Differences between Coumaric and Cinnamic Acids in Membrane Permeation As Evidenced by Time-Dependent Calorimetry

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We carried out an investigation by differential scanning calorimetry (DSC) on the effect of two structurally similar bioactive plant phenols, cinnamic and *p*-coumaric acids, on the phase transition of model membranes constituted by dimyristoylphosphatidylcholine (DMPC) vesicles. The aim was to evaluate how pH and molecular substituents influence liposolubility and thereby modify vesicle permeability. A change in permeability would result in modifications to the phase transition for DMPC liposomes such that the transition temperature would be lowered and the enthalpy changes should be little affected. Evidence of differences in permeability was obtained by comparing the effects exerted by the two compounds left in touch with unilamellar and multilamellar vesicles, at two different pHs (4 and 7.4). While the *p*-coumaric acid was unable to appreciably modify the thermotropic behavior of the model membrane, the cinnamic acid interacted with lipid vesicles at both pH's, even if at acidic pH the effect was greater than neutral. It can be hypothesized that the interaction between the cinnamic acid and the lipidic layers is due to the lack of a hydroxyl group. This interaction is enhanced by an acidic pH, where the carboxylic acid is in a protonated form.

Keywords: *Phosphatidylcholine; differential scanning calorimetry; membrane permeation, biophenols*

INTRODUCTION

Dietary habits corresponding to the "Mediterranean diet" are associated with a lower risk of many chronic diseases such as cancer and cardiovascular diseases. In addition to a high monounsaturated/saturated fatty acids ratio, the Mediterranean diet is characterized by a large intake of fresh fruit, vegetables, grain, and olive oil. All these foods may exert beneficial effects on human health mainly because they are rich in "minor" bioactive ingredients, such as vitamins and polyphenols, which are able to reduce lipid peroxidation and/or enzyme-mediated oxidation. Cinnamic acid and its derivatives (or phenylpropanoids), in particular, are widely found in edible plants as products from the shikimate pathway (Herrmann, 1989) and their biological activities are well described in the literature (Rice-Evans et al., 1996). In fact these biophenols are very effective peroxyl radical scavengers and protect low-density lipoproteins from oxidative modification (Castelluccio et al., 1995).

The interaction of biophenols with biological membranes plays an important role in their transport, distribution, action, selectivity, and toxicity (Kaneko et al., 1994). Small organic acids are compounds sometimes able to interact with and penetrate biological membranes (Lohner, 1991), but their ability to exert such an interaction with lipid membranes may be deeply influenced by substituents present on their main structure. As a consequence, structurally similar compounds should interact with biological membranes in a different way, due to the complex relationship (which may be modulated by pH variations) between liposolubility and permeability.

Techniques employed to investigate drug−membrane interactions represent useful tools to obtain preliminary information about membrane permeation pathways or bioavailability of a drug (Seydel, 1991). Moreover, by means of modulation of certain experimental conditions (for example, by lowering pH) they can allow us to make interesting considerations about the importance of physicochemical properties of a bioactive compound in determining its permeation across biological barriers, particularly in some body compartments (e.g., at the gastrointestinal tract).

The interaction of a compound with, and its permeation through, the lipid bilayer of biomembranes can cause variations in membrane structure and fluidity. In the present study, we examined the effects on the thermotropic behavior of L-α-dimyristoylphosphatidylcholine (DMPC) vesicles, exerted by two structurally similar phenylpropanoids, cinnamic acid (I) (CN) and *p*-coumaric acid (II) (CUM), differing only for the presence of a −OH substituent *para* with respect to the carboxylic group. When the temperature is increased, the DMPC shows a reorientation of acyl chains from an ordered structure to a disordered configuration, known as the $L_{\beta}-L_{\alpha}$ phase transition (Lee, 1977; Houslay and Stanley, 1983; Tenchov, 1991; Marsh, 1996). This thermotropic behavior may be investigated by differential scanning calorimetry (Mabrey-Gaud, 1981; Bach, 1984; Silvius, 1991). In fact, liposomes are generally accepted to be a suitable model for the study of membrane

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structure and properties, given that they are surrounded by a lipid bilayer, structurally similar to the lipid matrix of cell membranes. Furthermore, DSC is a powerful and nonperturbing thermodynamic technique that allows characterization of the thermotropic-phase behavior of the lipid bilayer in liposomal structures and convenient and sensitive determination of the interaction of drugs with artificial membranes. The presence of drug molecules dissolved in the ordered lipid bilayer can cause, depending on their structural features, significant variations in the thermodynamic parameters associated with the lipid phase transition, such as the transition temperature (T_m) and enthalpy changes (ΔH) (Jain and Wu, 1977; Jain, 1988; Castelli et al., 1989).

