

# Effect of bis(hydroxymethyl) alkanoate curcuminoid derivative MTH-3 on cell cycle arrest, apoptotic and autophagic pathway in triple-negative breast adenocarcinoma MDA-MB-231 cells: An *in vitro* study

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**Abstract.** Curcumin has been shown to exert potential antitumor activity *in vitro* and *in vivo* involved in multiple signaling pathways. However, the application of curcumin is still limited because of its poor hydrophilicity and low bio-availability. In the present study, we investigated the therapeutic effects of a novel and water soluble bis(hydroxymethyl) alkanoate curcuminoid derivative, MTH-3, on human breast adenocarcinoma MDA-MB-231 cells. This study investigated the effect of MTH-3 on cell viability, cell cycle and induction of autophagy and apoptosis in MDA-MB-231 cells. After 24-h treatment with MTH-3, a concentration-dependent decrease in MDA-MB-231 cell viability was observed, and the IC<sub>50</sub> value was 5.37±1.22 μM. MTH-3 significantly triggered G<sub>2</sub>/M phase arrest and apoptosis in MDA-MB-231 cells. Within a 24-h treatment, MTH-3 decreased the CDK1 activity by decreasing CDK1 and cyclin B1 protein levels. MTH-3-induced apoptosis was further confirmed by morphological assessment and Annexin V/PI staining assay. Induction of apoptosis caused by MTH-3 was accompanied by an apparent increase of DR3, DR5 and FADD and, as well as a marked decrease of Bcl-2 and Bcl-xL protein expression. MTH-3 also decreased the protein

levels of Erol, PDI, PERK and calnexin, as well as increased the expression of IRE1α, CHOP and Bip that consequently led to ER stress and MDA-MB-231 cell apoptosis. In addition, MTH-3-treated cells were involved in the autophagic process and cleavage of LC3B was observed. MTH-3 enhanced the protein levels of LC3B, Atg5, Atg7, Atg12, p62 and Beclin-1 in MDA-MB-231 cells. Finally, DNA microarray was carried out to investigate the level changes of gene expression modulated by MTH-3 in MDA-MB-231 cells. Taken together, our results suggest that MTH-3 might be a novel therapeutic agent for the treatment of triple-negative breast cancer in the near future.

## Introduction

Breast cancer is the second leading cause of death in women and has approximately 1 million new cases per year worldwide (1,2). Breast cancer patients develop metastasis eventually leading to poor prognosis (3). Triple-negative breast cancer (TNBC) accounts for 12-20% of all breast cancer (4). It has more aggressive disease progress and worse prognosis (5). TNBC characteristics are the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and the lack of over-expression of HER-2 (4,6). TNBC is resistance to anti-hormone therapies and HER-2-aiming target therapies (7,8). Treatment of TNBC remains a great clinical challenge because of the lack of targeting agents and limited therapeutic options (8,9).

Curcumin has been used in traditional Chinese medicine for a long time in Taiwan, China and India (10). The pharmacological effects of curcumin include anti-amyloid (11), anti-bacterial (12), anti-depressant (13), anti-inflammatory (14), anti-oxidant (15), anti-diabetes (16) and anticancer properties (17,18). In addition, curcumin has been found to affect several anticancer signaling pathways such as inhibition of cancer cell proliferation (19,20) and induction of cell cycle arrest (21), apoptosis (22) or autophagy (23). Specifically,

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**Key words:** bis(hydroxymethyl) alkanoate curcuminoid derivative, MTH-3, apoptosis, autophagy, breast cancer MDA-MB-231 cells

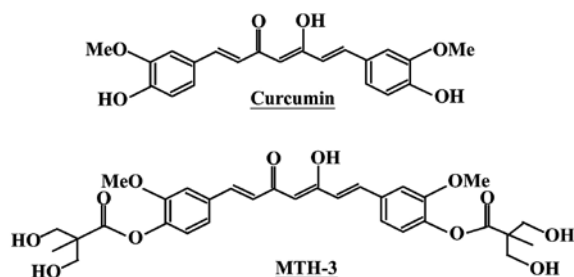


Figure 1. The chemical structures of curcumin (upper panel) and MTH-3 (bottom panel).

the phase II and III clinical trial of curcumin was advocated for use in patients with colon and pancreatic cancers (24,25), but its low water solubility exerts poor bioavailability and primary limiting factors (low efficacy and safety) (26,27). To improve these issues, we designed and developed a novel bis(hydroxymethyl) alkanooate curcuminoid derivative, MTH-3 (Fig. 1). In our previous studies, novel bis(hydroxymethyl) alkanooate curcuminoid derivatives were shown to exhibit antitumor effects on triple-negative breast cancer cells and in a xenograft animal experiment (28). The aim of the present study was to characterize the property of MTH-3 and to clarify the molecular mechanism of MTH-3 in human breast adenocarcinoma MDA-MB-231 cells *in vitro*.

## Materials and methods

**Chemicals and reagents.** MTH-3 was synthesized as previously described (28) (patent pending). The purity of MTH-3 is 98.7, and its molecular weight is 600.61. Leibovitz's L-15 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, Premo Autophagy Sensor LC3B-GFP (BacMam 2.0) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

**Cell culture.** The human breast adenocarcinoma cell line MDA-MB-231 was purchased from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan). Cells were cultured in Leibovitz's L-15 medium with 10% FBS and 1% penicillin-streptomycin (100 Units/ml penicillin and 100 µg/ml streptomycin) in an incubator under 95% air and 5% CO<sub>2</sub> at 37°C.

