

Quercetin-mediated Cell Cycle Arrest and Apoptosis Involving Activation of a Caspase Cascade through the Mitochondrial Pathway in Human Breast Cancer MCF-7 Cells

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(Received July 18, 2009/Revised April 1, 2010/Accepted April 9, 2010)

Dietary polyphenols have been correlated with a reduced risk of developing cancer. Quercetin (a natural polyphenolic compound) induced apoptosis in many human cancer cell lines, including breast cancer MCF-7 cells. However, the involvement of possible signaling pathways and the roles of quercetin in apoptosis are still undefined. The purpose of this study was to investigate the effects of quercetin on the induction of the apoptotic pathway in human breast cancer MCF-7 cells. When MCF-7 cells were treated with quercetin for 24 and 48 h and at various doses (10-175 μ M), cell viability decreased significantly in time- and dose-dependent manners. Exposure of MCF-7 cells to 10-175 μ M quercetin resulted in an approximate 90.25% decrease in viable cells. To explicate the mechanism underlying the antiproliferative effect of quercetin, cell cycle distribution and apoptosis in MCF-7 cells was investigated after exposure to 150 μ M quercetin for 6-48 h. Quercetin caused a remarkable increase in the number of S phase (14.56% to 61.35%) and sub-G1 phase cells (0.1% to 8.32%) in a dose- and time-dependent manner. Quercetin caused S phase arrest by decreasing the protein expression of CDK2, cyclins A and B while increasing the p53 and p57 proteins. Following incubation with quercetin for 48 h, MCF-7 cells showed apoptotic cell death by the decreased levels of Bcl-2 protein and $\Delta\Psi_m$ and increased activations of caspase-6, -8 and -9. Moreover, quercetin increased the AIF protein released from mitochondria to nuclei and the GADD153 protein translocation from endoplasmic reticulum to the nuclei. These data suggested that quercetin may induce apoptosis by direct activation of the caspase cascade through the mitochondrial pathway in MCF-7 cells.

Key words: Quercetin, Apoptosis, Cell cycle, Caspase-6, Mitochondria, Breast cancer MCF-7 cells

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INTRODUCTION

The occurrence of breast cancer, one of the major causes of death in woman, is increasing in women

throughout the world; this incidence has been partially attributed to environmental agents (Krieger, 1989; Perera, 1997; Davis et al., 1998). In Taiwan, 12 persons per 100 thousand die annually from breast cancer, based on reports from the People Health Bureau of Taiwan. The major treatment methods for breast cancer patients are surgery, radiotherapy and chemotherapy, however, the cure rates are not satisfactory. New agents acting on novel targets in breast cancer are currently under investigation.

Cell death can be divided into apoptosis and necrosis. Apoptosis is the most important processes leading to

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cell death and the use of chemotherapy agents to induce apoptotic cell death is one of the best strategies for killing tumor cells. Caspases-mediated apoptosis can be divided into two major signaling pathways including the cell death receptor, or extrinsic pathway, the mitochondrial or intrinsic pathway and the ER stress pathway (Gupta, 2003; Fan et al., 2005). The cell death receptor pathway is specifically mediated through FasL and Fas/CD95 receptors and then followed by active/cleaved caspase-8 which plays a fundamental role for maintaining tissue homeostasis in the immune system. The mitochondrial pathway is mediated through the changes in the ratios of Bax/Bcl-2 proteins, loss of mitochondrial membrane potential and then followed by active/cleaved caspase-9 which responds to extracellular cues and internal insults such as DNA damage (Herr and Debatin, 2001; Fan et al., 2005). It has been reported that induction of endoplasmic reticulum (ER) stress and the subsequent activation of the unfolded protein response (UPR) play a critical role in the induction of apoptosis by anticancer drugs in breast cancer cells (Wang et al., 2009). Glucose-regulated protein 78 (GRP78) has important roles in protein folding and assembly, by targeting misfolded proteins for degradation in ER Ca^{2+} -binding and in controlling the activation of transmembrane ER stress sensors. CHOP/GADD153 represents a critical executioner of the pro-apoptotic arm of the ER stress response (Schonthal, 2009). It has been reported that there are multiple targets for chemoprevention in carcinogenesis (Yang et al., 2001; Surh, 2003), but the induction of apoptosis is an important target approach for prevention of cancer.

Quercetin, a ubiquitous bioactive plant flavonoid, has been shown to induce cytotoxic effects including inhibition of cell proliferation and apoptosis in many human cancer cell lines. The molecular mechanism of quercetin induces cytotoxic effects including cell cycle arrest at G1, and/or G2/M arrest and induction of caspase-mediated apoptosis in colon (van Erk et al., 2005), leukemia (Mertens-Talcott et al., 2003; Shen et al., 2003; Lee et al., 2006), breast (Singhal et al., 1995), lung (Nguyen et al., 2004), hepatoma (Chi et al., 2001) and oral (Ong et al., 2004) cancer cells. Furthermore, several lines of evidence indicate that quercetin affects many factors in cellular signal transduction pathways which link to the pro-apoptotic (Bax) and antiapoptotic (Bcl-2) genes and cell survival or cell proliferation (MAPKs and Akt) processes (Ong et al., 2004; van Erk et al., 2005). Although, it was reported that quercetin induced cell cycle arrest and apoptosis in human breast cancer MCF-7 cells (Choi et al., 2001), the exact molecular mechanism of action remains

unclear. In this study, we investigate the molecular signaling pathways of quercetin-induced apoptosis in human breast MCF-7 cancer cells.

MATERIALS AND METHODS

Chemicals and reagents

Quercetin, trypan blue, propidium iodide (PI), dimethyl sulfoxide (DMSO), Tris-HCl, triton X-100, penicillin and streptomycin were purchased from Sigma Chemical. The fluorescent probes 2', 7'-dichlorofluorescein diacetate (DCFH-DA), Indo 1/AM and DiOC₆ were obtained from Calbiochem. Anti-caspase-6, anti-pro-caspase-8, anti-caspase-9, anti-pro-caspase-12 and PARP were obtained from Cell Signaling Technology. Anti-Bcl-2, anti-BID, anti-pro-ATF6, anti-PERK, anti-GRP78, anti-GADD153, anti-p57, anti-Thymidylate synthase, anti-cyclins A and E, anti-p53, anti-Fas, anti-TRAIL, anti-XIAP and anti-AIF were purchased from Santa Cruz Biotechnology. Caspase-6, -8 and -9 substrates were purchased from R&D system Inc. Materials and chemicals for electrophoresis were from BioRad. Cell culture dishes and cell culture medium were purchased from Falcon.