Moreover, evidence of membrane penetration can be obtained by comparing the thermotropic effects exerted by a compound put in contact with empty multilamellar or unilamellar vesicles and then following the perturbative effects on the two different layered membrane. If the perturbative effects (T_m shift) of the two systems are similar, reaching a value comparable to that observed when the compounds have been completely dispersed in the bilayer during the liposomal preparation, this indicates that membrane penetration has taken place. The time-evolving effect of bioactive compounds on the thermotropic behavior of different layered liposomes seems to be a valuable approach to estimate their ability to interact with and cross the biological membranes. This approach was previously reported in a experimental and theoretical study on Diflunisal penetration through lipid membranes (Raudino and Castelli, 1998). The penetration can be modified by protonation/deprotonation equilibrium since experiments were out carried also at two different pHs (4 and 7.4). Evaluation of all these results should give useful indications to understand the role of biophenol structure in interacting with the microenvironment of the model lipid bilayer and, thereby, allow us to speculate about the in vivo biovailability of the compounds investigated.

MATERIALS AND METHODS

Chemicals. 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. Lipid concentrations were determined by phosphorus analysis (Bartlett, 1959). Cinnamic acid and *p*-coumaric acid were obtained by Sigma Chemical Co. (St. Louis, MO). Tris, 50 mM solution, was adjusted to pH = 7.4 (buffered solution) and to pH = 4 (unbuffered solution) with hydrochloric acid (0.5 and 0.1 M).

Preparation of Liposomes. In the present study we employed multilamellar or unilamellar DMPC liposomes as model membranes due to their low transition temperature. Multilamellar vesicles (MLV) were prepared, either in the absence or in the presence of biophenols, by the following procedure: chloroform−methanol (1:1 v/v) lipid alone and biophenols solutions were transferred in a glass tube in amounts so to obtain the chosen molar fraction of biophenols (0.0 or 0.2). The solvents were removed under a nitrogen flow in a rotoevaporator and the resulting films were freeze-dried under vacuum to remove the residual solvents.

The samples (8 mg of lipid), empty liposomes as well as liposomes containing a 0.2 molar fraction of CN or CUM, were obtained by adding to the lipidic films 240 μL of 50 mM Tris-buffered (pH = 7.4) or unbuffered (pH = 4) solutions. The samples were then heated at a temperature (37 °C) above the gel−liquid crystalline phase transition and vortexed three times for 1 min. The samples were shaken for 1 h in a water bath (Dubnoff 721) at 37 °C to homogenize the liposomes. This temperature was chosen to avoid a probable degradation of the compounds and also to permit a better interaction between the compounds with the liposomes staying in a disordered fluid state.

The samples containing the biophenols dispersed in the multilamellar vesicles were directly examined in the DSC apparatus.

The empty large unilamellar vesicles (LUV) were prepared by submitting empty MLV to extrusion through polycarbonate membranes of 100 nm in an extruder system (Liposofast Avenstin) (Hope et al., 1985).

Aliquots of 120 μL (4 mg of lipid) of empty MLV or LUV at the two different pHs were transferred to 150 μL of DSC aluminum pans where aliquots of CN or CUM finely ground were present to ensure the presence of a 0.2 molar fraction of acid. Afterward, the samples, sealed, were submitted to DSC analysis.

