Phytoestrogens Induce Apoptosis *via* Extrinsic Pathway, Inhibiting Nuclear Factor-κB Signaling in HER2-overexpressing Breast Cancer Cells

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Abstract. *Background: Phytoestrogens are known to prevent tumor induction. But their molecular mechanisms of action are largely unknown. This study aimed to examine the effect of genistein and quercetin on proliferation and apoptosis in HER2-expressing breast cancer cells. Materials and Methods: The antiproliferative effects of phytoestrogens were tested by proliferation assays. Flow cytometry was performed to analyze the cell cycle. The effect of phytoestrogens on cell-signaling molecules was determined by Western blotting. Results: Genistein and quercetin inhibited the proliferation of MCF-7 vec and MCF-7 HER2 cells. This growth inhibition was accompanied with an increase of subG_0/G_1 apoptotic fractions. Genistein and quercetin induced extrinsic apoptosis pathway, up-regulating p53. Genistein and quercetin reduced the phosphorylation level of IκBα, and abrogated the nuclear translocation of p65 and its phosphorylation within the nucleus. Conclusion: Genistein and quercetin exert their antiproliferative activity by inhibiting NFκB signaling. Phytoestrogens could be potential useful compounds to prevent or treat HER2-overexpressing breast cancer.*

Apoptosis, or programmed cell death (PCD), plays an important role in various biological systems, such as embryonic development, cell turnover, and immune response against tumorigenic or virus-infected cells (1). When apoptosis occurs, different cell morphological changes including the shrinkage of cell and nuclei, membrane blebbing, loss of cell membrane asymmetry and attachment, nuclear fragmentation, and chromatin condensation appear (2). Apoptosis differs from necrosis in its morphological and biochemical features. In most tumor cells, apoptosis occurs *via* two different signaling pathways: the extrinsic and the intrinsic apoptosis pathways. The extrinsic pathway is related to the activation of the death receptors, such as Fas and tumor necrosis factor receptors (TNFR). Death domains (DD) of Fas are oligomerized and recruit Fas-associated death domain (FADD) and procaspase-8 to form death-inducing signaling complex (DISC). Procaspase-8 is cleaved and activated and released from the DISC into the cytoplasm where it activates caspase-3 to induce apoptosis (1, 3, 4). The intrinsic pathway is related to changes in mitochondrial membrane potential ($\Delta\psi m$) and mitochondrial permeability transition, resulting in mitochondrial release of apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF) into the cytoplasm (1, 5). Cytochrosome c binds to APAF1 and recruits procaspase-9 to form an apoptosome; caspase-9 activates effector caspases such as caspase-3 to induce apoptosis (4). Caspase-3 from both extrinsic and intrinsic pathways is responsible for the cleavage of Poly (ADP-ribose) polymerase (PARP) during cell death (6). Extracellular signal-regulated kinases (ERK) pathway and Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathway can inhibit this apoptotic signaling (4). The cross-talk between the extrinsic apoptosis pathway and the intrinsic apoptosis pathway exists; caspase-8 can cleave BID, a death-inducing member of the BCL2

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family. The truncated BID translocates to the mitochondria and induces cytochrome c release which subsequently results in caspase-9-dependent activation (1, 7).

Phytoestrogens are phenolic compounds derived from plants and that structurally mimic the principal mammalian estrogen (17β-estradiol), and bind to the estrogen receptor (ER) (8-10). There is also evidence that phytoestrogens have hormone-independent activities (10). Phytoestrogens are suggested to induce the prevention or the growth suppression of various cancers including breast (11, 12), prostate (13, 14), colorectal (15) and bladder cancer (16). Genistein is a small and biologically active flavonoid that is found in high amounts in soy and is reported to inhibit cancer cell invasion and metastasis (17). Quercetin is a plant-derived flavonoid found in fruits, vegetables, leaves and grains and is reported to inhibit carcinogen-induced rat mammary tumor (18).

The human epidermal growth factor receptor (HER2) oncogene encodes a 185-kDa transmembrane receptor tyrosine kinase (19). HER2 is overexpressed in breast cancer and other cancers (19) and HER2 overexpression is associated with poor disease-free survival (20). HER2-positive breast carcinomas are likely to be more aggressive and fast growing than other types of breast cancer (21). HER2-positive breast cancer patients are also less responsive to hormone treatment and have a shorter duration of response than HER2-negative breast cancer patients (22). Currently, trastuzumab (Hercepin) is one of the key drugs in the treatment strategy for HER2-positive breast cancer (21). It works against cancer by maximizing the expression and inhibitory effect of p27, which leads to cell cycle G1 arrest and growth inhibition (23). However, many women do not respond to trastuzumab or develop resistance (24). This has resulted in significant efforts to find other compounds which could effectively treat HER2-overexpressing breast cancer.

In the present study, we investigated whether phytoestrogens (genistein and quercetin) display growth-suppressive activity on HER2-overexpressing breast cancer cells. For this purpose, we tested the effects of phytoestrogens on proliferation and apoptosis of MCF-7 human breast cancer cells engineered to overexpress oncogenic HER2 (MCF-7 HER2) and control vector cells (MCF-7 vec). We also investigated the mechanism by which phytoestrogens regulate the growth of MCF-7 vec and MCF-7 HER2 cells analyzing the cell cycle and measuring the levels of apoptotic molecules and intracellular signaling molecules. We also verified whether phytoestrogens inhibit NFκB signaling pathway, leading to growth suppression of HER2-expressing breast cancer cells. Moreover, we examined the effect of phytoestrogens on calmodulin and cAMP response element-binding protein (CREB) activity.

Nutrition of soybean, tofu, vegetables, fruits, leaves and grains (rich in phytoestrogens) is well studied and appreciated. Phytoestrogen nutrition is known to reduce menopausal symptoms, risk of coronary heart disease, osteoporosis, help lower blood cholesterol and blood pressure levels, and protect against various types of cancer.

Materials and Methods

Compounds. Genistein (4',5,7-trihydroxyisoflavone) and quercetin (3,3',4',5,7-pentahydroxyflavone) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). These compounds were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the controls and each sample did not exceed 0.1%. We found that 0.1% DMSO did not affect the cell growth rate as compared to 0% DMSO (no treatment) in breast cancer cells (data not shown). JC-1 was obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

Cell cultures. MCF-7 human breast cancer cells (ATCC, American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 50 U/ml penicillin, 50mg/ml streptomycin and 10% FBS (Welgene, Daegu, Korea) at 37˚C in an atmosphere of 5% CO_2. MCF-7 vec and MCF-7 HER2 cells were generated as described elsewhere (25). Briefly, 293T human kidney cells were transfected with pBMN-vec-GFP or pBMN-HER2-GFP, pCMV-VSVG (encording vesicular stomatitis virus glycoprotein), and pCMV-gag-pol (containing the Moloney murine leukemia virus *gag* and *pol* genes). At 48 h post-transfection, retroviral particles were collected from 293T cell media and concentrated using amicon ultra (Millipore, Billerica, MA). MCF-7 cells were treated with the concentrated retrovirus particles and 8 μg/ml of polybrane (Sigma–Aldrich, St. Louis, MO, USA). After 48 h, the cells were selected by using flow cytometry.

