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Interaction of genistein benzyl derivatives with lipid bilayers—fluorescence spectroscopic and calorimetric study

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ABSTRACT

The purpose of the present paper was to assess the ability of genistein benzyl derivatives to interact with lipid bilayers. Calorimetric and fluorescence spectroscopic measurements revealed that, depending on the details of chemical structure, the studied compounds penetrated bilayers and affected their polar as well as hydrophobic regions. It was also found that physical state of bilayer played some role in flavonoid–lipid interactions.

1. Introduction

Genistein is presumably one of the most often studied flavonoids, its name appears in titles of more than 155 papers published only in 2008 (as found in December 2008 by PubMed). Genistein owes this popularity to the wide spectrum of biological activities and the beneficial effects on human health. The best known and also practically used genistein features are: estrogenic and antioxidant properties, anticancer activity, prevention of osteoporosis and cardiovascular diseases (for review see Dixon[1], Szkudelska and Nogowski[2]). Genistein shares some structural features with 17β-estradiol and binds to estrogen receptors ERα and ERβ.[3] Due to this property genistein can be used as an alternative to the hormonal therapy for post-menopausal women. On the other hand, presence of 4-oxo group conjugated with 2,3 double bond is the most important structural genistein feature responsible for its antioxidant properties.[4] Suppression of the oxidation processes induced by free-radicals is considered to be the main mechanism of such beneficial genistein effects as protection against cardiovascular disease or anticancer activity. Apart from the numerous studies on the molecular mechanisms underlying various genistein activities also some efforts are made to synthesize its derivatives characterized by better bioavailability[5] or higher efficiency and specificity of their actions.[6–8] The substitution of hydroxyl group in position 7 is one of the most common strategies. Recently Zhang et al.[8] have shown that genistein 7-*O*-heterocycle derivatives display relatively strong antimicrobial activity against fungi and Gram-positive as well as Gram-negative bacteria. In the present work we studied a series of 7-*O*-benzyl genistein derivatives. As we have just shown[9] such a modification of genistein significantly changes the properties of derivative molecules and alters their impact on lipid bilayers.

Interactions of flavonoids with lipids may be important for at least two reasons: (i) they may be involved in the mechanism of prevention of lipid oxidation and (ii) they have to be known when the preparation of liposomal or micellar formulation of certain flavonoids is planned. Flavonoid–lipid interactions were studied by several research groups. Using calorimetry Saija et al.[10] and Wójtowicz et al.[11] have demonstrated that quercetin, hesperidin and naringenin intercalated into the liposomal membranes. Interactions of daidzein and genistein with the liposomes composed of different lipids was studied by means of calorimetry and turbidity measurements.[12] Fluorescence polarization anisotropy measurements were used in the study on influence of several flavonoids on the fluidity of liposome membranes.[13] Also in our group the interactions of several flavonoids with the lipid bilayers were studied previously. Four *Sophora* isoflavonoids (formononetin, irisolidone, licoisoflavone A and 6,8-diprenylgenistein) were found to interact with different intensity with the liposome membranes.[14] Using calorimetry and fluorescence spectroscopy we have also shown that O-demethylation of isoflavones did not influ-

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ence the biophysical properties of the lipid bilayers.[15] Interactions of silybin with lipid bilayers were recently also studied in our laboratory.[16]

In the present paper we describe the results of the fluorescence spectroscopic and calorimetric investigation of the influence of a series of newly synthesized genistein benzyl derivatives (see Fig. 1 for chemical structures) on the phase behavior and fluidity of phospholipid bilayers. We show that these derivatives exert mild effects on the bilayer structure and induce no phase separation during the phase transition of dipalmitoylphosphatidylcholine.

2. Results

2.1. Microcalorimetry

In the calorimetric experiments we investigated the influence of three ethanol-soluble isoflavones (IFG71, IFG74 and genistein) on the thermal properties of DMPC bilayers. Since the pretransition was not detected in samples containing any of those compounds (for genistein see Fig. 2) we followed the dependencies of main transition temperature (T_m), enthalpy change (ΔH), and transition half-height width ($\Delta T_{1/2}$) on the isoflavone concentration.

