Calorimetric Evidence of Differentiated Transport of Limonin and Nomilin through Biomembranes

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The effect exerted by two structurally similar limonoids possessing antifeedant and anticancer activity, limonin and nomilin, on the thermotropic behavior of model membranes constituted by dimyristoylphosphatidylcholine (DMPC) vesicles was studied by differential scanning calorimetry. Attention was directed to evaluate modifications in phytochemical−lipid interaction induced by compound structure and lipophilicity and to evidence their different membrane penetration. The two examined compounds, when dispersed in liposomes during their preparation, were found to exert a very different action on the L_β−L_α gel-to-liquid crystal phase transition of DMPC multilamellar vesicles. Nomilin caused a detectable effect on the transition temperature (T_m), shifting it toward lower values with a concomitant small decrease of the associated enthalpy (ΔH) changes, while limonin was not able to modify the lipid vesicles thermotropic behavior. Modifications induced by nomilin were a function of phytochemical concentration, while the different behavior of limonin can be due to the different polarity induced by the presence of the single A ring in nomilin that possesses an acetyl group versus the A,A′ ring system of limonin. Solid limonoids and aqueous dispersions of multilamellar (MLVs) or unilamellar vesicles (LUVs) (limonoids molar fraction 0.045, 0.12, and 0.18) were left in touch for long incubation times at temperatures higher than T_m to detect their spontaneous transfer through the medium. By following this procedure, no interaction was detected for limonin with lipid vesicles. The rate of transfer and interaction of nomilin was a function of the kind of vesicle species (faster for LUV, slower for MLV). The interaction, monitored by compound transfer from the solid phytochemical to the lipidic species after several periods of incubation, was on the same order as that detected by preparation carried out in organic solvent. The obtained results can be explained in terms of compound hydrophobicity, and a relation between compound structure and membrane interaction can be suggested. This allows the membrane interaction with nomilin, but the low water solubility of limonin hinders or totally blocks its transfer through the aqueous medium.

Keywords: *Phosphatidylcholine; differential scanning calorimetry; membranes; limonin; nomilin*

INTRODUCTION

Limonoids are one of the main secondary metabolites found in the rutaceae and meliaceae families. They are tetranortriterpenoid derivates possessing a 4,4,8-trimethyl-17-furanyl steroid skeleton (Arnott et al., 1961; Roussef and Nagy, 1982; Hasegawa et al., 1994), which can be present as aglycons or glucosides. Limonoids are usually classified according to changes they undergo in one or more of their molecule's rings.

In the *Citrus* genus (Rutaceae), the limonoids have been mainly examined in relation to their contribution to the bitterness of citrus fruit and juice (Hasegawa and Maier, 1990; Hasegawa et al., 1996); these studies have led to the characterization of 38 limonoid aglycons and 17 glucosides. The most abundant aglycons are limonin and nomilin, and both of them are founded in very high concentrations (>1000 ppm) in many citrus plants, whereas the most common glucoside is the limonin-17β-D-glucopyranoside (Hasegawa et al., 1994).

Limonoids are known for their insecticidal properties, including growth inhibition, feeding inhibition, and molt inhibition; they are potentially natural pesticides (Champagne et al., 1992; Hasegawa et al., 1994; Miller et al., 1999). Beside these effects, a variety of medicinal properties in animals and humans have been shown, including antifungal, bactericidal, and antiviral effects. Of recent great interest is the potential use of limonoids, both in the aglycon and glucoside form, as anticarcinogenic agents. One of the most interesting uses of limonin and nomilin is their application in the treatment of specific forestomach, lung, and skin cancers; both of the limonoids induce increased activity of the enzyme glutathione-*S*-transferase, which is correlated with inhibition of carcinogenesis (Ahn, 1994). Nomilin appears to be able to inhibit the initiation stage of induced carcinoma, while limonin is more effective in inhibiting the promotion phase; these and other findings suggest citrus limonoids may be useful as cancer chemopreventative agents (Miller et al., 1994; Miyazawa et al., 1995). Their properties on "in vitro" human cancer cell lines and test animals are being explored, and many researchers are now working on the development of new foods enriched in limonoid glucosides (Miller et al., 1999).

