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## **Effects of the Flavonoids Biochanin A, Morin, Phloretin and Silymarin on P-glycoprotein-Mediated Transport**

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## ABSTRACT

Flavonoids are constituents of fruits, vegetables and plant-derived beverages, as well as components in herbal-containing dietary supplements. The objective of this investigation was to characterize the effect of flavonoids on P-glycoprotein (P-gp)-mediated cellular efflux, and to determine the molecular mechanism(s) of the flavonoid-drug interaction. Studies were conducted in the sensitive and MDR human breast cancer cell lines MCF-7 and MDA435/LCC6 and examined the effects of the flavonoids biochanin A, morin, phloretin and silymarin on daunomycin (DNM) accumulation and doxorubicin cytotoxicity. The potential mechanism(s) involved in the interaction were evaluated by determining flavonoid effects on a) P-gp ATPase activity, b)  $^3\text{H}$ -azidopine photoaffinity labeling of P-gp, and c) cellular P-gp levels. The flavonoids increased  $^3\text{H}$ -DNM accumulation in P-gp positive cells, but not P-gp negative cells, and these effects were both flavonoid-concentration and P-gp expression level dependent. Biochanin A and silymarin potentiated doxorubicin cytotoxicity in P-gp positive cells. Biochanin A and phloretin stimulated, while morin and silymarin inhibited P-gp ATPase activity, confirming that these flavonoids interact with P-gp. Morin and silymarin significantly inhibited  $^3\text{H}$ -azidopine photoaffinity labeling of P-gp, suggesting a direct interaction with P-gp substrate binding. A 24-hour preincubation with all flavonoids, followed by flavonoid removal, did not alter cellular P-gp level in P-gp positive cells. In conclusion, biochanin A, morin, phloretin and silymarin all inhibited P-gp-mediated cellular efflux and the mechanism of the interaction involved, at least in part, a direct interaction. The findings of this study indicate a potential for significant flavonoid-drug interactions with P-gp substrates.

## INTRODUCTION

Cancer chemotherapy has been of limited success because of the intrinsic or acquired resistance of cancer cells to a broad range of chemically and functionally distinct anticancer agents, a phenomenon termed Multidrug Resistance (MDR). The classical form of MDR involves the over-expression of drug efflux transporters such as P-glycoprotein (P-gp) (Juliano and Ling, 1976; Kartner et al., 1983) and Multidrug Resistance- Associated Protein 1(MRP1) (Cole et al., 1992) in the cell membrane, which pump anticancer drugs out of the cells, resulting in low intracellular drug concentrations. P-gp is a 170-180 kDa plasma membrane protein encoded by the human MDR1 and MDR3 genes and the murine *mdr1a*, *mdr1b*, and *mdr2* genes. P-gp belongs to the ATP-binding cassette (ABC) transporter superfamily and is responsible for the efflux of a broad array of hydrophobic compounds, including a number of important chemotherapeutic agents such as Vinca alkaloids, anthracyclines, epipodophyllotoxins and taxol (Germann, 1996). Structure analysis based on the P-gp sequence predicts that P-gp consists of two homologous halves, each containing six transmembrane domains and a cytoplasmic nucleotide-binding domain (Gottesman and Pastan, 1993). P-gp acts as an efflux pump by exporting them from the membrane or cell cytosol to the exterior of the cells, with ATP hydrolysis as the driving force (Gottesman and Pastan, 1993). Over-expression of this efflux protein has been associated with the clinical MDR phenotype and poor prognosis for many human cancers (Nooter and Sonneveld, 1994; List, 1996). Since the report that the calcium channel blocker, verapamil could reverse resistance by inhibiting P-gp-mediated drug efflux (Tsuruo et al., 1981), a wide panel of substances have been tested both in vitro and in clinical trials for their ability to reverse MDR. Although a variety of agents including calcium channel blockers, calmodulin antagonists, cyclosporins, quinolines and their analogs have been found to

be potent P-gp inhibitors in MDR cancer cells, the clinical results have been disappointing, due to the toxicities resulting from administration of the P-gp modulators and resulting from the pharmacokinetic interactions between the modulators and cytotoxic drugs (Volm, 1998).

Besides its role in conferring MDR, P-gp has also been found in many normal tissues, including the intestinal epithelium, blood brain barrier, hepatocytes and renal tubular cells, suggesting its important role in drug absorption, elimination and distribution (Yu, 1999). It has been shown that the oral absorption and brain penetration of P-gp substrates are significantly lower in normal mice compared with *mdr1a* (-/-) mice (Schinkel, 1998) and oral bioavailability and brain penetration of P-gp substrates can be significantly enhanced by simultaneous administration of P-gp modulators (Mayer et al., 1997; Fromm et al., 1999). Clearance of a P-gp substrate due to biliary excretion and renal secretion can be also significantly decreased in the presence of a P-gp inhibitor (Song et al., 1999; Kiso et al., 2000).

Flavonoids are the most abundant polyphenolic compounds present in human diet, such as fruits, vegetables and plant-derived beverages such as tea and red wine. Over 4000 naturally-occurring flavonoids have been described (Harborne, 1986). Certain plants and spices containing flavonoids represent traditional Eastern medicines, including the widely-used Ginkgo biloba and flavonoids are present in a plethora of dietary supplements. The daily intake of total flavonoids from the average US diet is estimated to be 1 gram, expressed as glycosides (Kuhnau, 1976), and could be higher when dietary supplements are taken. Flavonoids have long been associated with a variety of biochemical and pharmacological properties including antioxidant, antiviral, anti-carcinogenic and anti-inflammatory activities (Middleton et al., 2000), believed to be beneficial to human health. In a recent review, Middleton et al. (Middleton et al., 2000) concluded “the flavonoids appear to be remarkably safe nutrients with a wide range of

biochemical and pharmacological activities that strongly suggest their possible role as health-promoting, disease-preventing dietary supplements.”

The objectives of this investigation are 1) to determine the effects of some naturally occurring flavonoids, namely biochanin A, morin, phloretin and silymarin, on P-gp - mediated drug efflux and 2) to gain an understanding of the potential mechanism(s) underlying these interactions. These flavonoids have been shown to be able to increase the accumulation of <sup>3</sup>H-daunomycin (<sup>3</sup>H-DNM) in P-gp overexpressing MCF-7/ADR cells from our initial screening of 20 naturally occurring flavonoids (Tseng et al., 2001).

## MATERIALS AND METHODS

**Materials.** Verapamil, the flavonoids were purchased from Sigma-Aldrich (St. Louis, MO). Silymarin is comprised of silybinin, silydianin and silychristine and the molar concentration was calculated based on the molecular weight of silybinin since this is the major flavonoid present. <sup>3</sup>H-daunomycin (DNM) (14.4 Ci/mmol) was purchased from NEN Life Science products (Boston, MA). P-gp negative control membrane and human P-gp membrane were purchased from GENTEST (Woburn, MA). RPMI 1640, fetal bovine serum (FBS) and phosphate buffered saline (PBS) were from Gibco BRL (Buffalo, NY). MCF-7/sensitive and MCF-7/ADR cells were gifts from Dr. Ralph Bernacki (Roswell Park Cancer Institute). MDA435/LCC6 and MDA435/LCC6MDR cells were kindly provided by Dr. Robert Clarke (Georgetown University). Both MCF-7 and MDA435/LCC6 cell lines are human breast cancer cell lines; MCF-7 is estrogen-dependent and MDA435/LCC6 is estrogen-independent. <sup>3</sup>H-azidopine and biodegradable counting scintillate was purchased from Amersham Pharmacia Biotech. (Piscataway, NJ).

**Cell Culture.** MCF-7 and MDA435/LCC6 cells (both parent and MDR subtype) were cultured in 75-cm<sup>2</sup> flasks with RPMI 1640 culture media supplemented with 10% fetal bovine serum at 37<sup>0</sup>C in a humidified atmosphere with 10 % CO<sub>2</sub>/ 90 % air. For MCF-7 cells, culture media also contained 100 units / ml penicillin and 100 µg / ml streptomycin. MCF-7 cells with passage number of 16-24 and MDA435/LCC6 (both wild type and MDR subtype) cells with passage number of 16-28 were used for experiments. A solution of 0.05 % trypsin-EDTA was used to detach the cells. For the drug accumulation studies, cells were seeded onto 35-mm<sup>2</sup> dishes at a density of approximately 5×10<sup>5</sup> cells per dish. Confluence was reached after 2 days.



**Western Analysis of P-gp and MRP1.** Cells grown in 100 × 15 mm culture dishes were washed with PBS, and harvested using a rubber policeman. Total cell lysates were prepared by adding the lysis buffer (20 mM Tris pH 7.5, 120 mM NaCl, 100 mM NaF, 1% Nonidet P-40 (NP-40), 200 μM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), 50 mM β-glycerolphosphate, 10 mM sodium pyrophosphate (NaPPi), 4 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin) to the harvested cells. The cells were kept on ice for 30 min. The soluble extracts were obtained by centrifuging the cell lysates at 13,000 g for 20 min. The protein concentrations of the soluble extracts were obtained by BCA protein assay (Pierce, Rockford, IL). Proteins (30 μg) were electrophoresed on 7.5% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Invitrogen, Grand Island, NY). Membranes were then blocked overnight at 4°C in Tris-buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) fat-free dry milk (Bio-Rad, Hercules, CA), and then incubated first with primary antibody, and then with secondary antibody at room temperature for 2 and 1.5 hours, respectively. C219 (DAKO, Carpinteria, CA) and MRPM6 (Signet Laboratories, Inc., Dedham, MA) were used as primary antibodies to detect P-gp and MRP1, respectively. Anti-mouse IgG HRP (Amersham, Piscataway, NJ) was used as secondary antibody. As a loading control, some of the membranes were also incubated with anti-β-actin antibody (Alltech Associates, Deerfield, IL) and anti-mouse IgG to detect β-actin. After incubation with the antibodies, membranes were washed and detected with ECL detection reagent (Amersham, Piscataway, NJ). KODAK 1D Image Analysis Software (Eastern Kodak Company, Rochester, NY) was used to analyze the western blot results.

