Interaction of resveratrol analogues with biomembrane models: a calorimetric study on structural variations effects.

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Original Article

ABSTRACT

Differential Scanning Calorimetry was used to study the interaction of new resveratrol derivatives using dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (MLV) as biomembrane models. MLV prepared in the presence of increasing molar fractions of the following compounds: (A) 3,5,3',5'-tetramethoxystilbene, (B) 3,5,3',4'-tetramethoxystilbene, (C) 3,4,5,4'-tetramethoxystilbene, (D) 3,4,5,3',5'-pentamethoxystilbene and (E) 3,4,2',4',5'-pentamethoxystilbene were analyzed to study the effects exerted by the number and position of the substituents on the variations of thermotropic properties of the biomembrane model. The results showed that the number and the position of the substituent strongly affected the interaction between the compounds and the MLV based on DMPC. Kinetic experiments demonstrated that, the absorption of compounds A to E is limited in an aqueous medium. The presence of a lipophilic medium improves the absorption of the compounds by the biomembrane model.

INTRODUCTION

In recent years human diet has played an increasingly important role in the prevention of many medical conditions, such as cancer and cardiovascular and inflammatory diseases. Resveratrol (3,5,4'-trihydroxystilbene) has received particular attention because of its abundant presence in food and for its wide variety of biological activities including antioxidation (1) cyclooxygenase inhibition (2), platelet aggregation inhibition (3), and theneuroprotective activity for neurodegenerative diseases (4). After oral administration, resveratrol metabolises rapidly to several glucuronides and sulfates (5). The aim of this study was to synthesize novel analogues with the same structural backbone as resveratrol, modified to provide better bioavailability and activity. 3,5,3',4',5'-Pentamethoxystilbene has been shown to have antiproliferative effects on human breast cancer MCF-7 cells (6). Simoni *et.*

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al. (7) found that introducing methoxy groups at the stilbene motif of resveratrol confers cytotoxic and apoptotic activity to this class of compounds and, in some cases, the methylated derivatives were more active than the corresponding polyphenols. Further examples of polymethoxystilbenes related to resveratrol showing anti-tumor properties are reported in the literature (8). It has recently been shown that some methoxy stilbenes, in particular the 3,5,3',4'-tetramethoxystilbene (2) are potent inhibitors of a breast cancer resistant protein (9). The pharmacokinetic studies of these methoxylated stilbenoids show that their metabolic profile is more advantageous than that of the related natural polyphenols (10).

Previous studies have shown that 3,5,4'-trimethoxystilbene, obtained by permethylation of resveratrol, is favorable from the viewpoint of metabolism, and in addition methylation increases lipophilicity and enhances cell membrane permeability (11, 12). This study focused on the investigation of new analogues to examine the effects exerted by the number and position of the substituents. To gain additional information about the relationship between structure and activity, the interaction of different polymethoxystilbenes related to resveratrol with dimyristoyl-phosphatidylcholine (DMPC) multilamellar vesicles (MLV) using Differential Scanning Calorimetry (DSC) were studied. Generally, bioactive compounds which interact with the phospholipid bilayer act as impurities destabilizing the ordered structure which shifts the temperature of the gel-liquid crystal phase transition (T_m) toward lower values. DSC allows the monitoring of this process (13-16). As the decrease of T_m depends on the amount of compound dissolved in the phospholipid matrix (17, 18) it was determined that by preparing DMPC MLV the maximum interaction between the DMPC and the resveratrol analogues would increase molar fractions of each compound. Finally kinetic experiments were carried out to characterize the absorption of the compounds by the
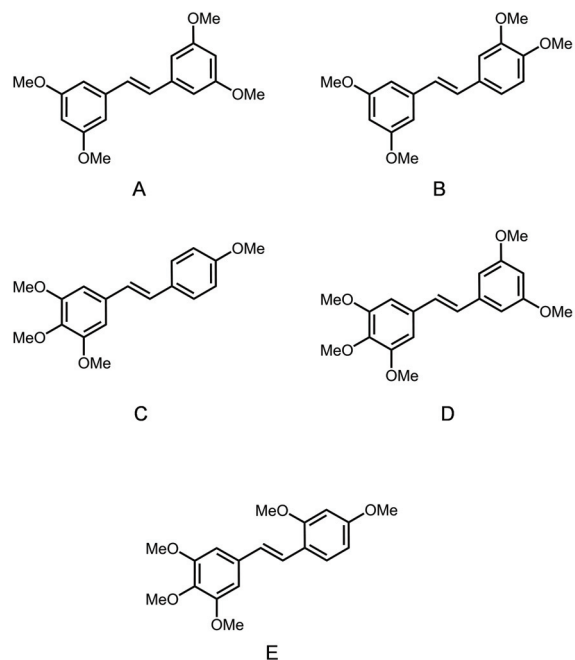

