The Role of Ca^{2+} in Baicalein-induced Apoptosis in Human Breast MDA-MB-231 Cancer Cells through Mitochondria- and Caspase-3-dependent Pathway

JAU-HONG LEE[1], YU-CHING LI[2], SIU-WAN IP[3], SHU-CHUN HSU[4], NAI-WEN CHANG[5], NOU-YING TANG[6], CHUN-SHU YU[7], SU-TZE CHOU[8], SONG-SHEI LIN[9], CHIN-CHUNG LIN[10], JAI-SING YANG[11] and JING-GUNG CHUNG[4,12]

[1]Department of Surgery, China Medical University Hospital, Taichung, Taiwan;
Departments of [2]Medical Laboratory Science and Biotechnology, and [9]Radiological Technology
Mid-Taiwan Technology Unuiversity, Takun, Taichung, Taiwan;
Departments of [3]Nutrition, [4]Biological Science and Technology,
[5]Biochemistry, [11]Pharmacology, Schools of [6]Chinese Medicine and
[7]Pharmacy, China Medical University, Taichung, Taiwan;
[8]Department of Food and Nutrition, Providence University, Taiwan;
[10]Fong-Yuan Hospital, Fong Yuan City 420, Taiwan;
[12]Department of Biotechnology, Asia University, Wufeng, Taichung County, Taiwan, R.O.C.

Abstract. *Baicalein was investigated for tumor cell-specific cytotoxicity, apoptosis-inducing activity and signal pathway against the MDA-MB-231 human breast cancer cell line. After the MDA-MB-231 cells had been treated with baicalein, trypan blue exclusion, propidium iodide (PI) assay and 4',6-diamidino-2-phenylindole (DAPI) were used to stain the dead cells and detect apoptosis, respectively. The effects of baicalein on the levels of reactive oxygen species (ROS), Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi_m$) on MDA-MB-231 cells were examined by flow cytometric assays. The ROS caused endoplasmic reticulum (ER) stress, confirmed by the increase of GADD153 and GRP78 in the examined cells. GADD153 and GRP78 increases were also confirmed by confocal laser microscopy examination and indicated that both proteins translocated to the nucleus. The effects of baicalein on the expression of apoptotic-regulated genes, such as Bcl-2 family and caspase, were detected by Western blotting. To further investigate the apoptotic pathway and the role of Ca^{2+} induced by baicalein, a caspase-3 inhibitor and Ca^{2+} chelator were used to block caspase-3 activity and Ca^{2+} in MDA-MB-231 cells. Baicalein induced apoptosis in a time-dependent effect through the inhibition of Bcl-2 expression, increased the levels of Bax, reduced the level of $\Delta\Psi_m$, and promoted the cytochrome c release and caspase-3 activation. MDA-MB-231 cells were pretreated with BAPTA which reduced the levels of Ca^{2+}, $\Delta\Psi_m$ and apoptosis. In conclusion, baicalein induced apoptosis via Ca^{2+} production, mitochondria-dependent and caspase-3 activation in MDA-MB-231 cells.*

Correspondence to: Jing-Gung Chung, Ph.D., Department of Microbiology, School of Medicine, China Medical University, No 91, Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C. Tel: +886422053366 ext. 2161, Fax: +886422053764, e-mail: jgchung@mail.cmu.edu.tw / Jai-Sing Yang, Ph.D., Department of Pharmacology, China Medical University, No 91, Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C. Tel: +886422053366 ext 2225, Fax: +886422053764, e-mail: jaising@mail.cmu.edu.tw

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Based on reports from the People Health Bureau of Taiwan, 12 persons per 100 thousand die annually from breast cancer. Surgery, radiotherapy and chemotherapy are the treatment options for breast cancer patients, but the cure rates are not satisfactory. New agents acting on novel targets of breast cancer are currently under investigation.

Apoptosis is one of the most important processes leading to cell death. The Bcl-2 family of proteins plays an important role as an intracellular checkpoint of apoptosis (1). It has been demonstrated that the impairment of apoptosis is a crucial step in the process of cancer development (2-4). Bcl-2 proteins include pro-apoptotic (Bax, Bak, Bid, Noxa) and anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, Bcl-w) members and the shifting of the Bax/Bcl-xL ratio in favor of apoptosis (5, 6). Therefore, if agents are able to cause apoptosis in cancer cells, they are deemed to be important in strategies for killing cancer cells (7).

Baicalein (5,6,7-trihydroxyflavone) is a naturally occurring polyphenolic pigment, derived from the root of

Scutellaria baicalensis Georgi, and is a traditional medicine used in Oriental populations (8). Baicalein has been demonstrated to be an antioxidant (9) and anti-inflammatory agent (10), an inhibitor of prostaglandin E_2 (11), to possess antioxidant activities (12-14) and cyto-protective effects (15-18). It was reported that baicalein induced apoptosis in human breast (20), hepatoblastoma (19), prostate (21), and gastric (22) cancer cells.

The role of Ca^{2+} in the induction of apoptosis by baicalein in MDA-MB-231 human breast cancer cells is unknown. Therefore, the present study aimed to investigate the role of Ca^{2+} and the signal pathway for baicalein-induced apoptosis in MDA-MB-231 human breast cancer cells.

Materials and Methods

Chemicals and reagents. Baicalein, propidium iodide (PI), Tris-HCl and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BAPTA, dimethyl sulfoxide (DMSO) and potassium phosphates were purchased from Merck Co. (Darmstadt, Germany). Eagle's minimum essential medium (MEM), penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS) and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kit was bought from OncoImmunin (MD, USA).

Human breast cancer line (MDA-MB-231). The MDA-MB-231 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were cultured for several generations and were checked for viability and morphology. The cells were placed into 75 cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 and 95% air at one atmosphere in MEM supplemented with 10% FBS, 1% penicillin-streptomycin (10 ng/ml penicillin and 10 ng/ml streptomycin) and 1% glutamine.

Phase-contrast microscopy and flow cytometry examination of morphological changes and viability of MDA-MB-231 cells. The MDA-MB-231 cells were plated in 6- and/or 12-well plates at a density of $3x10^5$ cells/well and grown for 24 hours. Baicalein was added at final concentrations of 0, 25, 50, 75 and 100 μM, while only adding DMSO (solvent) for the control, and the cells were grown at 37˚C, in 5% CO_2 and 95% air for different periods of time. A phase-contrast microscope was used for photography to determine morphological changes and flow cytometric assays were carried out to determine cell viability as described elsewhere (23-27).

