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Inhibition of P-Glycoprotein by Natural Products in Human Breast Cancer Cells

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Multidrug resistance (MDR) is one of the most significant obstacles in cancer chemotherapy. One of the mechanisms involved in the development of MDR is the over-expression of P-glycoprotein (P-gp). It is widely known that natural compounds found in vegetables, fruits, plant-derived beverages and herbal dietary supplements not only have anticancer properties, but may also modulate P-gp activity. Therefore, the purpose of this investigation was to examine the effects of naturally occurring products on P-gp function in human breast cancer cell lines, MCF-7 (sensitive) and MCF-7/ADR (resistant). The accumulation of daunomycin (DNM), a P-gp substrate, was greater in the sensitive cells compared to the resistant cells, while the efflux of DNM was higher in the resistant cells compared to the sensitive cells over a period of 2 h. The IC_{50} value of DNM in the resistant cells was about 22 times higher than that in the sensitive cells, indicating an over-expression of P-gp in the resistant cells, MCF-7/ADR. All of the compounds tested, with the exception of fisetin, significantly decreased the IC_{50} value of DNM. Biochanin A showed the greatest increase in [^{3}H]-DNM accumulation, increasing by 454.3 ± 19.5% in the resistant cells, whereas verapamil, the positive control, increased the accumulation by 229.4 ± 17.6%. Also, the accumulation of [^{3}H]-DNM was increased substantially by quercetin and silymarin while it was reduced by fisetin. Moreover, biochanin A, silymarin, and naringenin significantly decreased DNM efflux from MCF-7/ADR cells compared with the control. These results suggest that some flavonoids such as biochanin A and silymarin may reverse MDR by inhibiting the P-gp function.

Key words: P-glycoprotein, Natural Products, MCF-7/ADR Cells, Daunomycin

INTRODUCTION

At times chemotherapy has not been successful due to the broad-spectrum resistance of cancer cells to diverse chemotherapeutic agents. This is a phenomenon called multidrug resistance (MDR) by which tumor cells that have been exposed to one cytotoxic agent develop cross-resistance to a wide range of structurally and functionally unrelated cytotoxic compounds, such as anthracyclines, *vinca* alkaloids, epipodophyllotoxins, paclitaxel, doxorubicin, and actinomycin D (Gottesman and Pastan, 1993). These anticancer drugs associated with MDR are naturally occurring hydrophobic and amphipathic compounds.

The major form of MDR is the over-expression of a drug efflux transporter, P-glycoprotein (P-gp) (Juliano and Pastan, 1976; Kartner *et al.*, 1983). P-gp is a 170-kDa plasma membrane protein encoded by human MDR1 and MDR3 genes and is a member of the ATP-binding cassette superfamily of transport proteins (Doige and Ames, 1993; Higgins, 1992). P-gp is composed of two homologous halves, containing six transmembrane domains and an ATP binding domain (Ambudkar *et al.*, 1999). ATP binding and hydrolysis appear to be important in ensuring the P-gp, drug efflux pump to function properly (Horio *et al.*, 1988). Therefore, the reversal of P-gp activity may improve the outcome of cancer therapy (Shikic, 1993; Kasahara *et al.*, 1992; Yahanda *et al.*, 1992).

Since Tsuruo *et al.* initially described (1981) that verapamil, a calcium channel blocker, was able to down-modulate P-gp activity, many compounds that inhibit P-gp mediated transport have been identified (Ford *et al.*, 1990). Such modulators, also termed chemosensitizers or MDR reversing agents, include verapamil, cyclosporine, PSC-833, surfactants, and steroidal agents (Fardel *et al.*, 1996). These chemosensitizers were clinically dropped

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out by their own toxicities that occur at pharmacological doses, which are required to achieve a significant reversal of MDR (Raderer *et al.*, 1993).

It is widely known that natural compounds found in vegetables, fruits and some plants have anticancer, antiviral, and antioxidant properties. It has also been reported that some may modulate P-gp activity (Chieli *et al.*, 1995; Critchfield *et al.*, 1994; Ikegawa *et al.*, 2002; Phang *et al.*, 1993; Potter, 1997; Scambia *et al.*, 1994; Wargovich, 1997). P-gp inhibitors found in natural products, especially those found in traditional medicine and dietary supplements, have the potential to be developed as MDR reversing agents which could lead to more successful chemotherapy.