Figure 1 Chemical structures of compounds A to E.

biomembrane model and the influence of the aqueous and the lipophilic media.

MATERIAL AND METHODS

Materials

Synthetic 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, 99% pure) was obtained from Genzyme (Switzerland). A 50 mM tris buffer solution, adjusted to pH 7.4 with HCl, was used for the liposome production.

Synthesis of compounds

Compounds (A) *trans*-3,5,3',5'-tetramethoxystilbene (19), (B) *trans*-3,5,3',4'-tetramethoxystilbene (19), (C) *trans*-3,4,5,4'-tetramethoxystilbene (20), (D) *trans*-3,4,5,3',5'-pentamethoxystilbene (21) and (E) *trans*-3,4,5,2',4'-pentamethoxystilbene (10) were synthesized according to a general protocol based on an Arbuzov rearrangement followed by the Horner-Emmons-Wadsworth reaction (Figure 1). The methoxybenzylbromide (or methoxybenzylchloride) was heated with excess

triethylphosphite to 130°C to give diethyl (methoxybenzyl)phosphonate. The latter was cooled to 0°C and dry Dimethylformamide (DMF) and sodium methoxide were added. To this solution, methoxybenzaldehyde was added and the mixture was allowed to stand at room temperature for 1 hour. The solution was further heated to 100°C and kept at this temperature for 1 hour. After cooling, the mixture was stirred overnight and subsequently quenched with water-methanol (2:1) to produce the compunds.

The spectral data of the isolated compounds were in perfect agreement with those reported in the literature. The purity of the compounds was greater than 98%.

Preparation of the liposomes

Stock solutions of DMPC and compounds A to E were prepared in chloroform-methanol (1:1 v:v). Aliquots were mixed in glass flasks to obtain the same amount of DMPC (0.010325 mmol) and increasing molar fractions (0.00, 0.015, 0.03, 0.045, 0.06, 0.09, 0.12) for compounds A to E with respect to the DMPC. The solvents were removed under a nitrogen flow and the resulting films were freeze-dried under vacuum to remove any residual solvent. Lipid films were suspended in 168 µl of Tris 50 mM buffer, pH 7.4 and multilamellar vesicles (MLV) were prepared by heating to 37°C (above the gel-liquid crystalline phase transition) for 1 minute and vortexing for 1 minute, three times. To homogenize the liposomes the samples were kept for 1 hour in a water bath at 37 °C.

Differential Scanning Calorimetry analysis

A Mettler Toledo STAR® system equipped with a DSC-822® calorimetric cell together with Mettler TA-STAR^e software was used for analysis. The sensitivity was automatically set as the maximum possible and the reference pan was filled with Tris buffer solution. The DSC was calibrated for transition temperature and enthalpy changes, using indium, stearic acid and cyclohexane following the instructions of the DSC 822 Mettler TA STAR® instrument.

120 µl of each MLV suspension was transferred into a 160 µl aluminum DSC pan, sealed, and analysed as follows: (i) a heating scan between 5 and 37°C at 2°C/min, (ii) a cooling scan between 37 and 5°C at 4°C/min. Each scan was carried out a minimum of three times to confirm reproducibility. After the DSC analysis, aliquots of all samples were extracted from the calorimetric aluminum pans (22) and the exact amount of phospholipids present in each sample was determined using a phosphorus assay.