Flow cytometric analysis of DNA content for apoptosis from MDA-MB-231 cells treated with baicalein. Apporoximately $5x10^5$ cells/well of MDA-MB-231 cells in 12-well plates with 50 μM baicalein were incubated for 6, 12, 24, 48 and 72 hours, and the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) by putting them in 70% ethanol (in PBS) in ice overnight and were then re-suspended in PBS containing 40 μg/mL PI and 0.1 mg/mL RNase (Sigma, St. Louis, MO, USA) and 0.1% Triton X-100 in a dark room. After 30 minutes at 37˚C, the cells underwent analysis with flow cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at 488 nm. The cell cycle distribution and sub-G1 population (apoptosis) were determined and analyzed (23, 24).

Flow cytometric analysis for caspase-3 activity of MDA-MB-231 cells after treatment with baicalein. The MDA-MB-231 cells were plated in 12-well plates at a density of $5x10^5$ cells/well and grown for 24 hours. Various concentrations (0, 25, 50, 75 and 100 μM) of baicalein were added, with DMSO (solvent) alone added for the control, and the cells were grown at 37˚C in a humidified 5% CO_2 for 12 hours to detect the activity of caspase-3. The cells were harvested and washed twice, re-suspended in 50 μl of PhiPhiLux (50 μM), incubated at 37˚C for 30 min and then analyzed by flow cytometry (23, 24).

Inhibition of baicalein-induced caspase-3 activity and apoptosis by the caspase-3 inhibitor (Ac-DEVD-CHO) in MDA-MB-231 cells. In order to examine whether or not caspase-3 activation was involved in the apoptosis triggered by baicalein, the MDA-MB-231 cells were pretreated with the cell permeable caspase-3 inhibitor Ac-DEVD-CHO 3 hours prior to treatment with baicalein. Then, the caspase-3 activity and apoptosis were determined as described above.

Flow cytometric detection of reactive oxygen species (ROS) in MDA-MB-231 cells after treatment with baicalein. The level of ROS of the MDA-MB-231 cells was examined by flow cytometry (Becton Dickinson FACSCalibur), using 2,7-dichloro-dihydrofluorescein diacetate (DCFH-DA; Sigma). The MDA-MB-231 cells were treated with or without baicalein (0, 25, 50, 75 and 100 μM) for 24 hours to detect the changes of ROS. The cells were harvested and washed twice, re-suspended in 500 μl of DCFH-DA (10 μM), incubated at 37˚C for 30 min and then analyzed by flow cytometry (23, 24).

Flow cytometric detection of Ca^{2+} concentrations in MDA-MB-231 cells after treatment with baicalein. The level of Ca^{2+} of the MDA-MB-231 cells was determined by flow cytometry (Becton Dickinson FACSCalibur), using the Indo 1/AM (Calbiochem; La Jolla, CA USA). Cells were pre-treated with or without 10 μM BAPTA-AM (intracellular calcium chelator) for 3 hours, and then with baicalein (0, 25, 50, 75 and 100 μM) for 24 hours to detect the changes of Ca^{2+} concentration. The cells were harvested and washed twice, one for apoptosis analysis and the other for re-suspension in Indo 1/AM (3 μg/ml), incubated at 37˚C for 30 min, and then analyzed by flow cytometry (23, 24).

Flow cytometric detection of mitochondrial membrane potential ($\Delta\Psi_m$) in MDA-MB-231 cells after treatment with baicalein. The mitochondrial membrane potential ($\Delta\Psi_m$) of the MDA-MB-231 cells was determined by flow cytometry (Becton Dickinson FACSCalibur) using $DiOC_6$ (4 μmol/L). Cells were pre-treated with or without 10 μM BAPTA-AM for 3 hours and then with various concentrations (0, 25, 50, 75 and 100 μM) of baicalein for 24 hours to detect the changes of $\Delta\Psi_m$. The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 μmol/L) and incubated at 37˚C for 30 min before being analyzed by flow cytometry (23, 24).

Western blotting to examine the effect of baicalein on GADD153, GRP78, p53, Bax, Bcl-2, cytochrome c and caspase-3 of MDA-