**Cell viability assay and morphologic changes.** Cell viability was evaluated by the reduction in MTT to yield blue formazan. MDA-MB-231 cells (1×10<sup>4</sup> cells/well) in 96-well plates were allowed to attach overnight and then treated with different concentrations (1, 3, 5 and 10 µM) of MTH-3 for 24 h. After treatments, MTT solution was added to each well (a final concentration of 0.5 µg/ml), and then the plates were incubated for another 4 h. The medium was removed, blue formazan was dissolved in dimethyl sulfoxide (DMSO), and the absorbance was read at 570 nm as previously described (29). For trypan blue exclusion assay, cells were collected after 1, 3, 5 and 10 µM of MTH-3 exposure, stained with 0.4% trypan blue and then counted on a hemocytometer under a microscope.

For morphological observation, cells were visualized and photographed using a phase-contrast microscope equipped with a digital camera (Leica Microsystems GmbH, Wetzlar, Germany) as in previous reports (26,30).

**Distribution of cell cycle analysis.** MDA-MB-231 cells (2×10<sup>5</sup> cells/well) in 12-well plates were exposed to 10 µM MTH-3. After a 24-h treatment, cells were harvested and fixed gently by putting 70% ethanol at 4°C overnight before being stained with PI solution (40 µg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100) in the dark for 30 min as previously described (31). The cells were analyzed for the cell cycle distribution with a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA).

**CDK1 kinase assay.** CDK1 kinase activity was analyzed according to the manufacturer's protocol (CycLex Cdc2-Cyclin B Kinase Assay kit; MBL International Corp., Woburn, MA, USA). The ability of CDK1 kinase from MDA-MB-231 cell extracts prepared from each treatment of 10 µM MTH-3 for 4, 8, 16 and 24 h was measured as previously described (32,33).

**Apoptosis analysis.** MDA-MB-231 cells (2×10<sup>5</sup> cells/well) into 12-well plates were incubated in the presence and absence of 10 µM MTH-3 for 24 and 48 h. Subsequently, cells were harvested and stained with Annexin V and propidium iodide (PI) using the Annexin V-FITC apoptosis detection kit (BD Biosciences, San Diego, CA, USA) and subjected to flow cytometry (BD FACSCalibur; BD Biosciences). The percentage of apoptotic cells were quantified with BD CellQuest Pro software (BD Biosciences) (34,35).

**Cells lysate preparation and western blot analysis.** After 10 µM MTH-3 treatments at indicated intervals of time, MDA-MB-231 cells were harvested, washed and suspended in the PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Gyeonggi-do, Korea). Protein concentrations were estimated using the Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The samples were resolved with SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF) (EMD Millipore, Billerica, MA, USA). Each membrane was blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 for 1 h followed by individual incubation with specific primary antibodies [cyclin B1 (cat. no. 4138, 1:1,000), CDK1/Cdc2 (cat. no. 9116, 1:1,000), DR3 (cat. no. 4758, 1:1,000), DR5 (cat. no. 8074, 1:1,000), FADD (cat. no. 2782, 1:1,000), Bcl-2 (cat. no. 4223, 1:1,000), Bcl-xL (cat. no. 2764, 1:1,000), Erol (cat. no. 3264, 1:1,000), PDI (cat. no. 3501, 1:1,000), PERK (cat. no. 5683, 1:1,000), calnexin (cat. no. 2679, 1:1,000), IRE1α (cat. no. 3294, 1:1,000), CHOP (cat. no. 2895, 1:1,000), Bip (cat. no. 3177, 1:1,000), Atg5 (cat. no. 12994, 1:1,000), Atg7 (cat. no. 8558, 1:1,000), Atg12 (cat. no. 4180, 1:1,000), Beclin-1 (cat. no. 3495, 1:1,000), p62 (cat. no. 88588, 1:1,000), LC3A/B (cat. no. 12741, 1:1,000) and β-actin (cat. no. 3700, 1:5,000) (Cell Signaling Technology, Danvers, MA, USA)] at 4°C overnight. Each membrane was then incubated with anti-rabbit IgG (cat. no. 7074, 1:10,000) or anti-mouse IgG (cat. no. 7076, 1:10,000) horseradish peroxidase (HRP)-linked antibodies (Cell Signaling Technology) at room temperature for 1 h. The signal was detected with the Immobilon Western Chemiluminescent

HRP substrate (EMD Millipore) and visualized using the LAS 4000 imaging system (Fuji, Tokyo, Japan) as previously described (36-38). The quantitative densitometric analysis of immunoreactive band was employed by ImageJ bundled with 64-bit Java 1.6.0\_24 program for Windows from the National Institutes of Health (NIH; Bethesda, MD, USA).

**Immunofluorescence staining.** MDA-MB-231 cells ( $2 \times 10^6$  cells/dish) were grown on sterile coverslips placed in a 10-cm dish. After 10  $\mu$ M MTH-3 treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS). After blocking with 2% bovine serum albumin (BSA) in PBS, LC3B and p62 were detected using anti-LC3B and anti-p62 antibody followed by reaction with FITC- or PE-conjugated secondary antibody (BD Biosciences). Coverslips were mounted on glass slides with ProLong Gold Antifade reagents (Thermo Fisher Scientific) containing DAPI, and fluorescent image was taken on a Leica Microsystems TCS SP2 Confocal Spectral microscope as detailed by Lu *et al* (39).

**cDNA microarray analysis.** MDA-MB-231 cells were incubated with or without 10  $\mu$ M MTH-3 for 24 h. After exposure, cell pellets were collected, and the total RNA from each treatment was purified using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA purity was determined to check the quality at 260/280 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). mRNA was amplified and labeled using the GeneChip WT Sense Target Labeling and Control Reagents kit (Affymetrix, Santa Clara, CA, USA) for expression analysis. The synthesized cDNA was labeled with fluorescence and then hybridized for 17 h using GeneChip Human Gene 1.0 ST array (Affymetrix) to determine microarray hybridization following the manufacturer's protocols. The arrays were subsequently washed using GeneChip Fluidics Station 450 (Affymetrix), stained with streptavidin-phycoerythrin (GeneChip Hybridization, Wash and Stain kit; Affymetrix) and scanned on a GeneChip Scanner 3000 (Affymetrix). The localized concentrations of fluorescent molecules were quantitated and analyzed using Expression Console Software (Affymetrix) with default RMA parameters as previously described (40). The gene expression level of a 2.5-fold change ( $\log_2$  ratio) was considered a difference in MTH-3-treated cells *in vitro* (41,42).

