**Original article**

**SCREENING OF FLAVONOIDS FOR THEIR POTENTIAL INHIBITORY EFFECT ON P-GLYCOPROTEIN ACTIVITY IN HUMAN CERVICAL CARCINOMA KB CELLS**  

Orawan Khantamat, M.S., Wittaya Chaiwangyen, M.S., Porn-ngarm Limtrakul, Ph.D.  

*Department of Biochemistry, Faculty of Medicine, Chiang Mai University*

**Abstract** The 170 kDa plasma membrane, P-glycoprotein (Pgp), causes the efflux of chemotherapeutic drugs from cells and is believed to be an important mechanism in multidrug resistance (MDR) in human cancer. In this study, some well-known flavonoids from vegetables and fruit were tested for their potential ability to modulate the function of Pgp in the multidrug-resistant human cervical carcinoma cell line, KB-V1. The data demonstrated that kaempferol and daidzein stimulated vinblastine sensitivity of KB-V1 cells ($P<0.05$) and revealed that the inhibitory concentration at 50% growth ($IC_{50}$) of vinblastine was decreased markedly in the presence of these flavonoids in a dose dependent manner. These flavonoids did not affect in wild type KB-3-1 cells, which lack Pgp. Kaempferol also increased the intracellular accumulation, and reduced the efflux of rhodamine 123 (Rh123), which is known to be a good substrate of Pgp in KB-V1 cells. These findings provide evidence that the flavonoid, i.e. kaempferol, could reverse the vinblastine resistant phenotype by inhibiting Pgp activity in KB-V1 cells, and the ability to affect the Pgp activity could be of relevance to the chemosensitization of this flavonoid towards anticancer drugs.  

**Keywords**: Flavonoids, KB-V1 cells, P-glycoprotein, Multidrug resistance, Vinblastine, Rhodamine123

Resistance of cancer cells to multiple chemotherapeutic drugs (a mechanism termed MDR) is a major obstacle to the success of cancer chemotherapy and has been closely associated with treatment failure. One of the most studied mechanisms of drug resistance is characterized by a decrease in drug accumulation resulting from over-expression of the 170 kDa plasma membrane, P-glycoprotein (Pgp). The available evidence strongly suggests that Pgp is a pump that catalyzes the efflux of drugs from the cells, reducing drug accumulation and
hence the access of cytotoxic drugs to their targets.\textsuperscript{(1-3)} Since Pgp can confer MDR on cancer cells, the development of agents, which inhibit the Pgp-mediated efflux of drugs and thus reverse MDR, has been intensively pursued.

Flavonoids, 2-phenyl-benzo-\(\alpha\)-pyrones, are polyphenolic compounds that occur ubiquitously in food of plant origin.\textsuperscript{(4)} These substances are composed of a common phenylethromalonone structure (C6-C3-C6), with one or more hydroxyl substituents. A multitude of substitution patterns in the two benzene rings of the basic structure occur in nature. Variations in their heterocyclic ring give rise to flavones, flavonols, isoflavones, flavanols, flavanones, and anthocyanidins.

As flavonoids are abundant in plant food (Table 1) and have the advantage of being natural dietary compounds that are nontoxic in animals, this study considered whether well-known flavonoids from vegetables and fruit such as quercetin, kaempferol, genistein, genistin and daidzein could influence Pgp function \textit{in vitro}. Many reports have been published concerning the Pgp modulation of flavonoids, but the results of these compounds on Pgp modulation from various groups are controversial and complicated.\textsuperscript{(5-11)} Each result depended on the MDR cell models and the chemotherapeutic drugs used in the experiments. In this study, the flavonoids were tested for their ability to modulate Pgp function in the multidrug resistant human cervical carcinoma cell line, KB-V1 compared with the drug sensitive cell line, KB-3-1, which lacks Pgp.