Permeation experiments

The aim of these experiments was to detect the capability of the compounds to dissolve in aqueous medium, reach the MLV surface and penetrate the phospholipid bilayer. To obtain a 0.09 molar fraction with respect to the DMPC, an exact amount of each of the tested compounds were weighed and placed in the bottom of the DSC pan and 120 µl of MLV were added. The aluminum pan was hermetically sealed and analyzed as follows: (i) a heating scan between 5 and 37°C at the rate of 2°C/min, (ii) an isothermal period (1 hour) at 37°C and (iii) a cooling scan between 37 and 5°C at the rate of 4°C/min. This procedure was repeated eight times to follow the variations in the calorimetric curves, which indicated that an interaction between the tested compounds and the DMPC vesicles occurs.

Transmembrane transfer kinetics

60 µl of loaded MLV, prepared in the presence of each compound, were transferred into the aluminum pan and 60 µl of an equimolar unloaded MLV solution was added. The pan was sealed, and the sample was analyzed using the DSC as described previously in the *Permeation Experiments* section. If the compound completely migrates from the loaded to the unloaded MLV at the end of the process a concentration equilibrium will be reached.

RESULTS AND DISCUSSION

The aim of this research was to establish whether compounds A to E, interact with the lipid vesicles mimicking biological membranes, affecting the thermotropic properties of DMPC multilayers, such as phase transition, temperature and enthalpy variation (ΔH).

The implication of different structural elements on the interaction between the compounds and the biomembrane model was also investigated. The DMPC MLV were prepared in the presence of increasing molar fraction of

