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Genistein inversely affects tubulin-binding agent-induced apoptosis in human breast cancer cells

Cho-Hwa Liao[a], Shiow-Lin Pan[a], Jih-Hwa Guh[b], Che-Ming Teng[a,*]

[a]*Pharmacological Institute, College of Medicine, National Taiwan University, No. 1, Jen Ai Road, Section 1, Taipei 100, Taiwan*
[b]*School of Pharmacy, College of Medicine, National Taiwan University, No. 1, Jen Ai Road, Section 1, Taipei 100, Taiwan*

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Abstract

Genistein, a natural isoflavone phytoestrogen present in soybeans, has been extensively studied as a chemopreventive or therapeutic agent in several types of cancer. The traditional Asian diet is rich in soy products may explain in part why the incidence of breast cancer in Asian women is relatively low. To improve therapeutic benefits, we investigated the combination of genistein with chemotherapeutic agents in phenotypically dissimilar human breast cancer cells, MCF-7 and MDA-MB-231, in which estrogen receptor expression is positive and negative, respectively. In the present study, genistein significantly decreased cell apoptosis induced by tubulin-binding agents, paclitaxel and vincristine. FACScan analysis revealed that genistein also diminished the accumulation of the G2/M phase in the cell cycle caused by tubulin-binding agents. In situ staining of microtubules revealed that genistein could decrease paclitaxel-induced tubulin polymerization. However, in vivo tubulin polymerization assay revealed that simultaneous treatment of genistein did not change the tubulin/microtubule dynamic. Genistein reduced Bcl-2 phosphorylation triggered by paclitaxel and vincristine without changing Bax protein expression. p53 and p21 expression, monitored by Western blotting, was not altered by genistein. However, the expression of cyclin B1 and CDC2 kinase was markedly decreased in combination with genistein. In conclusion, genistein inversely affected tubulin-binding agent-induced apoptosis via down-regulation of cyclin B1/CDC2 kinase expression resulting in reduced Bcl-2 phosphorylation.

Keywords: Genistein; Paclitaxel; Vincristine; Cyclin B1; CDC2 kinase

Genistein (4′,5,7-trihydroxyisoflavone), a natural isoflavone phytoestrogen found in soybeans, exhibits multiple functions resulting in antitumor effects [1]. Genistein is a potent in vitro inhibitor of tyrosine kinase activity, especially that of the epidermal growth factor receptor. Genistein that possesses heterocyclic phenols is structural resemblance to estradiol and has mixed agonist/antagonist properties [2]. At the concentration above 10 μM, experimental data demonstrated that proliferation of various types of cancer cells was dose- and time-dependently inhibited by treatment with genistein, regardless of p53, estrogen receptor, and androgen receptor status [3,4]. However, at levels as low as 100 nM, genistein stimulates the growth of estrogen-dependent breast cancer tumors in vitro and in vivo at 1 μM and also increases the levels of the estrogen-responsive genes pS2 due to estrogenic activity [5,6].

The anti-proliferative effect of genistein may be due to cell cycle regulation. Genistein increases the expression of $p21^{WAF1}$ and reduces the expression of cyclin B1 [3,7,8]. In addition, apoptosis was induced in genistein-treated cancer cells with an increase in the ratio of Bax to Bcl-2 [4]. Genistein also possesses antioxidant properties and inhibits DNA topoisomerase II activity [9], angiogenesis, and invasion [10,11]. All these effects of genistein contribute to chemoprevention [12]. Experimental evidences prove that dietary soy has a biological effect on animal models of breast cancer, with several studies demonstrating a reduction in the incidence and size of radiation-induced and chemically induced mammary tumors when rats were fed a soy diet [12,14,15]. Epidemiological studies have shown convincing evidence that Asian women consuming a high-soybean diet have a low incidence of certain types of cancer, such as breast, prostate, and colon cancer [13–15]. Furthermore, westerners of Asian emigrants adopt a Western diet are at higher risk of breast cancer [16,17].

* Corresponding author. Tel.: +886-2-23221742; fax: +886-2-23221742.
E-mail address: cmteng@ha.mc.ntu.edu.tw (C.-M. Teng).

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Microtubules, the self-assembly of α- and β-tubulin heterodimers, are important cytoskeletal components involved in the regulation of cell proliferation, differentiation, and apoptosis. Tubulin-binding agents including polymerizing agents (paclitaxel and docetaxel) and depolymerizing agents (*vinca* alkaloids and colchicine) alter the polymerization dynamics of microtubules, blocking mitosis at the metaphase/anaphase transition, and consequently inducing cell death by apoptosis.

Disruption of the microtubule dynamic also changes the activity of several kinases, including Ras/Raf-1, PKC/PKA I/II, MAP, and p34CDC2 [18]. These kinases are involved in Bcl-2 phosphorylation either directly or indirectly. Several studies have demonstrated that Bcl-2 phosphorylation can be specifically induced by these tubulin-binding agents but not by DNA-damaging agents [19]. Induction phosphorylation of Bcl-2 results in its inactivation, thus promoting apoptosis, possibly with by releasing Bax from Bcl-2/Bax heterodimers [20]. There is also evidence that Bcl-2 phosphorylation is associated with normal mitotic progression [21]. Because of the potential role of Bcl-2 phosphorylation in apoptotic and mitotic regulation, signaling pathways and the kinases involved have been intensely studied. All of these tubulin-binding agents are widely used, either alone or in combination with other anticancer drugs.