**<sup>3</sup>H-DNM Accumulation Studies.** The accumulation studies were performed after the cells reached 90% confluence in 35-mm<sup>2</sup> dishes. Cells were washed twice with assay buffer (137 mM NaCl, 54 mM KCl, 2.8 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 10 mM Hepes, pH 7.4) and then incubated with 0.05 μM <sup>3</sup>H-DNM containing 50 μM flavonoids or vehicle (0.2% DMSO, used as control). Verapamil (100 μM) was added as a positive control. Preliminary studies demonstrated that steady state conditions were achieved by 2 hours for <sup>3</sup>H-DNM accumulation in MCF-7/ADR cells. The accumulation was stopped by rinsing the cells four times with ice-cold buffer (137 mM NaCl, and 14 mM Trisbase, pH 7.4). Cells were then solubilized using a solution of 0.3N NaOH and 1 % SDS, and aliquots were used to determine radioactivity by liquid scintillation counting (1900 CA, Tri-Carb Liquid Scintillation Analyzer, Packard) and protein content using the BCA protein assay. Results were normalized for the protein content of the cells in each dish and <sup>3</sup>H-DNM accumulation level was expressed as percent accumulation in control group. For each sample, three or four separate experiments were performed and each experiment had triplicate measurements.

**Cytotoxicity Studies.** Cytotoxicity studies were performed in 96-well plates. Cells (5×10<sup>3</sup>) were seeded in each well of 96-well plates. After cell attachment (24 hour incubation), culture medium in each well were replaced with fresh medium containing 0-300 μM doxorubicin as well as 100 μM flavonoids or vehicle (0.2% DMSO). Verapamil (100 μM) was used as a positive control. After incubation for another 24 hours, the drug-containing medium was aspirated and cells washed thrice with PBS buffer and fresh medium (without any drug) added. The incubation was then continued for an additional 24 hours and cell growth in each well was determined by sulforhodamine B (SRB) assay (Skehan et al., 1990). The absorbance values (OD570) from the

SRB assay indicate the cell number in each well of the 96-well plates. Growth inhibition by doxorubicin (IC<sub>50</sub> value) either alone or with 100 μM flavonoids was obtained by fitting the fraction of cell growth (F) by the equation:  $F=1-(I_{\max} \bullet C^{\gamma}) / (IC_{50}^{\gamma} + C^{\gamma})$  using WinNonlin (Pharsight, Mountain View, CA). The observed F values were calculated as the ratio of the cell growth (OD<sub>570</sub>-OD<sub>570</sub>(300)) to the maximum cell growth (OD<sub>570</sub>(0)-OD<sub>570</sub>(300)). OD<sub>570</sub>(0), OD<sub>570</sub>(300) are the absorbance values from cells treated with 0 and 300 μM of doxorubicin, respectively. C is the concentration of doxorubicin. In each experiment, quadruplicate measurements were performed for each sample. The extent of potentiation of the doxorubicin cytotoxicity is expressed as the ratio of IC<sub>50</sub> without flavonoids to that with flavonoids. Three to five separate experiments were performed.

**Initial Uptake Rate Studies.** The experiments were performed in a similar manner as the <sup>3</sup>H-DNM accumulation studies, but after the cells were washed with assay buffer, they were incubated with 50 μM flavonoids or vehicle (0.2% DMSO) for 1 ½ hours. Then 0.05 μM <sup>3</sup>H-DNM was added and the incubation continued for 7 min. The accumulation was then stopped and samples were processed as <sup>3</sup>H-DNM accumulation studies. Preliminary studies demonstrated that the accumulation of <sup>3</sup>H-DNM in MCF-7/sensitive cells is approximately linear within 10 min, and so the initial uptake rate was calculated as <sup>3</sup>H-DNM accumulation per min normalized by protein content. Results were expressed as percentage of the uptake rate of the control group. For each sample, three separate experiments were performed and each experiment had triplicate measurements.

**P-gp ATPase Activity Assay.** P-gp ATPase activity was measured using human P-gp membrane and P-gp negative control membrane, following the manufacturer's instructions. Briefly, human P-gp membrane or P-gp negative control membrane (30  $\mu$ g) were incubated at 37 °C in 60  $\mu$ l of Tris-MES buffer (50 mM (2-[N-morpholino] ethanesulfonic acid) hydrate (MES), 2mM ethylene glycol-bis (beta-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 2 mM dithiothreitol (DTT), 50 mM KCl, 5 mM NaN<sub>3</sub>, with pH adjusted to 6.8 with Tris-base) containing 100  $\mu$ M flavonoids, solvent control (0.3% DMSO) and/or 20  $\mu$ M verapamil. The ATPase reaction was initiated by the addition of 4 mM MgATP. The reaction was stopped by addition of 30  $\mu$ l of 10% SDS solution and the amount of phosphate was determined immediately by adding 200  $\mu$ l of detection solution (4 parts of 10% ascorbic acid, pH 5.0 and 1 part of 35 mM ammonium molybdate in 15 mM zinc acetate, pH 5.0) and incubating at 37 °C for 20 min in the dark. The amount of phosphate was then determined by measuring the absorbance at 800 nm wavelength with phosphate buffer (pH 7.4) as standards. Preliminary studies using phosphate buffer standards demonstrated that 100  $\mu$ M flavonoids or 20  $\mu$ M verapamil, as used in these experiments, did not interfere with the phosphate assay. Phosphate released by ATPase reaction was estimated by the difference in the phosphate level between 0 min (ATPase reaction was stopped immediately with SDS solution) and 20 min incubation periods and the ATPase activity was expressed as the rate of phosphate release per mg membrane protein. The difference between ATPase activity in the absence and presence of 100  $\mu$ M orthovanadate (orthovanadate-sensitive ATPase activity) (Urbatsch et al.1995), was determined.

**<sup>3</sup>H-Azidopine Photoaffinity Labeling:** Photoaffinity labeling of P-gp by <sup>3</sup>H-azidopine was performed as previously described (Castro and Altenberg, 1997) with certain modifications.

Briefly, 50  $\mu\text{g}$  of human P-gp membrane or control membrane was incubated with 100  $\mu\text{M}$  flavonoids, or solvent control (0.2% DMSO) for 30 min;  $^3\text{H}$ -azidopine was then added to a final concentration of 0.5  $\mu\text{M}$  and incubated in the dark for 60 min. Then the samples were irradiated with UV light at wavelength of 254-320 nm (Ultra-Violet Products, Inc., Upland, CA) for 20 min at a distance of 8 cm. Samples were then electrophoresed on a 7.5% SDS-PAGE. Gels were fixed in 10% acetic acid / 40% methanol for 1 hour and then incubated with AMPLIFY® (Amersham, Piscataway, NJ), and dried. Autoradiography was performed at  $-70\text{ }^\circ\text{C}$  for a week. Vinblastine (100  $\mu\text{M}$ ) was included as a positive control. The signal intensity was quantified using OptiQuant software (Packard BioScience) and expressed as % of intensity of the solvent control.

**Pre-incubation Studies:** MCF-7/sensitive and MCF-7/ADR cells were grown in 35- $\text{mm}^2$  dishes. After cells reached 80-90% confluence, the cell media were replaced with fresh media containing flavonoids (50  $\mu\text{M}$ ) or vehicle (0.2% DMSO) and the incubation ( $37\text{ }^\circ\text{C}$ ) was continued for another 24 hours. Cells were then washed twice with assay buffer and the 2-hour accumulation of  $^3\text{H}$ -DNM was determined as described for the  $^3\text{H}$ -DNM accumulation studies. Verapamil was used as a positive control and added during the 2-hour accumulation period instead of during the pre-incubation period. Two or three separate experiments were performed and each experiment was performed in triplicate.

**Statistical Analysis:** The differences between the mean values were analyzed for significance using one-way ANOVA followed by Dunnett test. P values  $< 0.05$  were considered statistically significant.

## RESULTS

**P-gp and MRP1 Expression Levels.** To investigate effects of flavonoids on P-gp mediated cellular efflux, we first characterized the cellular P-gp levels in the cells employed in this study using western blot analysis. As shown in Fig 1, the rank order of cellular P-gp level (lowest to highest) is MCF-7/sensitive, MDA435/LCC6WT, MCF-7/ADR and MDA435/LCC6MDR1 cells. MCF-7/sensitive cells have no detectable P-gp expression, which is consistent with previous observations (Fairchild et al., 1990). Over-expression of P-gp in MCF-7/ADR and MDA435/LCC6MDR1 cells have been well established and the cells are used extensively as model MDR cells over-expressing P-gp (Fairchild et al., 1990; Leonessa et al., 1996). MDA435/LCC6WT cells have moderate cellular P-gp. No significant expression of Multidrug Resistance Associated Protein 1 (MRP1) was found in any of the cell lines. This was determined with primary antibody MRPM6 and using H69/AR (Cole et al., 1992) and H69 cells as positive and negative controls, respectively (data not shown).

