Induction of Caspase-dependent Apoptosis by Apigenin by Inhibiting STAT3 Signaling in HER2-overexpressing MDA-MB-453 Breast Cancer Cells

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Abstract. Background: This study aimed to examine the effect of apigenin on proliferation and apoptosis in HER2-overexpressing MDA-MB-453 breast cancer cells. Materials and Methods: The antiproliferative effects of apigenin were examined by proliferation and MTT assays. The effect of apigenin on apoptotic molecules was determined by western blotting. RT-PCR was performed to measure mRNA levels of HIF-1α and VEGF. ELISA assay was performed to measure intracellular VEGF levels. Immunocytochemistry was performed to evaluate nuclear STAT3 level. Results: Apigenin inhibited the proliferation of MDA-MB-453 cells. Apigenin upregulated the levels of cleaved caspase-8 and caspase-3, and induced the cleavage of PARP. Apigenin induced extrinsic apoptosis and blocked the activation (phosphorylation) of JAK2 and STAT3. Apigenin inhibited CoCl2-induced VEGF secretion and decreased the nuclear staining of STAT3. Conclusion: Apigenin exerts its antiproliferative activity by inhibiting STAT3 signaling. Apigenin could serve as a useful compound to prevent or treat HER2-overexpressing breast cancer.

Cancer prevention/treatment through food may be largely feasible by increased consumption of fruits and vegetables. Considerable efforts have been put to identify plant-derived dietary agents which could prevent or treat cancer. A naturally-occurring plant flavone, (4', 5, 7-trihydroxyflavone), apigenin abundantly presents in common fruits and vegetables including parsley, onions, oranges, tea, chamomile, wheat sprouts and some seasonings (1, 2). Apigenin is known to exert anti-oxidant (2), anti-inflammatory (3), anti-telomerase (4), and anti-depressant activities (5). Notably, apigenin also possesses antitumor properties and, therefore, has special interest for the development of a novel chemopreventive and/or chemotherapeutic agents. It was previously demonstrated that apigenin reduces the tumor volume and mass of androgen-sensitive 22Rv1 and androgen-insensitive PC-3-implanted cells (6). Apigenin also suppresses inducible cyclooxygenase and inducible nitric oxide synthase in mouse macrophages (7) and inhibits ultraviolet light-induced skin carcinogenesis in SKH-1 mice (8). In addition, apigenin induces growth inhibition and apoptosis in a variety of cancer cell lines, including breast (9), lung (10), colon (11, 12), prostate (13), leukemia (14), and pancreatic (15) cells.

Apoptosis plays an important role in determining cellular fate; it participates in development, cellular homeostasis, and both physiological as well as pathological processes (16-18). Caspases are aspartate-specific cysteine proteases and members of the interleukin-1β-converting enzyme family and essential in cells for apoptosis (16, 17). The caspase cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals (16, 17). The apoptotic-signaling cascade is divided into two major pathways, extrinsic and intrinsic. The extrinsic pathway is related to the activation of death receptors, such as Fas and the tumor necrosis factor receptors (TNFRs) and the cleavage of caspase-8 and caspase-3 (19-21). The intrinsic pathway is related to changes in the mitochondrial membrane potential (Δψm), the mitochondrial permeability transition, and the cleavage of caspase-9 and caspase-3 (22). In both the extrinsic and intrinsic pathways, caspase-3 is responsible for the cleavage of poly (ADP-ribose) polymerase (PARP) during cell death (23).
Human epidermal growth factor receptor-2 (HER2)-positive breast cancers tend to grow more quickly than HER2-negative breast cancers. In HER2-positive breast cancers, hormonal receptors expression is associated with a poor prognosis despite the hormone therapy (24). HER2 amplification or overexpression is a risk factor for recurrence (24). Between 15 and 25 out of every 100 women with breast cancer (15-25%) have HER2-positive cancers (25). Effective treatments called targeted (or sometimes biological) therapies have been developed to treat HER2-positive breast cancer. The drug most commonly used is trastuzumab (Herceptin®) (26). Trastuzumab induces the down-regulation of HER-2/Neu, leading to the disruption of receptor dimerization and signaling (27). Trastuzumab also causes arrests during G1 phase of the cell cycle and inhibits the phosphorylation of p27Kip1, resulting in the suppression of cdk2 activity and reduced proliferation (28). Trastuzumab suppresses angiogenesis by both the induction of anti-angiogenic factors and the repression of pro-angiogenic factors. However, many women do not respond to trastuzumab or develop resistance to this drug (29). This has resulted in significant efforts to identify other compounds that can effectively treat HER-2-overexpressing breast cancer.