To examine the effect of flavonoids on Pgp activity, the experiments were evaluated in two ways: 1) their ability to potentiate anticancer drugs (vinblastine and doxorubicin) cytotoxicity on KB-V1 cells; and 2) their ability to restore the intracellular accumulation of rhodamine 123 (Rh123) in KB-V1 cells. In this report we demonstrate, for the first time, that flavonoid, i.e. kaempferol which inhibits Pgp-mediated drug efflux, result in an increase in the intracellular

<table>
<thead>
<tr>
<th>Class</th>
<th>Flavonoids</th>
<th>Food sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavones</td>
<td>Apigenin, Luteolin</td>
<td>Celery, parsley, thyme</td>
</tr>
<tr>
<td>Flavonols</td>
<td>Quercetin, Kaempferol, Myricetin</td>
<td>Onions, lettuce, broccoli, tomato, berries, olive oil, apple peels, tea, red wine, Kale, leek, broccoli, endives, grapefruit, black tea, Cranberry grapes, red wine</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Genistein, Genistin, Daidzein</td>
<td>Soy beans, legumes</td>
</tr>
<tr>
<td>Flavanols</td>
<td>Catechin, Epicatechin</td>
<td>Tea (camellia sinensis)</td>
</tr>
<tr>
<td>Flavanones</td>
<td>Hesperetin, Naringenin</td>
<td>Tea</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>Cyanidin</td>
<td>Berries, cherries, grapes</td>
</tr>
</tbody>
</table>

Table 1. Classification of flavonoids and their food sources.
accumulation and cytotoxicity of chemotherapeutic drugs in drug-resistant human cervical carcinoma cells in vitro.

Materials and methods

Chemicals

Quercetin, kaempferol, genistein, daidzein, genistin, verapamil, vinblastine, doxorubicin, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Rhodamine 123 (Rh123), HRP-conjugated goat anti-mouse IgG and mouse monoclonal anti-Pgp (MDR) clone F4 were obtained from Sigma Chemical Company (St Louis, Missouri, U.S.A). Dulbecco’s Modified Eagle’s Medium (DMEM) and Hanks’ balanced salt solution (HBSS) without phenol red were purchased from Gibco BRL (Grand Island, NY, U.S.A). A SuperSignal® detection kit was purchased from Pierce.

Cells and cell culture

A multidrug resistant cervical carcinoma cell line (KB-V1) and a drug sensitive cervical carcinoma cell line (KB-3-1) were generous gifts from Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, MD, USA). Both cell lines were cultured in DMEM with 4.5 g of glucose/L plus 10% fetal calf serum (FCS), 5 mM of L-glutamine, 50 units/mL of penicillin and 50 µg/mL of streptomycin; 1 µg/mL of vinblastine was added only to the KB-V1 culture medium. These two cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO2 at 37°C. When the cells reached confluency, they were harvested and plated for either consequent passages or drug treatment.

Measurement of cell growth and viability by MTT assay

The effects of flavonoids on cell growth were measured by means of the MTT colorimetric assay performed in 96-well plates. Briefly, KB-V1 and KB-3-1 cells, plated out at 2.0 x 10^3 cells in 100 µL of culture medium, were inoculated into each well. After 24-h incubation, 100 µL of various flavonoid concentrations were added and the plates were incubated for 48 h. Thereafter, 100 µL of condition medium were removed and 20 µL of MTT stock dye solution (5 mg/mL in phosphate buffered saline, PBS pH 7.4) were added to each well. The plates were incubated for a further 4 h. The resulting formazan was dissolved in 200 µL of dimethyl sulfoxide (DMSO) after aspiration of the medium. The plates were shaken mechanically for 10 min and read immediately at 540 nm, with a reference wavelength of 630 nm using an ELISA microplate reader. The fractional absorbance was calculated by the following formula: % Cell survival = (mean absorbance in test wells)/(mean absorbance in control wells) x 100.

