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Genistein derivatives decrease liposome membrane integrity — Calcein release and molecular modeling study

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ABSTRACT

The ability of newly synthesized genistein benzyl and glycosylated derivatives to permeabilize the liposome membrane was studied by calcein-leakage method. All studied derivatives appeared to be more effective than their parent compound — genistein. Comparing the experimental results with theoretical calculations we found that in the case of benzyl derivatives the dipole moment of added benzene ring (with its substitutions) might be important for the strength of flavonoids–lipid interactions. This conclusion may have some implications for QSAR studies in which mostly the dipole moments of entire molecules are considered.

1. Introduction

Peroxidation of lipids, as well as other membrane components, by reactive oxygen species — ROS — leads to the impairment of membrane structure and functions, and in consequence may cause many serious diseases like atherosclerosis, stroke, cardiovascular disease or cancer [1,2]. Detrimental effects of oxidative damage can be reduced by the presence of different molecules possessing antioxidant, ROS scavenging properties — flavonoids belong to the best known representatives of this group of substances. When considering the molecular mechanism of flavonoid antioxidant activity exerted on lipid membranes it seems obvious that interaction of flavonoids with lipids must be involved [3–6]. Since the position of flavonoids within lipid bilayer seemed to be essential for their activity several attempts were made to determine the localization of membrane-bound flavonoid molecules. In the early calorimetric study Saija et al. [3] suggested that quercetin, hesperetin, naringenin and rutin superficially interact with lipid bilayer. On the other hand penetration of hydrophobic bilayer core by a group of flavonoids and isoflavonoids was deduced by Arora et al. [4] on the basis of fluorescence spectroscopic experiments. Intrinsic fluorescence properties of fisetin were used by Sengupta et al. [7] to show that this molecule was localized and rigidly bound at the polar/apolar interfacial region of lipid bilayer. In a series of ^{1}H magic angel spinning NMR experiments it was shown that in fact flavonoid molecules are broadly distributed along the membrane normal with a maximal probability of localization close to polar/apolar interface [8]. After intercalation of flavonoids into membrane the antioxidant effects can be achieved by the two complementary mechanisms — i) direct scavenging of ROS by flavonoid molecules and/or ii) reduction of the ROS access to the interior of membrane caused by flavonoid-induced alteration of membrane fluidity. This last effect was recorded in the studies on the impact of different flavonoid types on biophysical properties of lipid bilayers: green tea catechins [6,9,10], flavonols [6] or isoflavones [4,6]. The consequences of flavonoid–membrane interactions are not limited to the antioxidant action of these drugs: alteration of bilayer properties may affect also the activity of integral proteins and thus influence transport and other membrane-related processes [2,11]. Such effects were found for erythrocyte membrane proteins [12,13] and also for gramicidin ion channels [14].

Calcein leakage from liposomes is a simple but efficient assay often used in the studies on the influence of various factors on membrane permeability or integrity [15–17]. This method was also applied to monitor the effects exerted by different flavonoids on vesicle [18] and giant unilamellar liposome membranes [19]. In the present paper the calcein-leakage assay was used to study the impact of a group of newly synthesized genistein derivatives on the liposome membrane permeability. The results of these experiments are discussed in context of different descriptors of the flavonoids

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molecular structure obtained by the computer-assisted molecular modeling.

2. Materials and methods

2.1. Chemicals

Genistein and its derivatives were synthesized at Department of Organic, Bioorganic Chemistry and Biotechnology of Silesian Technical University, their purity was checked by HPLC and NMR. The chemical structures of the isoflavones used in the present paper are shown in Fig. 1. Since studied flavonoids were almost insoluble in water, in experiments their DMSO stock (5 mM) solutions were used.

Egg yolk phoshphatidylcholine (EYPC) and fluorescent label — bis [*N*,*N*-bis(carboxymethyl) aminomethyl]fluorescein (calcein) were purchased from SIGMA (Poznań, Poland). Calcein before use was purified by chromatography on Sephadex G-50 column.