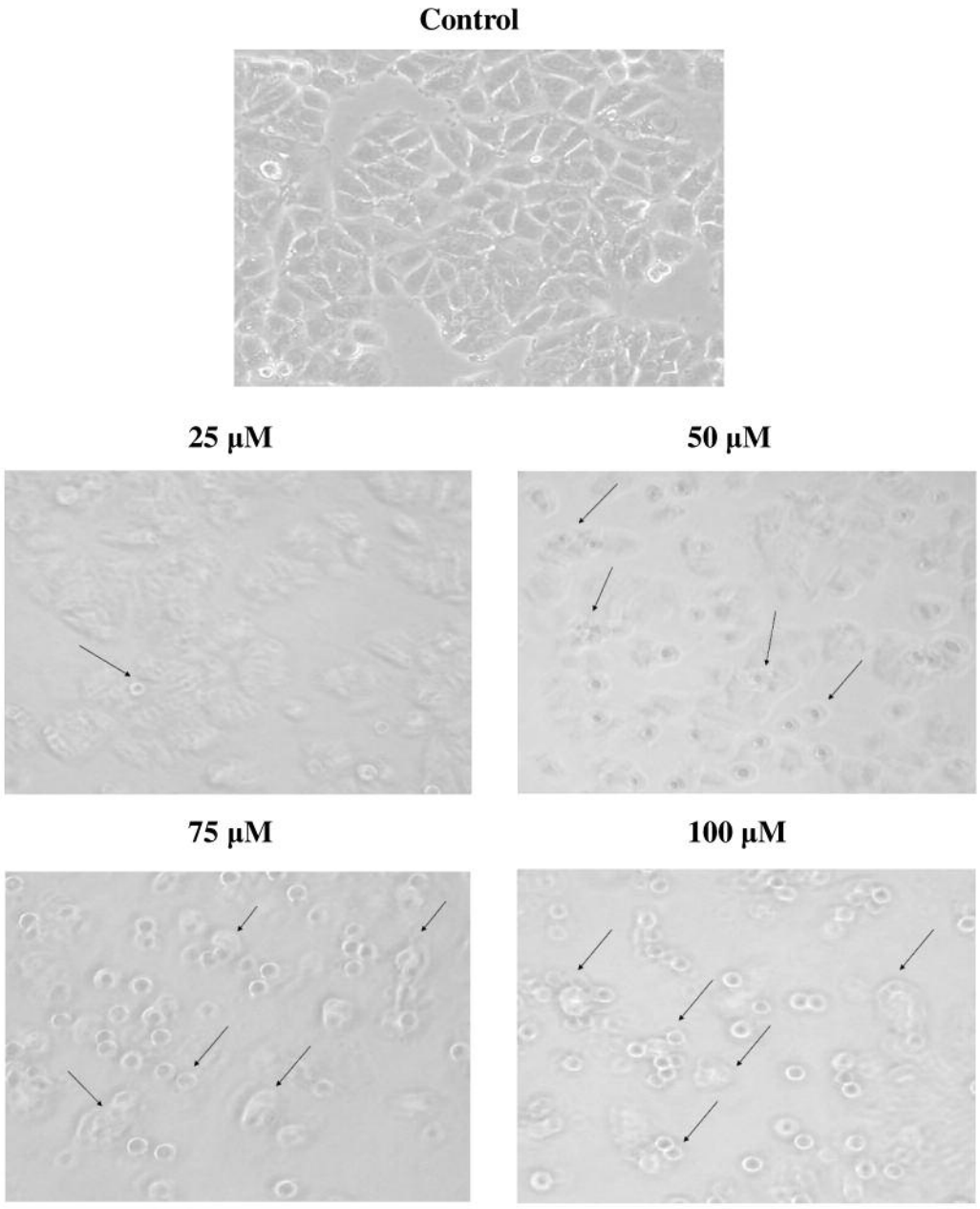

Figure 1. *Morphological changes of human breast cancer MDA-MB-231 cells in response to baicalein. MDA-MB-231 cells were treated with various concentrations of baicalein for 24 hours. Cells were examined and photographed under phase-contrast microscopy.*

MB-231 cells. Steady-state levels of GADD153, GRP78, Bax, Bcl-2, cytochrome *c* and caspase-3 proteins were determined by Western blotting. Briefly, the total proteins were collected from MDA-MB-231 cells treated with or without baicalein (0, 25, 50, 75 and 100 μM) for 48 hours. Protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (50 μg each) were boiled with gel loading buffer for 5 min. A 50 μg of total, 50 μg of cytosolic or 50 μg of mitochondrial protein extracts were separated on 10% SDS-polyacrylamide electrophoresis gels. The samples were transferred to enhanced chemiluminescence (ECL; Amersham-Pharmacia Biotech, Piscataway, NJ, USA) membranes and the membranes were blocked overnight at 4°C with Tris-buffered saline (TBS)–0.1% Tween 20 (TBS-T) containing 2% skim milk. The membranes were then incubated for 2 hours with anti-GADD153, -GRP78, -Bax, -Bcl-2, -cytochrome *c* and -caspase-3 antibody diluted to 1:200 in TBS. After washing five times with TBS-T, the membranes were incubated for 1 hour with peroxidase-labeled anti-rabbit IgG antibody (1:5000; Cappel, Aurora, OH, USA) in TBS-T. After washing five times with TBS-

T, the immune complex was visualized with an ECL Plus Western Blotting Detection Systems kit (Amersham-Pharmacia Biotech). The signals were detected using Kodak LAS-3000 as described elsewhere (23, 24).

RT-PCR detection of GADD153 mRNA from MDA-MB-231 cells after treatment with baicalein. The total RNA was extracted from the MDA-MB-231 cells after treatment with 0, 25, 50, 75 and 100 μM baicalein for 24 hours, using the Qiagen RNeasy Mini Kit as described elsewhere. The PCR reactions were performed with the following primers, as described elsewhere (25): GADD153: sense 5-GAAACGGAAACAG AGTGGTCATTCCCC-3 and antisense 5-GTGGGATTGAGGGTCACATCATTG GCA-3 to produce a 301-bp DNA fragment of GADD153. The internal control using the primers were: GAPDH: sense 5-CTCAGACACCATGGGGAAGGTGA-3 and antisense 5-ATGATCTTGAGGCTGTTGTCATA-3 to produce a 450-bp fragment of the GAPDH. Each assay was conducted at least twice to ensure reproducibility (25).

Confocal laser microscopy. MDA-MB-231 cells (5×10^4 cells/well) plated on 4-well chamber slides were treated with 50 μM baicalein for 24 hours then cells were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton-X 100 in PBS for 1 hour with blocking of non-specific binding sites using 2% BSA. The slides were then incubated with antihuman GADD153 and GRP78 antibody (1:100 dilution; Santa Cruz) overnight and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution; Santa Cruz), followed by DNA staining with PI. Photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope (26).

Densitometry and statistical analysis. The relative intensities of protein bands were analyzed using the ImageMaster 1D Elite v 4.00 densitometric analysis program (Amersham Biosciences). All data were expressed as mean±SEM from at least three separate experiments. Statistical calculations of the data were performed using an unpaired Student's *t*-test and Tukey's test. Statistical significance was set at $p<0.05$.

Results

Effects of baicalein on morphological changes and cell viability of MDA-MB-231 human breast cancer cells. The results from morphological examinations and PI staining experiments indicated that baicalein induced cell death (morphological changes) (Figure 1), and that the percentage of viable cells was significantly different between the baicalein-treated group and the control. Baicalein reduced the percentage of viable cells (Figure 2A) and the increase of the incubation time led to a decrease in the percentage of viable cells (Figure 2B). These effects were dose- and time-dependent (Figure 2A and B).

Effects of baicalein on caspase-3 activity in MDA-MB-231 cells. The result from flow cytometric analysis indicated that the percentage of caspase-3 activity was significantly different between baicalein-treated and control groups. Increasing the dose of baicalein led to an increase of the caspase-3 activity in MDA-MB-231 cells (Table I). These effects of baicalein on caspase-3 activation were dose-dependent.