Previously, we reported that genistein and queretin induce apoptosis via the extrinsic pathway inhibiting nuclear factor-kappa B (NF-kB) signaling in HER2-transfected MCF-7 cells (30). We also reported that apigenin promotes apoptosis via the extrinsic pathway, inducing p53 and inhibiting signal transducer and activator of transcription-3 (STAT3) and NF-kB signaling in HER2-transfected MCF-7 cells (31). In the present study, we investigated whether apigenin produces apoptosis-inducing cleavage of caspases and PARP and regulating STAT3 signaling. We also investigated whether apigenin regulates the levels of VEGF which is a STAT3 target gene. Our study will provide new insight on the role of phytoestrogens in the prevention or treatment of HER2-overexpressing breast cancer.

Materials and Methods

Compounds. Apigenin (4’-5,7-trihydroxyflavone) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Apigenin was 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 compared to 0% DMSO (no treatment) in the breast cancer cells (data not shown). The caspase-8 inhibitor Z-IEED-fmk and the caspase-9 inhibitor Z-LEHD-fmk were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). The STAT3 inhibitor S31-201 was obtained from Calbiochem (Billerica, MA, USA). An EZ-western chemiluminescent detection kit was purchased from Daecillab Service Co. (Seoul, Korea).

Cell culture. The human breast cancer cell line MDA-MB-453 (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI containing 50 U/ml penicillin, 50 mg/ml streptomycin and 10% FBS (Welgene, Daegu, Korea) at 37°C in an atmosphere of 5% CO₂.

Antibodies. Primary antibodies against FAS, cleaved caspase-8, caspase-3, cleaved caspase-3, PARP, and HER2 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies against Bcl-2, BAX, p53, phospho-p53 (Ser15), p21 and VEGF were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibodies against Bcl-xL and HIF-1α were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Primary antibodies against STAT3, phospho-STAT3 (Tyr705), phospho-JAK2 (Tyr1007/Tyr1008) were obtained from Upstate-Millipore (Billerica, MA, USA). The anti-tubulin antibody was from Sigma Chemical Co. Horseradish peroxidase (HRP)-conjugated secondary antibodies (mouse and rabbit) were purchased from Calbiochem (San Diego, CA, USA).

Cell proliferation assay. Cells were seeded in 12-well culture plates at a density of 8x10⁴ cells/well. After the cells were exposed to different concentrations of apigenin (0-60 μM) and incubated for 3 days, they were harvested by trypsinization, re-suspended in 1-2 ml medium, and counted using a hemocytometer.

MTT assay. Cells were seeded in 96-multiwell culture plates at densities of 5x10³ - 7x10³ cells/well and incubated overnight at 37°C. Then, they were treated with different concentrations of apigenin (0-60 μM) for 24 h, 48 h, or 72 h. After incubation, MTT reagent (0.5 mg/ml) was added to each well, and the plates were incubated in the dark at 37°C for 2 h. At the end of the incubation, the medium was removed, the resulting formazan was dissolved in DMSO, and the optical density was measured at 570 nm using an ELISA plate reader.

Cell-cycle analysis 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 cold 95% ethanol with 0.5% Tween-20, and stored at –20°C for at least 30 min. The cells were incubated in 50 μg/ml propidium iodide (PI) (including 1% sodium citrate and 50 μg/ml RNase A) at room temperature in the dark for 30 min. Analysis of the apoptotic cells was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA), and the data were analyzed using CellQuest software.

Immunocytochemistry. Cells (6x10⁴ cells/well) were seeded in 8-well chamber slides, incubated for 24 h at 37°C and treated with apigenin (60 μM) in the presence or absence of CoCl₂ for another 24 h. The cells were fixed with 4% paraformaldehyde for 30 min and treated with 3% hydrogen peroxide (H₂O₂) in methanol for 20 min to quench the endogenous peroxidase activity. The cells were washed with PBS, blocked with 5% BSA in PBS for 1 h and incubated with the anti-STAT3 primary antibody (1:100 dilution) overnight at 4°C. After washing with PBS, the cells were incubated with the anti-rabbit biotin-conjugated secondary antibody for 1 h at room temperature. Then, the cells were treated with Vectastain ABC reagent (Vector Laboratories, Inc. Burlingame, CA, USA) for 30 min at 4°C and stained with diaminobenzidine tetrachloride (DAB) and hematoxylin. The cells were mounted with mounting medium and subsequently analyzed by microscopy.