Therefore, the objective of the present investigation was to examine the effects of naturally occurring compounds on P-gp mediated drug efflux in MCF-7/ADR cells.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, 0.25% trypsin-1 mM EDTA, and penicillin (10,000 units/mL)-streptomycin (10,000 μg/mL) were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Characterized fetal bovine serum (FBS) was obtained from Hyclone (South Logan, UT, U.S.A.). Hanks' balanced salts without sodium bicarbonate (HBSS), L-glutamine, sodium bicarbonate ($NaHCO_3$), daunomycin (DNM), verapamil, quercetin, fisetin, berberine, naringenin, artemisinin, biochanin A, daidzein, morin, silymarin, cephalotaxine, dimethyl sulfoxide (DMSO), sulforhodamine B (SRB), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). *N*-(2-hydroxyethyl) piperazine-*N*'-2-ethanesulfonic acid (HEPES), Triton®X-100, and Tris base were supplied by USB (Cleveland, OH, U.S.A.). NaCl, KCl, $MgCl_2$ were obtained from Duksan pure chemical (Ansan, Korea), $CaCl_2$ was purchased from Showa chemical (Tokyo, Japan), and acetic acid was obtained from Daejung (Siheung, Korea). Microscint™40 (scintillation cocktail) was obtained from Packard Instrument Co. Inc. (Meriden, CT, U.S.A.), [^{3}H]-DNM (16 Ci/mmol) was supplied by Perkin Elmer Life Science (Boston, MA, U.S.A.). In addition, the following instruments were used in this study; cell incubator (3158, Forma Scientific Inc., Marietta, OH, U.S.A.), liquid scintillation counter (Topcount NXT, Packard Instrument Co. Inc., Meriden, CT, U.S.A.), orbital shaker (SLOS-20, SLB, Seoul, Korea) and ELISA reader (3550, Bio-Rad, Hercules, CA, U.S.A.).

Cell culture

MCF-7 (sensitive) and MCF-7/ADR (resistant) cells were generously provided by Dr. Marilyn E. Morris (State University of New York at Buffalo, New York, U.S.A.).

Both cell lines were maintained in a RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 24 mM $NaHCO_3$, 100 units/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere containing 5% CO_2 - 95% air at 37°C. Both cell lines were used between 16 and 30 passages.

Cytotoxicity assay

Cells were seeded in 96-well plates at a density of 5000 cells per well. Following 24 h of incubation at 37°C, various concentrations of daunomycin, 9×10^{-8} M to 7.2×10^{-5} M, were added to each well in the presence and absence of naturally occurring compounds; 100 μM of quercetin, fisetin, berberine, artemisinin, biochanin A, daidzein, morin, silymarin, cephalotaxine, and 50 μM of naringenin. Daunomycin with 100 μM of verapamil was used as a positive control. Following 2 h of incubation, the cells were washed twice with HBSS and fresh media were added. Following 72 h of incubation, cytotoxicity was measured by SRB staining assay as described previously (Skehan *et al.*, 1990) with certain modifications.

[^{3}H]-Daunomycin accumulation study

The daunomycin accumulation study was performed as described previously (Harker *et al.*, 1985) with modifications. For this study, cells (150,000 cells in 3 mL) were seeded in each well of a 6-well plate. After reaching 80~90% confluence, the cells were washed with 1 mL of the uptake buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES, pH 7.4). Then, the incubation buffer (0.05 μM of [^{3}H]-DNM dissolved in uptake buffer) was added to each well and the cells were incubated for the designated time periods at 37°C. At the end of the incubation, the accumulation of DNM was terminated by rinsing the cells with the ice-cold stop solution (137 mM NaCl and 14 mM Tris base, pH 7.4). Cells were then solubilized with 1% Triton®X-100, and the radioactivity of each aliquot was determined using the liquid scintillation counter. The effect of naturally occurring compounds (dissolved in DMSO, not greater than 0.4 v/v % for all the experiments and control) was examined by adding the compounds, at a final concentration of 100 μM and [^{3}H]-daunomycin at the same time and measuring daunomycin accumulation for 2 h. However, the final concentration of naringenin used in the study was 50 μM. Verapamil (100 μM), a representative P-gp inhibitor, was used as a positive control.