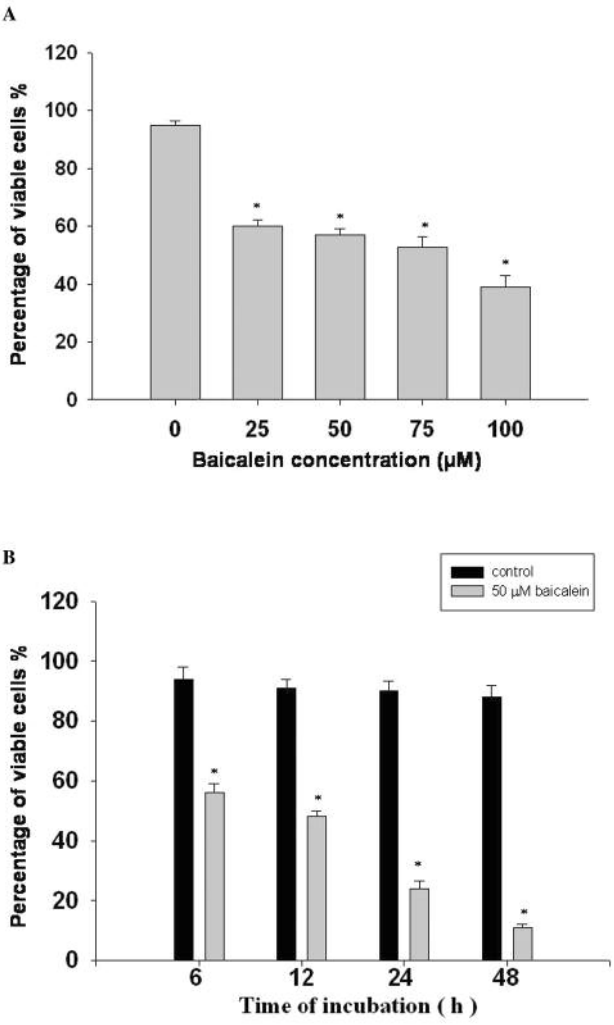

Figure 2. *The percentage of viable human breast cancer MDA-MB-231 cells after treatment with baicalein. MDA-MB-231 cells (2×10^5 cells/well; 12-well plates) were treated with different concentrations of baicalein for 24 hours or treated with 50 μM balcalein for 6, 12, 24 and 48 hours. The cells were collected by centrifugation and the viable cells were determined by trypan blue exclusion and flow cytometry as described in Materials and Methods. A: dose-dependent growth; B: time course of growth. Each point is mean±S.D. of three experiments. *Significantly different from the control at $p<0.05$.*

Effects of baicalein on the levels of reactive oxygen species (ROS) in MDA-MB-231 cells. The result from flow cytometric analysis indicated that the percentage of ROS was significantly different between baicalein-treated and control groups. Increasing the dose of baicalein led to an increase of the ROS activity in MDA-MB-231 cells (Table I). The ROS production was dose-dependent.

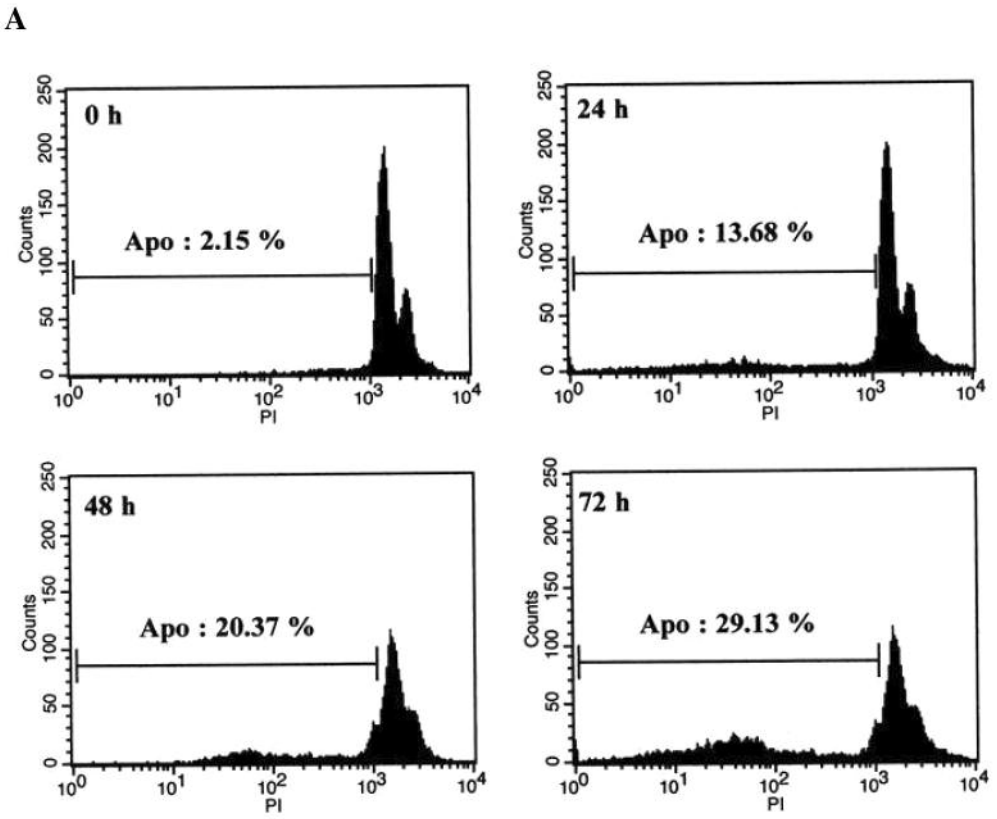

Figure 3. *PI staining for the effects of baicalein on human breast cancer MDA-MB-231 cell apoptosis. MDA-MB-231 cells were incubated with 50 μM baicalein for 6, 12, 24, 48 and 72 hours and apoptosis was determined by flow cytometric analysis as described in Materials and Methods. A: representative profiles; B: time course. Data represent mean±S.D. of three experiments. *Significantly different from the control at p<0.05.*

Effects of baicalein on the mitochondria membrane potential from MDA-MB-231 cells. The result from flow cytometric analysis indicated that the percentage of mitochondria membrane potential ($\Delta\Psi_m$) was significantly different between baicalein-treated and control groups. Increasing the dose of baicalein led to decrease in the $\Delta\Psi_m$ in MDA-MB-231 cells (Table I). The effects of baicalein on the levels of $\Delta\Psi_m$ were dose-dependent.

Table I. *Flow cytometric analysis of intracellular ROS and Ca^{2+} levels, MMP and caspase-3 activity in MDA-MB-231 cells with baicalein treatment.*

	% of control			
Baicalein (μM)	ROS	Ca^{2+}	$\Delta\Psi_m$	Caspase-3
Control	0.0±0.0	0.0±0.0	100.0±0.0	0.0±0.0
25	9.0±2.6 *	12.4±2.9*	90.4±9.8*	11.4±2.1 *
50	19.4±2.2 *	26.6±2.1*	82.6±8.8*	18.5±2.8*
75	31.8±3.6*	43.6±3.8*	72.4±8.3*	34.4±2.3*
100	58.6±5.4 *	69.1±4.6*	60.1±8.3*	49.8±3.2*

The MDA-MB-231 cells ($5x10^5$ cells/ml) were treated with different concentrations of baicalein. The zero concentration was defined as the control. The percentage of cells of stained by DCFH-DA, Indo-1/AM or DiOC6 dyes or PhiPhiLux substrate, respectively, for ROS, Ca^{2+} or MMP were determined by flow cytometry as described in the Materials and Methods section. Values are means±SD (n=3). Statistical calculations of the data were performed using an unpaired Student's *t*-test and Tukey's test. *Significantly different from the control at $p<0.05$.

