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Research article

PERTURBATION OF THE LIPID PHASE OF A MEMBRANE IS NOT INVOLVED IN THE MODULATION OF MRP1 TRANSPORT ACTIVITY BY FLAVONOIDS

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Abstract: The expression of transmembrane transporter multidrug resistance-associated protein 1 (MRP1) confers the multidrug-resistant phenotype (MDR) on cancer cells. Since the activity of the other MDR transporter, P-glycoprotein, is sensitive to membrane perturbation, we aimed to check whether the changes in lipid bilayer properties induced by flavones (apigenin, acacetin) and flavonols (morin, myricetin) were related to their MRP1 inhibitory activity. All the flavonoids inhibited the efflux of MRP1 fluorescent substrate from human erythrocytes and breast cancer cells. Morin was also found to stimulate the ATPase activity of erythrocyte ghosts. All flavonoids intercalated into phosphatidylcholine bilayers as judged by differential scanning calorimetry and fluorescence spectroscopy with the use of two carbocyanine dyes. The model of an intramembrane localization for flavones and flavonols was proposed.

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Abbreviations used: BCECF-AM – 2',7'-bis-(3-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; BCRP – breast cancer resistance protein; ΔH – enthalpy change of the main phospholipid phase transition; $DiOC_2(3)$ – 3,3'-diethyloxacarbocyanine iodide; $DiOC_6(3)$ – 3,3'-dihexyloxacarbocyanine iodide; DMPC – 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; DPPC – 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; EYPC – egg yolk L-α-phosphatidylcholine; MDR – multidrug resistance; MRP1 – multidrug resistance-associated protein 1; NBD – nucleotide binding domain; P-gp – P-glycoprotein; T_M – temperature of the main phospholipid phase transition

No clear relationship was found between the membrane-perturbing activity of flavonoids and their potency to inhibit MRP1. We concluded that mechanisms other than perturbation of the lipid phase of membranes were responsible for inhibition of MRP1 by the flavonoids.

Key words: Flavonoids, Multidrug resistance-associated protein 1 (MRP1), Lipid bilayer, Carbocyanine dyes

INTRODUCTION

The phenomenon known as multidrug resistance is the ability of cancer cells or pathogenic microorganisms to reduce the concentration of structurally and functionally unrelated drugs in the cell's interior to a level below the killing threshold. Typically, the appearance of the MDR phenotype is associated with the overexpression of proteins from the ABC-transporters family, such as P-glycoprotein (P-gp, ABCB1), multidrug resistance-associated protein 1 (MRP1; ABCC1) or breast cancer resistance protein (BCRP, ABCG2), which pump anticancer drugs out of the cell at the expense of energy obtained from ATP hydrolysis. MRP1 is a 190-kDa protein composed of two hydrophobic transmembrane domains, each followed by a cytoplasmic nucleotide-binding domain (NBD) (see [1] for review). Apart from conferring multidrug resistance to cancer cells, MRP1 plays an important role in the cellular detoxification system transporting glutathione, glucuronate and sulfate conjugates of many endogenous and xenobiotic substances [2]. Additionally, it was discovered that MRP1 is engaged in the transbilayer transport of NBD-labeled analogues of phospho- and sphingolipids [3-5].

Flavonoids are polyphenolic compounds found abundantly in many foods and beverages of plant origin. They are considered to be beneficial for human health, and the list of their biological activities is very long, including prevention of cancer and cardiovascular diseases, beneficial activities on postmenopausal syndromes (estrogenic activity), and anti-inflammatory and antimicrobial activities (see [6, 7] for review). Above all, flavonoids are known to be potent natural antioxidants (see [8] for review). The inhibitory effect of flavonoids against MRP1 activity was demonstrated both in cancer cell lines [9, 10] and human erythrocytes [11, 12]. Other protein transporters engaged in MDR, such as P-glycoprotein and BCRP, can also be modulated by flavonoids [13-15].

P-glycoprotein and MRP1 are transmembrane proteins, and their activity can be dependent on the properties of the surrounding lipids (see [16, 17] for review). The transport activity of P-gp can be modulated by detergents and other amphipathic compounds [18, 19], suggesting that perturbation of the membrane structure rather than direct binding to the protein is likely to be the mechanism of action of at least some P-glycoprotein inhibitors [20-23]. Although P-gp and MRP1 are both transmembrane proteins, much less is known about the influence of membrane properties on MRP1 transport activity. Previous studies demonstrated that detergents do not influence the binding and transport of

a substrate by MRP1 [24, 25]. In cells, MRP1 is associated with membrane microdomains enriched in glycosphingolipids, but it was shown that the changes in cholesterol [26] or glucosulceramide levels [27] did not directly modulate MRP1 function. However, cholesterol depletion below 40% caused MRP1 shift out of lipid rafts, associated with a decrease in its functionality [26].

Since flavonoids are known to affect lipid bilayers, and their antibacterial and antioxidant activities are believed to engage some membrane-dependent processes (see [28] for review), we decided to ask whether the perturbation of membrane biophysical properties is related to their MRP1-inhibitory activity. To further assess this issue, we studied the ability of two flavones, apigenin and acacetin, and two flavonols, morin and myricetin, to modulate MRP1 activity and to influence phosphatidylcholine model membranes. The flavonoids chosen differed mainly by the number and spatial orientation of the hydroxyl groups in the flavonoid's molecule (see Fig. 1. for chemical structures). We expected that these features would be responsible for the different localization of the studied compounds within membrane and would govern the effects exerted by them on the lipid bilayer properties.

In this study, we demonstrated that the flavonoids inhibited the MRP1-mediated efflux of fluorescent substrate both from human erythrocytes and MDA-MB-231 resistant breast cancer cells. Morin was also found to stimulate the ATPase activity of erythrocyte ghosts. All the studied compounds intercalated into model phosphatidylcholine membranes as judged by differential scanning calorimetry and fluorescence spectroscopy with the use of carbocyanine dyes. The membrane localization of flavones and flavonols was discussed in detail; however, no clear relationship was found between the membrane-perturbing potency of the flavonoids and their ability to inhibit MRP1. We concluded that mechanisms other than the perturbation of the lipid phase of biological membranes, probably direct interaction with MRP1 [29, 30], were responsible for the inhibition of MRP1 by the flavonoids.

