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Research Paper

Effects of Various Methoxyflavones on Vincristine Uptake and Multidrug Resistance to Vincristine in *P*-gp-Overexpressing K562/ADM Cells

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Purpose. Some methoxyflavones (MFs) are known to inhibit the function of *P*-glycoprotein. The aim of this study is to characterize the reversal of multidrug resistance (MDR) by MFs.
Methods. The effects of 19 MFs, including 3,5,6,7,8,3′,4′-heptamethoxyflavone, nobiletin, and tangeretin, and flavone on the uptake of [^{3}H]vincristine into an adriamycin-resistant variant of human chronic myelogenous leukemia (K562/ADM) cells were investigated. Potentiation of vincristine-induced growth inhibition by these MFs was also tested in K562/ADM cells by means of WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] assay.
Results. All MFs (20 μM) tested increased the uptake of [^{3}H]vincristine. 3,5,6,7,8,3′,4′-heptamethoxyflavone, nobiletin, tangeretin, quercetagetin and quercetin pentamethylether showed especially potent effects. The increase in the uptake of [^{3}H]vincristine was proportional to the number of methoxyl moieties. While substitution with a methoxyl moiety at the C3 position was the most influential, methoxyl substitution at both the C3′ and C5′ positions resulted in a decrease in the potentiation of uptake. Furthermore, there was a significant correlation between the potencies for increasing [^{3}H]vincristine uptake and for growth inhibition assessed by WST-1 assay.
Conclusions. MFs increased the uptake of [^{3}H]vincristine into MDR cells and exhibited MDR-reversing effects. Their potencies were influenced by the number and positions of the methoxyl moieties.

KEY WORDS: methoxyflavone; multidrug resistance; *P*-glycoprotein; structure-activity relationship.

INTRODUCTION

Multidrug resistance to anticancer drugs (MDR) is one of the important problems in cancer chemotherapy. Cancer cells with multidrug resistance show cross resistance to a range of structurally and pharmacologically unrelated compounds, such as Vinca alkaloids, anthrocyclines, epipodophillotoxins and actinomycin D (1). MDR is, at least in part, attributable to the function of *P*-glycoprotein (*P*-gp), a 170 kDa glycoprotein that is overexpressed on the plasma membranes of multidrug-resistant cancer cells (2). *P*-gp is a member of the ATP-binding cassette (ABC) transporter family, and functions to extrude anticancer drugs from the cells, resulting in a decrease in their intracellular concentration. Tsuruo *et al.* (3) found that a calcium channel blocker, verapamil, can overcome multidrug resistance by inhibiting the function of *P*-gp. Since then, various drugs, such as quinidine, cyclosporine A, PSC833 and so on, have been reported to have the potential to overcome MDR. However, most of these MDR-reversing agents have pharmacological effects other than *P*-gp inhibition and/or they potently inhibit the function of cytochrome P450 (CYP), thereby causing a range of drug interactions.

We have previously identified several natural *P*-gp-inhibitory compounds from citrus juices and found that some methoxyflavones (MFs) with many methoxy moieties, such as tangeretin, nobiletin and heptamethoxyflavone, selectively inhibit *P*-gp without affecting CYP3A4 (4). Tangeretin, nobiletin and heptamethoxyflavone have five, six and seven methoxyl moieties, respectively, and the rank order of activity for *P*-gp inhibition was tangeretin < heptamethoxyflavone ≈ nobiletin (5). Choi *et al.* have also investigated the MDR-reversing potency of sinensetin, a MF with five methoxyl moieties, and its analogues with two to five methoxyl moieties in *P*-gp-overexpressing cells and conclud-

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ed that sinensetin and pentamethoxyflavones most potently reversed MDR (6,7). Therefore, the inhibitory effects of MFs on *P*-gp may be related to the number of methoxyl moieties.

The aim of this study is to investigate the effects of 19 MFs and flavone (Fig. 1) on the uptake of [^{3}H]vincristine, a *P*-gp substrate, by human chronic myelogenous leukemia K562 cells and their adriamycin-resistant variant, K562/ADM cells, as well as their effects on the inhibition of cell growth by vincristine. We also investigated the structure-activity relationship of MFs for *P*-gp inhibition, as assessed by measuring [^{3}H]vincristine uptake.