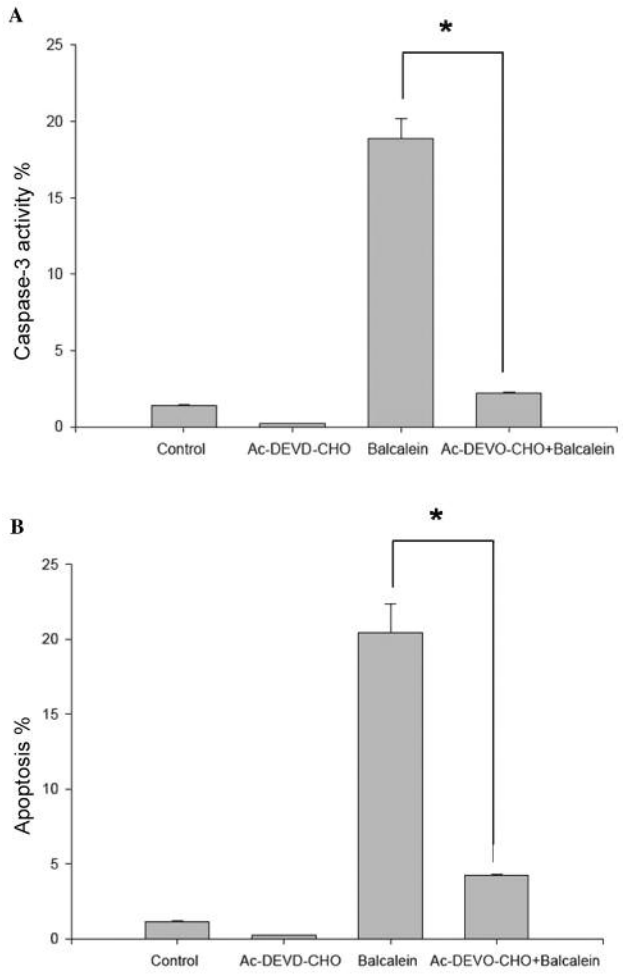

Figure 4. *Effects of caspase-3 inhibitor (Ac-DEVD-CHO) on baicalein-induced caspase-3 activity and apoptosis in human breast cancer MDA-MB-231 cells. The MDA-MB-231 cells were pre-treated with caspase-3 inhibitor (Ac-DEVD-CHO) for 3 hours then treated with 50 μM baicalein before cells were harvested for caspase-3 activity and apoptosis determinations as described in Materials and Methods. A: caspase-3 activity; B: apoptosis. Data represent mean ± S.D. of three experiments. *Significantly different from baicalein alone at p<0.05.*

Effects of baicalein on the production of Ca^{2+} from MDA-MB-231 cells. The result from flow cytometric analysis indicated that the Ca^{2+} production was significantly different between baicalein-treated and control groups. Increasing the dose of baicalein led to an increase of the Ca^{2+} production in MDA-MB-231 cells (Table I). The effects of baicalein on the levels of Ca^{2+} production were dose-dependent.

Effects of Ac-DEVD-CHO on baicalein-induced caspase-3 activity and apoptosis of MDA-MB-231 cells. As illustrated in Figure 4A and B, the percentage of caspase-3 activity and apoptosis were significantly different between the Ac-

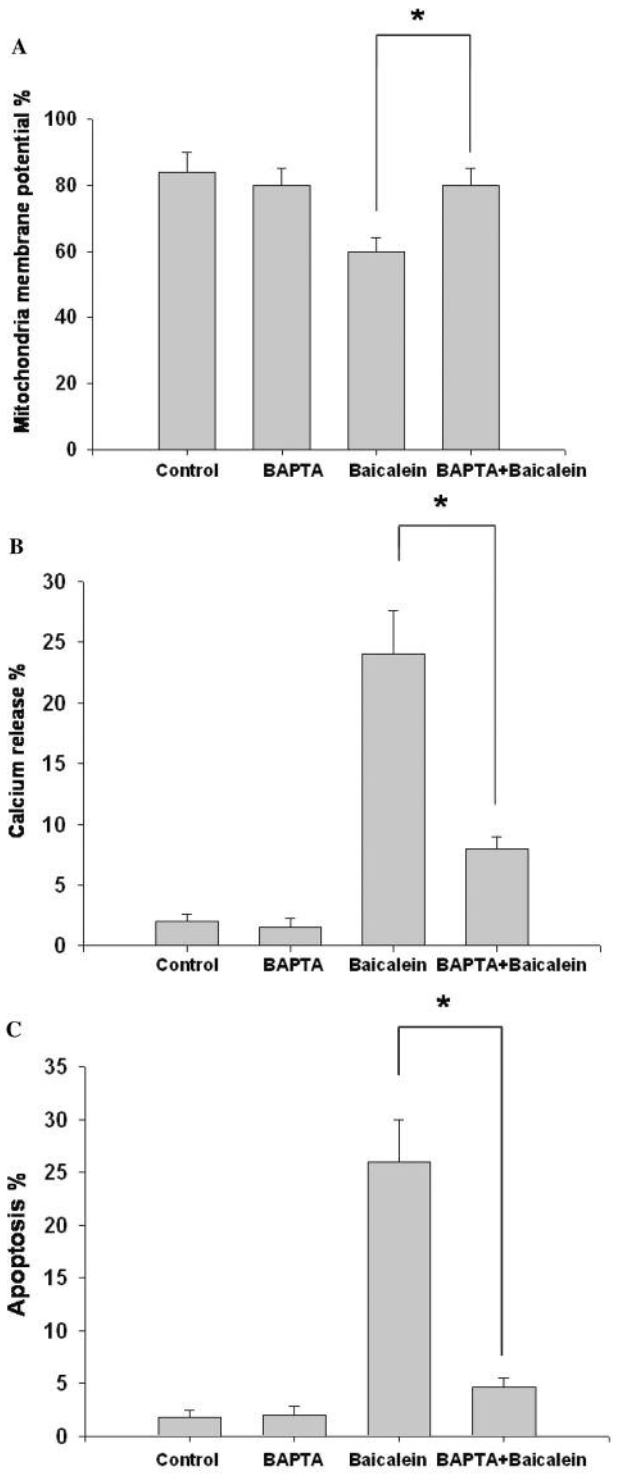

Figure 5. *Effects of calcium antagonist BAPTA (Ca^{2+} chelator) on baicalein affecting the levels of $\Delta\Psi_m$, Ca^{2+} and apoptosis in human breast cancer MDA-MB-231 cells. The MDA-MB-231 cells were pre-treated with BAPTA for 3 hours then treated with 50 μM baicalein before cells were harvested for $\Delta\Psi_m$ (A), Ca^{2+} (B) and apoptosis (C) determinations as described in Materials and Methods. Data represent mean±S.D. of three experiments. *Significantly different from baicalein alone at p<0.05.*

