Abstract. Phenethyl isothiocyanate (PEITC), one of many compounds found in cruciferous vegetables, has been reported as a potential anticancer agent. In earlier studies, PEITC was shown to inhibit cell growth and induction of apoptosis in many cancer cell lines. However, no report has shown whether PEITC can induce apoptosis in human prostate cancer cells. Herein, we aimed to determine whether PEITC has anticancer activity in DU 145 human prostate cancer cells. As a result, we found that PEITC induced a dose-dependent decrease in cell viability through induction of cell apoptosis and cell cycle arrest in the G2/M phase of DU 145 cells. PEITC induced morphological changes and DNA damage in DU 145 cells. The induction of G2/M phase arrest was mediated by the increase of p53 and WEE1 and it reduced the level of CDC25C protein. The induction of apoptosis was mediated by the activation of caspase-8-, caspase-9- and caspase-3-dependent pathways. Results also showed that PEITC caused mitochondrial dysfunction, increasing the release of cytochrome c and Endo G from mitochondria, and led cell apoptosis through a mitochondria-dependent signaling pathway. This study showed that PEITC might exhibit anticancer activity and become a potent agent for human prostate cancer cells in the future.

Prostate cancer is not a single disease and is an umbrella which under a plethora of heterogeneous diseases is included. These heterogeneous diseases include indolent localized tumors and aggressive metastatic diseases (1). In the developed world, prostate cancer is the primary male cancer, accounting for 24% of all diagnosed male cancer cases, and is the second leading cause of death from cancer (2). In Taiwan, prostate cancer is the seventh most common cancer in males and about 8.0 men per 100 thousand die annually from prostate cancer based on reports from the Department of Health, Executive Yuan, R.O.C (Taiwan) in
2009. About 90% of advanced prostate cancer patients developing skeletal metastases and in advanced prostate cancer patients is morbidity from bone metastasis is high (3), despite treatments including radiation, chemotherapy, or combination of radiotherapy with chemotherapy.

The majority of cancer-targeting drugs cause aberrant cellular growth through the induction of apoptosis (4-6). Apoptosis, programmed cell death type I, is regulated by multifactor and signaling pathways to remove unwanted cells (7, 8). Active caspases are known to be biologically important and associated with apoptosis (9). Poly-ADP-ribose polymerase (PARP) is cleaved by both caspase-3 and caspase-7, and is an abundant DNA-binding enzyme that detects and signals DNA strand breaks (10). Caspase-3 in particular is the main executor of apoptosis and caspase activation is considered to be a key hallmark of apoptosis (11). Cytochrome c release from the internal part of the mitochondrial membrane into the cytosol leads to the activation of caspase-9, -3, -6, and -7, and then to apoptosis (12-14).

Traditional drugs obtained from various plant types with active natural compounds such as licochalcone-A (15), xanthoangelol (16) and chalcones (17) have been reported to act as potential agents for the treatment of cancer. Dietary intake of cruciferous vegetables can reduce the risk of various types of malignancies (18). Phenethyl isothiocyanate (PEITC) is one of such important compounds from cruciferous vegetables. Several mechanisms have been shown to be involved in the antitumor effect of PEITC, including the inhibition of cytochrome p450 enzymes, the induction of phase II detoxification enzymes (19) and the reduction of azoxymethane-induced colon aberrant crypt foci formation (20). PEITC also inhibited 4-(methyleneimino)-1-(3-pyridyl)-1-butenedioic acid neoplasia in A/J mouse lung (21). In addition, PEITC can act as a cancer chemopreventive in rat (22). Recently, it was reported that PEITC inhibits oxidative phosphorylation to trigger reactive oxygen species-mediated death of human prostate cancer cells (23).

Although many reports stated that PEITC induced cell death through induction of apoptosis, there is no report to address whether this occurs in human prostate cancer cells. In this study, we investigated the effects of PEITC on cell growth of human prostate cancer cells.

Materials and Methods

Reagents. PEITC, RPMI-1640 medium, fetal bovine serum, and penicillin and streptomycin antibiotic mixture were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). Propidium iodide (PI), dimethyl sulfoxide (DMSO), RNase A, and 4,6-diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich Corp. (St. Louis, MO, USA). The antibodies against BAK, BID, BCL-2, cell division cycle 25C (CDC25C), and apoptosis inducing effect (AIF) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and the antibodies against cytochrome c, Glucose-regulated protein 78 (GRP78), and PARP were from BD PharMingen (Palo Alto, CA, USA). 6-Carbboxy-2,7-dichlorodihydrofluorescein diacetate (H2DCFDA), Flu-o-3/AM and 3,3′-dihexyloxycarbocyanine iodide (DiOC6) were purchased from Invitrogen Life Technologies. The caspase-9 inhibitor (Z-LEHD-FMK), caspase-8 inhibitor (Z-IETD-FMK) and caspase-3 inhibitor (Z-DEVD-FMK) were obtained from R&D Systems Inc. (Minneapolis, MN, USA). All chemicals were reagent grade.

