograms do not vary, generally, as the concentration of AmB is increased. The only exception is when 12.5 mol% cholesterol and AmB are present in the bilayer. In this case, the transition temperature associated to the first peak is decreased by about 1.5°C (1-Chol) while the decrease is about 0.6 and 0.9 for the second (2-Chol) and third peak (3-Chol), respectively. This was explained above by the important destabilisation of the organisation of the aliphatic chains of the phospholipid due to the presence of both AmB and cholesterol in the membrane.

Fig. 6B presents the cooperativity of the various transitions observed for all the systems studied. Inasmuch as the first component of the transitions is

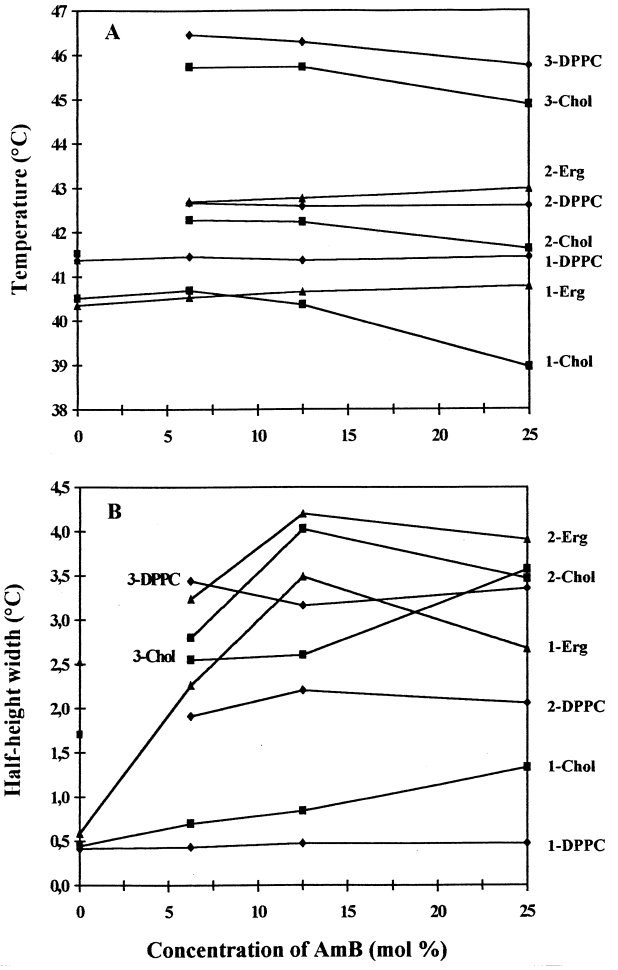

Fig. 6. The effect of AmB on the various components of the thermograms for all the systems studied plotted as a function of the concentration of AmB. (A) Transition temperature. (B) Half-height width. The curves are identified by a number referring to the various components of the decomposed thermograms and a code identifying the system under study. Thus 1-Chol refers to the peak centred at 40.5°C for the cholesterol-containing lipid vesicles (Fig. 3) while 3-DPPC refers to the peak centred at ~46°C for the sterol-free DPPC (Fig. 2).

concerned, Fig. 6B (1-DPPC, 1-Chol, 1-Erg) shows that the half-height width of the transition when no AmB is present is minimal and almost the same for the three systems, ~0.5°C, thereby implying that the cooperativity of the transitions is high, and similar. However, when AmB is introduced in these systems, the half-height width of the transition is changed dramatically (rising from 0.5°C to 3.5°C) for the ergosterol containing vesicles while it increases by less than 1°C for the cholesterol containing ones and does not vary at all for the sterol-free DPPC vesicles.

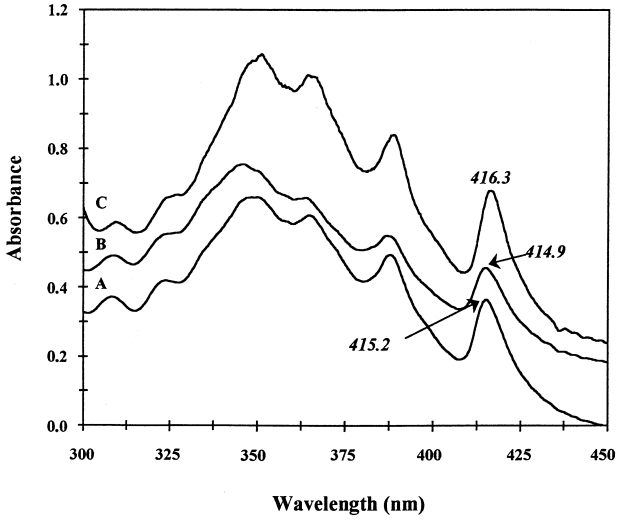

Fig. 7. UV-visible spectra of AmB incorporated into lipid vesicles. A, AmB+DPPC; B, AmB in cholesterol-containing DPPC vesicles; C, AmB in ergosterol-containing DPPC vesicles. In all cases, AmB was present as 6.25 mol% with respect to DPPC.