**Statistical analysis.** Data are presented as the mean  $\pm$  SD for three separate experiment. Differences among the groups were considered to be significant at  $P < 0.05$  using ANOVA followed by the Duncan's test.

## Results

**MTH-3 inhibits cell proliferation of human breast adenocarcinoma MDA-MB-231 cells.** At first, the effect of MTH-3 on the viability of MDA-MB-231 cells was investigated using the MTT and trypan blue exclusion assays. MTH-3 at 1, 3, 5 and 10  $\mu$ M significantly reduced the viability of MDA-MB-231 cells by  $98.94 \pm 2.26$ ,  $89.57 \pm 2.07$ ,  $69.57 \pm 4.13$  and  $59.6 \pm 4.04\%$ , respectively (Fig. 2A). Importantly, the cell viability reduction after 30  $\mu$ M MTH-3 challenge is  $34.23 \pm 3.31\%$ . This effect is in

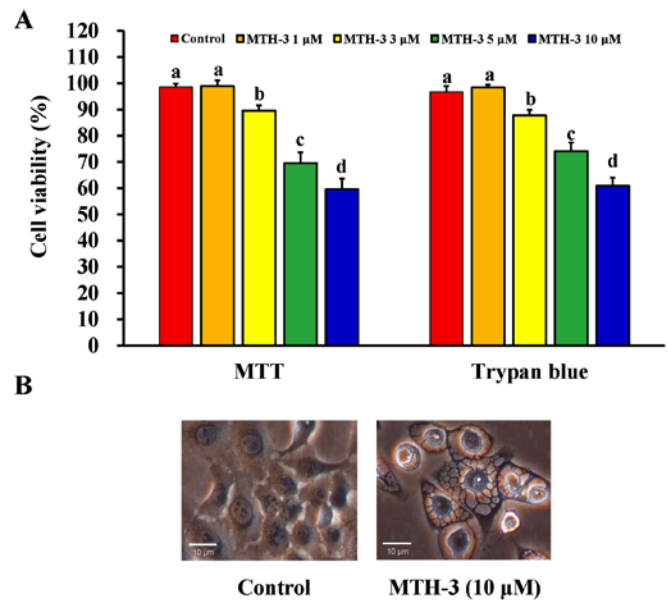


Figure 2. MTH-3 reduces cell viability and affects cell morphology in MDA-MB-231 cells. (A) Cells were incubated with or without various concentrations (1, 3, 5 and 10  $\mu$ M) of MTH-3 for 24 h, and the viable cells were monitored using an MTT and trypan blue exclusion methods. Data are presented as the mean  $\pm$  SD of three independent experiments. The different letters (a-d) show statistically significant differences ( $P < 0.05$ ) in each group by the Duncan's test. (B) Cells were examined after with or without 10  $\mu$ M MTH-3 for 24 h to photograph the changes in cell morphology using a phase-contrast microscope as described in Materials and methods. Scale bar, 10  $\mu$ m.

a concentration-dependent manner. Data from morphological observation revealed that MTH-3 treatment at 10  $\mu$ M caused obvious MDA-MB-231 cell apoptosis and autophagy with characteristics, including cytoplasmic membrane blebbing, cell shrinkage and autophagic vacuoles (Fig. 2B). Based on these findings and gaining effective evidence of cell death, we selected MTH-3 at 10  $\mu$ M for the majority of the experiments in MDA-MB-231 cells.

**MTH-3 triggers  $G_2/M$  phase arrest and reduces CDK1 activity in MDA-MB-231 cells.** To investigate the cell cycle distribution of treated and untreated MDA-MB-231 cells, cells were monitored after 10  $\mu$ M MTH-3 challenge. Results from flow cytometric analysis showed that MTH-3 treatment of MDA-MB-231 cells significantly increased  $G_2/M$  phase cell population at 24 h (Fig. 3A). The effects of MTH-3 on  $G_2/M$  phase-related proteins in MDA-MB-231 cells were investigated. Our results showed that MTH-3 effectively down-regulated the levels of cyclin B1 and CDK1 (Fig. 3B). We also tested the CDK1 kinase activity in MDA-MB-231 cells prior to MTH-3 treatment. MTH-3 markedly reduced CDK1 kinase activity at 4, 8, 12 and 24 h of treatment, respectively (Fig. 3C). Therefore, the finding showed that downregulation of CDK1 activity contributed to  $G_2/M$  phase arrest caused by MTH-3 in MDA-MB-231 cells.

**MTH-3 elicits cell apoptosis of MDA-MB-231 cells.** To further explore whether the inhibition of cell viability results from the induction of apoptosis in MDA-MB-231 cells, MTH-3-treated cells were detected with Annexin V/PI double staining (Fig. 4). Treatment with 10  $\mu$ M MTH-3 for 48 h significantly increased the population of Annexin V-positive cells (Fig. 4),