compounds A to E and analyzed using DSC. The calorimetric curves are compared with the calorimetric curve of MLV prepared without compound and shown in Figure 2. MLV of pure phospholipid, when submitted to heating, passes from a gel ordered phase to a ripple phase and, then, to a liquid-crystalline disordered phase. The transition from the gel to the ripple phase is evidenced by a pretransition peak, at approximately 16°C, whereas the transition from the ripple phase to the liquid-crystalline phase is evidenced by a transition peak, at approximately 24.8°C (23, 24). The MLV made with DMPC in this study agrees with results reported elsewhere (25, 26),
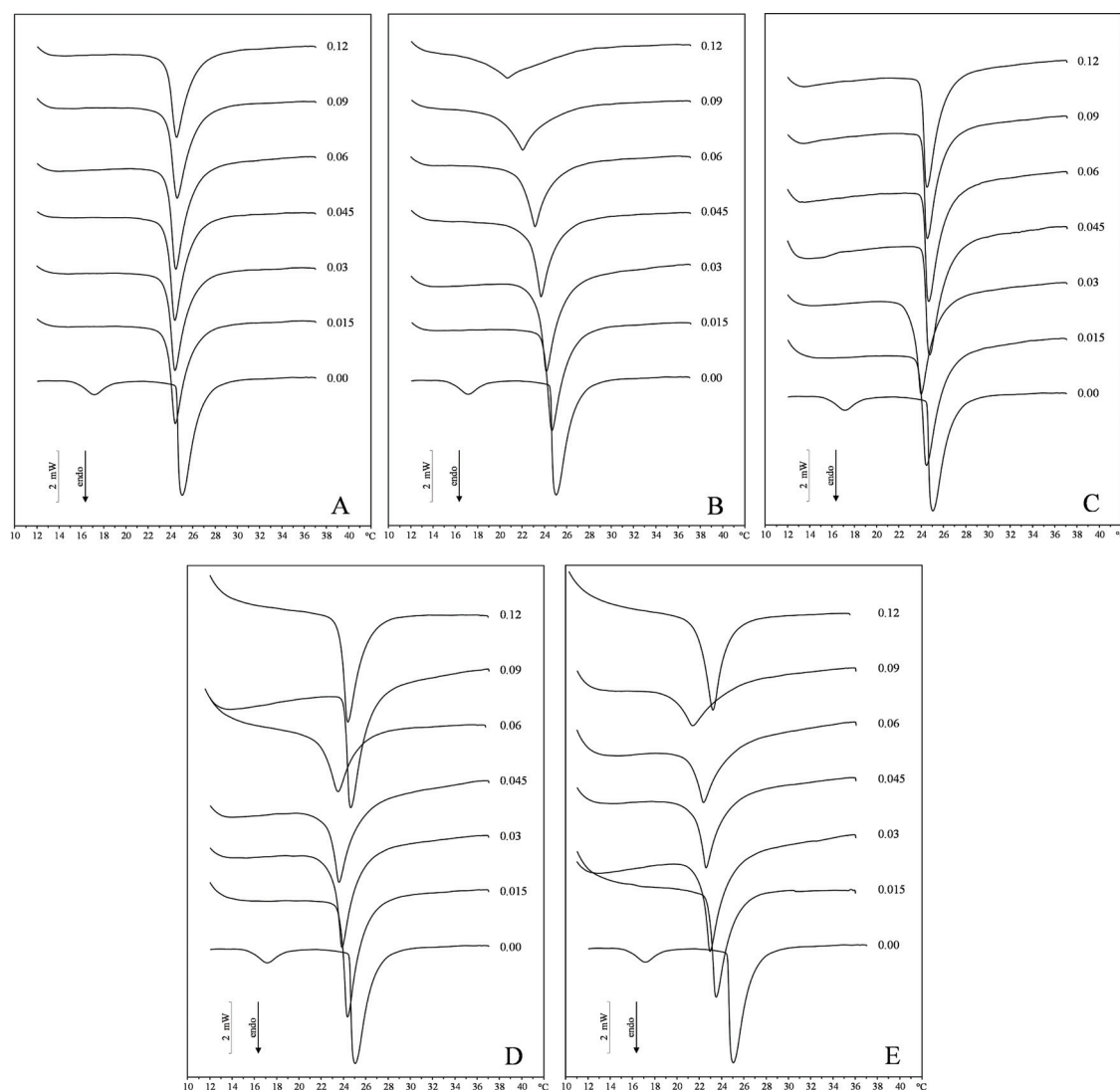

Figure 2 Calorimetric curves, in heating mode, of MLV prepared in the presence of increasing molar fractions of compounds A to E.

showing a pretransition peak at about 16°C, and a transition peak at about 24.8°C, with a ΔH of 7.16 Kcal/mol. Variations of the thermodynamic parameters of the phase transitions are caused by the presence of molecules dissolved in the lipid bilayers and are related to the amount of substance interacting with the bilayers (27, 28). The extent of the interaction between MLV and compounds was evaluated by the modifications in the MLV calorimetric curve. It was evident that compound B, 3,3',4',5-tetramethoxystilbene caused the greatest effect. The pretransition peak already at the lowest molar fraction disappeared and, with increasing amount of compound, the main peak shifted toward lower temperature and broadened. Furthermore, at 0.12 molar fraction, we observed a phase separation, and this behavior indicates a lower homogeneity in the distribution of the compound within the DMPC bilayers and the formation of compound-reach and compound-poor domains.

Compound A 3,5,3',5'-tetramethoxystilbene resulted in the disappearance of the pretransition peak, already at 0.015 molar fraction, and only a slight shift of the main peak toward lower temperatures. In comparison, compound D, bearing a further methoxy group in position 4 causes a stronger interaction and a greater decrease of T_m up to 0.06 molar fraction. At higher molar fractions the T_m increased almost overlapping the values observed for compound A. This is probably due to the formation of aggregates unable to interact with the MLV. The effect of the pentamethoxystilbene [5] is similar to that of compound D in causing a T_m decrease and, then, an increase of the T_m. However, the strong interaction between compound E and DMPC MLV proceeds up to 0.09 molar fraction, then the T_m increases again for 0.12 molar fraction. Interestingly, comparing the effect of compounds D and E with that of compound C, keeping in one ring only one methoxy group, it is observed that compound C interacts with MLV only for small molar fractions and the T_m increases from 0.045 molar