[^{3}H]-Daunomycin efflux study

For the daunomycin efflux study, cells (150,000 cells in 3 mL) were seeded in each well of a 6-well plate. After reaching 80~ 90% confluence, the cells were washed with the uptake buffer and 0.05 μM of [^{3}H]-daunomycin was

added to each well. After 1 h, the cells were washed again with the uptake buffer and the drug-free uptake buffer was then added to each well and incubated for the designated time periods at 37°C. Daunomycin efflux was terminated by washing the cells with the ice-cold stop solution. The cells were lyzed with 1% Triton®X-100 and the aliquots were used to determine radioactivity by liquid scintillation counter. The effect of naturally occurring compounds was examined by adding the compounds, at a final concentration of 100 μM, with the drug-free uptake buffer and measuring daunomycin efflux for 1 h. For naringenin, the final concentration of 50 μM was used in the study.

Statistical analysis

The data were analyzed using the unpaired Student's *t*-test between the control and compounds. P-values < 0.05 were considered to be statistically significant.

RESULTS

Characterization of MCF-7 and MCF-7/ADR cells

It has been reported that P-gp is over-expressed in the MCF-7/ADR cell line (Zhang and Morris, 2003). To confirm this, the inhibition of cell growth on increasing DNM concentrations was examined in MCF-7 (sensitive) and MCF-7/ADR (resistant) cells (Fig. 1). The IC_{50} value of DNM in the resistant cells was approximately 22 times higher than that in the sensitive cells (41.6 ± 0.90 μM vs. 1.79 ± 0.01 μM).

The accumulation of 0.05 μM DNM was examined in MCF-7 and MCF-7/ADR cells up to 2 h (Fig. 2A). The accumulation of DNM was significantly greater in MCF-7 cells compared to MCF-7/ADR cells for the entire 2 h. In addition, after loading MCF-7 and MCF-7/ADR cells with 0.05 μM DNM for 1 h, the efflux of DNM was determined for 2 h (Fig. 2B). The efflux of DNM was higher in MCF-7/ADR cells compared to MCF-7 cells for the entire 2 h. These results confirm the over-expression of P-gp in the resistant (MCF-7/ADR) cells.

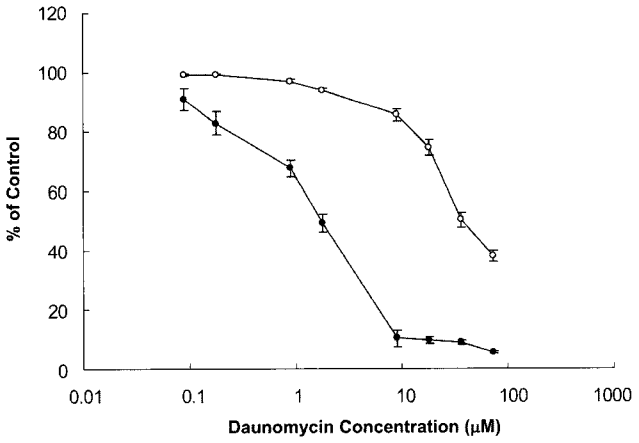

Fig. 1. Daunomycin cytotoxicity in MCF-7 (●; sensitive) and MCF-7/ADR (○; resistant) cells. The effect of various concentrations of DNM on cell growth in both cell lines was examined. Each data point represents mean ± S.D. from three wells in two experiments.

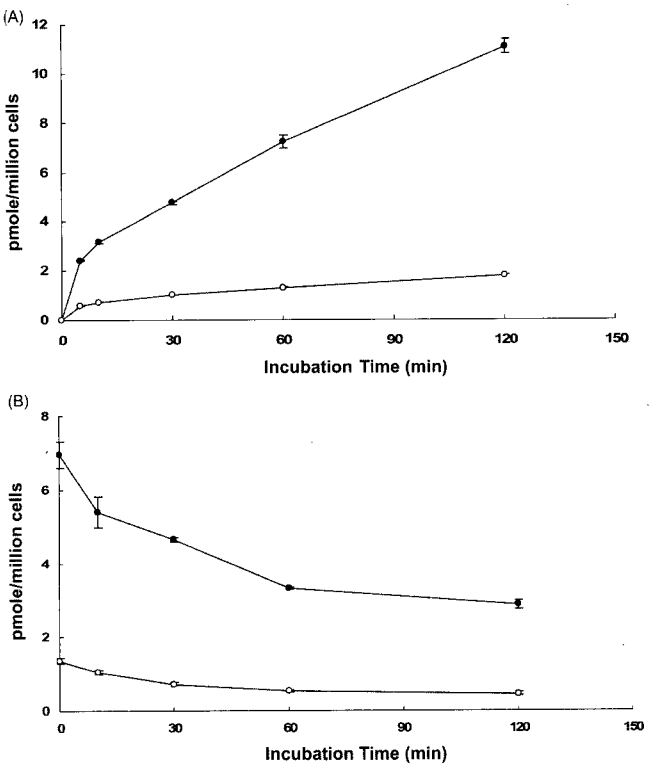

Fig. 2. (A) Time dependent accumulation of [^{3}H]-DNM in MCF-7 (●) and MCF-7/ADR (○) cells. (B) Time dependent efflux of [^{3}H]-DNM from MCF-7 (●) and MCF-7/ADR (○) cells. Each data point represents the mean ± S.D. from three wells in one representative study.