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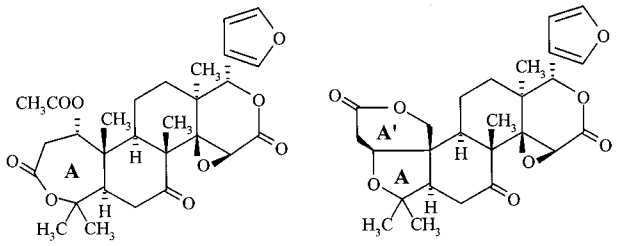

Figure 1. Structural formula of (a) nomilin and (b) limonin.

Limonoids are present in large amounts in seeds, peels, and juice molasses, which are waste products of citrus fruit processing. The large-scale production of limonoids can be economically profitable (Hasegawa and Maier, 1990; Ozaki et al., 1995).

A more extensive study of the organization and interaction of the limonoids in membrane systems is important to assess their potential anticarcinogenic effect. One of the techniques employed to carry out studies on the effect of increasing incorporation of limonoids on phosphatidylcholine membranes is differential scanning calorimetry (DSC). This nonperturbative tool permits the study of the effect exerted by a substance on the well-known phase transition gel-to-liquid crystal exhibited by phospholipid species (Mabrey-Gaud, 1981; Silvius, 1991; Bach, 1994). An amphiphilic substance dissolved in the lipidic sea acts as a solute in a solution and causes a decrease in the phase transitional temperature (Jain and Wu, 1977; Jain, 1988; Castelli and Valencia, 1989). This interaction is related to the amount of phytochemical dissolved in the lipidic structure, as well as matching of the length of the phospholipid with respect to the dissolved molecule and its hydrophobicity. By DSC it is also possible to monitor the uptake process of a compound in a model membrane surface. This process is modulated by the compound's lipophilicity and solubility in water, as well as the phospholipid membrane composition and the presence of a phase separation (Raudino and Castelli, 1998; Castelli et al., 1999a).

In this work we investigated the effects of the structural difference between the two limonoids molecules (Figure 1) on the interaction with DMPC (dimyristoylphosphatidylcholine) vesicles, similarly as described previously by studying the interaction of the structurally similar lutein and β-carotene with DMPC multilamellar vesicles (Castelli et al., 1999a). To study this interaction, we have used L-α-dimyristoylphosphatidylcholine vesicles (multilamellar, MLV, and unilamellar, LUV) as synthetic simplified model membranes which show a change in their thermotropic behavior if other molecules are dissolved in their ordered structure (Cater et al., 1974; Papahadjopoulos et al., 1975; O'Learly et al., 1986; Jain, 1988).

By comparing the results obtained by the "classical" interaction between the limonoid and the membrane (interaction in organic solvent before multilamellar vesicle preparation) with kinetic experiments of the transfer of compounds to empty membranes, the differences in the compounds ability to interact and to penetrate the lipid bilayer of biomembranes, which cause variations in their structure and fluidity, should be detected. In this way it is possible to examine the steric hindrance of these compounds, which is known to modulate the interaction and/or penetration of foreign molecules into cell membranes, as well as assessing the lipophilicity, which should be a factor in such penetration phenomena.

The results then will give a useful indication to understand the role of substituents present on a molecule in the interaction with the microenvironment of the lipid bilayer, determining the variation in their transport across natural membranes.

MATERIALS AND METHODS

Chemicals. Limonin and nomilin have been extracted from seeds of *Citrus limon* cv. Femminello variety Verdello, which have been kindly given by the Ditta Lucchesi, Acicatena, Catania, Italy. Synthetic L-α-dimyristoylphosphatidylcholine was obtained from Fluka Chemical Co. (Buchs, Switzerland). Solutions of the lipid were chromatographically pure as assessed by two-dimensional thin-layer chromatography (TLC). Lipid concentrations were determined by phosphorus analysis by the method of Bartlett (1959). Buffer solution consists of 50 mM Tris, adjusted to pH 7.4 with hydrochloric acid.