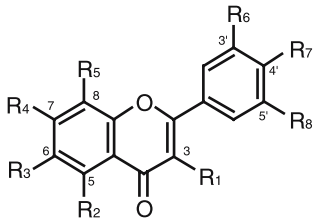
MATERIALS AND METHODS

Reagents

[^{3}H]Vincristine (5.3–6.6 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK). Vincristine was a kind gift from Eli Lilly Japan K.K. (Hyogo, Japan). Verapamil was purchased from Nacalai Tesque (Kyoto, Japan). We purchased following MFs with the highest quality commercially available, stored under manufacturer's instruction, and after dissolved we stored it under −80°C and used within three months. Tangeretin, sinensetin, 5,6,7,3′,4′,5′-hexamethoxyflavone, 5,7,3′,4′,5′-pentamethoxyflavone, scutellarein tetramethylether, 7,3′,4′-trimethoxyflavone, 3′,4′-dimethoxyflavone, chrysin dimethylether, 3-methoxyflavone, myricetin and quercetagetin were purchased from Extrasynthese (Genay, France). Fisetin and galangin were purchased from Aldrich Chem. (Tokyo, Japan). Luteolin, flavone, potassium carbonate and absolute acetone were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dimethyl sulfate was purchased from Kishida Chemical (Osaka, Japan). Cyclosporin A and nobiletin were kind gifts from Novartis Pharm (Basel, Switzerland) and Kanebo Yakuhin (Osaka, Japan), respectively. Heptamethoxyflavone and baicalein were extracted from *Aurantii* Pericarpium and *Scutellariae* Radix, respectively, by the method previously described and used after purification (8,9). Morin pentamethylether was synthesized by etherification of morin with alkyl halide/K_2CO_3 in refluxing dimethylformamide (DMF/acetone; 10). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, sodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Wako Pure Chemical Industries. All other reagents used were of the highest reagent grade commercially available.

Cell Culture

K562 cell line was provided by Dr. K. Ezaki (Cancer Chemotherapy Center, Tokyo), and a subline resistant to adriamycin (K562/ADM) was established by Tsuruo *et al.* (11). The cells were cultured by the method previously described (11). Briefly, cells are grown in suspension in RPMI



compounds	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Clog P
heptamethoxyflavone	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	H	1.51
quercetagetin	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	H	1.64
nobiletin	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	H	2.31
myricetin hexamethylether	OCH_3	OCH_3	H	OCH_3	H	OCH_3	OCH_3	OCH_3	2.31
5,6,7,3',4',5'-hexamethoxyflavone	H	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	OCH_3	2.31
tangeretin	H	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	H	2.44
quercetin pentamethylether	OCH_3	OCH_3	H	OCH_3	H	OCH_3	OCH_3	H	1.76
morin pentamethylether	OCH_3	OCH_3	H	OCH_3	H	OCH_3	H	OCH_3	1.76
sinensetin	H	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	H	2.44
5,7,3',4',5'-pentamethoxyflavone	H	OCH_3	H	OCH_3	H	OCH_3	OCH_3	OCH_3	2.92
scutellarein tetramethylether	H	OCH_3	OCH_3	OCH_3	H	H	OCH_3	H	2.57
fisetin tetramethylether	OCH_3	H	H	OCH_3	H	OCH_3	OCH_3	H	1.89
luteolin tetramethylether	H	OCH_3	H	OCH_3	H	OCH_3	OCH_3	H	3.28
7,3',4'-trimethoxyflavone	H	H	H	OCH_3	H	OCH_3	OCH_3	H	3.25
galangin trimethylether	OCH_3	OCH_3	H	OCH_3	H	H	H	H	2.02
baicalein trimethylether	H	OCH_3	OCH_3	OCH_3	H	H	H	H	2.69
chrysin dimethylether	H	OCH_3	H	OCH_3	H	H	H	H	2.82
3',4'-dimethoxyflavone	H	H	H	H	H	OCH_3	OCH_3	H	3.16
3-methoxyflavone	OCH_3	H	H	H	H	H	H	H	3.09
flavone	H	H	H	H	H	H	H	H	3.48

Fig. 1. Structures of flavone and methoxyflavones investigated in the present study. *Clog P* Calculated logarithm of octanol/water partition coefficient calculated by using Chem Ultra 10.0 program (CambridgeSoft MA).

1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum, 100 μg/ml gentamicin (Schering-Plough, Osaka, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. K562/ADM cells were cultured in the presence of 300 ng/ml adriamycin (Kyowa Hakko, Tokyo, Japan) once every 2 weeks.

Uptake Study

Uptake experiments were performed as described in a previous report (5). Briefly, K562 cells and K562/ADM cells were seeded at 1×10^6 cells on four-well multidishes (Nunc, Roskilde, Denmark) and were used after 2 h for uptake study. Uptake was initiated by adding growth medium containing 25 nM [^{3}H]vincristine in the presence or absence of 20 μM verapamil, cyclosporine A or MFs, and incubation was continued for 2 h at 37°C. The cells were then washed twice with 1 ml of ice-cold phosphate-buffered saline (PBS) to stop the uptake, dissolved in 1 M NaOH (200 μl) and neutralized with 1 M HCl (200 μl). To assay the radio-labeled compounds, all samples (200 μl) were transferred into 4 ml-plastic counting vials (Pony Push-ON-economY Vial; PerkinElimer Japan, Yokohama, Japan), mixed with 4 ml of scintillation fluid (Clearsol I; Nacalai Tesque, Kyoto, Japan), and measured with a liquid scintillation counter (LS6500; Beckman Instruments, Fullerton, CA). The amounts of protein in K562 and K562/ADM cells used in uptake studies were measured by the method of Lowry *et al.* (12). The uptake of [^{3}H]vincristine was expressed as cell/medium (C/M) ratio (μl/mg protein), i.e. the ratio of uptake amount into cells (pmol/mg protein) to the concentration of [^{3}H]vincristine in the medium (pmol/μl). Relative potency (%) for potentiation of the uptake of [^{3}H]vincristine was calculated by use of the following Eq. 1.