Antibodies. Primary antibodies directed against FAS, cleaved caspase-8, PARP, HER2, phospho-HER2 (Tyr1248), AKT, phospho-AKT (Ser473), phospho-MDM2 (Ser166), RSK, phospho-RSK (Thr359/Ser363), CREB, phospho-CREB (Ser133), phospho-NFκB (Ser536), and phospho-IκBα (Ser32/36) were purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). BCL2, BAX, p53, phospho-p53(Ser15), p16, p21, MDM2, Rb, ERK, phospho-ERK (Tyr204), JNK, phospho-JNK (Thr183/185), p38, phospho-p38 (Thr180/Tyr182), IKK α/β , NFκB, Lamin B, and α-actin were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz , CA, USA). BCL-XL and p27 were purchased from BD Biosciences (Franklin Lakes, NJ, USA). STAT3, phospho-STAT3 (Tyr705), and calmodulin were obtained from Upstate-Millipore (Billerica, MA, USA). Phospho-calmodulin (Thr79/Ser81) was from Abcam (Cambridge, UK). α-Tubulin was from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated secondary antibodies (mouse and rabbit) were purchased from Calbiochem (San Diego, CA, USA) and anti-goat secondary antibody was from Jackson ImmunoResearch (West Grove, PA, USA).

Cell proliferation assay. Cells were seeded in 12-well culture plates at a density of 5×10^4 cells/well. After the cells were exposed to different concentrations of phytoestrogens (genistein and quercetin) and incubated for 3 days, the cells were harvested by trypsinization, resuspended in 1-2 ml of medium, and counted using a hemocytometer.

Cell cycle analyses by flow cytometry. Cells were harvested with 0.25% trypsin and washed once with phosphate buffered saline (PBS). After centrifugation, the cells were fixed in 95% cold ethanol

with 0.5% Tween-20, and stored at –20˚C for at least 30 min. The cells were incubated in 50 μg/ml of propidium iodide (PI) (including 1% of sodium citrate and 50 μg/ml of RNase A) at room temperature in the dark for 30 min. The analysis of apoptotic cells were performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the data were analysed using CellQuest software.

Preparation of cytosolic and nuclear protein. Cells were incubated in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and 0.5% NP40). The cells were stood on ice for 5 min and then centrifuged at 3,000 rpm for 5 min. The supernatant was collected as cytosol extract. The pellet was then lysed with Buffer B (5 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 26% glycerol,) and centrifuged at 5,000 rpm for 5 min at 4˚C then 4.6 M NaCl was added to give 300 mM NaCl. The pellet was homogenized with 20 full strokes in Dounce or glass homogenizer on ice. The pellet was incubated on ice for 30 min, and then centrifuged at 24,000 rpm for 20 min at 4˚C. Nuclear proteins were obtained from the supernatant.

Western blot analysis. Cells were lysed in modified RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitor mixture). The lysates were cleared by centrifugation at 13,000 rpm for 15 min and the supernatants were collected. The protein concentration was quantified using a Bio-Rad Bradford protein assay (Bio-rad, Hercules, CA, USA). Equal amounts of protein lysates were used for Western blot analyses with the indicated antibodies. Immunoreactive protein bands were detected with an EZ-Western Detection kit (Daeillab service Co, Ltd, Seoul, Korea).

Analysis of mitochondrial transmembrane potential ($\Delta\Psi m$). Cells were seeded at a density of 1×10^6 cells/dish in 100 mm dishes and incubated for 24 h at 37˚C. After stabilization, the cells were treated with 100 μM phytoestrogens (genistein and quercetin) and DMSO for 72 h. After harvest by treatment of trypsin-EDTA, the cells were washed with cold PBS, centrifuged at 1,500 rpm for 5min and stained with 4 μg/ml JC-1 for 15min at 37˚C in the dark. The data were analyzed by FACSCalibur flow cytometry (BD Biosciences) measuring the green fluorescence and red fluorescence at 514/529 nm (FL-1) and 585/590 nm (FL-2), respectively.

Statistical analysis. The experiments were performed in triplicate. The data for cell proliferation assays are expressed as mean±standard deviation. Standard deviations for all measured biological parameters are displayed in the appropriate figures. Student's *t*-test was used for single variable comparisons, a *p*-value <0.05 being considered as statistically significant.

Results

Genistein and quercetin suppress the growth of MCF-7 vec and MCF-7 HER2 breast cancer cells. The effects of phytoestrogens (genistein and quercetin) on cell growth were measured by cell proliferation assay. As seen in Figure 1A, genistein had no significant effect on the proliferation rates of either MCF-7 vec or MCF-7 HER2 cells when used at low concentrations ranging from 1 to 25 μM, while it strongly suppressed the growth of both cell types when used at high concentrations (50 to 100 μM) after 72 h of treatment. As seen in Figure 1B, quercetin had no effect on the proliferation rates of either MCF-7 vec or MCF-7 HER2 cells when used at low concentrations ranging from 1 to 10 μM, while it mildly suppressed the growth of both cell types at 25 μM and strongly suppressed the growth of the two cell types when used at high concentrations (50 to 100 μM) after 72 h of treatment. We expected that MCF-7 HER2 cells would exhibit resistance to phytoestrogens due to the presence of the *HER2* oncogene. However, we did not observe such resistance in our experiments. Overexpression

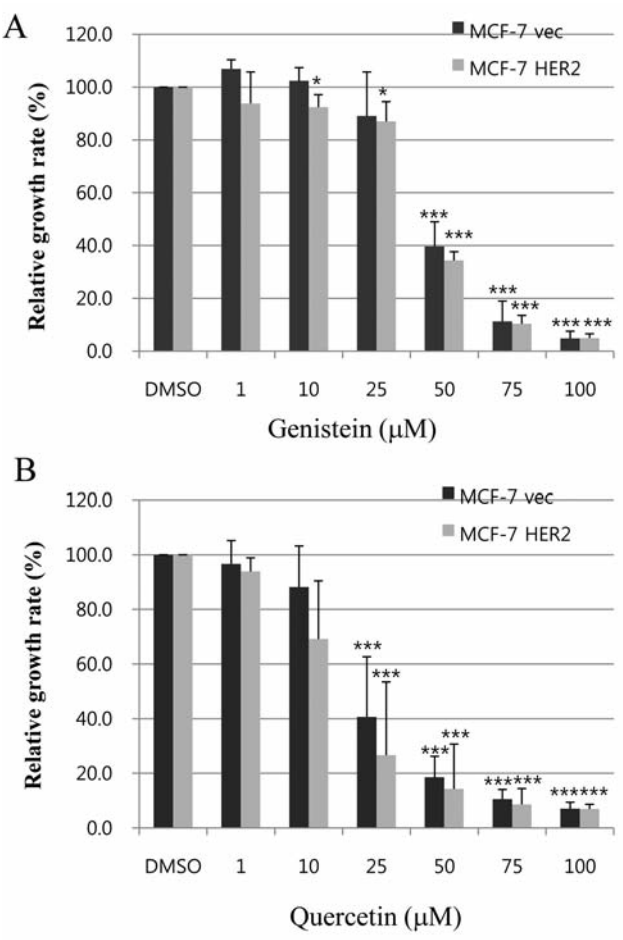

Figure 1. *Effect of phytoestrogens (genistein and quercetin) on the growth of MCF-7 vec and MCF-7 HER2 cells MCF-7 vec and MCF-7 HER2 cells were treated with different doses of genistein (A) and quecetin (B). After 72 h, the cell viability was assessed with proliferation assay. The value from vehicle-treated cells was set to 100% and relative decrease in the cell viability by phytoestrogen treatment was expressed as a percentage of the control. Data are shown as the mean of three independent experiments (error bars are mean±standard deviation (SD)) (*p<0.05 , **p<0.01, ***p<0.001).*

of HER2 did not confer resistance to phytoestrogens in MCF-7 cells. It seems that quercetin is more effective at suppressing the growth of MCF-7 vec and MCF-7 HER2 cells compared to genistein since growth inhibition was apparent at 25 μM quercetin. To have maximal effects of phytoestrogens, we used the highest concentration of the compound (100 μM) for the experiment. These growth inhibitions were verified by microscopic observation. The results in Figure 2 show that genistein and quercetin effectively inhibited the growth rate of both MCF-7 vec and MCF-7 HER2 monolayer cells after 72 h treatment. Of note, phytoestrogens also induced morphological changes in the cells after exposure (Figure 2).