Extraction of Limonin and Nomilin. Freeze-dried and ground seeds (150 g) were extracted for 3 h with hexane (1 L) by a Soxhlet apparatus. The residue was successively extracted with CH_2Cl_2 (1 L) by soxhlet for 3 h. The extract was evaporated to dryness to give a residue, which was suspended in hexane and kept at 5 °C overnight. The precipitate was separated by filtration, washed with hexane, and dried under vacuum. TLC analysis of the mixture (silica gel, 5% CH_2Cl_2−Et_2O) showed that the mixture consisted of limonin, nomilin, and obacunone.

Purification of Limonoids. The total limonoids extract was subjected to chromatography on a silica gel column and eluted with CH_2Cl_2 with an increasing amount (until 10%) of Et_2O. Fractions with similar TLC profiles were combined, and the chromatography was repeated with the same eluant system to yield 366 mg of limonin (0.24% dry wt), 306 mg of nomilin (0.20%), and 197 mg of obacunone (0.13%).

Limonin and nomilin were identified by direct comparison of their physical and spectral properties with those reported in the literature (Dreyer, 1965; Dreyer et al., 1976; Pifferi et al., 1993).

Electron impact mass spectrometry (EIMS) was determined at 70 eV on a Kratos MS-50S instrument. UV and IR spectra were recorded on Beckman model DU-65 and Perkin-Elmer model 1720X spectrophotometers, respectively. NMR spectra were measured on a Bruker AC-250 instrument, operating at 250 and 62.9 MHz for ^1H and ^{13}C, respectively. Optical rotations were performed on a Jasco DIP-370 polarimeter. Thin-layer chromatography (TLC) was carried out on silica gel 60 F_{254} plates (Merck).

Liposome Preparation. Multilamellar liposomes were prepared in the presence and absence of increasing concentrations of limonin or nomilin by the following procedure. Chloroform stock solutions of lipid and phytochemicals were mixed to obtain the chosen molar fraction of phytochemicals. Solvent was removed under nitrogen flow and the resulting film was freeze-dried to remove the residual solvents.

Liposomes were prepared by adding to the film 50 mM Tris buffer solution (pH 7.4), heating the mixture at 37 °C, the temperature above the gel−liquid crystalline phase transition, and then vortexing the preparation three times for 1 min.

The samples were shaken for 1 h in a water bath at 37 °C to homogenize the liposomes. Then, aliquots of 120 μL (5 mg of lipid) were transferred in a 150 μL DSC aluminum pan, hermetically sealed, and submitted to DSC analysis.

Unilamellar liposomes were prepared by extrusion of pure DMPC−MLV dispersion through polycarbonate membranes (diameter = 19 mm, pore diameter = 100 nm; Avestin Inc., Ottawa Canada) in a extruder system (LiposoFast Basic, Avestin Inc.) equipped with two Hamilton syringes (Hamilton, Reno, NV) (Hope et al., 1985; MacDonald et al., 1991).

Differential Scanning Calorimetry. DSC was performed by using a Mettler TA 4000 system equipped with a DSC-30

cell and a TC-11 processor. The scan heating rate employed was 2 °C/min in the temperature range 5−37 °C. The sensitivity was 1.5 mW, and the reference pan was filled with Tris buffer solution. After the factory-suggested calibration of the calorimetric system, a narrow range of calibration temperatures was determined by using indium, stearic acid, and cyclohexane. Indium was employed to calibrate the transitional enthalpies (ΔH). Temperature and enthalpies were checked also by using palmitic acid. Enthalpies were evaluated from the peak areas using the integration program of the TC11 processor or by using the software Mettler TA72, permitting the choice of different baselines and ranges of integration. The areas calculated with these different methods lie within the experimental error (±5%).

The samples were cooled and heated four times to cheek the reproducibility of the results. All samples, after calorimetric scans, were extracted from the pan, and aliquots were used to determine the amount of phospholipid by the phosphorus assay (Barlett, 1959).

