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Study on the Mechanism of P-glycoprotein Inhibitory Activity of Silymarin in Human Breast Cancer Cell

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ABSTRACT – Silymarin showed P-glycoprptein (P-gp) inhibitory activity as much as verapamil, a well-known P-gp inhibitor, by decreasing IC_{50} value of daunomycin (DNM) (16.0±0.7 μM), increasing the DNM accumulation (224.9±3.2%), and decreasing DNM efflux (58.5±6.7%), concurrently. In this study, we clarified the mechanism of action of silymarin for P-gp inhibitory function. First, silymarin may bind to the ATP-binding site and thus, prevent ATP hydrolysis. Second, the P-gp inhibitory activity of silymarin is not related to changing the cellular P-gp level. Third, the cytotoxicity of silymarin was increased in the presence of verapamil, reflecting that silymarin is a competent P-gp substrate against verapamil in the P-gp-overexpressed adriamycin-resistant MCF-7 breast cancer (MCF-7/ADR) cells. Conclusively, silymarin had the P-gp inhibitory activity through the action of competent binding to the P-gp substrate-binding site. Therefore, silymarin can be a good candidate for safe and effective MDR reversing agent in clinical chemotherapy by administering concomitantly with anticancer drugs.

Key words – P-glycoprotein, Silymarin, MCF-7/ADR cells, Daunomycin

The overexpression of the plasma membrane transporter, P-glycoprotein (P-gp), is one of the main causes of multidrug resistance (MDR).[1] The function of P-gp, associated with MDR in cancer cell, is an ATP-driven efflux pump, resulting in decrease of intracellular anticancer drug concentration.[2] The P-gp overexpression in the chemotherapy-treated patients implicates a treatment failure. In order to overcome the failure of cancer chemotherapy induced by MDR, numerous researchers have been making many efforts to search better chemo-sensitive compound, P-gp modulator, among biological active components such as natural medicines and functional foods.[3] Especially, flavonoids have been focused due to lack of toxicity and cancer preventive properties, and it has been reported that a wide range of flavonoids have strong cytotoxicity against disease-outbreaking cells with effectiveness and safety.[4]

Our laboratory tested several well-known flavonoids, such as biochanin A, morin, naringenin, quercetin, and silymarin to examine their activities against P-gp mediated MDR using human breast cancer cell lines, MCF-7 (sensitive) and MCF-7/ADR (resistant).[5] Silymarin significantly decreased the IC_{50} value of daunomycin (DNM) and increased the [^{3}H]-DNM accumulation by 225±3% and decreased [^{3}H]-DNM efflux by 58.5±6.7%, whereas verapamil, the positive control, increased the accumulation by 229±18% and decreased the efflux by 56.4±9.5%. These results suggested the possibility for silymarin to function as a MDR reversal chemosensitizer by inhibiting the P-gp activity.

There are several possible mechanisms of chemosensitizers in modulation of MDR. Most chemosensitizers show their activity to modulate MDR through direct blocking of P-gp activity by binding to chemotherapeutic drug binding sites, or by binding to other allosteric binding sites which cause inhibition of drug binding or transport later.[6] The other approach for MDR modulation is down-regulation of P-gp expression.[7] In the present study, these possible mechanisms have been examined to clarify how silymarin acts for inhibition of P-gp function.

Materials and Methods

Materials

Characterized fetal bovine serum (FBS) was purchased from Hyclone (South Logan, UT, USA), and RPMI 1640 medium, 0.25% trypsin-1 mM EDTA, and penicillin (10,000 units/mL)-streptomycin (10,000 μg/mL) were from Invitrogen (Carlsbad, CA, USA). Hanks' balanced salts without sodium bicarbonate (HBSS), L-glutamine, sodium bicarbonate ($NaHCO_3$), DNM, verapamil, silymarin, dimethyl sulfoxide (DMSO), adenosine 5-triphosphate (ATP), sodium azid (NaN_3), amonium molybdate tetrahydrate, atifoam A concentrate, anti β-actin clone

