Abstract. Allyl isothiocyanate (AITC), a member of the isothiocyanate (ITC) family found in a constituent of cruciferous vegetables, possesses anticancer activity and induces apoptosis in various types of human cancer cell lines. However, no available information showed antitumor effects in human breast adenocarcinoma cells. The current study was focused on exploring the mechanisms underlying AITC-induced apoptosis in MDA-MB-468 human breast cancer cells in vitro. We found that AITC reduced the cell number and viability using trypan blue staining with the Countess Automated Cell Counter and the MTT assay, respectively. AITC also was found to induce apoptotic cell morphological changes by a contrast-phase microscope and cell cycle arrest at G2/M phase by flow cytometric assay in MDA-MB-468 cells. Intrinsic apoptosis-associated factors such as caspase-9 and caspase-3 activities were performed, and reactive oxygen species (ROS) production, loss of mitochondrial membrane potential (ΔΨm) occurred in AITC-treated MDA-MB-468 cells. AITC also stimulated mitochondria-related signaling, including p-Bcl-2 (Ser-70), cytochrome c and Apaf-1 in MDA-MB-468 cells. We found that the p-ERK signal was upregulated in AITC-treated cells. Importantly, NAC (a ROS scavenger) and U0126 (an ERK inhibitor) abolished AITC-reduced viability in MDA-MB-468 cells. AITC downregulated CDK1 activity and altered the expression of G2/M phase-modulated associated protein levels by western blotting in MDA-MB-468 cells. In summary, our findings demonstrated that AITC-promoted G2/M phase and AITC-triggered apoptosis correlate with the activation of phosphorylation of ERK in MDA-MB-468 cells. AITC is a potential agent for application in the treatment of human breast cancer.

Introduction

Allyl isothiocyanate (AITC) is a compound of the natural isothiocyanates found in cruciferous vegetables such as brussels sprouts, cauliflower, cabbage, kale, horseradish and wasabi (1-4). AITC is known to have multiple effects such as antipathogenic bacteria (5), anti-inflammatory (6), antifungicidal (7) and anticancer activities (4). Previous studies have demonstrated that AITC exhibits significant antitumor activities against human prostate (8), colorectal (9,10), bladder (11,12), cervical cancer cells (13) and leukemia cells (14). The anticancer activities by AITC are involved in the induction of cell cycle arrest and apoptosis as well as inhibition of cell metastasis (15-17). Our previous study demonstrated that AITC triggers G2/M phase arrest and apoptosis in human brain malignant glioma GBM 8401 cells (16). However, there is no report addressing whether or not AITC inhibits cell proliferation, promotes cell cycle arrest and induces apoptosis in human breast adenocarcinoma cells.

Breast cancer is one of the leading causes of death in women worldwide (18). According to statistical results from GLOBOCAN in 2008 year, about 1.38 million new patients were diagnosed in breast cancer, and 458,400 people died from breast cancer in the worldwide (19). In Taiwan, 14.8 per 100,000 women die from breast cancer each year according to the Department of Health in 2010. Breast cancer cells that lack of estrogen receptor (ER), progesterone receptor (PR), and human EGF receptor 2 (HER-2/neu) expressions are known as
triple negative breast cancer (TNBC) (20,21). TNBC is the most clinically invasive breast cancer and the characteristics are more invasive, less responsive to chemotherapy agents and associated with poorer prognosis (22). The current therapy for TNBC includes surgery and systemic chemotherapy but the clinical treatment is still unsatisfactory (23). Discovering TNBC therapeutic agents from dietary natural products provides a useful application and chemo-preventive or chemotherapeutic effectiveness on TNBC (24,25). The goal of this study was to explore whether the anti-TNBC activity of AITC mediates through the direct cytotoxic effects and to understand the molecular mechanisms in human breast adenocarcinoma MDA-MB-468 cells. This study is focused on the cell cycle arrest and apoptotic cell death-induced by AITC in the MDA-MB-468 cells. Our data demonstrated that AITC inhibits cells viability, induces apoptotic death, and simultaneously causes cell cycle arrest in G2/M phase through the extracellular signal-regulated kinase (ERK) signaling pathway in MDA-MB-468 cells.

Materials and methods

Chemicals. AITC, propidium iodide (PI), dimethyl sulfoxide (DMSO), RNase A, N-acetyl-L-cysteine (NAC), U0126 and Triton X-100 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The fluorescent probes 2',7'-dichlorofluorescin diacetate (H$_2$DCF-DA) and 3,3'-dihexyloxacarbocyanine iodide DiOC$_{3}$(5), Dullbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Life Technologies (Carlsbad, CA, USA). Anti-p-ERK, anti-ERK, anti-Bcl-2, anti-p-Bcl-2 (Ser-70), anti-cytochrome c, anti-Apaf-1, anti-cyclin B and anti-CDK1 and second antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-p21/WAF-1 (Cat. 05-345) and Immobilon Western Chemiluminescent HRP substrate (Cat. WBKLS0500) were bought from Merck Millipore Corp. (Bedford, MA, USA).

