A Quantitative Structure–Activity Relationship for the Modulation Effects of Flavonoids on P-Glycoprotein-Mediated Transport

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P-Glycoprotein accounts for multidrug resistance in chemotherapy patients and contributes to reduced oral bioavailability and distribution of drugs in the brain. The aim of this study was to establish the qualitative and quantitative structure–activity relationships (QSAR) of flavonoid modulation effects on P-glycoprotein (P-gp)'s function. Using human colorectal adenocarcinoma (HCT15) cells as an *in vitro* model and fexofenadine as a P-gp substrate, the modulation effects on P-gp at three concentration levels of 22 representative compounds from four flavonoid families were evaluated. Results showed that the modulation (enhanced or inhibitory) effects could be divided into three ranges designated as enhanced (<100%) as compared to the control, low inhibitory (100—217%) and high inhibitory (>217%) as compared to verapamil. An optimal QSAR was constructed for Y1 (adjusted R^2=0.4798), Y2 (adjusted R^2=0.6809), and Y3 (adjusted R^2=0.5902), respectively. This was further confirmed by a highly correlated plot of the predicted percent inhibition against observed values from a respective QSAR equation.

Key word flavonoid; P-glycoprotein; quantitative structure–activity relationship; multidrug resistance; fexofenadine

Cancer chemotherapy has been of limited success because of intrinsic or acquired resistance of cancer cells to a broad range of chemically and functionally distinct anticancer agents, a phenomenon termed multidrug resistance (MDR). Tumor cells develop drug resistance through various mechanisms, such as overexpression of drug efflux transporters like P-glycoprotein (P-gp), changes in topoisomerase activity, modifications to glutathione *S*-transferase, and altered expressions of apoptosis-associated protein, Bcl-2, and tumor suppressor protein, p53. Of these, overexpression of P-gp is the critical factor.

P-gp (ABCB1), a 170-kDa plasma glycoprotein encoded by the human *MDR1* gene and murine *mdr1a*, *mdr1b*, and *mdr2* genes, belongs to the ATP-binding cassette family of transporters.[1] It is an integral membrane protein with two homologous halves, each consisting of one hydrophobic loop with six transmembrane domains (TMDs) and one hydrophilic nucleotide-binding domain (NBD). Binding and transport of substrates is mediated by TMD using energy derived from the hydrolysis of ATP at the NBD.[2,3]

Numerous studies suggest that the principal physiological role of P-gp is to protect the organism from toxic substances. This efflux pump is also present in many normal tissues including the epithelium of the gastrointestinal tract, renal proximal tubules, canalicular surface of hepatocytes, and the endothelial cell surfaces comprising the blood–brain barrier. P-gp in the intestines, liver, and kidneys may play important roles in the absorption, distribution, or excretion of drugs.[4] P-gp acts as an energy-dependent efflux pump with a broad specificity for chemically unrelated hydrophobic compounds, such as paclitaxel, anthracyclines, Vinca alkaloids, dexamethasone, lidocaine, erythromycin, and protease inhibitors. Therefore, its overexpression is a major cause of failure in human cancer chemotherapy.

MDR cells can be sensitized to anticancer drugs when treated with a P-gp inhibitor, otherwise known as a chemosensitizer. Compounds such as verapamil, dihydropyridine analogs, quinidine, and cyclosporin A are capable of suppressing P-gp function due to their inhibitory properties. Meanwhile, these compounds have an intrinsic toxicity because they are pharmacologically active. They cannot be used safely at dosages required for MDR reversal. The search for chemosensitizers, which have the advantage of being a non-transportable inhibitor without side effects, has led to a great deal of research in plant-derived flavonoids.

Flavonoids are constituents of fruits, vegetables, and plant-derived beverages, such as coffee, tea, and red wine as well as components of herbal-containing dietary supplements (*e.g., Silybum marianum*, *Alpina officinarum*, and *Hypericum perforatum*). More than 4000 naturally occurring flavonoids have been discovered,[5] arising from the various combinations of multiple hydroxyl and methoxyl group substituents of the basic flavonoid skeleton. The classes of flavonoids include chalcones, flavones, flavonols, flavanones, flavanols, anthocyanins, and isoflavones. Flavonoids have long been associated with a variety of biochemical and pharmacological properties, including antioxidative, antiviral, anticarcinogenic, and anti-inflammatory activities,[6] believed to be beneficial to human health. Flavonoids may also be regarded as xenobiotics in the body. Efflux transporters, such as P-gp are also involved in flavonoid handling. Various *in vitro* studies on the effects of flavonoids on P-gp have been reported, with some flavonoids like kaempferol and quercetin shown to be potent stimulators of P-gp-mediated efflux in tumor cells.[7] On the other hand, it was reported that flavonoids such as quercetin, genistein, and morin inhibit P-gp-mediated transport.[8] Information about flavonoid effects on P-gp activity, however, remains poorly understood.

Flavonoids are recognized as a new class of bifunctional modulators capable of binding with both the ATP-binding site (ABS) and vicinal steroid-binding hydrophobic region (SBHR) within the cytosolic domain of P-gp.[9] Further studies indicate that flavonoids tend to bind directly to drug-binding sites (DBSs), and do not inhibit ATP hydrolysis unless a high concentration (200-fold) is reached. In fact, both non-prenylated flavonoids and prenylated derivatives were found to interact with the hydrophobic region or outside the NBDs.[10—12] Based on measurements of binding affinity to-