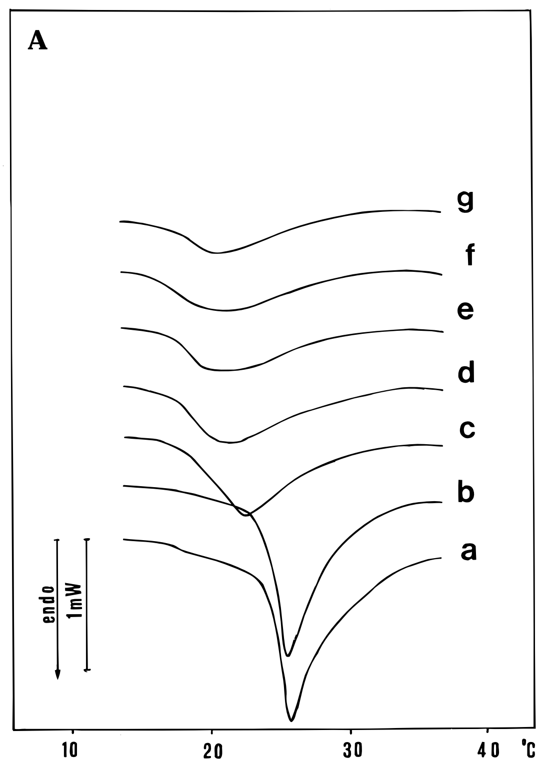
Differential Scanning Calorimetry. DSC was performed using a Mettler TA 3000 system equipped with a DSC-30 cell and a TC-10 processor. The scanning rate employed was 2 °C/min in the temperature range 2−37 °C. The sensitivity was 1.72 mW, and the reference pan was filled with Tris buffer solution. Indium was employed to calibrate the transitional enthalpies (ΔH). Temperature and enthalpies were checked also by using palmitic acid (61.82 °C, 42.04 kJ/mol). After a routine temperature calibration in a large range, indium, palmitic acid, and water were employed for a better temperature calibration within a narrow range. Enthalpies were evaluated from the peak areas by use of the integration program of the TA processor. After the calorimetric scans, all samples were extracted from the pan and aliquots were used to determine the amount of phospholipid by the phosphorus assay (Bartlett, 1959).

Permeation Experiments. To study the capacity of the two examined biophenols to permeate the model membrane, a kinetic experiment was carried out by putting the DMPC liposome suspension (LUV or MLV) in contact with a fixed amount of finely powdered biophenol (0.2 molar fraction) placed in the bottom of the DSC crucible. The crucible was gently shaken for 10 s and submitted to the following heating, isothermal, and cooling calorimetric cycles: (1) a first scan between 2 and 37 °C, to detect the interaction between the compound and model membrane; (2) an isothermal period of 1 h at 37 °C to permit to the biophenol to permeate (if able) the lipid layer(s) in a disordered state at a temperature above the lipid transitional temperature; and (3) a cooling scan between 37 and 2 °C to restart the heating program. This procedure was run at least five times.

RESULTS AND DISCUSSION

A brief methodological comment is needed as a prelude to the discussion of the results obtained. In the present study, to investigate the interaction of CN and CUM with phospholipid bilayers, the biophenols were put in contact with empty MLVs or LUVs. The general result of such a process is the extraction of molecules from the solution, enriched by the solid biophenol, by the external lipidic bilayer of both MLVs and LUVs. This can cause a decrease in the T_m of the lipid fraction (external) into which the molecules have been extracted. However, the lipid lamellar structure of internal layers of MLVs, that represents the lipid fraction, which is free of extracted material, is not perturbed. The result is a slower decrease in T_m compared to that exhibited by LUVs, which are more easily and quickly permeated. By comparing the perturbative effects on the two different layered membranes, we can obtain interesting information about the capability of the material of interest to cross biomembranes.

Figures 1 and 2 show the calorimetric heating curves of DMPC liposomes (LUV, Figure 1; MLV, Figure 2) in the presence of 0.2 molar fraction of CN at two different



