Antiproliferative and Apoptosis-inducing Activity of Nobiletin Against Three Subtypes of Human Breast Cancer Cell Lines

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Abstract. *Although nobiletin has a potent antitumor activity against several types of human cancers, its inhibitory effects and possible mechanisms of action on breast cancer cells with different hormone receptor and HER2 status remains unknown. Materials and Methods: Using hormone receptor-positive MCF-7, HER2-positive SK-BR-3, and triple-negative MDA-MB-468 cell lines, we investigated the antitumor mechanisms of nobiletin. Results: Nobiletin exhibited dose- and time-dependent antitumor activity against these different subtypes of cell lines, with the greatest inhibition observed against the MDA-MB-468 cell line. Nobiletin induced cell-cycle arrest at the G_0/G_1 phase by suppressing ERK1/2 activity, with concomitant cyclin-D1 suppression and p21 up-regulation. Nobiletin induced apoptotic cell death by reducing Bcl-xL expression, without affecting Bax levels, and inhibited the activity of AKT and downstream mTOR in MDA-MB-468 cells, but not in other cell lines. Conclusion: The predominant anticancer activity of nobiletin in MDA-MB-468 cells suggests a potential role of nobiletin for the prevention of triple-negative breast cancer.*

Breast cancer is the most common malignancy among women and the first leading cause of cancer-related death worldwide (1). Breast cancer is a heterogeneous disease and is classified into different subtypes based on estrogen receptor (ER), progesterone receptor (PR), and HER2 expression. Therapeutic options for patients with advanced breast cancer are limited and depend on breast cancer subtypes. Although the survival of patients with breast cancer is prolonged by molecular-targeted therapy, triple-negative breast cancer, a subtype of breast cancer clinically-negative for expression of ER/PR and HER2 protein, has been considered as one of the disease with poor prognosis (2). In view of the current situation where no curative therapeutic approaches exist for advanced breast cancer, prevention of this disease is extremely important.

It is well-recognized that breast cancer can be largely-prevented by minimizing a variety of dietary, hormonal and lifestyle risks (3). Among a number of dietary items, fruit and vegetable intake have been epidemiologically shown to reduce overall breast cancer risk (4), and differentially affect ER-negative and -positive breast cancer incidence rates, preferentially lowering the risk of ER-negative breast cancer (5). Specifically, dietary flavonoids, which are ubiquitously found in fruits and vegetables, have been suggested as potential cancer-preventive components of fruits and vegetables (6, 7).

Nobiletin is a polymethoxy flavone extracted from citrus depressa *Hayata* (Rutaceae), a popular citrus fruit in Okinawa, Japan. Nobiletin has been shown to exert anticancer activity against several types of human cancer cell lines including HT-29 human colorectal cells, MCF-7 human breast cancer cells, four gastric adenocarcinoma cell lines, and SMMC-7721 hepatocellular carcinoma cells (8-11). Moreover, in animal studies, nobiletin has been reported to reduce the development of chemically-induced colon carcinogenesis in rodents (12-14), as well as adenocarcinoma of the prostate in transgenic rats (15). Although these preventive and anticancer activities of nobiletin both *in vivo* and *in vitro* have been suggested to be mediated by its antioxidant activity as well as interference with biological inflammatory processes (16, 17), other potential anticancer mechanisms such as cell-cycle arrest and/or apoptosis induction through inhibition of signal transduction cascades have been suggested in several cancer cells (9-11). Moreover, it has been reported that nobiletin can suppress invasion and migration through suppression of phosphoinositide 3-kinase (PI3K)/AKT in AGS gastric cancer cells (18), inhibit metastasis in human fibrosarcoma HT-1080 cells through protein kinase MEK inhibition (19), and suppress cell proliferation by inhibiting Ras activity and mitogen-activated protein kinase (MAPK) kinase/ERK signaling in C6 rat

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glioma cells (20). However, the molecular mechanisms whereby nobiletin induces growth inhibition and apoptosis still remains poorly-understood for breast cancer cells. Furthermore, there is no report to address differences in nobiletin activity among subtypes of breast cancer cells. In the present study, we investigated the cellular and molecular mechanisms of growth-inhibitory activity of nobiletin against three human breast cancer cell lines that differ in their hormone receptor and HER2 status.