Cell culture

Human breast adenocarcinoma MCF-7 cell line was obtained from the Food Industry Research and Development Institute. The cells were placed into 75 cm² tissue culture flasks under humidified 5% CO₂ and 95% air and cultured at 37°C and one atmosphere in RPMI 1640 medium (Gibco/Invitrogen) supplemented with 10% FBS, 1% penicillin-streptomycin (100 units/mL penicillin and 100 µg/mL streptomycin) and 2 mM L-glutamine. Subconfluent cells (80%) (Gibco/Invitrogen) were passaged with a solution containing 0.25% trypsin and 0.02% EDTA.

Viable cells determination

Approximately 2×10^5 cells/well of MCF-7 cells in a 12-well plate was treated with 0, 10, 50, 100, 150 and 175 µM quercetin and was incubated for 24 and 48 h. For determining cell viability, the trypan blue exclusion and PI- exclusion methods were employed using the flow cytometric protocol described by (Chiang et al., 2006; Lee et al., 2008). Viable cells were calculated by counting the cells in a Neubauer chamber. An aliquot of the total cell suspension was mixed with an equal volume of trypan blue in PBS and incubated for 5 min at room temperature. Dead cells were turned to light blue and live cells showed white color. For the PI exclusion method, cells were collected, resuspended in PBS containing 4 µg/mL PI and then analyzed by a

flow cytometer (FACSCalibur, Becton Dickinson). All experiments were performed in triplicate. The percentage of cell viability was calculated as a ratio between drug-treated cells and 0.1% DMSO vehicle-control cells.

Cell cycle and sub-G1 group assays

Approximately 2×10^5 cells/well of MCF-7 cells in 12-well plates were treated with 150 μM quercetin and then incubated in an incubator for 0, 6, 12, 24 and 48 h. Medium containing float cells was removed using pipetman and trypsin was added to the cells in plates for 3 min, after which the cells were harvested by centrifugation at 110 g for 5 min. Pellets were washed twice with cold PBS and then fixed by using 70% ethanol (in PBS) at 4°C overnight. The cells were then washed twice with cold PBS and re-suspended in PBS containing 40 $\mu\text{g}/\text{mL}$ PI, 0.1 mg/mL RNase and 0.1% triton X-100 in a dark room for 30 minutes at 37°C; the cells were then analyzed by flow cytometry. Then the cell cycle and sub-G1 (apoptosis) groups were determined and analyzed (Lin et al., 2007a, 2007b).

Apoptosis determination by DAPI staining

Approximately 2×10^5 cells/well of MCF-7 cells in a 12-well plate were treated with 0, 50, 100, 150, 200 and 250 μM quercetin and then incubated in an incubator for 48 h, while only adding DMSO (solvent) for the control regimen, and cultured in 5% CO_2 and 95% air at 37°C. Cells in each treatment and control were stained by DAPI and then examined and photographed under fluorescence microscopy as described elsewhere (Lin et al., 2007a, 2007b).

Apoptosis determination by DNA fragmentation detection

Approximately 1×10^6 cells/well of MCF-7 cells in a 6-well plate were treated with 0, 100, 200, 300 and 400 μM quercetin and then incubated in an incubator for 48 h. Total DNA was isolated by using the DNA ladder kit (Genemark Technology Co.) and aliquots from each sample then were resolved using 1.5% agarose gel containing 0.3 $\mu\text{g}/\text{mL}$ ethidium bromide in 1X TAE buffer (pH 8.5, 20 mM Tris-acetic acid, 2 mM EDTA) after electrophoresis for 45 min. The DNA bands were visualized, examined and photographed by PhotoDoc-It Imaging system (Lee et al., 2008).

Determination of reactive oxygen species, Ca^{2+} concentrations and mitochondrial membrane potential

Approximately 2×10^5 cells/well of MCF-7 cells in a 12-well plate were treated with 150 μM quercetin, and

incubated for indicated time periods to determine the changes of reactive oxygen species (ROS) production, levels of Ca^{2+} concentrations and mitochondrial membrane potential ($\Delta\psi_m$). Cells were harvested by centrifugation, washed twice in PBS, and then re-suspended in 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA; 10 μM), Indo 1/AM (3 $\mu\text{g}/\text{mL}$) and DiOC₆ (4 μM). The cells were then incubated at 37°C in a dark room for 30 min and immediately analyzed by flow cytometry as previously described (Lin et al., 2007a, 2007b).

Determination of apoptotic associated proteins by Western blotting

Approximately 1×10^6 cells/well of MCF-7 cells in a 6-well plate were treated with 150 μM of quercetin and incubated for 0, 6, 12, 24 and 48 h to examine the various proteins correlated with ER stress, cell cycle arrest and apoptosis. Harvested cells were washed with cold PBS. The total proteins were collected from MCF-7 cells before the pro-ATF6, IRE-1, PERK, GRP78, GADD153, pro-caspase-12, p53, CDK2, p57, Thymidylate synthase, cyclin A, cyclin E, Fas, TRAIL, BID, pro-caspase-8, Bcl-2, AIF, PARP, caspase-9, caspase-6 and XIAP were detected. About 50 μg protein from each sample was resolved using 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The blot was soaked in blocking buffer (5% non-fat dry milk/0.05% Tween 20 in 20 mM TBS at pH 7.6) at room temperature for 1 h and then incubated overnight with individual monoclonal antibodies in blocking buffer at 4°C. This incubation was followed by secondary antibody horseradish peroxidase conjugate and detection by chemiluminescence and autoradiography using X-ray film (20, 21). To ensure equal protein loading, each membrane was stripped and reprobed with anti- β -actin antibody (Lin et al., 2007a, 2007b).