It has been documented that genistein can reverse the apoptotic effect of paclitaxel without alternation of cell cycle arrest [22,23]. Although these data indicated the involvement of tyrosine kinase and Bcl-2 protein, the detailed mechanism of involvement was not clear. In the present study, the reversed effects of genistein were further investigated. Genistein specifically inhibited the apoptotic effect induced by the tubulin-binding agents. Also, the mitotic arrest caused by paclitaxel and vincristine was diminished to the action of genistein. Although the in situ staining microtubule showed that genistein could partially alter the effects of paclitaxel and vincristine, the distribution of soluble and polymerized forms of microtubule were not changed in presence of genistein. Hence, genistein did not affect the dynamics of tubulin/microtubule. Nevertheless, the Bcl-2 phosphorylation was markedly decreased without altering the expression of Bax protein. And the expression of cyclin B1 and CDC2 kinase was significantly reduced to the action of genistein. In conclusion, genistein reversed tubulin-binding agents, paclitaxel and vincristine, induced apoptosis and mitosis via reducing the expression of cyclin B1 and CDC2 kinase.

1. Materials and methods

1.1. Materials

Paclitaxel, vincristine, genistein were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All compounds were dissolved in dimethylsulfoxide (DMSO) and equal volume of vehicle was added in control experiments. RPMI-1640 medium, fetal bovine serum (FBS) and all the other cell culture reagents were obtained from Gibco BRL life technologies (Grand Island, NY, USA). Propidium iodide, anti-β-tubulin monoclonal antibody and secondary antibodies (mouse or rabbit IgG horseradish peroxidase conjugate and FITC-conjugated mouse antihuman antibody) were bought from Sigma.

1.2. Cell lines and cell culture

Human breast carcinoma cells, MCF-7 and MDA-MB-231 were purchased from American Type Culture Collection (Rockville, MD, USA). MCF-7 cells and MDA-MB-231 cells propagated in RPMI-1640 were supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Both cell lines were cultured at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Cell culture medium was renewed 2–3 times a week.

1.3. Cell viability assay

The breast cancer cells were inoculated in 96-well plates. After drug treatment, cells were incubated with calcain-AM (1 μM) at 37 °C for 1 h and then washed twice with PBS. Fluorescence data was read on Multiwell Fluorscence Plate Reader (Millipore CytoFluor 2300) with λ_{EX}: 485 nm and λ_{EM}: 535 nm. The percentage of cell survival was defined as treated wells/untreated control wells × 100%.

1.4. Apoptosis assay

For quantitative assessment of oligonucleosomal DNA fragmentation, 10^4 cells were incubated with medium containing agents tested. Apoptosis was detected by Cell Death ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany). Spectrophotometric data at a wavelength of 405 nm, with a reference of 490 nm, was acquired on a microplate reader. The enhancement of apoptosis was calculated in relation to control cells receiving vehicle alone.

1.5. Flow cytometric cell cycle analysis

Following drug treatment, cell were harvested by trypsinization, washed with PBS, then pellets were resuspended and fixed in ethanol (70%, v/v) and incubated at −20 °C for 6 h. Cells were stained with propidium iodide staining buffer containing Triton X-100 (0.1%, v/v), RNase A (100 μg/ml), propidium iodide (80 μg/ml) for 30 min. Cell cycle distribution was performed using a FACScan flow cytometry with CellQuest software (Becton Dickinson, San Jose, CA, USA).

1.6. Immunohistochemistry and confocal microscopy

Cells grown and treated on eight-chamber slides were fixed with 4% glutaldehyde for 10 min, washed twice with

PBS, blocked with 2% BSA and 0.1% Triton X-100 in PBS for 1 h at room temperature. After washed twice with PBS, cells were stained with anti-β-tubulin monoclonal antibody (1:500 dilution, Sigma, UK) at 4 °C overnight and then FITC-conjugated mouse antihuman antibody (1:100 dilution, Sigma, UK) at room temperature for 1 h. Nuclear staining was performed with 1 μg/ml 4,6-diamidino-2-phenylindole. Cells were washed three times with PBS and imaged with Leica TCS SP2 Spectral Confocal System.