2.2. Calcein release from liposomes

Measurement of calcein release from liposomes was performed using the method developed by Gubernator et al. [16]. In brief, the samples containing chloroform solution of lipid were dried under the stream of nitrogen to prepare a thin lipid film on the wall of a test-tube. Residual amounts of organic solvent were removed under vacuum for at least 2 h. The lipid film was hydrated with 35 mM calcein solution in buffer (10 mM Hepes, pH 7.4). The obtained multilamellar liposomes were calibrated by extrusion (seven times) through 200 nm nucleopore polycarbonate filters (Whatman Millipore, USA). Afterwards, the liposome suspension was purified from non-encapsulated fluorescent dye by gel filtration on a Sepharose 4B column (1 cm×20 cm) equilibrated with Hepes buffer (10 mM Hepes, 150 mM NaCl, 1 mM EDTA, pH=7.4). Stock solutions of studied compounds were prepared in DMSO (5 mM). Flavonoids were added to calcein-loaded liposomes (10 μM of lipid per sample) to achieve the appropriate range of concentrations for the planned experiments. The liposomes containing calcein were incubated with studied flavonoids for 15 min in darkness at room temperature. The degree of calcein release was determined with Perkin Elmer spectrofluorimeter LS 50B. The excitation wavelength was 490 nm and the emission wavelength was 520 nm. The total amount of calcein in liposomes was assessed by measuring the fluorescence of liposome lysate obtained by the addition of Triton X-100 to a final concentration of 0.1%.

The permeability of liposome membranes was calculated using the following formula:

$$P = \frac{F_t - F_0}{F_\infty - F_0} \cdot 100[\%]$$

where P is liposome membrane permeability, F_t is calcein fluorescence intensity in the sample, F_0 is initial fluorescence, and F_∞ is maximal fluorescence of the sample after lysis by Triton X-100.

Since in our experiments DMSO was used to obtain stock solution of flavonoids we additionally checked if there was any significant influence of this compound on liposome permeability. We found that DMSO added alone at the same amount as in the rest of experiments caused only slight increase of calcein leakage (P was increased by less than 5%).

2.3. Molecular modeling

Theoretical calculations were performed using Titan 1.0.8 software (Wavefunction, Inc., Irvine, USA & Schrodinger, Inc., Portland, USA). The properties of studied isoflavonoids were modeled using AM1 semi-empirical molecular orbital method.

3. Results and discussion

In present paper we describe the results of the studies on the effect of a group of newly synthesized flavonoids on the membrane permeability. The alteration of calcein fluorescence intensity was used to monitor the bilayer destabilization induced by increasing flavonoid concentrations. Calcein was entrapped in unilamellar

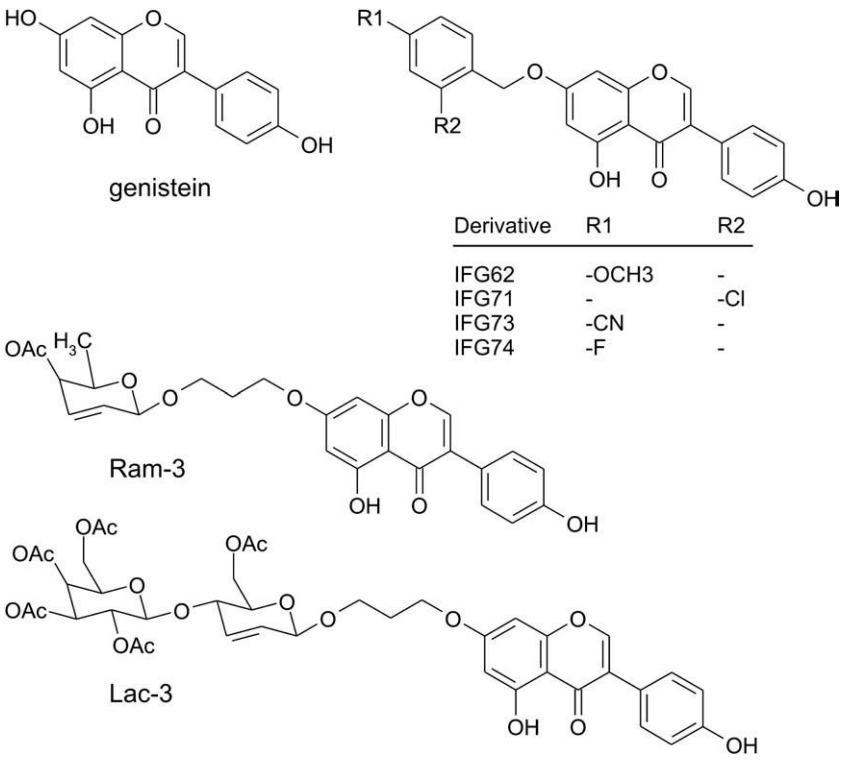

Fig. 1. Chemical structures of genistein and its derivatives.