In Figure 2, showing the thermograms of DMPC mixed with genistein at different molar ratios, the impact of this isoflavone on the lipid thermal behavior is exemplified. The addition of genistein caused the disappearance of the DMPC pretransition (even at the lowest molar ratio used) and concentration-dependent shift of the main transition temperature towards lower values accompanied by the decrease of the transition peaks area and the peak broadening. The dependencies of T_m, relative transition enthalpy change ($\Delta H/\Delta H_0$, where ΔH_0 is transition enthalpy change for pure lipid and ΔH for isoflavone-lipid mixture) and $\Delta T_{1/2}$ on the isoflavone/lipid molar ratio obtained for mixtures of DMPC with IFG71, IFG74 and genistein are shown in Figure 3A–C, respectively. All examined isoflavones decreased the lipid main transition temperature in a concentration-dependent manner (see Fig. 3A). Generally the influence of IFG74 on T_m was stronger than that of genistein, which, on the other hand, decreased T_m more effectively than IFG71. Additionally IFG74 seemed to exert a biphasic effect on T_m: in lower concentrations it caused the decrease of T_m but at highest used molar ratio to lipid (0.2) this effect was reversed and slight back-increase of transition temperature was observed.

The addition of the examined compounds to DMPC resulted also in broadening of the transition peaks, what was observed as the increase of transition half-height width (Fig. 3C). This process followed shifting the T_m towards lower values and also depended on the concentration. Transition profiles were more effectively broadened by IFG74 and genistein than by IFG71. Figure 3B shows the influence of the studied isoflavones on the enthalpy change on DMPC main phase transition. Isoflavones caused the concentration-dependent decrease of transition enthalpy change. In case of ΔH the most pronounced effects were found for IFG71, while genistein and IFG74 altered the transition enthalpy change to a lesser extent.

2.2. Fluorescence spectroscopy

In spectroscopic experiments we assessed the influence of examined isoflavones on Laurdan generalized polarization and DPH fluorescence polarization anisotropy. First, using EYPC liposomes, we measured the dependence of Laurdan GP on the concentration of genistein and its derivatives. As shown in Figure 4 the effects exerted by isoflavones are scattered, however the general tendency for concentration-dependent increasing of Laurdan GP was observed for all compounds. In higher concentrations IFG71, IFG62 and IFG73 showed much bigger influence on Laurdan GP than IFG74, IFG43 and genistein. Since the IFG62 effects exerted on Laurdan GP did not depend monotonically on concentration we have chosen IFG71 and IFG73 for further experiments in which