1.7. Western blot analysis

The breast cancer cells were cultured in 100-mm tissue culture Petri dishes and allowed to attach for 24 h followed by drug treatment. To harvest the cells, they were washed twice with PBS and scraped from culture dishes with lysis buffer containing 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethyl-sulfonyl fluoride, 1 mM $NaVO_4$ and 1 μg/ml leupeptin and 1 μg/ml aprotinin in 20 mM Tris–HCl buffer, pH 7.5. Cell lysates were centrifuged at 13,000 × *g* for 30 min. Total protein was determined and equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis. After electrophoresis, the gel was then transferred to a nitrocellulose membrane. p53, Bcl-2, Bax, p21, cyclin B1, and CDC2 kinase were detected by immunoblotting. Membranes were then incubated with mouse or rabbit IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 25 °C. Proteins were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

1.8. In vivo tubulin polymerization assay

To quantitate of the degree of in vivo tubulin polymerization in response tubulin-binding agents was as previously described [24]. After treatment, cells were washed twice with phosphate-buffered saline and lysed at 37 °C for 5 min in the dark, with 200 μl of hypotonic buffer containing 1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40, 2 mM phenylmethylsulfonyl fluoride, 200 unit/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 5.0 mM -amino caproic acid, 1 mM benzamidine, and 20 mM Tris–HCl, pH 6.8. The cell lysate in which the cytosolic and cytoskeletal fractions containing soluble (S) and polymerized (P) tubulin, respectively, was separated by centrifugation, resolved by electrophoresis through 10% SDS–polyacrylamide gels, and immunoblotted with an antibody against β-tubulin.

1.9. Statistical analysis

Results are expressed as means ± S.E.M. of the indicated number of independent experiments. *P*-values lower than 0.05 were considered significant.

2. Results

2.1. Combination of genistein increased breast cancer cell viability in cells treated with paclitaxel and vincristine

Paclitaxel and vincristine caused a dose-dependent decline of cell viability in MDA-MB-231 and MCF-7 cells (Fig. 1). At 100 nM, paclitaxel and vincristine decreased cell viability to 48.1 ± 5.0% and 37.2 ± 3.2% of control values in MDA-MB-231 cells after 24-h treatment. The IC_{50} values of paclitaxel and vincristine were 0.16 ± 0.08 μM and 0.07 ± 0.04 μM. Simultaneous exposure to 100 μM genistein increased cell survival by 75.8 ± 9.1% and 55.8 ± 8.0% and their IC_{50} values were 6.82 ± 0.29 μM and 1.61 ± 1.48 μM. A similar effect was also observed in MCF-7 cells. Paclitaxel and vincristine exhibited a greater effect on MCF-7 cells, and cell viability markedly declined at 10 nM. With the combination of genistein, the effect of paclitaxel and vincristine apparently decreased. However, viability of cells treated with doxorubicin, a DNA-damaging agent, was decreased by genistein (77.8 ± 14.4% and 43.8 ± 7.6%, data not shown). In the following assays, the concentration of genistein was stationary at 100 μM while paclitaxel and vincristine were 100 nM.

2.2. Genistein protected MCF-7 cells from paclitaxel- and vincristine-induced apoptosis

To further confirm the possibility that genistein might compromise the proapoptotic effect of paclitaxel and vincristine, a quantitative evaluation was performed with the Cell Death Detection ELISA^{PLUS} kit by detection of histone-associated mono- and oligo-nucleosome DNA fragments. As compared with vehicle-treated cells, paclitaxel and vincristine induced significant apoptosis with 9.59 ± 2.23-fold and 11.36 ± 2.17-fold increase (Fig. 2). However, in combination with genistein, apoptosis was only increased to 2.34 ± 0.56-fold and 2.15 ± 0.69-fold, respectively. Genistein by itself did not enhance apoptosis (0.89 ± 0.26-fold).

2.3. Genistein abrogated paclitaxel- and vincristine-induced G2/M arrest of cell cycle distribution

To establish general characteristics of the cell cycle response to tubulin-binding agents in MDA-MB-231 and MCF-7 cells, cells were exposed to varying concentrations of paclitaxel or vincristine in the range of 1–1000 nM. MDA-MB-231 cells treated with paclitaxel or vincristine were pronounced in G2/M phase accumulation at the concentration of 100 nM (53.8 and 52.0% compared to vehicle treatment, 28.9%, Table 1). The sub-G1 phase, which represents apoptotic cells, was also augmented by paclitaxel or vincristine treatment (20.9 and 20.3%