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AC-74, DL-dithiothreitol (DTT), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 2-[N-morpholino]ethanesulfonic acid (MES) hydrate, sodium orthovanadate, sulforhodamine B (SRB) and trichloroacetic acid (TCA) were supplied by Sigma-Aldrich (St. Louis, MO, USA). N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES), Triton®X-100 and Tris base were obtained from USB (Cleveland, OH, USA), $CaCl_2$ was from Showa Chemical (Tokyo, Japan), and NaCl, KCl, and $MgCl_2$ were from Duksan Pure Chemical (Ansan, Korea), and acetic acid was from Daejung (Siheung, Korea). Microscint™40 (scintillation cocktail) was purchased from the Packard Instrument Co. Inc. (Meriden, CT, USA), and [^{3}H]-DNM (16 Ci/mmol) was from Perkin Elmer life science (Boston, MA, USA). Human P-gp membrane and P-gp negative control membrane was purchased from BD Bioscience (Woburn, MA, USA). Ammonium persulfate and ECL plus western blotting detection reagents were obtained from Amersham Biosciences (Buckinghamshire, UK). Monoclonal mouse anti-human P-gp clone (C219) was supplied by DakoCytomation Inc. (Carpinteria, CA, USA). EIL grade affinity purified gout anti-mouse IgG horseradish peroxidase conjugate was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). In addition, the following instruments were used in this study; cell incubator (3158, Forma Scientific Inc., Marietta, OH, USA), liquid scintillation counter (Topcount NXT, Packard Instrument Co. Inc., Meriden, CT, USA), orbital shaker (SLOS-20, SLB, Seoul, Korea) and ELISA reader (3550, Bio-Rad, Hercules, CA, USA).

Cell line and cell culture

P-gp-overexpressing MCF-7/ADR cells were generous gift from Dr. Marilyn E. Morris (State University of New York at Buffalo, USA). The P-gp overexpression in this cell line was checked by western blot (data not shown) and also confirmed by examining the inhibition of cell growth upon increasing DNM in MCF-7 (sensitive) and MCF-7/ADR (resistant) cells. The IC_{50} value of DNM in the resistant cells was approximately 22 times higher than that in the sensitive cells.[5] Cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 24 mM $NaHCO_3$, penicillin (100 units/mL), and streptomycin (100 μg/mL) and maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cytotoxicity assay

The cells were incubated with various concentrations of DNM in the range from 0.09 to 72 μM with and without 100 μM silymarin for 2 hrs. The cells were then washed and incubated for 72 hrs with fresh media. The *in vitro* cytotoxicity of DNM was measured using a SRB staining assay as previously reported.[5,8,9]

In order to test whether silymarin has a direct interaction with P-gp substrate binding site, the change in the cytotoxicity of silymarin with the concentration from 10 μM to 1 mM was monitored in the presence and absence of 100 μM verapamil, which is the non-cytotoxic concentration but can inhibit P-gp activity in MCR-7/ADR cells.

[^{3}H]-daunomycin accumulation and efflux study

The DNM accumulation and efflux study was conducted as reported previously.[5] The cells were washed with 1 mL of the solution containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4, uptake buffer) and incubated with uptake buffer containing 0.05 μM of [^{3}H]-DNM (incubation buffer) in the presence and absence of 100 μM silymarin for 2 hrs at 37°C. The final concentration of 100 μM verapamil, a representative P-gp inhibitor, was used as a positive control. The extent of DNM accumulation was determined by measuring the radioactivity of [^{3}H]-DNM using a liquid scintillation counter (Topcount NXT, Packard Instrument Co. Inc., Meriden, CT, USA). For DNM efflux study, the cells were washed with 1 mL of uptake buffer and incubated with 1 mL of incubation buffer for 1 hr. Cells were then washed with 2 mL of uptake buffer and incubated again in uptake buffer with and without 100 μM silymarin for an additional 1 hr to determine the DNM efflux effect. The amount of DNM effluxed for 1 hr was calculated by subtracting the amount of DNM remained in the cells at 1 hr after treated with silymarin from total pre-accumulated amount of DNM.

Human P-glycoprotein ATPase assay

P-gp ATPase activity was determined using human P-gp membrane and P-gp negative control membrane according to the manufacturer's instructions. Briefly, the ATPase activities of human P-gp membrane and P-gp negative control membrane were estimated by measuring the amount of inorganic phosphate (Pi) liberated from the hydrolysis of ATP. The potassium phosphate standards were prepared in the solution containing 50 mM MES hydrate, 2 mM EGTA, 2 mM dithiothreitol, 50 mM KCl, and 5 mM NaN_3 (pH 6.8, Tri-MES buffer), and added into each well in duplicate to achieve the final concentrations of 0, 3, 9, 30, 60, 90, 120 and 150 nM. Then, 100 μM of silymarin, dissolved in DMSO, was added to the plate. The final concentration of DMSO in the assay solution was not greater than 0.2 v/v % for all the experiments. Verapamil (100 μM), a well-known P-gp ATPase stimulator

