Structure–Activity Relationships of the Inhibitory Effects of Flavonoids on P-Glycoprotein-Mediated Transport in KB-C2 Cells

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We studied the effects of flavonoids, naringenin (flavanone), baicalein (flavone), kaempferol, quercetin, myricetin, morin, and fisetin (flavonols) as well as two glycosides of quercetin on P-glycoprotein (P-gp) function in multidrug-resistant P-gp overexpressing KB-C2 cells. Flavonoids such as kaempferol and quercetin increased the accumulation of rhodamine-123 dependent on their chemical structure. Analysis by flow cytometry indicated that the increase in substrate accumulation was due to the inhibition of substrate efflux. Naringenin, which lacks the 2,3-double bond in the C ring, had no effect, although it was more hydrophobic than myricetin, fisetin and morin. Therefore, the planar structure of the flavonoids seemed to be important for their interaction with P-gp. The effects of other flavonoids on the accumulation of daunorubicin were in the order of kaempferol>quercetin, baicalein>myricetin>fisetin, morin. Quercetin-3-*O*-glucoside and rutin had no effect. The order of the effects corresponded with that of the partition coefficients. Difference in the number and position of hydroxyl groups in flavonoid molecules by themselves seemed to have little effect. These results suggested that hydrophobicity as well as planar structure is important for the inhibitory effects of flavonoids on P-gp-mediated transport.

Key words flavonoid; P-glycoprotein; KB-C2 cell; partition coefficient

Overexpression of P-glycoprotein (P-gp), a plasma membrane transporter which extrudes chemotherapeutic agents out of cells, has been associated with the multidrug resistance of cancer cells. Compounds such as verapamil, dihydropyridine analogs, quinidine and cyclosporin A reversed this P-gp-mediated multidrug resistance (MDR) due to their inhibition of transporter activity.[1,2] In addition to these compounds, it has been revealed that flavonoids and other polyphenols modulate P-gp activity.[3—6] Although the structure–activity relationships of flavonoids on P-gp functions have been studied,[5,7,8] they are still not clear, especially concerning their effects in whole cells. Recently, we revealed that tea catechins inhibited P-gp function dependent on their chemical structure in multidrug-resistant human epidermal carcinoma cell line KB-C2 cells,[9] which overexpress P-gp.[10] Among the tea catechins, we found the maximum activity in the (−)epigallocatechin gallate whose chemical structure is shown in Fig. 1. We clarified that the presence of the galloyl moiety in the C ring, and the presence of the trihydric pyrogallol group as the B ring instead of the dihydric catechol group, markedly increased its activity on P-gp, although their effects on the partition coefficients between *n*-octanol and phosphate-buffered saline (PBS) were opposite to each other.[9]

In this study, we examined the structure–activity relationships of seven flavonoids, naringenin (flavanone), baicalein (flavone), kaempferol, quercetin, myricetin, morin, and fisetin (flavonols) (Fig. 1), which do not have large substituents like the galloyl group, as well as two glycosides of quercetin, for their effects on P-gp-mediated drug extrusion in KB-C2 cells. We analyzed the effects from the standpoints of chemical structure, conformation and hydrophobicity. We studied the importance of the 2,3-double bond in the C ring, in relation to the stereoscopic structure of flavonoids. Furthermore, we studied the effects of hydrophobicity of the flavonoids on the inhibitory activity to P-gp function. For this study, we used fluorescent rhodamine-123 and daunorubicin as the P-gp substrates; these substrates have often been used for the study of various P-gp transport modulators including flavonoids.[3,6,11] Although daunorubicin has also been revealed to be a substrate of multidrug resistance-associated protein 1 (MRP1),[12] since MRP1 is rarely found in KB-C2 cells,[13] its involvement in substrate efflux is negligible.