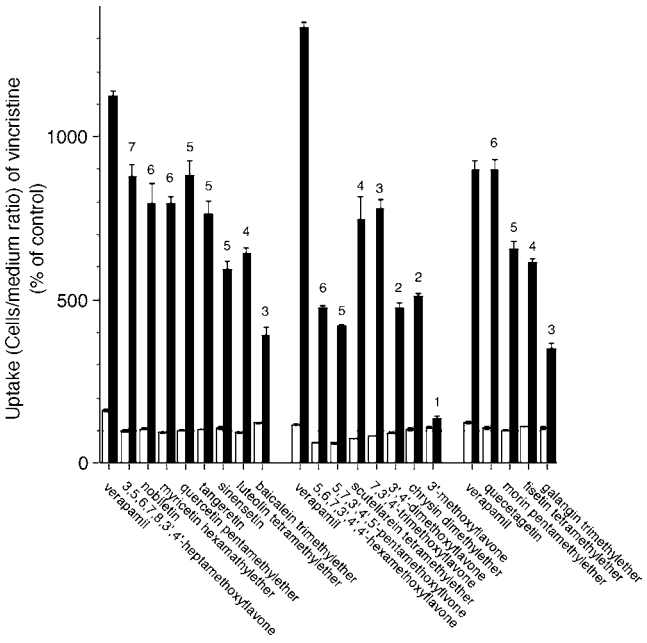


Fig. 2. The effects of 20 μM methoxyflavones, flavone and verapamil on the uptake of [^{3}H]vincristine by K562 (*open column*) and K562/ADM cells (*closed column*) in 2 h. Relative uptakes to that in the absence of test compounds are shown. Each column represents the mean±SEM from four experiments. The *number on the top of each column* represents the number of methoxyl moieties in the compound.

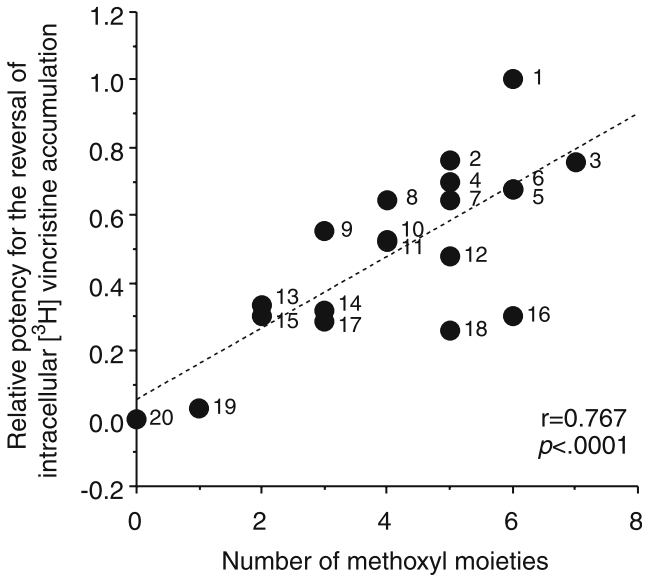


Fig. 3. Relationship between the number of methoxyl moieties and relative potency for the reversal of multidrug resistance, as assessed in terms of increase of [^{3}H]vincristine uptake into K562/ADM cells. Each *point* represents the mean potency (n=4) of respective compound and is shown as the ratio to that of verapamil. (*1* quercetagetin, *2* quercetin pentamethylether, *3* heptamethoxyflavone, *4* morin pentamethylether, *5* nobiletin, *6* myricetin hexamethylether, *7* tangeretin, *8* fisetin tetramethylether, *9* 7,3′,4′-trimethoxyflavone, *10* luteolin tetramethylether, *11* scutellarein tetramethylether, *12* sinensetin, *13* chrysin dimethylether, *14* galangin trimethylether, *15* 3′,4′-dimethoxyflavone, *16* 5,6,7,3′,4′,5′-hexamethoxyflavone, *17* baicalein trimethylether, *18* 5,7,3′,4′,5′-pentamethoxyflavone, *19* 3-methoxyflavone, *20* flavone) SDs are omitted for clarity.

$$\text{Relative potency} = \frac{(C/M \text{ ratio in the presence of test drug})/(C/M \text{ ratio in the absence of test drug})}{(C/M \text{ ratio in the presence of verapamil})/(C/M \text{ ratio in the absence of verapamil})} \quad (1)$$

WST-1 Assay

Growth inhibition was evaluated with the WST-1 method, which is a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (13). K562 and K562/ADM cells were seeded in 96-well plates (5×10^4 cells/ml, 100 μl) and cultured for 24 h at 37°C. Then the cells were treated with 20 μM test compound in the presence of 1 μM vincristine for 48 h. Cell cultures were spiked with 10 μl of WST-1 solution (containing 3.3 mg/ml WST-1 and 70 μg/ml 1-methoxy-5-methylphenazinium methylsulfate) and incubated for 2 h at 37°C. The absorbance was measured with a microplate reader (Bio-Rad Laboratories, CA, USA) at wavelengths of 415 nm (test) and 630 nm (reference).