Permeation Experiments. To study the capacity of the two examined limonoids to permeate the model membrane a kinetic experiment was carried out. DMPC aqueous suspension (MLV or LUV) was left in contact with fixed amounts of finely powdered phytochemicals (to obtain 0.045, 0.12, and 0.18 molar fractions with respect to the phospholipid aqueous dispersion) and placed in the bottom of the DSC crucible. The samples, after having been hermetically sealed in the pans, were gently shaken for 10 s and then submitted to subsequent calorimetric cycles by using the following step procedure: (1) a scan between 5 and 37 °C, to detect the interaction between the compound, dissolved in the aqueous medium and transferred to membrane surface, and the model membrane during the heating of the sample; (2) an isothermal period (1 h) at 37 °C to permit to the phytochemical to permeate the lipid layers staying in a disordered state at a temperature over the lipid transitional temperature; and (3) a cooling scan between 37 and 5 °C, at the rate of 4 °C/min, before restarting the heating program (point 1).

This procedure was run at least five times to detect variations in the temperature of the calorimetric peak and repeated for a long incubation time (12 h), to observe eventual further shifts in the peak temperature.

RESULT AND DISCUSSION

As previously reported, using DSC it is possible to investigate the potential of molecules to interact with a model membrane's surface and to permeate lipid liposomes passing through bilayers (Castelli and Valencia, 1989; Castelli et al., 1998, 1999b). To study the interaction of limonin or nomilin with DMPC liposomes, these compounds were dissolved in chloroform and added to a phospholipid system, obtaining, after liposome preparation, different molar fractions of the two limonoids in MLV aqueous dispersion.

Figure 2 shows the calorimetric heating curves of DMPC−MLV in the presence of increasing nomilin molar fractions (the calorimetric curves of limonin are not reported, as this molecule does not effect the L_β−L_α gel-to-liquid crystal phase transition of DMPC liposomes). It is evident that nomilin exerts interesting interactions with DMPC vesicles, as the typical DMPC liposomes pretransition peak disappears. Furthermore, by increasing the amount of nomilin, the transitional temperature (T_m) was linearly shifted toward lower values. This effect is showed in the Figure 3, in which temperature shifts are reported as $(\Delta T \times 10^3)/T^0_m$ ($\Delta T = T_m - T^0_m$, where T_m is the transition temperature obtained for increasing nomilin molar fractions and T^0_m is the transition temperature of pure DMPC).

The data were obtained from experiments carried out in triplicate, and for each value reported the standard deviation was less than 1.5%; thus, no statistical treatment of the data is reported.

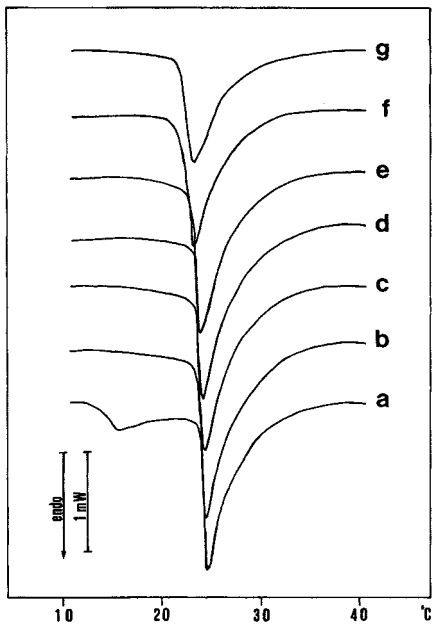

Figure 2. Differential scanning calorimetry heating curves of hydrated DMPC containing nomilin, obtained by starting from organic solvent solutions, at a phytochemical molar fraction of (a) 0.0, (b) 0.015, (c) 0.045, (d) 0.06, (e) 0.09, (f) 0.12, (g) 0.18.