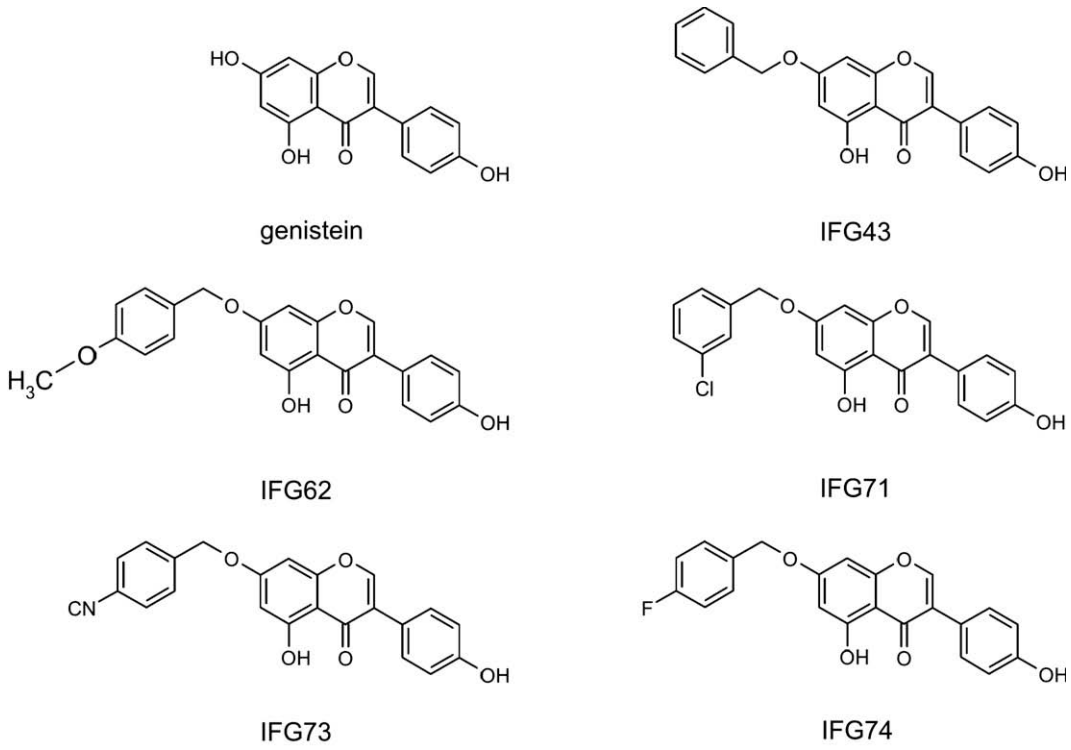

Figure 1. Chemical structures and abbreviations of studied genistein derivatives.

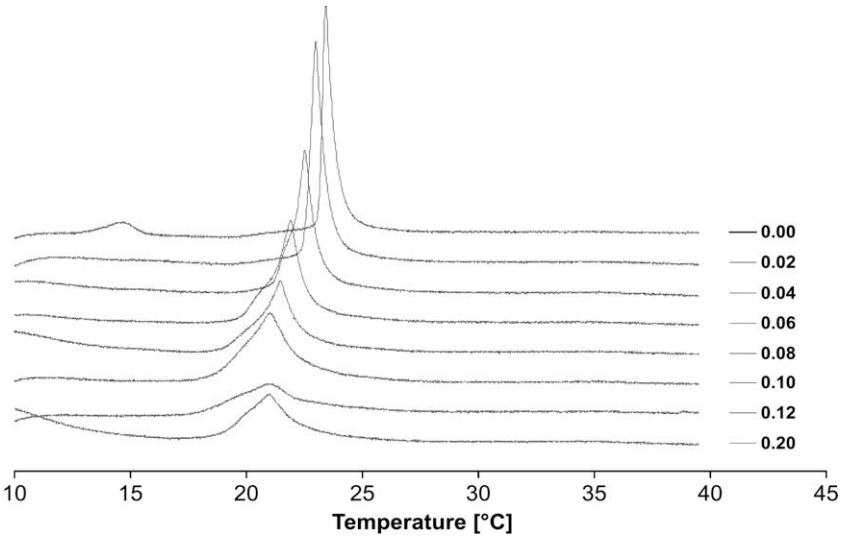

Figure 2. The example thermograms obtained for DMPC mixed with genistein at different molar ratios (given at the right side of each thermogram).

we measured their impact on the dependence of Laurdan generalized polarization on temperature in DPPC liposomes. The results of these measurements are shown in Figure 5. For both examined isoflavones as well as for pure DPPC in temperatures below the main phase transition we observed a high and almost constant GP values. In the temperature range corresponding to the main phase transition the abrupt decrease of GP values was recorded. In this temperature region IFG73 increased the GP values, while IFG71 decreased it (when compared to the pure lipid). For temperatures higher than T_m we observed further (but less dramatic) decrease of GP. In this temperature range GP was decreased less effectively by IFG71 then by other compounds.

In the last part of experiments, in which we used Laurdan as fluorescent dye, we checked if IFG71 and IFG73 affected the dependence of GP on the excitation wavelength at three different temperatures: below, close to and above the main phase transition of DPPC. At 24 °C and 42 °C GP was not dependent on the excitation wavelength while at 48 °C GP(λ_{ex}) dependence was descending for liposomes formed from pure DPPC as well as with addition of IFG71 and IFG73 (data not shown).