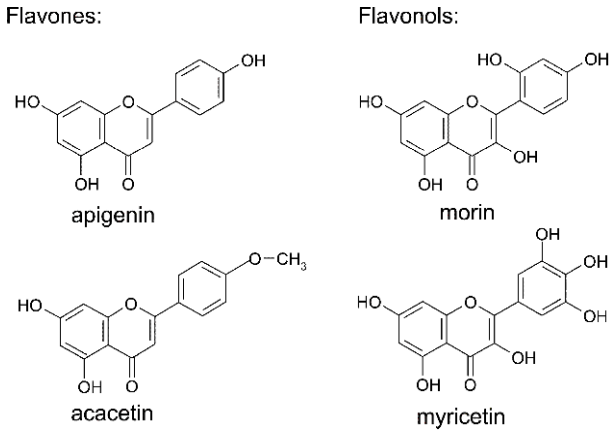

Fig. 1. The chemical structures of the studied flavonoids.

MATERIALS AND METHODS

Morin and myricetin were purchased from Sigma (Poznan, Poland). Apigenin and acacetin were from Fluka (Poznan, Poland). The fluorescent probes 2',7'-bis-(3-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), and 3,3'-diethyloxacarbocyanine iodide $DiOC_2(3)$ were from Molecular Probes (Eugene, USA). Rhodamine 123 and 3,3'-dihexyloxacarbocyanine iodide $DiOC_6(3)$ were from Sigma (Poznan, Poland). Egg yolk L-α-phosphatidylcholine (EYPC), 1,2-dimyristoyl-*s*n-glycero-3-phosphatidylcholine (DMPC) and 1,2-dipalmitoyl-*s*n-glycero-3-phosphatidylcholine (DPPC) were from Sigma (Poznan, Poland). The flavonoids and all fluorescent probes were dissolved in DMSO, with the exception of rhodamine 123, which was dissolved in water. All the other chemicals used were of analytical grade.

Preparation of erythrocytes and erythrocyte ghosts

Fresh human venous blood from healthy volunteers was obtained by vein puncture with EDTA as an anticoagulant. The blood was centrifuged at 2000 x g and 4ºC for 5 min to remove plasma. The erythrocytes were isolated from the blood cell mixture using an α-cellulose column eluted with 20 mmol/l Tris-HCl, 155 mmol/l NaCl, pH 7.0. Subsequently, the erythrocytes were washed three more times. The cells were stored at 4ºC and used within 36 h.

Erythrocyte ghosts were prepared according to procedure of Steck and Kant [31] with modifications. Briefly, erythrocytes were lysed in 40 volumes of ice-cold hemolytic buffer (20 mmol/l Tris-HCl, 1 mmol/l EDTA-Na_2, 0.575 mmol/l phenylmethylsulphonylfluoride, pH 7.4) and centrifuged (23000 x g, 12 min, 4ºC). The pellet was resuspended in a hemolytic buffer and washed three more times. The erythrocyte ghosts were snap-frozen and stored at -80ºC until use. The protein concentration was determined according to Lowry *et al.* [32] with bovine serum albumin as a protein standard.

BCECF transport studies in erythrocytes

A functional test developed by Rychlik *et al.* [33] was used to monitor the MRP1-mediated efflux of BCECF out of the erythrocytes. Briefly, erythrocytes (at 5% hematocrit) were incubated with flavonoids (15 min, room temperature, in the dark) in a buffer containing 6.1 mmol/l Na_2HPO_4, 1.4 mmol/l NaH_2PO_4, 138 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, and 5.6 mmol/l glucose, pH 7.4. Next, the samples were mixed with an equal volume of ice-cold 2 µmol/l BCECF-AM solution in the same buffer and erythrocytes were allowed to load for 10 minutes on ice. After loading, the samples were transferred into a water bath (37ºC) for 0, 20, 40 and 60 min. The rate of BCECF efflux remained constant during at least the first 60 min of the experiment. The incubation was stopped by putting the samples on ice, and then the samples were centrifuged (14000 x g, 3 min, 4ºC). The fluorescence intensity of free BCECF pumped out of the erythrocytes was measured in the supernatant (the excitation and emission wavelengths were 475 and 525 nm, respectively). The percentage of MRP1

inhibition was calculated by comparing the slopes of fluorescence intensity versus time dependencies for the control sample and the samples containing flavonoids. The DMSO concentration in the samples was maintained below 0.5%. No erythrocyte haemolysis occurred under the experimental conditions. It was checked that the flavonoids themselves did not influence the fluorescence of BCECF. The experiments were performed in triplicate.

ATPase activity studies

The ATPase activity of erythrocyte ghosts was measured in a buffer containing 100 mmol/l Tris-HCl, 10 mmol/l $MgCl_2$, pH 7.4, with 1 mmol/l ATP as a substrate. Additionally, the reaction medium contained 1 mmol/l ouabain, 2 mmol/l sodium azide, and 50 μmol/l EGTA to inhibit Na^+/K^+-ATPase, F-type ATPases and Ca^{2+}-ATPase. Then the test compounds (3-500 μmol/l) were added and the samples were placed on ice. Next, the erythrocyte membranes were added (protein concentration 0.5 mg/ml) and the samples were transferred into a water bath (37ºC, 30 min). The reaction was stopped by the addition of equal volume of ice-cold 0.6 mol/l trichloroacetic acid, and the samples were centrifuged (18000 x g, 5 min, 4ºC). The amount of inorganic phosphate (P_i) in the supernatant was determined as the complex of phosphomolybdate and malachite green by method of Van Veldhoven and Mannaerts [34]. To estimate ATPase activity, we calculated the difference between the absorbance (at 595 nm) of studied and control samples incubated on ice instead of at 37ºC. The experiments were repeated at least three times. The differences between the mean values were tested for significance using Student's t-test ($p < 0.05$).