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ward mouse NBD2 of P-gp, the structure–activity relationship analysis of flavonoid-P-gp concluded that flavonols, chalcones, and flavones are the most active, and flavanones and isoflavones have lower activities, and the presence of the 5-OH group, 3-OH group, and two or three double bonds are required for activity.[10,13] A quantitative structure–activity relationship (QSAR) analysis by stepwise regression was performed to construct the equation $\log(1/K_d)=0.335c\log P-0.007H_f-0.114N_{lc}+4.342$, ($r^2=0.742$; K_d, dissociation constant toward NBD2; $c\log P$, calculated logarithm of the octanol/water partition coefficient; H_f, heat of formation; and N_{lc}, number of hydrogen-suppressed atoms of the longest chain length) for quantitatively evaluating the most significant structural factors on the interaction between flavonoids and P-gp.[14] A structure–activity relationship determined for inhibitory effects of flavonoids along with other polyphenols on P-gp-mediated transport of KB-C2 cells was reviewed.[15] Recently, a 3D linear solvation energy model was reported that quantified the affinity of flavonoid derivatives toward mouse NBD2 of P-gp, identifying shape parameters and hydrophobicity as the major physicochemical parameters of this activity, with hydrogen-bonding capacities acting as minor modulators.[16]

It is imperative to understand the actions of flavonoids on P-gp functions and to qualitatively clarify and establish the qualitative and quantitative structure–activity relationships of their modulation activities on P-gp's function; however, only a few studies, as disclosed above, have examined this topic. Therefore, the objectives of this investigation were to (1) determine the effects of naturally occurring flavonoids on P-gp-mediated MDR; and (2) gain an understanding of the potential mechanisms and construct a QSAR underlying these interactions. Human colorectal adenocarcinoma (HCT-15) cells were used as an *in vitro* model and fexofenadine as the P-gp substrate; the modulation effects on P-gp of several representatives from each of the four flavonoid families (flavones, flavonols, flavanols, and isoflavones) were evaluated to empirically establish a QSAR.

Experimental

Materials Fexofenadine HCl, verapamil, baicalein, apigenin, luteolin, diosmin, quercetin, kaempferol, morin, naringin, naringenin, hesperidin, hesperetin, daidzein, genistein, acacetin, and galangin were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Quercetin 3-β-D-glucoside, formononetin, eriodictyol, myricetin, fisetin, flavanone, and taxifolin were purchased from Fluka Chemie (Buchs, Switzerland). The Bio-Rad dye reagent and bovine serum albumin (BSA) were purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). HPLC-grade acetonitrile was purchased from Labscan Asia (Bangkok, Thailand). Dimethyl sulfoxide (DMSO) was obtained from Mallinckrodt Baker (Phillipsburg, NJ, U.S.A.). The RPMI-1640 cell culture medium was purchased from Sigma. Fetal bovine serum (FBS) was obtained from PAA Laboratories (Pasching, Austria).

Cells and Cell Culture A human colorectal adenocarcinoma cell line (HCT-15) was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and used between passages 10 and 20. HCT-15 cells were cultured in RPMI-1640 medium supplemented with 10% v/v FBS at 37 °C in a humidified incubator with 5% CO_2; the medium was changed every second day. A solution of 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) was used to detach the cells. Cells were maintained in an exponential growth phase by periodic subculturing. HCT-15 cells were cultured in T-75 flasks for subculture and seeded in 12-well cell culture plates at a density of 6×10^5 cells/well/ml medium for the *in vitro* accumulation test. Confluence was reached after 2 d.

Construction of an *in Vitro* Accumulation Model HCT-15 cells and fexofenadine, a P-gp substrate, were used to construct an *in vitro* accumulation model for this study. The step-by-step construction of the model is described as follows. First, fexofenadine was dissolved in DMSO as a stock solution at a concentration 50 mM and diluted to designated concentrations with the fresh medium. The final concentration of DMSO was $<0.1\%$. After cell attachment and 90% confluence (2 d of incubation), every culture medium was replaced with a fresh medium containing 50, 100, 150, 200, 300, or 400 μM fexofenadine in the presence or absence of 100 μM verapamil. Then measurements were taken at different incubation times (30, 60, 90, 120 min). Verapamil (100 μM) was used as the positive control. Accumulation was stopped by rinsing the cells twice with phosphate buffer solution (PBS). Cells were then solubilized using 200-μl aliquots of PBS. One hundred-microliter aliquots of HCT-15 cell samples were added to 200-μl aliquots of acetonitrile and centrifuged at $8000\times g$ for 10 min at 4 °C. The supernatant was subsequently transferred to a new 1.5-ml Eppendorf tube to determine the intracellular concentration of fexofenadine at different substrate concentrations and incubation times by liquid chromatography mass spectrometry/mass spectroscopy (LC-MS/MS). Results were normalized to the protein content of cells in each well. The protein content was estimated by the Bradford method.

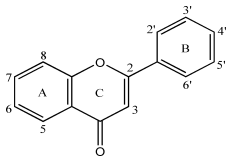
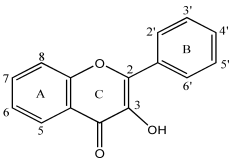
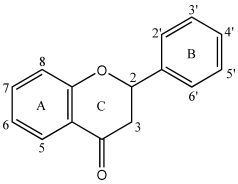
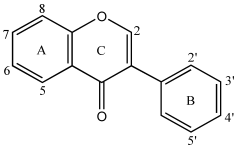
Evaluation of the Inhibitory Activity of Flavonoids Toward P-gp A total of twenty-two representative compounds from each of the four flavonoid families including flavones, flavonols, flavanones, and isoflavones as listed in Table 1 were used as the model compound. Stock solutions of these model compounds were prepared at a concentration 100 mM in DMSO. Flavonoids were dissolved in a culture medium with final solvent concentrations not exceeding 1% v/v. Control cells were treated with the same solvent amount. To examine the effects of flavonoids on P-gp activity, cells were preloaded with flavonoids or verapamil for 10 min. Verapamil (100 μM) was used as the positive control. Cells were treated with a fresh medium containing 100 μM fexofenadine at different concentrations (0, 20, 50, 100 μM) of flavonoids in each cell well of the 12-well plates, and then incubated for specified times. Plates were placed in a shaking incubator at 37 °C during the experiments. Cells were washed twice with PBS to stop the experiment and intracellular concentrations of fexofenadine were determined by LC-MS/MS.