liposomes composed of egg yolk phosphatidylcholine (EYPC), which in the temperature of experiments (25 °C) was in a liquid-crystalline phase. The initial concentration of calcein in liposomes was high enough to ensure the self-quenching of its fluorescence. Decrease of the dye concentration occurring when the fluorescence probe leaked from the vesicles into the surrounding buffer caused the increase of its fluorescence. All flavonoids (except genistein) were tested in the same concentration range (from 0.5 μM up to 61 μM), their influence on liposome permeability is shown in Fig. 2a. As follows from this figure, we observed similar pattern of relation between fluorescence intensity and flavonoid concentration for all tested compounds. For low concentrations (0.5 μM–5 μM) we recorded the rapid increase of the calcein fluorescence, while for higher concentrations this effect was saturated and almost constant values of fluorescence were observed. This pattern of permeability-drug concentration dependence is in keeping with our previous studies [20] in which we have observed that the silybin — major biologically active flavanolignan extracted from milk thistle (*Sylibum marianum*) — was able to increase the permeability of EYPC liposomes for calcein in a similar way. The saturation level of the permeability increase varied for different studied flavonoids. The highest permeability was observed for liposomes modified with IFG74 and IFG62 (almost 80%), on the other hand the lowest increase of permeability (70%) was found for Lac-3. Genistein itself caused the weakest effect since at saturation the permeability increase was 45%–50% and the saturation was observed at higher concentration (25 μM) than in case of its studied herein derivatives. The reasons for the saturation of calcein release observed at higher concentrations of studied flavonoids are unclear. This effect might be caused by the limited interactions of flavonoids with lipid membranes which may result from the self-aggregation of flavonoid molecules at high enough concentrations. The aggregation effect was found for morin [21] and intuitively is supported by our observations of very poor solubility of studied flavonoids in water.

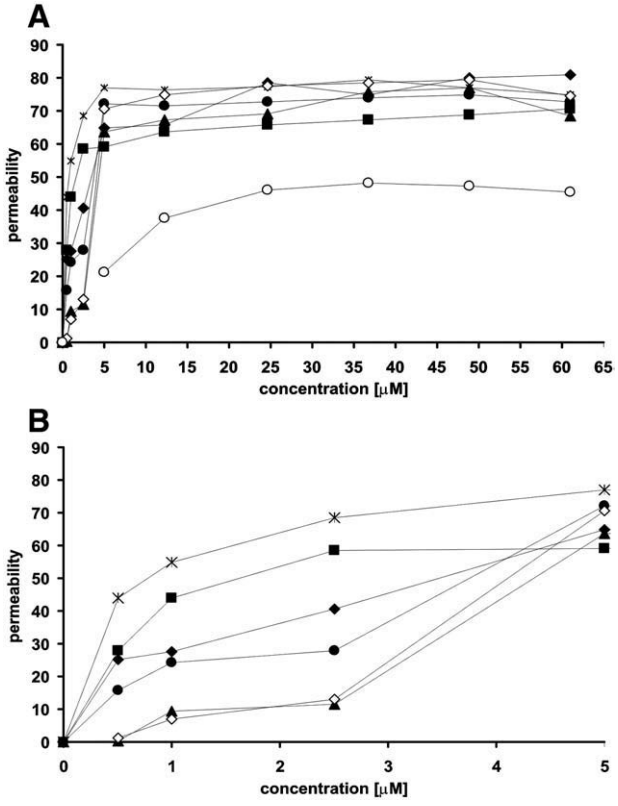

Fig. 2. The dependence of liposome permeability on the concentration of genistein (○) and its derivatives (IFG62 — ◇, IFG71 — ▲, IFG73 — ●, IFG74 —⨳, Ram-3 — ◆, Lac-3 — ■). Whole range of studied concentrations is presented in part A, the impact of low concentrations of genistein derivatives is shown in part B.