Measurement of VEGF secreted from MDA-MB-453 cells by ELISA. To assess the level of VEGF in the MDA-MB-453 cell supernatants, the cells were treated with apigenin (0-60 μM) in the presence or absence of CoCl₂ (100 μM) to mimic hypoxia. After 24 h, the media were collected, centrifuged to remove the cellular debris, and stored
Statistical analysis. All experiments were performed in triplicate. The data for the cell proliferation assay, MTT assay and ELISA assay are expressed as the mean±standard deviation (SD). The standard deviations for all of the measured biological parameters are displayed in the appropriate Figures. A student’s t-test was used for single variable comparisons, and a p-value of <0.05 was considered statistically significant.

Table 1. The sequence of primers used.

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<td></td>
<td>Reverse 5’CCA GCA AAG TTA AAG CAT CAG GTT CC 3’</td>
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<tr>
<td>VEGF</td>
<td>Forward 5’AAG GCC CAC AGG GAT TTT CT 3’</td>
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<td>Reverse 5’AGG AGG GCA GAA TCA TCA CG 3’</td>
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<td>GAPDH</td>
<td>Forward 5’CGG CCA TCA CGC CAC AGT TT 3’</td>
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<td>Reverse 5’CGT CTT CAC CAC CAT GGA GA 3’</td>
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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 Na3VO4, and a 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 Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein lysates were used for western blot analysis with the indicated antibodies. The immunoreactive protein bands were detected using an EZ-Western Detection kit (Daebillab Service Co., Seoul, Korea).

Results

Apigenin suppresses the growth of MDA-MB-453 cells. We investigated the growth suppressive activity of three different phytoestrogens (apigenin, genistein and quercetin) in MDA-MB-453 cells using a cell proliferation assay. As shown in Figure 1A, apigenin, genistein and quercetin significantly inhibited MDA-MB-453 cell proliferation in a dose-dependent manner (0-100 μM) after 72 h of treatment. In addition, the time-dependent growth suppressive activity of apigenin was measured by the MTT assay, as shown in Figure 1B. It seems that the proliferation assay was more sensitive than the MTT assay with respect to measuring the intensity of the cell growth inhibition, as shown in Figure 1A and 1B. Moreover, the growth inhibition induced by apigenin was verified by microscopic observation. The results in Figure 1C show that apigenin effectively inhibited the growth rate of MDA-MB-453 monolayer cells after 72 h of treatment. Of note, apigenin also induced morphological changes in these cells (Figure 1C).

The growth-suppressive activity of apigenin is accompanied by an increase in the sub G0/G1 apoptotic population in MDA-MB-453 cells. To investigate whether apigenin inhibits cell proliferation by promoting changes in cell-cycle progression, the effect of apigenin on the cell-cycle profile was assessed in MDA-MB-453 cells. For this purpose, cells were treated with apigenin (0-60 μM) for 72 h and then analyzed for cell-cycle stage by flow cytometry. The results demonstrated that apigenin induced an increase in the sub G0/G1 apoptotic population in MDA-MB-453 cells (Figure 2).

Apigenin induces apoptosis via a caspase-dependent pathway in MDA-MB-453 cells. In this step, we investigated whether apigenin activates caspase-dependent apoptosis by measuring the expression of caspase-8, caspase-3, and PARP. We observed that apigenin up-regulated the levels of cleaved caspase-8 and caspase-3, and induced the cleavage of PARP in SKBR3 cells (Figure 3A). We also found that the cleavage at –70°C until assayed for VEGF. The amount of VEGF secreted into the culture medium was measured by ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Briefly, 96 well plates were coated with capture antibody in ELISA coating buffer and incubated overnight at 4°C. The plates were then washed with PBS with 0.05% Tween 20 (PBS-T) and subsequently blocked with 10% PBS in PBS for 1 h at 20°C. Serial dilutions of standard antigen or sample in dilution buffer (10% FBS in PBS) were added to the plates, and the plates were incubated for 2 h at 20°C. After washing, biotin-conjugated anti-mouse IgE and streptavidin-conjugated horseradish peroxidase (SAv-HRP) were added to the plates, and the plates were incubated for 1 h at 20°C. Finally, the tetramethylbenzidine (TMB) substrate was added to the plates, and after 15 min of incubation in the dark, 2 N H2SO4 was added to stop the reaction. The optical density was measured at 450 nm on an automated ELISA reader.