Materials and Methods

Cell culture and chemicals. The study was performed on three subtypes of breast cancer cell lines, including hormone receptor (ER/PR) positive MCF-7, hormone receptor negative but HER2-positive SK-BR-3, and triple-negative MDA-MB-468, all purchased from American Type Culture Collection (Manassas, VA, USA). Origins of the cell lines and their hormone receptor and HER2 status have been described elsewhere (21). These cell lines were cultured in RPMI1640 (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. Nobiletin (more than 98.0% of purity) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and stored at –20˚C. A 100 mM solution was prepared by dissolving original nobiletin with dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) immediately before experimental use. The final concentration of DMSO for all experiments and treatments (including controls, where no drug was added) was maintained at less than 0.05%. These conditions were found to be non-cytotoxic for at least 168 h.

Determination of growth inhibition. The antiproliferative effects of nobiletin on these breast cancer cells were assessed by WST assay as previously described (22). Briefly, 100 μl suspension of these breast cancer cells was seeded into each well of a 96-well plate (BD Falcon, Franklin Lakes, NJ, USA) at a density of 2,000 cells per well. This inoculation density was determined by the growth curves so that the non-treated cells did not reach confluency up to 7 days. After overnight incubation, 100 μl nobiletin solution at different concentrations was added and cells further cultured up to 168 h. At various times after treatment, cell viability was measured by the Premix CCK-8 Cell Proliferation Assay System (Dojindo, Kumamoto, Japan). The relative number of viable cells was determined by comparing the absorbance of the treated cells with the corresponding absorbance of vehicle-treated cells taken as 100%. Each experiment was performed using six replicate wells for each nobiletin concentration and was carried out independently for three times. The IC_{50} value was defined as the concentration required for a 50% reduction in the absorbance.

Cell cycle analysis and apoptosis measurement. At various times following treatment with 50 μM nobiletin, floating and trypsinized adherent cells were combined, and fixed in 70% ethanol and stored at 4˚C prior to cell cycle analysis. After removal of ethanol by centrifugation, cells were washed with phosphate buffered saline and stained with a solution containing RNase A and propidium iodide (Sigma-Aldrich). Cell cycle analyses were performed on a Beckman Coulter Gallios Flow Cytometer using the Kaluza ver. 1.2 software packages (Beckman Coulter, Brea, CA, USA), and the extent of apoptosis was determined by measuring the sub-G_0/G_1 population.

Immunoprecipitation and Western blot analysis of signaling proteins. Immunoprecipitation and Western blot were performed as previously described (22). Equal amounts of proteins or immunoprecipitated target proteins were resolved by 4-15% sodium dodecylsulfate–polyacrylamide gel electrophoresis (Bio Rad, Hercules, CA, USA) and electrotransferred onto a polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ, USA). Non-specific binding sites were blocked by incubating the membranes in blocking buffer (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min. The membranes were then incubated with primary antibodies against either phospho-mTOR (Ser2448) (Abcam, Cambridge, UK), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), phospho-AKT (Ser473), cyclin D1 (2922), p21 (C19) (Santa Cruz Biotech, Dallas, Texas, USA), Bcl-xL (2762), or Bax (2772). The membranes were hybridized with horseradish peroxidase-conjugated secondary antibody (7074). Immunoblots were developed with the enhanced chemiluminescence system (GE Healthcare) and were then quantitated using the LAS-3000 Luminescent Image Analyzer (Fuji Film, Tokyo, Japan). The blots were striped and reprobed with primary antibodies against mTOR (9964), MAPK (9102), AKT (9272), and β-actin (4967). All primary and secondary antibodies except antibody against p21 and phospho-mTOR were purchased from Cell Signaling Technology (Danvers, MA, USA). For reblotting, membranes were incubated in stripping buffer (Thermo, Rockford, IL, USA) for 30 min at room temperature before washing, blocking, and incubating with antibody. Triplicate determinations were made in separate experiments.