Chemosensitivity testing

The effect of flavonoids on the sensitivity of the KB-V1 cells to the anticancer drugs, vinblastine and doxorubicin; was determined using the MTT microtiter plate assay as described for cell survival measurement. Non toxic doses (>80% cell survival) of flavonoids were used in this study. The cells were treated
with a low (10 µM) and high (30 µM) concentration of flavonoids in combination with several concentrations of anti-cancer drugs. Within each experiment, determinations were carried out in triplicate. Relative resistance was calculated as the ratio of inhibitory concentration at 50% growth (IC₅₀) value of the KB-V1 cells to the IC₅₀ value of the KB-3-1 cells.

**Rh123 accumulation**

Rh123 accumulation was performed as previously described. Briefly, KB-V1 cells (5x10⁵ cells per sample) were incubated with 1 µg/mL of Rh123 in the dark at 37°C in 5% CO₂ for 60 min. Flavonoids (dissolved in DMSO) were added to cell cultures at the same time as Rh123. The final concentration of 0.4% DMSO (v/v) was used for all experiments and controls. Following Rh123 accumulation, the cells were washed twice with ice-cold HBSS, then placed in HBSS with 10% FCS on wet ice and analyzed on a FACScan flow cytometer (Becton-Dickinson, Sydney, Australia) equipped with a 488-nm argon laser. The green fluorescence of Rh123 was measured by a 530 nm band-pass filter (hLi). Samples were gated on forward and side scatter to exclude debris and clumps. A minimum of 10,000 events was collected for each sample.

**Rh123 efflux**

For determination of Rh123 efflux, cells were loaded with Rh123 in the presence of flavonoids for 60 min. Then the cells were washed with ice-cold HBSS and placed in Rh123-free medium containing flavonoids, verapamil (positive MDR reversal agent), or the vehicle, DMSO. Following efflux intervals of 30 min, the medium was removed and the cells were washed twice with ice-cold HBSS and prepared for the Flow cytometer, as described in Rh123 accumulation.

**Western blot analysis**

For Western blotting, plasma membrane from KB cells were prepared according to Anuchapreeda et al. The amount of protein was measured by the Folin-Lowry method using bovine serum albumin (BSA) as a standard. The plasma membrane proteins (20 µg/lane) were separated on a 7.5% SDS-polyacrylamide gel and immunoblotted overnight onto nitrocellulose filters (GIBCO-BRL). The filters were incubated sequentially with mouse monoclonal anti-Pgp clone F4 at a 1:5,000 dilution, and HRP-conjugated goat anti-mouse IgG at a 1:20,000 dilution. Proteins were visualized by the SuperSignal® protein detection kit and quantitated by scanning densitometry.

**Statistical analysis**

Data were the mean±SD from duplicate or triplicate samples of three independent experiments. Differences between the mean were analyzed by one-way analysis of variance. Statistical significance was considered when p was < 0.05.

**Results**

**The expression of Pgp/MDR1 on KB cells**

The study of Pgp expression in KB-3-1
Reversal of MDR by flavonoids and KB-V1 cells by Western blot analysis showed that a Pgp-170 kDa immunoblottable amount expressed significantly in KB-V1 cells that were maintained in both 0.5 and 1 µg/mL of vinblastine, and the expression level of Pgp correlated well with the elevated concentration of the drug. KB-3-1 cells did not express Pgp at a level detectable by the method used in this experiment (Figure 1).

**Effect of flavonoids on the viability of KB cells**

Using the MTT assay, the cytotoxicity of flavonoids on KB-V1 and KB-3-1 cells are shown in Figure 2 (A). The data indicated that the cytotoxicity of flavonoids on both drug-resistant KB-V1 and drug-sensitive KB-3-1 cells were dose-dependent. The flavonoids, i.e. quercetin, kaempferol, genistein, genistin and daidzein are similarly non-toxic to the parental and resistant cells (IC$_{50}$ > 200 µM). The inhibitory concentration at 20% growth (IC$_{20}$) and IC$_{50}$ values of the flavonoids on cell viability are summarized in Figure 2 (B).