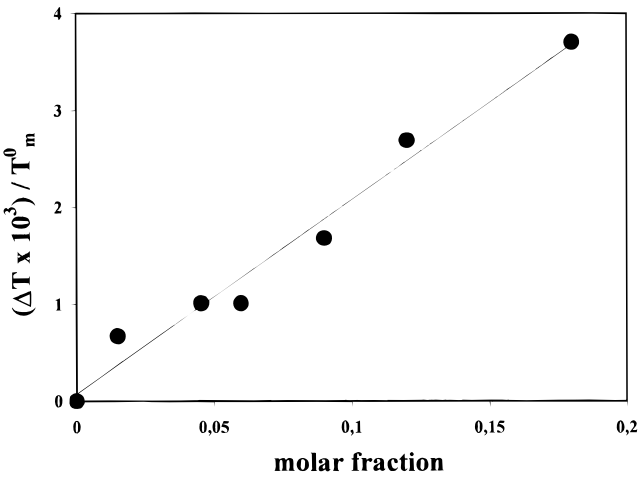

Figure 3. Transitional temperature variations (as $\Delta T/T^0_m$) values (average of at least three runs), in heating mode, as a function of nomilin molar fraction.

The enthalpy change (ΔH), calculated from the calorimetric peak area, is negligibly affected (data not reported); actually a slight decrease was observed in the ΔH, which is possible to see looking at the calorimetric curves shape (Figure 2).

The interaction between limonoids and MLV or LUV vesicles can be explained in terms of a "*fluidifying*" effect due to the introduction of lipophillic molecules into the ordered structure of the lipid bilayer. These molecules, acting as spacers in such a structure cause a destabilization of the lipid mosaic with a decrease in the T_m of the gel-to-liquid crystal phase transition. The slight variation in the ΔH, which occurs as in the case of nomilin, can be explained as a superficial interaction between molecules and lipids. Molecules can interact with lipids in liposomes as "substitutional impurities" of a membrane, taking the place of lipid molecules, and

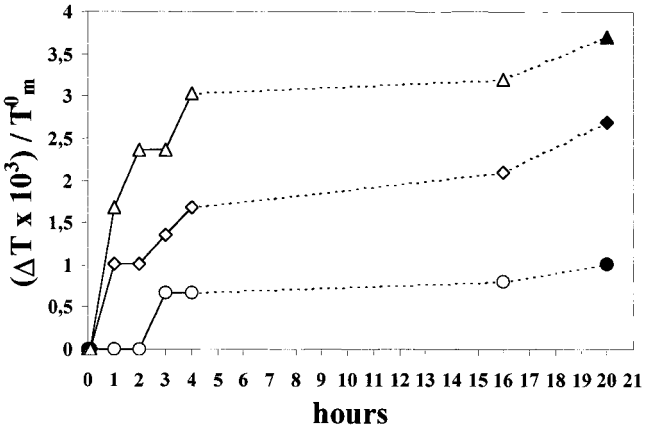

Figure 4. Transitional temperature variations (as $\Delta T/T^0_m$) of DMPC multilamellar vesicles in the presence of different nomilin molar fraction (○ = 0.045, ◇ = 0.12, △ = 0.18) for increasing incubation times. The full points refer to samples prepared for direct interaction between the examined compounds and phospholipid, by organic solvent dissolution and MLV preparation, to be considered as the maximum interaction between compound and vesicles.

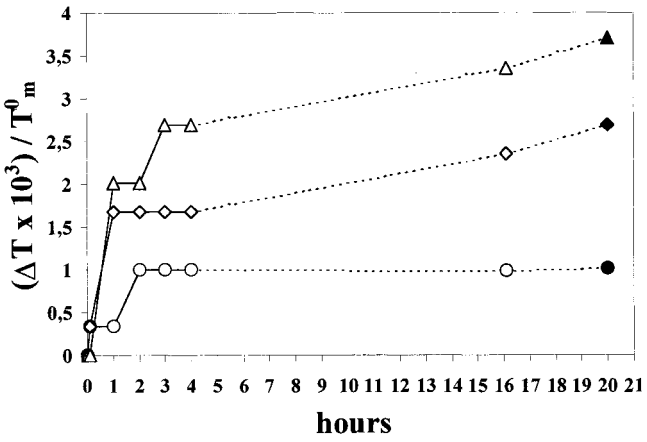

Figure 5. Transitional temperature variations (as $\Delta T/T^0_m$) of DMPC unilamellar vesicles in the presence of different nomilin molar fraction (○ = 0.045, ◇ = 0.12, △ = 0.18) for increasing incubation times. The full points refer to samples prepared for direct interaction between the examined compounds and phospholipid, by organic solvent dissolution and MLV preparation, to be considered as the maximum interaction between compound and vesicles.