The growth-suppressive activity of genistein and quercetin was accompanied by an increase in sub-G_0/G_1 apoptotic fractions and a decrease in the cell population in the S phase. To investigate whether phytoestrogens inhibited cell proliferation through changes in cell cycle progression, the effect of phytoestrogens on the cell cycle profile was assessed in MCF-7 vec and MCF-7 HER2 cells. To induce the maximum effect, cells were treated with 100 μM phytoestrogens for 72 h and then analyzed for cell cycle location by flow cytometry. The data demonstrated that genistein and quercetin induced an increase in the sub G_0/G_1 apoptotic fractions in both MCF-7 vec and MCF-7 HER2 cells (Figure 3A and 3B). Moreover, phytoestrogen treatment reduced the cell population in the S phase in both MCF-7 vec and MCF-7 HER2 cells (Figure 3A and 3B).

Genistein and quercetin do not induce apoptosis via the intrinsic mitochondrial apoptosis pathway in MCF-7 vec and MCF-7 HER2 breast cancer cells. Next, we investigated whether apoptosis induced by phytoestrogens occurs *via* intrinsic mitochondrial apoptosis pathway in MCF-7 vec and MCF-7 HER2 cells. For that purpose, we measured the levels of BCL2 family members (BCL-XL, BAX, and BCL2). We found that both genistein and quercetin slightly reduced the level of BCL-XL, but failed to regulate the levels of BAX and BCL2 in both cell types as seen in Figure 4A. We also measured the loss of mitochondrial transmembrane potential ($\Delta\Psi m$) using JC-1. JC-1 is able to selectively enter mitochondria and reversibly transforms color from red to green when the membrane potential decreases. In non-apoptotic cells with high mitochondrial $\Delta\Psi m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic cells (especially mitochondria-mediated apoptotic cells) with low $\Delta\Psi m$, JC-1 remains in the monomeric form, which shows only green fluorescence. In our study, genistein and quercetin did not induce a low mitochondrial transmembrane potential ($\Delta\Psi m$), maintaining red fluorescence from 99.99% (DMSO; MCF-7 vec) to 99.24% (genistein; MCF-7 vec) and 99.88% (quercetin; MCF-7 vec) and from 100% (DMSO; MCF-7 HER2) to 95.01% (genistein; MCF-7 HER2) and 99.55% (quercetin; MCF-7 HER2) (Figure 4B). These results demonstrate that both genistein and quercetin do not induce apoptosis *via* the intrinsic mitochondrial apoptosis pathway in MCF-7 vec and MCF-7 HER2 breast cancer cells.

Genistein and quercetin induce apoptosis via the extrinsic apoptosis pathway in MCF-7 vec and MCF-7 HER2 breast cancer cells. In this step, we investigated whether phytoestrogens activated the extrinsic apoptosis pathway by measuring the expression levels of death receptor signaling-related proteins including FAS, caspase-8, and PARP. We observed that both genistein and quercetin up-regulated the levels of FAS receptor, and cleaved caspase-8, and induced the cleavage of PARP in MCF-7 vec and MCF-7 HER2 cells (Figure 5). We did not detect caspase-3 which is important in the extrinsic apoptosis pathway since MCF-7 cells do not contain caspase-3 due to a genomic deletion (26). This suggests that MCF-7 cells induce the cleavage of PARP *via* caspase-3-independent pathway. These results indicate that phytoestrogens induce apoptosis *via* extrinsic FAS receptor-dependent apoptosis pathway in MCF-7 vec and MCF-7 HER2 cells.

Genistein and quercetin increase the expression of p53 and p16 in MCF-7 vec and MCF-7 HER2 breast cancer cells. We next examined the expression levels of protein related to apoptosis by Western blot analysis. As seen in Figure 6, both genistein and quercetin did not change the tyrosine phosphorylation of HER2 (phospho-HER2 level) in MCF-7 vec and MCF-7 HER2 cells. On the other hand, both genistein and quercetin increased p53, as well as phospho-p53 (p-p53), in MCF-7 vec and MCF-7 HER2 cells. We observed that both genistein and quercetin increased the p21 level in MCF-7 vec cells, while they did not in MCF-7 HER2 cells, suggesting that phytoestrogens can act differently in different cell lines. We also observed that quercetin increases the p27 level in MCF-7 HER2 cells. To investigate the p53-related apoptosis mechanism, we measured the levels of MDM2, p-MDM2, p16 (INK4A), and retinoblastoma (Rb). Both genistein and quercetin increased the level of p16, suggesting cross-talk between the ARF-MDM2-p53 pathway and INK4A-Rb-E2F pathway. The levels of MDM2, p-MDM2 and Rb slightly decreased in MCF-7 HER2 cells after phytoestrogen treatment. The p-MDM2 level also increased in MCF-7 vec cells after phytoestrogen treatment.

Genistein and quercetin demonstrated their antiproliferative activity via AKT, ERK, JNK and p38-independent manner. Next, we investigated whether phytoestrogens exert their antiproliferative activity *via* intracellular signaling molecules such as AKT, ERK, JNK, p38, RSK, and signal