Statistical analysis. To determine the significance of observed differences, analysis of variance (ANOVA) was applied to the data using statistical software (version 12.0.1 for Windows, SPSS Inc., USA). The mean values were compared by Dunnett *t*-test. A *p*-value less than 0.05 was considered significant.

Results

Effects of nobiletin on proliferation and survival. Nobiletin treatment exhibited dose-dependent growth inhibitory activities against these three breast cancer cells, with the greatest growth inhibition observed in MDA-MB-468 and the lowest in SK-BR-3 (Figure 1). As shown in Table I, the IC_{50} values decreased as incubation time with nobiletin increased, indicating time-dependent growth inhibition. MDA-MB-468 cells treated with nobiletin for 168 h showed significantly lower IC_{50} value (20.3 μM), compared to MCF-7 cells (39.6 μM) and SK-BR-3 (59.3 μM), being approximately 2-fold and 3-fold more sensitive to nobiletin than MCF-7 and SK-BR-3 cells, respectively (Table I). HER2-positive SK-BR-3 was significantly more resistant to nobiletin than MCF-7 and MDA-MB-468 cells even at 72 h treatment with nobiletin (Table I).

Time-course analysis of the effect of nobiletin on cell-cycle progression and apoptosis. When MCF-7 cells were treated with nobiletin, the proportion of cells in the G_0/G_1 phase

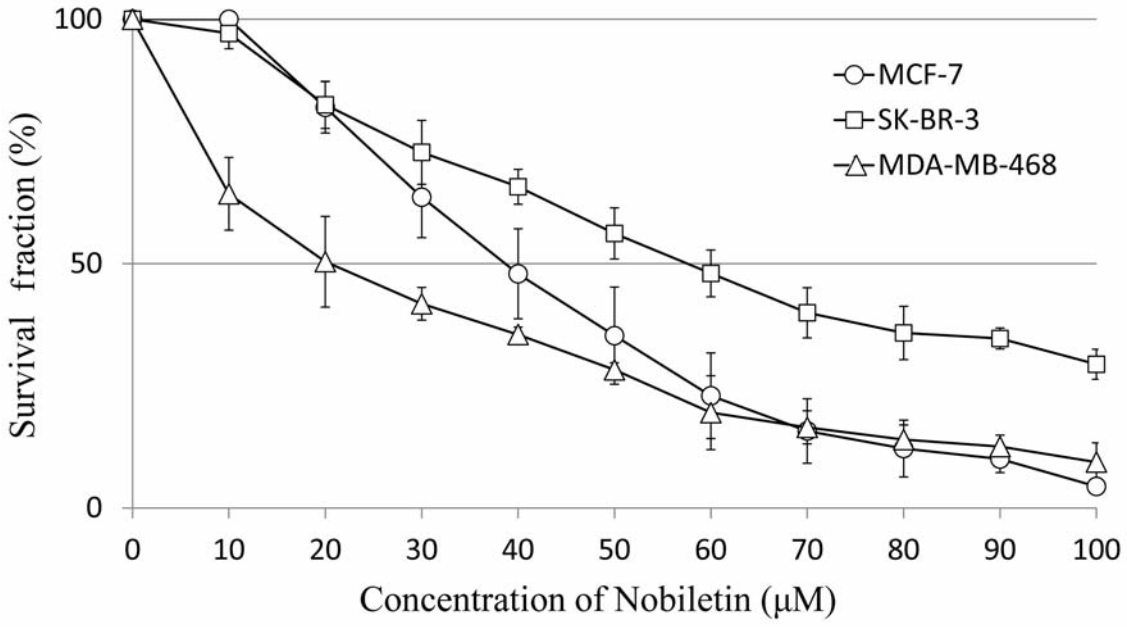

Figure 1. *Survival curves for nobiletin against MCF-7 (○), SK-BR-3 (□), and MDA-MB-468 (△) cells. Cells were treated with various concentrations of nobiletin for 168 h and assessed for viability by the WST-1 assay, as described in Materials and Methods. Data are the means from three independent experiments. Bars, standard deviation (SD).*