and P-gp inhibitor, was used as a positive control. And then, 20 μL of human P-gp membrane or P-gp negative control membrane (40 μg) with or without 100 μM sodium orthovanadate were loaded to all the wells except for those containing the phosphate standards, followed by incubation of the plate at 37°C for 5 minutes. Continuously, 20 μL of the MgATP (4 mM in final) was added for initiating the ATPase reaction and then incubated at 37°C for 0 or 20 minutes. The reaction was quenched by addition of 10% SDS solution containing antifoam A. The ATPase activity was estimated by the difference in Pi obtained between the 0-min and the 20-min incubation periods. Pi was measured by colorimetric reaction as follows. After APTase reaction was quenched, 200 μL of detection reagent (10% of ascorbic acid (pH 5.0):35 mM ammonium molybdate in 15 mM zinc acetate (pH 5.0)=4:1) was immediately added and then incubated for 20 minutes at 37°C. Then, Pi was detected by reading the absorbance at 655 nm with ELISA reader and quantified by comparing the absorbance to a phosphate standard curve. The difference between ATPase activity in the absence and presence of 100 μM orthovanadate was also determined. The ATPase activity was expressed as the rate of phosphate release per milligram of membrane protein.

Western blot analysis

Cells were seeded in 6-well plate at a density of approximately 150,000 cells per well. The cells were washed twice with 3 mL of uptake buffer when reached 80~90% confluence, and treated with uptake buffer with or without 100 μM of silymarin for 2 hrs at 37°C. And then, the cells were washed twice with 2 mL of d-PBS and harvested using a cell scraper. A 60 μL of the lysis buffer, containing 150 mM NaCl, 50 mM Tris·HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 200 μM sodium orthovanadate, and 1% protease inhibitor cocktail, was added to the harvested cells to prepare the cell lysate. The cell lysate was kept on ice with shaking for 30 minutes and then centrifuged at 12,000 g for 20 minutes at 4 to obtain the soluble extracts. The soluble extracts were stored at −70°C unless it was used. The protein concentrations of soluble extracts were obtained by bicinchoninic acid protein assay.[10] The proteins (20 μg) were electrophoresed using 8% SDS-Polyacrylamide gel and transferred overnight onto nitrocellulose membrane. Membranes were then blocked for 1 hr at room temperature in Tris-buffered saline containing 0.2% Tween 20 (TTBS) with 5% skim milk, and then incubated with first antibodies, followed by incubation of second antibody at room temperature for 2 hrs and 1.5 hrs, respectively. The primary antibodies used were C219 monoclonal antibody (1:1000 in TTBS with 3% skim milk) for P-gp and anti-β-actin (1:5000 in TTBS with 3% skim milk) for β-actin, which was used as loading control. Anti-mouse IgG horseradish peroxidase was used as secondary antibody. After incubation with each antibody, membranes were washed three times with 37°C-prewarmed TTBS with shaking for 5 minutes. And then, membranes were detected with ECL plus western blotting detection reagent. The western blot images were analyzed using Multi-Gauge Software (Fuji photo film Co., Ltd., Tokyo, Japan).

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

Effect of silymarin on P-glycoprotein function

Silymarin and verapamil at a concentration of 100 μM were not cytotoxic for the MCF-7/ADR cells. As shown in Table I, silymarin distinctly decreased IC_{50} values of DNM, supporting that it increased the efficacy of DNM in the resistant cells.[5]

The effect of silymarin on [^{3}H]-DNM accumulation and efflux using MCF-7/ADR cells depicted in Figure 1 is redrawn from the data published previously by our group.[5] Silymarin increased DNM accumulation by 225±3% which was similar to the increase of 229±18% by the positive control, verapamil.

Silymarin extruded the pre-accumulated [^{3}H]-DNM by 58.5 ±6.7%, suggesting that silymarin decreased the P-gp-mediated drug efflux efficiently as much as verapamil (56.4±9.5%).

Effect of silymarin on P-glycoprotein ATPase activity

Silymarin showed no significant change on orthovanadate-sensitive ATPase activity in P-gp negative control membrane. However it seemed to show the inhibitory effects on orthovanadate-sensitive ATPase activity in P-gp positive membrane

Table I–*Effect of Silymarin on Daunomycin Cytotoxicity in MCF-7/ADR Cells after 2 hrs of incubation*

Compounds	IC_{50} values (μM)
Control (DNM)	40.8±2.63
Verapamil	7.99±0.49*
Silymarin	16.0±0.68*

Values are from data published previously by our group[5] and present mean±S.D. from triplicate experiments.
*p<0.001 compared with control.