The results of DPH fluorescence polarization anisotropy measurements (presented in Fig. 6) allowed us to divide the examined isoflavones into two groups: those which increased anisotropy (IFG71, IFG74 and genistein), and those which did not cause any significant changes of this parameter (IFG43, IFG62 and IFG73). Two representatives of the first group (IFG71 and IFG74) and one of the second group (IFG73) were used in further experiments in which we assessed their influence on the dependence of DPH fluorescence polarization anisotropy on temperature in DPPC liposomes. The results of these investigations are shown in Figure 7. In temperatures below the DPPC main phase transition temperature the values of DPH fluorescence polarization anisotropy for IFG73 and IFG74 were lower than for pure lipid, while anisotropy values obtained for IFG71 were similar to those obtained for pure DPPC. Above the T_m the DPH fluorescence polarization anisotropy for IFG71 and IFG74 was higher than for DPPC and values for IFG73 were similar to those obtained for pure lipid.

3. Discussion

As shown by the example thermograms presented in Figure 2 genistein as well as its two benzyl derivatives (IFG 71 and IFG 74, not shown) eliminate the pretransition and substantially affect the main phase transition of DMPC in a concentration-dependent manner. The pretransition is very sensitive to the presence of molecules that alter packing of the phospholipid molecules and their hydration in gel state.[17,18] Therefore pretransition vanishing induced by genistein and its derivatives is the first marker of the interaction of the studied isoflavones with the lipid bilayers. Simultaneously the dependencies of the main transition temperature (T_m), enthalpy change (ΔH) and half-height width ($\Delta T_{1/2}$) on the isoflavone concentration, shown in Figure 3, allow to conclude that the main phase transition of DMPC was altered by IFG 74 more effectively than by genistein, while IFG 71 was less effective transition modifier than its parent compound. According to the standard interpretation of calorimetric data proposed by Jain and Wu[19] the decrease of both transition temperature and enthalpy change suggests that lipid polar-heads as well as hydrocarbon chains regions were affected by the studied compounds. The character of observed changes (decrease of T_m, ΔH and increase of $\Delta T_{1/2}$) allows to conclude also, that interactions between lipid molecules in the gel state became weaker in the presence of isoflavones. Similar influence of genistein on the thermal properties of DPPC bilayers has been already observed in our laboratory.[15] Comparing the effects observed for the same isoflavone/lipid molar ratios that were obtained in the previous and the present study it is worth noticing that the magnitude of changes induced by genistein in DMPC bilayers is bigger, presumably due to generally weaker interactions between shorter acyl chains of this lipid (when compared to DPPC). The effects of genistein and its derivatives on the thermal behavior of phosphatidylcholines observed in our experiments differ from those recorded by Saija et al.[10] for quercetin, hesperetin and naringenin, which caused exclusively a concentration-dependent decrease of the transition temperature. Discussing their results these authors suggested only superficial interaction between drugs and lipids. Since genistein and naringenin possess the same number of hydroxyl groups, similarly located around the flavonoid ring system, one can conclude that some other structural features like position at which ring B is bound to chromone moiety of flavonoid may be important for the interaction of flavonoids with the lipid bilayers.

In spectroscopic measurements we used two fluorescence dyes which are located in different membrane regions. DPH penetrates the hydrophobic core of the bilayer[20] and therefore its fluorescence polarization anisotropy directly depends on the mobility of lipid acyl chains. Laurdan fluorophore due to its location at the hydro-