**Effect of flavonoids on anticancer drug cytotoxicity**

Two widely used anticancer drugs, vinblastine and doxorubicin, were chosen to investigate the ability of quercetin, kaempferol, genistein, genistin and daidzein in sensitizing MDR KB-V1 cells to the cytotoxicity effects of anticancer drugs. In initial experiments with the flavonoids alone, it was found that the KB-V1 cells were not resistant to these compounds, which were related to drug-sensitive KB-3-1 cells. Moreover, the IC$_{20}$ for flavonoids was approximately 30 µM and, consequently, this concentration of flavonoids was used in subse-

![Figure 1](image_url)

*Figure 1.* P-glycoprotein expression in the drug sensitive cells, KB 3-1, and drug resistant cells, KB-V1. KB-3-1 cells were cultured in DMEM medium without vinblastine. KB-V1 cells were cultured in DMEM medium with 0.5 or 1 µg/mL of vinblastine. Cells were then grown to 80% confluence in a T-75 cm$^2$ culture flask, harvested by scraping and homogenized. The plasma membrane proteins (20 µg/lane) were separated on a 7.5% SDS-PAGE. The P-glycoprotein was determined by Coomassie blue staining (A) and Western blotting (B) using Mab F4 (Sigma-Aldrich) at a 1:5,000 dilution, HRP conjugated goat anti-mouse IgG at a 1:20,000 dilution and detected by Enhance chemiluminescence (ECL).
quent combination experiments with anticancer drugs. Quercetin, kaempferol, genistein and daidzein at 30 µM increased the sensitivity of KB-V1 cells to vinblastine, and also decreased the relative resistance of cell to this anticancer drugs (Table 2). Both kaempferol and daidzein, at a concentration of 30 µM, had a significant decrease of vinblastine IC50 (p<0.05) in the resistant KB-V1 cells. The reversing effects of these flavonoids were concentration dependent (Figure 3). Quercetin and genistein were found to slightly, but not significantly,
Reversal of MDR by flavonoids

Reversal of MDR by flavonoids 51

reverse the resistance of KB-V1 cells to vinblastine. In contrast, incubation of KB-V1 cells, with increasing amounts of vinblastine, resulted in a decrease of cell growth that was not affected by genistin (data not shown). The flavonoids, at a concentration of 30 µM, had no change in cytotoxic effects on doxorubicin sensitivity of the KB-V1 cells (Table 2). None of the flavonoids had a statistically significant effect on the anticancer drug sensitivity of the wild-type KB-3-1 cells (data not shown).

**Effect of flavonoids on Rh123 accumulation and efflux**

To study the effect of flavonoids on Pgp function, the activity of Pgp was assessed by measuring the intracellular retention of Rh123 fluorescence in MDR KB-V1 cells as compared with wild-type KB-3-1 cells. Quercetin, kaempferol, genistein and daidzein, at the concentration of 10 to 200 µM, showed a tendency to stimulate the accumulation, and reduce the efflux of Rh123 in KB-V1 cells in a dose dependent manner (Figure 4 and 5), but they did neither in KB-3-1 cells, which lack Pgp. Only kaempferol increased \( p<0.05 \) intracellular accumulation significantly, and reduced the efflux of Rh123 in KB-V1 cells. There was no change of intracellular Rh123 after cells were incubated with genistin in both accumulation and efflux studies (data not shown). The flavonoid concentrations (10-200 µM) used in these

![Graphs showing effect of flavonoids on cell survival](image)

**Figure 3.** Effect of flavonoids on vinblastine cytotoxicity in KB-V1 cells. KB-V1 cells were incubated in the presence and absence of flavonoids in combination with vinblastine. The number of viable cells was determined by the MTT assay. None of the flavonoids had a statistically significant effect on anticancer drug sensitivity of the wild-type KB-3-1 cells and these data have been omitted for clarity. Data points represent the mean (± S.D.) of three independent experiments performed in triplicate.
Figure 4. Effect of flavonoids on Rh123 accumulation in KB-V1 and KB-3-1 cells. The amount of intracellular Rh123 fluorescence was determined by flow cytometry. Each point represents the mean value for three independent experiments performed in triplicate. Asterisks denote values that were significantly different from the vehicle control ($P<0.05$).