Reversal of MDR in MDA-MB-231 cell line

The human breast cancer cell line MDA-MB-231 (American Type Culture Collection accession number HTB-26) was grown in Leibovitz's L-15 medium, supplemented with 10% fetal bovine serum, L-glutamine and antibiotics, at 37ºC without CO_2. This cell line has been demonstrated by immunocytochemistry to express both P-glycoprotein (MDR1) and MRP1 [35]. However, further functional studies have shown that P-gp was inactive in these cells [35]. Increased expression of MRP1 in the MDA-MB-231 cell line was also demonstrated by Northern blotting [36].

Prior to the experiment, cells were seeded onto a 6-well plate ($2.5x10^5$ cells per well containing 5 ml of medium) and grown for 72 h. For the experiment, the culture medium was changed to 1 ml of serum-free medium per well. Next, flavonoids at a 0.004 g/l concentration were added to the cells (0.004 g/l equalled 14.8 μmol/l for apigenin, 14.1 μmol/l for acacetin, 13.2 μmol/l for morin, and 12.6 μmol/l for myricetin). After incubation (10 min, room temperature) BCECF-AM was added to a final concentration of 0.24 μmol/l, and the cells were further incubated (20 min, 37ºC). In the next step, the cells were washed with PBS and detached with a trypsin-EDTA treatment. The harvested cells from each well were transferred to centrifuge tubes, washed twice with

PBS, and resuspended in 0.5 ml of PBS for analysis. The fluorescence of the cell population was measured by flow cytometry using a Beckton Dickinson FACScan instrument equipped with an argon laser. The fluorescence excitation and emission wavelengths were 488 nm and 520 nm, respectively. The mean geometric channel of fluorescence was calculated for the evaluation of cell fluorescence intensity. Indomethacin was used as a positive control. The influence of DMSO on the cells was also monitored. The fluorescence intensity ratio (FIR) was the ratio of fluorescence intensity values for the treated and untreated samples.

Reversal of MDR in the mouse T lymphoma cell line

The L5178Y mouse T lymphoma parent cell line was transfected with the pHa MDR1/A retrovirus as previously described [37]. MDR1-expressing cell lines were selected by culturing the infected cells with $6x10^{-6}$ g/l colchicine to maintain uniform expression of the MDR phenotype in all the cells of the population. The L5178 MDR cell line and the L5178Y parent cell line were grown in McCoy's 5A medium with 10% heat inactivated horse serum, L-glutamine and antibiotics, at 37ºC and 5% CO_2. For the experiment, the cells were adjusted to a concentration of $2x10^6$/ml in serum-free medium. Then flavonoids were added (final concentration 0.004 g/l) and the samples were incubated for 10 min at room temperature. Next, the cells were incubated (20 min, 37ºC) with the fluorescent indicator rhodamine 123 (final concentration 5.2 μmol/l). After two washes with PBS, the cells were resuspended in 0.5 ml PBS for analysis by flow cytometry. Verapamil was used as a positive control. The influence of DMSO on the cells was also monitored.

Differential scanning calorimetry

To prepare the sample, 0.002 g of DMPC or DPPC were dissolved in ethanol and mixed with an appropriate amount of flavonoid stock solution (5 mmol/l, in ethanol) to obtain a drug:lipid molar ratio of 0.06. DSC measurements are very sensitive to the presence of any organic solvent in the samples, so ethanol solutions of flavonoids were added to the lipids during model membrane formation. The samples were then dried under a stream of nitrogen, and the residual solvent was removed under a vacuum for at least 2 h. The dry samples were hydrated by adding 15 μl of 20 mmol/l Tris-HCl buffer (150 mmol/l NaCl, 0.5 mmol/l EDTA, pH 7.4). The hydrated mixtures were heated to a temperature around 10ºC higher than the main phase transition temperature of a given lipid and vortexed until homogeneous dispersion was obtained. Samples were sealed in aluminum pans and scanned at a rate of 1.25ºC/min immediately after preparation. Calorimetric measurements were performed using a Rigaku calorimeter that had been partially rebuilt in our laboratory. Samples were prepared in duplicate, and each sample was scanned at least four times. The calorimetric data was collected and processed off-line using software developed in our laboratory.

Fluorescence spectroscopy

As model membranes, we used small unilamellar liposomes, prepared by sonicating 2 mmol/l phospholipid suspensions in 10 mmol/l Tris-HCl buffer (pH 7.5). Liposomes (200 μmol/l of lipid) were incubated with 5 μmol/l of $DiOC_2(3)$ or $DiOC_6(3)$ for 10 min (room temperature, darkness). Then the flavonoids were added and the samples were incubated for a further 15 min (darkness, room temperature). The appropriate incubation time of liposomes with both carbocyanine probes and flavonoids was chosen so that the equilibrium state was reached before measurements were taken. The fluorescence emission spectra of $DiOC_2(3)$ and $DiOC_6(3)$ were collected using a LS 50B spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfiled, UK) equipped with a xenon lamp. The excitation wavelengths were 480 nm and 490 nm for $DiOC_2(3)$ and $DiOC_6(3)$, respectively. Both the excitation and emission slit widths were 5 nm. It was checked that the flavonoids themselves did not influence $DiOC_2(3)$ and $DiOC_6(3)$ fluorescence.

Molecular modeling

The theoretical calculations were performed with Titan 1.0.8 software (Wavefunction, Inc., Irvine, USA & Schrodinger, Inc., Portland, USA). The properties of the flavonoids were modelled using the AM1 semi-empirical molecular orbital method. The octanol/water partition coefficients (logP) of the flavonoids were calculated according to the procedure of Ghose *et al.* [38].