Caspase-6, caspase-8 and caspase-9 activity assay

Approximately 1×10^4 cells/well of MCF-7 cells in a 96-well plate were pretreated with or without pan-caspase inhibitor, then were treated with 150 μM quercetin and incubated for 24 h to detect the activities of caspase-6, -8 and -9, which were assessed according to manufacture's instructions for the Caspase colorimetric kit (R&D system Inc.). Cells were harvested and lysed in 50 μl lysis buffer which containing 2 mM DTT for 10 min. After centrifugation, the supernatants containing 200 μg protein were incubated with caspase-6, caspase-8 and caspase-9 substrate in reaction buffer. All samples were then incubated in a 96 well flat bottom microplate at 37°C for 1 h. Levels

of released pNA were measured with ELISA reader (Anthos Reader 2001, Anthos Labtec) at 405 nm wavelength (Ying and Sanders, 2001).

Confocal laser microscopy

The detailed protocol was previously described (Lee et al., 2008). Briefly, approximately 5×10^4 cells/well of MCF-7 cells were plated on 4-well chamber slides and then treated, with or without (control), 150 μM quercetin for 24 h. Cells were then washed and fixed with 4% formaldehyde in PBS for 15 min and permeabilized for 1 h using 0.3% Triton-X 100 in PBS containing 2% BSA to block non-specific binding sites.

The fixed cells were then incubated overnight with anti-human AIF or anti-human GADD153 antibody (1:100 dilution, respectively), washed twice with PBS and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution) for 40 min, followed by DNA staining with PI. Photomicrographs were taken using a Leica TCS SP2 Confocal Spectral Microscope, as previously described (Lee et al., 2008).

Statistical analyses

The differences between the quercetin-treated and control groups were analyzed using the Student's *t*-test; a probability of $p < 0.05$ being considered significant.

RESULTS

The effects of quercetin on the percentage of viable MCF-7 cells

In order to examine the effects of quercetin on the percentage of viable MCF-7 cells, the cells were treated with different concentrations of quercetin for 24 and 48 h and the viable cells were then determined using a flow cytometric assay and confirmed by the trypan blue exclusion assay (data not shown). As shown in Fig. 1A and B, quercetin decreased the percentage of viable cells by approximately 12–90% in dose- and time-dependent manners, with an IC_{50} (50% inhibitory concentration) value of 92.4 μM after 48 h of treatment.

The effects of quercetin on the cell cycle distribution and sub-G1 phase from MCF-7 cells

To determine whether quercetin-decreased the percentage of viable cells via cell cycle arrest and/or apoptosis, the distribution of cells in different phases was examined. Cells with DNA content were designated as being in the G0/G1, S or G2/M phase of the cell cycle. The number of cells in each compartment of the cell cycle is expressed as a percentage of the total

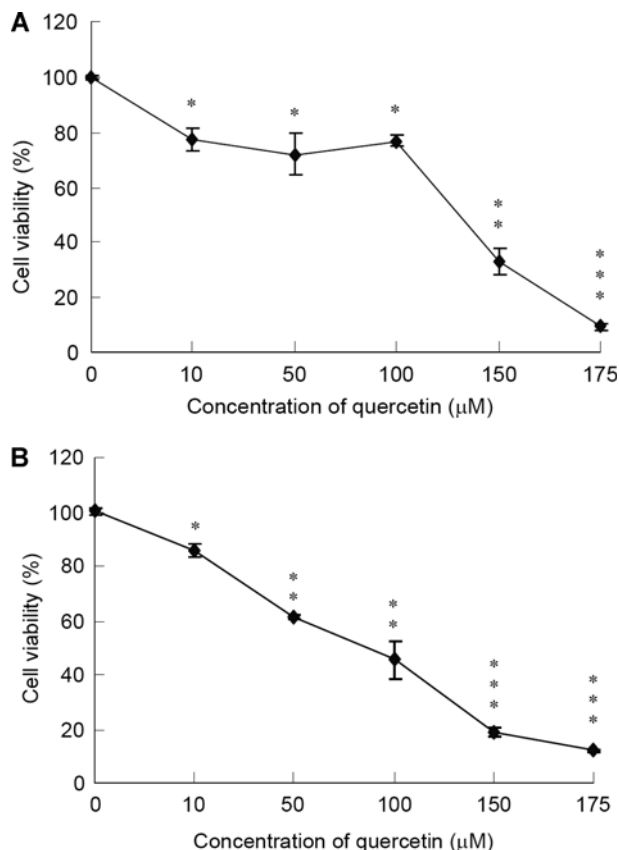


Fig. 1. The effects of quercetin on the percentage of total viable MCF-7 cells. MCF-7 cells (2×10^5 cells/well; 12-well plates) in RPMI 1640 medium with 10% FBS were treated with different concentrations of quercetin for 24 h (A) and 48 h (B). Cells were collected by centrifugation and the viable cells were counted by flow cytometric assay as described in Materials and Methods. Each point is mean \pm S.D. of three experiments. *, **, ***Significantly different from the control at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

number of cells examined. As shown in Fig. 2A and B, the number of S phase profiles for MCF-7 cells was significantly increased and number of cells in the G0/G1 phase were significantly decreased after the treatment of quercetin. Thus, quercetin caused cell cycle arrest at S phase in the MCF-7 cells. Next, we characterized apoptotic cell death in the quercetin-treated cells. As shown in Fig. 2A and B, clear sub-G1 group (apoptosis) occurred after treatment with quercetin for 48 h.