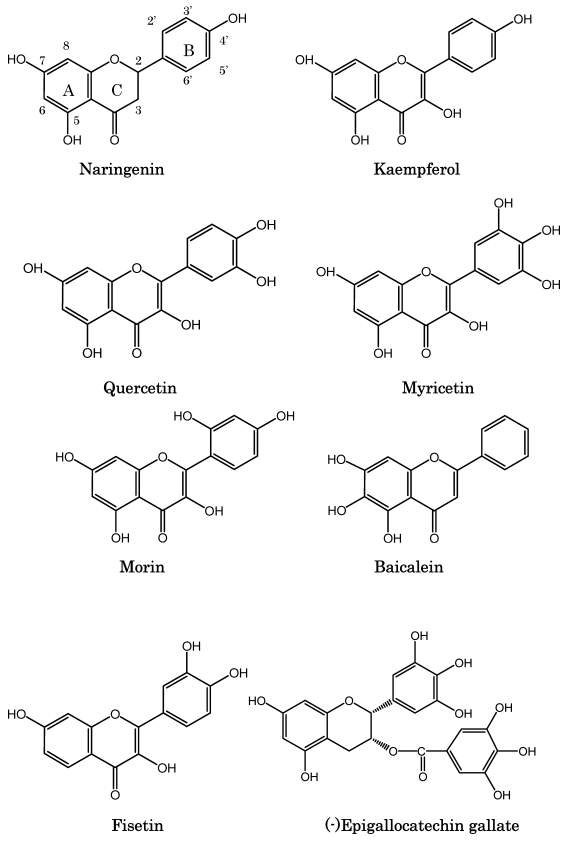

Fig. 1. Chemical Structures of Flavonoids Tested and (−)Epigallocatechin Gallate

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MATERIALS AND METHODS

Materials Quercetin, myricetin, morin and rutin were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and kaempferol was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Naringenin and fisetin were from Sigma-Aldrich Japan Co. (Tokyo, Japan). Baicalein and quercetin-3-*O*-glucoside were from Funakoshi Co. (Tokyo, Japan). Rhodamine-123 was from Molecular Probe (Junction City, OR, U.S.A.). Dulbecco's Modified Eagle Medium (D-MEM) and fetal bovine serum were from Invitrogen Co. (Carlsbad, CA, U.S.A.). Daunorubicin hydrochloride and all other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). KB-C2 cells and drug sensitive cell line KB-3-1 cells were kindly provided by Prof. Shin-ichi Akiyama (Kagoshima University, Japan).

Cell Cultures KB-C2 cells were cultured in D-MEM culture medium supplemented with 10% fetal bovine serum and 2 μg/ml colchicine. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2–95% air. KB-3-1 cells were cultured similarly in the absence of colchicine.

Measurements of the Cellular Accumulations of Substrates Cellular accumulations of P-gp substrates were measured as described previously.[9] Cells were plated at 2.5×10^5 cells/35-mm dish and cultured for 24 h in a CO_2 incubator. Cells were then washed with D-MEM without serum and the medium was exchanged to D-MEM without serum. After the addition of flavonoids, either 20 μM rhodamine-123 or 50 μM daunorubicin was added, and cells were incubated for another 2 h in a CO_2 incubator to reach a steady state level. Cells were then washed twice with an excess volume of ice-cold phosphate-buffered saline (PBS). Either 0.1% Triton X-100 (for rhodamine-123) or 1% SDS (for daunorubicin) was used to lyse the cells and solubilize each substrate completely. The fluorescence intensity was measured with an F-4010 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan), and the accumulated amounts of the probes were calculated. The excitation and emission wavelengths used for rhodamine-123 and daunorubicin were 485 and 527 nm, and 502 and 588 nm, respectively.

FACS Flow Cytometry Fluorescence measurements of individual cells were performed as described previously[9,14] using a Becton-Dickinson FACScalibur fluorescence-activated cell sorter (San Jose, CA, U.S.A.) equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530±15 nm band-pass filters). A total of 10000 cells were analyzed, and non-viable cells were gated out based on forward and side scatter characteristics. Log fluorescence was collected and displayed as single-parameter histograms representing the distribution of cells with different levels of fluorescence substrates.

For the efflux experiment, cells were incubated with 20 μM rhodamine-123 in the absence of flavonoids for 2 h at 37 °C. Then, cells were washed with ice-cold PBS, the medium was replaced with rhodamine-free medium in the presence or absence of flavonoids, and further incubated for 30 min at 37 °C. After washing cells with ice-cold PBS, rhodamine 123 retained in the cells was measured.