significantly increased from 64.9% at the beginning of the treatment to 79.5% after 24 h treatment, with corresponding decrease in cells in S and G_2/M phases, and the proportions of cells in each cell cycle phase did not significantly change afterwards (Figures 2A and 3A). The percentages of sub-G_0/G_1 cell population which represents apoptotic cells slightly increased, although not significant, with 24 h (1.7%), 48 h (2.5%) and 72 h (3.7%) exposures (Figure 3A). When SK-BR-3 cells were treated with nobiletin, the proportions of cells in each cell-cycle phase did not significantly change, and the percentages of sub-G_0/G_1 cell population did not change at all (Figures 2B and 3B). By contrast, when MDA-MB-468 cells were treated with nobiletin, the sub-G_0/G_1 apoptotic cell population increased from 1.4 % to 5.4% after 24-h exposure, with a subsequent increase up to 14.7% afterwards (Figure 2C), while the proportion of cells in G_0/G_1 phase increased from 62.1% (0 h) to 86.1% (24 h) (Figure 3C). Therefore, it appears that nobiletin-induced growth decline would be mediated by the G_0/G_1 arrest of the cell cycle as well as apoptosis induction for triple-negative MDA-MB-468 cells.

Effects of nobiletin on activations of signaling molecules. Upon treatment with 100 μM nobiletin, the constitutive activity of ERK1/2 was reduced 2-6 h after initiation of treatment in these 3 cell lines, and suppressed over 24 h, although the suppression of ERK1/2 was slight in SK-BR-3 cells (Figure 4A). By contrast, phosphorylation of AKT and downstream mTOR were clearly inhibited by nobiletin in triple-negative breast cancer cells, but not in MCF-7 and SK-BR-3 cells where apoptosis induction was not detected or extremely low (Figure 4A). Cyclin-D1 functioning as a key regulator of G_0/G_1 cell-cycle checkpoint was inhibited with subsequent increase of p21 protein in all cell lines (Figure 4A), indicating that nobiletin arrests the cell cycle at G_0/G_1 through decrease in cyclin-D1 expression and concomitant up-regulation of p21 regardless of subtypes of breast cancer.

Effects of nobiletin on expression of pro- and anti-apoptotic proteins. To clarify the apoptotic mechanism induced by nobiletin, we examined the expression of Bcl-xL, the anti-

Table I. *IC_{50} values of nobiletin treated for different time periods*

Cell line	72 h	120 h	168 h
MCF-7	59.8±9.7	39.7±5.7	39.6±6.3
SK-BR-3	86.9±4.4	69.6±10.7	59.3±5.7
MDA-MB-468	51.3±9.5	34.1±6.6	20.3±5.8

Cells were treated with various concentrations of nobiletin for 72, 120, and 168 h. The IC_{50} values were determined by the WST-1 assay, as described in Materials and Methods. Values are mean±SD of 3 independent experiments. $^{**}p<0.01$.