Table 1
Molecular descriptors of genistein and its benzyl derivatives

Flavonoid	Ovality	Log *P*	Polariz. [Å^3]	HOMO [eV]	LUMO [eV]	M.D$_x$ [D]	M.D$_y$ [D]	M.D$_z$ [D]	M.D. [D]
Genistein	1.46	1.74	58.31	−8.80	−0.70	0.60	0.27	−1.06	1.25
IFG62	1.67	3.61	67.90	−8.73	−0.60	0.76	−2.21	−1.16	2.60
IFG71	1.65	4.30	66.90	−8.77	−0.66	1.80	−1.21	−0.62	2.26
IFG73	1.65	3.77	67.31	−8.83	−0.77	1.05	−0.69	1.52	1.98
IFG74	1.63	3.90	66.19	−8.79	−0.70	0.63	0.30	0.08	0.71
IFG74head	–	–	–	–	–	−0.03	0.0	−1.86	1.86

Log *P* is a logarithm of octanol–water partition coefficient, Polariz. — the electronic polarizability of molecule, HOMO — highest occupied molecular orbital energy, LUMO — lowest unoccupied molecular orbital energy, M.D$_x$, M.D$_y$, M.D$_z$, M.D. — spatial components and total dipole moment, respectively. Spatial components of IFG74 head were calculated assuming that *x*–*y* plane lies in the plane of its benzene ring and do not include the fact that this ring is twisted with the respect to the plane of genistein molecule.

Since the most profound changes of membrane permeability were observed in narrow flavonoid concentration range of 0.5–5 μM, then Fig. 2b shows how particular genistein derivatives differed in the induction of calcein leakage from liposomes. Analyzing the dependence of liposome permeability on flavonoid concentration one may divide the studied compounds into three groups: IFG62 and IFG71 showing only moderate dynamics of changes, IFG73 and Ram-3 characterized by medium activity, Lac-3 and IFG74 presenting the most dynamic effects. As shown in Fig. 2b IFG 62 and IFG71 at 0.5 μM concentration did not induce a significant calcein leakage but at 5 μM concentration the compounds induced much intensive dye efflux from liposomes: 70% and 65% for IFG62 and IFG71, respectively. The effects of IFG 73 and Ram-3 were stronger than those of IFG62 and IFG71 but weaker than IFG74 and Lac-3. Significant increase of calcein leakage was observed after the addition of IFG74 even at concentration lower than 1 μM. On the other hand, the second of the most active compounds (Lac-3) caused the saturation of calcein leakage at relatively low concentration — 2.5 μM. Generally, comparing the influence of genistein benzyl derivatives on liposome membrane permeability one can classify them in the following order: IFG 74>IFG 73>IFG 71>IFG 62. When comparing the effect of glycosylated derivatives on membrane permeability it was recognized that for Lac-3 increase of permeability appeared at lower concentrations but the maximal increase (saturation level) of the calcein leakage observed at high flavonoid concentrations was stronger for Ram-3.

The experimental results described above should be discussed concerning the chemical structure of studied compounds. The case of the most effective genistein benzyl derivative — IFG 74 seems to be particular. This compound has a fluorine atom at the position 4 of additional benzyl ring. The molecules containing in their structure fluorine, the most electronegative halogen, display a number of physical properties that are unmatched by any other organic molecules of medicinal interest. The electronegativity and size of fluorine orbitals are similar to those of carbon and therefore the carbon-fluorine bond is one of the most energetic in which a carbon atom can participate. Due to the big difference in electronegativity between carbon and fluorine the bond between these atoms is characterized by a large dipole moment. Presence of this dipole moment together with the distribution of charge in a specific molecule, may significantly enhance the molecule's ability to engage in intramolecular interactions. This way of reasoning holds also for aromatic systems, where the presence of fluorine changes the electrostatic distribution of the molecular surface. It may lead to the enhancement of the ability of aromatic fluorine to bind to different molecules [22]. This indicates that fluorine in the IFG74 aromatic system may play an important role in the interaction of this flavonoid with lipid molecules. This suggestion seems to be confirmed by the

example of another flavonoid IFG73, which structurally is similar to IFG74 but substituted by –CN instead of fluorine at 4-th position of additional benzyl ring. As shown in Fig. 2b IFG73 at concentration 1.0 μM induces less intensive dye efflux from liposomes (24.37%) than IFG 74 at the same concentration (54.81%).