LC-MS/MS Instrument and Conditions for Analysis of Fexofenadine Reversed-phase HPLC and electrospray ionization mass spectrometry (LC-MS/MS) were performed on a Bruker esquire 3000 plus (Bruker Daltonics, Billerica, MA, U.S.A.). Analyses of the data from MS of test samples were performed with a Bruker Compass 1.0 (Bruker Daltonics). A 200-μl aliquot of acetonitrile was added to a 100-μl aliquot of the cell sample. After mixing and centrifugation at $8000\times g$ for 10 min, 200 μl of the supernatant was transferred to a clean Eppendorf tube. A 20-μl aliquot of the supernatant was directly injected into the reversed-phase HPLC column (Inertsil, C18; 2.1 mm×50 mm; particle size, 3 μm; Tokyo, Japan). Gradient elution was performed using acetonitrile (eluent A) and 0.1% formic acid (eluent B). The gradient elution initial condition was 10% A with a linear gradient to 60% in 3 min. The mobile phase was then returned to the initial condition at 3.5 min. The total run time was 4.5 min. An ion spray interface operating in the positive ionization mode was used. Nebulizer (nitrogen) pressure was set to 20 psi and the temperature of the probe was set to 300 °C. Detection was based on multiple-reaction monitoring (MRM). The precursor/product ion pairs monitored were m/z 502.2/466.3 for fexofenadine. The method was successfully validated to determine the concentration of fexofenadine in both cell and plasma samples.

Preparation of Cell Samples and Plasma Samples Cell samples were solubilized using 200-μl aliquots of PBS. One hundred-microliter aliquots of HCT-15 cell samples incubated at a variety of concentrations and time intervals were added to 200-μl aliquots of acetonitrile. After mixing and centrifugation at $8000\times g$ for 10 min at 4 °C, 200 μl of the supernatant was transferred to a clean Eppendorf tube. A 20-μl aliquot of the final solution was injected into HPLC-MS/MS system to determine the fexofenadine concentration. Results were normalized to the protein content of cells in each well.

All animal experiments were performed under an institutionally approved protocol (Taipei Medical University). Three New Zealand white rabbits (*ca.* 3.0 kg) were used for each of the two treatments in a parallel design. Treatment I was oral administration of ground powder of one Allegra® tablet (fexofenadine, 60 mg/tablet) suspended in 30 ml de-ionized water and treatment II was the co-administration of ground powder of one Allegra® tablet suspended in 30 ml de-ionized water with 10 mg/kg of baicalein, 30 min before which was pretreated with 10 mg/kg of baicalein. Blood sampling (0.2 ml) began before dosing and at 0, 5, 10, 20, 30, 45, 60, 75, 90, 120, 180, 240, 360, 480, 600 min, and 24 h after dosing *via* a marginal ear vein of the rabbit. Blood samples were prevented from coagulating with heparin

Table 1. Structures of the Four Major Flavonoid Subclasses and Their Physicochemical Properties

Type	Compound	Free OH substituent			Glycosylated position	M.W.	$c\log P/\delta$
		A-ring	B-ring	C-ring			
Flavones	Acacetin	5,7	4′-OMe	—	—	284.27	2.215/12.15
	Baicalein	5,6,7	—	—	—	270.24	1.629/13.73
	Apigenin	5,7	4′	—	—	270.24	1.629/13.73
	Luteolin	5,7	3′,4′	—	—	286.24	0.962/14.52
	Diosmin	5	3′,4′-OMe	—	7-rutinose	608.55	−0.188/15.36
Flavonols	Quercetin	5,7	3′,4′	3	—	302.24	0.295/15.81
	Quercetin 3-β-D-glucoside	5,7	3′,4′	—	3-glucose	464.38	−2.256/16.98
	Kaempferol	5,7	4′	3	—	286.24	0.962/15.20
	Morin	5,7	2′,4′	3	—	302.24	0.295/15.81
	Galangin	5,7	—	3	—	270.25	1.629/14.52
	Myricetin	5,7	3′,4′,5′	3	—	318.24	−0.372/16.35
	Fisetin	7	3′,4′	3	—	286.25	2.539/15.20
Flavanones	Flavanone	—	—	—	—	224.25	2.539/10.19
	Naringin	5	4′	—	7-rhamnoglucose	580.53	−1.291/15.79
	Naringenin	5,7	4′	—	—	272.25	0.538/13.49
	Hesperidin	5	3′,4′-OMe	—	7-rutinose	610.56	−1.499/15.21
	Hesperetin	5	3′,4′-OMe	—	—	302.28	0.262/11.96
	Eriodictyol	5,7	3′,4′	—	—	288.25	−0.129/14.28
	Taxifolin	5,7	3′,4′	3	—	304.26	−1.310/14.97
Isoflavones	Formononetin	7	4′-OMe	—	—	268.26	2.882/11.16
	Daidzein	7	4′	—	—	254.24	2.296/12.82
	Genistein	5,7	4′	—	—	270.24	1.629/13.73

(100 IU/ml) and centrifuged at 8000×***g*** for 15 min. Plasma was separated and stored at −30 °C until analysis. Plasma samples (100 μl) were supplemented with 10 μl of the internal standard of sildenafil (15 μg/ml) in a phosphate-buffered solution (PBS, 500 mM KH_2PO_4, pH 6.0) and then precipitated with additional 200 μl of acetonitrile. The mixture was vortex-mixed for 30 s and centrifuged at 8000×***g*** at 4 °C for 5 min. An aliquot of the supernatant (20 μl) was subjected to analysis of the LC-MS/MS system as validated above for fexofenadine.