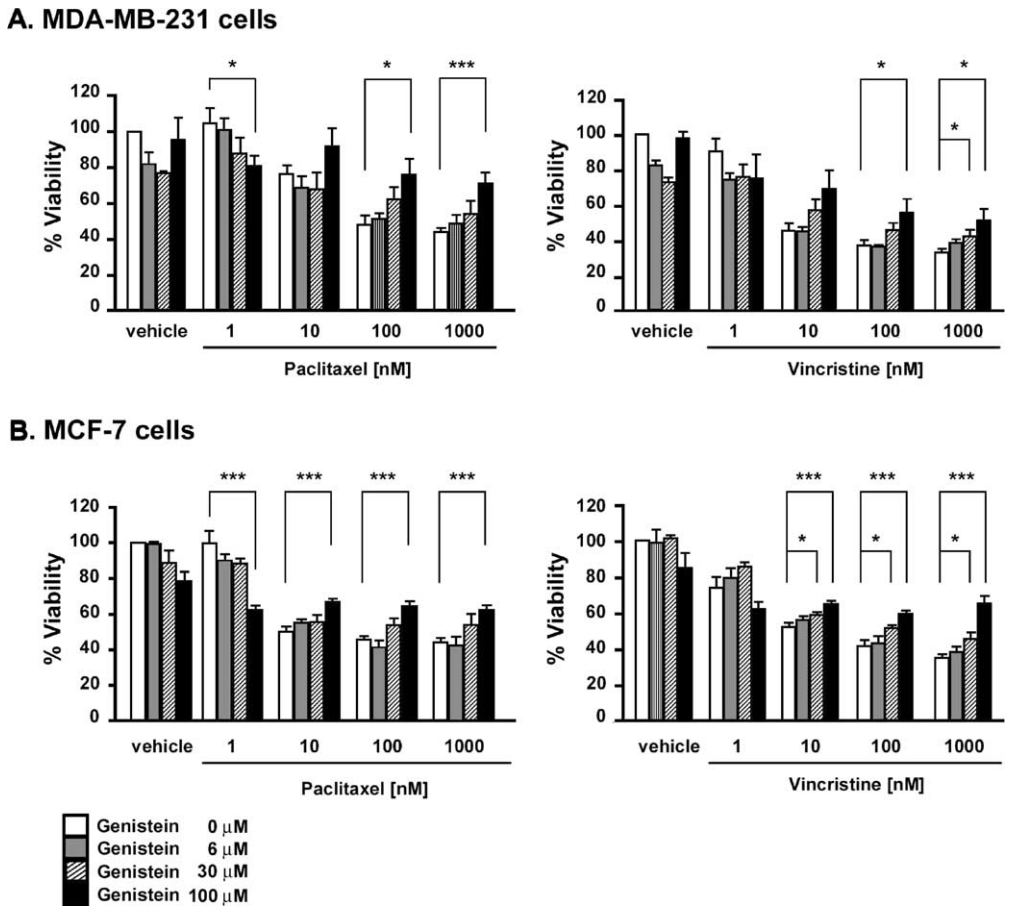

Fig. 1. Genistein increased survival of breast cancer cells with paclitaxel and vincristine treatment. MDA-MB-231 (A) and MCF-7 (B) cells were grown in 96-well plate and treated for 24 h with increasing concentrations (1, 10, 100, and 1000 nM) of paclitaxel and vincristine in the absence (empty bars) or presence of genistein (6 μM, gray bars; 30 μM, slashed bars; 100 μM, black bars). Cell viability was evaluated by calcein-AM assay. The percentage of viable cells was fluorescence of treated cells/untreated cells × 100%. Each value represented the mean ± S.E.M. of five independent experiments. The statistical probability (P-value) compared with the indicated bar is expressed as: $^{*}P < 0.05$ and $^{***}P < 0.001$.

compared to vehicle treatment, 6.1%). Combination with genistein (100 μM) markedly inhibited paclitaxel- or vincristine-induced apoptosis and cell cycle arrest as manifested by a reduced number of cells with hypodiploid DNA and G2/M arrest of living cells. Typical DNA histograms obtained from MDA-MB-231 are shown in Fig. 3. A similar effect was also observed in MCF-7 cells (data not shown).

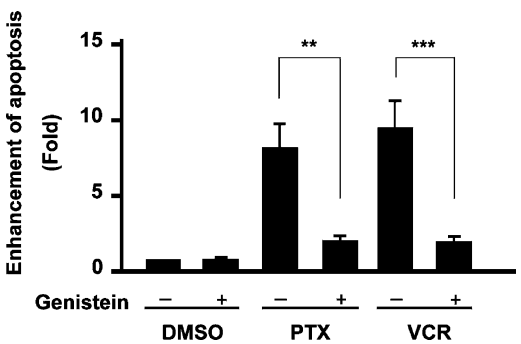

Fig. 2. Genistein protected MCF-7 cells from paclitaxel and vincristine-induced apoptosis. After MCF-7 cells were treated with vehicle, paclitaxel (PTX; 100 nM) or vincristine (VCR; 100 nM) in the absence or combination with genistein (Gen; 100 μM), apoptosis was evaluated using the Cell Death Detection ELISA^{PLUS} kit. Results are the mean and S.E.M. from four experiments expressed as the fold increase in apoptosis compared with vehicle control, arbitrarily set at 1.0. The statistical probability (P-value) compared with the indicated bar is expressed as: $^{**}P < 0.01$ and $^{***}P < 0.001$.