RESULTS

Modulation of MRP1 transport activity in human erythrocytes

Human erythrocytes physiologically express several ABC transporters from the MRP family. The expression of MRP1 [39], MRP4 [40] and MRP5 [41] has thusfar been demonstrated for this cell type. Western blot analysis of erythrocyte membrane preparations performed by Rychlik *et al.* [33] revealed a much higher expression of MRP1 than MRP5, and no P-glycoprotein was recorded in erythrocytes. Therefore, human erythrocytes constitute a good model to study the modulation of MRP-mediated transport by different compounds. A functional test based on the ability of MRP1 to pump its fluorescent substrate BCECF out of erythrocytes has been developed [33]. Briefly, the cells are loaded with a non-fluorescent ester BCECF-AM, which diffuses passively into the erythrocytes. After hydrolysis by intracellular esterases, fluorescent BCECF is formed. It is hydrophilic and negatively charged, and the only way it can leave the erythrocyte interior is by being pumped out by MRP1. This test was previously found to be indicative of MRP1 transport activity [33, 42]. In our previous study, we demonstrated that indomethacin, probenecid and MK-571 inhibited MRP1 in human erythrocytes (with respective IC_{50} values of 7, 220 and 3.5 μmol/l) [12]. MK-571 inhibited MRP1 by up to 97%, while the maximal inhibition of indomethacin and probenecid was around 70% [12]. MRP5 was

shown to be able to transport BCECF [43], but typical MRP5 substrates (such as cGMP, thioguanine or 6-mercaptopurine) had no effect on BCECF efflux out of human erythrocytes [33].

All the flavonoids studied were found to inhibit BCECF efflux out of the erythrocytes (see the data presented in Tab. 1). Acacetin and morin were the most effective inhibitors of MRP1 transport activity. The IC_{50} values recorded for apigenin and myricetin were much higher. The inhibition was concentration-dependent for a lower concentration of flavonoids, but it reached a maximum above 25 μmol/l for morin and acacetin, 75 μmol/l for apigenin, and 150 μmol/l for myricetin. It is worth noting that both flavonols (morin and myricetin) were able to inhibit MRP1 almost completely, while for flavones (apigenin and acacetin), inhibition higher than 70% was not observed.

Tab. 1. Flavonoid inhibition of MRP1-mediated BCECF efflux out of human erythrocytes (mean of three experiments ± S.D.).

Compound	IC_{50} (μmol/l)	Maximal inhibition (%)
Apigenin	40.0 ± 16[a]	66 ± 4[a]
Acacetin	6.5 ± 4	65 ± 5
Morin	7.0 ± 1[a]	95 ± 1[a]
Myricetin	52.6 ± 2	89 ± 1

[a] data taken from [12].

The ATPase activity of erythrocyte ghosts

To further elucidate the interactions of flavonoids with the MRP1 protein, we studied their influence on the ATPase activity of erythrocyte ghosts. Since the erythrocyte membrane constitutes a complex model system with many proteins possessing ATPase activity, we decided to employ a battery of ATPase inhibitors (sodium azide, ouabain and EGTA) in order to eliminate as much of non-MRP1 ATPase activity as possible. When this was done, virtually 100% of remaining ATPase activity was sensitive to orthovanadate and beryllium fluoride. The basal ATPase activity averaged 236 ± 36 nmole of P_i released per mg of total protein per 60 min.

Morin stimulated the ATPase activity in the erythrocyte ghosts but the effect was not monotonically dependent on the flavone concentration (Fig. 2). Strong stimulation was observed for low concentrations of the flavonol, whereas higher concentrations of morin induced lower levels of stimulation. The stimulation of ATPase activity by apigenin and acacetin was very weak (data not shown), and was found to be statistically insignificant as judged by Student's t-test ($p < 0.05$). Unfortunately, myricetin could not be tested because it was unstable under the conditions of the experiment, as judged by the rapid colour change of the myricetin solution when added to the reaction buffer.

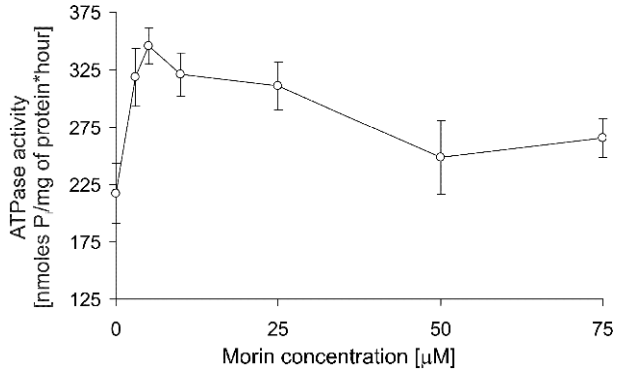

Fig. 2. Stimulation of the ATPase activity of erythrocyte ghosts by morin. The errors are given as the standard deviation values of three experiments.

The effect of flavonoids on multidrug-resistant cancer cells

The ability of modulators to affect the transport activity of MRP1 can be estimated using two approaches. In the first (used for erythrocytes), the amount of exported substrate is measured, while in the second, the accumulation of fluorescent substrate inside the cells is monitored. The influence of flavonoids on MRP1 transport activity in the human breast cancer cell line MDA-MB-231 was studied using the flow cytometric functional test. The amount of BCECF, the fluorescent substrate of MRP1, accumulated by the cells was measured in the presence and absence of the flavonoids. Indomethacin, used as a positive control (at a 0.05 g/l concentration), gave a fluorescence intensity ratio (FIR) value of 1.38 ± 0.07 (the mean ± SD of three experiments). When MDA-MB-231 cells were treated with the studied flavonoids at a concentration of 0.004 g/l, more BCECF was retained inside the cells than inside the control cells. Morin was identified as the most effective inhibitor of MRP1 transport activity (FIR = 1.14). The activity of acacetin and apigenin was slightly smaller (FIR = 1.13 and 1.11, respectively), whereas the activity of myricetin was negligible at the concentration tested (FIR = 1.01).

The ability of apigenin, acacetin, morin and myricetin to inhibit P-glycoprotein-mediated transport of rhodamine 123 in resistant mouse lymphoma cell line L5178 MDR was also tested. The fluoresecence intensity value (FIR) recorded for verapamil, used as a positive control (at 0.01 g/l) was 4.74 ± 0.90 (the mean ± SD of three experiments). None of the studied compounds was an active P-glycoprotein inhibitor when applied at 0.004 g/l (FIR = 0.88 for apigenin, 0.99 for acacetin, 1.00 for morin, and 1.06 for myricetin).