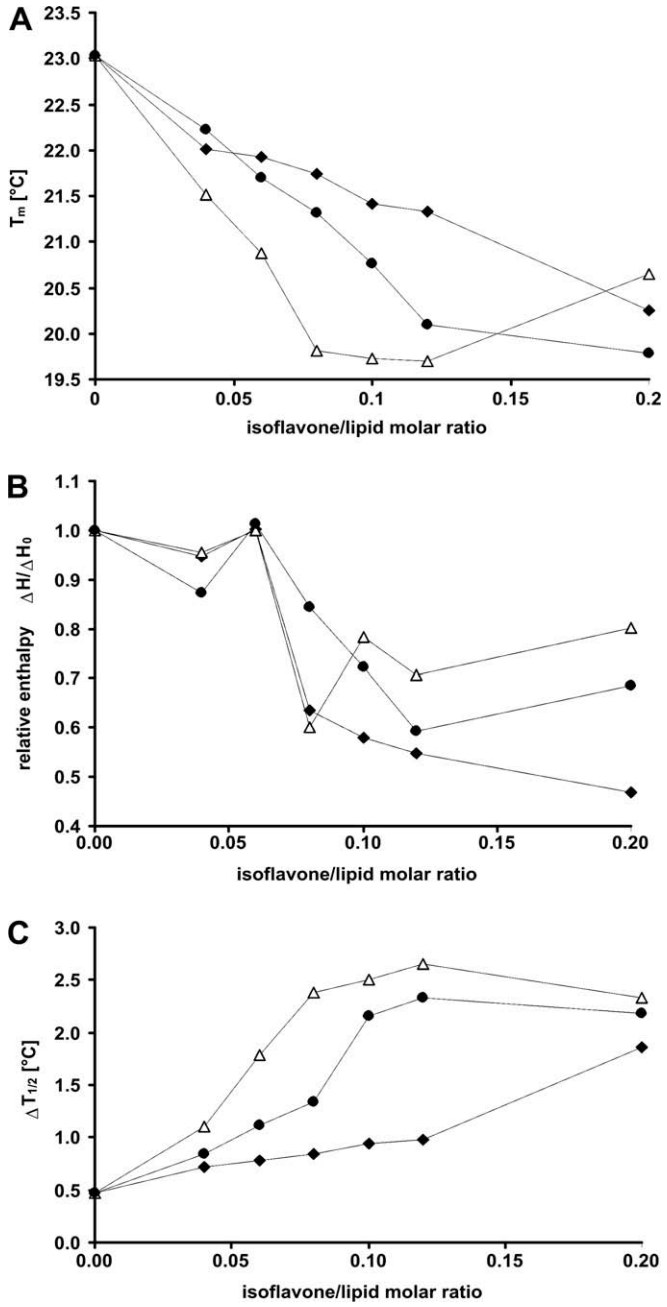

Figure 3. Influence of genistein (●), IFG71 (♦) and IFG74 (Δ) on the parameters of DMPC main phase transition: temperature (A), relative enthalpy change—$\Delta H/\Delta H_0$, where ΔH_0 is transition enthalpy change for pure lipid and ΔH for isoflavone-lipid mixture (B) and peak half-height width (C).

philic–hydrophobic interface of the bilayer reports on the polarity of this region.[21] Generalized polarization (GP) of Laurdan fluorescence is sensitive to the presence of water molecules in the vicinity of the dye molecule and therefore depends also on the packing of lipid polar head-groups.[22] Keeping above in mind we may thus conclude that the results of spectroscopic studies are in general agreement with calorimetric ones. As it follows from Figuers 4 and 6 IFG71 effectively increased both Laurdan GP and DPH fluorescence polarization anisotropy in EYPC liposomes, what can be interpreted as a rigidifying effect exerted by this compound on both polar-heads (Laurdan) and hydrocarbon chains (DPH) regions of lipid bilayer. Other compounds caused some increase of either GP (IFG62 > IFG73 > IFG43) or anisotropy (IFG74), so we may

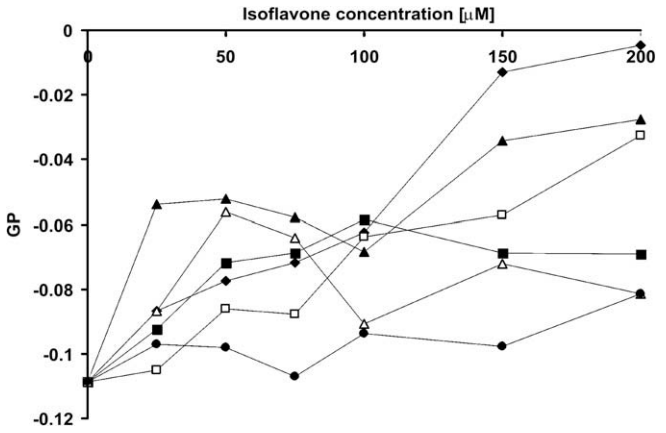

Figure 4. The dependence of Laurdan generalized polarization (GP) on the concentration of isoflavones: ●—genistein, ■—IFG43, ▲—IFG62, ♦—IFG71, □—IFG73, Δ—IFG74. Liposomes were prepared from EYPC. Lipid concentration in the liposome suspension was 200 μM.