Cell culture. Human breast adenocarcinoma MDA-MB-468 cell line was made by Dr Wei-Chien Huang (Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan). Cells were placed into 75-cm$^2$ tissue culture flasks under humidified 5% CO$_2$ and 95% air grown at 37˚C and one atmosphere in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Subconfluent cells (80%) were passaged with a solution containing 0.25% trypsin and 0.02% EDTA.

Determinations of cell number and cell viability. Cells at a density of 2x10$^5$ were seeded in 12-well plates and then exposed to 5, 10, and 20 µM AITC or 0.1% DMSO (as a vehicle control) for 24 and 48 h, then the cells were harvested and the cell number determined using trypan blue stain by Countess Automated Cell Counter (Invitrogen/Life Technologies) (26,27). Cells were incubated with or without 5, 10 and 20 µM of AITC for 24 and 48 h in presence and absence of NAC (a ROS scavenger) or U0126 (an ERK inhibitor). Cells were determined for viability utilizing thiazolyl blue tetrazolium bromide (MTT) assay as previously described (28,29). Each data point was represented from three independent experiments.

Cell morphological analysis. Approximately 2x10$^5$ cells/well of MDA-MB-468 cells in 12-well plates were incubated with or without 20 µM AITC and equal amount of DMSO as a control for 24 h at 37˚C. At the end of treatment, cells were examined and photographed under a phase-contrast microscope at a x200 magnification for examining the cell morphological changes (30,31).

Analysis for cell cycle distribution and sub-G$_1$ population. Approximately 2x10$^5$ cells/well of MDA-MB-468 cells in 12-well plates were incubated in presence and absence of 5, 10 and 20 µM AITC then were placed in an incubator for 24 h, and then cells were harvested by centrifugation at 1,000 x g for 5 min, pellet were washed twice with cold PBS then fixed gently by 70% ethanol at 4˚C overnight. Then cells were washed twice with cold PBS then resuspended in PBS containing 40 µg/ml PI and 0.1 mg/ml RNase A and 0.1% Triton X-100 in the dark for 30 min at 37˚C then the cells were analyzed with a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA) equipped with an argonion laser at 488 nm wavelength. Then the cell cycle and sub-G$_1$ (apoptosis) group were determined and analyzed (32,33).

Caspase-9 and -3 activity assays. About 5x10$^5$ cells of MDA-MB-468 cells in 75-T flasks were treated with or without 5, 10, 15 and 20 µM of AITC, then incubated for 12 h to detect the activity of caspase-9 and -3 which was assessed according to manufacturer's instruction of the Caspase colorimetric kit (R&D Systems Inc., Minneapolis, MN, USA). Cells were harvested and lysed in 50 µl lysis buffer containing 2 mM DTT, for 10 min. After centrifugation, the supernatant containing 200 µg protein were incubated with caspase-9 and -3 substrates in reaction buffer. Then all samples were incubated in a 96-well flat bottom microplate at 37˚C for 1 h. Levels of released pNA were measured with ELISA reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 405 nm wavelength (34,35).

Detections of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta$Ψ$m$). MDA-MB-468 cells (2x10$^5$ cells/well) in 12-well plates with 0, 5, 10, 15 and 20 µM of AITC were incubated for 12 h to determine the changes of ROS production and $\Delta$Ψ$m$ levels. The cells were harvested by centrifugation and were washed twice by PBS, then were resuspended in 500 µl of H$_2$DCF-DA (10 µM) and 500 µl of DiOC$_{3}$(3) (1 µmol/l) and incubated at 37˚C in the dark for 30 min and were analyzed immediately by flow cytometry as described previously (32,33).

Western blot analysis. MDA-MB-468 cells at a density of 5x10$^5$ cells/ml seeded into T-75 flasks were treated with 5, 10 and 20 µM of AITC for 2 and 12 h. Cells were harvested from each treatment then were washed with cold PBS and then scraped and washed twice by centrifugation at 1,000 x g for 5 min at 4˚C. All pellets were individually resuspended in the PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam-si, Korea) for 3 h at -20˚C as described previously (36,37). The lysate from each sample was collected by centrifugation at 12,000 x g for 30 min at 4˚C, and the supernatant was stored at -20˚C. Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels were used to separate proteins before
Each sample was incubated with the primary antibodies (Santa Cruz Biotechnology Inc.) followed by secondary antibodies. These blots were then detected by Immobilon Western Chemiluminescent HRP substrate (Merck Millipore Corp.) and autoradiography using X-ray film (GE Healthcare, Piscataway, NJ, USA) (38,39). Each Immobilon-P transfer membrane (Cat. IPVH00010, Merck Millipore) was stripped and reprobed with anti-β-actin antibody as the loading control for ensuring that equal proteins were loaded (40,41).