Effect of natural products on daunomycin cytotoxicity

Nine naturally occurring compounds and verapamil were not cytotoxic when the MCF-7/ADR cells were treated with the compounds at a concentration of 100 μM. Naringenin was used for the study at a concentration of 50 μM, which was not cytotoxic (data not shown).

The growth inhibition of MCF-7/ADR cells was examined in the presence of naturally occurring compounds. As shown in Table I, all the compounds except fisetin significantly reduced the IC_{50} value of DNM. Among them, biochanin A and silymarin strongly potentiated cytotoxicity of DNM, with results similar to that of the P-gp inhibitor, verapamil.

Effect of natural products on [^{3}H]-daunomycin accumulation

The effect of various natural compounds on 0.05 μM

Table I. Effects of natural products on the IC_{50} values of DNM in MCF-7/ADR cells after 2 h of incubation

Compounds	IC_{50} values (μM)
Control (DNM)	40.8 ± 2.6
Verapamil	8.0 ± 0.5**
Quercetin	29.8 ± 4.0*
Fisetin	40.5 ± 2.0
Berberine	26.4 ± 4.3*
Naringenin	32.6 ± 0.1*
Artemisinine	32.0 ± 6.0*
Biochanin A	11.0 ± 1.1**
Daidzein	33.8 ± 1.5*
Morin	26.9 ± 2.7*
Silymarin	16.0 ± 0.7**
Cephalotaxine	21.8 ± 3.7**

* p< 0.01, ** p< 0.001 compared with control
Values are presented as mean ± S.D. from triplicate experiments.

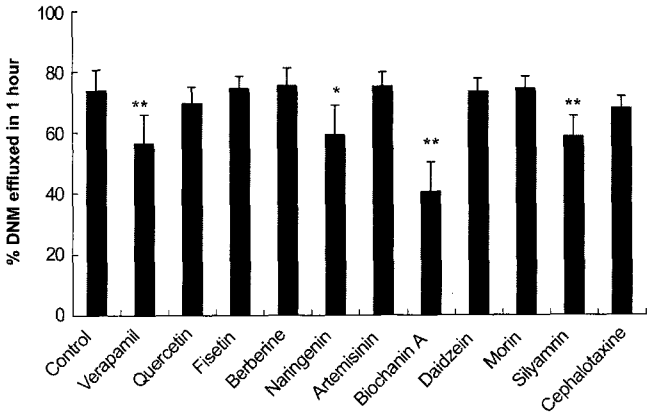

Fig. 4. Effects of natural products on [^{3}H]-DNM efflux from MCF-7/ADR cells. The efflux of DNM was examined in the presence of naturally occurring compounds for 1 h. Control represents DNM efflux in the absence of the compounds. Each data point represents the mean ± S.D. from triplicate measurements in three experiments (* p<0.05 and ** p<0.01).

[^{3}H]-DNM accumulation was examined in MCF-7/ADR cells (Fig. 3). All compounds were used at a concentration of 100 μM while naringenin was used at a concentration of 50 μM. As shown in Fig. 3, biochanin A, quercetin and silymarin displayed a dramatic increase in DNM accumulation. Biochanin A showed the greatest increase in DNM accumulation (454.3 ± 19.5%) while DNM accumulation in the presence of quercetin (201.8 ± 16.4%) and silymarin (224.9 ± 2.8%) was similar to that seen with the positive control, verapamil (229.4 ± 17.6%). Also, cephalotaxine, morin, and naringenin significantly increased the 2 h accumulation of DNM by 129.1 ± 4.6%, 129.9 ± 2.6%, and 130.6 ± 0.9%, respectively, while fisetin significantly reduced DNM accumulation. On the other hand, artemisinine, berberin, and daidzein had no effect on DNM accumulation.