Measurement of Partition Coefficients of Flavonoids between *n*-Octanol and PBS Partition coefficients of flavonoids between *n*-octanol and PBS were also measured as also described previously.[15] PBS solution (3 ml) of flavonoids (0.1—1.0 mM) was mixed with 3 ml *n*-octanol in test tubes with glass stoppers. The PBS and *n*-octanol solutions used were pre-saturated with either *n*-octanol or PBS and de-oxygenized with a nitrogen stream. The test tubes were set at 37 °C for 18 h in a shaking water bath. After stopping the shaking, the incubation continued for another 1 h. The concentration of flavonoids in both the PBS phase and the *n*-octanol phase was determined by HPLC (L-6000; Hitachi, Tokyo, Japan) with an L-4000 UV detector (Hitachi) at 365 nm (kaempferol), 373 nm (myricetin), 324 nm (baicalein), 362 nm (fisetin) and 291 nm (naringenin). Separation was achieved on a reversed-phase column (Mightysil RP-18 GP, 4.6 mm i.d., 250 mm) using a mobile phase consisting of methanol, water and phosphoric acid (100 : 100 : 1) at a flow rate of 0.68 ml min^{-1}. Ferulic acid was used as an internal standard.

Statistical Analysis One way analysis of variance and Bonferroni's *post-hoc* test were used to analyze differences between the sets of data. A *p*-value less than 0.05 was considered significant.

RESULTS

Effects of Flavonoids on the Accumulation and Efflux of P-gp Substrates We first examined the effects of flavonoids on the accumulation of rhodamine-123 in KB-C2 cells. As shown in Fig. 2 for the effects of kaempferol and quercetin at 100 μM, these flavonoids increased the cellular accumulation of the fluorescent substrate. The increase by kaempferol, which has one hydroxyl group in B ring, was more prominent than that by quercetin, which has the ortho-diphenolic catechol group as B ring. On the other hand, the two glycosides of quercetin, quercetin-3-*O*-glucoside and rutin, had no effect, as also shown in Fig. 2. No effects of the flavonoids were observed in the drug sensitive KB-3-1 cells (relative accumulative amount of rhodamine-123 in the presence of 100 μM kaempferol was 1.07±0.11). Increased accumulation of rhodamine-123 in individual cells in the pres-

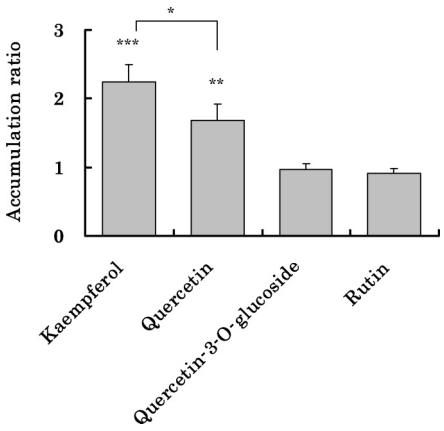

Fig. 2. Effects of 100 μM Kaempferol, Quercetin, Quercetin-3-*O*-glucoside and Rutin on the Relative Accumulative Amounts of Rhodamine-123 in Multidrug-Resistant P-Glycoprotein Overexpressing KB-C2 Cells

** $p<0.01$, *** $p<0.001$ compared with the value in the absence of flavonoids. Statistical significance of difference between the data of kaempferol and those of quercetin (* $p<0.05$) was also listed. Data are means±S.D. of six experiments. The control value of accumulation, which was $(4.46\pm0.73)\times10^8$ molecules/cell for six experiments, was defined as 1.00.