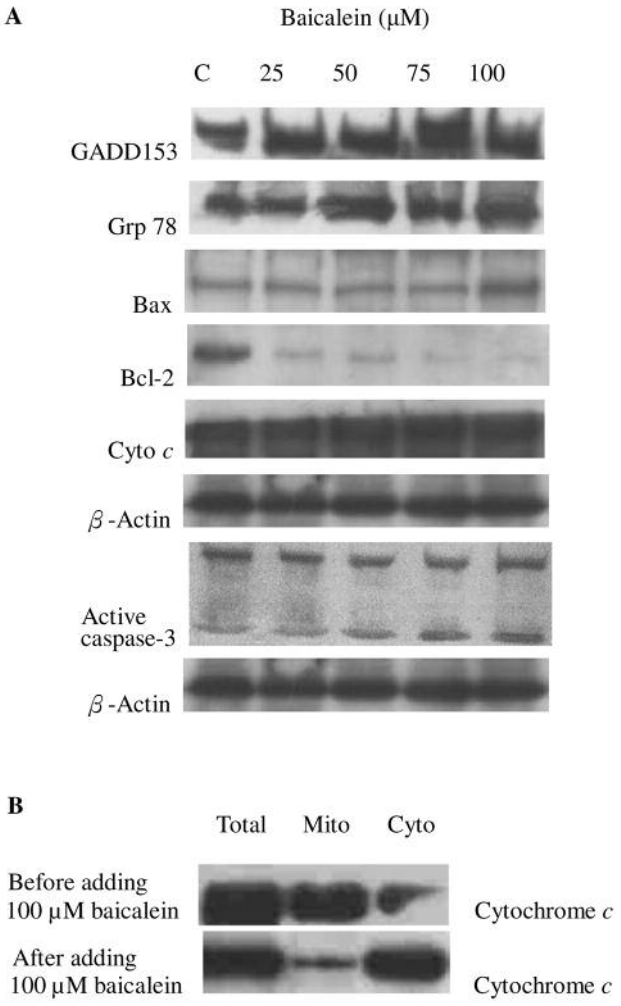

Figure 6. *Representative Western blot showing changes in the levels of GADD153, GRP78, bax, Bcl-2, cytochrome c and caspase-3 expression in human breast cancer MDA-MB-231 cells after treatment with baicalein. The MDA-MB-231 cells (5×10^6/ml) were treated with 0, 25, 50, 75 and 100 μM baicalein for 48 hours then the cytosolic fraction and total protein were prepared and determined as described in Materials and Methods. Evaluation of the associated protein levels was then carried out by Western blotting as described in Materials and Methods. A: GADD153, GRP78, Bax, Bcl-2, cytochrome c, caspase-3; B: total, cytosolic (Cyto) and mitochondrial (Mito) cytochrome c.*

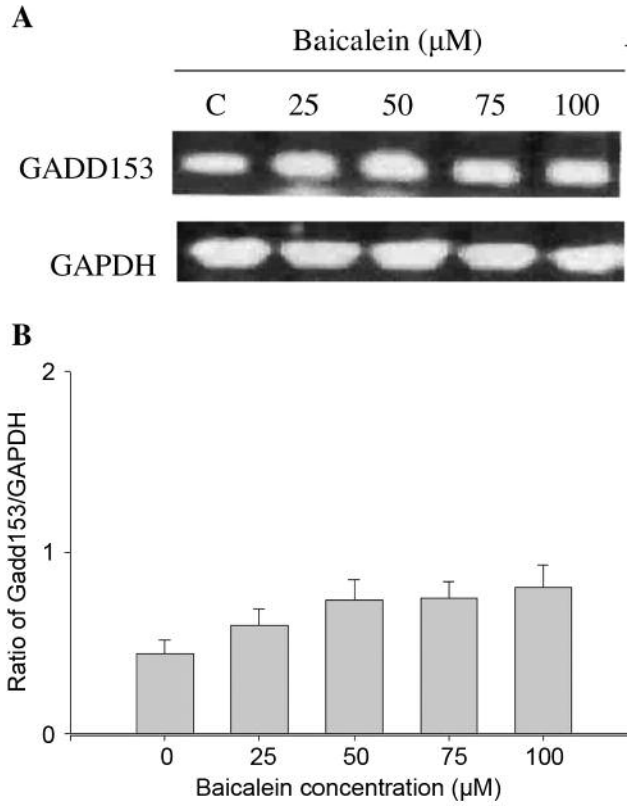

Figure 7. *Effect of baicalein on the expression of GADD153 mRNA in MDA-MB-231 cells. MDA-MB-231 cells (5×10^5 cells/well; 12-well plates) were incubated with 0, 25, 50, 75 and 100 μM baicalein for 24 h. The cells were collected and RNA was extracted. The extracted RNA was subjected to RT-PCR analysis using specific primers for GADD153 and GAPDH then PCR-amplified cDNA derived from mRNA (A) was analysed by agarose gel-electrophoresis and the ratio of GADD153 to GAPDH (B) was determined.*

DEVD-CHO pretreated before baicalein treated group and the baicalein only treated group in MDA-MB-231 cells. Ac-DEVD-CHO significantly inhibited the caspase-3 activity and reduced the apoptosis of baicalein-treated groups.

Effects of BAPTA on baicalein-induced production of Ca^{2+}, $\Delta\Psi_m$ and apoptosis of MDA-MB-231 cells. As illustrated in Figure 5A and B, the Ca^{2+} production, $\Delta\Psi_m$ and apoptosis levels were significantly different between baicalein alone and/or pretreated with BAPTA groups and the control group. MDA-MB-231 cells pre-treated with BAPTA exhibited lower levels of $\Delta\Psi_m$, Ca^{2+} production and apoptosis (Figure 6A, B and C) induced by baicalein.