2.4. *Effects of genistein on the cellular organization of microtubule network in MDA-MB-231*

Because genistein could abrogate apoptosis and G2/M arrest of the cell cycle induced by tubulin-binding agents, we used immunocytochemical analysis to investigate whether the microtubule network in MDA-MB-231 cells was affected (Fig. 4A). The microtubule network and nuclear morphology in control cells exhibited normal organization and arrangement. Treatment with paclitaxel (100 nM) enhanced microtubule polymerization as visualized with an increased density of cellular microtubules and formation of microtubule bundles around the nucleus. In the presence of genistein, microtubule polymerization trigger by paclitaxel was diminished. Meanwhile, vincristine treatment caused microtubule depolymerization and cells exhibited dispersed fluorescence. Combination with genistein counteracted effect of vincristine on microtubule

Table 1
Effect of genistein on cell cycle progression in MDA-MB-231 cells

	Sub-G1	G0/G1	S	G2/M
Vehicle (DMSO)	6.1 ± 1.4	57.5 ± 3.0	7.6 ± 0.6	28.9 ± 2.0
Genistein	9.8 ± 1.8	40.9 ± 2.3***	16.8 ± 1.4***	32.4 ± 1.9
Paclitaxel	20.9 ± 2.5***	18.6 ± 2.7***	8.4 ± 1.1	53.8 ± 2.9***
Paclitaxel + genistein	10.5 ± 2.3$^{\#\#}$	34.8 ± 2.2$^{***, \#\#\#}$	18.4 ± 1.4$^{***, \#\#\#}$	36.7 ± 1.8$^{***, \#\#\#}$
Vincristine	20.3 ± 2.3***	17.1 ± 2.9***	10.0 ± 0.7**	52.0 ± 3.9***
Vincristine + genistein	12.9 ± 2.7$^{*, @}$	37.6 ± 1.7$^{***, @@@}$	16.4 ± 1.5$^{***, @@@}$	30.9 ± 1.9$^{@@@}$

Results were expressed as mean ± S.E.M. of five independent experiments. MDA-MB-231 cells were incubated with vehicle (DMSO), paclitaxel (100 nM), or vincristine (100 nM) with or without genistein (100 μM) for 24 h. Then, cells were harvested and stained DNA with propidium iodide for flow cytometric analysis. Statistical probability (P-value) compared with respective treatment was shown as: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with paclitaxel; $^{@}P < 0.05$, $^{@@}P < 0.01$, and $^{@@@}P < 0.001$ compared with vincristine.

network. However, the morphology of cells co-treated with genistein and tubulin-binding agents was not similar with that of control cells. To further confirmation of the distribution of tubulin/microtubule, the cytosolic (soluble) and cytoskeletal (polymerized) forms of tubulin were separated from whole cell lysate by centrifugation (Fig. 4B). Paclitaxel caused tubulin polymerization in which a shift in tubulin from soluble to polymerized form while vincristine caused tubulin depolymerization that the majority of tubulin shifts to soluble form. However, simultaneously treated with genistein did not alter the distribution of tubulin/microtubule as treated with paclitaxel and vincristine without genistein.

2.5. Genistein abrogated paclitaxel- and vincristine-induced-Bcl-2 phosphorylation

To define the molecular mechanism by which genistein protects breast cancer cells from apoptosis induced by tubulin-binding agents, the expression of Bcl-2 family proteins involved in apoptosis was evaluated by Western blot analysis. Previous studies showed that tubulin-binding agents induced Bcl-2 protein phosphorylation, resulting in Bcl-2 protein inactivation and induction of apoptosis [19]. As shown in Fig. 5, exposure to 100 nM paclitaxel and vincristine induced Bcl-2 phosphorylation. Simultaneous treatment with genistein diminished this paclitaxel- and vincristine-induced Bcl-2 phosphorylation. Therefore, genistein inhibited the apoptotic effect of paclitaxel and vincristine by decreasing phosphorylation of Bcl-2 protein. On the contrary, the expression of Bax protein, which counteracts the survival action of Bcl-2 protein, was not altered by combination treatment with genistein and paclitaxel or vincristine. Doxorubicin, a DNA-damaging agent, could not induce Bcl-2 phosphorylation, even in combination with genistein.

2.6. Genistein decreased the expression of cyclin B1 and CDC2 kinase in MDA-MB-231

Tubulin-binding agents induced mitotic arrest, which led to apoptosis. Because of microtubule damage, the spindle-