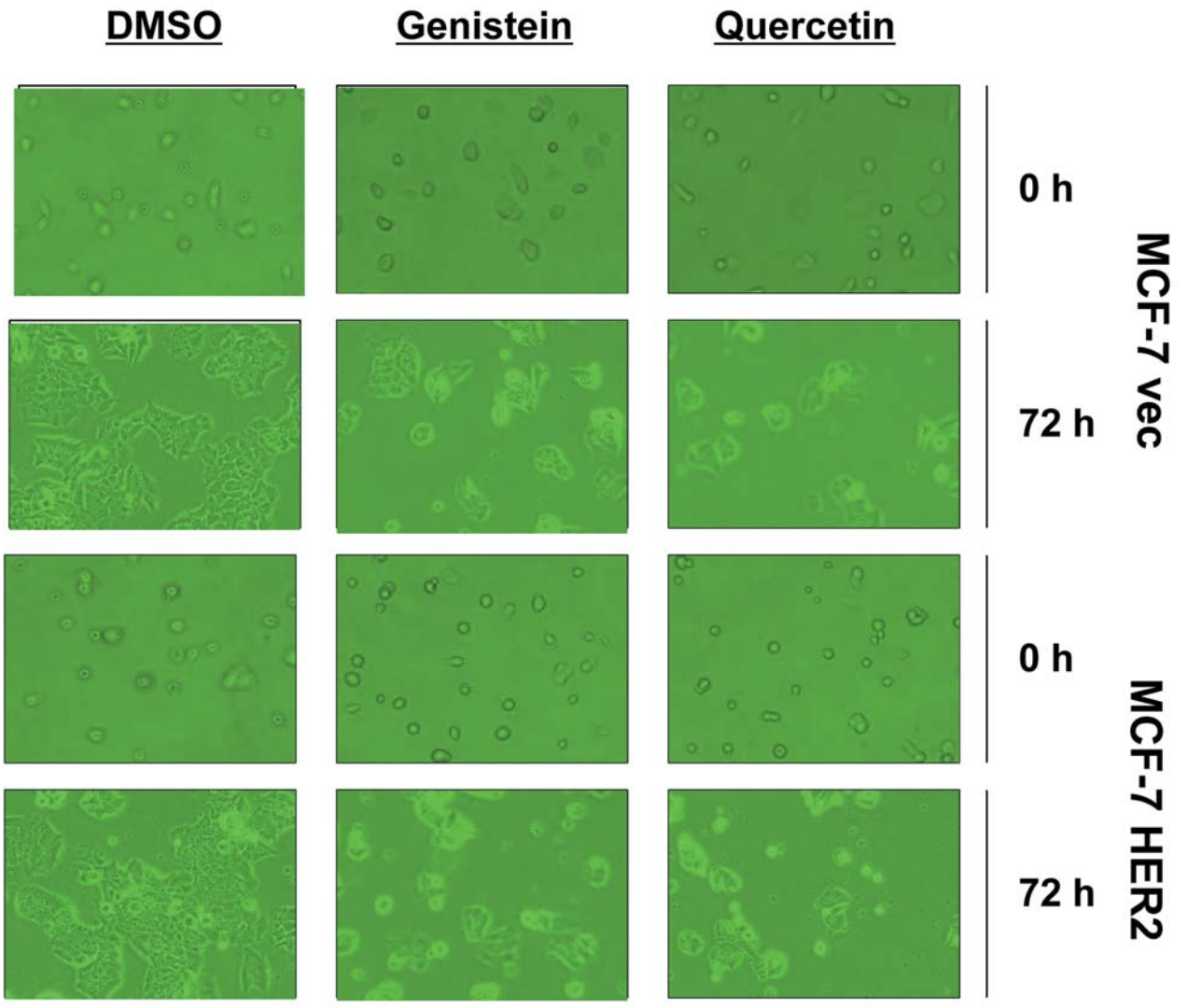

Figure 2. *Phytoestrogens (genistein and quercetin) inhibited the proliferation of MCF-7 vec and MCF-7 HER2 cells. MCF-7 vec and MCF-7 HER2 cells were seeded at a density of 1×10^6 cells on a 100 mm dish. The following day, the cells were treated with 100 μM phytoestrogens for 72 h and photographed by phase contrast microscopy (magnification of 40X). Control groups of cells were treated with DMSO alone.*

transducer and activator of transcription 3 (STAT3). As seen in Figure 7, Western blot analysis demonstrated that phytoestrogen failed to reduce the level of AKT, ERK, JNK, p38 and their active forms (phospho forms) in MCF-7 vec and MCF-7 HER2 cells; quercetin increased the level of p-ERK in MCF-7 vec and MCF-7 HER2 cells and genistein and quercetin increased phospho-ribosomal S6 kinase (p-RSK) in MCF-7 vec cells. This suggests that genistein and quercetin exert their antiproliferative activity *via* AKT, ERK, JNK, and p38-independent manner. Whereas genistein and quecetin reduced the level of p-STAT3 suggesting that they are dependent on the STAT3 signaling pathway.

Genistein and quercetin inhibit the NFκB signaling in MCF-7 vec and MCF-7 HER2 breast cancer cells. To investigate whether phytoestrogens demonstrate their antiproliferative activity by inhibition of NFκB signaling pathway, we measured the NFκB signaling molecules by Western blot analysis. As seen in Figure 8A, we found that both genistein and quercetin inhibited the phosphorylation of IκBα in total cell extracts, suggesting that two compounds abrogate the dissociation of IκBα from NFκB heterodimer (p65 and p50) to suppress NFκB signaling. Both genistein and quercetin also inhibited the nuclear translocation of p65 (subunit of NFκB heterodimer) and its phosphorylation within the nucleus, leading to the inhibition of the transactivation of NFκB target