Detection kit (Daeillab Service Co., Seoul, Korea).

RT-PCR was performed by coamplification of the genes with a reference gene by use of the cDNA template and corresponding gene-specific primer sets.

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of caspase-8, caspase-3, and PARP was inhibited by the caspase-8 inhibitor Z-IETD-fmk and the caspase-9 inhibitor Z-LEHD-fmk (Figure 3B), but apigenin prevented this inhibition and was able to induce the cleavage of caspase-3 and PARP in the presence of Z-IETD-fmk and Z-LEHD-fmk (Figure 3B). Moreover, the caspase-8 and caspase-9 inhibitors did not suppress cell growth, but apigenin was able to induce apoptosis even in their presence (Figure 3C). These results confirm that apigenin strongly promotes apoptosis via a caspase-dependent mechanism.

![Figure 1. Effect of apigenin on MDA-MB-453 cell growth. (A) MDA-MB-453 cells were treated with different doses of apigenin, genistein and quercetin (0-100 μM). After 72 h, cell viability was assessed using a cell proliferation assay. (B) MDA-MB-453 cells were treated with different doses of apigenin (0-100 μM). The relative cell growth rate was measured by the MTT assay after 24 h, 48 h and 72 h. The growth rate of the vehicle-treated cells was set to 100%, and the relative decrease in cell viability resulting from the phytoestrogen treatment was expressed as a percentage of the control. (C) MDA-MB-453 cells were treated with different doses of apigenin (0-60 μM) for 72 h and photographed by phase-contrast microscopy (original magnification, ×40). Control cells were treated with DMSO-alone. Data are shown as the means of three independent experiments (error bars denote SD). *p<0.05, **p<0.01, ***p<0.001.](image-url)
Figure 2. Effect of apigenin on the cell cycle and subG0/G1 apoptotic population of MDA-MB-453 cells. (A) MDA-MB-453 cells were treated with apigenin (0-60 μM) and fixed for 72 h for flow cytometry. Propidium iodide-labeled nuclei were analyzed for DNA content. (B) The subG0/G1 apoptotic population and the G1, S and G2/M phase populations were quantified using DNA histograms. The data shown are representative of three independent experiments that gave similar results.
Apigenin induces extrinsic apoptosis in MDA-MB-453 cells. Next, we investigated whether apoptosis induced by apigenin occurs via the extrinsic apoptosis pathway in MDA-MB-453 cells. For this purpose, we measured the levels of BCL2 family members (BAX, Bcl-2, and Bcl-xl) which are important in non-extrinsic (intrinsic mitochondrial) apoptosis pathway. We found that apigenin failed to regulate the levels of BAX, Bcl-2, and Bcl-xl in MDA-MB-453 cells as seen in Figure 4A and 4B. We also measured the loss of mitochondrial transmembrane...
Figure 4. Apigenin induces apoptosis via the extrinsic pathway in MDA-MB-453 cells. (A) and (B) analysis of intrinsic apoptosis-related molecules. MDA-MB-453 cells were treated with apigenin (0-60 μM) for 24 h. Total proteins were analyzed by western blotting with anti-Bcl-2, anti-BAX, anti-Bcl-xL, and anti-tubulin antibodies. (C) MDA-MB-453 cells were incubated with apigenin (0-60 μM) 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. The data shown are representative of three independent experiments that gave similar results.
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