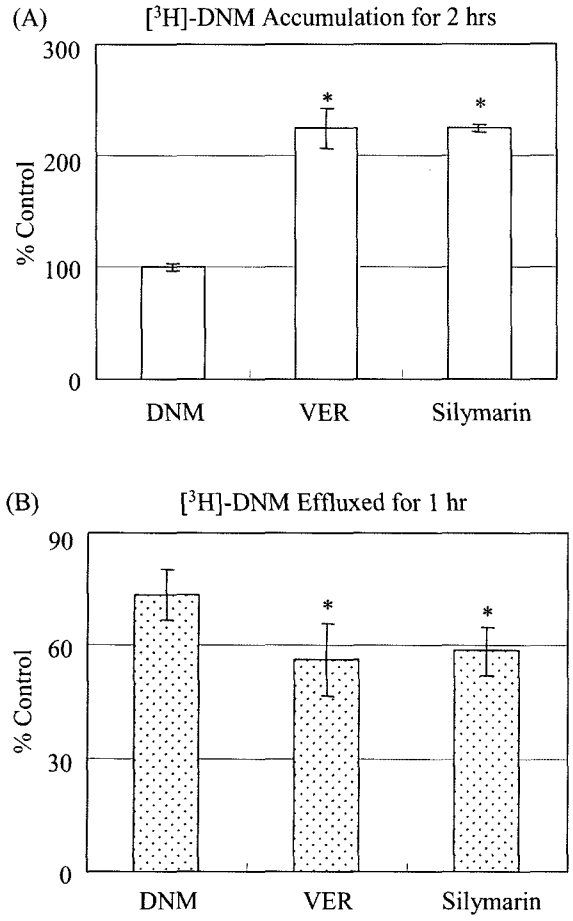

Figure 1–Effect of silymarin on [^{3}H]-DNM accumulation (A) and efflux (B) in MCF-7/ADR cells. (A) DNM accumulation was examined in the presence of silymarin for 2 hrs. Control represents DNM accumulation in the absence of silymarin. Each bar represents the mean±S.D. from triplicate measurements in three experiments (*$p<0.001$). (B) The efflux of DNM was examined in the presence of silymarin for 1 hr. Control represents DNM efflux in the absence of silymarin. Each bar represents the mean±S.D. from triplicate measurements in three experiments (*$p<0.01$). Verapamil, a typical P-gp inhibitor, was used as a positive control. These results were adopted from the data published previously by our group.[5]

Table II–*Effect of Silymarin on the P-glycoprotein ATPase Activity (mol/min/mg protein)*

Compounds	P-gp negative membrane	P-gp positive membrane
Control	4.36±3.12	5.94±1.28
Verapamil	4.89±5.28	33.0±1.92*
Silymarin	4.96±3.20	0.65±3.85

Control represents P-gp ATPase activity in the absence of the compounds. Values present mean±S.D. from triplicate experiments. *$p<0.001$ compared with control.

(Table II). The positive control, verapamil, significantly stimulated ATPase activity in P-gp positive membrane.

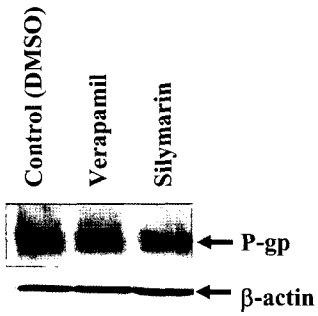

Figure 2–Effect of silymarin on P-glycoprotein expression in MCF-7/ADR cells. The cellular P-gp level was determined by western blot analysis with anti-P-gp monoclonal antibody C219. Each lane contained 20 μg of protein and β-actin was used as a loading control.

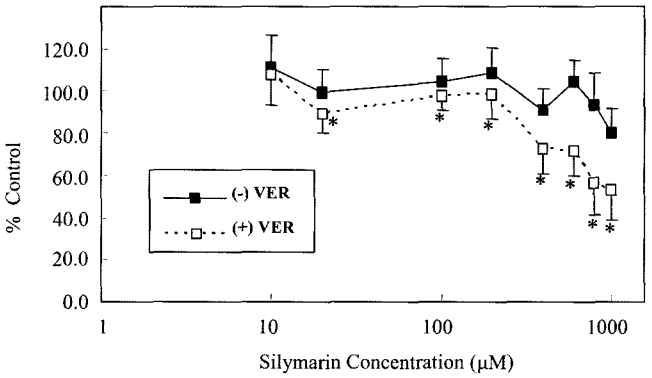

Figure 3–Effect of P-gp inhibition on the silymarin cytotoxicity. The silymarin cytotoxicity with and without verapamil (100 μM) was examined using a SRB staining assay in MCF-7/ADR cells. Each data point represents the mean±S.D. from three experiments (*$p<0.05$).