**Determination of CDK1 kinase activity.** MDA-MB-468 cells were seeded onto 75-T flask and then treated with 0, 5, 10 and 20 µM of AITC for 12 h. MDA-MB-468 cells were suspended in a final volume of 0.2 ml buffer containing 20 mM Tris-HCl (pH 8.5), 150 mM NaCl, 0.2% NP-40, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 µg/ml pepstatin, 0.5 µg/ml leupeptin, 5 mM β-glycerophosphate, 5 mM NaF, 1 mM Na3VO4 and 5 mM β-mercaptoethanol. Cell suspensions were sonicated and centrifuged at 10,000 x g for 30 min. CDK1 kinase activity condition was determined by using MV Peptide (CycLex Cdc2-Cyclin B kinase assay kit, Medical & Biological Laboratories Co Ltd, Nagoya, Japan) and measuring OD492 as described previously (42,43).

**Statistical analysis.** Our data were performed as means ± SD of at least in triplicate. The difference between the AITC-treated and control groups were analyzed by Student’s t-test, p<0.05 was considered significant.

**Results**

**AITC reduces cell viability and affects cell morphological changes and cell cycle arrest in MDA-MB-468 cells.** In order to examine the biological effects of AITC, MDA-MB-468 cells were treated with varying concentrations of AITC at 0, 5, 10 and 20 µM for 24 and 48 h. Cells were harvested and the cell number was determined using trypan blue stain by Countess Automated Cell Counter. The data are representative of three independent experiments.
of AITC are accompanied by its effect on cell cycle progression and/or apoptotic cell death. We found that AITC promoted G2/M phase arrest and sub-G1 population in MDA-MB-468 cells. Also, these effects were dose- and time-course dependent (Fig. 2B).

AITC stimulates the activities of caspase-9 and -3 in MDA-MB-468 cells. Cells were exposed to various concentrations of AITC for 12 h treatment to determine caspase-9 and -3 activities. Our results indicated that the caspase-9 and -3 activities were time- and concentration-dependently stimulated in AITC-treated MDA-MB-468 cells (Fig. 3). Thus, we suggest that AITC-triggered apoptosis is carried out through caspase-9 and -3-dependent signaling in MDA-MB-468 cells.

AITC promotes the reactive oxygen species (ROS) production and loss of \( \Delta \Psi_m \) levels in MDA-MB-468 cells. The results of flow cytometric analysis for ROS production and \( \Delta \Psi_m \) levels are shown in Fig. 4A and B. AITC-treated MDA-MB-468 cells with DCF were observed with increased intracellular ROS (Fig. 4A). We further explored if mitochondrial depolarization contributed to AITC-induced apoptosis of MDA-MB-468 cells. The treated and un-treated cells were exposed to AITC to investigate the change in \( \Delta \Psi_m \) in MDA-MB-468 after being stained with DiOC6(3), a mitochondria-specific and voltage-dependent dye. Results shown in Fig. 4B display that AITC significantly decreased the levels of \( \Delta \Psi_m \) in MDA-MB-468 cells (Fig. 4B). Based on these observations, we found that AITC-provoked cell
apoptosis is involved in ROS production and intrinsic signaling pathway in MDA-MB-468 cells.

AITC upregulates p-ERK signaling and alters mitochondria-dependent apoptotic pathway in MDA-MB-468 cells. It is widely reported that ERK/MAPK positively regulated phosphorylation of Bcl-2 at Ser-70, causing antiapoptotic function to suppress Bcl-2 expression (44,45). To clarify whether AITC influences intrinsic apoptotic signaling through ERK pathway, the results from western blotting are shown in Fig. 5A and B indicating that the protein levels of p-ERK and ERK (Fig. 5A), p-Bcl-2 (Ser-70), cytochrome c and Apaf-1 (Fig. 5B) and p21/WAF-1 (Fig. 7B) were upregulated in MDA-MB-468 cells. Many reports have shown that apoptosis is associated with the loss of ∆Ψm which is an endpoint of apoptosis (46). Our findings indicated that antiapoptosis signaling involving Bcl-2 phosphorylation is involved in ERK/MAPK pathway in AITC-treated MDA-MB-468 cells.