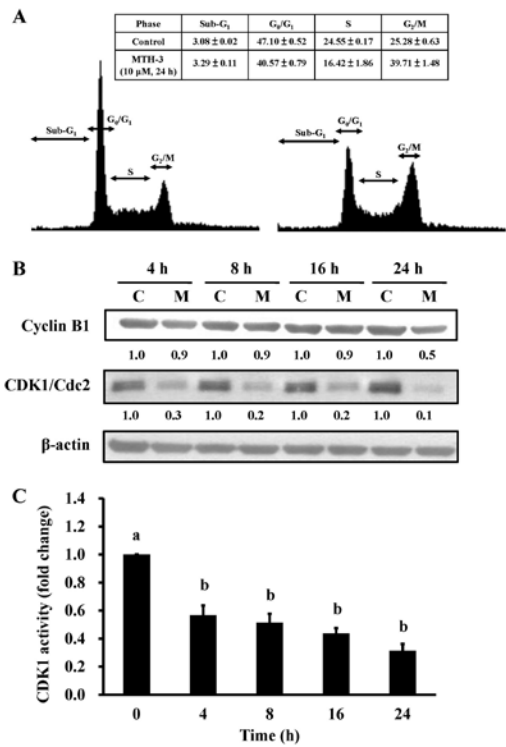


Figure 3. MTH-3 induces G<sub>2</sub>/M phase arrest of MDA-MB-231 cells. (A) Cells were exposed to 10 μM MTH-3 for 24 h. The cell cycle distribution was detected using flow cytometric analysis and cell cycle distribution was quantified. (B) Cells were exposed to 10 μM MTH-3 and then incubated for 0, 4, 8, 16 and 24 h. The protein levels of cyclin B1, CDK1 and β-actin were determined by western blotting. C, control; M, MTH-3 exposure. (C) CDK1 activity was examined as described in Materials and methods. Data are presented as the mean ± SD of three independent experiments. The different letters (a-b) show statistically significant differences (P<0.05) in each group by the Duncan's test.

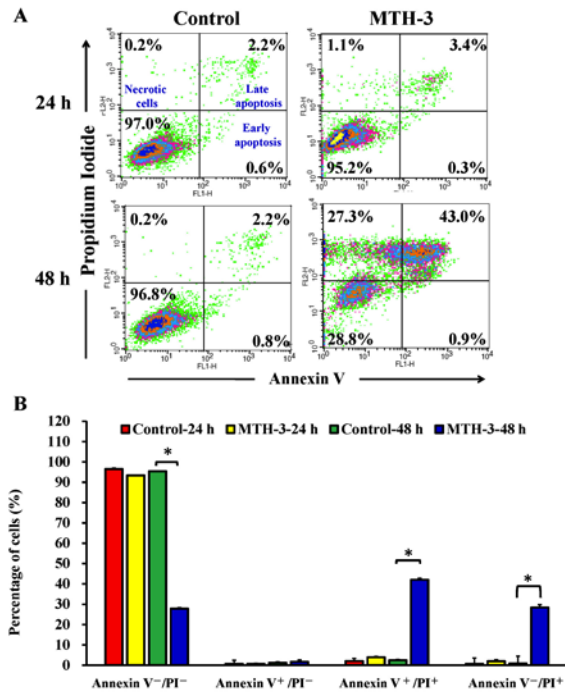


Figure 4. MTH-3 induces apoptosis of MDA-MB-231 cells. Cells were incubated with 10 μM MTH-3 for 24 and 48 h. Cells were collected and stained with Annexin V/propidium iodide (PI) before analysis with flow cytometry. The Annexin V-positive cells were counted, and data are presented as the mean ± SD of three independent experiments. \*P<0.05 indicates statistically significant differences by the Duncan's test.

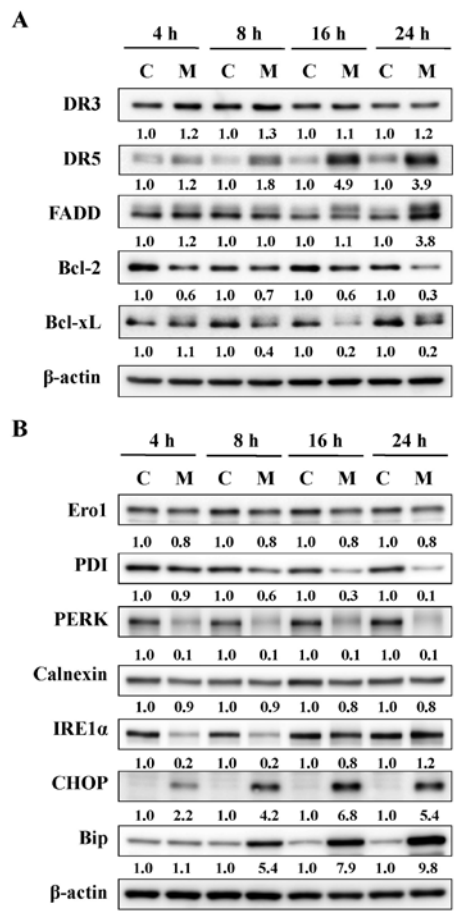


Figure 5. MTH-3 activates death receptor-mediated, mitochondrial and ER stress-regulated apoptosis pathways in MDA-MB-231 cells. Cells were exposed to 10 μM MTH-3 for 0, 4, 8, 16 and 24 h, and cell lysates were collected for western blot analysis. (A) Death receptor-mediated (DR3, DR5 and FADD) and mitochondrial (Bcl-2 and Bcl-xL) apoptosis pathways, and (B) ER stress (Ero1, PDI, PERK, calnexin, IRE1α, CHOP and Bip) were performed. β-actin served as an internal control. C, control; M, MTH-3 exposure.

indicating that MTH-3 induced apoptosis in MDA-MB-231 cells. However, the necrotic cells (Annexin V<sup>+</sup>/PI<sup>+</sup>) increased rapidly after 48 h of 10 μM MTH-3 exposure.

*MTH-3 activates death receptor, mitochondrial and ER stress-mediated apoptotic pathways in MDA-MB-231 cells.* The effects of MTH-3 on apoptosis-related proteins in MDA-MB-231 cells were investigated. Our results demonstrated that MTH-3 upregulated the levels of DR5 and FADD, and it downregulated the levels of Bcl-2 and Bcl-xL (Fig. 5A). Furthermore, our findings also revealed that MTH-3 markedly increased the levels of CHOP and Bip, as well as decreased the levels of Ero1, PDI, PERK, calnexin and IRE1α (Fig. 5B). These results suggest that MTH-3 induced apoptosis through death receptor (extrinsic pathway) and mitochondria (intrinsic pathway)-dependent pathways and possibly by modulating ER stress mechanism in MDA-MB-231 cells.