QSAR Model Construction A stepwise regression analysis was used to determine statistically significant relationships between the dependent variable of the modulation activity and the independent variables of the structural units, structural skeletons, and physicochemical properties. The statistical analyses were performed using SAS statistical software (Version 8.0; SAS Institute Inc., Cary, NC, U.S.A.). The following model statistical criteria were noted: n, the number of observations; adjusted (adj.) R^2, adj. squared correlation coefficient; s, the standard deviation; F, the Fisher statistic; and the p value. The raw data or logarithmic transformation of three modulation activities at three flavonoid levels (25, 50, 100 μM) were set as the dependent variables, Y1 ($\log$ Y1), Y2 ($\log$ Y2), and Y3 ($\log$ Y3), respectively. The structural units (x_1—x_{11}) and skeletons (x_{14}—x_{17}), and physicochemical properties of $\log$ MW (x_{12}) and $\log\delta$ (or $c\log P$, x_{13}) were empirically chosen as independent variables: x_1=A-ring-5-OH, x_2=A-ring-6-OH, x_3=A-ring-7-OH, x_4=B-ring-2′-OH, x_5=B-ring-3′-OH, x_6=B-ring-4′-OH, x_7=B-ring-4′-OMe, x_8=B-ring-5′-OH, x_9=C-ring-3-OH, x_{10}=A-ring-3-Glu, x_{11}=A-ring-7-Glu, x_{12}=$\log$ MW, x_{13}=$\log\delta$ (or $c\log P$), x_{14}=C2-ring (or flavones); x_{15}=C2-double bond (or flavonols); x_{16}=C3-OH (or flavanols); and x_{17}=C3-ring (or isoflavones). $c\log P$ (the calculated octanol–water partition coefficient) was calculated using Molsuit 2000 Molecular Modeling Pro Plus (ChemSW®, Fairfield, CA, U.S.A.) based on the fragmental method. The solubility parameter (δ) was calculated based on Fedor's approach.[17]

Statistical Analysis Values are given as the mean±standard error (S.E.) of more than six experiments. Statistical comparisons were performed by Student's t-test. A p value of <0.05 was considered statistically significant.

Results and Discussion

Fexofenadine Assay Performance and Validation To optimize the detection of the analyses, the operational parameters of tandem mass spectrometry (LC-MS/MS) were optimized based on the response from the MRM channels for quantification of fexofenadine. The MRM chromatogram and the product ion mass spectrum of fexofenadine were examined (data not shown). The most prominent ions (m/z 466.3) resulted from the loss of two fragments with m/z 18 (m/z 502.2→466.3), a result which is most likely attributable to the loss of 2 mol of water from the parent compound. The linearity of the calibration curve of fexofenadine was well correlated ($r^2>0.998$) within a range of 300—4500 ng/ml with an accuracy and precision of −5.73—15.07% and 4.30—9.54%, and 2.84—9.10% and −6.47—9.85%, respectively, for the intra- and inter-day assays. The method is precise and sensitive enough for its intended purpose of assaying the fexofenadine concentrations in both cell and plasma samples.

Construction of an *in Vitro* Accumulation Model In terms of the effect of flavonoids on P-gp function, different studies illustrated an inverse effect for the same flavonoids.

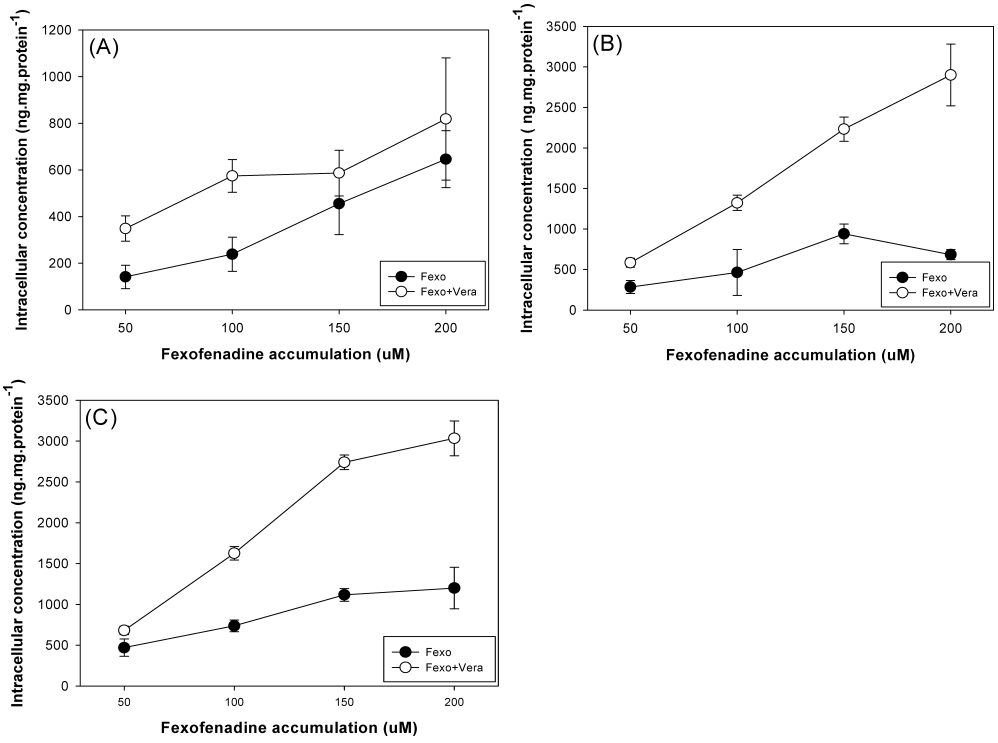


Fig. 1. Fexofenadine Solutions in the Presence or Absence of 100 μM Verapamil Were Added to HCT-15 Cells to Final Concentrations of 50, 100, 150, and 200 μM, and Cells Were Incubated for (A) 30, (B) 60, and (C) 90 min at 37 °C

The data are the mean±S.E., $n=6$.