Statistics

Data were expressed as the mean±SEM. The number of sample ('n') represents the number of individual wells

investigated. The significance of differences of the mean values between groups was evaluated with Student's *t* test or ANOVA, followed by Dunnett's test, and a *P* value of less than 0.05 was considered to represent statistical significance. The relationship between the relative potency for the potentiation of vincristine uptake and the numbers of methoxy moieties was analyzed by linear regression. The effects of the number and position of methoxy moieties on the potentiation of vincristine uptake were analyzed by multiple factor analysis based on Hayashi's quantification theory type I (14). Hayashi's quantification is one of multivariate analyses and can deal with category data as explanatory variables instead of quantitative measure. Statistical significance of the correlation was determined by calculating the 95% confidential interval of population correlation coefficient.

RESULTS

The Effects of MFs on the Uptake of [^{3}H]Vincristine into K562 and K562/ADM Cells

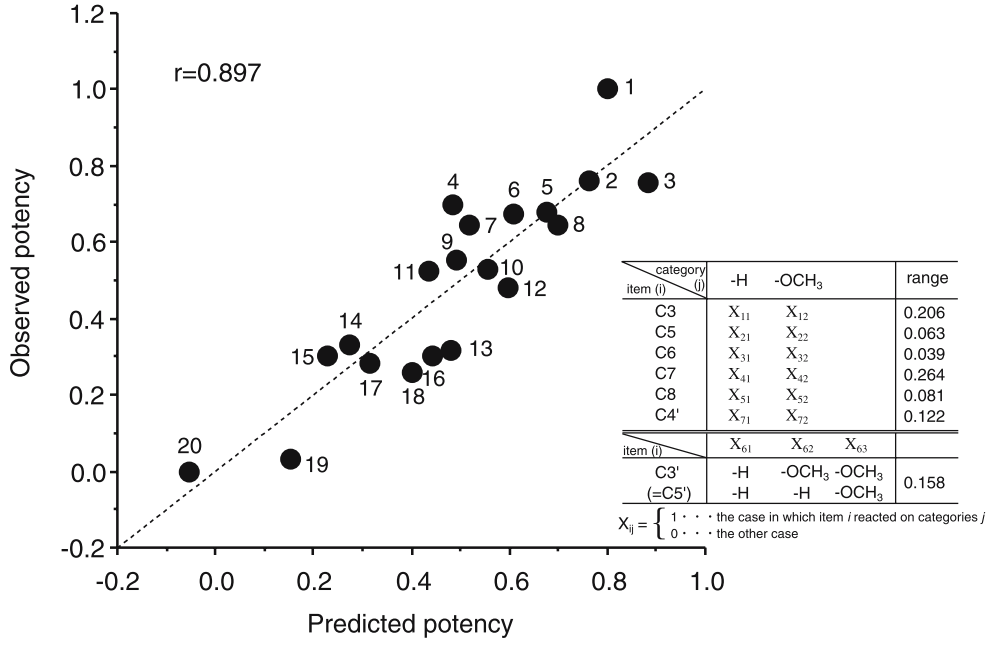
The uptake of [^{3}H]vincristine into K562 and K562/ADM cells in the absence of drugs amounted to 101.7±3.1 and 5.5±0.7 μl/mg protein (mean±SEM, n=16), respectively. The effects of 20 μM verapamil, flavone and MFs on the uptake of [^{3}H]vincristine are shown in Fig. 2. While the uptakes into parental K562 cells were essentially unaffected, those into K562/ADM cells were significantly increased by verapamil and all the MFs tested (Fig. 2). The effects of 3,5,6,7,8,3′,4′-heptamethoxyflavone, nobiletin, tangeretin, quercetagetin and quercetin pentamethylether were potent and comparable to that of verapamil.

Relationship Between Potentiation of Uptake and the Number of Methoxyl Moieties

Figure 3 shows the relationship between the number of methoxyl moieties and the relative potency for the reversal of multidrug resistance as assessed in terms of the increase in [^{3}H]vincristine uptake into K562/ADM cells. There was a statistically significant correlation with a correlation coefficient of 0.767 (p<0.0001).