**Effects of Flavonoids on  $^3\text{H}$ -DNM Accumulation.** After characterization of the cellular P-gp levels, the 2-hour accumulation of  $^3\text{H}$ -DNM was determined in these cells in the presence or absence of flavonoids (50  $\mu\text{M}$ ). Verapamil (100  $\mu\text{M}$ ), a well characterized P-gp inhibitor, was used as a positive control. The results are shown in Fig 2. The  $^3\text{H}$ -DNM accumulation in MCF-7/sensitive cells (Fig 2A), which has no detectable P-gp expression, was not significantly changed by any flavonoids or verapamil. In MDA435/LCC6WT cells (Fig. 2C), which have moderate P-gp expression, and MCF-7/ADR (Fig 2B) and MDA435/LCC6MDR1 (Fig 2D) cells, both of which have substantial P-gp expression,  $^3\text{H}$ -DNM accumulation is significantly increased in the presence of all the tested flavonoids and verapamil (except for the  $^3\text{H}$ -DNM accumulation

in MDA435/LCCMDR1 cell in the presence of phloretin, which was  $277 \pm 93\%$  of the control, but not statistically significant). In addition, the increase in  $^3\text{H-DNM}$  accumulation in the presence of both flavonoids and verapamil was qualitatively correlated with the cellular P-gp levels in these cells (Fig 2) with higher effects in cells expressing higher levels of P-gp. To further characterize the effects of flavonoids on the accumulation of  $^3\text{H-DNM}$  in P-gp positive cells, we also investigated  $^3\text{H-DNM}$  accumulation in MDA435/LCC6MDR1 cells in the presence of different concentrations (5-100  $\mu\text{M}$ ) of flavonoids.  $^3\text{H-DNM}$  accumulation was found to be flavonoid concentration-dependent. The enhancement of  $^3\text{H-DNM}$  accumulation increased with increasing flavonoid concentration and significant changes were observed following treatment with biochanin A ( $502 \pm 16\%$ ,  $p < 0.001$ ), morin ( $604 \pm 32\%$ ,  $p < 0.001$ ) at 30  $\mu\text{M}$  concentrations and phloretin ( $431 \pm 28\%$ ,  $p < 0.001$ ), silymarin ( $445 \pm 12\%$ ,  $p < 0.001$ ) at 50  $\mu\text{M}$  concentrations (Data not shown).

**Effects of Flavonoids on Doxorubicin Cytotoxicity.** The effects of flavonoids on doxorubicin cytotoxicity in MDA435/LCC6MDR1 and MDA435/LCC6WT cells were investigated.

Verapamil (100  $\mu\text{M}$ ) was used as a positive control. As shown in Fig 3 and Table 1, verapamil significantly enhanced doxorubicin cytotoxicity in both MDA435/LCC6MDR1 ( $p < 0.05$ ) and MDA435/LCC6WT cells ( $p < 0.001$ ) and the extent of cytotoxicity potentiation was related to the level of P-gp expression, being more pronounced in MDA435/LCC6MDR1 cells ( $\text{IC}_{50}$  ratio = 70.23) compared with that in MDA435/LCC6WT cells ( $\text{IC}_{50}$  ratio = 7.76). Biochanin A and silymarin enhanced doxorubicin cytotoxicity in MDA435/LCC6MDR1 cells (Fig 3A and Table 1) ( $\text{IC}_{50}$  ratio = 37.83 ( $p > 0.05$ ) and 3.45 ( $p > 0.05$ ) respectively) although the effects were not statistically significant when assessed by one-way ANOVA followed by Dunnett test ( $p < 0.01$

and  $p < 0.05$ , respectively if assessed by student t-test). The trend of doxorubicin cytotoxicity enhancement by biochanin A and silymarin was consistent with the previous observation that both flavonoids can inhibit P-gp-mediated drug efflux and that biochanin A is more effective than silymarin in doing so. Morin and phloretin, however, were not able to potentiate doxorubicin cytotoxicity; instead, there was a trend towards decreased doxorubicin cytotoxicity ( $IC_{50}$  ratio = 0.75 and 0.53, respectively), although the effects were not statistically significant. Biochanin A significantly enhanced doxorubicin cytotoxicity in MDA435/LCC6WT cells ( $IC_{50}$  ratio = 10.30,  $p < 0.05$ ) although the extent of cytotoxicity potentiation was less than that in MDA435/LCC6MDR1 cells (Fig 3B and Table 1). As shown in Fig 4, the effects of biochanin A in MDA435/LCC6MDR1 cells were concentration-dependent.

**Effects of Flavonoids on  $^3\text{H}$ -DNM Initial Uptake Rate in MCF-7/sensitive Cells.** To characterize the effects of flavonoids on passive membrane permeability, we investigated the effects of flavonoids (50  $\mu\text{M}$ ) on the initial uptake rate of  $^3\text{H}$ -DNM in P-gp negative cells (MCF-7/sensitive), which is a measure of DNM passive membrane permeability assuming the uptake process in drug sensitive cells is solely a diffusion process. None of the tested flavonoids significantly increased the initial uptake rate of  $^3\text{H}$ -DNM in MCF-7/sensitive cells, suggesting that none of these compounds increased DNM passive membrane permeability (data not shown). Instead, morin, phloretin and silymarin slightly decreased the initial uptake rate of  $^3\text{H}$ -DNM in MCF-7/sensitive cells ( $82 \pm 17\%$ ,  $82 \pm 17\%$ , and  $81 \pm 15\%$  respectively, with  $p < 0.01$  for all of these compounds).



**Effects of Flavonoids on P-gp Expression Following a 2-hour Incubation.** The effects of flavonoids on cellular P-gp expression in MDA435/LCC6MDR1 cells following a 2-hour incubation period, as used in the  $^3\text{H}$ -DNM accumulation studies, were investigated. Cells grown in 35 mm<sup>2</sup> dishes were washed, and then incubated with the assay buffer used in the  $^3\text{H}$ -DNM accumulation studies containing 50  $\mu\text{M}$  flavonoids or vehicle (0.2% DMSO) for 2 hours. The cellular P-gp level of these cells was then examined by western blot analysis. As shown in Fig 5, none of the tested flavonoids significantly changed the cellular P-gp level in MDA435/LCC6MDR1 cells after a 2-hour incubation and the relative P-gp expression levels after biochanin A, morin, phloretin and silymarin treatment were  $104.5 \pm 33.7\%$ ,  $102.7 \pm 22.0\%$ ,  $110.4 \pm 18.4\%$  and  $95.7 \pm 10.2\%$  of the control, respectively.

**Effects of Flavonoids on P-gp ATPase Activity.** To further understand the interactions between flavonoids and P-gp, we examined the effects of flavonoids (100  $\mu\text{M}$ ) on both P-gp constitutive ATPase activity and verapamil (20  $\mu\text{M}$ )-induced ATPase activity. As shown in Table 2, the presence of verapamil (20  $\mu\text{M}$ ) and flavonoids (100  $\mu\text{M}$ ) had no significant effects on orthovanadate-sensitive ATPase activity of the P-gp negative control membrane; however, the orthovanadate-sensitive ATPase activity of the P-gp membrane was significantly increased by both verapamil (a well known P-gp ATPase stimulator,  $p < 0.001$ ) and biochanin A ( $p < 0.001$ ) from  $5.16 \pm 2.37$  to  $31.7 \pm 5.94$  and  $18.73 \pm 3.28$  nmol/min/mg, respectively, but not changed significantly by morin, phloretin and silymarin. This observation suggested that biochanin A and verapamil can stimulate P-gp constitutive ATPase activity, but morin, phloretin and silymarin are not effective. We also investigated the effects of flavonoids (100  $\mu\text{M}$ ) on the P-gp ATPase activity induced by verapamil (20  $\mu\text{M}$ ) by incubating the P-gp membrane in the assay system

with both flavonoids (100  $\mu$ M) and verapamil (20  $\mu$ M). As shown in Table 2, the orthovanadate-sensitive ATPase activity of the P-gp membrane was significantly stimulated by 20  $\mu$ M verapamil (from  $3.40 \pm 1.97$  to  $32.60 \pm 6.83$  nmol/min/mg,  $p < 0.001$ ), consistent with the above observation. In the co-presence of biochanin A, orthovanadate-sensitive ATPase activity is further stimulated (from  $32.60 \pm 6.83$  to  $61.04 \pm 5.30$  nmol/min/mg,  $p < 0.01$ ). In the co-presence of phloretin, orthovanadate-sensitive ATPase activity was also significantly increased (from  $32.60 \pm 6.83$  to  $52.74 \pm 8.91$  nmol/min/mg,  $p < 0.05$ ), although phloretin was not able to stimulate the P-gp constitutive ATPase activity (Table 2). In the co-presence of silymarin, the verapamil-induced P-gp ATPase activity was significantly decreased (from  $32.60 \pm 6.83$  to  $1.97 \pm 6.44$  nmol/min/mg,  $p < 0.01$ ). In addition, it seems that silymarin (100  $\mu$ M) was able to completely inhibit verapamil-induced P-gp ATPase activity because in the co-presence of verapamil and silymarin, the orthovanadate-sensitive ATPase activity ( $1.97 \pm 6.44$  nmol/min/mg) was not significantly different from the P-gp constitutive ATPase activity ( $3.40 \pm 1.97$  nmol/min/mg). Morin (100  $\mu$ M) also decreased verapamil-induced P-gp ATPase activity (from  $32.60 \pm 6.83$  to  $14.72 \pm 6.27$  nmol/min/mg,  $p > 0.05$ ), although the effect was not statistically significant.