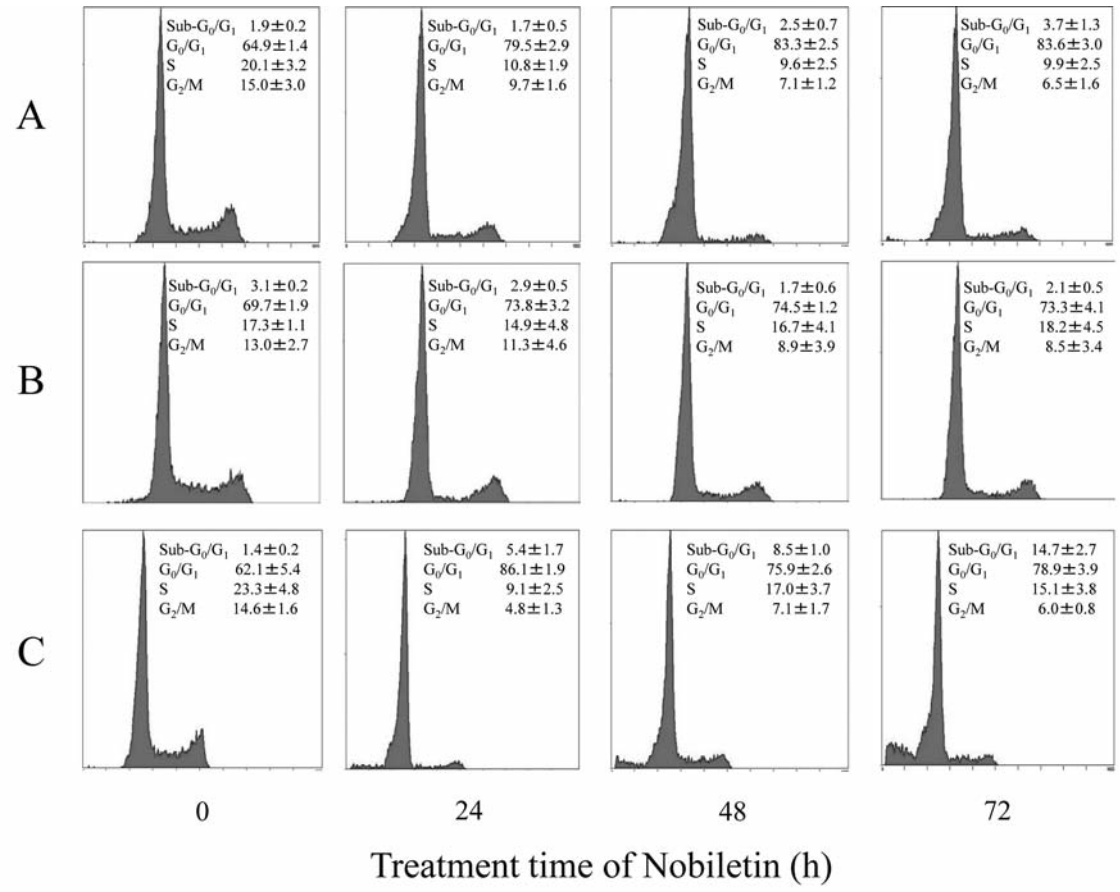

Figure 2. *Time-course analysis of the effect nobiletin on cell-cycle progression and apoptosis as determined by flow cytometry for MCF-7 (A), SK-BR-3 (B), and MBA-MB-468 (C). Representative cell-cycle distributions after exposure to 50 μM nobiletin for 0 h, 24 h, 48 h, and 72 h were shown. The percentage of the total cell population in the different phases of cell cycle was determined with curve fitting using Kaluza v. 1.2 software. Mean values±SD of 3 independent experiments are shown on the top right of each panel.*

apoptotic homolog of Bcl-2, and pro-apoptotic Bax protein (Figure 4B). Upon treatment with 100 μM nobiletin, expression of Bcl-xL was suppressed in MDA-MB-468, but not in MCF-7 and SK-BR-3 cells (Figure 4B), where apoptosis was not evident. Expression of Bax protein did not change with incubation time in all cell lines. Therefore, reduced expression of Bcl-xL would be responsible for nobiletin-induced apoptotic induction in MDA-MB-468 cells. Both Bcl-xL and Bax appear not to be involved in nobiletin-induced apoptosis in MCF-7 and SK-BR-3 cells, providing explanations for less or no amounts of apoptosis induction in these cells compared to MDA-MB-468 cells.

Discussion

In the present study, we investigated the growth-inhibitory activity of nobiletin against three different subtypes of human breast cancer cell lines that differ in their hormone receptor and HER2 status. Nobiletin showed a time- and dose-dependent growth inhibitory activity, with the greatest inhibition observed in triple-negative MDA-MB-468 cells. HER2-positive SK-BR-3 cells were least sensitive to nobiletin, while ER-positive MCF-7 cells are moderately sensitive. Correspondingly, a recent meta-analysis of epidemiological studies showed that the risk of overall breast cancer was significantly reduced in women with high intake of flavones (23). Therefore, our results support the hypothesis that nobiletin may be potentially useful as a preventive phytochemical for overall breast cancer, and more specifically triple-negative breast cancer.

Nobiletin induced a substantial delay of the cell cycle at the G_0/G_1 phase and suppressed the ERK1/2 activity, with concomitant cyclin-D1 suppression and p21 up-regulation in the three cell lines. By contrast, nobiletin induced apoptotic cell death by reducing the expression of Bcl-xL, without affecting Bax levels in triple-negative breast cancer cells.