such an effect can cause T_m variation and ΔH change. They can, also, interact as "interstitial impurities", by intercalating among the flexible acyl chain of lipids and causing T_m variations without ΔH change, according to the temperature depression of melting point for ideal solution (Cater et al., 1974; Lee, 1977; Estep et al., 1984; Mouritsen and Bloom, 1984; Cevc and Marsh, 1987; Jorgensen et al., 1991; Castelli et al., 1992). Nomilin is able to react with the membrane model and is transferred to the lipidic layers (MLV or LUV) causing, on the gel-to-liquid crystal phase transition, a shift of the T_m.

Temperature shifts versus different nomilin molar fractions are compared with those relative to the effects of solid nomilin (molar fraction 0.045, 0.120, and 0.180) left in contact with DMPC−MLV (Figure 4) or LUV (Figure 5) aqueous dispersion. This comparison is essential to understand nomilin's ability to pass through the medium, reaching and reacting with membrane models and, successively, to permeate the lipidic layer (Raudino and Castelli, 1998; Castelli et al., 1999a). By comparing Figures 4 and 5 it is evident that the data obtained from interaction of solid nomilin with MLV or LUV dispersion are both close to each other and close to values representing the maximal interaction between nomilin and DMPC vesicles. (Data are obtained from the interaction of different nomilin molar fractions prepared in organic phase, in which nomilin is compelled to stay inside the multilamellar vesicle.) The data obtained for three nomilin molar fractions (0.045, 0.120, and 0.180) are reported in Figures 4 and 5 by full signs (as values at 20 h).

The rate of transfer of nomilin and its interaction with the lipid bilayer depend on the type of liposomes species. The increased effects on LUV or MLV is different, as observed in Figures 4 and 5. The reported data show that the transition temperature shift increases with time both when nomilin is in contact with MLV (Figure 4) and LUV (Figure 5). This augmentation continues, even if with a different trend, depending on the liposome species, until it reaches a constant value close to that obtained by direct mixing of nomilin with the lipid component during the vesicle preparation.

By comparing the two figures, we can conclude that the transition temperature shift tends to reach the same values observed for direct mixing of nomilin and DMPC, even if nomilin took longer in reaching equilibrium (maximal interaction) with MLV than for LUV. The analysis of this behavior permits us to make some considerations about nomilin permeation and distribution into membranes; this observation reflects a slower transfer kinetic through a multilamellar system compared with a single bilayer. Furthermore, we can suppose that nomilin reacts with the vesicles bilayer, obtaining a constant molar fraction on the MLV or LUV membrane surface; it is also able to dissolve itself into lipid membranes, penetrating progressively inside the other internal bilayers, where it reaches the same molar fraction of superficial bilayer.

By following the same experimental procedure, limonin does not show the same effects on the calorimetric peak nor capacity to transfer itself through the aqueous−lipid phase (data not reported).

The present findings clearly demonstrate that nomilin, but not limonin, is able to permeate and cross biological membranes. The different behavior of the two compounds with lipid vesicles can find justification in their physiochemical and structural features, due to the presence of a limonin A,A′ ring system versus the single A ring in nomilin (Figure 1). In fact, the different backbone structure of the two limonoids, and their consequent different polarity, should modulate limonin or nomilin interaction with DMPC−bilayer and their incorporation into model membranes.

These findings are in agreement with the literature, where the influence of structural changes on the molecular backbone can modify the interaction with model membranes by affecting the shift of lipid phase transition temperature and enthalpy change (Castelli and Valencia, 1989; Cater et al., 1974).

The results obtained suggest that probably limonoids insecticidal and medicinal properties are not directly dependent on interaction or passive transport through biological membranes, but they can be correlated with another mechanism of action, at least for limonin. Finally, we have shown the importance of the calorimetric technique to detect an indirect transport process through biological membranes, trying to clarify the

factors influencing the possibility to absorb a substance by the biological membranes.

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