Relationship Between the Number and Position of Methoxyl Moieties, and Potentiation of Uptake

Figure 4 shows the influence of the number and position of methoxyl moieties on the potentiation of [^{3}H]vincristine



Predicted potency = {488.6 - 82.6X_{11}+123.9X_{12}-47.2X_{21}+15.7X_{22}-15.7X_{31}+23.57X_{32}-224.8X_{41}+ 39.7X_{42}-12.2X_{51}+68.9X_{52}-72.1X_{61}+86.3X_{62}-68.0X_{63}-86.0X_{71}+36.9X_{72}} / 1000

Fig. 4. Predictability of the relative potency for the reversal of multidrug resistance by multiple factor analysis based on quantification theory type I. The observed relative potency of each drug was plotted against the predicted one, which was calculated by the equation shown on the bottom of the figure. In the equation, category scores of dummy variables (*Xij*) were shown as their respective factors. Inset shows the definition of dummy variable (*Xij*) for each category (*j*) and item (*i*) along with the range of category score. Each *point* represents the mean potency (n=4) of respective compound and is shown as the ratio to that of verapamil (*1* quercetagetin, *2* quercetin pentamethylether, *3* heptamethoxyflavone, *4* morin pentamethylether, *5* nobiletin, *6* myricetin hexamethylether, *7* tangeretin, *8* fisetin tetramethylether, *9* 7,3′,4′-trimethoxyflavone, *10* luteolin tetramethylether, *11* scutellarein tetramethylether, *12* sinensetin, *13* chrysin dimethylether, *14* galangin trimethylether, *15* 3′,4′-dimethoxyflavone, *16* 5,6,7,3′,4′,5′-hexamethoxyflavone, *17* baicalein trimethylether, *18* 5,7,3′,4′,5′-pentamethoxyflavone, *19* 3-methoxyflavone, *20* flavone) SDs are omitted for clarity.

Table I. The Effects of 20 μM Methoxyflavones and Verapamil on the Growth of K562/ADM Cells Treated with 1 μM Vincristine for 48 h as Assessed by using WST-1 Assay

Compound	Growth (%)[a]
Control (*n*=22)	85.3±3.5
Verapamil	42.3±18.2[b]
Quercetagetin	79.4±7.3
Quercetin pentamethoxylether	59.8±9.9
3,5,6,7,8,3′,4′-Heptamethoxyflavone	37.9±6.5[b]
Morin pentamethylether	82.8±10.8
Nobiletin	25.3±4.0[b]
Myricetin hexamethylether	71.9±12.1
Tangeretin	24.7±1.4[b]
Fisetin tetramethylether	102.5±15.1
7,3′,4′-Trimethoxyflavone	89.2±5.4
Luteolin tetramethylether	68.6±10.2
Scutellarein tetramethylether	91.2±9.3
Sinensetin	84.5±6.4
Chrysin dimethylether	96.1±12.9
Galangin trimethylether	86.6±10.1
3′,4′-Dimethoxyflavone	65.3±8.8
5,6,7,3′,4′,4′-Hexamethoxyflavone	55.6±11.9
Baicalein trimethylether	99.6±27.2
5,7,3′,4′,5′-Pentamethoxyflavone	96.2±8.2
3-Methoxyflavone	95.8±9.6
Flavone	111.1±8.9

Each value indicates the mean±SEM from six experiments if not indicated otherwise.
[a] Ratio of cell growth in the presence of both vincristine and test drug to that in the absence of vincristine
[b] $p<0.01$ vs control by ANOVA followed by Dunnett's test (results of ANOVA, $F=5.47$, $p<0.0001$)

uptake analyzed by multiple factor analysis. A methoxyl moiety at the C3 or C7 position appeared to have the greatest influence. On the other hand, the presence of methoxyl moieties at both C3′ and C5′ seemed to decrease the potency.

Potentiation of Vincristine-Induced Growth Inhibition of K562/ADM Cell by MFs

The effects of 20 μM verapamil and MFs on the growth of K562/ADM cells in the presence of vincristine were assessed by using WST-1 assay, and the results are summarized in Table I. Heptamethoxyflavone, nobiletin and tangeretin (20 μM) potentiated the growth inhibition of K562/ADM cells by 1 μM [^{3}H]vincristine as well as 20 μM verapamil. Figure 5 shows the concentration dependency of the growth inhibition of parental K562 and K562/ADM cells by vincristine and its potentiation by 2 and 20 μM heptamethoxyflavone, nobiletin, tangeretin, verapamil and cyclosporine A. The inhibitory curve of vincristine for cell growth was shifted leftward by three methoxyflavones, verapamil and cyclosporine A in a concentration-dependent manner.