fraction yet. These different behaviors are to be correlated with the number and the position of the methoxy groups, as shown in Figures 3a and 3b, where the T_m variation, as $\Delta T/T^0_m$, and the ΔH, as $\Delta\Delta H/\Delta H^0$, are plotted as a function of the molar fraction of compounds in the MLV. Primarily two different trends for both T_m and ΔH were observed. The first is characterized by a gradual decrease of the T_m and the ΔH as a function of the compound molar fraction. The second is characterized by a decrease of the T_m and ΔH up a certain molar fraction of compound followed by a T_m and ΔH increase. This suggests that the extent of interaction is
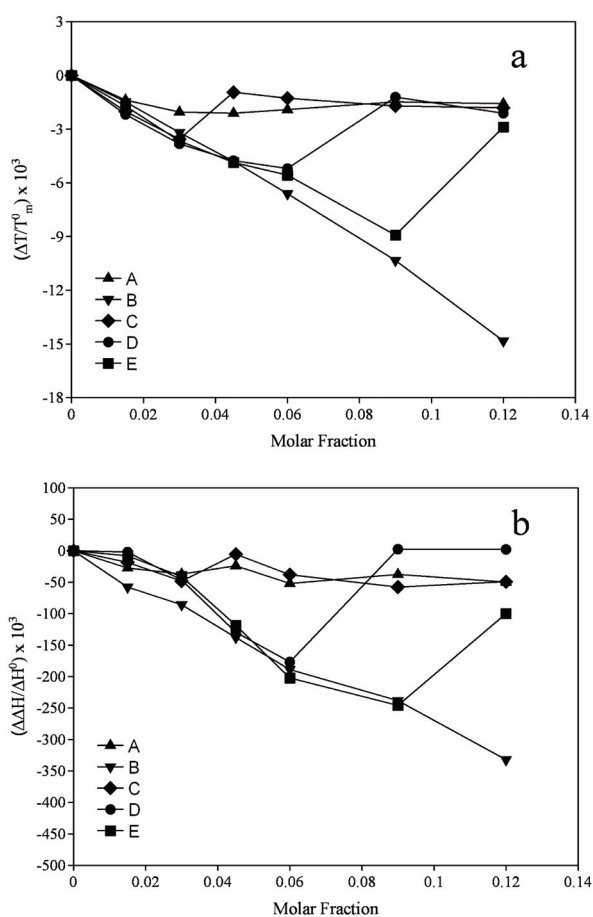

Figure 3 (a) Transition temperature variation, reported as $\Delta T/T^0_m$, of DMPC MLV as a function of resveratrol analogues molar fraction. $\Delta T = T_m - T^0_m$; where T_m is the transition temperature of loaded MLV and T^0_m is the transition temperature of unloaded MLV, (b) Enthalpy change, reported as $\Delta\Delta H/\Delta H^0$, of DMPC MLV as a function of resveratrol analogues molar fraction. $\Delta\Delta H = \Delta H - \Delta H^0$, where ΔH is the enthalpy change of loaded MLV and ΔH^0 is the enthalpy variation of unloaded MLV.

not linearly related to the molar fraction but is dependent on the compounds ability to interact with DMPC MLV. Certainly, the different behaviors of the MLV are to be correlated with the structure of the particular compound. In fact, the first behavior is shown by MLV containing compounds A and B bearing two methoxy group on the first ring, whereas the second behavior is exhibited by MLV containing compounds C, D and E with three methoxy groups on the first ring. Compounds A and B can interact with MLV phospholipid for all molar fractions. On the other hand compounds C, D and E can interact with MLV up to a defined molar fraction (0.03 for compound C, 0.06 for compound D and 0.09 for compound E, then the third methoxy group hinders the interaction probably because the compound molecules aggregate each other and do not interact with the phospholipid molecules of MLV. Another plausible hypotheses is related to the preparation of the dispersion samples with amounts exceeding the solubilisation capacity of the DMPC dispersion. In other words it is possible that the MLV coexist with insolubilized material in their structure. In summary, compounds A and B exert a fluidifing effect (T_m decrease) and diminish hydrophobic interactions between the phospholipid acyl chains themselves (ΔH decrease) due to their intercalation and, therefore, their interaction with the phospholipid molecules for all the molar fractions used. Whereas compounds C, D and E exerts these effect only up to a certain concentration.