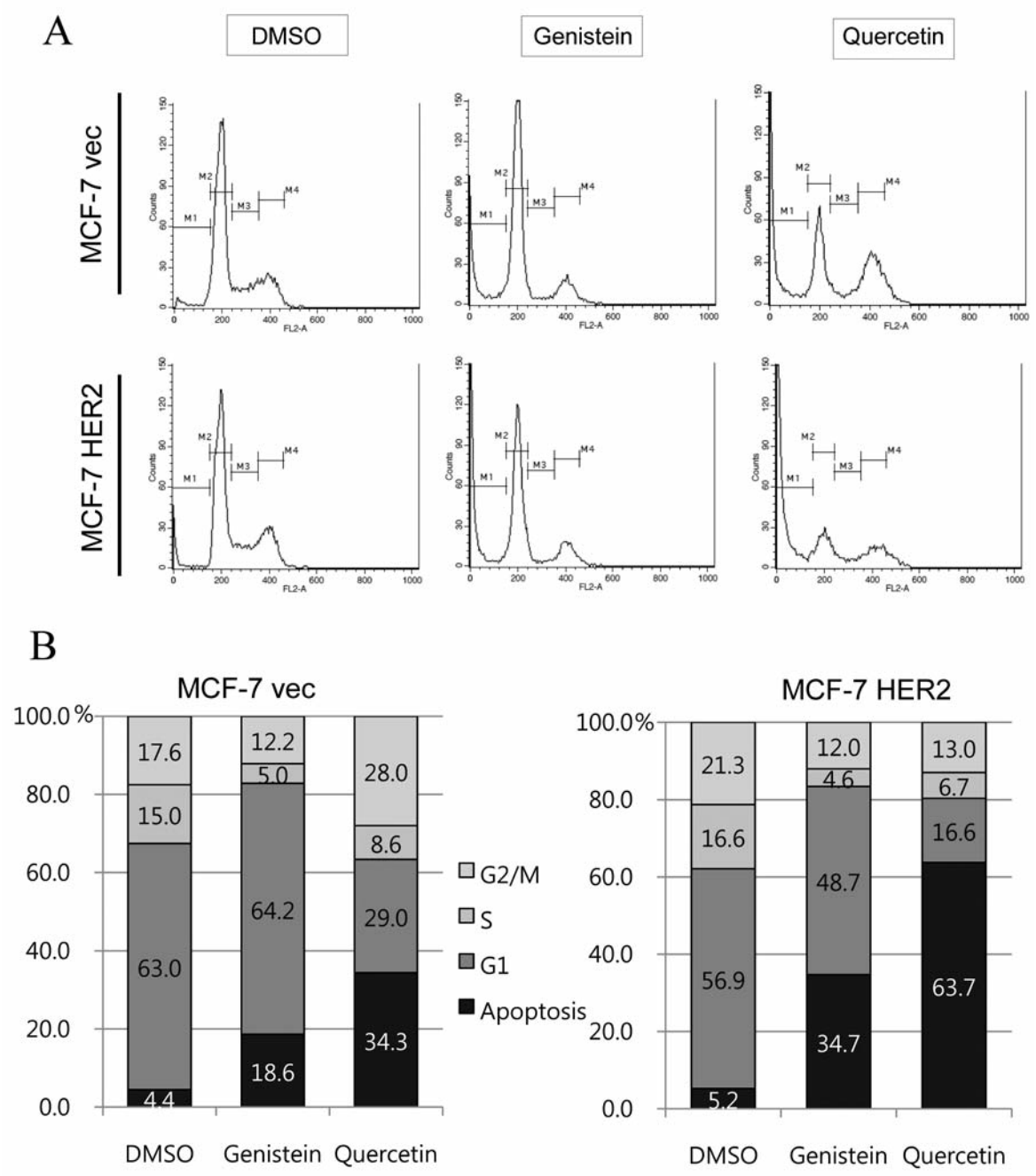

Figure 3. *Effect of phytoestrogens (genistein and quercetin) on the cell cycle and sub G_0/G_1 apoptotic fractions of MCF-7 vec and MCF-7 HER2 cells. A: MCF-7 vec and MCF-7 HER2 cells were treated with 100 μM phytoestrogens and the cells were fixed at 72 h for flow cytometry analyses. Propidium iodide-labeled nuclei were analyzed for DNA content. Representative of two independent experiments. B: Sub G0/G1 apoptotic fractions, G_1, S and G_2/M phase fractions were quantified from DNA histograms data in (A). Representative of two independent experiments.*

genes (Figure 8B). The levels of p65, and phospho-p65 were not significantly changed in the cytosol extract (Figure 8C)

Genistein and quercetin increase the levels of CREB and p-CREB in MCF-7 vec and MCF-7 HER2 breast cancer cells. To determine if phytoestrogens regulate calmodulin activity, we measured the expression levels of calmodulin and p-calmodulin. We found that genistein and quercetin hardly regulate calmodulin and p-calmodulin, while they increased the levels of CREB and p-CREB (Figure 9).

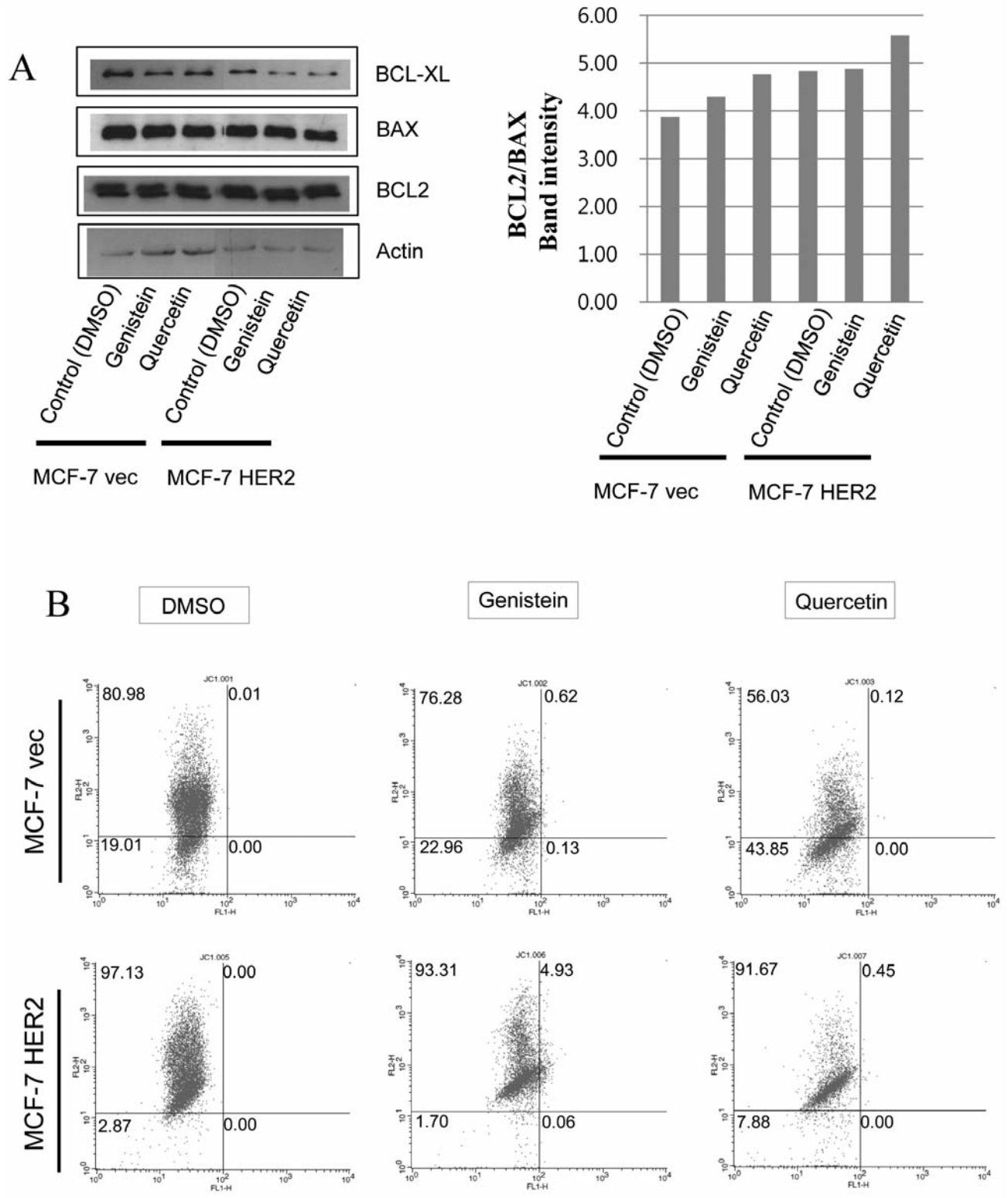

Figure 4. *Phytoestrogens (genistein and quercetin) do not induce apoptosis via the intrinsic mitochondrial apoptosis pathway in both MCF-7 vec and MCF-7 HER2 cells. A: Analysis of intrinsic apoptosis-related molecules. MCF-7 vec and MCF-7 HER2 cells were treated with 100 μM phytoestrogens for 24 h. Total proteins were analyzed by Western blotting with anti-BCL2, -BAX and -actin antibodies. B: MCF-7 vec and MCF-7 HER2 cells were incubated with 100 μM phytoestrogens for 72 h and were dyed with JC-1 (4 μg/ml). The data were analyzed by FACSCalibur flow cytometry measuring the green fluorescence and red fluorescence at 514/529 nm (FL-1) and 585/590 nm (FL-2), respectively.*

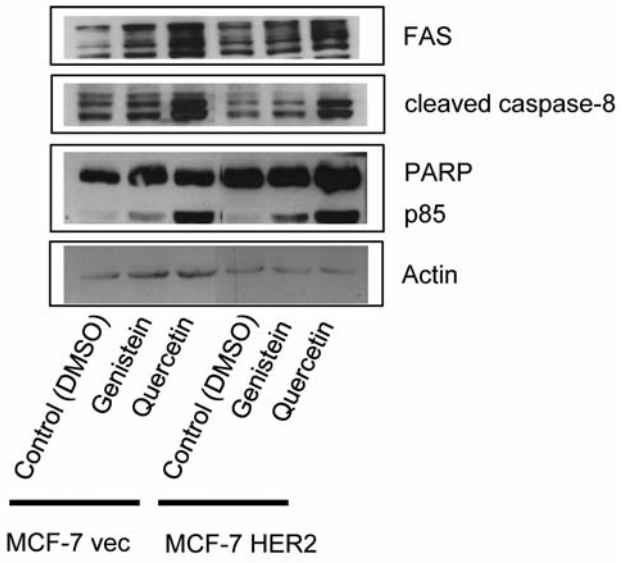

Figure 5. *Phytoestrogens (genistein and quercetin) induce apoptosis via FAS receptor-mediated extrinsic apoptosis pathway in both MCF-7 vec and MCF-7 HER2 cells Analysis of intrinsic apoptosis-related molecules. MCF-7 vec and MCF-7 HER2 cells were treated with 100 μM phytoestrogens for 24 h. Whole-cell lysates were analyzed by Western blotting with anti-FAS, -cleaved caspase-8,-PARP and -actin antibodies.*