Relationship Between the Increase in [^{3}H]Vincristine Uptake and Growth Inhibition

There was a significant correlation between the relative potencies for increase of [^{3}H]vincristine uptake and for growth inhibition as assessed by WST-1 assay (Fig. 6, $p<0.05$)

DISCUSSION

We have previously identified three MFs, tangeretin, nobiletin and heptamethoxyflavone, from orange juice as *P*-gp inhibitors; they are as potent as verapamil and cyclosporin A (4,5), while *P*-gp inhibitory effects of grapefruit juice are considered to be attributable to furanocoumarin derivatives such as dihydroxybergamottin (15). Choi *et al.* (6,7) have investigated the cytotoxic profiles of eight MFs and potentiation of rhodamine accumulation by six MFs in a *P*-gp-overexpressing cell line (AML-2/D100 cells). It appeared that inhibitory potency for *P*-gp tends to increase with an increase in the number of methoxyl moieties. Choi *et al.* have calculated the 'chemosensitizing index' of MFs by comparing the cytotoxicities of MFs in the presence and absence of vincristine. Contrarily, we compared the cytotoxicity of vincristine in the presence and absence of each MF, in order to assess MDR reversing effect of each compound. The reports of Choi *et al.* dealt already with eight MFs. However, the MDR-reversing effects of MFs and the structure-activity relationship of these compounds remain to be further investigated. Therefore, in the present study, we investigated the interaction of 19 natural and semisynthetic MFs with *P*-gp to further clarify the relationship between structure and inhibitory potency, in order to assess the feasibility of using MFs as MDR-reversing agents.

The uptake of [^{3}H]vincristine into K562/ADM cells was significantly increased in the presence of MFs (Fig. 2), and 3,5,6,7,8,3′,4′-heptamethoxyflavone, nobiletin, tangeretin, quercetagetin and quercetin pentamethylether exhibited potent inhibition of *P*-gp. As the potency of verapamil was different between three experiments (Fig. 2), we introduced 'relative potency' corrected by the potency of verapamil to normalize the effects of MFs. Although the reason for the between-day variations remains uncertain, it does not always imply the lack of reproducibility for *P*-gp function in K562/ADM cells because the uptake and the effects of verapamil in parental K562 cells also vary considerably.

We have previously shown that an ethyl acetate extract of orange juice inhibits *P*-gp function (4). The concentrations of tangeretin, nobiletin, quercetagetin and heptamethoxyflavone in orange juice have been reported to be 0.26, 1.38, 0.27 and 0.50 mg/L, respectively (16). Indeed, we succeeded in isolating tangeretin, nobiletin and heptamethoxyflavone as *P*-gp inhibitors (4). Although we did not isolate quercetagetin from the juice as a *P*-gp inhibitor, we did note that the inhibitory potency of orange juice extract could not be fully explained by the potencies and putative contents of tangeretin, nobiletin and heptamethoxyflavone alone (4). Other MFs with potent *P*-gp-inhibitory effects, such as quercetagetin, probably contribute to the inhibitory effects of orange juice on *P*-gp.

Besides *P*-gp, another transporter RLIP76 has been shown to transport vincristine and to be induced by adriamycin (17). While we did not investigated the expression of RLIP76 in K562/ADM cells, further study is required to clarify whether RLIP76 contributes significantly to the resistance of K562/ADM cells to vincristine.

As shown in Fig. 3, the *P*-gp-inhibitory potency of MFs, as assessed in terms of enhanced uptake of [^{3}H]vincristine into K562/ADM cells, increased in proportion to the number of methoxyl moieties ($r=0.767$, $p<0.0001$). Flavone, which lacks