Western blotting for the effect of baicalein on GADD153, GRP78, Bax, Bcl-2, cytochrome c and caspase-3 of MDA-MB-231 cells. The results (Figure 6) from the Western blotting indicated that baicalein increased the expressions of GADD153, GRP78, Bax, cytochrome *c*, caspase-3 and reduced the expression of Bcl-2 which may have led to apoptosis in these cells. The results also showed that baicalein affected the ratio of Bax/Bcl-2 which led to the dysfunction of mitochondria, such as the decrease of the levels of $\Delta\Psi_m$.

Effect of baicalein on GADD153 mRNA expression in MDA-MB-231 cells. The mRNA gel image and the ratio of

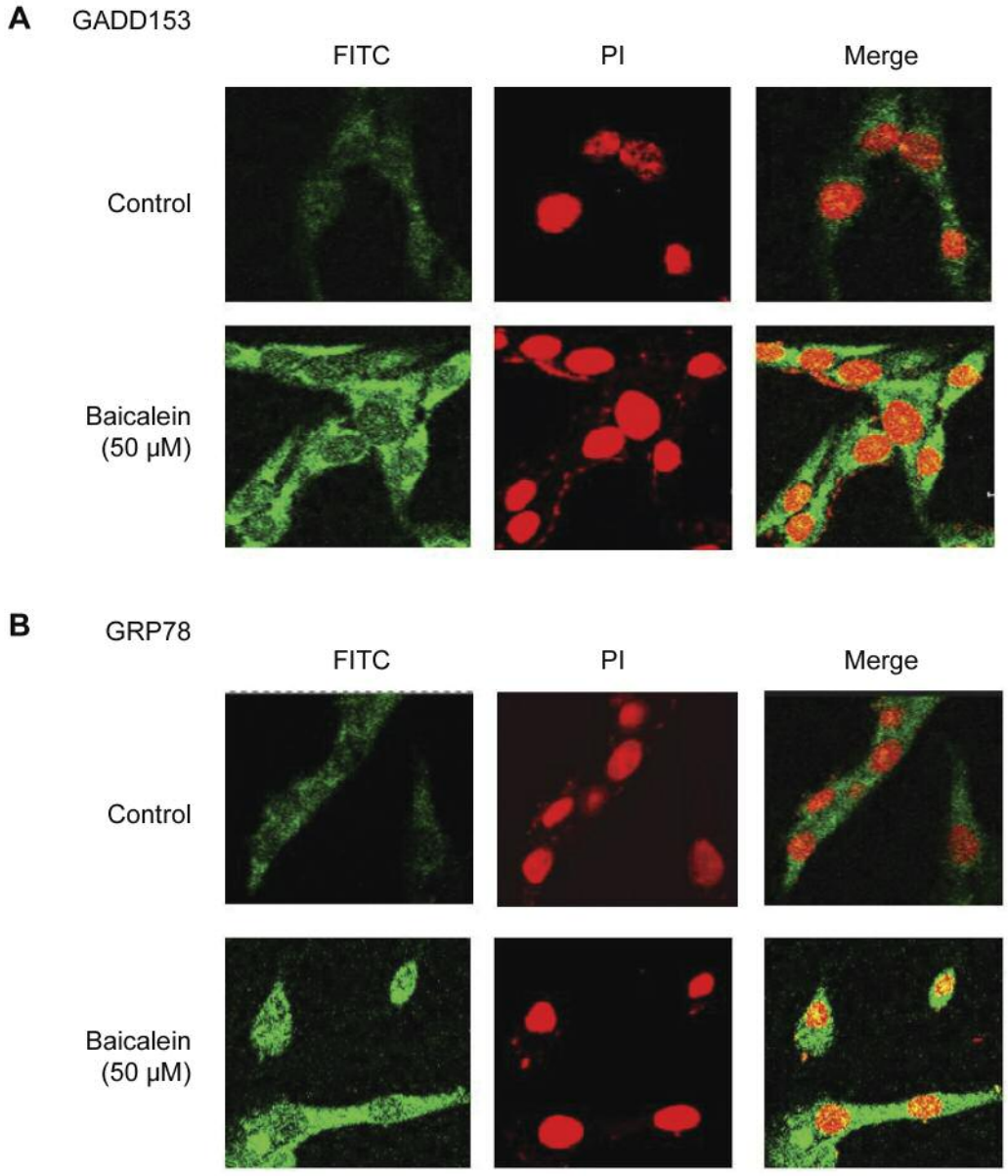

Figure 8. *Effects of baicalein on GADD1153 and GRP78 nuclear translocation in MDA-MB-231 cells. MDA-MB-231 cells (5×10^5 cells/well; 12-well plates) were incubated with 50 μM baicalein for 24 hours. Cells were fixed and stained with primary antibodies to GADD1153 (A) FITC-labeled secondary antibodies 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 GADD153 and/or GRP78 expressions and nuclei in the merged panels are yellow. Scale bar, 10 μm.*

GADD153 mRNA level versus GAPDH in response to the effect of 0, 25, 50 and 100 μM baicalein on MDA-MB-231 cells were examined (Figure 7A) and quantified (Figure 7B). The figure shows that GADD153 mRNA levels increased after the MDA-M-231 cells were treated with baicalein for 24 hours.

Immunofluorescence microscopy for examining the levels of GADD153 and GRP78. As illustrated in Figure 8A, baicalein-treated MDA-MB-231 cells reacted with GADD153 antibodies and PI staining results showed that baicalein treatment for 24 h increased the levels of GADD153; it was also translocated to nuclei. The results shown in Figure 8B also showed that baicalein treatment for 24 h increased the levels of GRP78; it was also translocated to nuclei. However, higher levels of GADD153 than GRP78 were translocated to nuclei (Figure 8A and B).