**<sup>3</sup>H-Azidopine Photoaffinity Labeling.** To understand the interaction of flavonoids with P-gp and its substrate binding, we investigated <sup>3</sup>H-azidopine photoaffinity labeling of P-gp in the presence of flavonoids (100  $\mu$ M) using human P-gp membrane and P-gp negative control membrane. Vinblastine (100  $\mu$ M) was included as a positive control. As shown in Fig 6, vinblastine inhibited <sup>3</sup>H-azidopine photoaffinity labeling of P-gp by  $60.0 \pm 2.7$  % ( $p < 0.001$ ), similar to previously reported results (Castro and Altenberg, 1997), in which vinblastine (100  $\mu$ M) inhibited <sup>3</sup>H-azidopine photoaffinity labeling of P-gp by  $75 \pm 4$ %. All the tested flavonoids

inhibited  $^3\text{H}$ -azidopine photoaffinity labeling of P-gp but to different extents; the effect of biochanin A and phloretin (inhibited  $23.2 \pm 6.3\%$  and  $30.2 \pm 11.6\%$ , respectively) was not statistically significant. The effect of morin was moderate but statistically significant (inhibited  $37.9 \pm 28.4\%$ ,  $p < 0.05$ ), which might be comparable to that reported for genistein, which at a higher concentration (200  $\mu\text{M}$ ), inhibited  $^3\text{H}$ -azidopine photoaffinity labeling of P-gp by  $42 \pm 15\%$  (Castro and Altenberg, 1997). The effect of silymarin was more substantial ( $61.2 \pm 20.7\%$ ,  $p < 0.001$ ) and comparable to that of vinblastine.

**24-hour Preincubation Studies.** To investigate the effects of a longer incubation with flavonoids on P-gp-mediated efflux, we investigated the 2-hour accumulation of  $^3\text{H}$ -DNM in both P-gp negative (MCF-7/sensitive) and P-gp positive (MCF-7/ADR) cells after pre-treatment of the cells with 50  $\mu\text{M}$  flavonoids for 24 hours. Verapamil was included as a positive control and was added with  $^3\text{H}$ -DNM for the accumulation study (2 hour incubation period). As shown in Fig 7A,  $^3\text{H}$ -DNM accumulation in MCF-7/sensitive cells was not changed significantly by the pre-treatment with any of the flavonoids. In MCF-7/ADR cells (Fig 7B),  $^3\text{H}$ -DNM accumulation was significantly increased by a 24-hour pre-treatment with 50  $\mu\text{M}$  silymarin, but the change ( $38 \pm 5\%$ ,  $p < 0.001$ ) was moderate.  $^3\text{H}$ -DNM accumulation was not changed significantly by pre-treatment of cells with biochanin A, morin or phloretin. As shown in Fig 8, none of the tested flavonoids significantly changed cellular P-gp level in MCF-7/ADR cells following a 24-hour pre-treatment with 50  $\mu\text{M}$  of these flavonoids, and the cellular P-gp level after biochanin A, morin, phloretin and silymarin pre-treatment was  $106 \pm 27\%$ ,  $83 \pm 29\%$ ,  $83 \pm 34\%$  and  $97 \pm 11\%$ , respectively. Data are expressed as mean  $\pm$  SD of 5-6 separate experiments.

## DISCUSSION

Flavonoids are present in diet and in a variety of dietary supplements such as the widely used ginkgo biloba, milk thistle and soy isoflavones. With the burgeoning interest in alternative medicine, herbal products are ingested by about 10% or more of the general population and 30-70% of individuals with specific disease states (Duggan et al., 2001; Ni et al., 2002). Since dietary supplements are not classified as drugs and do not require FDA approval to be marketed, potential drug interactions are not extensively evaluated. However, there is increasing evidence indicating that use of herbal products could result in clinically important drug interactions. For example, it has been shown that St John' wort reduced the AUC of indinavir by 57% (Piscitelli et al., 2000) and garlic supplements reduced the AUC of saquinavir by 51% (Piscitelli et al., 2002) in human subjects and the proposed mechanism(s) include induction of drug metabolizing enzyme (CYP3A4) and/or P-gp (Durr et al., 2000).

From an initial screening of 20 naturally occurring flavonoids, we found that biochanin A, morin, phloretin and silymarin can substantially increase the accumulation of  $^3\text{H}$ -DNM in MCF-7/ADR cells (Tseng et al., 2001). Thus we decided to further study these flavonoids in four different breast cancer cell lines with different levels of P-gp expression. Although P-gp expression in MDA435/LCC6WT cells was not detected in a previous study (Leonessa et al., 1996) using Ab-1 polyclonal anti-gp170 antibody, we observed moderate P-gp expression in this cell line using the C219 antibody. The expression of P-gp in MDA435/LCC6WT cells was also consistent with our findings in the  $^3\text{H}$ -DNM accumulation and doxorubicin cytotoxicity studies, in which verapamil, a well known P-gp inhibitor, significantly increased  $^3\text{H}$ -DNM accumulation and potentiated doxorubicin cytotoxicity in these cells. The differences between our observation and that of Leonessa et al. (Leonessa et al., 1996) may be due to the different antibodies and

experimental conditions used. The flavonoids, biochanin A, morin, phloretin and silymarin were able to increase  $^3\text{H}$ -DNM accumulation in all the P-gp positive cells (MCF-7/ADR, MDA435/LCC6WT and MDA435/LCCMDR1) but not in P-gp negative cells (MCF-7/sensitive) and the increase of DNM accumulation by these flavonoids was shown to be both flavonoid-concentration dependent and P-gp expression level dependent, strongly suggesting that these flavonoids inhibited P-gp mediated cellular efflux. The increase of DNM accumulation by 50  $\mu\text{M}$  biochanin A or morin in all the P-gp positive cells is comparable to that with 100  $\mu\text{M}$  verapamil, indicating that biochanin A and morin are fairly potent P-gp inhibitors. The possibility that the increase of DNM accumulation by these flavonoids may be due to modulation of MRP1, another drug efflux transporter, instead of P-gp, can be excluded by the following evidence: First of all, we were unable to detect MRP1 in all the cell lines with western blot analysis using the MRPM6 antibody, indicating MRP1 expression in these cell lines must be very low, if present at all. In a previous study, Benderra et al. (Benderra et al., 2000) detected some expression of MRP1 in MCF-7/ADR cells by flow cytometry analysis using the same antibody; however, the expression level must be low enough to be functionally insignificant compared to P-gp because in the same study, it was shown that treatment of MCF-7/ADR cells with buthionine sulphoximine (BSO), a GSH biosynthesis inhibitor, had no effects on daunorubicin cytotoxicity in these cells; however, BSO completely sensitized MCF-7/VP cells, which express relatively high levels of MRP1, to daunorubicin (Benderra et al., 2000). Second, the increase of DNM by these flavonoids has been shown to positively correlate with the cellular P-gp level of the cells. It appears likely that the cellular efflux of DNM by MRP1 in these cells is insignificant compared with that of P-gp.

In the cytotoxicity study, biochanin A and silymarin increased doxorubicin cytotoxicity in MDA435/LCC6MDR1 cells, consistent with their inhibitory effects on P-gp-mediated efflux. Biochanin A was also able to enhance doxorubicin cytotoxicity in MDA435/LCC6WT cells, but the effect was less than that in MDA435/LCC6MDR1 cells, suggesting that enhancement of doxorubicin cytotoxicity by biochanin A is mediated through P-gp inhibition. Phloretin and morin, however, were not able to potentiate doxorubicin cytotoxicity, although they were shown to inhibit P-gp-mediated efflux. A number of reasons could explain this inconsistency, although the exact reason is currently unknown. One possible explanation could be related to the stability of the various flavonoids. We found that biochanin A was stable when incubated with MDA435/LCC6MDR1 cells for 24 hours (data not shown). However, the stability of morin and phloretin in culture media and in incubations with MDA435/LCC6MDR1 cells is not known. Drug accumulation studies were performed over a relatively short period of time (2 hours) but the cytotoxicity studies involved 24-hour drug incubations. Therefore, it is possible that phloretin and morin are degraded or metabolized by the cells during a 24-hour incubation and thus had no effect on doxorubicin cytotoxicity.

To gain some understanding of the underlying mechanism(s) by which flavonoids alter P-gp-mediated cellular efflux, we examined the effects on a) passive membrane permeability, b) P-gp expression, c) P-gp ATPase activity and d) direct binding using the photoaffinity label <sup>3</sup>H-azidopine. An increase in passive membrane permeability could lead to an increase in DNM steady state accumulation in P-gp positive cells but not P-gp negative cells. In P-gp negative cells, <sup>3</sup>H-DNM accumulation at equilibrium will not be altered by the increased passive membrane permeability because the intracellular free DNM concentration will be always equal to the extracellular free DNM concentration at equilibrium, assuming the uptake of DNM in P-gp

negative cells is solely a diffusion process. However, in P-gp positive cells, this is not true: the increased passive membrane permeability will result in an increased steady state accumulation of  $^3\text{H}$ -DNM. A number of agents including some flavonoids and P-gp modulators (Drori et al., 1995; Arora et al., 2000) have been shown to be able to change membrane lipid packing order and thus change membrane fluidity or permeability. So, it is at least theoretically possible that the observed effects of flavonoids on  $^3\text{H}$ -DNM accumulation may be due to their non-specific interaction with the cell membrane, resulting in an increased passive membrane permeability, instead of a specific inhibition of P-gp. To exclude this possibility, we investigated the effects of flavonoids on the initial uptake rate of  $^3\text{H}$ -DNM in P-gp negative cells (MCF-7/sensitive). The uptake of anthracyclines in drug sensitive cells, such as MCF-7/sensitive cells, can be described adequately by passive diffusion model (Frezard and Garnier-Suillerot, 1991), and so the initial uptake rate of  $^3\text{H}$ -DNM in MCF-7/sensitive cells represents a measure of passive membrane permeability. None of the tested flavonoids increased DNM initial uptake rate, indicating that the observed effects of these flavonoids are not the result of an increased passive membrane permeability.