The effects of quercetin on apoptosis in MCF-7 cells, as examined by DAPI staining and DNA gel electrophoresis

Flow cytometric analysis showed that the sub-G1 group was present in MCF-7 cells after quercetin treatment and these findings were needed to reconfirm apoptotic cell death in MCF-7 cells. The cells

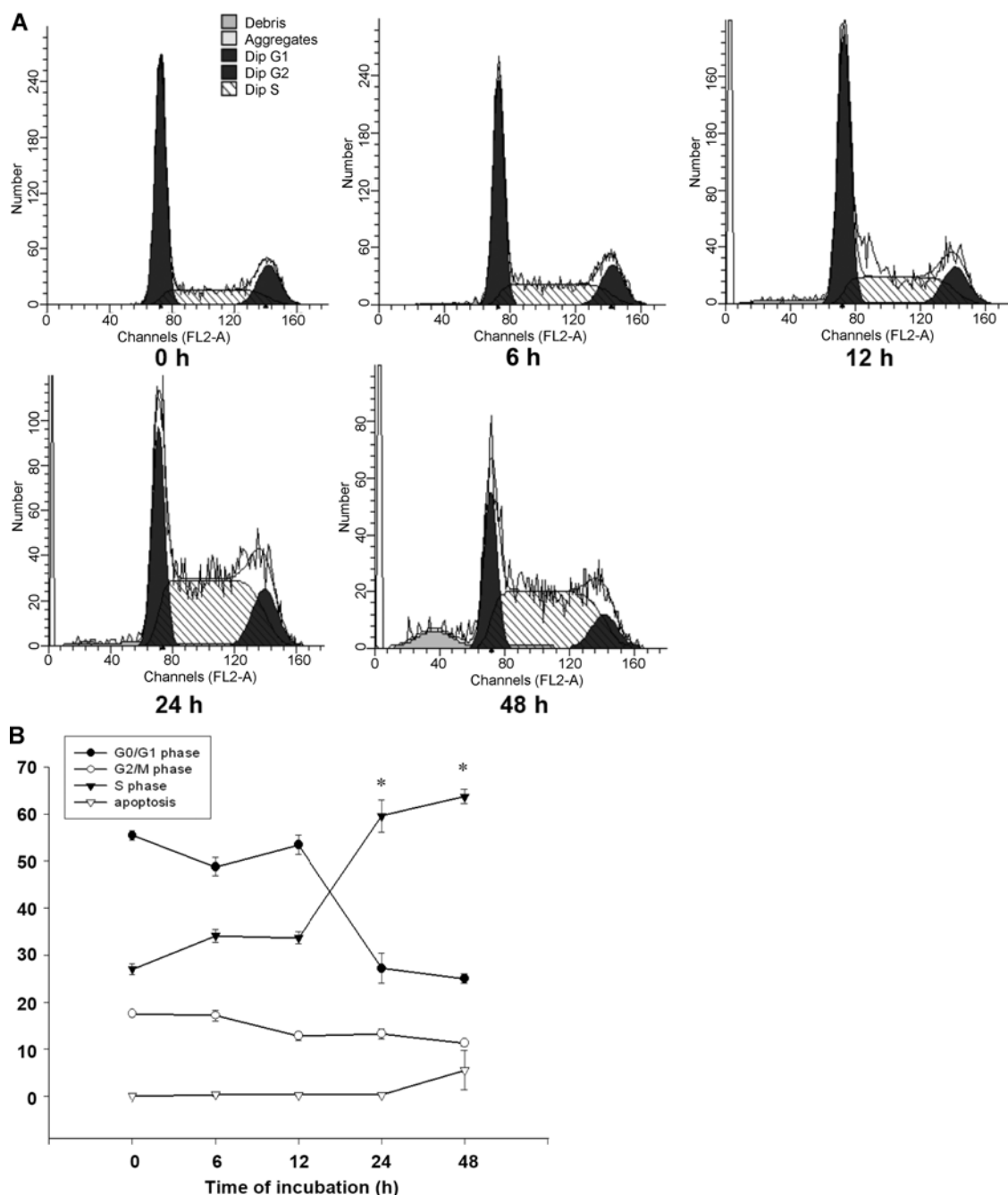


Fig. 2. The effects of quercetin on the cell cycle distribution and sub-G1 phase in MCF-7 cells. Cells (2×10^5 cells/well; 12-well plates) in RPMI 1640 medium with 10% FBS were treated with 150 μ M quercetin for 0, 6, 12, 24 and 48 h. The cells were collected by centrifugation and the cell cycle and sub-G1 phase were examined by flow cytometric assay as described in Materials and Methods. Each point is mean \pm S.D. of three experiments. *Significantly different from the control at $p < 0.05$.

were treated with various concentrations of quercetin for 24 h. As shown in Fig. 3A and B, compared with controls, both assays demonstrated that quercetin induced DNA damage, which was examined by DAPI staining (Fig. 3A), and DNA fragmentation which was

examined by DNA gel electrophoresis (Fig. 3B) in MCF-7 cells. Taken together, we conclude that quercetin decreased the percentage of viable MCF-7 cells through induction of cell cycle arrest and apoptotic cell death.