*MTH-3 stimulates autophagy in MDA-MB-231 cells.* To confirm if autophagy is involved in the inhibition of MDA-MB-231 cell viability, cells with or without MTH-3 exposure were detected with LC3B and p62 double immunostaining. MTH-3 at 10 μM increased the LC3B (FITC; green



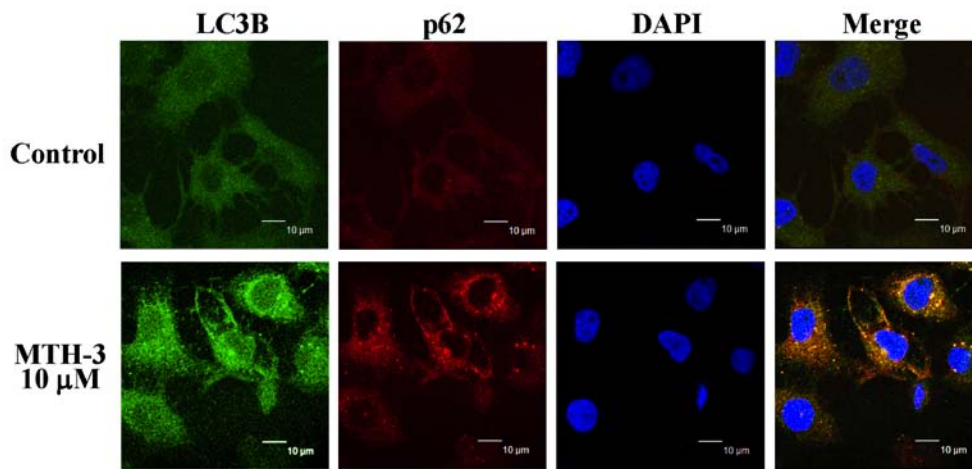


Figure 6. MTH-3 induces LC3B and p62 expression of MDA-MB-231 cells. Cells were treated with 10  $\mu$ M MTH-3 for 24 h. Cells were collected and stained with LC3B-FITC antibody (green color) and p62-PE antibody (red color) and analyzed with confocal microscope. DAPI dye (blue color) is for nuclear acid (nuclear) staining. Scale bar, 10  $\mu$ m.

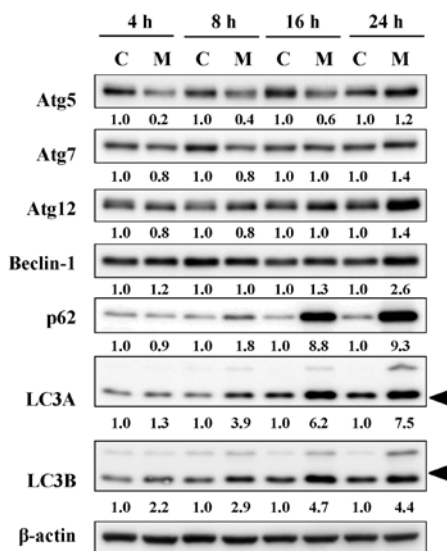


Figure 7. MTH-3 alters the protein levels of autophagy-related proteins in MDA-MB-231 cells. Cells were incubated with 10  $\mu$ M MTH-3 for 4, 8, 16 and 24 h, and cell lysates were collected for western blot analysis to probe autophagic signals (Atg5, Atg7, Atg12, Beclin-1, p62, LC3A and LC3B).  $\beta$ -actin was an internal control. C, control; M, MTH-3 exposure.

color) and p62 (PE; red color) protein expression (Fig. 6), indicating that MTH-3 induced autophagy through increasing LC3B/p62 signaling in MDA-MB-231 cells.

**MTH-3 alters the levels of autophagy-associated proteins in MDA-MB-231 cells.** Based on the results of autophagy, its related signals were further employed by immunoblotting analysis. MTH-3 treatment induced the levels of Atg5, Atg7, Atg12, Beclin-1, p62 and LC3B in a time-dependent manner (Fig. 7). These data demonstrated that MTH-3 induced autophagy by activating Atg family proteins in MDA-MB-231 cells.

**MTH-3 modulates cell death-related gene expression in MDA-MB-231 cells by cDNA microarray analysis.** After treatment with 10  $\mu$ M MTH-3 for 24 h, cells were collected,

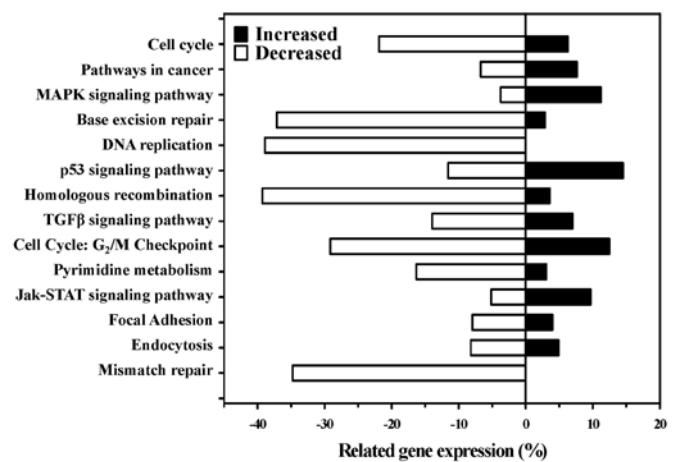


Figure 8. The possible canonical pathways from MDA-MB-231 cells after exposure to MTH-3 by cDNA microarray. Cells were treated with 10  $\mu$ M MTH-3 for 24 h, and then were harvested before total RNA was extracted for cDNA microarray assay. The changes in gene expression scored by the number of pathways from GeneGo analysis.

and cDNA microarray analysis was performed. The analysis showed that 97 genes (69 genes, upregulated; 28 genes, down-regulated) were expressed at least by 2.5-fold compared with the untreated control (Table I). The top alteration in gene expression scored by the number of pathway networks from GeneGo analysis program (Fig. 8). These genes may also be involved in cell death and cytotoxic responses in MTH-3-treated MDA-MB-231 cells.