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**Figure 5.** Effect of apigenin on STAT3 activation in MDA-MB-453 cells. (A) MDA-MB-453 cells were treated with apigenin (0-60 μM) for 24 h. Whole-cell lysates were analyzed by western blotting with anti-HER2, anti-p-p53, anti-p53, anti-p21 and anti-tubulin antibodies. (B) MDA-MB-453 cells were treated with apigenin (0-60 μM) for 24 h. Whole-cell lysates were analyzed by western blotting with anti-p-JAK2, anti-p-STAT3, anti-STAT3, anti-VEGF, and anti-tubulin antibodies. (C) MDA-MB-453 cells were treated with apigenin (60 μM) for 24 h in the presence or absence of CoCl$_2$ (4 h). Whole-cell lysates were analyzed by western blotting with anti-phospho-STAT3, anti-HIF-1α, anti-STAT3, and anti-tubulin antibodies. (D) MDA-MB-453 cells were treated with apigenin (60 μM) for 24 h in the presence or absence of CoCl$_2$ and then submitted to immunocytochemistry for detection of nuclear STAT3. The data shown are representative of three independent experiments that gave similar results.
shows only green fluorescence. In our study, apigenin did not induce a low mitochondrial transmembrane potential ($\Delta \Psi_m$), showing relatively weak green fluorescence (DMSO: 5.90%, Api 20 μM: 7.49%, Api 40 μM: 6.88%, Api 60 μM: 17.39%) as compared to FCCP (positive control: 51.91%) (Figure 4C). These results demonstrate that apigenin does not induce apoptosis via the intrinsic mitochondrial pathway but induces apoptosis via the extrinsic pathway in MDA-MB-453 cells.

**Effect of apigenin on STAT3 activation in MDA-MB-453 cells.** Figure 5A shows that MDA-MB-453 cells express HER2 and apigenin slightly up-regulates phospho-p53 (p-p53) and p21 (p53 target gene). In Figure 5(B), we investigated whether apigenin affects STAT3 signaling by measuring levels of p-STAT3 and VEGF (STAT-3 target gene). We found that apigenin reduced the expression of p-STAT3 as well as p-JAK2 (an upstream kinase of STAT3) (Figure 5B). Apigenin also reduced the level of VEGF (Figure 5B). Since STAT3 is a potential modulator of HIF-1α, we observed the relationship between STAT3 and HIF-1α. We found that apigenin suppressed the expression of p-STAT3 and Hif-1α that was up-regulated by CoCl2 (hypoxia mimic) (Figure 5C). Immunocytochemical staining indicated
that apigenin decreased the nuclear localization of STAT3 in the presence and absence of CoCl$_2$ (Figure 5D). As seen in Figure 6A, apigenin decreased mRNA levels of HIF-1α and VEGF that was slightly up-regulated by CoCl$_2$. Moreover, apigenin strongly decreased the CoCl$_2$-induced up-regulation of intracellular VEGF (Figure 6B). These results suggest that apigenin decreases the cell growth rate by inhibiting the JAK2-STAT3-VEGF signaling pathway.

**Effect of S31-201 on STAT3 activation in MDA-MB-453 cells.** Finally, we investigated whether the STAT3 inhibitor S31-201 inhibits cell proliferation and STAT3 activation in MDA-MB-453 cells. As shown in Figure 7A and B, S31-201 decreased cell growth in a dose- and time-dependent manner. Furthermore, S31-201 reduced the expression of p-STAT3 and VEGF (Figure 7C). These results demonstrate that STAT3 inhibition induces cell growth inhibition and represses the expression of oncogenic molecules.

**Discussion**

In the present study, we investigated the anti-proliferative activity of apigenin and its mechanisms of action in HER2-overexpressing breast cancer cells (Figure 8). Apigenin suppressed the growth of MDA-MB-453 cells in a dose- (0-100 μM) and time- (0-72 h) dependent manner. The growth inhibition induced by apigenin was related to an increase in the sub-G$_0$/G$_1$ apoptotic population in MDA-MB-453 cells. Apigenin increased the number of apoptotic cells in a dose-dependent manner, as assessed by FACS analysis. Interestingly, apigenin induced apoptosis via a caspase-dependent pathway by enhancing the cleavage of caspase-8, caspase-3, and PARP. To confirm whether the apoptosis induced by apigenin occurs via a caspase-8- and caspase-3-dependent pathway, we treated MDA-MB-453 cells with the caspase-8 inhibitor Z-IETD-fmk and the caspase-9 inhibitor Z-LEHD-fmk and performed western blot analysis. We found that the cleavage of caspase-8, caspase-3, and PARP was inhibited by the caspase-8 and caspase-9 inhibitors (Figure 3B). However, this inhibition was abrogated by apigenin, suggesting that this phytochemical contains a strong apoptotic capacity. The caspases, a family of cysteine-dependent aspartate-directed proteases, are common death proteases (32). Caspases are synthesized as relatively inactive zymogens that become activated by scaffold-mediated transactivation or by cleavage via upstream proteases in an intracellular cascade (32). Once activated, they cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery, and a number of protein kinases (32).