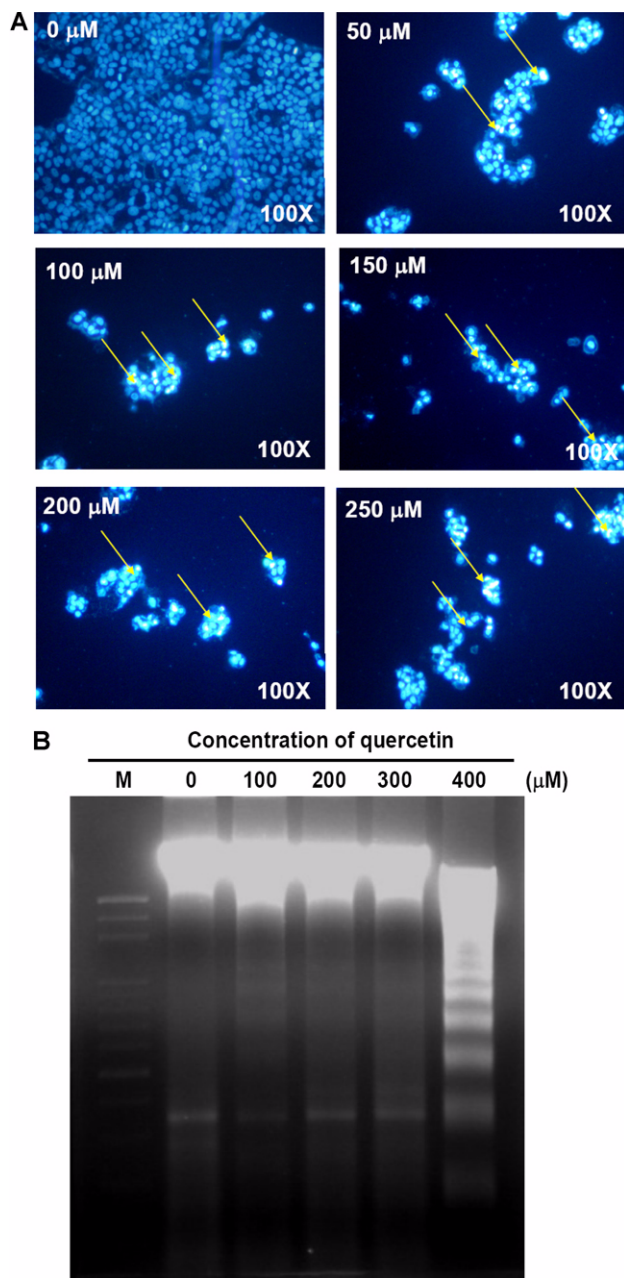


Fig. 3. The effects of quercetin on apoptosis were examined by DAPI staining and DNA gel electrophoresis in MCF-7 cells. Cells were incubated with various doses of quercetin for 48 h. Cells were examined by DAPI staining (**A**) and also DNA was isolated for gel electrophoresis (**B**), both for examining the occurrence of apoptosis as described in Materials and Methods.

The effects of quercetin on reactive oxygen species (ROS), Ca^{2+} concentrations and $\Delta\psi_m$ levels in MCF-7 cells

The results of ROS production, Ca^{2+} concentrations and levels of $\Delta\psi_m$ from flow cytometric analysis are shown in Fig. 4A, B and C. No significant increase in intracellular ROS was observed in the quercetin-

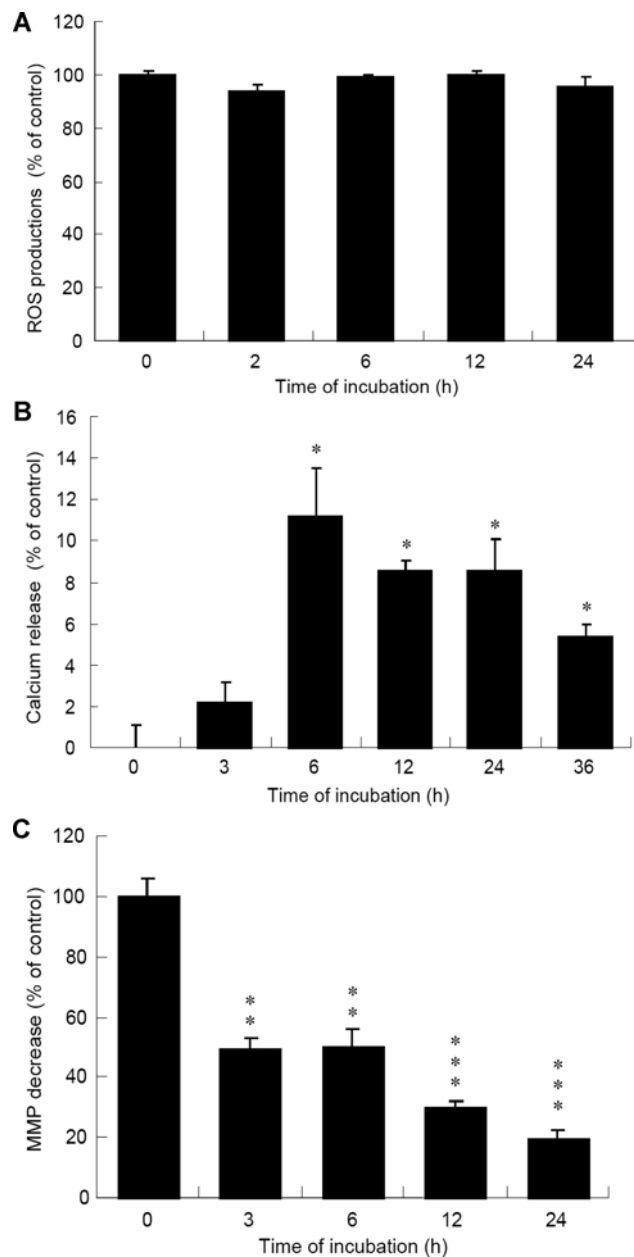


Fig. 4. The effects of quercetin on the production of reactive oxygen species (ROS), Ca^{2+} , and $\Delta\psi_m$ levels in MCF-7 cells. Cells were incubated with 150 μM quercetin for various time periods. The percentage of cells that were stained individually by DCFH-DA dye for ROS (**A**), Indo 1/AM dye for Ca^{2+} (**B**), and DiOC₆ for $\Delta\psi_m$ (**C**). The stained cells were examined and quantitated by flow cytometry as described in the Materials and Methods. *, **, ***Significantly different from the control at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

treated cells with DCFH-DA (Fig. 4A). Indo 1/AM was used to determine the changes of Ca^{2+} concentrations in MCF-7 cells treated with quercetin. Significant changes in Ca^{2+} concentrations were observed from 3 h up to 36 h following treatment (Fig. 4B). Many reports have shown that apoptosis is associated with