Permeation experiments

Previous studies have shown that methylation decreases water solubility and prevents the absorption by a biomembrane model (11, 12). The solid compounds at 0.09 molar fraction were placed in contact with the DMPC MLV dispersion, at increasing incubation times. This molar fraction was chosen in order to have a great interaction between the compound and the MLV, but also to obtain a good calorimetric peak, without phase separation. The

calorimetric curves showed for all the tested compounds the shift of the pretransition peak towards a lower temperature and its broadening, whereas the main peak underwent only a slight shift towards the lower temperature, even after eight hours of incubation at 37°C. In Figure 4 the variation of the transition temperature, as $\Delta T/T^0_m$, as function of calorimetric scans, recorded at increasing incubation time, is reported. All compounds resulted in only a slight decrease of T_m and the r value, related to the MLV prepared in the presence of 0.09 molar fraction of compounds, (the maximum interaction) is not reached. These results indicated that the compounds cannot dissolve in the aqueous medium to reach the MLV surface and interact with the vesicles.

Transmembrane transfer kinetics

In these experiments, loaded MLV and unloaded MLV, were put in contact to determinate if a transfer of the compounds between the two lipophilic systems occurs. Loaded MLV contained 0.09 molar fraction of compound A 3,5,3',5'-tetramethoxystilbene, compound B 3,5,3',4'-tetramethoxystilbene and Compound E 3,4,5,2'4'-pentamethoxystilbene and 0.06 molar fraction of compound C
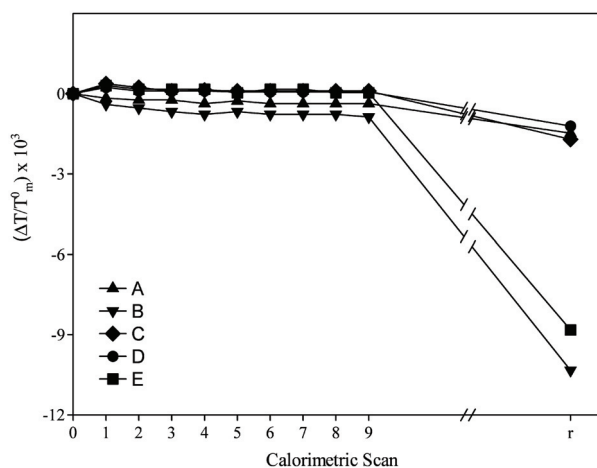

Figure 4 Transition temperature variation, reported as $\Delta T/T^0_m$, of DMPC MLV left in contact with resveratrol analogues at 0.09 molar fraction as a function of the calorimetric scans. The r values belong to DMPV MLV prepared in the presence of compounds at 0.09 molar fraction.

3,4,5,4'-tetramethoxystilbene, compound D 3,4,5,3',5'-pentamethoxystilbene. Figure 5 shows the calorimetric curves of this experiment. Figure 5 shows a reference curve (curve r) which is the curve obtained from the preparation of MLV in the presence of 0.045 or 0.03 molar fraction of compounds, respectively (see section "Preparation of Liposome"). If the compounds transfer from loaded to unloaded MLV a curve similar to curve r should be observed. With regard to 3,5,3',5'-tetramethoxystilbene (compound A) we observe the disappearance of the pretransition peak and the shift of the transition peak toward