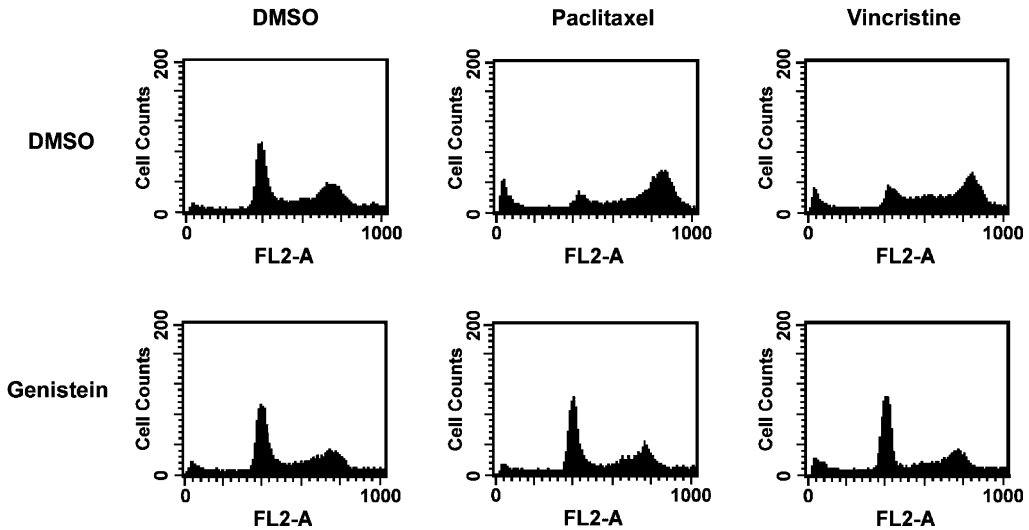

Fig. 3. Effect of genistein on paclitaxel and vincristine-induced G2/M arrest of cell cycle. MDA-MB-231 cells were incubated with vehicle (DMSO), paclitaxel (100 nM), and vincristine (100 nM) in the absence (upper panel) or presence of genistein (100 μM; lower panel) for 24 h. Cells were then harvested and stained DNA with propidium iodide for flow cytometric analysis. FL2-A was fluorescence 2-area.

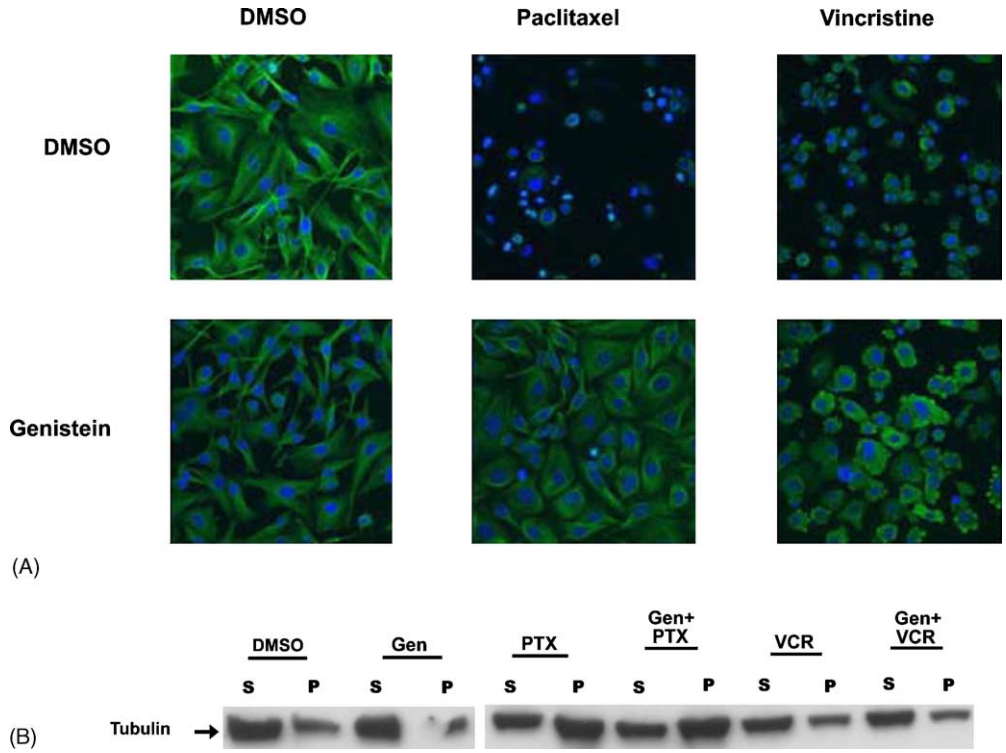

Fig. 4. (A) Effects of genistein on the cellular organization of microtubule network in MDA-MB-231. MDA-MB-231 cells were treated with vehicle (DMSO), paclitaxel (100 nM), or vincristine (100 nM) in the absence or combination with genistein (100 μM) for 24 h. Cells were then fixed with 4% glutaldehyde. After washed twice with PBS, blocked with 2% BSA and 0.1% Triton X-100 in PBS for 1 h at room temperature, cells were stained with anti-β-tubulin monoclonal antibody (1:500 dilution in PBS) at 4 °C overnight and then FITC-conjugated mouse antihuman antibody (1:100 dilution in PBS) at room temperature for 1 h. Nuclear staining was performed with 1 μg/ml 4,6-diamidino-2-phenylindole. Cells were washed three times with PBS and imaged with Leica TCS SP2 Spectral Confocal System. (B) MDA-MB-231 cells were treated with vehicle (DMSO), paclitaxel (PTX; 100 nM), or vincristine (VCR; 100 nM) in the absence or combination with genistein (Gen; 100 μM) for 24 h. The following procedure was as previously described in Section 1. The soluble (S) and polymerized (P) forms of microtubule were indicated.