Discussion

In the present study, we investigated the antiproliferative activity of phytoestrogens (genistein and quercetin) and their mechanism of action in HER2-overexpressing breast cancer cells. Phytoestrogens suppressed the growth of MCF-7 vec and MCF-7 HER2 cells at concentrations ranging from 50 μM to 100 μM (genistein) and from 25 μM to 100 μM (quercetin). We did not find any resistance of HER2 to genistein and quercetin. Genistein is known to inhibit total HER2 protein expression and tyrosine phosphorylation in BT-474 breast cancer cells (27). However, in our system, both genistein and quercetin failed to inhibit the expression of HER2 or its tyrosine phosphorylation.

Interestingly, both genistein and quercetin increased the expression of p53 and active p53 (p-p53), suggesting that these two phytoestrogens suppress HER2-overexpressing cancer cell growth *via* a p53-dependent manner. The p53 tumor suppressor inhibits cellular proliferation, by inducing cell cycle arrest and apoptosis in response to cellular stresses including DNA damage, growth factor deprivation, hypoxia, and oncogene activation (28, 29). p53-dependent apoptosis is produced by the caspase proteinases and related to pro-apoptotic proteins such as BAX, NOXA and PUMA (28). p53-independent apoptosis is associated with the presence of p73 and the transcription factor E2F-1 (30). p53 and E2F-1 are partners for apoptosis (31). p53 induces growth arrest and

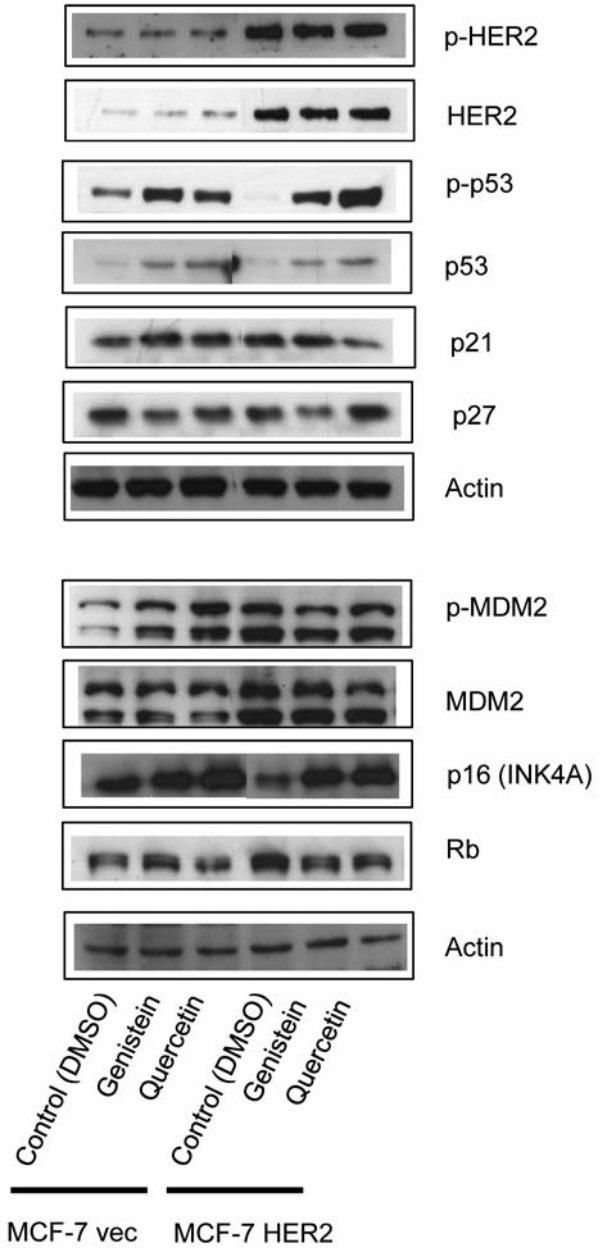

Figure 6. *Phytoestrogens (genistein and quercetin) increase the expression of p53 and p16 (INK4A) in MCF-7 vec and MCF-7 HER2 cells. MCF-7 vec and MCF-7 HER2 cells were treated with 100 μM phytoestrogens for 24 h. Whole-cell lysates were analyzed by Western blotting with anti-p-HER2, -HER2, -p-53, -p21, -p27, -p-p53, -p-MDM2, -MDM2, -p16, -Rb, and -actin antibodies.*

cell death *via* negative regulation of MDM2 (MDM2 inhibits the tumor suppressive activity of p53) and positive regulation of ARF (ARF inhibits MDM2 activity) (ARF-MDM2-p53 pathway). In the INK4A-Rb-E2F pathway, Rb suppresses E2F activity and INK4A helps Rb activity, inhibiting cyclin

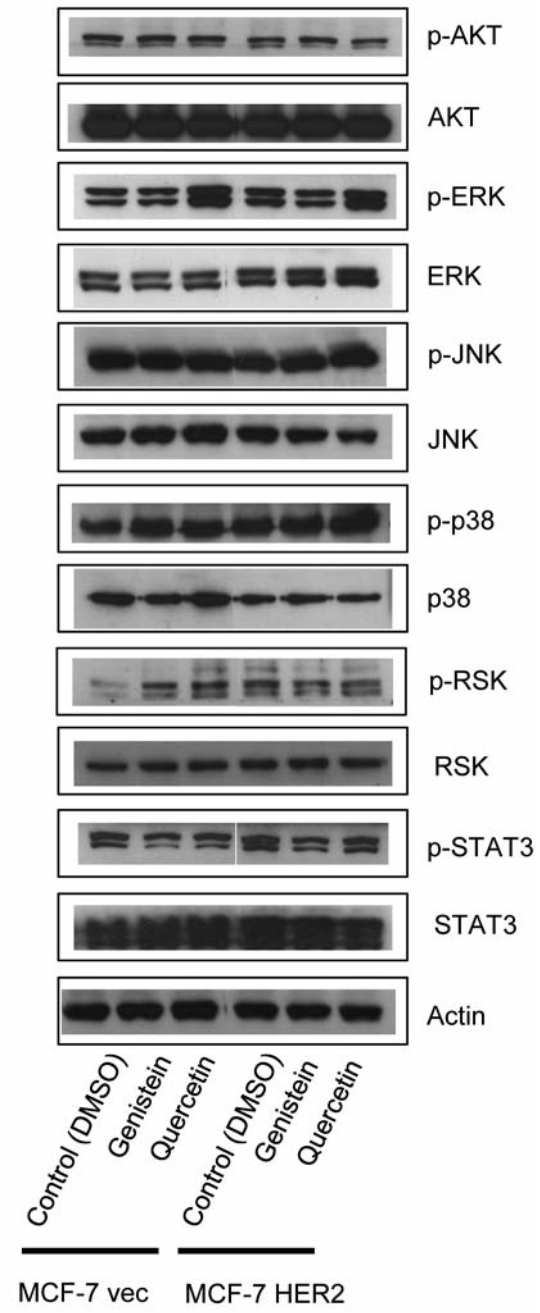

Figure 7. *Effect of phytoestrogens (genistein and quercetin) on the expression of intracellular signaling molecules in MCF-7 vec and MCF-7 HER2 cells. MCF-7 vec and MCF-7 HER2 cells were treated with 100 μM phytoestrogens for 24 h. Whole-cell lysates were analyzed by Western blotting for the detection of specific protein, as indicated (p-AKT, AKT, p-ERK, ERK, p-JNK, JNK, p-p38, p38, p-RSK, RSK, p-STAT3, STAT3, and actin).*