An increase in DNM accumulation in P-gp positive cells could be due to a flavonoid-induced decrease in P-gp expression. P-gp expression was examined in MDA435/LCC6MDR1 cells following a 2-hour incubation. Our data indicate that none of the tested flavonoids significantly changed the P-gp expression in these cells, suggesting that the observed enhancement of DNM accumulation by flavonoids is not due to their reduction of P-gp expression.

P-gp has been demonstrated to be an ATPase (al-Shawi and Senior, 1993) and many P-gp substrates and modulators have been shown to interact with P-gp ATPase activity (both

stimulation and inhibition). For example, the P-gp inhibitor verapamil has been reported to be one of the best stimulators of P-gp ATPase (Shapiro and Ling, 1994; Shapiro and Ling, 1997a). Flavonoids have been reported to modulate P-gp by interacting bi-functionally with the vicinal ATP-binding site and the steroid-binding site (Conseil et al., 1998). Thus, one potential mechanism responsible for the flavonoid inhibition of P-gp-mediated efflux may be the inhibition of P-gp ATPase by binding to the ATP binding site. In this study, we demonstrated that silymarin significantly inhibited P-gp ATPase activity induced by verapamil; however, biochanin A and phloretin stimulated P-gp ATPase activity. These findings indicate that the interactions of these flavonoids with P-gp may differ. Under our experiment conditions, we could not detect the inhibitory effects of morin and silymarin on P-gp constitutive ATPase activity, indicating that they may be not effective in modulating P-gp constitutive ATPase activity. However, it is also likely that this lack of effect on P-gp constitutive ATPase activity may be due to the limited sensitivity of the assay system, since the P-gp constitutive ATPase activity is low and further inhibition can be difficult to detect due to the experimental variability. Therefore, it is possible that the flavonoids silymarin and probably morin, which inhibit P-gp ATPase activity induced by verapamil, may bind to the ATP binding site and vicinal steroid binding site and thus prevent ATP binding. However, it is also possible, especially for silymarin, that these flavonoids bind to the P-gp substrate binding site and thus prevent binding of verapamil and inhibit verapamil-induced ATPase activity. This latter hypothesis is supported by the results of our photoaffinity labeling studies, in which silymarin significantly inhibited <sup>3</sup>H-azidopine labeling of P-gp (inhibited  $61.2 \pm 20.7\%$ ,  $p < 0.001$ ). It is interesting that phloretin increased P-gp ATPase activity in the presence of verapamil; however, it had no effect on P-gp constitutive ATPase activity (in the absence of verapamil). The underlying mechanism is not



clear at this time, but one explanation could be that binding of phloretin itself to P-gp may be not enough to affect P-gp ATPase activity, but phloretin binding could change the protein conformation and enhance the ATPase stimulatory effects upon the binding of verapamil. It has been proposed that P-gp possesses at least two substrate binding sites and an allosteric site (Shapiro and Ling, 1997b; Shapiro et al., 1999). It is therefore possible that phloretin could bind to the allosteric site or other sites and enhance the ability of verapamil to stimulate ATPase activity. Biochanin A effects differed from those of phloretin in that it stimulated P-gp ATPase activity both in the presence and in the absence of verapamil. In addition, the ATPase activity in the presence of both biochanin A and verapamil was shown not to be significantly different from the sum of the ATPase activities in the presence of biochanin A alone and verapamil alone. These data suggested that biochanin A itself can stimulate P-gp ATPase and the stimulatory effects of verapamil and biochanin A may be additive. The ATPase stimulatory effects indicate that biochanin A, as well as phloretin, probably do not bind to the ATP binding site of P-gp, as has been reported for the flavonoids quercetin, apigenin, kaempferide, naringenin, genistein and rutin (Conseil et al., 1998; Shapiro and Ling, 1997a).

To understand the interaction of flavonoids with P-gp and its substrate binding, we investigated the effects of flavonoids on  $^3\text{H}$ -azidopine photoaffinity labeling of P-gp. The inhibition of  $^3\text{H}$ -azidopine photoaffinity labeling of P-gp has been commonly used to indicate direct interaction with P-gp substrate binding (Castro and Altenberg, 1997). Both P-gp substrates and modulators such as vinblastine and verapamil have been shown to inhibit azidopine photoaffinity labeling of P-gp (Tamai and Safa, 1991; Christensen and LeBlanc, 1996). Flavonoids silymarin and morin were shown to significantly inhibit  $^3\text{H}$ -azidopine photoaffinity labeling of P-gp, but to different extents, suggesting that these flavonoids may interact with P-gp

substrate binding directly either by competitive binding to the substrate binding site or by binding to other drug binding sites and changing protein conformation that results in decreased photoaffinity labeling. The inhibition by 100  $\mu$ M biochanin A, morin and phloretin is moderate ( $23.2 \pm 6.3\%$ ,  $p > 0.05$ ,  $37.9 \pm 28.4\%$ ,  $p < 0.05$  and  $30.2 \pm 11.6\%$ ,  $p > 0.05$  respectively) and might be comparable to that reported for genistein ( $42 \pm 15\%$  at 200  $\mu$ M concentration) (Castro and Altenberg, 1997). Silymarin showed stronger inhibition ( $61.2 \pm 20.7\%$ ,  $p < 0.001$ ) which is comparable to that produced by 100  $\mu$ M vinblastine.

In the pre-incubation studies, we have shown that preincubation with biochanin A, morin or phloretin had no effect on  $^3\text{H}$ -DNM accumulation in MCF-7/ADR cells; consistent with this observation is our finding that the cellular P-gp level also showed no significant changes after preincubation with these flavonoids. Preincubation with silymarin was shown to increase  $^3\text{H}$ -DNM accumulation in MCF-7/ADR cells slightly but significantly ( $38 \pm 5\%$ ,  $p < 0.001$ ); however, western blot analysis was not able to detect any significant changes in P-gp level following preincubation with silymarin.

In conclusion, the naturally occurring flavonoids, biochanin A, morin, phloretin and silymarin can inhibit P-gp-mediated drug efflux, and biochanin A and silymarin can potentiate doxorubicin cytotoxicity in P-gp positive cells. The underlying mechanism(s) may involve direct interaction with P-gp as evidenced by flavonoid modulation of P-gp ATPase activity, inhibition of  $^3\text{H}$ -azidopine photoaffinity labeling and the observation that flavonoids do not change cellular P-gp level. In addition, the interactions of these flavonoids with P-gp may be different for specific flavonoids since these flavonoids demonstrated different effects on P-gp ATPase activity. Inferring the exact mechanism(s) by which flavonoids inhibit P-gp is difficult due to the complexity of the P-gp-substrate / modulator interactions. It is possible that some of these

flavonoids (for example, biochanin A) might be used alone, or in combination with other P-gp inhibitors, to reverse MDR in the treatment of certain cancers. In addition, dietary supplements containing the flavonoids investigated in this study, such as red clover extracts (biochanin A as one of the main components) and milk thistle (silymarin), are widely used and it is possible that they may interact with coadministered drugs that are P-gp substrates, resulting in altered bioavailability and / or clearance.

## REFERENCES

- al-Shawi MK and Senior AE (1993) Characterization of the adenosine triphosphatase activity of Chinese hamster P-glycoprotein. *J Biol Chem* **268**:4197-4206.
- Arora A, Byrem TM, Nair MG and Strasburg GM (2000) Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. *Arch Biochem Biophys* **373**:102-109.
- Benderra Z, Trussardi A, Morjani H, Villa AM, Doglia SM and Manfait M (2000) Regulation of cellular glutathione modulates nuclear accumulation of daunorubicin in human MCF7 cells overexpressing multidrug resistance associated protein. *Eur J Cancer* **36**:428-434.
- Castro AF and Altenberg GA (1997) Inhibition of drug transport by genistein in multidrug-resistant cells expressing P-glycoprotein. *Biochem Pharmacol* **53**:89-93.
- Christensen JG and LeBlanc GA (1996) Reversal of multidrug resistance in vivo by dietary administration of the phytochemical indole-3-carbinol. *Cancer Res* **56**:574-581.
- Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM and Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258**:1650-1654.
- Conseil G, Baubichon-Cortay H, Dayan G, Jault JM, Barron D and Di Pietro A (1998) Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. *Proc Natl Acad Sci U S A* **95**:9831-9836.
- Drori S, Eytan GD and Assaraf YG (1995) Potentiation of anticancer-drug cytotoxicity by multidrug-resistance chemosensitizers involves alterations in membrane fluidity leading to increased membrane permeability. *Eur J Biochem* **228**:1020-1029.
- Duggan J, Peterson WS, Schutz M, Khuder S and Charkraborty J (2001) Use of complementary and alternative therapies in HIV-infected patients. *AIDS Patient Care STDS* **15**:159-167.