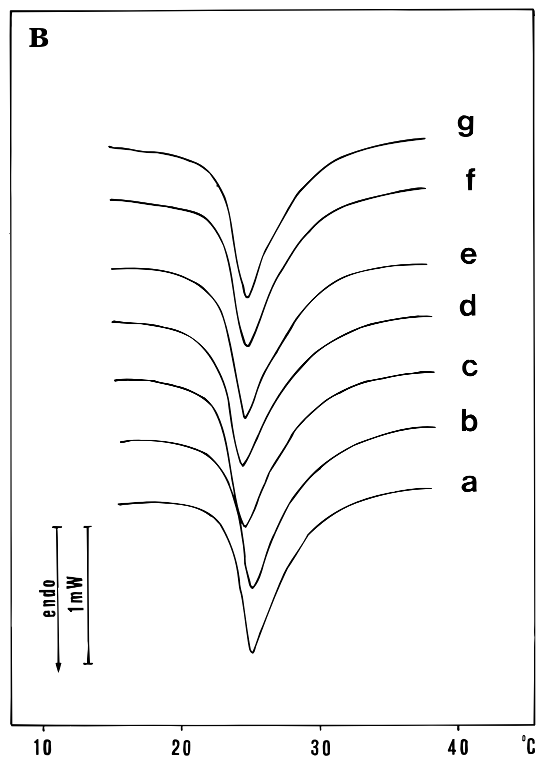
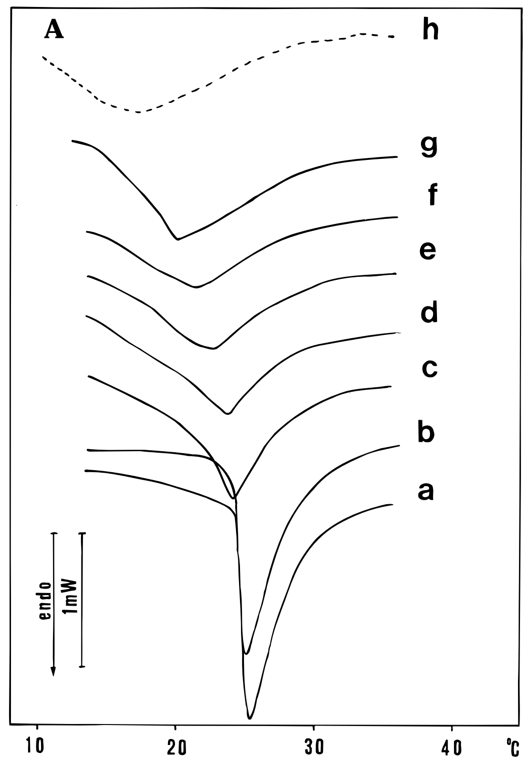


Figure 1. Calorimetric curves of DMPC unilamellar vesicles in the presence of CN, 0.2 molar ratio, for increasing incubation times (hours) at (A) pH = 4 or (B) pH = 7.4. (a) Pure DMPC; (b) = first scan after 10 min; (c) second scan; (d) third scan; (e) fourth scan; (f) fifth scan; (g) sixth scan.

pHs (pH = 4, Figures 1A and 2A; pH = 7.4, Figures 1B and 2B) for increasing incubation times.

CN interacts with DMPC liposomes (both LUV and MLV) by causing a shift toward lower values of the transition temperatures (T_m) associated with the gel to liquid-crystal phase transition.



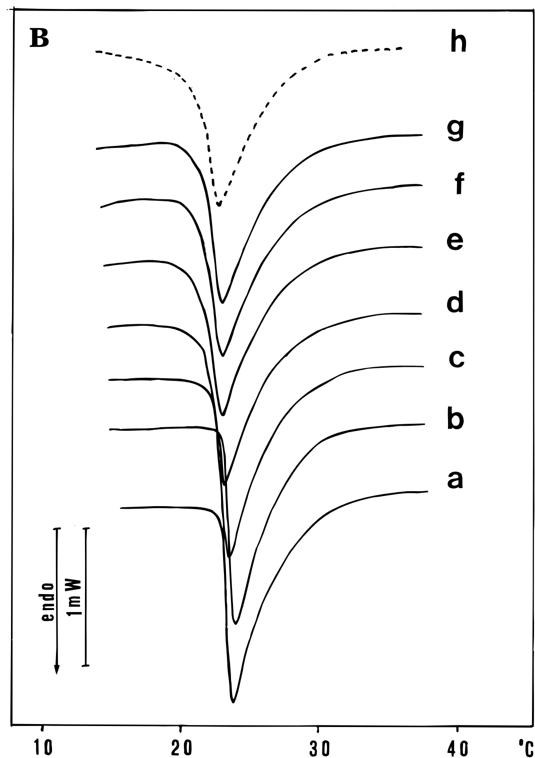


Figure 2. Calorimetric curves of DMPC multilamellar vesicles in the presence of CN, 0.2 molar ratio, for increasing incubation times (hours) at (A) pH = 4 or (B) pH = 7.4. (a) Pure DMPC; (b) first scan after 10 min; (c) second scan; (d) third scan; (e) fourth scan; (f) fifth scan; (g) sixth scan; (h) sample prepared by direct contact of the components in organic solvent.