Therefore, the cooperativity of the transition for the ergosterol containing vesicles is much lower than that of the cholesterol or sterol free vesicles. As was explained above, this can be rationalised by the fact that AmB has a high affinity for ergosterol, with the consequence that the peak (centred at 40.5°C) attributed to the lipid in interaction with the sterol is broadened and almost disappears. In addition, the fact that for the ergosterol-containing vesicles the cooperativity increases when concentrations of AmB are higher than 12.5% may suggest that aggregation of the AmB/ergosterol complexes occurs within the lipid matrix, as it is often the case when proteins are present in a bilayer [41].

The second component of the transitions has a different behaviour. In this case, the half-height widths for the sterol-containing bilayers (Fig. 6B, 2-Chol and 2-Erg) are similar, both being in the range of 3–4°C, but in the presence of ergosterol, the values are slightly higher. With the sterol-free lipid vesicles (2-DPPC) the half-height width is lower, around 2°C. Thus, when either cholesterol or ergosterol are present within the lipid matrix together with AmB, the cooperativity of the transition is lower than when AmB alone is dispersed within the lipid matrix. As noted above, however, the perturbing effect is more important with ergosterol than with cholesterol. Finally, Fig. 6B also presents the half-height widths of the third component of the transitions (3-DPPC, 3-Chol). However, the magnitude of these transition components is small and thus the changes of the half-height widths when the concentration of AmB is varied are more difficult to interpret.

In order to probe the interactions of AmB with DPPC, ergosterol and cholesterol, we have also used UV-visible spectroscopy. This latter technique is particularly useful since it has been shown to be particularly sensitive to the aggregation state of AmB as well as to the interaction of the drug with its molecular environment. For instance, AmB in the monomeric state has a specific spectrum with a maximum of absorption at 409 nm, while in the aggregated state this maximum is shifted to 421 nm [45]. Fig. 7A presents the UV-visible spectra for DPPC mixed with AmB (6.25 mol%) while Fig. 7B and C present the absorption spectra for cholesterol- and ergosterol-containing DPPC vesicles, respectively, incorporating the same amount of AmB with respect to the lipid. In the last two mixtures ergosterol and cholesterol were present at 12.5 mol% with respect to the lipid, as it was the case for the calorimetric studies. It is clear from Fig. 7A that the band at 415.2 nm, which is not observed in the spectrum of either the monomeric or aggregated forms of AmB, results from the interaction of the drug with its lipid environment. In this sense, the spectrum confirms the interaction of AmB with the aliphatic chains shown in Fig. 2. Furthermore, Fig. 7B and C show the presence of a band at 414.9 and at 416.3 nm for the cholesterol- and ergosterol-containing liposomes, respectively. Control spectra of binary mixtures of AmB mixed with cholesterol showed an absorption band at 414.9 nm while spectra of binary mixtures of AmB mixed with ergosterol presented an absorption band at 416.3 nm (results not shown). Thus the band observed at 416.3 nm in the ergosterol-containing DPPC vesicles (Fig. 7C) is associated to the interactions of AmB with ergosterol, a result consistent with the calorimetric data discussed above. On the other hand the band at 414.9 nm in the cholesterol-containing vesicles (Fig. 7B) cannot be unambiguously attributed to the cholesterol–AmB interactions due to the proximity of the band attributed to the AmB–lipid interactions positioned at 415.2 nm.

In conclusion, our calorimetric data have shown

that AmB, owing to both its rigidity and the way it is positioned within the membrane structures the aliphatic chains of DPPC. As a consequence these chains interact more importantly, through van der Waals interactions, with AmB than the aliphatic chains of the lipid with themselves. Thus, in this sense, one can say that the aliphatic chains of the lipid are rigidified upon their interaction with the drug. These results are consistent with the idea that AmB may form aggregates or pores within the bilayer, even in the absence of sterols. On the other hand, we have shown an important selectivity of the interactions of AmB with ergosterol- as compared to cholesterol-containing bilayers. This implies that the action of AmB on cholesterol- with respect to ergosterol-containing membranes results from different mechanisms, notably due to the fact that only the ergosterol- but not the cholesterol-containing membranes show the presence of pores, as suggested by Bolard et al. [25]. Current experiments now being pursued in our laboratory using other techniques (FTIR and ^{2}H-NMR), together with further DSC experiments using a larger range of concentrations of both AmB and sterols, shall soon confirm the structurisation effect of AmB on the aliphatic chains of its lipid environment.

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