- Durr D, Stieger B, Kullak-Ublick GA, Rentsch KM, Steinert HC, Meier PJ and Fattinger K (2000) St John's Wort induces intestinal P-glycoprotein/MDR1 and intestinal and hepatic CYP3A4. *Clin Pharmacol Ther* **68**:598-604.
- Fairchild CR, Moscow JA, O'Brien EE and Cowan KH (1990) Multidrug resistance in cells transfected with human genes encoding a variant P-glycoprotein and glutathione S-transferase-pi. *Mol Pharmacol* **37**:801-809.
- Frezard F and Garnier-Suillerot A (1991) Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cells. *Eur J Biochem* **196**:483-491.
- Fromm MF, Kim RB, Stein CM, Wilkinson GR and Roden DM (1999) Inhibition of P-glycoprotein-mediated drug transport: A unifying mechanism to explain the interaction between digoxin and quinidine [seecomments]. *Circulation* **99**:552-557.
- Germann UA (1996) P-glycoprotein--a mediator of multidrug resistance in tumour cells. *Eur J Cancer* **32A**:927-944.
- Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62**:385-427.
- Harborne J (1986) Nature, distribution and function of plant flavonoids, in *Plant Flavonoids in Biology and Medicine: Biochemical Pharmacological, and Structure-Activity Relationships*. pp 17-28, Alan R. Liss, Inc., New York.
- Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455**:152-162.
- Kartner N, Riordan JR and Ling V (1983) Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science* **221**:1285-1288.

- Kiso S, Cai SH, Kitaichi K, Furui N, Takagi K, Nabeshima T and Hasegawa T (2000) Inhibitory effect of erythromycin on P-glycoprotein-mediated biliary excretion of doxorubicin in rats. *Anticancer Res* **20**:2827-2834.
- Kuhnau J (1976) The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev Nutr Diet* **24**:117-191.
- Leonessa F, Green D, Licht T, Wright A, Wingate-Legette K, Lippman J, Gottesman MM and Clarke R (1996) MDA435/LCC6 and MDA435/LCC6MDR1: ascites models of human breast cancer. *Br J Cancer* **73**:154-161.
- List AF (1996) Role of multidrug resistance and its pharmacological modulation in acute myeloid leukemia. *Leukemia* **10**:937-942.
- Mayer U, Wagenaar E, Dorobek B, Beijnen JH, Borst P and Schinkel AH (1997) Full blockade of intestinal P-glycoprotein and extensive inhibition of blood-brain barrier P-glycoprotein by oral treatment of mice with PSC833. *J Clin Invest* **100**:2430-2436.
- Middleton E, Jr., Kandaswami C and Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* **52**:673-751.
- Ni H, Simile C and Hardy AM (2002) Utilization of complementary and alternative medicine by United States adults: results from the 1999 national health interview survey. *Med Care* **40**:353-358.
- Nooter K and Sonneveld P (1994) Clinical relevance of P-glycoprotein expression in haematological malignancies. *Leuk Res* **18**:233-243.
- Piscitelli SC, Burstein AH, Chaitt D, Alfaro RM and Falloon J (2000) Indinavir concentrations and St John's wort. *Lancet* **355**:547-548.

- Piscitelli SC, Burstein AH, Welden N, Gallicano KD and Falloon J (2002) The effect of garlic supplements on the pharmacokinetics of saquinavir. *Clin Infect Dis* **34**:234-238.
- Schinkel AH (1998) Pharmacological insights from P-glycoprotein knockout mice. *Int J Clin Pharmacol Ther* **36**:9-13.
- Shapiro AB, Fox K, Lam P and Ling V (1999) Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Evidence for a third drug-binding site. *Eur J Biochem* **259**:841-850.
- Shapiro AB and Ling V (1994) ATPase activity of purified and reconstituted P-glycoprotein from Chinese hamster ovary cells. *J Biol Chem* **269**:3745-3754.
- Shapiro AB and Ling V (1997a) Effect of quercetin on Hoechst 33342 transport by purified and reconstituted P-glycoprotein. *Biochem Pharmacol* **53**:587-596.
- Shapiro AB and Ling V (1997b) Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *Eur J Biochem* **250**:130-137.
- Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82**:1107-1112.
- Song S, Suzuki H, Kawai R and Sugiyama Y (1999) Effect of PSC 833, a P-glycoprotein modulator, on the disposition of vincristine and digoxin in rats. *Drug Metab Dispos* **27**:689-694.
- Tamai I and Safa AR (1991) Azidopine noncompetitively interacts with vinblastine and cyclosporin A binding to P-glycoprotein in multidrug resistant cells. *J Biol Chem* **266**:16796-16800.

- Tseng E, Liang W and Morris ME (2001) Effect of flavonoids on P-glycoprotein-mediated transport in a human breast cancer cell line, Poster presentation at the Annual Meeting of the American Association of Pharmaceutical Scientists, October 21-25, 2001, Denver CO. *Pharmaceutical Research* **3**.
- Tsuruo T, Iida H, Tsukagoshi S and Sakurai Y (1981) Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* **41**:1967-1972.
- Urbatsch IL, Sankaran B, Weber J and Senior AE (1995) P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site. *J Biol Chem* **270**:19383-19390.
- Volm M (1998) Multidrug resistance and its reversal. *Anticancer Res* **18**:2905-2917.
- Yu DK (1999) The contribution of P-glycoprotein to pharmacokinetic drug-drug interactions. *J Clin Pharmacol* **39**:1203-1211.



## FOOTNOTES

a) footnote to title:

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Table 1

Effect of flavonoids (100  $\mu$ M) on doxorubicin cytotoxicity in MDA435/LCC6 cells

<i>Flavonoids</i>	<b>MDA435/LCC6MDR1</b>			<b>MDA435/LCC6</b>		
	<i>IC<sub>50</sub></i>	<i>Ratio</i>	<i>N</i>	<i>IC<sub>50</sub></i>	<i>Ratio</i>	<i>N</i>
control	30.18 $\pm$ 12.18	1	4	2.27 $\pm$ 0.61	1	5
Biochanin A	0.80 $\pm$ 0.20	37.83	3	0.22 $\pm$ 0.14***	10.3	3
morin	40.36 $\pm$ 18.72	0.75	3			
phloretin	56.67 $\pm$ 28.91	0.53	3			
silymarin	8.74 $\pm$ 5.88	3.45	3			
verapamil	0.43 $\pm$ 0.13*	70.23	4	0.29 $\pm$ 0.02***	7.76	3

\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; N: number of independent experiments.

Doxorubicin cytotoxicity was studied in MDA435/LCC6MDR1 and MDA435/LCC6 cells with 100  $\mu$ M flavonoids or vehicle (0.2% DMSO) (control).  $IC_{50}$  values were obtained by modeling the cell growth data using WinNonlin as described in Materials and Methods. Data are expressed as mean  $\pm$  SD from 3-5 separate experiments. Each experiment had quadruplicate measurements.

Table 2

Effects of flavonoids (100  $\mu$ M) on P-gp ATPase activity

	<i>P-gp negative control membrane</i> (nmol/min/mg)	<i>P-gp positive membrane</i> (nmol/min/mg)	<i>P-gp positive membrane</i> (nmol/min/mg)	
<b>control</b>	1.22±5.62	5.16±2.37	control	3.40±1.97
<b>biochanin A</b>	-0.72±5.69	18.73±3.28***	verapamil	32.60±6.83a
<b>morin</b>	-0.12±4.99	1.40±3.03	verapamil+biochanin A	61.04±5.30**a
<b>phloretin</b>	-2.99±3.28	5.03±3.02	verapamil+morin	14.72±6.27
<b>silymarin</b>	2.38±5.57	1.16±1.79	verapamil+phloretin	52.74±8.91*a
<b>verapamil</b>	2.04±3.15	31.7±5.94***	verapamil+silymarin	1.97±6.44**

\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; a: statistically different from control group.

The ATPase activity was measured using P-gp negative control and P-gp membranes. The effects of flavonoids on P-gp constitutive ATPase activity and verapamil-induced ATPase activity were studied as described in Materials and Methods. Verapamil (a known P-gp ATPase activity stimulator) was included as a positive control. Presented is the data from one typical experiment with triplicate measurements. Data are expressed as mean  $\pm$  SD. Two independent experiments were performed for each sample and similar results were obtained in the two experiments.

## LEGENDS FOR FIGURES

Fig 1. Western blot analysis of P-gp expression in MCF-7 and MDA435/LCC6 cells  
Cellular P-gp level in 1. MCF-7/sensitive; 2. MCF-7/ADR; 3. MDA435/LCC6; and 4. MDA435/LCC6MDR1 cells were examined by western blot analysis as described in Materials and Methods. P-gp was detected with the primary antibody C219.  $\beta$ -actin was used to confirm equal protein loading. Two separate experiments were conducted and similar results were obtained.

Fig 2.  $^3\text{H}$ -DNM 2-hour accumulation in cells expressing different levels of P-gp  
The 2-hour accumulation of  $^3\text{H}$ -DNM in A. MCF-7/sensitive; B. MCF-7/ADR; C. MDA435/LCC6 and D. MDA435/LCC6MDR1 cells in the presence or absence (control) of 50  $\mu\text{M}$  flavonoids was investigated as described in Materials and Methods. Verapamil (100  $\mu\text{M}$ ) was included as a positive control. Data are expressed as mean  $\pm$  SD, n = 9 to 12. (*Abbreviation:* CON: control; BA: biochanin A; MO: morin; PH: phloretin; SI: silymarin; VER: verapamil).