## Discussion

Previous studies have demonstrated the anticancer potential of curcumin in regulating cell cycle, autophagy, apoptosis and survival, proliferation, angiogenesis, invasion and metastasis (19-23). Guan *et al* (43) demonstrated that curcumin reduced Akt kinase in MDA-MB-231 cells accompanied by a decrease in cell proliferation and migration as well as an increase in autophagic activity; moreover, AMPK-mediated activation

Table I. The >2.5-fold changes in mRNA levels in MDA-MB-231 cells following a 24-h treatment with 10  $\mu$ M MTH-3 as identified using DNA microarray.

ID	log2 (ratio)	Gene_symbol	Description
PH_hs_0049600	6.643856	HSPA6	Heat shock 70 kDa protein 6 (HSP70B')
PH_hs_0006387	6.274261	ZFAND2A	zinc finger, AN1-type domain 2A
PH_hs_0004421	5.381376	PPP1R15A	Protein phosphatase 1, regulatory subunit 15A
PH_hs_0000305	4.941673	MMP10	Matrix metalloproteinase 10 (stromelysin 2)
PH_hs_0046245	4.763129	RN7SK	RNA, 7SK small nuclear
PH_hs_0000076	4.587356	IL12A	Interleukin 12A
PH_hs_0027902	4.286664	ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2
PH_hs_0010276	4.189167	DUSP1	Dual specificity phosphatase 1
PH_hs_0031719	4.146525	CCL26	Chemokine (C-C motif) ligand 26
PH_hs_0000156	4.093858	DUSP2	Dual specificity phosphatase 2
PH_hs_0011943	4.063702	HMOX1	Heme oxygenase (decycling) 1
PH_hs_0045501	4.039442	EID3	EP300 interacting inhibitor of differentiation 3
PH_hs_0004561	3.997336	GEM	GTP binding protein overexpressed in skeletal muscle
PH_hs_0042334	3.931415	MT4	Metallothionein 4
PH_hs_0048553	3.866096	MYCT1	myc target 1
PH_hs_0000684	3.853854	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9
PH_hs_0035404	3.763571	SAT1	Spermidine/spermine N1-acetyltransferase 1
PH_hs_0000057	3.698185	ATF3	Activating transcription factor 3
PH_hs_0025319	3.562429	C3orf52	Chromosome 3 open reading frame 52
PH_hs_0033101	3.555868	DDIT3	DNA-damage-inducible transcript 3 (CHOP)
PH_hs_0002700	3.513438	OSGIN1	Oxidative stress induced growth inhibitor 1
PH_hs_0037472	3.480422	MALAT1	Metastasis associated lung adenocarcinoma transcript 1
PH_hs_0035765	3.427173	GDF15	Growth differentiation factor 15
PH_hs_0002492	3.366024	SAT1	Spermidine/spermine N1-acetyltransferase 1
PH_hs_0062199	3.356707	AKR1C1 LOC101060798	Aldo-keto reductase family 1, member C1 aldo-keto reductase family 1 member C2-like
PH_hs_0000852	3.324182	SESN2	Sestrin 2
PH_hs_0023008	3.242113	FRS2	Fibroblast growth factor receptor substrate 2
PH_hs_0004751	3.219326	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)
PH_hs_0031143	3.213328	VIMP	VCP-interacting membrane protein
PH_hs_0025525	3.198476	CLU	Clusterin
PH_hs_0024315	3.075314	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4
PH_hs_0035614	3.062771	RC3H1	Ring finger and CCCH-type domains 1
PH_hs_0027152	3.037995	RMND5A	Required for meiotic nuclear division 5 homolog A ( <i>S. cerevisiae</i> )
PH_hs_0021974	3.010862	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3
PH_hs_0061784	2.967357	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
PH_hs_0035466	2.962064	AKR1C3 AKR1C1	Aldo-keto reductase family 1, member C3 aldo-keto reductase family 1, member C1
PH_hs_0027162	2.960759	SLC3A2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
PH_hs_0022919	2.960552	CLCF1	Cardiotrophin-like cytokine factor 1
PH_hs_0000255	2.916655	SRGN	Serglycin
PH_hs_0024155	2.904033	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
PH_hs_0043719	2.894684	HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)
PH_hs_0045838	2.838192	SLC6A6	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6

Table I. Continued.