Figure 5. Effect of flavonoids on Rh123 efflux in KB-V1 and KB-3-1 cells. The amount of intracellular Rh123 fluorescence was determined by flow cytometry. Each point represents the mean value for three independent experiments performed in triplicate. Asterisks denote values that were significantly different from the vehicle control ($P<0.05$).
experiments did not affect cell viability when observed by examining the morphology of cultures with an inverted phase contrast microscope. The positive control, 30 μM of verapamil, was found to effectively increase the intracellular Rh123, with a statistical difference (p <0.05) in both accumulation and efflux studies of KB-V1 cells (data not shown).

Discussion

Most of the acquired resistance of cancer cells to anticancer agents is considered to be related with MDR1 gene expression and the presence of Pgp, which pumps out the intracellular drug.\(^{18}\) KB-V1 cells showed the over-expression of Pgp/MDR1, which is known as a major factor in changing intracellular drug concentration. These cells also showed a high resistance to vinblastine when compared with KB-3-1 cells. The flavonoids, i.e. quercetin, kaempferol, genistein, daidzein and genistin showed the same inhibition of KB-V1 and KB-3-1 cells. Therefore, it could be suggested that these flavonoids might not be substrates for Pgp, and the cell treatment with flavonoids and anticancer drugs did not create cross-resistance in MDR cells.

In this study, the efficiency of flavonoids on the antineoplastic activity of vinblastine from the biochemical modulation approach was evaluated. When combined with 30 μM of kaempferol or daidzein, the anticancer activity of vinblastine on KB-V1 cells was significantly increased (p<0.05), but no reversal effect was found in KB-3-1 cells. Moreover, none of flavonoids had any effect on doxorubicin sensitivity of both KB-V1 and KB-3-1 cells. Vinblastine is known to be a more specific substrate for Pgp than doxorubicin, which indicates that the reversal of MDR phenotype in KB-V1 cells by these flavonoids might correlate with Pgp, the drug efflux pump.

The intracellular drug target of vinblastine and doxorubicin are different. Drug mechanistic action of vinblastine is in the cytoplasmic level of cells by inhibition of tubulin formation,\(^{(19-21)}\) whereas doxorubicin acts at the DNA level in the cell nucleus.\(^{(22)}\) Therefore, inhibition of the Pgp efflux pump by kaempferol and daidzein might not interfere with the nuclear membrane transport of doxorubicin. It is likely that these flavonoids could inhibit drug efflux from the cytosol via the Pgp molecule, which is located on the cell plasma membrane. Doxorubicin could also be a Pgp substrate as well as other multidrug related proteins, however, it is premature to explain from our data why the flavonoids had no effect on doxorubicin sensitivity of KB-V1 cells.

In studying of the effect of flavonoids on Pgp-mediated Rh123 transport, the experiments revealed that only kaempferol had a significantly increased intracellular accumulation and reduced efflux of Rh123 in KB-V1 cells (p<0.05), but not in KB-3-1 cells, which lack Pgp. It appears that the induced Rh123 accumulation in response to kaempferol is due to acceleration of Rh123 efflux. Since Rh123 is known to be a good substrate for Pgp, it was concluded that kaempferol modulates intracellular drug levels.
by inhibiting Pgp. Although, the exposure to this flavonoid in either Rh123 accumulation or Rh123 efflux experiments of KB-V1 cells was about 1 to 1.5 h, it is unlikely that kaempferol acts by reducing the amount of cellular Pgp. Nevertheless, the protein level of Pgp was examined and the results showed that Pgp expression of KB-V1 cells was not affected by treating cells with the kaempferol for a period of 2 h. (data not shown). Thus, these data indicated that the kaempferol in the high micromolar range, with a short time period for incubation, is able to modulate Pgp activity in vitro, not expression, and the interference with the transporter is related to its concentration.