Differential scanning calorimetry

The influence of flavonoids on the thermotropic properties of DPPC and DMPC model membranes was investigated via the microcalorimetric method. All the studied compounds proved to intercalate into lipid bilayers (at a flavonoid:lipid molar ratio of 0.06). At such a flavonoid concentration, pretransition was

completely abolished in all cases. Both the temperature of the main phospholipid phase transition (T_M) and the enthalpy change (ΔH) were lowered in the presence of flavonoids (Fig. 3A and B, respectively) as compared to the values for pure lipid. The extent of flavonoid-induced changes was greater in phosphatidylcholine species possessing shorter acyl chains (DMPC) than in the one with longer chains (DPPC). In both lipids, the transition enthalpy was decreased by apigenin and acacetin more effectively than by morin and myricetin. T_M was reduced similarly by all studied compounds. The only exceptions were acacetin, which was the least effective in both DMPC and DPPC bilayers, and morin, whose effect on DMPC bilayers was stronger than the effect exerted by the other flavonoids. The presence of flavonoids in phosphatidylcholine model membranes also caused a broadening of transition peaks that was more pronounced for flavonols than for flavones (Fig. 3C). Again, the effect of acacetin was the least pronounced, and morin exerted an especially strong effect on the cooperativity of main phase transition of DMPC.

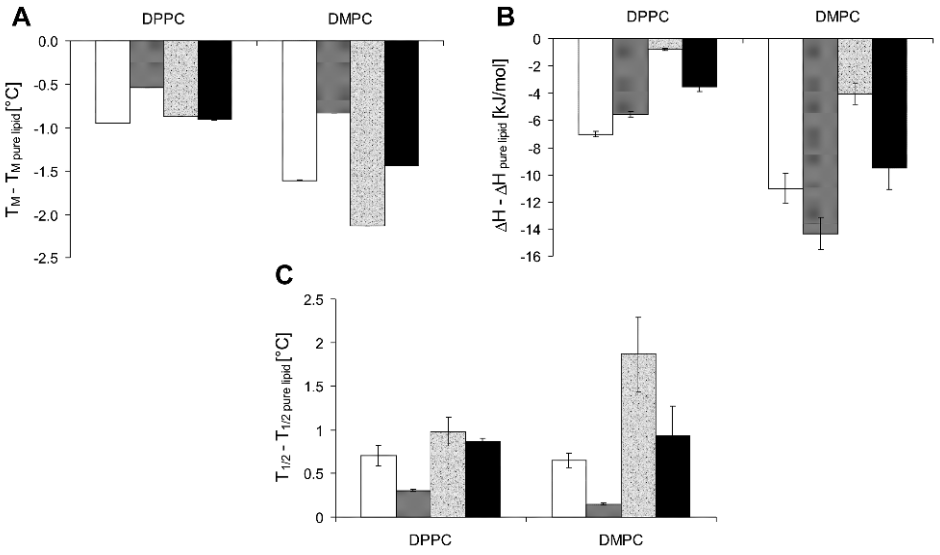

Fig. 3. The effect of apigenin (white bars), acacetin (grey bars), morin (dotted bars), and myricetin (black bars) on A – the transition temperature, B – enthalpy and C – half height of the main phase transition of DPPC and DMPC. The flavonoid/lipid molar ratio was 0.06. Errors are given as standard deviation values of eight scans.

Fluorescence spectroscopy

The interactions of apigenin, acacetin, morin and myricetin with phosphatidylcholine model membranes were also studied by means of fluorescence spectroscopy. All the studied flavonoids strongly absorbed light in the wavelength region of 300-400 nm. At the same time, this is the spectral region in which the majority of the fluorescent probes commonly used in membrane study (such as DPH or Laurdan) are excited. To avoid overlap

between the absorption spectra of the flavonoids and the fluorescent probe, two carbocyanine dyes ($DiOC_2(3)$ and $DiOC_6(3)$) were employed that were excited by wavelengths over 480 nm.

All the tested compounds strongly affected the fluorescence intensity of $DiOC_2(3)$ and $DiOC_6(3)$ (see Figs. 4 and 5, respectively). When flavonoids were added to the phosphatidylcholine liposomes, the fluorescence maxima of both carbocyanine dyes underwent a slight red shift (up to 5 nm). Both morin and myricetin caused concentration-dependent quenching of $DiOC_2(3)$ fluorescence. The effect of the flavones apigenin and acacetin was biphasic: at concentrations below 25 μmol/l, a slight increase of $DiOC_2(3)$ fluorescence was visible, whereas at higher flavone concentrations, the fluorescence intensity returned to the control values. The greatest influence of apigenin and acacetin on $DiOC_2(3)$ fluorescence was observed in EYPC liposomes; the effect was weaker in DMPC bilayer, and the smallest in DPPC model membranes. The type of phosphatidylcholine species used to form liposomes had no influence on the magnitude of the effect exerted by flavonols on $DiOC_2(3)$ fluorescence.

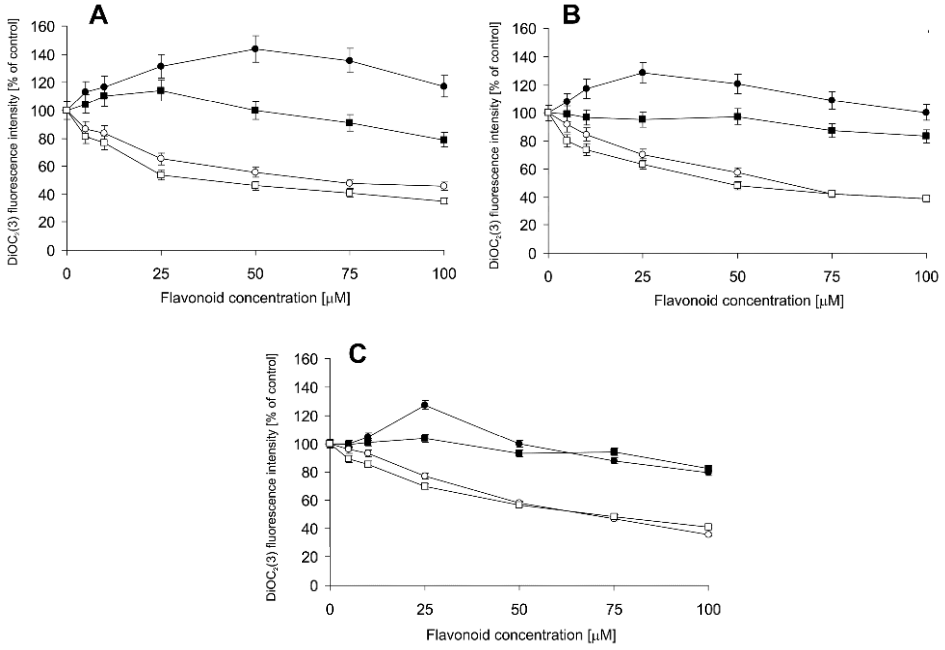

Fig. 4. The influence of apigenin (black circles), acacetin (black squares), morin (white circles), and myricetin (white squares) on the $DiOC_2(3)$ fluorescence intensity in A – EYPC, B – DMPC and C – DPPC liposomes. The errors are given as the standard deviation values of three experiments.