ID	log2 (ratio)	Gene_symbol	Description
PH_hs_0014155	2.836392	HSPA1B	Heat shock 70 kDa protein 1B
PH_hs_0044272	2.829317	CLK1	CDC-like kinase 1
PH_hs_0048881	2.809371	FKBP4	FK506 binding protein 4, 59 kDa
PH_hs_0020147	2.803912	CLK1	CDC-like kinase 1
PH_hs_0028987	2.768552	TCF21	Transcription factor 21
PH_hs_0042409	2.76703	DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1
PH_hs_0001262	2.748306	SENP5	SUMO1/sentrin specific peptidase 5
PH_hs_0060828	2.734692	TRIB3	Tribbles homolog 3 ( <i>Drosophila</i> )
PH_hs_0023556	2.733421	C21orf91	Chromosome 21 open reading frame 91
PH_hs_0061012	2.731293	ZBTB21	Zinc finger and BTB domain containing 21
PH_hs_0029660	2.695316	AKR1C1	Aldo-keto reductase family 1, member C1aldo-keto reductase family 1
PH_hs_0037242	2.683231	MALAT1	Metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)
PH_hs_0002812	2.667718	C18orf25	Chromosome 18 open reading frame 25
PH_hs_0027209	2.665362	GADD45B	Growth arrest and DNA-damage-inducible, $\beta$
PH_hs_0002971	2.664712	ZNF77	Zinc finger protein 77
PH_hs_0003180	2.646292	SMIM13	Small integral membrane protein 13
PH_hs_0000694	2.625719	RND3	Rho family GTPase 3
PH_hs_0023711	2.599232	HSPA5	Heat shock 70 kDa protein 5
PH_hs_0023894	2.583817	TRIB3	Tribbles homolog 3 ( <i>Drosophila</i> )
PH_hs_0060053	2.574976	ZNF121	Zinc finger protein 121
PH_hs_0014119	2.571605	BRF2	BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like
PH_hs_0033027	2.547837	SIK1	Salt-inducible kinase 1
PH_hs_0024236	2.547678	ATP2A2	ATPase, Ca <sup>++</sup> transporting, cardiac muscle, slow twitch 2
PH_hs_0042225	2.541029	DUSP5	Dual specificity phosphatase 5
PH_hs_0044921	2.534876	HSPA1A	Heat shock 70 kDa protein 1A
PH_hs_0000566	2.528881	SLC25A25	Solute carrier family 25, member 25
PH_hs_0030976	2.516291	NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, $\beta$
PH_hs_0014995	-3.653241	METTL7A	Methyltransferase like 7A
PH_hs_0023845	-3.269308	BBS2	Bardet-Biedl syndrome 2
PH_hs_0009437	-3.05235	TOP2A	Topoisomerase (DNA) II $\alpha$ 170 kDa
PH_hs_0047352	-3.043277	MARCKS	Myristoylated alanine-rich protein kinase C substrate
PH_hs_0047965	-2.959225	PHLDA1	Pleckstrin homology-like domain, family A, member 1
PH_hs_0040619	-2.891495	MXD3	MAX dimerization protein 3
PH_hs_0012629	-2.890238	H1FO	H1 histone family, member 0
PH_hs_0004988	-2.878231	LMNB1	Lamin B1
PH_hs_0035609	-2.788184	ETV1	Ets variant 1
PH_hs_0049449	-2.729758	GPR39	G protein-coupled receptor 39
PH_hs_0027843	-2.724437	FAM20C	Family with sequence similarity 20, member C
PH_hs_0027863	-2.718276	LRRC45	Leucine rich repeat containing 45
PH_hs_0007383	-2.717289	F2R	Coagulation factor II (thrombin) receptor
PH_hs_0036878	-2.71449	PIF1	PIF1 5'-to-3' DNA helicase homolog ( <i>S. cerevisiae</i> )
PH_hs_0047697	-2.688182	ARF6	ADP-ribosylation factor 6
PH_hs_0048993	-2.677322	NRP1	Neuropilin 1
PH_hs_0031540	-2.66121	GNG2	Guanine nucleotide binding protein (G protein), gamma 2

Table I. Continued.

ID	log2 (ratio)	Gene_symbol	Description
PH_hs_0010634	-2.659899	TXNIPLOC101060503	Thioredoxin interacting protein/thioredoxin-interacting protein-like
PH_hs_0028935	-2.621805	CCDC85B	Coiled-coil domain containing 85B
PH_hs_0000866	-2.612763	OMA1	OMA1 zinc metallopeptidase homolog ( <i>S. cerevisiae</i> )
PH_hs_0030800	-2.552826	FANCF	Fanconi anemia, complementation group F
PH_hs_0025966	-2.55207	CTDSP1	CTD small phosphatase 1
PH_hs_0023862	-2.551096	CBY1	Chibby homolog 1 ( <i>Drosophila</i> )
PH_hs_0047571	-2.546813	PDP1	Pyruvate dehydrogenase phosphatase catalytic subunit 1
PH_hs_0028200	-2.537288	CENPI	Centromere protein I
PH_hs_0003147	-2.533627	PDGFC	Platelet derived growth factor C
PH_hs_0035337	-2.514458	OMA1	OMA1 zinc metallopeptidase homolog ( <i>S. cerevisiae</i> )
PH_hs_0038982	-2.502536	LOC100134259	Uncharacterized LOC100134259

of autophagy contributes to anticancer effects through Akt degradation. In the present study, we also checked the growth inhibition effect of curcumin on MDA-MB-231 cells. Our data indicated that the half maximal inhibitory concentration (IC<sub>50</sub>) value of curcumin on MDA-MB-231 cells is 38.77±3.35  $\mu$ M. Strikingly, the IC<sub>50</sub> value of MTH-3 on MDA-MB-231 cells is 5.37±1.22  $\mu$ M (data not shown). Our results demonstrated that the MTH-3 had highly cytotoxic effects on MDA-MB-231 cells. Moreover, we also found that MTH-3 was non-cytotoxic on non-tumorigenic epithelial mammary MCF10A cells and human skin fibroblast Detroit 551 cells, respectively (data not shown). These are only preliminary data and further study is needed to validate the findings.

There are no reports regarding that the effects of MTH-3 on cell cycle arrest, autophagy and apoptosis and associated gene expression in human breast cancer cells. This study is first to demonstrate that MTH-3 induced cytotoxic effect on induction of G<sub>2</sub>/M phase arrest, autophagy and apoptosis in human breast adenocarcinoma MDA-MB-231 cells. The data demonstrated that MTH-3 induced growth inhibitory effects through G<sub>2</sub>/M phase arrest, apoptosis and autophagy in MDA-MB-231 cells. Our results showed that MTH-3 induced G<sub>2</sub>/M phase arrest through regulating cyclin B1 and CDK1 signaling. G<sub>2</sub>/M phase progression has been reported to regulate CDK1 and CDK2 kinases that are activated primarily in association with cyclins A and B (44). Furthermore, MTH-3 inhibited the CDK1 activity and the protein expression of CDK1 in MDA-MB-231 cells. However, neither effect is positively correlated because CDK1 activity might be involved in kinase activation rather than CDK1/cdc2 protein level (32,33). Previous studies also demonstrated that curcumin inhibited cell proliferation through induction of G<sub>0</sub>/G<sub>1</sub> phase arrest of cancer cells (45,46), but our finding indicated that MTH-3 induced G<sub>2</sub>/M phase arrest upon different types of cancer cell lines. However, the results are in agreement with previous studies to show that curcumin inhibited cell proliferation by inducing G<sub>2</sub>/M phase arrest in human glioblastoma U87 cells (47) and in Bcl-2 overexpressed MCF-7 cells (48). Further research is required to verify the mechanism of MTH-3 action in different breast cancer cell lines (such as MCF-7 and MDA-MB-453 cells).