Flavonoids are included in the extensive list of potential cancer preventive micronutrients. Some of these compounds, classified by Wattenberg (23) as “blocking agents”, have been shown to depress the bioactivation of liver carcinogens including aflatoxin B1, by selective inhibition of CYP-450 isozymes. (24,25) Some modulations of phase II detoxification enzymes by flavonoids have also been observed. This study described that possible interference on Pgp-mediated drug extrusion ought to be taken into account when estimating the cancer chemotherapeutic combination of these dietary compounds. These findings suggest that consumption of fruit and vegetables, which contain flavonoid, i.e. kaempferol, could improve the efficacy of cancer treatment by increasing the accumulation of chemotherapeutic drugs in cancer cells by blocking Pgp function. Considering quality of life, the researchers hope that this study will contribute some clinical benefits to cancer chemotherapy.

Acknowledgments

This work was supported by a grant from the Faculty of Medicine Research Fund, Faculty of Medicine, Chiang Mai University, Thailand. We also thank Dr. Michael M. Gottesman and Dr. Suresh V. Ambudkar (National Cancer Institute, NIH) for the gift of KB-3-1 and KB-V1 cell lines and some chemicals applied in this study.

References


การทดสอบสารฟลาโวนอยด์ที่มีคุณสมบัติยับยั้งการทำงานของฟี-กลไกโปรตีนในเซลล์มะเร็งปากมดลูกของมนุษย์ชนิด KB

ออรรเวน, กันธมาทน, วท.ม., วิทยา ขันวัญยื, ว.ม., พรงม อิ่มตราด, Ph.D.

ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

บทคัดย่อ ฟี-กลไกโปรตีนที่มีหน้าที่ป้องกันเซลล์ขนาด 170 กิโลdalton เป็นสาเหตุของการเจริญเติบโตของเซลล์มะเร็งปากมดลูกของมนุษย์ ใน การศึกษาครั้งนี้ได้ทดสอบความสามารถของสารฟลาโวนอยด์ที่รู้จักกันเมื่อพิจารณาแล้วนี้ ต่อการเปลี่ยนแปลงการทำงานของฟี-กลไกโปรตีน ในเซลล์มะเร็งปากมดลูกของมนุษย์ชนิด KB-V1 ผลการทดลองแสดงให้เห็นว่าเคมิออลและไดซิอีน สามารถรู้จักกันได้เซลล์มีความไวต่อวินบรุสติน (P<0.05) ทำให้ค่าความเข้มข้นของวินบรุสตินที่ยับยั้งการเจริญเติบโตของเซลล์ได้ร้อยละ 50 (IC50) ลดลงตามความเข้มข้นของสารฟลาโวนอยด์ดังกล่าวเพิ่มขึ้น โดยไม่ส่งผลต่อเซลล์ KB-3-1 ซึ่งไม่มีฟี-กลไกโปรตีน นอกจากนี้เคมิออลยังสามารถเพิ่มการสะสมและลดการขับโรดามิน123 ซึ่งเป็นสัญญาณที่รู้จักกันดีซึ่งพบในเซลล์ KB-V1 ได้จากผลที่พบแสดงให้เห็นว่าสารฟลาโวนอยด์ดังกล่าวสามารถเปลี่ยนแปลงการทำงานของฟี-กลไกโปรตีนที่เล็กลำดับสองได้ในเซลล์ KB-V1 โดยการยับยั้งการทำงานของฟี-กลไกโปรตีน ซึ่งส่งผลให้ลดการดื้อยาของวินบรุสตินในเซลล์ KB-V1 เมื่อใช้ร่วมกับยาฟี-กลไกโปรตีน.

คำที่เกี่ยวข้อง: สารฟลาโวนอยด์, เซลล์ KB-V1, ฟี-กลไกโปรตีน, การคัดกรอง, วินบรุสติน, โรดามิน123