assembly checkpoint was activated, which blocked the proteasome-dependent degradation of cyclin B, leading to prolonged activation of cyclin B/CDC2 kinase and resulting in mitotic arrest [25]. As shown in Fig. 3, G2/M arrest induced by tubulin-binding agents was significantly decreased in with genistein co-treatment. To clarify the molecular mechanism, cell cycle regulatory proteins, such as p53, p21, cyclin B1, and CDC2, were examined by Western blotting analysis. Treatment with the tubulin-binding agents, paclitaxel and vincristine, slightly increased the expression of cyclin B1 and CDC2 protein, whereas the expression of p53 and p21 proteins was not altered (Fig. 6). Combination treatment with genistein and tubulin-binding agents attenuated G2/M arrest of cell cycle, which was concomitant with down-regulation of cyclin B1 and CDC2 kinase. However, expression of p53 and p21 protein was not significantly changed either in the presence or absence of genistein. Doxorubicin, which was used as a positive control, significantly induced expression of p53 and p21. Combined treatment with genistein and doxorubicin did not alter the expression of p53 and p21 protein, though the expression of cyclin B1 and CDC2 kinase was decreased.

3. Discussion

In the present study, we found that genistein could specifically abrogate the effects of two tubulin-binding agents, paclitaxel and vincristine, in two human breast cancer cell lines, MCF-7 and MDA-MB-231 cells. Apoptosis, G2/M arrest of cell cycle distribution, and Bcl-2 phosphorylation induced by paclitaxel or vincristine were all diminished in the presence of genistein. However, viability of cells treated with doxorubicin, a DNA-damaging agent, was even more decreased by genistein. Although in situ staining of microtubules revealed that tubulin network affected by paclitaxel and vincristine was partially reversed in the presence of genistein, in vivo tubulin polymerization assay indicated that tubulin/microtubule dynamics was not changed. Cyclin B1/CDC2 kinase exhibited an important role in the cell cycle progression and was also a possible kinase of Bcl-2 phosphorylation. The expression of cyclin B1/CDC2 kinase and Bcl-2 phosphorylation were decreased in parallel. Thus, genistein might attenuate the effects of tubulin-binding agents through down-regulation of cyclin B1 and CDC2 kinase expression resulting in diminished Bcl-2 phosphorylation.

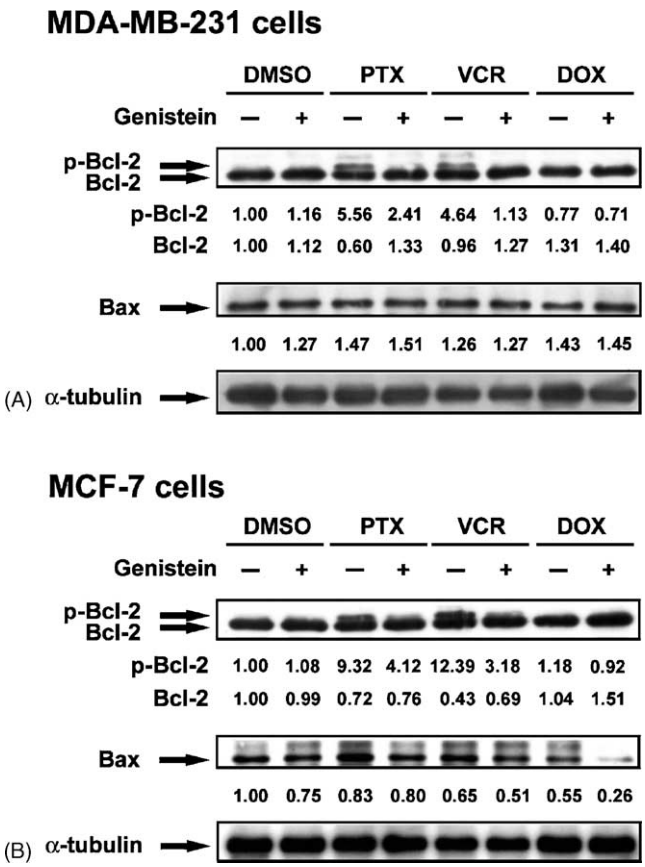

Fig. 5. Genistein reduced Bcl-2 phosphorylation induced by paclitaxel and vincristine in MDA-MB-231 (A) and MCF-7 (B) cells. Both cells were treated with vehicle, paclitaxel (PTX; 100 nM), vincristine (VCR; 100 nM), or doxorubicin (DOX; 1 μM) in the absence or combination with genistein (100 μM) for 24 h. Cells were then harvested, and cell extract was prepared and loaded on 12% SDS–PAGE as described in Section 1. After electrophoresis, protein blots were transferred on the nitrocellulose membrane. Bcl-2 and Bax protein were probed by monoclonal anti-Bcl-2 and anti-Bax antibody. The signals of Bcl-2, phosphorylated Bcl-2 (p-Bcl-2) and Bax proteins were as indicated. The relative amount of Bcl-2, p-BCL-2, and Bax protein was quantified and normalized to the corresponding α-tubulin protein amounts. The quantitative data is shown under each protein, respectively.