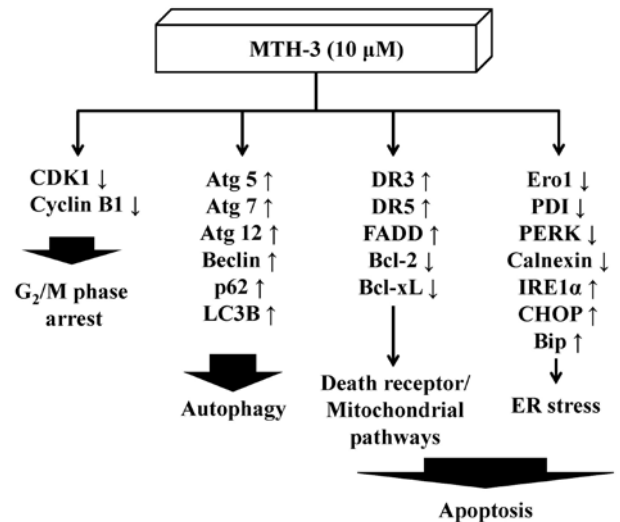


Figure 9. The proposed model shows that MTH-3 induces G<sub>2</sub>/M phase arrest, autophagy and apoptotic cell death (death receptor/mitochondrial pathways and ER stress) in human breast adenocarcinoma MDA-MB-231 cells.

It is well documented that apoptosis plays an important role in the maintenance of tissue homeostasis for the elimination of excessive cells (49,50). Induction of apoptosis of cancer cells by anticancer drugs such as etoposide, cisplatin and paclitaxel have been used for treatment of cancer in target cells (51). Apoptosis-associated signaling pathways include extrinsic (death receptor), intrinsic (mitochondria-dependent) and ER stress (unfolded protein response) signals (52,53). Our results demonstrated that MTH-3 promoted the protein levels of DR5, and FADD and downregulated the levels of Bcl-2 and Bcl-xL in MDA-MB-231 cells. MTH-3 also promoted the protein levels of CHOP and Bip, and it reduced the levels of Ero1, PDI, PERK, calnexin and IRE1 $\alpha$  in MDA-MB-231 cells. Our novel findings suggest that both extrinsic and intrinsic pathways, and ER stress signals were involved in MTH-3-treated cells *in vitro*. This agrees with a previous study reporting that the major targets of apoptotic initiation are mediated by dysfunction of cellular organelles (mitochondria, ER, lysosomes and golgi apparatus) (54).



Autophagy is another major clearance route for intracellular protein (55). Recently, curcumin can induce autophagy in cancer cells (56,57). Our results showed that MTH-3 significantly increased protein expression of autophagy markers LC3B, Atg complex (Atg5, Atg7 and Atg12) and Beclin-1, as well as GFP-LC3 puncta formation, suggesting that LC3B was recruited to the autophagosomal membrane during autophagosome formation. Our data strongly suggest that MTH-3 activated autophagy in MDA-MB-231 cells.

From gene expression profiles by DNA microarray, we found that cellular and molecular responses to MTH-3 treatment are multi-faceted and mediated by various regulatory pathways in MDA-MB-231 cells. MTH-3 regulated the expression of important genes in cell cycle, pathways in cancer, MAPK signaling, base excision repair, DNA replication, p53 signaling, homologous recombination, TGF- $\beta$  signaling, G<sub>2</sub>/M checkpoint, pyrimidine metabolism, Jak-STAT signaling, focal adhesion, endocytosis and mismatch repair pathways. The gene regulation may be responsible for inhibiting the proliferation of MDA-MB-23 cells. Cyclins associate with cyclin-dependent protein kinases (CDKs) and CDK inhibitor (CKI) can control the procedure of cell cycle to arrest the cell cycle and inhibit the cell growth of cancer cells (44,58). Our results from gene expression profiles indicated that MTH-3 changed the expression of cyclin and cyclin-dependent kinase inhibitor gene *CDKN1A*, suggesting a change in cyclin, cyclin-dependent kinase inhibitors which could finally lead to cell cycle G<sub>2</sub>/M phase arrest.

Heme oxygenase-1 (HO-1) has been implicated in cellular defense against oxidative stress and has anti-inflammation function (59,60). A recent study has demonstrated that curcumin inhibits apoptosis-induced apoptosis by upregulating HO-1 expression in SH-SY5Y cells (61). Curcumin-induced HO-1 expression also prevents H<sub>2</sub>O<sub>2</sub>-induced cell death in wild-type and HO-2 knockout adipose-derived mesenchymal stem cells (62). In this study of the gene expression profiles, MTH-3 upregulated the expression of heme oxygenase 1 (*HMOX1*) gene, suggesting that MTH-3 might have anti-inflammation and cell protection function.

In conclusion, the molecular signaling pathways are summarized in Fig. 9. This study is the first report to provide an approach regarding the bis(hydroxymethyl) alkanolate curcuminoid derivative, MTH-3 tends to inhibit human breast adenocarcinoma MDA-MB-231 cells. Based on the presented novel findings, the efficacy of MTH-3 might be sufficient to further investigate the potential of breast cancer treatment.

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