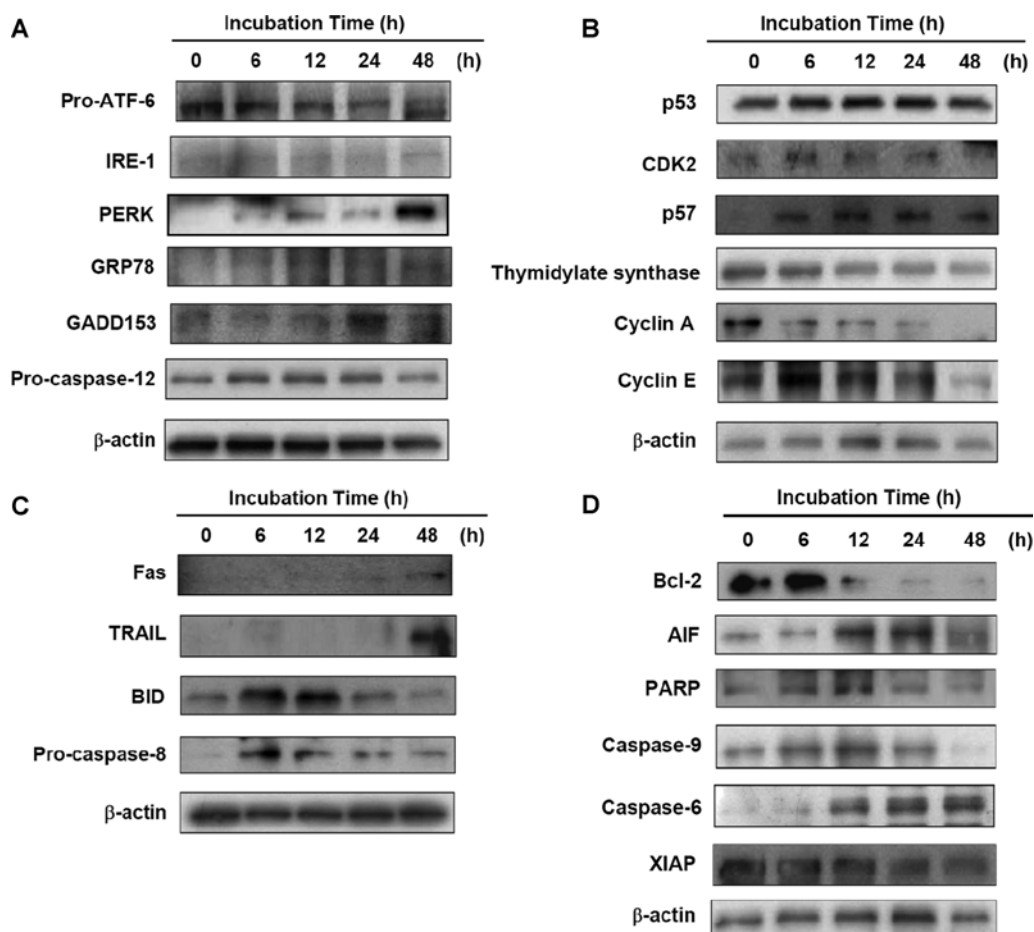


Fig. 5. The effects of quercetin on the levels of cell cycle arrest and apoptosis associated protein levels in human breast cancer MCF-7 cells were examined by Western blotting. Cells (1×10^6 /mL) were treated with 150 μ M quercetin for 0, 6, 12, 24 and 48 h and the total protein was prepared and determined. The evaluation of the associated protein levels was carried out by Western blotting as described in Materials and Methods. (A) pro-ATF-6, IRE-1, PERK, GRP78, GADD153, and pro-caspase-12; (B) p53, CDK2, p57, Thymidylate synthase, cyclins A and B; (C) Fas, TRAIL, BID, and pro-caspase-8; (D) Bcl-2, AIF, PARP, caspase-9, caspase-6, and XIAP

the loss of $\Delta\Psi_m$, which is an endpoint of apoptosis. To investigate the change in $\Delta\Psi_m$ of MCF-7 cells exposed to quercetin, DiOC₆, a mitochondria-specific and voltage-dependent dye, was employed. The results indicated that quercetin significantly decreased the levels of $\Delta\Psi_m$ in MCF-7 cells in a time-dependent manner (Fig. 4C).

Proteins levels in human breast cancer MCF-7 cells after treatment with quercetin, as examined by Western blotting

The results from Western blotting are shown in Fig. 5A, B, C and D. As shown in Fig. 5, the protein levels of pro-ATF-6, pro-caspase-12 (Fig. 5A), CDK2, Thymidylate synthase, cyclins A and B (Fig. 5B), pro-caspase-8 (Fig. 5C), Bcl-2, PARP, XIAP (Fig. 5D) in MCF-7 cells treated with quercetin were down-regulated, but the protein levels of PERK, GRP78 and GADD153 (Fig. 5A), p53 and p57 (Fig. 5B), Fas, TRAIL, BID

(Fig. 5C), AIF, caspase-9 and caspase-6 (Fig. 5D) were up-regulated. Quercetin increased the levels of ATF6, PERK, GRP78 and GADD153 (Fig. 5A) and those levels are correlated with the ER stress. However, quercetin decreased the levels of Thymidylate synthase and cyclins A and E (Fig. 5B) which are correlated with the S phase arrest. Quercetin increased the levels of Fas, TRAIL, BID, caspase-8, AIF, caspase-9, caspase-6 and decreased the levels of Bcl-2, XIAP and PARP (Fig. 5C and D), which may lead to apoptosis.

Quercetin promoted the activations of caspase-6, caspase-8 and caspase-9

In order to investigate whether caspase-6, -8 and -9 are involved in quercetin-induced apoptosis, the enzymatic activity of caspases were detected by using three fluorogenic peptide substrates - (Ac-VEID-AFC, Ac-IETD-AMC and Ac-LEHD-AMC) for caspases-6, -8

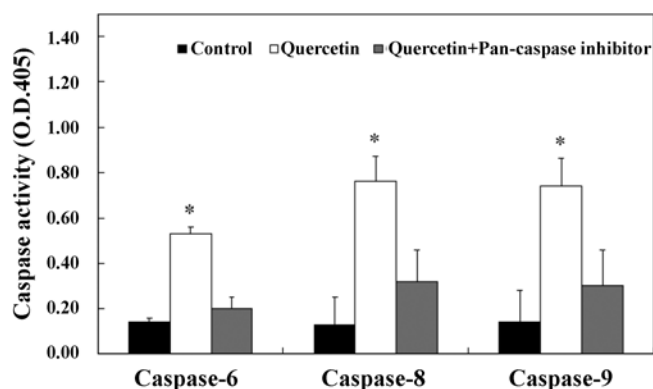


Fig. 6. The effects of quercetin on the activities of caspase-6, -8 and -9 in MCF-7 cells. Approximately 1×10^4 cells/well of MCF-7 cells in a 96-well plate were pretreated with or without pan-caspase inhibitors, then were treated with 150 μM quercetin, and were incubated for 24 h to detect the activities of caspase-6, -8 and -9 as described in Materials and Methods. *Significantly different from the control at $p < 0.05$.