The temperature shift is expressed as $(\Delta T/T^\circ_m) \times 10^3$ (Figure 3, curves A–D) ($\Delta T = T^\circ_m - T_m$, where T°_m is the transition temperature of the pure DMPC LUV or MLV at the two different pHs and T_m is the transition temperature obtained for increasing incubation times

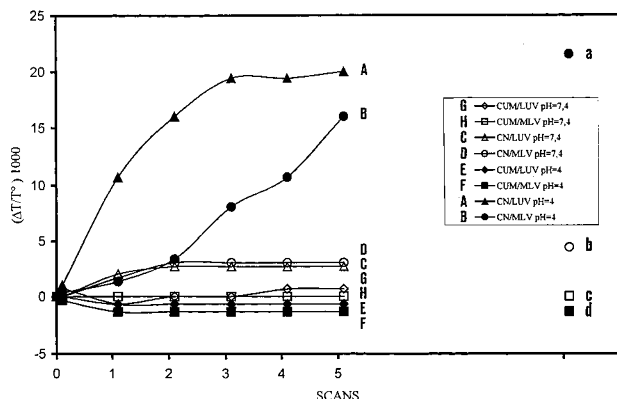


Figure 3. Transitional temperature variations (as $\Delta T/T°_m$) of DMPC unilamellar and multilamellar vesicles in the presence of 0.2 molar ratio of CN and CUM for increasing incubation times. Points a−d refer to samples prepared for direct interaction between the examined compounds (respectively CN, pH 4; CN, pH 7.4; CUM, pH 7.4; and CUM, pH 4) and MLV, by organic solvent dissolution and MLV preparation. These values are to be considered as the maximum interaction between compounds and vesicles.

in the presence of biophenols). 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 was reported.

The enthalpy changes (ΔH), calculated from the calorimetric peak area, remained nearly constant for all examined samples (data not reported). Only for the CN interaction with unilamellar vesicles at pH = 4 was a slight decrease in the ΔH observed, during the first two scans; afterward it remained constant, as it is possible to see by looking at the shapes (Figure 1A). The discrepancy with Figure 2A, where the ΔH was higher than that observed for the LUV (Figure 1A), should find justification in the different structure of the two kind of vesicles (dimension, lamellae curvature, etc.) (Biltonen and Lichtenberg, 1993).

The effect exerted by compounds interacting with model membranes can be summarized as follow. Molecules acting as spacers in such a structure can cause a destabilization of the lipid mosaic with a decrease in the T_m of the gel to liquid crystal phase transition. The negligible variation in the ΔH, when occurring, can be explained as a superficial interaction between molecules and lipids. In fact, molecules can interact with lipids in liposomes as "substitutional impurities" of a membrane, taking the place of lipid molecules, and such an effect can cause T_m variations and ΔH decrease; or they can interact as "interstitial impurities" intercalating between the flexible acyl chains of lipids and causing T_m variations without ΔH change (Jorgensen et al., 1991), according to the temperature depression of melting point for ideal solutions (Lee, 1977; Guggenhein, 1952).

The T_m shift caused by the CN at pH = 7.4 for both LUV and MLV vesicles is nearly the same (Figure 3, curves C and D), indicating that this perturbative effect is exerted in the same way. This affirmation takes on importance mainly if compared with the value b (Figure 3) obtained by the deep interaction between CN and MLV liposomes. In this sample, prepared following direct dissolution in organic phase and hydration of the lipidic film containing the biophenol, the biophenol was compelled to stay inside the multilamellar vesicle, so that value can be considered as coming from the interaction at infinite time.