lower temperature until overlapping the curve r. It is a sign of the transfer of the compound from loaded to unloaded MLV. As far as the 3,5,3',4'-tetramethoxystilbene (compound B) is concerned, the first calorimetric scan shows two peaks, the first relative to loaded MLV and the second to unloaded MLV. In the subsequent scans, the first peak decreases whereas the second peak moves toward lower temperature reaching the temperature of the curve r. It indicates that even this compound can migrate from loaded to unloaded MLV. For 3,4,5,4,4'-tetramethoxystilbene (compound C), 3,4,5,3',5'-pentamethoxystilbene (compound D) and
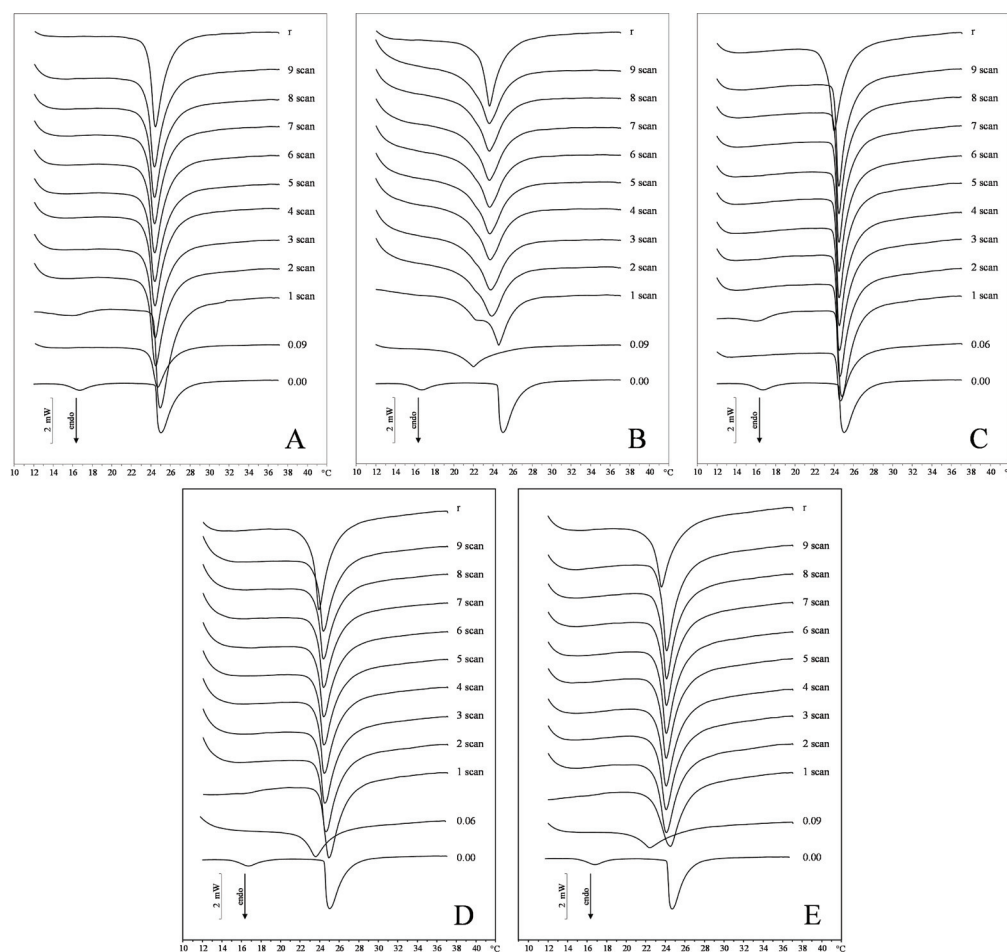

Figure 5 Calorimetric curves, in heating mode, of pure DMPC MLV left in contact with an equimolar amount of DMPC MLV prepared in the presence of resveratrol analogues derivatives (0.09 molar fraction of 3,5,3',5'-tetramethoxystilbene (A), 3,5,3',4'-tetramethoxystilbene (B) and 3,4,5,4'-tetramethoxystilbene (C) and 0.06 molar fraction of 3,4,5,3',5'-pentamethoxystilbene (D), 3,4,5,2',4'-pentamethoxystilbene (E). The curves r belongs to DMPV MLV prepared in the presence of 3,5,3',5'-tetramethoxystilbene (A), 3,5,3',4'-tetramethoxystilbene (B) and 3,4,5,4'-tetramethoxystilbene (C) at 0.045 molar fraction and 3,4,5,3',5'-pentamethoxystilbene (D) and 3,4,5,2',4'-pentamethoxystilbene (E) at 0.03 molar fraction.