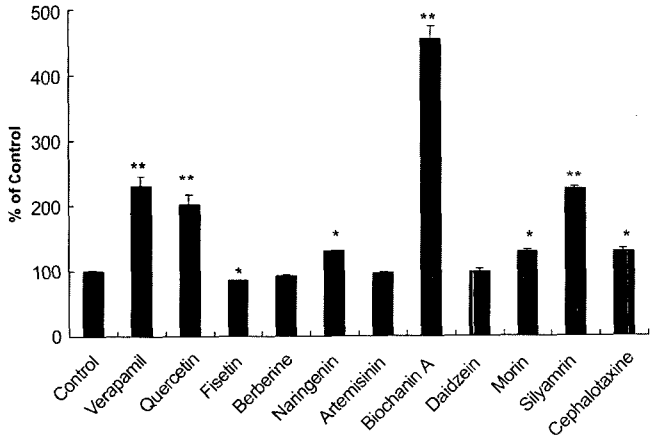

Fig. 3. Effects of natural products on [^{3}H]-DNM accumulation in MCF-7/ADR cells. DNM accumulation was examined in the presence of naturally occurring compounds for 2 h. Control represents DNM accumulation in the absence of the compounds. Each data point represents the mean ± S.D. from triplicate measurements in three experiments (* p<0.01 and ** p<0.001).

Effect of natural products on [^{3}H]-daunomycin efflux

The effect of various natural products on [^{3}H]-DNM efflux was examined (Fig. 4). MCF-7/ADR cells were loaded with 0.05 μM [^{3}H]-DNM for 1 h and then allowed to efflux of [^{3}H]-DNM for more than one hour in the presence of naturally occurring compounds (50 μM naringenin and 100 μM other compounds).

Of the compounds examined, biochanin A, silymarin, and naringenin, which were active in DNM accumulation, significantly decreased the efflux of DNM whereas cephalotaxine, morin, and quercetin, which increased DNM accumulation, did not show any effect on DNM efflux.

DISCUSSION

The intracellular concentration of many anticancer drugs is decreased by P-gp, the ATP-dependent efflux pump, which is over-expressed in tumor cells (Juliano and Pastan, 1976; Kartner *et al.*, 1983). Compounds that inhibit the expression and functional activity of P-gp will increase cellular accumulation of many chemotherapeutic agents and reverse MDR. However, the first-generation of P-gp inhibitors have proven to be too toxic to be used clinically at pharmacologically active doses (Raderer *et al.*, 1993).

With increasing interest in alternative medicine, herbal products are taken by about 10% or more of the general

population and 30 to 70% of individuals with specific diseases (Duggan *et al.*, 2001; Ni *et al.*, 2002). Moreover, many studies have shown that vegetables and fruits are associated with lower risk of developing cancers (Potter, 1997; Wargovich, 1997). Recently, flavonoids have been considered to be a new class of chemosensitizers, which interact with both the ATP binding site and its vicinal steroid-interacting hydrophobic sequence of P-gp (Conseil *et al.*, 1998). According to a review on flavonoids by Kuhnau, flavonoids are ubiquitously occurring non-toxic plant products (Kuhnau, 1976). Most of the compounds examined in this study were flavonoids, including biochanin A, diadzein, fisetin, morin, naringenin, quercetin, and silymarin.

In the present study, biochanin A and silymarin significantly decreased the IC_{50} value of DNM, potentiating the cytotoxicity of DNM. In addition, quercetin, cephalotaxine, berberine, morin, artemisinin, and daidzein significantly reduced the IC_{50} value of DNM (Table I). Biochanin A exhibited the greatest increase in DNM accumulation while DNM accumulation with quercetin and silymarin was similar to that of a well-known P-gp inhibitor, verapamil (Fig. 3). Moreover, biochanin A, silymarin, and naringenin significantly decreased DNM efflux (Fig. 4). These results suggest that biochanin A and silymarin appear to be potent and safe P-gp inhibitors that can increase the efficacy of chemotherapeutic agents when administered concomitantly. It has been reported that quercetin and morin derivatives accumulated vincristine into adriamycin-resistant human myelogenous leukemia cell, K562/ADM (Ikegawa *et al.* 2002). From this point of view, compounds like naringenin, cephalotaxine, quercetin, and morin may also be developed as powerful P-gp inhibitors with some modifications to their functional groups.

In conclusion, of the compounds tested, biochanin A and silymarin substantially potentiated DNM cytotoxicity, increased DNM accumulation and decreased DNM efflux in the resistant cell line, MCF-7/ADR. It is suggested that these two naturally occurring products can be used as safe and potent MDR reversing agents in clinical chemotherapy by administering concomitantly with anticancer drugs.

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