and -9, respectively. The results presented in Figure 6, indicating the kinetic activity of various caspases, show that quercetin induced a rapid rise in caspase-6, -8 and -9 activities. Co-treatment of MCF-7 cells with pan-caspase inhibitor led to significant activity decreases for the three examined caspases. The increases in caspase-6, -8 and -9 activities by quercetin are closely correlated with the processing of pro-caspase-6, -8 and -9, respectively, as demonstrated in Fig. 6.

Effects of quercetin on AIF and GADD153 nuclear translocation in MCF-7 cells

As illustrated in Fig. 7, quercetin-treated MCF-7 cells reacted with AIF and GADD153 antibodies separately and PI staining results indicated that quercetin treatment for 24 h increased the levels of AIF and GADD153; it was also showed that both proteins translocated to nuclei.

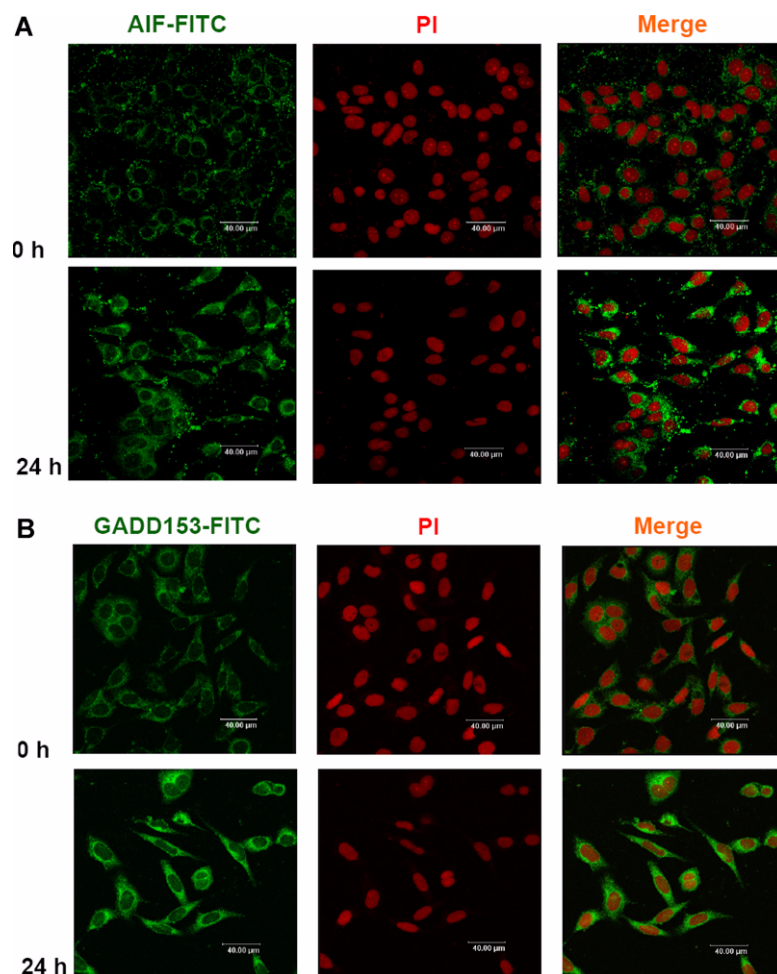


Fig. 7. Effects of quercetin on AIF and GADD153 nuclear translocations in MCF-7 cells. The MCF-7 cells (5×10^4 cells/well; 4-well chamber slides) were incubated with 150 μM quercetin for 24 h. Cells were fixed and stained with primary antibodies to AIF (A)-or GADD153 (B)-labeled secondary antibodies were used (green fluorescence) and the proteins were detected by a confocal laser microscopic system. The nuclei were stained by PI (red fluorescence). Areas of colocalization between AIF expressions and nuclei in the merged panels are yellow. Scale bar, 40 μm

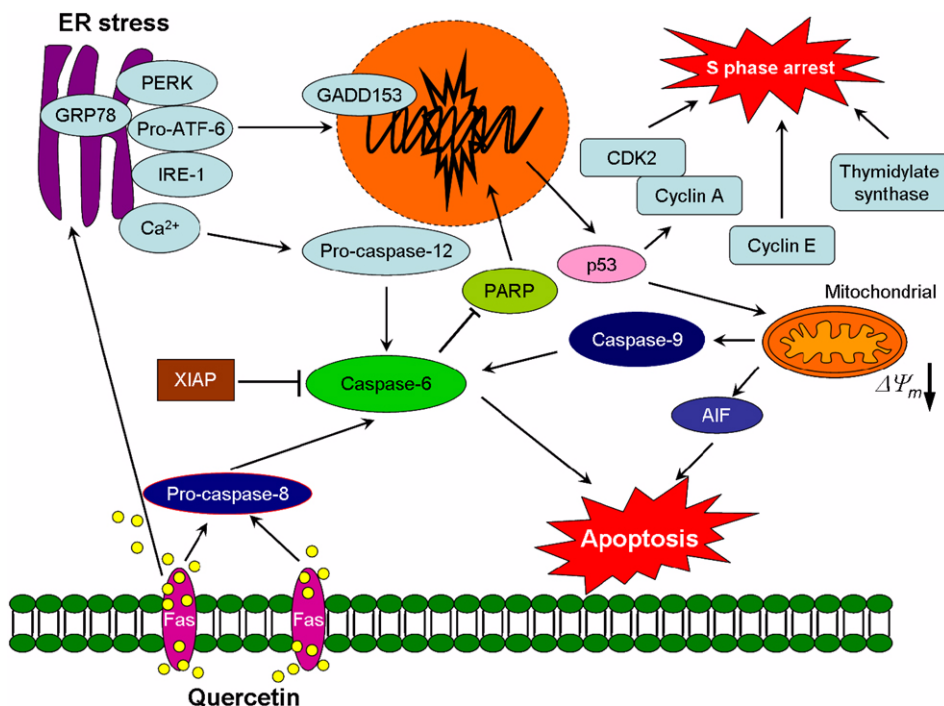


Fig. 8. Proposed model for the molecular mechanisms of quercetin induced apoptosis in human breast cancer MCF-7 cells. Quercetin induced ER stress (up levels of GADD153) through the production of Ca^{2+} and reduced $\Delta\Psi_m$ levels, leading to caspase-9 and caspase-6 activation before causing apoptosis in MCF-7 cells.