The main difficulty in all this research, which involved various conditions, was that we could not evaluate the structure–activity relationships of the effects of flavonoids on P-gp function. Construction of an *in vitro* accumulation model to compare the relationships between the structures of a great number of flavonoids and P-gp function is necessary. In this study, we used P-gp-expressing HCT-15 colon cells, an established MDR cell line, and fexofenadine as the model substrate of P-gp and reported to be present in cancer and HCT-15 cells.[18] The influence of the time course and concentration of fexofenadine in the presence or absence of a known inhibitor, verapamil, is evaluated in Fig. 1. As a positive control, the MDR reversal agent, verapamil (100 μM), was tested and found to effectively increase the time- and concentration-dependent accumulation of fexofenadine relative to the control. Taken together, the intracellular accumulation of fexofenadine was linear with respect to time (up to 90 min) and concentration (up to 200 μM). The concentration of fexofenadine was 470.4 ng·mg protein^{-1} in the absence of verapamil when HCT-15 cells were treated with 100 μM fexofenadine for 90 min. Relative to the case with 100 μM verapamil, the intracellular concentration increased almost 2.5-fold. Therefore, the inhibitory effects of flavonoids in drug-resistant HCT-15 cells were evaluated with a fixed fexofenadine concentration of 100 μM and incubation time of 90 min.

Inhibitory Effects of Flavonoids in Drug-Resistant HCT-15 Cells The study examined the ability of four flavonoid families, including 22 compounds whose structures are shown in Table 1, to modulate fexofenadine accumulation. Verapamil (100 μM) was used as a positive control. Effects of different concentrations of flavonoids (20, 50, 100 μM) on the relative cellular accumulation of fexofenadine in HCT-15 cells are shown in Fig. 2. The accumulation of fexofenadine increased to 217±10% of the control value by verapamil. Therefore, the modulation (enhanced or inhibitory) effects could be divided into three ranges designated as: enhanced (<100%) as compared to the control, low inhibitory (100—217%) and high inhibitory (>217%) as compared to verapamil. These were used to indicate the relative modulation potencies of the flavonoids on P-gp-expressing HCT-15 cells. As shown in Fig. 2, flavonoids with an enhanced potency included diosmin, luteolin, taxifolin, quercetin, daidzein, quercetin-3-β-D-glucoside, and genistein; a low inhibitory effect included kaempferol, morin, flavanone, naringenin, naringin, fisetin, formononetin, eriodictyol, myricetin, hesperetin, and hesperidin; and a high inhibitory effect included baicalein, apigenin, acacetin, and galangin, which might induce a strong binding affinity towards P-gp.

Those flavonoids with a high inhibitory effect on P-gp activity among 22 compounds examined appeared to possess hydroxyl groups attached at the 5- and 7-positions of the A-ring, with the total number of hydroxyl group at an optimal of three. However, flavonoids with increasing hydroxyl number such as kaempferol (3,5,7,4′-tetrahydroxyflavone) and fisetin (3,7,3′,4′-tetrahydroxyflavone) were contrary to the decrease in the inhibitory effect of P-gp activity, whereas flavonoids with an excess number of hydroxyl groups such as quercetin (3,5,7,3′,4′-pentahydroxyflavone) and taxifolin (3,5,7,3′,4′-pentahydroxyflavanone) were even able to enhance P-gp activity.

Regarding the hydrophobicity expressed by $c\log P$ as indicated in Table 1, those flavonoids with negative $c\log P$ values, such as quercetin 3-β-D-glucoside (−2.256), naringin

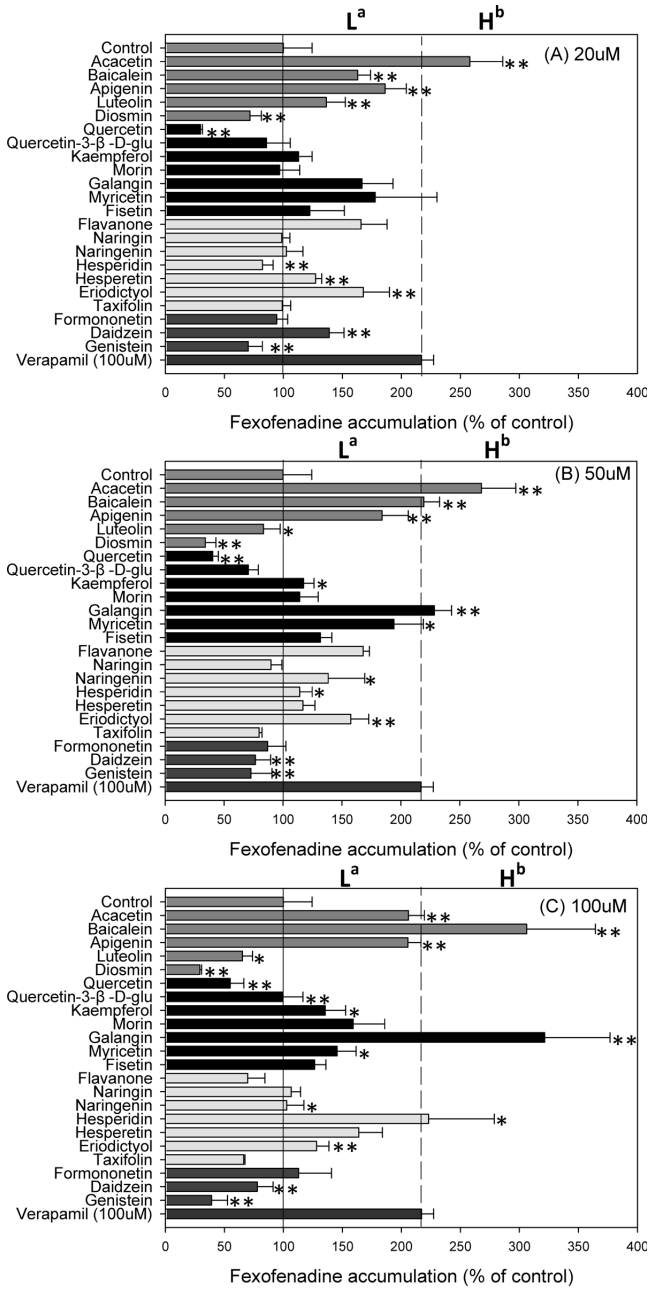


Fig. 2. Effects of (A) 20, (B) 50, and (C) 100 μM Flavonoids on the Relative Cellular Accumulation of Fexofenadine in Multidrug-Resistant P-Glycoprotein (P-gp)-Overexpressing HCT-15 Cells

$*p<0.05$; $**p<0.01$, *vs.* the control. *a*) Low inhibitory effect (100—217%) and *b*) high inhibitory effect (>217%) as compared to verapamil.