Effect of silymarin on cellular P-glycoprotein level

The cellular P-gp expression levels of MCF-7/ADR cells after treated with verapamil and silymarin, respectively, for 2 hrs were examined by western blot analysis (Figure 2). As shown in Figure 2, silymarin did not change P-gp expression level of which relative to control were 99.0±5.1% for verapamil, 97.6±25.6% for silymarin, respectively.

Effect of P-glycoprotein inhibition on silymarin cytotoxicity

As shown in Figure 3, silymarin itself without verapamil showed almost no cytotoxicity whereas it showed the decrease of % control in the presence of 100 μM verapamil, which was not cytotoxic to MCF-7/ADR cells. This indicates that silymarin is likely to be a competitive substrate for P-gp against verapamil.

Discussion

MDR has been recognized as one of major problems for

patients under treatment of anticancer drugs. P-gp overexpression in tumor cells is one of major reasons to cause the MDR due to its action of extruding cytotoxic drugs via ATP-dependent efflux pump.[11,12] Thus, compounds that suppress the P-gp expression and functional activity may increase cellular accumulation of chemotherapeutic agents and enhance their efficacies. The P-gp inhibitors have been focused to reverse MDR for successful chemotherapy. However, the P-gp inhibitors, such as verapamil and cyclosporine, have been demonstrated to have undesirable and toxic effects at the pharmacologically active doses.[13] Therefore, the natural products like flavonoids have been of extensive interest for many researchers to develop safe and efficient P-gp inhibitors because of several reasons as follows; flavonoids have been intensively studied and already broadly used as dietary supplements or alternative herbal medicines, reflecting that their safety problems are proved; recently, it has been found that some flavonoids possessing P-gp modulating activity interact with its vicinal ATP-binding site, substrate-binding site, and steroid-binding site.[14,15]

With this matter, we examined some flavonoids, including biochanin A, diadzein, fisetin, morin, naringenin, quercetin, and silymarin, for testing their P-gp inhibitory activity. It turned out silymarin possessed P-gp inhibitory activity as much as verapamil, a well-known P-gp inhibitor, by decreasing IC_{50} value of DNM (16.0±0.7), increasing the DNM accumulation (225±3%), and decreasing DNM efflux (58.5±6.7%), concurrently.[5]

In this study, we clarified the mechanism of action of silymarin for P-gp inhibitoty function. ATP hydrolysis by P-gp is highly coupled with the function of P-gp and drug transport.[16,17] P-gp ATPase activity was measured by a colorimetric assay. Since orthovanadate inhibits P-gp activities by inducing trap on a catalytic site of nucleotide,[18] the ATPase activity measured in the presence of orthovanadate indicates non-P-gp ATPase activity. The activity of non-P-gp ATPase was subtracted from the activity generated in the absence of orthovanadate to yield orthovanadate-sensitive ATPase activity. Silymarin showed inhibitory effects (Table II) on orthovanadate-sensitive ATPase activity in P-gp positive membrane, suggesting that silymarin may bind to the ATP-binding site or steroid-binging site of P-gp, and thus, prevent ATP hydrolysis. After 2 hrs of treatment with silymarin, cellular P-gp expression level was evaluated by western blot analysis because the regulation of P-gp expression level is likely to be another possible mechanism involved in P-gp inhibitory activity of silymarin. However, no change in cellular P-gp levels by silymarin was detected (Figure 2). To figure out whether the P-gp inhibitory activity of silymarin originates from binding to P-gp substrate binding site, the cytotoxicity change of silymarin in the presence and absence of verapamil was monitored as shown Figure 3. Silymarin showed the increased cytotoxicity in the presence of verapamil, suggesting that silymarin may directly interact with P-gp substrate binding site.

Conclusively, silymarin had the P-gp inhibitory activity through the action of competent binding to the P-gp substrate binding site, and thus, can be a good candidate for safe and effective MDR reversing agent in clinical chemotherapy by administering concomitantly with anticancer drugs.

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