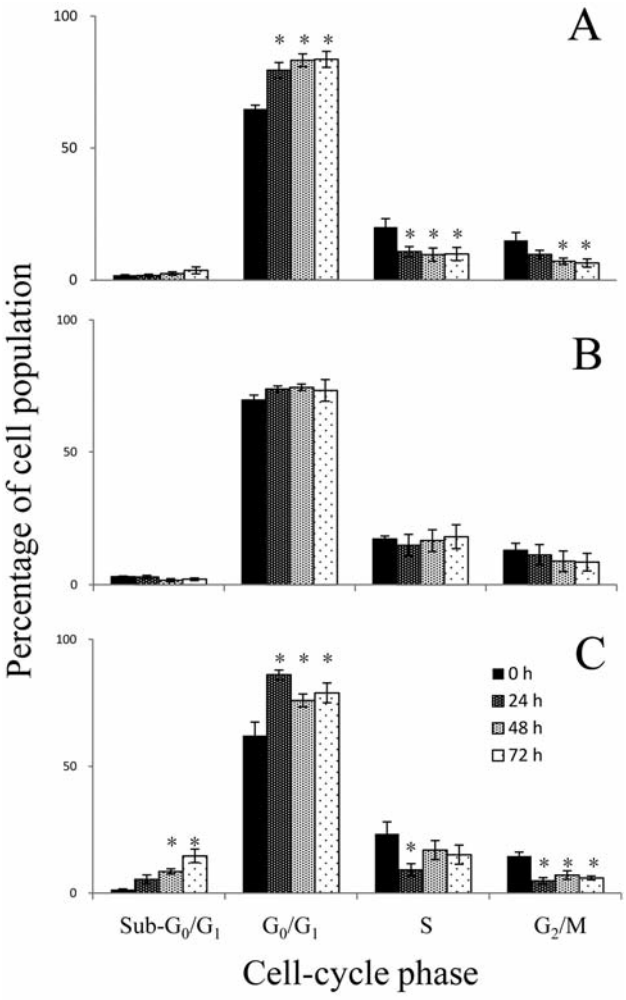

Figure 3. *Time-course analysis of the effect nobiletin on cell-cycle progression and apoptosis as determined by flow cytometry for MCF-7 (A), SK-BR-3 (B), and MBA-MB-468 (C). The percentage of the total cell population in the different phases of cell cycle is shown as column bars after exposure to 50 μM nobiletin for 0 h, 24 h, 48 h, and 72 h. Mean±SD of three independent experiments. *Significant difference versus control (0 h), p<0.05.*

These data suggest that anticancer activity of nobiletin against MDA-MB-468 cells could be brought by the arrest of cell-cycle progression at the G_0/G_1 phase as well as apoptosis induction presumably through suppression of Bcl-xL. In contrast, HER-2-positive cells that are 3-fold more resistant to nobiletin than triple-negative MDA-MB-468cells, did not show any apoptotic effects. Nobiletin has been shown to inhibit both RAS/MAPK and PI3K/AKT pathways in human and rodent tumor cells (18-20). In the present study, nobiletin did not suppress AKT/mTOR activity, while inhibiting activation of ERK1/2 in HER2-positive SK-BR-3 cells. The reduced sensitivity of nobiletin to HER2-overactivated cells, therefore, might be due to the inability of nobiletin to suppress the activities of AKT and downstream mTOR. Since signaling cascades downstream of HER2 including RAS/MAPK and PI3K/AKT/mTOR pathways are considered to be activated in HER2-overexpressed cells (24), it is conceivable that nobiletin could not override HER2-driven AKT activation in SK-BR-3 cells. Conversely, in nobiletin-sensitive triple-negative MDA-MB-468 cells, AKT and downstream mTOR were clearly inhibited. Therefore, simultaneous inhibition of both ERK1/2 and PI3K/AKT pathways would be important for the execution of nobiletin-induced anti-proliferative and apoptosis-inducing activity, as reported in the treatment of non-small cell lung cancer with gefitinib, an epidermal growth factor receptor inhibitor (25).