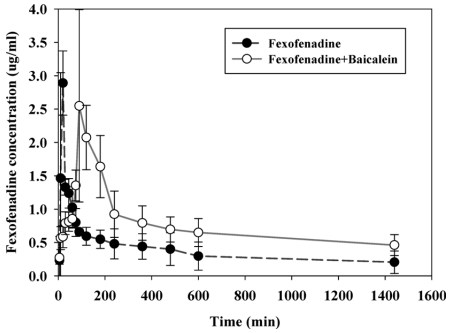


Fig. 3. Mean Plasma Concentration–Time Profiles of Fexofenadine after the Oral Co-administration of Fexofenadine (60 mg) with Baicalein to the Rabbits

Bars represent the standard deviation, (●) fexofenadine control; (○) co-administered with baicalein 10 mg/kg (n=3).

(−1.291), and taxifolin (−1.310), seemed to be so hydrophilic as to have a lesser inhibitory effect on P-gp. It was not taken as granted that those flavonoids with a larger positive value of $c\log P$ had a greater inhibitory effect (formononetin, with the largest $c\log P$ value of 2.88, did not possess the greatest inhibitory effect). Taking the backbone structure of flavonoids into consideration, the 2,3 double bond confers a special structure on flavone molecules that was largely planar such that flavones may have been more readily intercalate between the hydrophobic amino acid residues of P-gp. Since there is a single bond at the 2,3-position of the C-ring in the backbone structure of flavanones, a planar structure does not form for optimal intercalation, leading to an inhibitory effect of flavanones less strong than that of flavones. On the other hand, flavonols commonly demonstrated lower inhibitory effects on P-gp than flavones, suggesting that the negative role of the additional hydroxyl group at the 3-position of the C ring. The attachment of the B-ring to the 3-position of the C-ring for isoflavones seems to have a twisted structure and greatly hindered interactions to make the inhibitory effect worse.

From the above results, we should take the following elements into consideration for qualitatively clarification about the relationship between the structure and modulation effects of flavonoids; specifically, the number of hydroxyl groups, the backbone structure, and the hydrophobicity. The results confirm that flavonoids have bifunctional interactions in their A and C rings at the ATP-binding site and a hydrophobic B-ring at a steroid-interacting hydrophobic sequence of P-gp.[10] Of the flavonoids tested in our study, it was discovered that baicalein, apigenin, and galangin possess the highest inhibitory effects. These three flavonoids have a planar structure, a suitable $c\log P$ value, and an appropriate number of hydroxyl groups, which is consistent with the criteria described above, so they are able to significantly inhibit P-gp activity. Specifically, the effect of 50 μM baicalein was found to be comparable to that of 100 μM verapamil, while 100 μM baicalein was more significantly potent than 100 μM verapamil (Fig. 2C). As shown by Fig. 3, co-administration of fexofenadine with baicalein increased the oral bioavailability of fexofenadine up to twofold ($AUC_{\text{with baicalein}}/AUC_{\text{without baicalein}}$=1082.22/546.91) compared to that without co-administration with baicalein. This indicates that P-gp activity was inhibited to a greater extent by baicalein, leading to an increase in the oral bioavailability of fexofenadine as high as twofold. Additionally, it confirms the inhibitory effect of baicalein on P-gp activity determined by the *in vitro* accumulation model. A longer $T_{\max}$ (90 min *vs.* 20 min) was observed in the plasma profile for co-administration of fexofenadine with baicalein. It might be attributed to the inhibition of the intestinal motility by baicalein, whose effect has been reported for apigenin as belonging to the same flavone family as baicalein with three hydroxyl groups at different positions.[19]

Based on the empirical model, the QSAR for modulation effects on P-gp of HCT-15 cells was obtained by a regression

of the percent inhibition at three levels (either raw data, Y1, Y2, and Y3, or logarithmically transformed log Y1, log Y2, and log Y3) with respect to the structural units (x_1—x_{11}) or skeletons (x_{14}—x_{17}) with drug parameters of log MW (x_{12}) and log δ (or $c\log P$, x_{13}). The regression results are summarized in Table 2 and show a slight improvement in the regression when using log δ to regress the percent modulation effect at the three levels (using either raw or logarithmically transformed data). Furthermore, a better correlation with a higher adjusted R^2 value was found for the QSAR model by regressing the raw data of the modulation effects at the three levels with respect to either structural units (x_{14}=C2-ring; x_{15}=C2-double bond; x_{16}=C3-OH; x_{17}=C3-ring) or structural skeletons (x_{14}=flavones; x_{15}=flavonols; x_{16}=flavanols; x_{17}=isoflavones) with the Y2 level of 50 μM being the most highly correlated (adj. R^2=0.6809), followed by the Y3 level (100 μM) with adj. R^2=0.5902, and finally the Y1 level (20 μM) with adj. R^2=0.4798. This indicates that the raw data of the modulation effects at the three levels were an appropriate data type for constructing the QSAR with the Y2 level being the most optimal. Furthermore, since both log δ and $c\log P$ are indicators of hydrophobicity, only slight differences in the correlations with the percent modulation effect at these three levels (Y1, Y2, and Y3) would be drawn. However, only when log δ was included in the regression at the Y2 level respective to either structural unit or skeleton, was it

Table 2. List of Mathematical Equations and Statistical Parameters for the Quantitative Structure–Activity Relationship (QSAR)