Fig 3. The effects of flavonoids on doxorubicin cytotoxicity in MDA435LCC6 cells  
Doxorubicin cytotoxicity was investigated in A: MDA435/LCC6MDR1 and B: MDA435/LCC6 cells in the presence of 100  $\mu\text{M}$  flavonoids or vehicle (0.2% DMSO) as described in Materials and Methods. Verapamil (100  $\mu\text{M}$ ) was included as a positive control. Presented is a typical experiment and the data are expressed as mean  $\pm$  SD, Three to five separate experiments were performed and each experiment had quadruplicate measurements.  $\circ$  : MDA435/LCC6;  $\triangle$  : MDA435/LCC6 + 100  $\mu\text{M}$  biochanin A;  $\nabla$  : MDA435/LCC6 + 100  $\mu\text{M}$  verapamil;  $\bullet$  : MDA435LCC6/MDR;  $\blacksquare$  : MDA435/LCC6MDR1 + 100  $\mu\text{M}$  phloretin;  $\blacktriangledown$  : MDA435/LCC6MDR1

+ 100  $\mu$ M verapamil;  $\blacktriangle$ : MDA435/LCC6MDR1 + 100  $\mu$ M biochanin A;  $\blacklozenge$ :  
MDA435/LCC6MDR1 + 100  $\mu$ M morin;  $\bullet$ : MDA435/LCC6MDR1 + 100  $\mu$ M silymarin.

Fig 4. The concentration-dependent effects of biochanin A on doxorubicin cytotoxicity in MDA435/LCC6MDR1 cells.

The cytotoxicity of doxorubicin in MDA435/LCC6MDR1 cells in the presence of different concentrations of biochanin A (10-100  $\mu$ M) was investigated. The cytotoxicity study was performed as described in Materials and Methods. Verapamil (100  $\mu$ M) was included as a positive control. Data are expressed as mean  $\pm$  SD, n = 4 wells.  $\circ$ : MDA435/LCC6;  $\bullet$ : MDA435/LCC6MDR1;  $\blacksquare$ : MDA435/LCC6MDR1 + 10  $\mu$ M biochanin A;  $\blacktriangledown$ : MDA435/LCC6MDR1 + 30  $\mu$ M biochanin A;  $\blacktriangle$ : MDA435/LCC6MDR1 + 50  $\mu$ M biochanin A;  $\blacklozenge$ : MDA435/LCC6MDR1 + 100  $\mu$ M biochanin A;  $\bullet$ : MDA435/LCC6MDR1 + 100  $\mu$ M verapamil.

Fig 5. The effects of flavonoids on P-gp expression in MDA435/LCC6MDR1 cells following a 2-hour incubation.

Cells grown in 35 mm<sup>2</sup> dishes were washed, and incubated with the assay buffer used for <sup>3</sup>H-DNM accumulation study containing 50  $\mu$ M flavonoids or vehicle (0.2% DMSO). After incubation for 2 hours, cells were harvested and P-gp expression level was analyzed by western blot analysis as described in Materials and Methods. The P-gp expression level is expressed as percentage of control (vehicle treatment). Data are mean  $\pm$  SD from four separate experiments. 1. MCF-7/sensitive (P-gp negative control); 2. control (vehicle), 100%; 3. Biochanin A, 104.5  $\pm$  33.7%; 4. Morin, 102.7  $\pm$  22.0%; 5. Phloretin, 110.4  $\pm$  18.4%; 6. Silymarin, 95.7  $\pm$  10.2%.

Fig 6. <sup>3</sup>H-azidopine photoaffinity labeling of P-gp

<sup>3</sup>H-azidopine photoaffinity labeling of P-gp in the presence of flavonoids was performed as described in Materials and Methods. Vinblastine (100 μM) was used as a positive control. Three separate experiments were performed and the image is from one representative experiment. 1. P-gp negative membrane; 2. P-gp membrane; 3-7: P-gp membrane with vinblastine, biochanin A, morin, phloretin and silymarin, respectively; 8: P-gp membrane, but UV irradiation was done before instead of after <sup>3</sup>H-azidopine incubation. The <sup>3</sup>H-azidopine photoaffinity labeling of P-gp in the presence of flavonoids (100 μM) and vinblastine is: biochanin A, 76.8 ± 6.3%, p > 0.05; morin, 62.1 ± 28.4%, p < 0.05; phloretin, 69.8 ± 11.6%, p > 0.05; silymarin, 38.8 ± 20.7%, p < 0.001; vinblastine, 39.9 ± 2.7% of the control, p < 0.001.

Fig 7. <sup>3</sup>H-DNM accumulation in MCF-7 cells preincubated with flavonoids for 24 hours

<sup>3</sup>H-DNM accumulation was performed in A: MCF-7/sensitive and B: MCF-7/ADR cells after the cells were treated with 50 μM flavonoids for 24 hours. The residual flavonoids were removed prior to the accumulation study. Verapamil was included as a positive control and was added with <sup>3</sup>H-DNM for the accumulation study. Data are expressed as mean ± SE, n=6 or 9.

Fig 8. Western blot analysis of P-gp expression in MCF-7/ADR cells following a 24-hour preincubation with flavonoids.

MCF-7/ADR cells were treated with flavonoids or vehicle (0.2% DMSO) for 24 hours. The P-gp expression level in these cells was then examined by western blot analysis, as described in

Materials and Methods. Presented are the results from a typical experiment and 5 to 6 separate experiments were performed. Data are expressed as mean  $\pm$  SD. 1. MCF-7/sensitive; 2. MCF-7/ADR; 3. MCF-7/ADR treated with biochanin A ( $106 \pm 27\%$ ); 4. with morin ( $83 \pm 29\%$ ); 5. with phloretin ( $83 \pm 34\%$ ) and 6. with silymarin ( $97 \pm 11\%$ ).

Fig 1

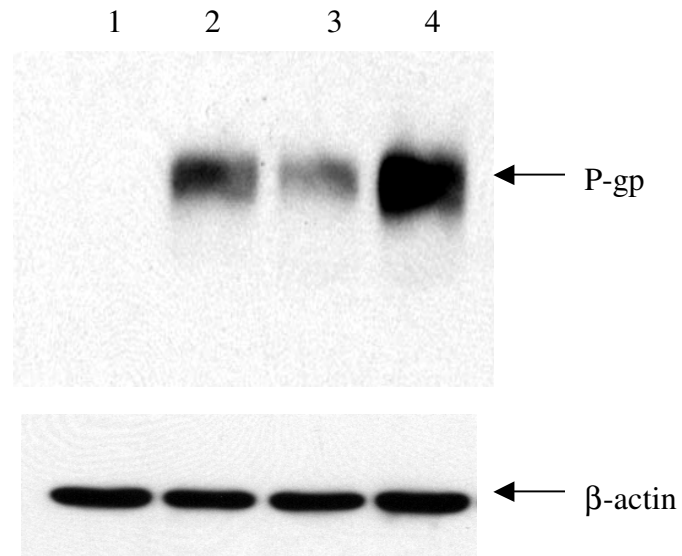
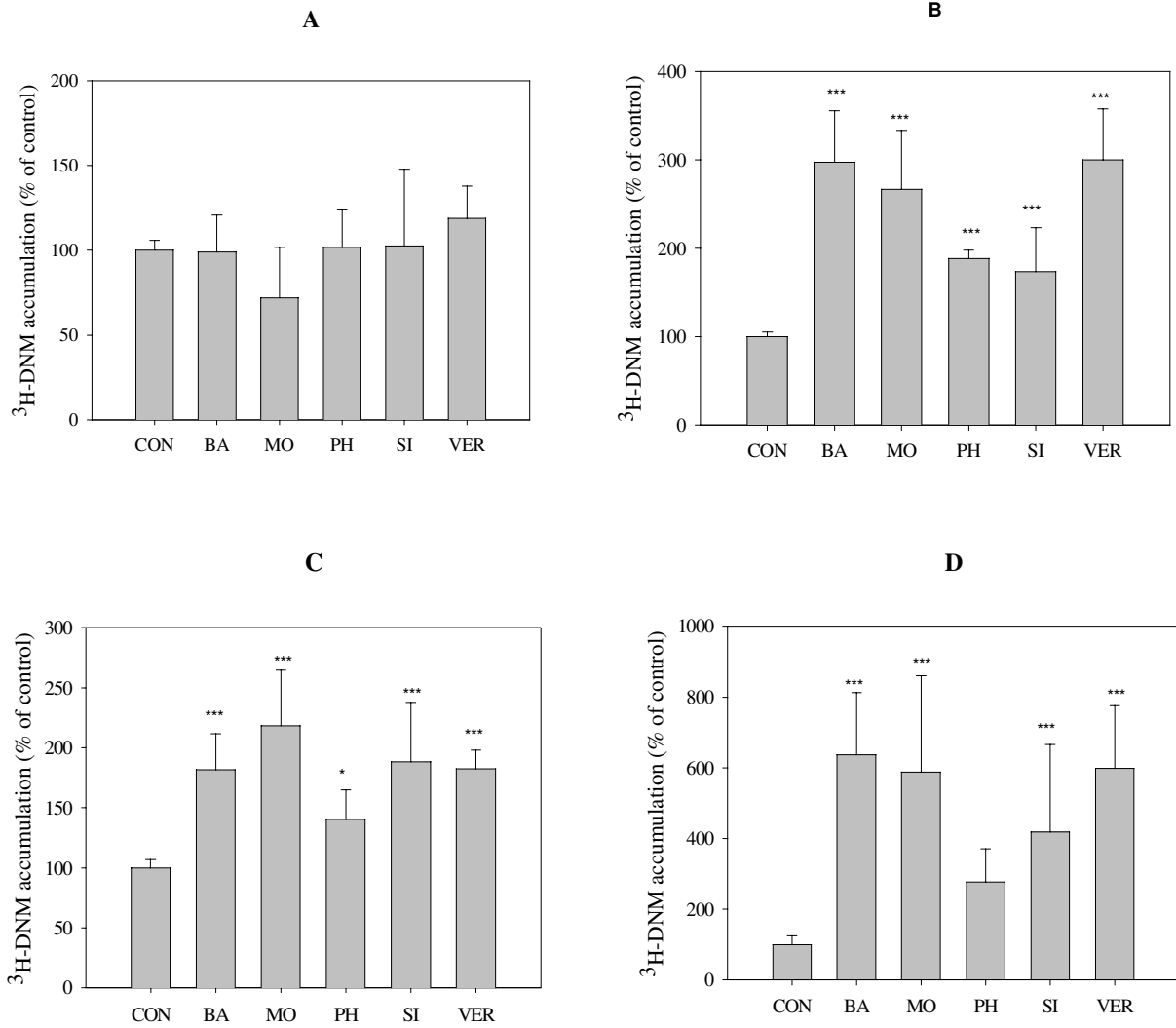