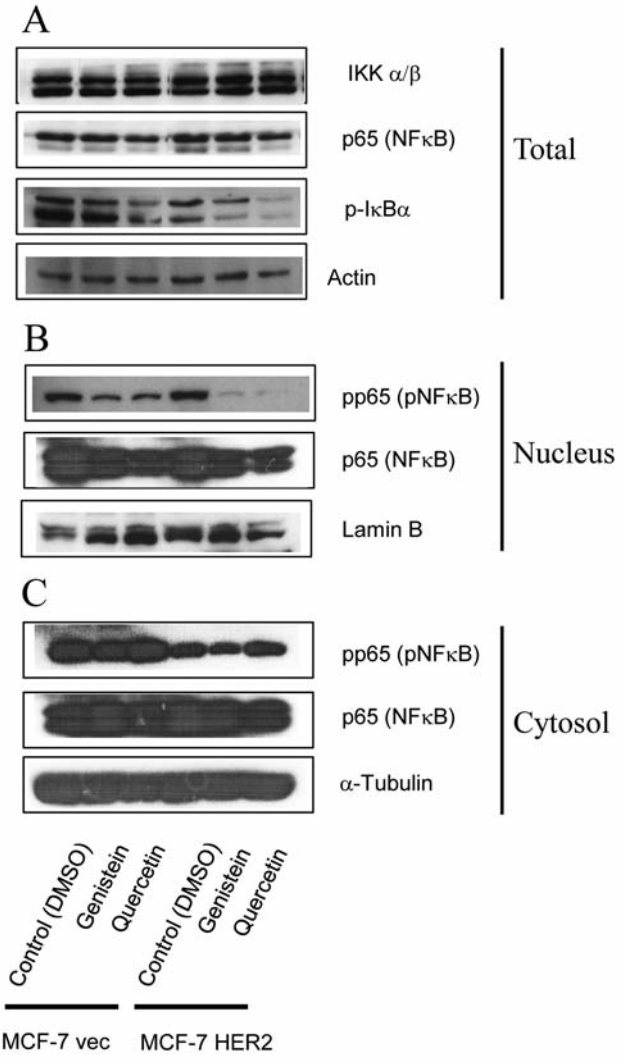

Figure 8. *Effect of phytoestrogens (genistein and quercetin) on NFκB signaling pathway in MCF-7 vec and MCF-7 HER2 cells. MCF-7 vec and MCF-7 HER2 cells were treated with 100 μM phytoestrogens for 24 h. A: Whole-cell lysates were analyzed by Western blotting for the detection of specific protein, as indicated (p-p65, p65, p-IκBα, IKKα/β, and actin). B and C: Nuclear and cytosol extract were prepared as indicated in Methods and Materials and were analyzed by Western blotting for the detection of specific protein, as indicated (p-p65, p65, Lamin B, and α-tublin).*

D, CDK4 or CDK6 to induce apoptosis. We found that genistein and quercetin significantly increased the p16 (INK4A) level to induce apoptosis, suggesting cross-talk between ARF-MDM2-p53 pathway and INK4A-Rb-E2F pathway as reported elsewhere (31).

The growth inhibition by genistein and quercetin was related to the increase of the sub G_0/G_1 apoptotic fraction and the decrease of the cell population in the S phase. Interestingly, genistein and quercetin did not induce apoptosis *via* the intrinsic mitochondrial apoptosis pathway since the two compounds did not reduce the mitochondrial membrane potential, maintaining red flouorescence, and did

not affect the levels of BCL2 and BAX, although they slightly reduced the level of BCL-XL. BCL2 family includes both anti-apoptotic protein (BCL2, BCL-XL, MCL-1, and CED-9 etc) and pro-apoptotic protein (BAX, BAK, DIVA, BCL-Xs, BIK, and BIM etc) (32). All members possess at least one of four conserved motifs known as BCL2 homology domains (BH1 to BH4) (32). Pro- and anti-apoptotic family members can heterodimerize and regulate each other's functions; their relative concentration may be important in modulating apoptosis (32). On the other hand, genistein and quercetin induced the extrinsic apoptosis pathway: genistein and quercetin activated death receptor-related apoptosis signaling, enhancing the cleavage of caspase 8, and PARP as seen in Figure 10.

Interestingly, genistein and quercetin were unable to inhibit the cell survival signaling such as AKT, ERK, JNK and p38, suggesting that they inhibit proliferation of MCF-7 vec and MCF-7 HER2 cells *via* a survival signaling-independent manner. However, genistein and quercetin slightly reduced the level of p-STAT3 in both cell lines, indicating that they induce growth-suppressive activity in a STAT3-dependent manner. STAT3 is a transcription factor and regulates the expression of various genes in response to cell stimuli and plays an important role in cell growth and apoptosis. STAT3 acts usually as a tumor promoter, although its tumor-suppressor role is recently reported (33, 34). Phytoestrogen (resveratrol) is known to inhibit STAT3 signaling and induces the apoptosis of malignant cells containing activated STAT3 (35).

NFκB is a transcription factor and plays an important role in cell proliferation, survival, inflammation, immune response, tumor formation, and tumor progression. The NFκB family consists of p50/p105 (NFκB1), p52/p100 (NFκB2), REL A (p65), c-REL and REL B. In cells, the p65-p50 heterodimer is maintained in an inactive state due to binding of IκB. However, when IκB is phosphorylated *via* IκB kinase, phospho-IκB is detached from p65-p50 dimer to be degraded. Free p65-p50 heterodimer then can enter the nucleus, and bind to specific DNA sequences (κB site) to induce the transcription of target genes related to tumor promotion, cell survival signaling, and inflammation (Figure 10). In our study, both genistein and quercetin suppressed the growth of MCF-7 vec and MCF-7 HER2 cells, inhibiting the NFκB signaling pathway. Clearly, both compounds abrogated the phosphorylation of IκBα, and nuclear translocation of p65, as well as its phosphorylation within the nucleus.