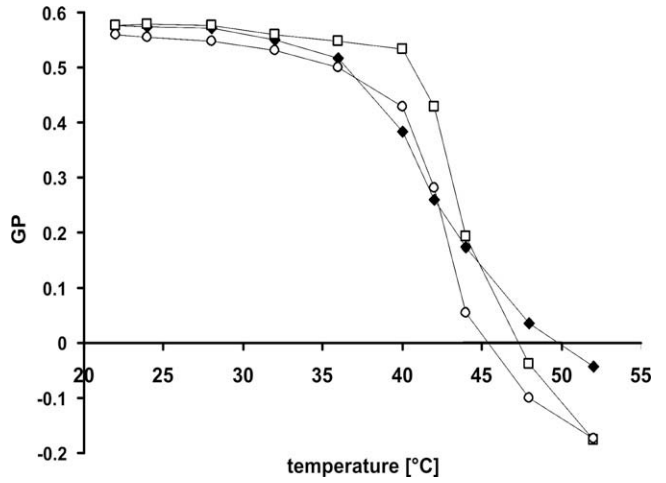

Figure 5. The dependence of Laurdan generalized polarization (GP) on temperature for liposomes formed from DPPC (○) with addition of 100 μM IFG71 (♦) and IFG73 (□). Lipid concentration in the samples was 200 μM.

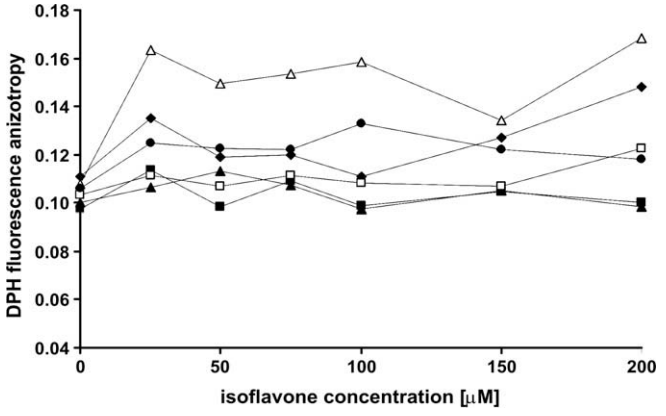

Figure 6. The dependence of DPH fluorescence polarization anisotropy on the concentration of isoflavones: ●—genistein, ■—IFG43, ▲—IFG62, ♦—IFG71, □—IFG73, Δ—IFG74. Liposomes were prepared from EYPC. Lipid concentration in the liposome suspension was 200 μM.

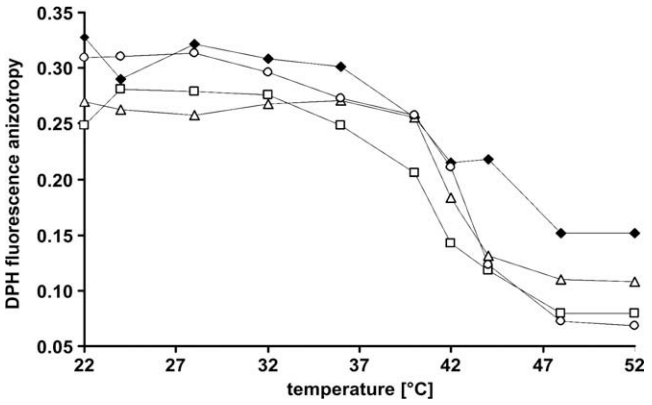

Figure 7. The dependence of DPH fluorescence polarization anisotropy on temperature measured in DPPC liposomes (○) and with addition of 100 μM IFG71 (♦), IFG73 (□) and IFG74 (Δ). Lipid concentration in the samples was 200 μM.