DISCUSSION

Quercetin decreased the viability of MCF-7 cells in a dose-dependent manner with an EC_{50} of approximately $92.4 \mu M$ quercetin for 48 h. Quercetin-inhibited MCF-7 cell growth may be a result of an arrest during the cell cycle. Therefore, we examined cell cycle distributions in quercetin-treated MCF-7 cells. Cell cycle analysis revealed that quercetin-induced S phase arrest in MCF-7 cells was accompanied by the alteration of various cell cycle-regulated proteins. In this study, we found that CDK2, plus cyclins A and E were decreased. According to our data, quercetin decreased the protein levels of T.S. and p57 (Fig. 5B) which may result in S phase arrest. The activation of the CDK2/cyclin E complex in the nucleus drives the progression from G2- to M-phase of the cell cycle.

In Fig. 2A and B, quercetin induced S phase arrest and decreased the percentage of viable cells; we suggested that quercetin-treated cells may also be due to apoptotic cell death. The major point is that results from Fig. 2 and 3 demonstrated that quercetin induces the apoptosis of MCF-7 cells. These observations were verified by morphological change of the nucleus (Fig. 3A), the increase of sub-G1 DNA content (Fig. 2A), DNA fragmentation (Fig. 3B) and PARP cleavage (Fig. 5D).

In order to further understand the molecular mechanism involved in apoptosis caused by quercetin, the expression of the apoptosis-related proteins such as Fas, Bcl-2, caspase-6, -8 and -9 and changes in mitochondrial membrane potential ($\Delta\Psi_m$) were assessed in MCF-7 cells. The ratio of Bax/Bcl-2 plays an important role in apoptosis. A high ratio of Bax to Bcl-2 caused $\Delta\Psi_m$ loss, Apaf-1, procaspase-9, cytochrome *c* and AIF proteins to be released from mitochondria into cytosol or nuclei, thus causing apoptosis (Yang et al., 1997). We showed quercetin decreased the level of $\Delta\Psi_m$, by using flow cytometry (Fig. 4C) and suggested it may result from changes in Bax and Bcl-2 proteins. We also suggested that quercetin-mediated apoptosis occurs via a mitochondrial-dependent pathway.

There are two signaling pathways involved with apoptosis; the mitochondrial pathway and the cell death receptor pathway (Ashkenazi and Dixit, 1998; Budihardjo et al., 1999; Shi, 2002; Gupta, 2003; Fan et al., 2005). The mitochondrial pathway is involved in the efflux of Apaf-1, procaspase-9 and cytochrome *c* from mitochondria into cytoplasm, thus leading to the activation of the caspase-3, -6, -7 (Mehmet, 2000). The cell death receptor pathway, mediated distinctively by active/cleaved caspase-8, plays a fundamental role in the maintenance of tissue homeostasis, is characterized by binding cell death ligands and cell death receptors,

and subsequently activates caspase-8, caspase-3, -6, -7 (Hengartner, 2000; Liu et al., 2004) and cleaved PARP.

Our results showed quercetin increased cleaved caspase-8 and -9 (Fig. 5C and D) in MCF-7 cells. Moreover, the increase of cleaved caspase-9 led to subsequent activation of the downstream caspase-6, an apoptotic executioner (Fig. 5D). The IAP family proteins include cIAP-1, cIAP-2, XIAP and survivin (Shen et al., 2003; Vaux and Silke, 2003). IAPs interact with and inhibit the activity of processed caspases that can impede the apoptotic process once it begins. Caspase-9 and caspase-3, -6 are IAP targets, whereas caspase-8 is not (Deveraux et al., 1998). Our results showed that quercetin inhibited XIAP protein, leading to promotion of caspase-6 activation, which caused apoptosis. It was reported that MCF-7 cells lack caspase-3, so we strongly suggested that quercetin-induced apoptosis was caspase-3 independent (Mc Gee et al., 2002).

In order to determine whether or not caspases are required for the induction of apoptosis by quercetin, we incubated quercetin-treated MCF-7 cells with a caspase inhibitor. Quercetin-induced apoptosis was significantly attenuated in the presence of inhibitors, in view of sub-G1 and annexin V positive staining (data not shown). Pan-caspase inhibitor prevented apoptosis induction in quercetin-treated MCF-7 cells. In addition, it was noted that inhibition of Fas (anti-FasL mAb, pre-treated MCF-7 cells) and a caspase-8 inhibitor, significantly decreased apoptosis in quercetin-treated cells, suggesting that apoptosis triggered by quercetin was in part mediated by the cell death receptor pathway.

As shown in Figure 8, our results proposed a model of quercetin-induced S phase arrest and apoptosis in human breast cancer MCF-7 cells. Quercetin-induced S phase arrest was associated with reduction of CDK2 and cyclins A and B proteins. It provided further evidence that adaptor proteins (Fas/CD95) acted as a receptor for quercetin, that promoted the activation of initiator caspases such as caspase-8 and caspase-9 and activated the effector caspase such as caspase-6. Therefore, we demonstrated that a model of quercetin-induced apoptosis operated through multiple pathways in human breast cancer MCF-7 cells.

ACKNOWLEDGEMENTS

This research was supported by grant 97-CCH-CSMU-13 from Changhua Christian Hospital, Taiwan.

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