3,4,5,2',4'-pentamethoxystilbene (compound E) the pretransition peak is lost and the transition peak moves toward lower temperature, however, not reaching the curve r indicating that the compounds is able to transfer from loaded to unloaded MLV but not completely.

A better visulization of the data is obtained by plotting a graph of the transition temperature, as $\Delta T/T^0_m$, as a function of the calorimetric sequential scan number (shown in Figure 6). The transfer process is rapid and complete for two compounds, 3,5,3',5'-tetramethoxystilbene (compound A) and 3,5,3',4'-tetramethoxystilbene (compound B), whereas is more gradual and incomplete for three compounds, 3,4,5,3',5'-pentamethoxystilbene (compound c), 3,4,5,3',5'-pentamethoxystilbene (compound D) and 3,4,5,2',4'-pentamethoxystilbene (compound E).

Figure 6 Transition temperature variation, reported as $\Delta T/T^0_m$, of pure DMPC MLV left in contact with compounds loaded with DMPC MLV as a function of the calorimetric scans. The r values belong to DMPC MLV containing the average molar fraction between loaded and unloaded MLV.

CONCLUSION

This study examined the interaction of 3,5,3',5'-tetramethoxystilbene (compound A), 3,5,3',4'-tetramethoxystilbene (compound B) 3,4,5,4'-tetramethoxystilbene (compound C), 3,4,5,3',5'-pentamethoxystilbene (compound D) and 3,4,2',4',5'-pentamethoxystilbene (compound E) with a biomembrane model in order to evaluate the relationship between compound structure and the effect on the biomembrane model. The experimental results showed that the number and the position of substituent strongly affected the interaction between compounds and DMPC MLV. In fact, the 3,5,3',4'-tetramethoxystilbene (compound B) that presents three substituents in the same position as resveratrol have the strongest interaction, whereas the 3,5,3',5'-tetramethoxystilbene (A) that have only two substituents in the same positions of resveratrol reveal the lowest effect on the thermotropic parameters of DMPC vesicles. Moreover the 3,4,5,4'-tetramethoxystilbene (C) 3,4,5,3',5'-pentamethoxystilbene (D) interact with biomembrane model only at low concentrations. Furthermore, kinetic experiments showed that in aqueous medium
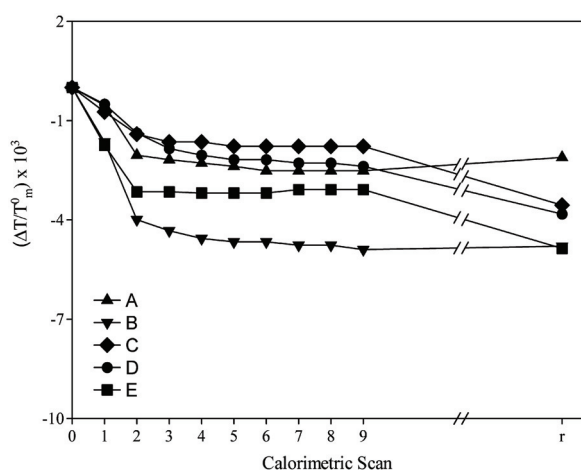
the compounds are not absorbed by the biomembrane model whereas when a lipophilic medium is present the compounds are strongly up taken by the biomembrane model. The obtained results highlight the usefulness of the DSC technique used in this study in helping to understand drug solubilization in lipid assemblies and to have information on the role of a medium to help the uptake of a bioactive molecule by biomembrane model.

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