Drug and structural parameters[a]		Mathematical equations	Adjusted R^2 (overall F-test)
With log δ	$Y1_{(unit)}$	=101.22*(A-7OH)+45.81*(B-5′OH)−94.83*(C3-ring)+76.03*(A-7glu)−949.20*(log δ)+1136.74	0.4798 ($F_{5,21}$=4.87, p<0.01)
	$Y2_{(unit)}$	=70.35*(A-7OH)−52.49*(B-3′OH)−52.66*(B-4′OH)+54.93*(B-5′OH)−114.11*(C3-ring)−335.13*(log δ)+525.55	0.6809 ($F_{6,21}$=8.47, p<0.01)
	$Y3_{(unit)}$	=63.11*(A-7OH)−63.66*(B-3′OH)−105.40*(B-4′OH)+128.71*(C2-ring)+74.87	0.5902 ($F_{4,21}$=8.56, p<0.01)
	$Y1_{(skeleton)}$	=41.08*(B-5′OH)−200.23*(log MW)+69.23*(flavones)+31.76*(flavanols)+596.77	0.4018 ($F_{4,21}$=4.53, p<0.02)
	$Y2_{(skeleton)}$	=70.35*(A-7OH)−52.49*(B-3′OH)−52.66*(B-4′OH)+54.93*(B-5′OH)−114.11*(isoflavones)−335.13*(log δ)+525.55	0.6809 ($F_{6,21}$=8.47, p<0.01)
	$Y3_{(skeleton)}$	=153.27*(A-6OH)−70.54*(B-4′OH)+56.59*(flavonols)+152.85	0.3411 ($F_{3,21}$=4.62, p<0.02)
	log $Y1_{(unit)}$	=−0.1290*(B-3′OH)+0.1928*(B-5′OH)+0.2989*(C2-ring)−2.5475*(log δ)+4.7856	0.3625 ($F_{4,21}$=3.99, p<0.02)
	log $Y2_{(unit)}$	=−0.2035*(B-3′OH)−0.1246*(B-4′OH)+0.1834*(B-5′OH)−0.7685*(log MW)−0.3327*(C3-ring)+4.1724	0.5168 ($F_{5,21}$=5.49, p<0.01)
	log $Y3_{(unit)}$	=−0.3348*(A-5OH)−0.2703*(B-3′OH)−0.2405*(B-4′OH)+0.8015*(C2-ring)−0.2192*(A-7glu)+1.9603	0.5214 ($F_{5,21}$=5.58, p<0.01)
	log $Y1_{(skeleton)}$	=0.1703*(B-5′OH)+0.1547*(flavones)−1.5704*(log δ)+3.8150	0.2533 ($F_{3,21}$=3.37, p<0.05)
	log $Y2_{(skeleton)}$	=−0.2035*(B-3′OH)−0.1246*(B-4′OH)+0.1834*(B-5′OH)−0.7685*(log MW)−0.3327*(isoflavones)+4.1724	0.5168 ($F_{5,21}$=5.49, p<0.01)
	log $Y3_{(skeleton)}$	=−0.2263*(B-3′OH)−0.4773*(isoflavones)+2.2043	0.2163 ($F_{2,21}$=3.90, p<0.05)
With $c\log P$	$Y1_{(unit)}$	=70.83*(B-5′OH)−41.48*(C3-OH)−82.35*(C3-ring)+24.42*(clogP)+128.17	0.4498 ($F_{4,21}$=5.29, p<0.01)
	$Y2_{(unit)}$	=81.22*(A-7OH)−61.11*(B-3′OH)−67.72*(B-4′OH)+59.35*(B-5′OH)−31.99*(C2-double bond)−112.10*(C3-ring)+154.85	0.6760 ($F_{6,21}$=8.30, p<0.01)
	$Y3_{(unit)}$	=63.11*(A-7OH)−63.66*(B-3′OH)−105.40*(B-4′OH)+128.71*(C2-ring)+74.87	0.5902 ($F_{4,21}$=8.56, p<0.01)
	$Y1_{(skeleton)}$	=41.08*(B-5′OH)−200.23*(log MW)+69.23*(flavones)+31.76*(flavanols)+596.77	0.4018 ($F_{4,21}$=4.53, p<0.02)
	$Y2_{(skeleton)}$	=81.34*(A-7OH)−50.37*(B-3′OH)−65.93*(B-4′OH)+58.81*(B-5′OH)+40.83*(flavanols)−101.90*(isoflavones)+12.13*($c\log P$)+115.81	0.7126 ($F_{7,21}$=8.44, p<0.01)
	$Y3_{(skeleton)}$	=153.27*(A-6OH)−70.55*(B-4′OH)+56.59*(flavonols)+152.85	0.3411 ($F_{3,21}$=4.62, p<0.02)
	log $Y1_{(unit)}$	=−0.5909*(log MW)+3.5388	0.0989 ($F_{1,21}$=3.30, p<0.1)
	log $Y2_{(unit)}$	=−0.2035*(B-3′OH)−0.1246*(B-4′OH)+0.1834*(B-5′OH)−0.7685*(log MW)−0.3327*(C3-ring)+4.1724	0.5168 ($F_{5,21}$=5.49, p<0.01)
	log $Y3_{(unit)}$	=−0.5002*(A-5OH)−0.3743*(B-3′OH)−0.3106*(B-4′OH)+0.8245*(C2-ring)−0.3694*(A-7glu)−0.0928*($c\log P$)+2.2535	0.5638 ($F_{6,21}$=5.52, p<0.01)
	log $Y1_{(skeleton)}$	=−0.6125*(log MW)+0.1583*(flavones)+3.5569	0.1831 ($F_{2,21}$=3.35, p<0.1)
	log $Y2_{(skeleton)}$	=−0.2035*(B-3′OH)−0.1246*(B-4′OH)+0.1834*(B-5′OH)−0.7685*(log MW)−0.3327(isoflavones)+4.1724	0.5168 ($F_{5,21}$=5.49, p<0.01)
	log $Y3_{(skeleton)}$	=−0.2263*(B-3′OH)−0.4773*(isoflavones)+2.2043	0.2163 ($F_{2,21}$=3.90, p<0.05)

a) Subscription of unit and skeleton indicates that Y or log Y values were regressed with respective to the independent variable involving the structural unit and skeleton, respectively.