Flavonols also induced strong quenching of $DiOC_6(3)$ fluorescence (Fig. 5). The strongest quenching was observed in the DPPC liposomes, while it was weaker

in the DMPC ones, and the weakest in the EYPC model membranes. Apigenin and acacetin caused a slight quenching of the $DiOC_6(3)$ fluorescence. The effect of apigenin on the DPPC liposomes was, however, biphasic. An initial slight fluorescence increase, visible at low concentrations of flavone, was accompanied by fluorescence quenching in apigenin concentrations above 25 μmol/l. It is worth noting that a stronger influence of morin and myricetin on the fluorescence of $DiOC_6(3)$ than on that of $DiOC_2(3)$ was observed. On the other hand, the biphasic effect exerted by flavones (especially apigenin but also visible for acacetin in EYPC) was recorded only for $DiOC_2(3)$, while a slight quenching was the typical influence of flavones on $DiOC_6(3)$ fluorescence.

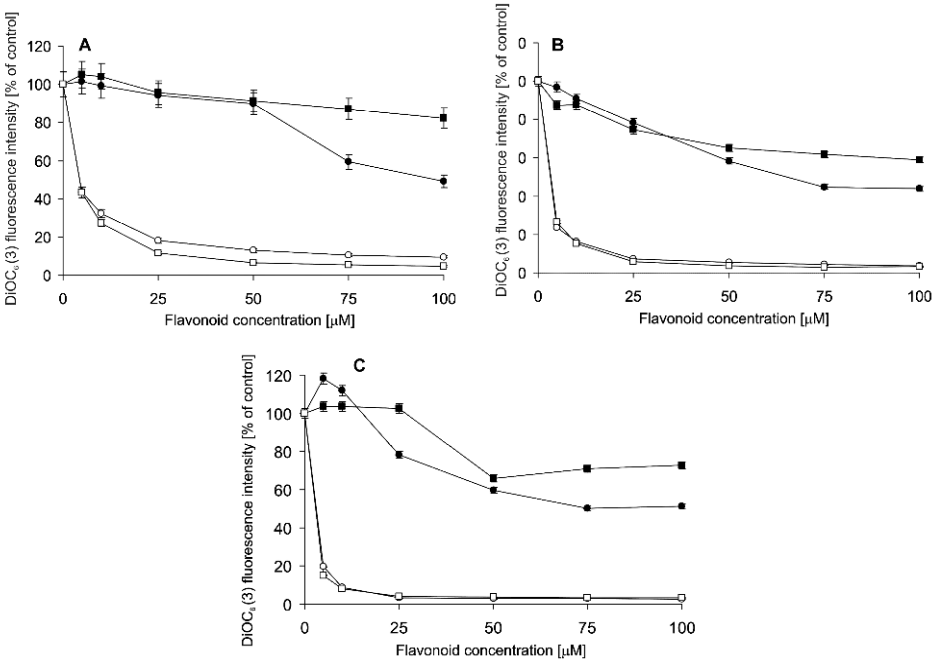

Fig. 5. The influence of apigenin (black circles), acacetin (black squares), morin (white circles), and myricetin (white squares) on the $DiOC_6(3)$ fluorescence intensity in A – EYPC, B – DMPC and C – DPPC liposomes. The errors are given as the standard deviation values of three experiments.

Molecular modeling

Molecular modelling was applied to elucidate by which physico-chemical properties the studied flavonoids differed (see Tab. 2 for results). Calculating the octanol/water partition coefficients revealed that acacetin was the most hydrophobic of all the compounds, followed by apigenin. The value of logP close to zero obtained for morin suggested that it would partition between the water and octanol phases almost equally. Myricetin was the most hydrophilic of all the flavonoids studied. Myricetin also possessed a significantly larger dipole

moment than the other compounds under investigation. Morin was characterized by the largest dihedral angle between the AB and C rings, which indicated that this compound was less planar than the other flavonoids under study.

Tab. 2. Selected physico-chemical properties of the studied flavonoids, calculated using Titan software.

Compound	log P	Dipole moment (D)	AC-B dihedral angle (°)
Apigenin	1.40	2.340	26.84
Acacetin	1.66	2.991	26.59
Morin	-0.07	2.024	37.38
Myricetin	-0.46	5.791	27.58

DISCUSSION

Human erythrocytes were previously demonstrated to constitute a good model to study the transport activity of MRP1 [33, 39, 44-46]. In this study, we showed that the flavones apigenin and acacetin and the flavonols morin and myricetin were effective inhibitors of MRP1-mediated BCECF efflux from human erythrocytes and also from the MRP1-expressing resistant cancer cell line MDA-MB-231. The weak activity of myricetin observed in MDA-MB-231 cells was probably the result of the low flavonoid concentration used in this test (0.004 g/l, which in case of myricetin means 12.5 μmol/l). The ability of the flavonoids studied here to inhibit MRP1 transport activity was reported previously [9-11, 47, 48]; however, the reported IC_{50} values for MRP1 inhibition varied depending on the model system used (different cancer cell lines or erythrocytes) and on the transported substrate (daunorubicin, vinblastine, leukotriene C_4, BCECF or calcein). An interesting case was reported by Nguen *et al.* [10]. They showed that morin increased the accumulation of both daunorubicin and vinblastine in MRP1-expressing Panc-1 cells, myricetin decreased the accumulation of both substrates, and apigenin acted as an MRP1 inhibitor when vinblastine was used as a substrate but stimulated daunorubicin efflux by MRP1.