assume that they influenced more effectively the polar head or acyl chains regions, respectively. Very similar effects of flavonoids and isoflavonoids with respect to membrane interior were found by Arora et al.[13], who recorded genistein and its derivatives-induced increase of the fluorescence polarization anisotropy of fluorescent probes located at different depths of the membrane hydrophobic core. It is worth emphasizing that in our experiments as well as in those presented in[13] liposomes were made of lipids that were in liquid-crystalline phase at room temperature. The role of bilayer physical state in flavonoid-bilayer interactions can be seen when one analyses the results of experiments on Laurdan GP and DPH fluorescence polarization anisotropy dependence on the temperature in DPPC liposomes (Figs. 5 and 7, respectively). Since GP was not altered by presence of IFG71 and IFG73 in the temperatures below T_m, we may conclude that the region of DPPC polar-heads in gel state was not affected by these genistein derivatives. The hydrocarbon core of the bilayer in gel phase, however, was fluidized by IFG73 and IFG74 as indicated by the decrease of DPH fluorescence polarization anisotropy. On the other hand the rigidifying effects of the isoflavones were found for both fluorescent dyes in liquid-crystalline DPPC bilayer, what is consistent with previously discussed results recorded in case of EYPC liposomes.

In Laurdan GP(λ_{ex}) experiments we additionally checked if genistein derivatives (IFG71 and IFG73) caused any phase separation in DPPC liposomes in gel and liquid-crystalline states as well as during the main phase transition. Since for any of the temperatures used we have not obtained an ascending GP(λ_{ex}) dependency, then according to Parassassi et al.[21,23] no phase separation was present in the studied system. The horizontal GP(λ_{ex}) lines recorded at 24 °C and 42 °C are characteristic for the gel phase while the descending GP(λ_{ex}) dependence obtained at 48 °C is typical for the liquid-crystalline phase of the bilayer.

As it was proposed earlier by research group of Saija et al.[10,24] the ability of different flavonoids to penetrate and interact with the lipid bilayers may be essential for their capacity to modify membrane-dependent processes, such as membrane lipid oxidation induced by free-radicals. On the other hand in the previous report[15] we have demonstrated that the alteration of lipid bilayer fluidity induced by some isoflavones (including genistein) correlates with their ability to inhibit the activity of MRP1 (an ABC transporter involved in the phenomenon of multidrug resistance of cancer cells). Taking into account the results presented above we may conclude that also some of the genistein benzyl derivatives studied herein can penetrate membranes and in this way they may perturb the oxidation or transport processes. Similarly to other flavonoids[25] the ability of genistein benzyl derivatives to interact with membranes depends on the details of their chemical structure. Presence of the additional benzene ring (not substituted by any additional group—IFG43) seems to give a molecule which is able to perturb polar-heads and acyl chains regions only very weakly. Membrane perturbing potency of genistein derivatives in which benzene ring is substituted by halogen atoms depends on the position of such a modification. Chloride in the position 3 (IFG71) gives molecule much less active than derivative with fluoride in the position 4 (IFG74). It is also worth emphasizing that the difference of effects exerted by these two compounds was bigger when observed in the bilayer interior (calorimetry and DPH experiments) than close to the polar region (Laurdan experiments). On the other hand the presence of cyanide in the position 4 of additional benzene ring (IFG73) yields a compound slightly more efficiently interacting with bilayers then IFG43.

In the present work we have used calorimetry and fluorescence spectroscopy to study the interactions of genistein benzyl derivatives with lipid bilayers. We have shown that these interactions depend on the structure of individual derivatives as well as on the physical state of a bilayer. In our opinion these interactions should be responsible for at least some of the membrane-dependent effects of flavonoids, however the understanding of their importance is far from being complete. Therefore we plan the future studies in which the influence of genistein derivatives on the membrane integrity as well their ability to inhibit the active transport carried out by MRP1 will be investigated.

4. Experimental

4.1. Chemicals

Genistein and its benzyl derivatives were synthesized at Department of Organic, Bioorganic Chemistry and Biotechnology of Silesian Technical University. Their purity was checked by HPLC and NMR. Chemical structures and abbreviations of genistein derivatives used in the present paper are shown in Figure 1. Since these isoflavonoids were almost insoluble in water, their DMSO or ethanol solutions were used for the experiments.

Egg yolk phoshphatidylcholine (EYPC), 1,2-dimyristoyl-*n*-glycero-3-phosphatidylcholine (DMPC) and 1,2-dipalmitoyl-*n*-glycero-3-phosphatidylcholine (DPPC) were purchased from SIGMA (Poznań, Poland). All lipids were used as delivered, without further purification.