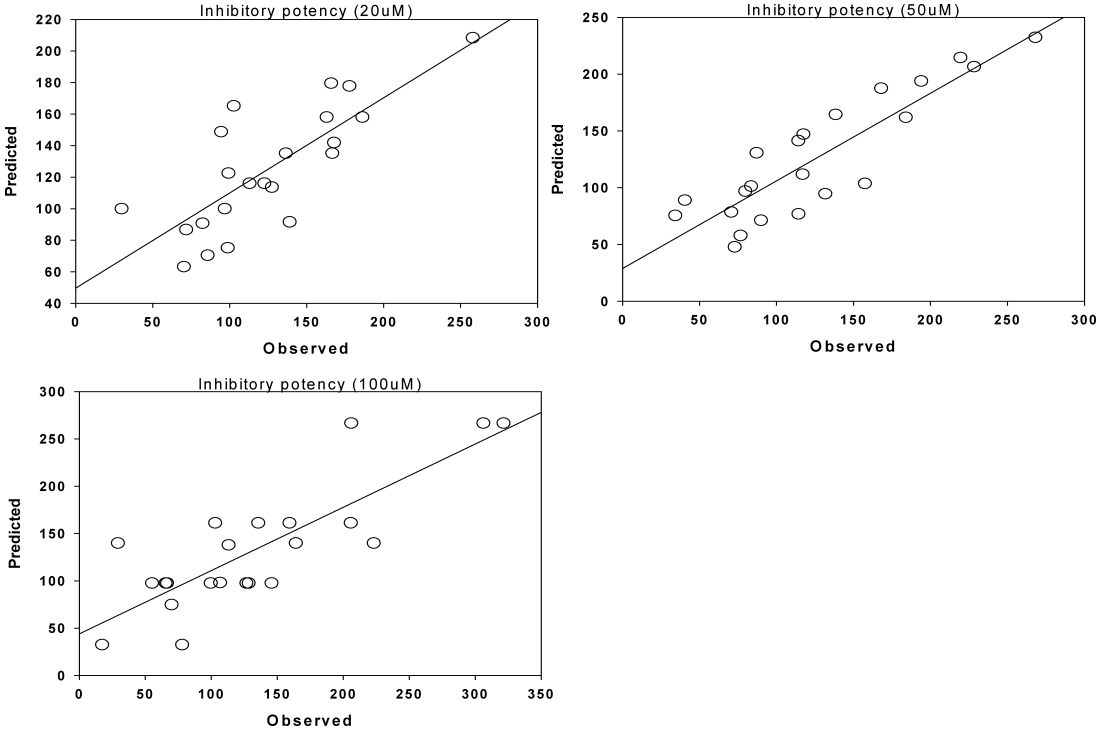


Fig. 4. The Predicted *versus* Observed Plots for Three Modulation Potencies of Flavonoids on P-Glycoprotein (P-gp)

found to be a significant and influential factor in the same QSAR correlation for $Y2_{(unit)}$ and $Y2_{(skeleton)}$ (both adjusted R^2 for $Y2_{(unit)}$ and $Y2_{(skeleton)}$ are equal to 0.6809). It was concluded that the establishment of statistically significant QSAR for flavonoids on the modulation of P-gp activity in HCT-15 cell *in vitro* models using fexofenadine as a substrate might be most appropriate at concentration levels of 50 μM with the inclusion of solubility parameters as an indicator of drug hydrophobicity.

The final QSAR equation for the modulation effects at the Y2 level for flavonoids was constructed as shown by the $Y2_{(unit)}$ and $Y2_{(skeleton)}$ equations listed in Table 2. It reveals that structural units of B-ring-3′-OH group, B-ring-4′-OH group, C3-ring (or structural skeleton of isoflavones), and $\log \delta$ negatively contributed to the modulation effect of flavonoids on P-gp activity, while the A-ring-7OH group and B-ring-5′-OH group tended to enhance their inhibitory effects. Among them, the coefficient for the variable of (C3-ring) or (isoflavones) was the same and the largest indicating the most influential factor for regulating the modulation effect of flavonoids on P-gp's function. The regression results of the QSAR equations obviously indicate that the underlying mechanisms for controlling the modulation effects on P-gp was most appropriate to clarify at the Y2 level of modulators. The B-ring attached to the C3 position (C3-ring) was the most unfavorable structural unit and isoflavones were the most unfavorable structural skeleton for an inhibitory effect on P-gp function. This conforms to results reported by Conseil *et al.* and Boumendjel *et al.* who used the dissociation constant (K_d) toward NBD2 as a dependent variable.[10—13] A plot of the predicted percent modulation effect from the respective QSAR equations (the first three equations in Table 2) against observed values, given in Fig. 4, shows that the predicted percent inhibition values for all flavonoids were highly correlated with the observed values and <2-fold off from the observed percent inhibition values observed for the Y2 level, suggesting that this model does not have a tendency to over- or under-predict the percent inhibition values and thus is useful for predicting percent inhibition values of flavonoids.

Conclusion

With regard to flavonoid structure–activity relationships, we identified structural features associated with positive or negative effects on the ability of flavonoids to increase cellular accumulation of fexofenadine. It therefore seems reasonable to predict that other flavonoids not tested in this experiment will also possess appreciable activity. It is believed that these QSAR results can be taken into account when developing flavonoids with high inhibitory effects on P-gp. In the present study, our results showed that baicalein, with its high inhibitory activity on P-gp, can be used as a modulator of multiple drug resistance on cancer cells that overexpress P-gp.

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