In contrast to MRP1, which, with a few exceptions, is inhibited by flavonoids, P-glycoprotein was reported to be either stimulated [13, 49] or inhibited [14, 50, 51] by flavonoids. In this study, we have shown that none of the studied compounds inhibited P-glycoprotein at a concentration of 0.004 g/l. In previous studies, morin was reported either to have almost no effect (at 20 μmol/l) on vincristine uptake into resistant leukaemia cells [52] or to increase (at 50 μmol/l) daunorubicin accumulation in four cell lines expressing different levels of P-gp. On the other hand, Critchfield *et al.* [13] reported that doxorubicin accumulation by HTC-15 colon cells was reduced in the presence of 100 μmol/l apigenin, morin and myricetin, respectivelty to 54%, 71% and 75% of control value.

Since ability of flavonoids to modulate the activity of both P-gp and MRP1 was postulated to involve their interaction with the nucleotide-binding domains (NBDs) of ABC transporters [29, 53], we decided to measure the effect of

apigenin, acacetin, morin and myricetin on the ATPase activity of erythrocyte ghosts. Apigenin was previously demonstrated to stimulate ATPase activity in drug-resistant small cell lung cancer cell line GLC_4/ADR by 30% when used at a concentration of 50 μmol/l [54], and also to bind to isolated NBD2 of P-glycoprotein [55] and to both NBDs of MRP1 [29]. In erythrocyte ghosts, we recorded significant stimulation of ATPase activity only by morin, whereas the influence of apigenin and acacetin was weak and statistically insignificant. Due to the effects described in the Results section, myricetin could not be tested. Morin was found to effectively inhibit the transport of fluorescent substrate by MRP1 and to stimulate the ATPase activity of erythrocyte ghosts. We should keep in mind that the erythrocyte membrane constitutes a complex model system, and many proteins possessing ATPase activity are present in the membranes of red blood cells. To get rid of as much of non-MRP1 ATPase activity as possible, we employed a battery of ATPase inhibitors in concentrations that were reported not to affect MRP1 [56]. Although the profile of substances that inhibited/stimulated ATPase activity that we measured in the erythrocyte ghosts was in general agreement with the one reported by other authors for the ATPase activity of MRP1 [9, 57, 58], there was no guarantee that the ATPase activity recorded from the erythrocyte membranes was pure MRP1 activity without any contribution from other proteins. Despite these caveats, we concluded that morin was likely to influence the ATPase activity of MRP1 in human erythrocytes. The effect exerted by morin on the MRP1-stimulation of ATPase activity and inhibition of substrate transport might seem confusing. Such apparently contradictory effects were previously also observed for other flavonoids, e.g. kaempferol, apigenin and flavopiridol [9, 59]. At present, there is no satisfactory explanation of such observations (see Leslie *et al.* [9] and discussion within). Stimulation of the ATPase activity of MRP1 protein was observed for its substrate leukotriene C_4 [57], and there are clues suggesting that some flavonoids may be transported by MRP1. Moreover, kaempferol was identified to be a competitive inhibitor of leukotriene C_4 transport by MRP1 [9].

Apart from the effect on ATPase activity, flavonoids could possibly modulate MRP1 by inducing changes in the lipid bilayer in which this protein transporter is embedded. Previous studies have indicated that MRP1-mediated transport is not sensitive to perturbations of lipid bilayer [24-27]. To investigate the ability of the four flavonoids to change the biophysical properties of lipid bilayers, we studied their interactions with model membranes formed from phosphatidylcholine. The calorimetrically recorded decrease in the lipid main phase transition temperature, and the lowering of the transition enthalpy and cooperativity indicated that all the studied flavonoids intercalated into phosphatidylcholine bilayers. The changes in transition parameters were more pronounced in DMPC than in DPPC membranes, presumably due to the weaker interactions between DMPC acyl chains, which are shorter than those in DPPC. According to the classification of Jain and Wu [60], lowering of T_M, ΔH and transition cooperativity are characteristic for the compounds that locate near the

polar/apolar interface of the membrane. Also, other flavonoids (naringenin, quercetin and hesperetin) [61] and isoflavonoids [62] were previously observed to exert similar effects on the thermotropic properties of DPPC bilayers. Such a localization of flavonoids inside the membrane could be deduced on the basis of their chemical structures: a hydrophobic ring system facilitated interaction with lipid acyl chains, while hydroxyl groups could interact with the polar headgroup region of the bilayer. The ability of apigenin (three OH) and acacetin (two OH groups) to reduce ΔH to a greater extent than flavonols did, indicated the deeper membrane insertion of flavones. A few years ago, acacetin and apigenin were demonstrated to interact similarly with HPLC RP-18- and DPPC-coated columns, whereas morin (five OH) and myricetin (six OH) remained bound to the DPPC column for a significantly longer time than to the RP-18 column [63]. This retention delay of morin and myricetin, as compared to the flavones, resulted from their higher hydrogen bonding capacity, which could govern their interactions with the lipid membrane interface region.

To further characterize the influence of flavonoids on phosphatidylcholine membranes, fluorescence spectroscopy was employed. We observed that in the presence of the flavonoids, the emission maximum of carbocyanine probes: $DiOC_2(3)$ and $DiOC_6(3)$ was shifted to longer wavelengths. The fluorescence maximum of carbocyanine dyes was previously shown to be red-shifted in apolar solvents [64]. Additionally, flavonols caused a concentration-dependent quenching of fluorescence, while the effect of flavones was biphasic. In the presence of low concentrations of apigenin and acacetin, an increase in carbocyanine fluorescence intensity was observed, while a decrease in intensity was recorded when higher concentrations of flavones were added to the liposomes. Moreover, the biphasic effect of apigenin and, to a lower extent, acacetin, was more pronounced when $DiOC_2(3)$ was employed as a fluorescent probe. In contrast to flavones, flavonols exerted a stronger effect on the fluorescence of $DiOC_6(3)$ than on that of $DiOC_2(3)$.