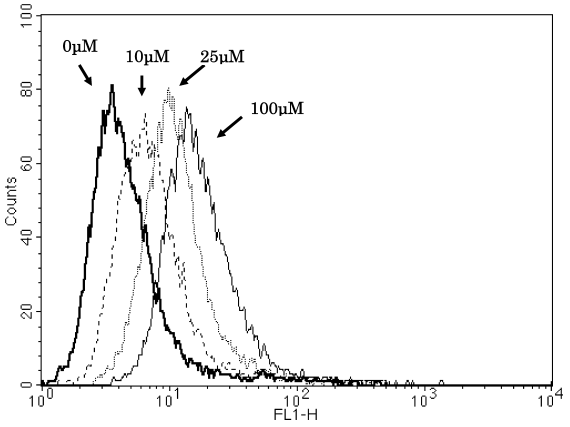

Fig. 3. Analysis by Flow Cytometry of the Effects of Kaempferol (0—100 μM) on the Intracellular Retention of Rhodamine-123 in the Accumulation Phase of Multidrug-Resistant P-Glycoprotein Overexpressing KB-C2 Cells

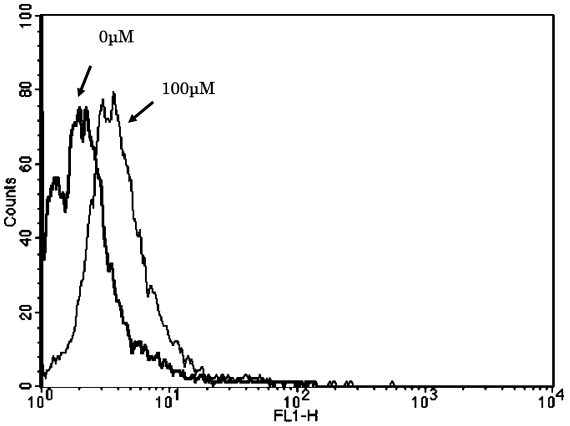

Fig. 4. Analysis by Flow Cytometry of the Effects of 100 μM Kaempferol on the Intracellular Retention of Rhodamine-123 in the Efflux Phase of Multidrug-Resistant P-Glycoprotein Overexpressing KB-C2 Cells

ence of these flavonoids was also confirmed by flow cytometry, as shown in Fig. 3 for kaempferol. These results are consistent with the previous findings in other cell lines, such as CH^RC5 cells and MDA435/LCC6 cells.[4,6]

The enhanced accumulation of rhodamine 123 in the presence of the flavonoids mentioned above seemed to be due to their inhibition of the P-gp-mediated efflux of the substrate. Therefore, we examined the effects of kaempferol on the efflux of rhodamine 123. As shown in Fig. 4 for the result at 100 μM, the amount of rhodamine-123 remaining in KB-C2 cells was higher in the presence of kaempferol than in its absence, suggesting that kaempferol decreased the efflux of rhodamine-123 by blocking P-gp. Similar results were obtained for other flavonoids which increased the cellular accumulation of rhodamine-123.

Relationship of the Effects of Flavonoids with a Planar Structure of the B-Ring and Their Hydrophobicity Next, we examined the structure–activity relationship of the effects of flavonoids by using daunorubicin as the P-gp substrate, because daunorubicin is more sensitive to inhibitory effects on P-gp-mediated efflux than rhodamine-123.[16] Therefore, the effects of flavonoids are expected to be more clearly detectable. Firstly we examined the effect of the double-bond between the 2- and 3-position in the C ring. As

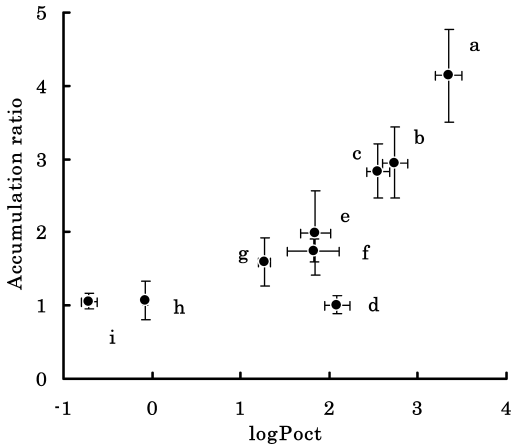

Fig. 5. Relationship between Logarithm Values of Partition Coefficients P_{oct} of Flavonoids and Relative Accumulative Amounts of Daunorubicin in the Presence of 100 μM Flavonoids