Calmodulin is a calcium-binding protein which plays a certain role in inflammation, apoptosis, metabolism, intracellular movement, and memory. It was reported that phytoestrogens increased the expression of calmodulin in male rat (36, 37). In our study, genistein and quercetin barely regulated the expression of calmodulin and p-calmodulin, suggesting that the antiproliferative activity of phytoestrogens is not related to calmodulin activity. On the other hand,

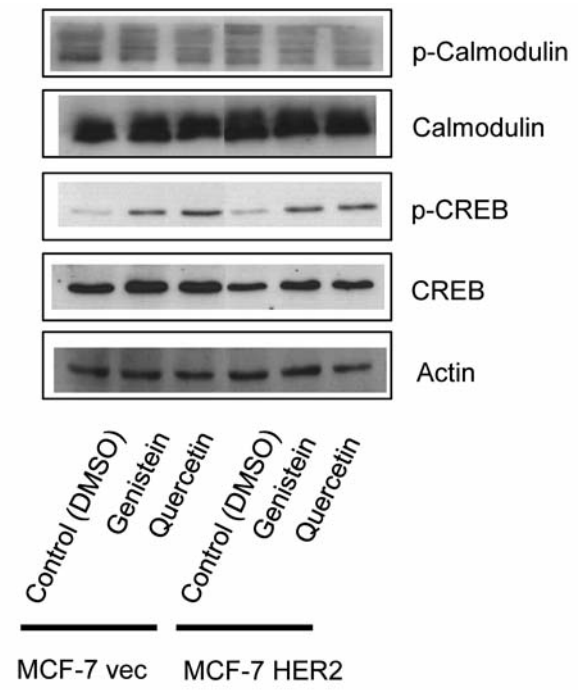

Figure 9. *Effect of phytoestrogens (genistein and quercetin) on Calmodulin and CREB activity in MCF-7 vec and MCF-7 HER2 cells. MCF-7 vec and MCF-7 HER2 cells were treated with 100 μM phytoestrogens for 24 h. Whole-cell lysates were analyzed by Western blotting for the detection of specific protein, as indicated (p-calmodulin, calmodulin, p-CREB, CREB, and actin).*

genistein and quercetin increased CREB and p-CREB level. CREB is a transcription factor which binds to cAMP response element (CRE) to regulate target genes which are implicated in differentiation, cell cycle progression, apoptosis suppression, proliferation, and tumorigenesis (38-42). CREB is activated *via* multiple signaling pathways (43, 44) including cAMP/protein kinase A, phosphatidylinositol 3-kinase (PI3K)/AKT, ERK/RSK, p38/mitogen- and stress-activated protein kinase pathways, and calmodulin kinase II and IV. It was reported that genistein increases CREB phosphorylation in neonatal hypothalamus (45). In agreement with this data, we also observed that genistein and quercetin increased the levels of CREB and p-CREB.

It should be noted that genistein and quercetin displayed the same effects on the apoptotic cell death of MCF-7 vec and MCF-7 HER2 cells, with a similar mechanism, probably due to their similar chemical structure.

About 20 to 25% of invasive breast carcinomas have *HER2* gene amplification (46). A normal breast cell has 20,000 HER2 receptors but a breast cancer cell could have 1.5 million. HER2 is a member of HER/ErbB2/Neu protein, which also includes HER1/EGFR, HER3 and HER4. HER2 cross talks with estrogen receptor (ER) signal transduction

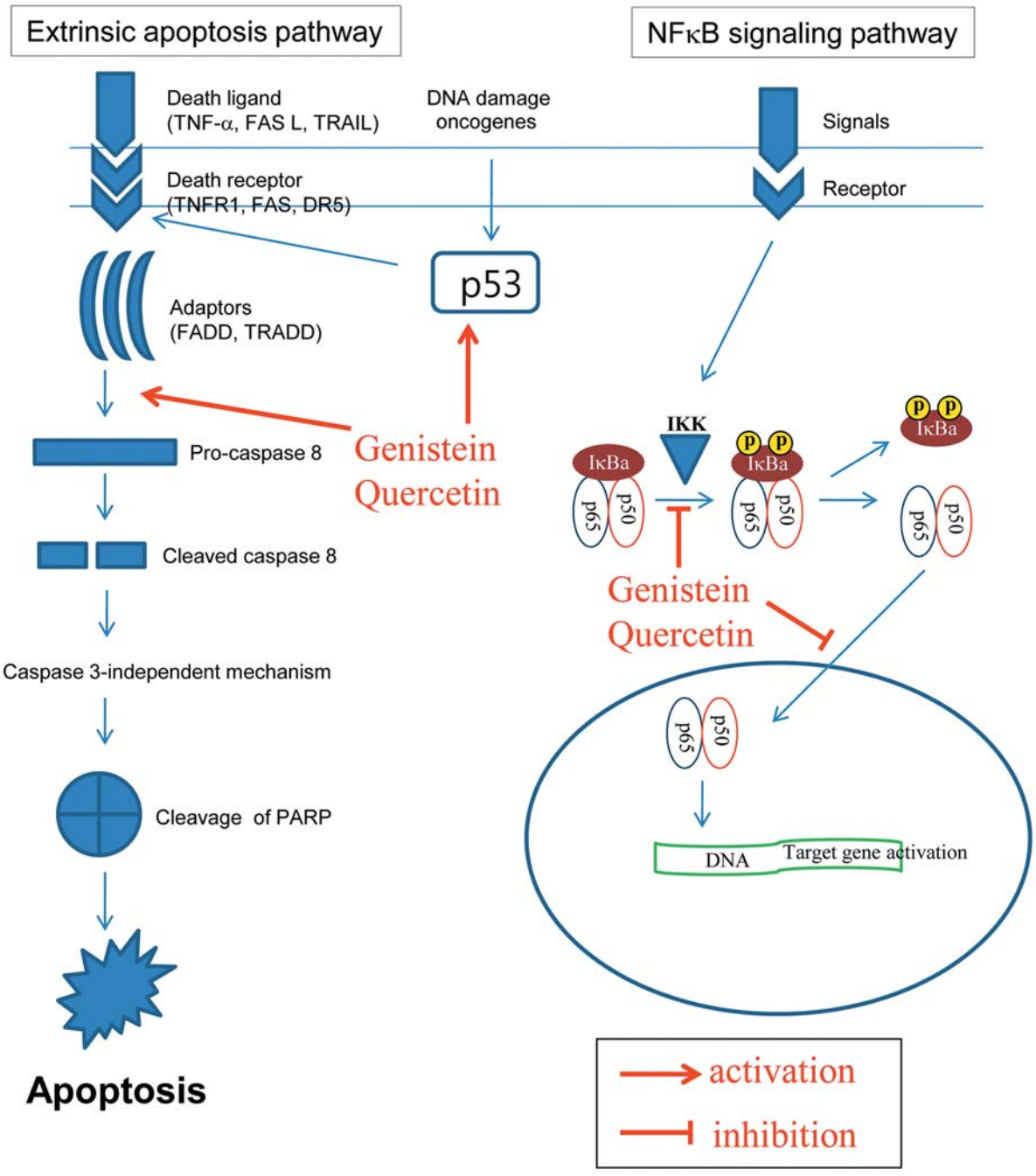

Figure 10. *Molecular mechanism of actions of phytoestrogens (genistein and quercetin) on apoptosis and NFκB signaling pathway in both MCF-7 vec and MCF-7 HER2 cells.*

pathway (47) and its expression level can be regulated by ER. Trastuzumab is effective in breast cancer where HER2 is overexpressed. It works against cancer, maximizing the expression and inhibitory effect of p27, which leads to cell cycle G_1 arrest and growth inhibition (23). However, many women do not respond to trastuzumab or generate resistance (24). This results in significant efforts to find other compounds which could be effective for HER2-overexpressing breast cancer. In our study, we found that phytoestrogens (genistein and quercetin) significantly inhibited the growth of MCF-7 HER2-overexpressing cancer cells. This indicates that genistein and quercetin could be a useful natural therapy that

inhibits HER2-overexpressing breast cancer. Genistein and quercetin could be promising products to treat and prevent HER2-overexpressing breast cancer.

Declaration of interest

The Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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