A very different case appears at pH = 4 where the protonation of the carboxylic group allows the substance to better penetrate the lipid layers. In fact, it is very clear that the CN transfer in the unilamellar vesicle is faster than that observed for the multilamellar vesicles at pH = 4, and both MLV and LUV for pH = 7.4, even if both values tend to the maximum values reachable. This is represented by the value a (Figure 3) obtained for the organic preparation of the MLV vesicles in the presence of CN at pH = 4.

The analysis of this behavior permits us to make some considerations about biophenol permeation and distribution into membranes. We can suppose that CN can dissolve into lipid membranes and penetrate them by migration from the aqueous phase. This process will continue until it reaches a constant molar fraction first on the membrane surface and then progressively inside the other internal bilayers. At the end of this process the thermotropic behavior is close to that obtained by direct mixing of the biophenol with the lipid component during the vesicle preparation (points a and b in Figure 3).

The fact that it took longer to reach equilibrium (maximal interaction) with the MLV (curve 3B) than for the LUV at pH = 4 (curve 3A) reflects a slower transfer kinetic through a multilayer system compared to a single bilayer.

A very different effect on the thermotropic behavior of DMPC vesicles (LUV and MLV) is exerted by CUM at two different examined pHs (calorimetric curves not reported). There was no evidence of interaction between CUM and the DMPC liposomes in this study. No shift of the calorimetric peaks toward lower values was observed (Figure 3, curves E−H). For both pHs and for all kinds of experimental models employed, no evident interaction is shown, suggesting that the −OH group influences the ability of this compound to penetrate the membrane, even if we cannot exclude a surface interaction with the lipidic layers.

The presence of different substituents in the backbone structure of biophenols should modulate their incorporation. In fact CUM is more hydrophilic and unable to penetrate the model membrane, whereas CN, which possesses a hydrophobic portion, is able to solubilize into membrane and to permeate it. These findings are in agreement with 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 (Cater et al., 1974; Castelli and Valencia, 1989). These authors reported, for opioid drugs, that changes in the length of the alkyl chain of the alcohol residue on C-7 (morphine structure) can allow drug penetration in the lipid bilayer, causing a transition temperature decrease and/or lowering the heat of the lipid phase transition by depending on lipophilicity variations.

It is then possible to presume the existence of a structure-dependent relationship between biophenols and their potential for affecting membrane fluidity. In fact, the compound (CUM) bearing an −OH group in the *para* position with respect to the carboxylic group is not soluble inside the lipid bilayer; conversely, when biophenol has an amphipathic character, as is the case with CN present, complete miscibility can occur, by considering the different solubilities between lipidic and Tris phases at the two examined pH values. The lipophilicity was measured, obtaining for cinnamic acid a lipid solubility at pH = 4 3 times higher than that of

p-coumaric acid. Conversely, at pH = 7.4, only slight differences in the lipid/Tris partition of the two examined acids were founds. These findings compared with the calorimetric results, showing a deeper interaction of CN with lipidic membranes in comparison to CUM, can suggest a different (higher) capability of CN to find collocation inside the bilayer by traversing it, while the CUM should remain anchored only at the bilayer surface without perturbing the lipidic structure (no effect on the T_m), by considering the high dipole (23 D) of the choline group.

CONCLUSION

The present findings clearly demonstrate that CN, but not CUM, is able to permeate through, and cross, biological membranes. Physicochemical and structural features of these biophenols may be taken into consideration to justify their different capability to interact with model membranes. Penetration of a drug into cells is often the first step necessary for its biological activity; furthermore, the degree of incorporation and the uniform distribution into lipid bilayers, and the rate of transport into cells, are important factors influencing the biological profile of a drug (Saija et al., 1995). This leads us to suppose that biological properties of these biophenols, particularly their effect on membrane-dependent processes, might be related, partially at least, to their capability to penetrate into the cell membrane. Besides, in our experiments, an acidic pH appears to improve CN penetration through the lipidic layers, by increasing, very likely, the lipophilic character of the protonated acid with respect to the deprotonated form. A suggested hypothesis is that physiological conditions needed for protonation of the carboxylic group (e.g., the low pH at gastric level) might allow CN (which represents a significant component of human diet) to better penetrate and cross the lipid bilayer of biological barriers. Of course this hypothesis is merely speculative and needs further in vivo experimentation.

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