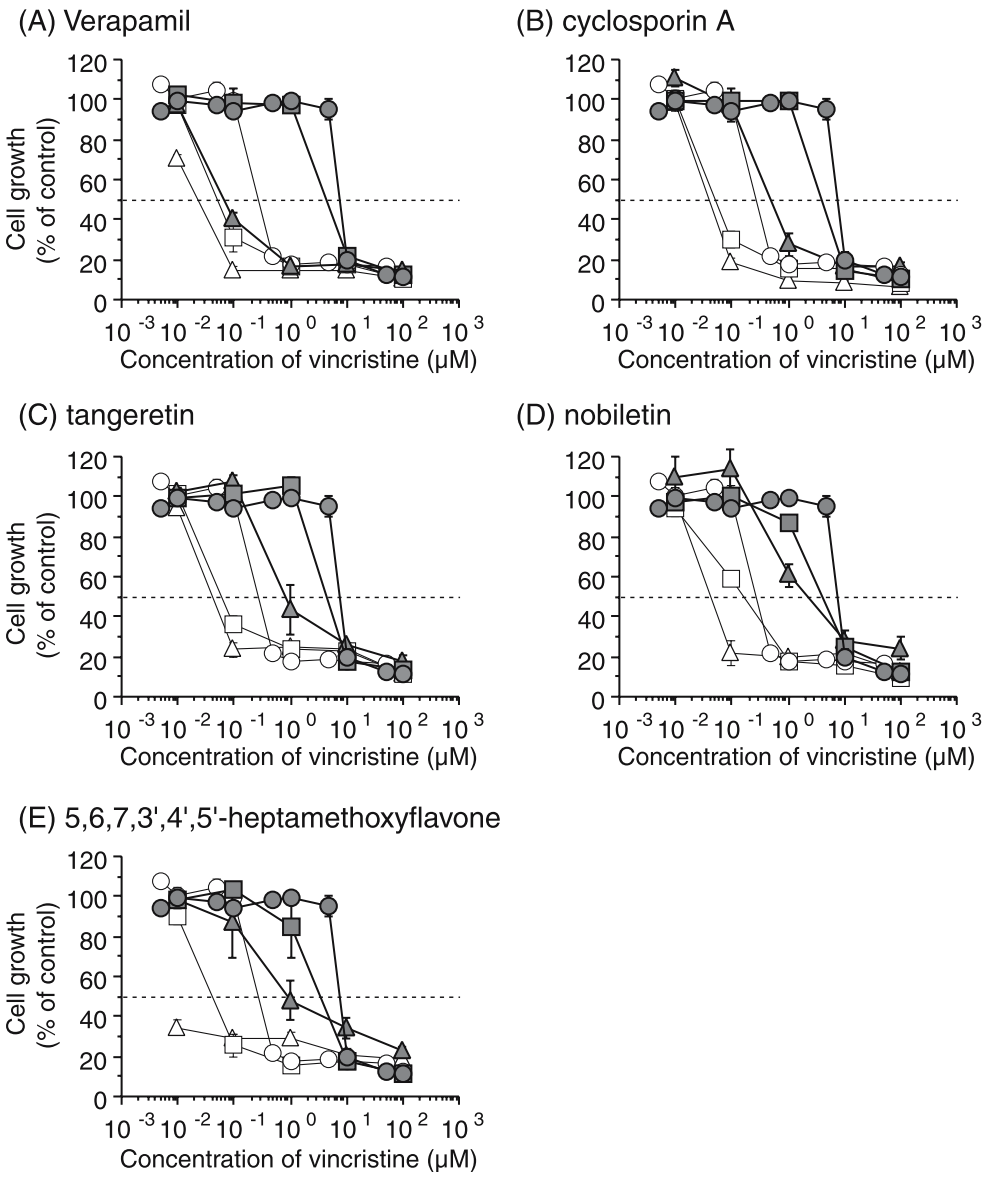


Fig. 5. Effects of verapamil (**A**), cyclosporine A (**B**), tangeretin (**C**), nobiletin (**D**) and 5,6,7,3′,4′,5′-heptamethoxyflavone (**E**) on the growth inhibition of vincristine in K562 (*open symbols*) and K562/ADM (*filled symbols*) cells. K562 and K562/ADM cells were treated with vincristine at the indicated concentrations in the presence or absence of verapamil, cyclosporine A, tangeretin, nobiletin and heptamethoxyflavone. Concentrations of each compound were 0 (*circle*), 2 (*square*) or 20 μM (*triangle*). Each point represents the mean±SEM from four experiments.

a methoxyl moiety, did not inhibit *P*-gp. These results are consistent with the findings of Choi *et al.* (6,7). Besides flavone derivatives, various other *P*-gp inhibitors bearing methoxy moieties have been reported, i.e., verapamil, dilazep, diltiazem, FK-506, reserpine analogs, cepharanthine, quinidine, NA-382, GF120918, and so on (3,18–25). Compounds that interact with *P*-gp are expected to include hydrophobic drugs with two or more planar aromatic rings and a tertiary amine carrying a positive charge at physiological pH (26). On the other hand, Ferté *et al.* (27) have investigated in detail the inhibitory potencies of flavonoid derivatives, *N*-benzylpiperazinyl flavones, with or without a methoxyl moiety, and they reported that lipophilicity is not the only determinant of *P*-gp inhibition. Our present results suggest that various MFs devoid of amine can also interact potently with *P*-gp, and the number of methoxyl moieties is one of the determinants of *P*-gp-inhibitory potency. However, the effect of the number of methoxyl moieties is observed only within nineteen structurally similar compounds, so that it remains to be investigated whether this relationship holds true for compounds structurally unrelated to flavonoids.

With regard to the drug binding sites on *P*-gp, Shapiro *et al.* (28) have suggested the existence of H and R sites that are

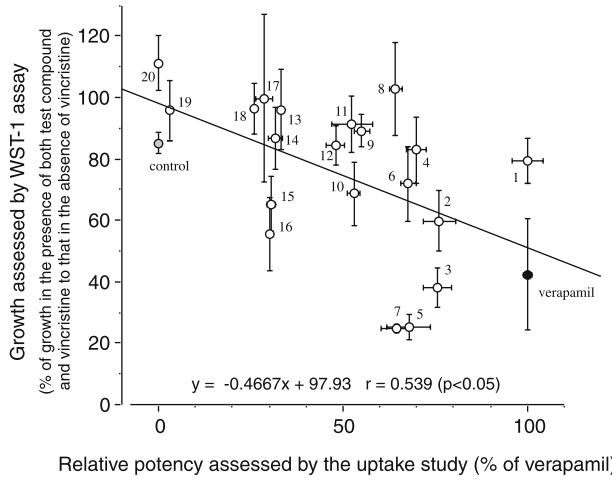


Fig. 6. Relationship between relative potency for the reversal of multidrug resistance as assessed by the increase in [^{3}H]vincristine uptake and growth inhibition as assessed by WST-1 assay. Each *point*

capable of drug transport and another allosteric P site devoid of transport activity, to which steroids (e.g. progesterone) bind. Conseil *et al.* (29) investigated the binding site of flavonoids on *P*-gp and demonstrated that flavonoids bind to vicinal ATP- and steroid-binding sites. Choi *et al.* (7) have implies that pentamethoxyflavone is not transported by *P*-gp. Thus, methoxyflavone is considered to inhibit allosterically the function of *P*-gp. The structure-activity relationship of the inhibition of methoxyflavone on the transport of *P*-gp substrates other than vincristine may possibly differ from the present observation and remains to be investigated.