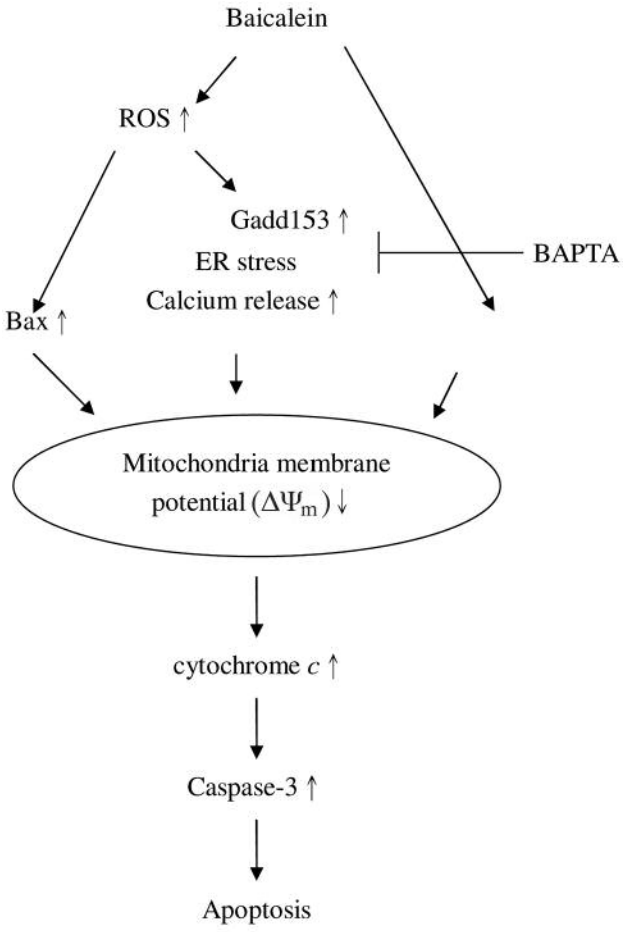

Figure 9. *Proposed model of baicalein mechanism of action for apoptosis in human breast cancer MDA-MB-231 cells. Baicalein increased the production of ROS and Ca^{2+} and reduced MMP levels leading to cytochrome c release and caspase-3 activation before causing apoptosis in MDA-MB-231 cells.*

Discussion

Baicalein has been found to be active against a wide variety of cancer cells, has been shown to induce apoptosis in many cancer cell lines (19-22), and has been demonstrated to be an antioxidant (12-14). *In vivo*, treatment of mice with baicalein demonstrated a statistically significant prostate tumor volume reduction ($p<0.01$) when compared to the control (27). Baicalein can directly inhibit proliferation of myeloma cells and down-regulate 12-LOX protein expression which is related to the effects of baicalein on myeloma cells (28). Other investigators demonstrated that baicalein protects neurons from the deleterious effects of 6-hydroxydopamine (OHDA) *via* the attenuation of oxidative stress, mitochondrial dysfunction, caspase activity, and JNK activation (29). However, the exact pathway of baicalein-induced apoptosis in MDA-MB-231 human breast cancer cells remains unknown.

It was reported that the Bcl-2 family of proteins is central in apoptosis regulation (30, 31). Our study demonstrated that baicalein induced apoptosis in MDA-MB-231 human breast cancer cells *via* altering the Bcl-2 family expression, especially in decreasing the protein levels of Bcl-2 and increasing the protein levels of Bax, before leading to the dysfunction of mitochondria ($\Delta\Psi_m$) followed by cytochrome *c* release and activation of caspase-3. These findings suggest that baicalein can induce apoptosis *via* a mitochondrial pathway, a mechanism that targets the Bcl-2 family, in particular, Bcl-2 protein, which is associated with the mitochondrial pathway. Overexpression of Bcl-2, Mcl-1 and Bcl-xL abort the apoptotic response while an increase in the activity of Bax, Bid and Bak promotes cell death (32).

However, our results also showed that baicalein-induced ROS led to the release of Ca^{2+}. Dysfunction followed (reduced levels of $\Delta\Psi_m$) as MDA-MB-231 cells pretreated with BAPTA (a chelator of Ca^{2+}) and then treated with baicalein led to decreasing levels of Ca^{2+} and $\Delta\Psi_m$ and subsequently the decrease of apoptosis. Our results also showed that baicalein induced an increase in the levels of GADD153 and GRP78; both proteins have been proven to be associated with ER stress. We used confocal microscopy to demonstrate that baicalein promoted GADD153 and GRP78 expressions and that both were also translocated to the nuclei (Figure 8A and B). Apparently ROS caused ER stress and then Ca^{2+} was released from the ER. GADD153 was originally discovered as a gene that was induced to express in response to genotoxic stress. GADD153 was shown to mediate apoptosis induced by ER stress in pancreatic cells and by ischemic stress induced in brain neuronal cells in animal knockout models (33, 34). The overexpression of GADD153 in cultured macrophages and Cos-7 cells also resulted in apoptotic cell death (35). GADD153 was shown to induce the expression of the pro-apoptotic Bcl protein, Bak, and the translocation of Bax from the cytosol to the mitochondria (36, 37), which may lead to the dysfunction of mitochondria.

Our results demonstrated that baicalein induced ROS production in MDA-MB-231 cells and these effects were dose-dependent. This is also in agreement with other reports indicating that baicalein may trigger an apoptotic death program through a ROS-mediated mitochondrial dysfunction pathway (38). Our results also indicated that baicalein induced caspase-3 activity and increased the level of caspase-3 in MDA-MB-231 cells. This is also in agreement with other studies demonstrating that baicalein-induced apoptosis also involved the activation of caspase-3 of CH27 human lung squamous carcinoma cells (39). Therefore, in order to investigate whether baicalein induced apoptosis through caspase-dependent and/or -

independent pathways, we used an inhibitor of caspase-3 and -9 (Ac-DEVD-CHO) which first led to the decrease of caspase-3 and then finally led to a decrease in the percentage of apoptosis, while it also led to an increase in the percentage of viable MDA-MB-231 cells (Figure 5B and C). Therefore, our results are consistent with those of others (32) who indicated baicalein-induced apoptosis in human lung cancer cells, not only through a mitochondrial pathway but also *via* the caspase-3-dependent pathway (activation of caspase-3) (39). However, our major findings are that baicalein also induced ER stress based on the expression and localization of GADD153 and GRP78.

In conclusion, baicalein may have chemopreventive benefits by enhancing cancer cell apoptosis. These data suggest that Ca^{2+} release, reduced Bcl-2 and increased Bax expression may lead to the disruption of the mitochondrial membrane potential, followed by cytochrome *c* release and the activation of caspases under baicalein treatment could be a possible mechanism of baicalein-caused apoptosis in MDA-MB-231 cells (Figure 9). Studies examining the mechanisms of baicalein in down-regulating Bcl-2 and in DNA damage in breast cancer cells *in vitro* and *in vivo* are ongoing in our laboratory.

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