a, kaempferol; b, quercetin; c, baicalein; d, naringenin; e, myricetin; f, fisetin; g, morin; h, quercetin-3-*O*-glucoside; i, rutin. Data of daunorubicin accumulation are means±S.D. of six experiments. The control value of accumulation, which was $(2.72\pm0.50)\times10^9$ molecules/cell for fifteen experiments, was defined as 1.00. $p<0.01$ (g), $p<0.001$ (a, b, c, e, f) compared with the control value of accumulation. $p<0.001$ (a) compared with the accumulation values of b and c. $p<0.05$ (b, c) compared with the values of e, f and g.

Table 1. Partition Coefficients P_{oct} of Flavonoids between *n*-Octanol and PBS at 37 °C

Flavonoids	$\log P_{oct}$
Kaempferol	3.35±0.15
Quercetin	2.74±0.14[a)]
Baicalein	2.55±0.13
Naringenin	2.09±0.14
Myricetin	1.84±0.17
Fisetin	1.82±0.29
Morin	1.27±0.07[a)]
Quercetin-3-*O*-glucoside	−0.076±0.026[a)]
Rutin	−0.71±0.09[a)]

Data are means±S.D. of nine experiments at three different concentrations. *a*) Cited from ref. 15.

shown in Fig. 5, naringenin, which lacks the 2,3-double bond in the C ring, had no effects on the accumulation of daunorubicin, although naringenin was more hydrophobic than myricetin, fisetin and morin. These findings suggested the importance of the double bond in that position.

To observe the effect of the number and position of hydroxyl groups in the B ring, we next examined the effects of 3,5,7-hydroxyflavones (kaempferol, quercetin, myricetin, and morin), because the most prominent effect was found for kaempferol on the accumulation of rhodamine-123 as described above. We also examined the effects of baicalein and fisetin which differ in their number and position of hydroxyl groups. Baicalein is a flavone, which lacks 3-OH in C ring, and has no hydroxyl group in B ring, but has an additional hydroxyl group at 6-position in the A ring. Fisetin, which is a flavonol, lacks 5-OH and has the ortho-dihydric catechol group as the B ring. Since a hydrophobic interaction has been suggested for P-gp modulators,[5,8] we measured the partition coefficients P_{oct} of the flavonoids tested between *n*-octanol and PBS at 37 °C, which are listed in Table 1. We examined the relationship between their inhibitory activity on P-gp function and their partition coefficients.

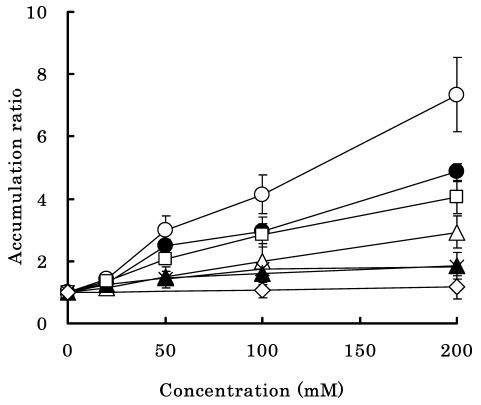

Fig. 6. Dose-Dependent Effects of Flavonoids on the Accumulation of Daunorubicin in Multidrug-Resistant P-Glycoprotein Overexpressing KB-C2 Cells

○, kaempferol; ●, quercetin; □, baicalein; △, myricetin; ×, fisetin; ▲, morin; ◇, quercetin-3-*O*-glucoside. Data are means±S.D. of six experiments. The control value of accumulation, which was $(2.72\pm0.50)\times10^9$ molecules/cell for fifteen experiments, was defined as 1.00.