Fluorescent labels: 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-2-dimethylaminonaphtalene (Laurdan) were purchased from Sigma (Poznań, Poland) and Molecular Probes (Eugene, OR, USA), respectively. All other chemicals used in this study were of analytical grade.

The sets of the compounds studied by each of the techniques used in this work are not the same because of the different solubility of individual genistein derivatives. None of them was soluble in water, compounds used in the calorimetric experiment were soluble in ethanol and DMSO while those studied only by the spectroscopic methods were soluble exclusively in DMSO. Since DMSO affects the thermotropic phase transitions of phospholipids[26–28] only ethanol-soluble compounds were studied by calorimetry.

4.2. Calorimetry

For each calorimetric sample 1.5 mg DMPC was dissolved in the appropriate amount of ethanol stock solution (5 mM) of the studied isoflavone. The isoflavone/lipid molar ratios in the samples were: 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.20. Than the solvent was evaporated by a stream of nitrogen and the residual solvent was removed under vacuum for 2 h. Samples were hydrated by 15 μl

of Tris–EDTA–NaCl (20 mM Tris, 0.5 mM EDTA, 150 mM NaCl) buffer (pH 7.4). Hydrated mixtures were heated to the temperature 10 C higher than the main phase transition temperature of DMPC and vortexed until homogeneous dispersion was obtained. After that, the samples were transferred into aluminum sample pans and sealed. Calorimetric measurements were performed using Unipan (Warsaw, Poland) microcalorimeter type 600 equipped with the measuring unit rebuilt in our laboratory. The samples were scanned at a rate of 1 °C/min. Data were stored on hard disk and analyzed off-line using software developed in our laboratory.

4.3. Fluorescence spectroscopy

Unilamelar EYPC or DPPC liposomes were obtained by sonication of 2 mM phospholipid suspension in Tris–NaCl–EDTA (20 mM Tris, 0.5 mM EDTA, 150 mM NaCl) buffer (pH 7.4) using UP 200s sonicator (Dr Hilscher, GmbH, Berlin, Germany). Laurdan stock solution (1 mM) was prepared in DMSO. DPH stock solution (1 mM) was prepared in tetrahydrofuran. Stock solutions of the studied isoflavones (30 mM) were prepared in DMSO. Liposomes were diluted and incubated with the fluorescent dye in darkness for 30 min at room temperature, then, the studied modulator was added and liposomes were incubated for another 15 min (also in darkness, at room temperature).

In all experiments the final phospholipid concentration was 200 μM. The concentration of the fluorescent dye (DPH or Laurdan) was 5 μM. The isoflavonoid concentration in samples was 25–200 μM in concentration-dependence experiments and 100 μM in all temperature-dependence experiments. In the latter measurements we used liposomes prepared from DPPC (T_m = 41.5 °C) to be sure that also during the preparation stage (at room temperature) two types of liposomes (EYPC or DPPC) were in different physical state. The measurements were carried out in a temperature range from 22 °C to 52 °C. All other spectroscopic experiments were carried-out at room temperature.

Fluorescence experiments were carried out with LS 50B spectrofluorimeter (Perkin–Elmer Ltd., Beaconsfield, UK) equipped with a xenon lamp using emission and excitation slits of 5 nm. Temperature was controlled by cooling-heating circuit. The actual temperature was measured directly in the sample cuvette using the platinum thermometer. The excitation wavelength for DPH was 380 nm and for Laurdan—390 nm. The emission wavelength for DPH was 450 nm, and Laurdan emission spectra were measured for wavelengths ranging from 410 nm to 510 nm, in 10 nm steps. Data were processed with FLDM Perkin–Elmer software. In DPH experiments the anisotropy of fluorescence polarization (a) was calculated using the following formula:

$$a = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}}. \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the fluorescence measured with analyzer parallel and perpendicular to the polarization of the incident beam, respectively. G is an instrumental correction factor, automatically calculated by FLDM software for each experiment. Laurdan generalized polarization (GP) was calculated according to the formula introduced by Parasassi et al.[25]:

$$GP = \frac{I_B - I_R}{I_B + I_R}. \quad (2)$$

where I_B and I_R represent the Laurdan fluorescence intensities recorded at 440 nm and 490 nm, respectively.

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