It should be noted that apigenin did not induce apoptosis via the intrinsic mitochondrial apoptosis pathway since this
The compound did not reduce the mitochondrial membrane potential, maintaining red fluorescence, and did not affect the levels of Bcl-2 and BAX. BCL2 family includes both anti-apoptotic proteins (Bcl-2, Bcl-XL, MCL-1, and CED-9 etc) and pro-apoptotic proteins (BAX, BAK, DIVA, BCL-Xs, BIK, and BIM etc.) (33). All BCL2 members possess at least one of four conserved motifs known as BCL2 homology domains (BH1 to BH4) (33). Pro- and anti-apoptotic family members can heterodimerize and regulate each other’s functions; their relative concentration may be important in modulating apoptosis (33). On the other hand, apigenin induced the extrinsic apoptosis pathway: apigenin activated death receptor-related apoptosis signaling, enhancing the cleavage of caspase-8, caspase-3 and PARP.

Our western blot data also indicated that apigenin slightly increased the expression of p53, active p53 (p-p53) and p21, suggesting that this compound suppresses HER2-overexpressing cancer cell growth via a p53-dependent manner. In agreement with our data, apigenin has been shown to induce G1 or G2/M arrest and apoptosis in human prostate carcinoma cells (34, 35), human cervical carcinoma cells (36), and human hepatoma cells (37) through 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 (38, 39). p53-dependent apoptosis is produced by the caspase proteinases and is related to pro-apoptotic proteins such as BAX, NOXA and PUMA (38).

Interestingly, apigenin reduced the expression of p-STAT3 and p-JAK2 in MDA-MB-453 cells. The VEGF promoter contains various transcription factor binding sites, including sites for STAT3 (40) and HIF-1 (41). The physical interaction

Figure 8. Molecular mechanism of action for apigenin.
of STAT3 with HIF-1 controls VEGF transcriptional activation by their binding to the VEGF promoter (42). In our study, we found that apigenin inhibited VEGF expression/production as well as STAT3 expression/nuclear localization in the presence or absence of CoCl₂. The STAT3 inhibitor S31-201 decreased the expression of p-STAT3 and VEGF. In our experiments, culturing of MDA-MB-453 cells under conditions that mimicked hypoxia or normoxia did not induce the expression or activation of MMP-2 or MMP-9 (data not shown), as indicated in a previous report (43). The co-culture of MDA-MB-453 with another cell line or tumor-associated macrophages induces the expression and activation of MMP-2 and MMP-9 (43). Our data clearly demonstrate that apigenin suppresses cell growth by inhibiting the STAT3-VEGF signaling pathway in MDA-MB-453 cells (Figure 8). STAT3 is a transcription factor that regulates the gene expression in response to various cellular stimuli and plays an important role in cell growth and apoptosis. STAT3 usually acts as a tumor promoter, although its role as a tumor-suppressor has been recently reported (44, 45). STAT3 accelerates cell proliferation and angiogenesis, inhibits apoptosis, and drives invasion and metastasis (36-38). STAT3 in melanoma tumors is associated with poor prognosis (46-48). Constitutive STAT3 phosphorylation is mediated by several upstream kinases (Jak and Src) and is thought to be a key component of the oncogenic process (49, 50). Another phytoestrogen (resveratrol) is known to inhibit STAT3 signaling and induces the apoptosis of malignant cells containing activated STAT3 (51).

Approximately 20-25% of invasive breast carcinomas show HER2 overexpression (52). A normal breast cell has 20,000 HER2 receptors, but a breast cancer cell could have up to 1.5 million. HER2 is a member of the HER/ErbB2/Neu protein family, which also includes HER1/EGFR, HER3 and HER4. HER2 cross-talks with the estrogen receptor (ER) signal transduction pathway (53), and its expression levels can be regulated by ER. In our study, we found that apigenin significantly inhibited the growth of HER2-overexpressing cancer cells. This indicates that apigenin could be a useful natural therapy that inhibits HER2-overexpressing breast cancer. Apigenin could be a promising target for the treatment and prevention of 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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HYE SOOK SEO performed most of the experiments and wrote the manuscript. JIN MO KU performed RT-PCR and Elisa assay. HAN SEOK CHOI performed FACS analysis. JONG-KYU WOO, BO-HYOUNG JANG and YONG CHEOL SHIN advised the discussion. SEONG-GYU KO designed, supervised the experiments and corrected the manuscript.

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