Cell-cycle checkpoints are important control mechanisms that ensure the proper execution of cell cycle events. We have shown that nobiletin induces cell cycle arrest at the G_0/G_1 phase in 3 subtypes of human mammary cancer cells when cells are treated at a dose of 50 μM, although the cell cycle arrest at G_0/G_1 phase were not significant in HER2 positive SK-BR-3. This is because SK-BR-3 cells are resistant to nobiletin, having higher IC_{50} value more than 50 μM. Upon treatment with nobiletin at a dose of 100 μM, the cell cycle progression was completely arrested at G_0/G_1 phase in all cell lines, almost completely diminishing the population of S and G_2/M phases (data not shown). Similar nobiletin-induced G_0/G_1 cell cycle arrests have been reported in MCF-7 cells (9, 26). The molecular mechanism whereby nobiletin arrests the cells in the G_0/G_1 phase remains unclear. However, suppression of constitutive activity of ERK1/2 may cause nobiletin-induced G_0/G_1 block. ERK1/2 is an important sub-family of MAPK that controls a broad range of cellular activities and physiological processes (27). In the present study, continuous treatment with nobiletin suppressed the constitutive activity of ERK1/2 from 2-6 h and sustained at low level over 24 h in these 3 cell lines, with concomitant decrease in cyclin D1 expression and p21 up-regulation. Therefore, nobiletin-induced strong and sustained inhibition of the ERK1/2 pathway may cause a G_0/G_1 cell-cycle blockade in breast cancer cells regardless of hormone receptor and HER2 status, suggesting that the arrest of cell cycle at G_0/G_1 might be a universal event triggered by nobiletin treatment.

In triple-negative breast cancer cells, nobiletin induced apoptotic cell death by reducing the expression of anti-apoptotic Bcl-xL, without affecting Bax levels. Suppression of Bcl-xL was not observed in MCF-7 and SK-BR-3 cells where apoptosis is not detected or extremely low. It has been shown that apoptosis could be induced by down-regulation of the expression levels of Bcl-xL through inhibition of ERK1/2 activity in human pancreatic cancer cells and human dermal keratinocytes (28, 29). Despite suppression of ERK1/2, Bcl-xL was not reduced and apoptosis did not