The changes in the fluorescence intensity of carbocyanine dyes in the presence of flavonoids could be the result of several phenomena. Fluorescence quenching could be caused by direct interactions (collisions) between flavonoid and fluorescent probe molecules. Besides, the spectral properties of carbocyanine dyes depend strongly on the degree of aggregation of the dye molecules. Monomers are highly fluorescent, while dimers exhibit no fluorescence at all [65]. The type and number of carbocyanine aggregates that exist in the particular model system depend on the lipid used to form the bilayer, as well as on its physical properties [66]. Any flavonoid-induced changes in the model membrane that would produce a change in carbocyanine monomer-dimer equilibrium could be the cause of either fluorescence quenching or enhancement. However, the analysis of the excitation spectra of carbocyanines recorded in the presence of flavonoids (data not shown) did not show any signs of formation of carbocyanine dimers in the presence of any of the studied compounds.

Steady state and time-resolved fluorescence measurements led Krishna and Periasamy [67] to the conclusion that two populations of $DiOC_2(3)$ existed in lipid membranes. A short fluorescence lifetime population of dye molecules was bound near the membrane surface (parallel to it), whereas the other, long fluorescence lifetime population, was bound in the interior of membrane (perpendicular to its surface). In short chain and in unsaturated phosphatidylcholines, the fraction of the dye in the interior of the membrane was higher than the fraction of dye located close to membrane surface. In the case of $DiOC_6(3)$, a shallow membrane localization was postulated, near the polar head region of DPPC [68]. At the same time, the prevalence of the population of dye molecules with a parallel orientation to the membrane surface was demonstrated. As the effect exerted by morin and myricetin on the fluorescence of $DiOC_6(3)$ was much stronger than on $DiOC_2(3)$, we assumed that the flavonols were located closer to the surface of the lipid bilayer. On the other hand, flavones influenced the fluorescence of both $DiOC_6(3)$ and $DiOC_2(3)$, suggesting that both deep and shallow regions of the membrane were affected by apigenin and acacetin. However, the biphasic effect on carbocyanine fluorescence (the increase of intensity in low concentrations of compound followed by the decrease in higher concentrations) was recorded only for $DiOC_2(3)$ in the presence of flavones. This effect was seen most clearly in EYPC, more weakly in DMPC and it was the least pronounced in DPPC bilayers. It prompted us to the conclusion that this effect was caused by the interaction of flavones with $DiOC_2(3)$ molecules bound deeply inside membrane, as the highest fraction of such molecules could be expected in EYPC. In the presence of low amounts of flavones (especially apigenin), the fluorescence quantum yield of $DiOC_2(3)$ increased. That could be the result of a change in lipid packing or an altered water amount around the fluorescent probe. However, the detailed description of the mechanism in which flavones influence fluorescence of carbocyanine dye in lipid bilayers requires further studies, especially fluorescence lifetimes measurements.

Using NMR spectroscopy, Scheidt *et al.* [69] showed that the spatial distribution of flavonoids inside phosphatidylcholine membranes could be quite broad. In the presence of flavonoids, the signals of almost all the protons of the lipid molecule were changed. The most affected were the signals of protons associated with the lipid glycerol backbone and a few upper groups of lipid acyl chains. A similar localization was reported for genistein in DMPC membranes [70]. Additionally, it was shown that the spatial orientation in the bilayer depended on the position of the polar centre of the flavonoid molecule [69]. According to the model of Scheidt *et al.* [69], we postulate that the flavonols morin and myricetin were located with their B rings pointing towards the aqueous phase, while their A rings were inserted deeper into the acyl chain region of the membrane. On the other hand, apigenin and acacetin were more likely to be oriented with their B rings heading for membrane centre and A rings located closer the surface of the lipid bilayer.

The results obtained in this study show clearly that all the studied flavonoids interacted with lipid bilayers, although their hydrophobicity as predicted by logP values was not very high. Similarly, the hemolytic and toxic effects of efficient flavonoid MRP1 inhibitors indicate their partition into and interaction with the lipid bilayer [11, 71]. A given flavonoid's hydrophobicity seemed to determine, at least to some extent, its membrane binding site. The position of a flavonoid inside the lipid bilayer might be important for its biological activity. For example, the antioxidant activity of flavonoids was often attributed to their ability to interact with biological membranes [72]. On the other hand, the ability of flavonoids to perturb lipid bilayers was not related to their affinity for MRP1 as judged by their respective IC_{50} values. However, the similar membrane localization of apigenin and acacetin might be related to the comparable values of maximal inhibition of MRP1 obtained for both flavones. The flavonols, morin and myricetin, also inhibited MRP1 transport activity to a similar extent. It was likely that membrane position of flavonoids affected the availability of MRP1 for interaction with the studied compounds. It seemed that there was the portion of MRP1 molecules that could not be inhibited by flavones, while almost the whole population of MRP1 was inhibited by morin and myricetin. The process of binding the fluorescent substrate by MRP1 was not likely to be influenced by the flavonoids present near the polar/apolar interface of the membrane. Whether BCECF enters the transporter from the cytoplasm or has to interact with the membrane prior to being recognized by MRP1 is not known, but the first possibility seems more probable taking into account the acidic character of BCECF.

The results obtained in this study clearly demonstrated that flavonoids interact with model lipid membranes influencing their biophysical properties. Apigenin, acacetin, morin and myricetin were also shown to modulate the transport function of MRP1 in human erythrocytes and in cancer cell lines. The membrane-perturbing activity of flavonoids and their affinity for MRP1 are not, however, correlated with each other. This study showed that perturbation of the lipid phase of the membrane by flavonoids is not likely to constitute the mechanism of MRP1 inhibition by these compounds. It seems therefore probable that flavonoids inhibit MRP1 transport activity by directly interacting with the protein, as previously indicated [29, 30]. Further studies are required to fully understand the influence of flavonoids on multidrug resistance-associated protein 1. The interaction of flavonoids with the ATP- or drug-binding domains of MRP1 could be investigated by studying their effect on the binding of the photoreactive ATP (e.g. [^{32}P]8-azido ATP) or drug (e.g. [^{125}I]iodoaryl azido-rhodamine123) analogues to cell membranes containing MRP1.

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