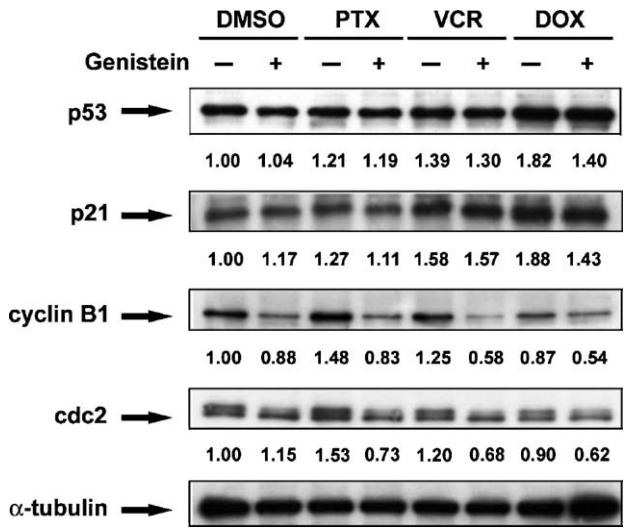

Fig. 6. Effect of genistein on cell cycle regulatory proteins in MDA-MB-231 cells. Cells were treated with vehicle, paclitaxel (PTX; 100 nM), vincristine (VCR; 100 nM), or doxorubicin (DOX; 1 μM) in the absence or combination with genistein (100 μM) for 24 h. Cells were then harvested, and cell extract was prepared and loaded on 12% SDS–PAGE as described in Section 1. After electrophoresis, protein blots were transferred on the nitrocellulose membrane. p53, p21, cyclin B1, and CDC2 protein were probed by specific antibodies. The relative amount of p53, p21, cyclin B1, and CDC2 protein was quantified and normalized to the corresponding α-tubulin protein amounts. The quantitative data is shown under each protein, respectively.

Previous studies showed that genistein reversed apoptotic effect of paclitaxel in ovarian cancer cell line by inhibiting tyrosine kinase activity and *bcl-2* gene expression [22]. However, it was also documented that the reversed effect of genistein was not correlated with Bcl-2 protein expression [23]. Both assays revealed that G2/M arrest of cell cycle distribution induced by paclitaxel was not altered in combination with genistein. In this study, we firstly found that all effects, including apoptosis, mitotic arrest, and Bcl-2 phosphorylation, induced by tubulin-binding agents, both polymerization agents and depolymerization agents, were abrogate in the presence of genistein.

Genistein possessed estrogenic activity. However, genistein inhibited effects of tubulin-binding agents via an estrogen-independent pathway because the effects of estradiol (100 nM) were not similar (data not shown), and genistein at higher concentration ($\geqq$10 μM) did not induce PS2, an estrogen-regulated antigen [6].

There is a speculation that genistein might act on a target of tubulin/microtubule. However, both tubulin-toxins, paclitaxel and vincristine, which bind to distinct site of tubulins, were inhibited by genistein, suggesting that the primary target to genistein action is not tubulin/microtubule. The tubulin polymerization assay shown in Fig. 4B was also quite sympathetic to this proposal.

Cell cycle progression is controlled by several different Cdk regulatory mechanisms [26]. Binding with its catalytic subunit of cyclin activates Cdk/cyclin complexes and promotes cell growth and proliferation, whereas binding with its Cdk inhibitors inactivates Cdk/cyclin complexes and decreases cell growth and proliferation, followed by cell death or apoptosis. Microtubule damage activates the spindle-assembly checkpoint which blocks the proteasome-dependent degradation of cyclin B, leading to a prolonged activation of cyclin B/CDC2 kinase and resulting in mitotic arrest [25]. Genistein was reported to regulate cell cycle progression by reducing cyclin B1, followed by induction of $p21^{WAF1/CIP1}$ expression [3,7,8]. In the current study, genistein decreased cell cycle arrest in G2/M phase induced by tubulin-binding agents. Expression of cyclin B1 and CDC2 kinase was significantly decreased without an observed change in other cyclins (data not shown). Expression of p53 and its downstream protein, p21, were not different with combination treatment with tubulin-binding agents and genistein. Similar effects were also found in

PC-3 cells, a p53-null prostate cancer cell line (data not shown). Thus, genistein reversed the effects of tubulin-binding agents via a p53- and p21-indpendent pathway.

There are several kinases involved in Bcl-2 phosphorylation, including the cyclin B/CDC2 complex [21,27]. Ling et al. [21] showed that short-term exposure (90 min) to genistein did not alter Bcl-2 phosphorylation after release from M phase blockade by nocodazole. The possible mechanism might be that cyclin B1 protein was highly accumulated in nocodazole M phase-arrested cells, resulting in failure of genistein in Bcl-2 dephosphorylation. In our study, simultaneous treatment with genistein and tubulin-binding agents diminished Bcl-2 phosphorylation and inhibited cyclin B1 and CDC2 kinase expression. Post-treatment of genistein at 12 h after addition of tubulin-binding agents could still diminish G2/M arrest of cell cycle distribution (data not shown). This result demonstrated that the expression of cyclin B1/CDC2 kinase complex plays an important role in signaling activated by tubulin-binding agents and also the likely action mechanism of genistein inhibit the effects of tubulin-binding agents.

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