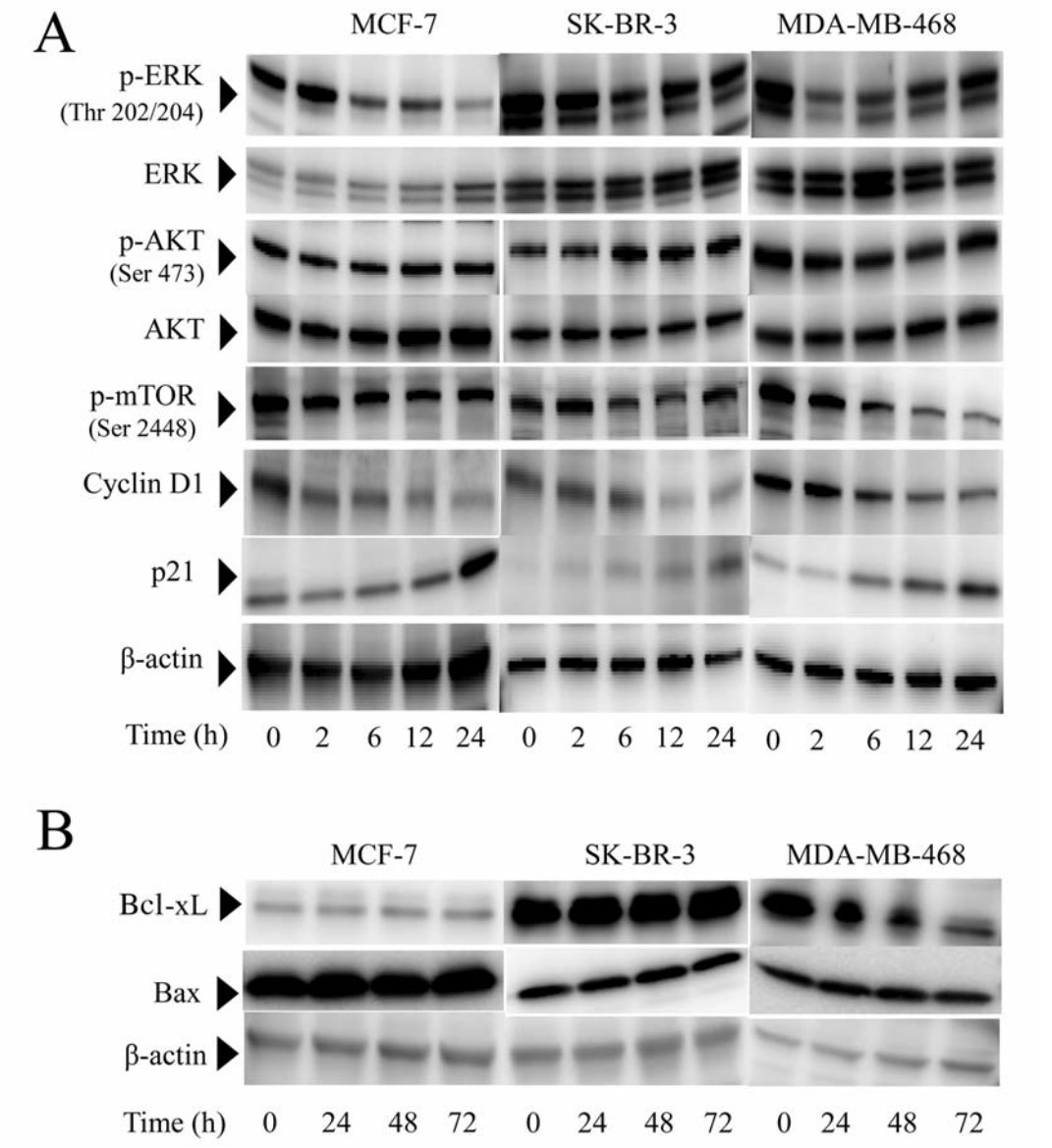

Figure 4. *Effects of nobiletin on activations of signaling molecules for cell proliferation and survival. Cells were treated with 100 μM nobiletin for the indicated times and harvested for western blot. A: Western blots are shown for phosphorylated and total extracellular signal-regulated kinase (ERK) 1/2 and AKT. Phosphorylated mammalian target of rapamycin (p-mTOR), cyclin-D1 and p21 are also shown. B: Effects of nobiletin on the pro-apoptotic Bax and anti-apoptotic Bcl-xL proteins. beta-actin was used as an internal control.*

occur in MCF-7 and SK-BR-3 cells. This is probably because AKT cannot be inactivated by nobiletin in these cells. AKT plays a critical role in controlling survival and apoptosis by directly phosphorylating mTOR, thereby inducing signals leading to anti-apoptotic pathways (30). Indeed, we have found that in triple-negative breast cancer cells, AKT and downstream mTOR phosphorylation were clearly inhibited by nobiletin, but not in MCF-7 and SK-BR-3 cells. These data suggest that nobiletin may execute its apoptotic effects primarily through blocking AKT/mTOR-mediated signaling pathways in triple-negative breast cancer cells, and that suppression of RAS/ERK pathway alone is insufficient for the induction of apoptosis. Taken together, the cytotoxic effects of nobiletin seem to be determined by the extent of both blockade of the cell cycle at the G_0/G_1 phase and induction of apoptotic cell death, depending on subtypes of breast cancer.

In conclusion, triple-negative breast cancer cells were significantly more sensitive to nobiletin than either hormone receptor-positive or HER2-positive cells. This

predominant anticancer activity of nobiletin in triple-negative breast cancer cells could be brought not only by an arrest of cell-cycle progression at the G_0/G_1 phase, but also by apoptosis induction through Bcl-xL, suggesting a potential role of nobiletin for the prevention of this subtype of breast cancer.

Disclosure Statement

Authors have no conflicts of interest to disclose.

Acknowledgements

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