ROS and ERK are associated with the induction of apoptosis in AITC-treated MDA-MB-468 cells. To elucidate the possible signaling pathways of AITC-reduced viability of MDA-MB-468 cells, we determined whether ROS and ERK mediated AITC-regulated apoptotic signaling. Cells were pretreated with or without NAC (a ROS scavenger) and U0126 (an ERK inhibitor) and then exposed to AITC (10 µM) for 24 h. Cells were then determined for measuring cell viability by MTT assay. As shown in Fig. 6A, cells after treatment with AITC in presence and absence of NAC were observed to protect reduction of viability of MDA-MB-468 cells when compare with only AITC treated sample. Fig. 6B displays that reduction of cell viability in MDA-MB-468 cells by AITC was dramatically reversed by U0126 in comparison to AITC-treated only cells. Thus, ROS and ERK play central roles in AITC-induced apoptosis of MDA-MB-468 cells.

AITC decreases CDK1 activity and alters G2/M phase-modulated protein levels in MDA-MB-468 cells. We further assessed if G2/M phase arrest is involved in CDK1 activity, and data showed that AITC at 5-20 µM dramatically reduced CDK1 activity in MDA-MB-468 cells (Fig. 7A). We also explored the possible molecular mechanisms in the modulation of G2/M phase arrest. Fig. 7B indicates that AITC caused an increase of p21/WAF-1 expression and a decrease of cyclin B and CDK1 protein levels in MDA-MB-468. Hence, we suggest that AITC-provoked G2/M phase arrest is mediated through activating p21/WAF-1 and suppressing CDK1/cyclin B expression in in vitro cultivation of MDA-MB-468 cells.

Discussion

Many studies in new drug discovery have focused on breast adenocarcinoma therapeutic agents through the promotion
with the growth arrest in cells (53). Enhanced p21/WAF1 mRNA expression occurs through both p53-dependent and -independent mechanisms (54). Furthermore, ERK MAPK pathway has recently been reported to cooperate to cause sustained cell cycle arrest requiring p21/WAF1 expression (55,56). Our results from cell cycle analysis indicated that AITC induced G_{2}/M phase arrest (Fig. 2) in MDA-MB-468 cells. The CDK1 and cyclin B proteins were decreased (Fig. 7B), and phospho-ERK (Fig. 5A) and p21/WAF-1 (Fig. 7B) were increased by AITC treatment in a concentration-dependent manner. AITC also inhibited the CDK1 activity (Fig. 7A). Our study revealed that the novel molecular mechanism by which AITC induces G_{2}/M phase arrest and apoptosis in triple negative breast cancer MDA-MB-468 cells is through ERK-dependent p21/WAF-1 upregulation.

Two major signaling pathways are involved in apoptotic cell death (57,58). The extrinsic pathway (also called death receptor pathway) through the activation of the cell surface [Fas/Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL)] then promotes caspase-8 activation. The intrinsic pathway (also called mitochondria pathway) through death signals to mitochondria result in the release of mitochondria inter-membrane proteins such as cytochrome c, which associate with apoptotic protease-activating factor-1 (Apaf-1) and pro-caspase-9 to form the apoptosome and then active caspase-3. The caspase-independent pathway is involved in the mitochondria which led to releases of apoptosis inducing factor (AIF) or endonuclease G (Endo G) from mitochondria causing cell death (59). Our results showed that AITC induced apoptotic death (sub-G_{1} phase) of MDA-MB-468 cells and this action is concentration-dependent (Fig. 2B). AITC treatment in MDA-MB-468 cells concentration-dependently promoted the activations of caspase-9 and caspase-3 (Fig. 3). Cells were pretreated with NAC (a ROS scavenger) and U0126 (an ERK inhibitor) and exposed to AITC, leading to increase the percentage of viable cells when compared to the AITC-treated only cells (Fig. 6). The results in Fig. 5 show that the protein expressions of p-ERK, p-Bcl-2 (Ser-70), cytochrome c and Apaf-1 were upregulated in MDA-MB-468 cells after treatment with AITC. Our results suggested that AITC upregulated ERK signaling and altered mitochondria-dependent associated apoptotic pathway in MDA-MB-468 cells. Bcl-2 phosphorylation is known to affect antiapoptotic activity (44,45). Induction of apoptosis associated with Bcl-2 phosphorylation by anticancer agents has been linked with altering a variety of cellular signaling pathways, such as Ras/Raf, protein kinase C, protein kinase A, mitogen-activated protein kinase, ERK and CDK1 (35). Our results are in agreement with previous studies (60,61) indicating that that AITC-induced apoptotic cell death was caused by Bcl-2 phosphorylation and ERK activation.

The molecular signaling pathways are summarized in Fig. 8. Our results demonstrate that the ERK signaling pathway modulated intrinsic signaling and G_{2}/M phase arrest in AITC-treated MDA-MB-468 cells. These findings implied that AITC may be used as a novel therapeutic agent for the treatment of human breast cancer.

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