As shown in Fig. 5 for the effects at 100 μM, and for their dose-dependent effects in Fig. 6, when comparing the effects of kaempferol, quercetin and myricetin, the accumulation was in the order of kaempferol>quercetin>myricetin. The increase in the number of hydroxyl groups in the B ring decreased the effect. The effect of morin, which has the methadiphenolic resorcinol group as the B ring, was smaller than that of quercetin, which has the ortho-diphenolic catechol moiety as B ring, and even smaller than that of myricetin at 200 μM ($p<0.01$). The effect of baicalein was similar with that of quercetin. On the other hand, that of fisetin was smaller than that of baicalein, and even smaller than that of myricetin at 200 μM ($p<0.01$). The order of the effects of these flavonoids including quercetin-3-*O*-glucoside and rutin, which had no effects even at 200 μM, were well corresponded with the order of their P_{oct} values as shown in Fig. 5. The glycosides of quercetin might also have lost their activities due to the steric hindrance of the sugar moieties. Although these flavonoids differ in the number and position of hydroxyl groups in flavonoid molecules, the difference seems to affect their activity to P-gp *via* modifying their hydrophobicity.

DISCUSSION

Flavonoids are the most abundant polyphenols present in fruits, vegetables, and plant-derived beverages. Due to their inhibitory activities on P-gp function and their physiological safety, they are candidates for modulators of MDR. To determine the suitable candidates, it is important to clarify structure–activity relationships of their inhibitory activities on P-gp function. This is also meaningful because the intake of flavonoids may also alter drug pharmacokinetics and pharmacodynamics *via* inhibition of P-gp-mediated drug efflux in tissues such as the intestinal epithelium, blood-brain barrier, hepatocytes and renal tubular cells.

From the present findings, structure requirements were suggested for the inhibitory effects of flavonoids. Firstly, the planar structure of flavonoids seems to be important for their interaction with P-gp. For flavanones, which lack the double-bond between the 2- and 3-position in the C ring, the stereoscopic relationship of the B ring with other A and C rings is different from that of flavones which have the double-bond in that position. It has been reported that the torsion angles of B ring in flavones are smaller than those of flavanones.[17,18] Therefore, the double bond confers a special structure on flavone molecules that are largely planar so that they may more readily intercalate between the hydrophobic amino acid residues of P-gp. The importance of the 2,3-double bond has recently been suggested for the interaction of the flavonoids with MRP1 and MRP2.[18] It has also been revealed for the interaction of flavonoids with other proteins such as 15-lipoxygenases.[19]

Another point suggested from the present study is that the hydrophobicity of flavonoids is important for their inhibitory effects on substrate efflux by P-gp. The effects of flavonoids tested in this study were in the order of kaempferol> quercetin, baicalein>myricetin>fisetin, morin. Quercetin-3-*O*-glucoside and rutin had no effect. The order of the effects corresponded with their order of hydrophobicity. This finding is different from the previous finding on tea catechins, which indicated the importance of the trihydric pyrogallol ring structure of the B ring.[9] The difference in the results is probably due to the presence of the galloyl moiety in (−)epicatechin gallate and (−)epigallocatechin gallate (Fig. 1), which had marked effects on P-gp function. Since they do not have planar structures, their interactions with P-gp may be different from those of the relatively planar flavonoids tested in this study. Concentration of the flavonoids in the cytoplasm and their metabolism should also be clarified, to determine the precise structure–activity relationships of the inhibitory effects of the flavonoids on P-gp function.

The inhibitory mechanisms of flavonoids have been studied. Flavonoids such as morin and silymarin have been reported to inhibit azidopine binding in MDA435/LCC6 cells.[6] As for the mechanisms of modification, flavonoids like kaempferide have also been revealed as modulators with bifunctional interactions at vicinal ATP-binding sites and steroid-interacting hydrophobic regions within the cytoplasmic domain of P-gp.[5] For the flavonoids which do not have large substituents such as the galloyl group, the planar structure of the flavonoids and their hydrophobicity seem to be important, especially for their interaction with the hydrophobic region of P-gp. The results obtained in this study are consistent with the recent findings by three dimensional quantitative structure activity relationship model studies.[20]

Flavonoids seem to have the potential to overcome the multidrug resistance that results from the active efflux of anti-tumor drugs by P-gp. On the basis of this study, further studies are currently in progress to clarify the structural requirements for the inhibitory effects of a wide variety of flavonoids, including those which have large substituents as well as other polyphenols such as alkyl gallates.[21]

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