To study more deeply the role of flavonoid structure in interactions of these derivatives with the liposome membrane we calculated several molecular descriptors that characterize the studied compounds. The values of these descriptors are presented in Table 1. Comparing the order of genistein benzyl derivatives permeabilizing abilities with order of certain descriptor values it is easy to find that among the ovality, octanol/water partition coefficient (Log P), molecular polarizability, HOMO and LUMO energies, and molecular dipole moment only this last property seems to be somehow related with experimentally determined flavonoids activity. This conclusion is based on observation that the order of membrane permeabilization (IFG74>IFG73>IFG71>IFG62) represents the inversed order of molecular dipole moments.

Dipole moment is one of the molecular descriptors that are often used in different QSAR studies, which were performed among others to predict antiparasitic properties of thieno[2,3-b]pyridine derivatives [23], cyclooxygenase-2 inhibition by 3-phenoxypyran-4-one [24], and also anticancer effects of flavonoids [25]. Benzyl genistein derivatives studied in present work differed only by one fragment of the molecule (by group substituting additional benzene ring). This prompted us to ask a question if the dipole moment of entire molecule is important for observed effect or, maybe the dipole moment of molecule's fragment has bigger impact on the interaction of flavonoids with lipid bilayer. To answer this question we have calculated the dipole moment and its spatial components also for the "head" of IFG74 (benzene ring substituted by methyl group and fluorine in *para* position). Considering the vectors of dipole moments of genistein, IFG74 and IFG74 head we concluded that small value of IFG74 dipole moment results presumably from the summing-up of the genistein and IFG74 head dipole moment vectors (schematically presented in Fig. 3). This conclusion is in keeping with the presented above discussion of the role of fluorine atom. Further calculation and spatial analysis of the dipole moment vectors of the "heads" of other genistein benzyl derivatives (data not shown) have demonstrated that they are oriented in space similarly to IFG74 head and the order of their values repeats the order of permeabilizing effects exerted by these compounds on liposome membranes. Final argument confirming the role of local dipole moment of the molecule's fragment is provided by the comparison of the dipole moments and liposome permeabilizing activity of parent genistein and its benzyl derivatives. According to the value of the dipole moment of the entire molecule genistein (1.25 D) should be only slightly less effective than IFG74 (0.71 D) and more effective than IFG73 (1.98 D). Experimental results show, however, that genistein is much less active than any of its benzyl derivatives. Therefore we conclude that the ability of genistein benzyl derivatives to interact with liposome membrane and permeabilize it is governed by the dipole moment of the additional fragment of the molecule.

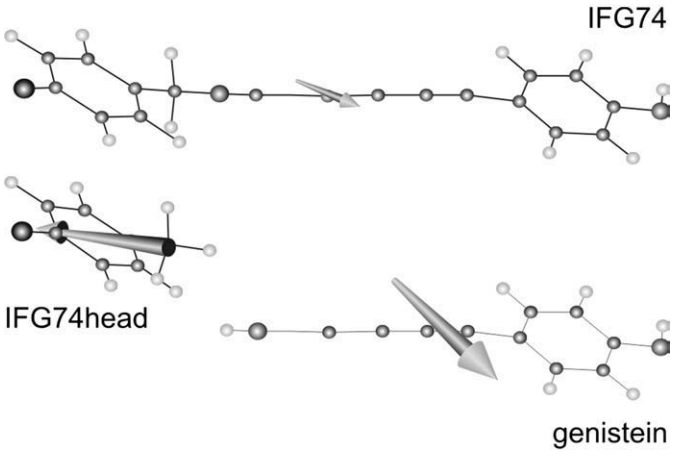

Fig. 3. Dipole moment vector of IFG74 as a sum of genistein and IFG74 head dipole moments. Figure is redrawn after the images of dipole moments were generated by Titan to enhance the clarity of vector presentation. Flavonoid molecules are oriented parallel with the planes of their A and C rings perpendicular to the paper plane.

Ram-3 and Lac-3 are of course excluded from the above way of reasoning because their sugar moieties are highly hydrophilic and they could not intercalate into the lipid bilayer like benzyl derivatives. Despite of this they are able to destabilize the liposome membrane and permeabilize it as efficiently as benzyl derivatives.

In the present work we have shown that benzyl and glycosylated derivatives of genistein are able to interact with liposome membrane and permeabilize it. Comparing the experimental results obtained for benzyl derivatives with theoretical calculations we suggested also that local dipole moment of additional benzene ring (with its substitutions) might play an important role in flavonoid–membrane interactions. This suggestion may be of some importance for QSAR studies in which mostly the dipole moments of entire studied molecules are taken into account.

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