It is generally agreed that lipophilicity is one of the important factors to determine the inhibitory potency on *P*-gp (30,31). However, *P*-gp inhibition was potentiated with an increase in the number of methoxyl moieties in this study, although substitution of the hydrogen atom with a methoxyl group is considered to lead to a decrease in lipophilicity (Fig. 1). Therefore, with regard to methoxyflavones, lipophilicity is not the primary determinant factor of *P*-gp-inhibitory potency. The *P*-gp-inhibitory effects of 5,7,3′,4′,5′-pentamethoxyflavone and 5,6,7,3′,4′,5′-hexamethoxyflavone were less potent than would be expected from the numbers of methoxyl moieties. Therefore, not only the number of methoxyl moieties, but also their positions may be important. To investigate further the structure-activity relationship, we employed multiple factor analysis based on Hayashi's quantification theory type I (13). The observed relative potencies were well regressed by multiple factor analysis (Fig. 4), and the results suggested that methoxyl substitution does not always lead to an increase of inhibitory potency. Although the effect of methoxyl substitution position on *P*-gp-inhibitory potency were in the rank order of C3′ (=C5′) > C7 > C3 ≫ C5 > C4′ > C6 > C8, the potency was decreased when both C3′ and C5′ were substituted. Indeed, heptamethoxyflavone and quercetagetin, which meet these criteria for potent *P*-gp inhibitors, were as potent as verapamil, a well-known *P*-gp inhibitor. These MFs may be candidates for MDR-reversing agents.

We have previously shown that orange juice-derived MFs, heptamethoxyflavone, nobiletin and tangeretin, do not inhibit CYP3A4 (4). Although we did not investigate the CYP3A4-inhibitory effects of the other 16 MFs, it seems unlikely that they potently inhibit CYP3A4. Nevertheless, the inhibitory effects of these MFs on CYP3A4 should be investigated if they are to find clinical application. On the other hand, we have recently reported that an ethyl acetate extract of orange juice, as well as its components heptamethoxyflavone, nobiletin and tangeretin, inhibits the multidrug resistance associated protein 2(MRP2)-mediated transport of saquinavir (8). The inhibitory effects of MFs on MRP2 and other ABC transporters should also be further investigated.

We evaluated the MDR-reversing effects of MFs by growth inhibition assay using the WST-1 assay. Growth inhibition was significantly correlated with the *P*-gp-inhibitory potency assessed in terms of [^{3}H]vincristine uptake. However, quercetagetin, which most potently enhanced the uptake of [^{3}H]vincristine into K562/ADM cells, failed to exhibit the most potent growth inhibition in WST-1 assay. On the other hand, heptamethoxyflavone, nobiletin and tangeretin potentiated cell growth in the presence of vincristine as potently as verapamil, although their effects on the uptake of [^{3}H]vincristine were weaker than that of verapamil. The reason for this discrepancy remains unclear, but it is possible that heptamethoxyflavone, nobiletin and tangeretin have some other effect that is independent of *P*-gp inhibition.

We investigated the potentiation of uptake and cell growth inhibition by MFs at 20 μM in the present study. At lower concentration, some MFs may be potent but others may be impotent to reverse MDR. We have previously investigated the concentration-dependency of the potentiation of vincristine accumulation by heptamethoxyflavone, nobiletin and tangeretin at concentrations from 0.1 to 20 μM and found that all these MFs at 0.2 μM significantly potentiated vincristine accumulation (5). In the present study, we have demonstrated that the growth inhibition curve by vincristine was shifted leftward by these MFs at 2 μM (Fig. 5). Taken together, these MFs may be candidates for clinical application as MDR-reversing agents.

In the present study, we assessed the effects of MFs on *P*-gp by measuring the potentiation of cellular accumulation and growth inhibition. These measures are not suitable for estimating the inhibitory constant (Ki values) or inhibitory manners of MFs. Analysis of the kinetics of ATP-dependent uptake of *P*-gp substrate into *P*-gp-expressing inside-out vesicles may be necessary to definitely estimate the inhibitory manner and inhibitory constant of MFs for *P*-gp.

In conclusion, MFs increased the uptake of vincristine into MDR cells and exhibited MDR-reversing effects. Their potency was influenced by the number and positions of their methoxyl moieties. As MFs are contained in a wide range of foods, beverages and supplements, they may be candidates for clinical application as MDR-reversing agents.

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