Fig 2



\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$

Fig 3

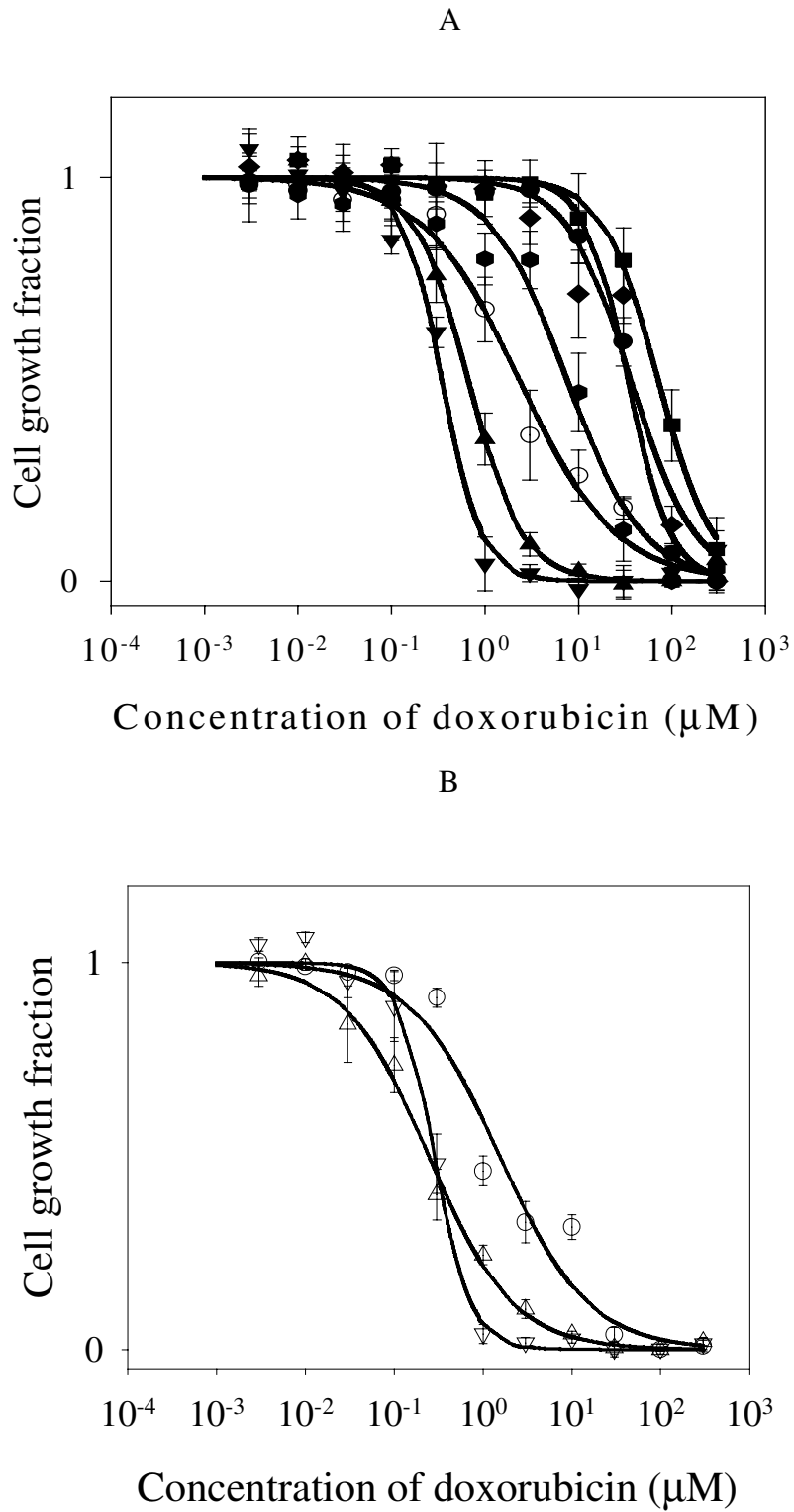


Fig 4

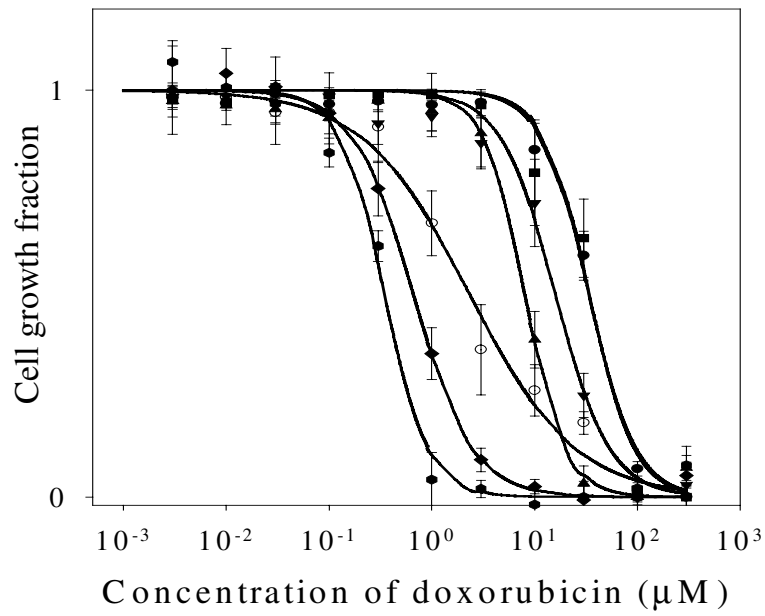


Fig 5

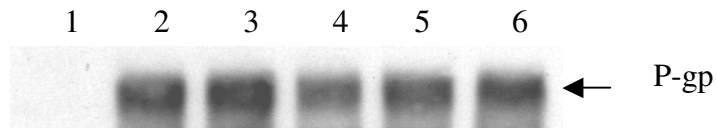


Fig 6

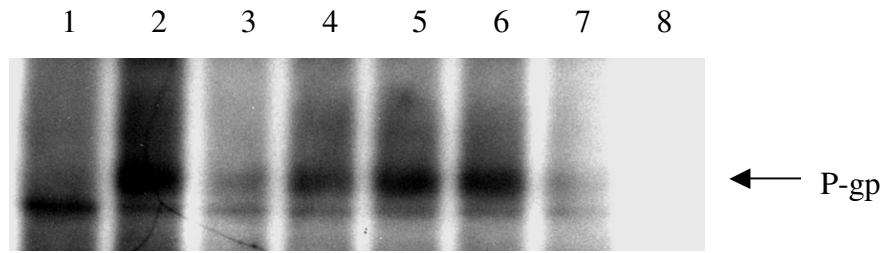
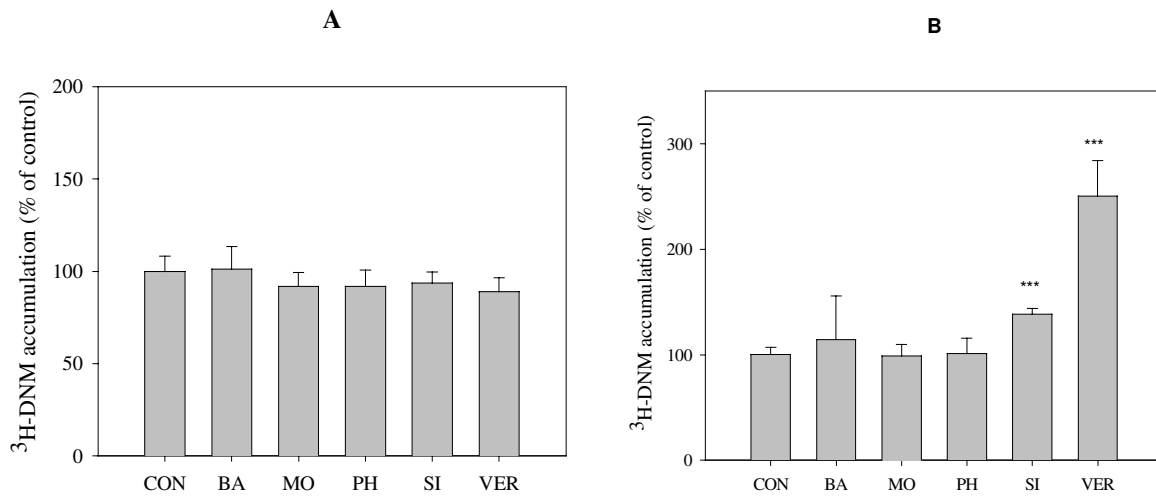


Fig 7



\*\*\*:  $p < 0.001$

Fig 8