Cell line. The human prostate carcinoma cell line (DU 145) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). DU 145 cells were maintained in RPMI-1640 medium with 2 mM L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin and 100 U/ml streptomycin. Cells (1×10^5 cells/ml) were maintained on 12-well plates and then incubation with different concentrations (0, 1, 5, 10, 15 and 20 μM) of PEITC for different time periods (24 and 48 h). For examination of cell morphological, treated and negative-control cells were observed at 24 h incubation under a phase-contrast microscope and photomicrographs were taken. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The DU 145 (1×10^6 cells/well/100 μl) cells were cultured in 96-well plates. After incubation overnight, the RPMI-1640 medium in each well was replaced with different concentrated solutions of PEITC and cells further incubated for 24 and 48 h. At the end of incubation, 10 μl of MTT (5 mg/ml in phosphate-buffered saline (PBS)) (Sigma-Aldrich Corp.) were added individually to each well for 4 h incubation at 37°C, and then removed media after 100 μl of DMSO were added. The absorbance was then measured by Microplate Reader (Bio-Rad, Hercules, CA, USA) at 570 nm (25, 26). In addition, cultured DU 145 cells with or without treatment with 25 μM of caspase-8 inhibitor (Z-IETD-FMK), caspase-9 inhibitor (Z-LEHD-FMK) or caspase-3 inhibitor (Z-DEVD-FMK), respectively, for 3 h prior to the addition 10 μM of PEITC were then analyzed for cell viability as described previously (26, 27).

Flow cytometric analysis for DNA content. DU 145 cells were plated at a density of 2×10^5 cells on 12-well plates for 24 h, and exposed to 0, 1, 5, 10, 15 and 20 μM of PEITC for different time periods. At the end of incubation, cells were trypsinized and centrifuged then the cell pellet from each treatment was resuspended with 1 ml of PI staining buffer containing 4 mM sodium citrate, 0.1% Triton X-100, 50 μg/ml PI and 200 μg/ml RNase and incubated for 30 min at 37°C in the dark. All samples were analyzed for cell cycle distribution and sub-G1 proportion (apoptosis) by using a FACSCalibur flow cytometer and BD CellQuest Acquisition software (San Jose, CA, USA) as described previously (24, 27). Data in each phase were reported as the percent age cells in each phase of the cell cycle.

DAPI staining. Morphological changes of apoptosis were examined by staining DU 145 cell nuclei with DAPI. Cells (5×10^4 cells) were incubated in chamber slides and treated with 0, 1, 5, 10, 15 and 20 μM
of PEITC for 24 h. The cells were washed twice with PBS then were fixed with 4% paraformaldehyde-PBS solution for 15 min and stained with DAPI (300 nM) for 30 min at room temperature. The cells were washed with PBS and mounted. Images of DAPI fluorescence were examined and photographed under a fluorescence microscope as described previously (28, 29).

**Comet assay.** DU 145 cells (1×10^5 cells) were treated with PEITC (0, 1, 5, 10, 15 and 20 μM) for 24 h and then cells were embedded onto 0.6% low melting point agar then immersed in ice-cold cell lysis solution (2.5 mM NaCl, 100 mM ethylenediaminetetra acetic acid (EDTA), 10 mM Tris (pH 10), 1% N-laurylsarcosine, 1% Triton X-100 and 10% DMSO). Cells were then washed and digested with

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**Figure 1.** PEITC affected on cell morphology and viability in DU 145 cells. Cells were cultured in RPMI-1640 medium and 10% FBS with PEITC for 24 and 48 h. Morphological changes were examined and cells photographed under a phase-contrast microscope (×200) (A). Cells viability was measured by flow cytometric assays as described in the Materials and Methods (B). *p<0.05 Significantly different from the control.
two units of endonuclease III in the same buffer for 1 h at 37˚C then slides were denatured with 0.3 mM NaOH, 1 mM EDTA (pH 13.4) for 20 min and electrophoresis was carried out at 25 V, 300 mA for 25 min. Cellular DNA was stained with 4 μg/ml PI and examined under a fluorescence microscope as described previously (26, 30).

Flow cytometric detection of reactive oxygen species (ROS), mitochondrial membrane potential (ΔΨm) and intracellular Ca^{2+} levels. DU 145 cells were plated at a density of 2×10^5 cells on 12-well plates for 24 h, and then PEITC (10 μM) was added to the well and cells incubated for different time periods. The cells from each treatment were trypsinized and centrifuged, washed twice with PBS and were re-suspended in 50 μl of 10 μM substrate solution (PhiPhiLux-G1D1 for caspase-3, CaspaLux8-L1D2 for caspase-8 and CaspaLux9-M1D2 for caspase-9, OncoImmunin, Inc. Gaithersburg, MD, USA) before being incubated at 37˚C for 60 min. The cells were washed again by PBS and were analyzed by flow cytometry as described previously (29, 30).

Western blotting analysis. Western blotting was carried out as previously described (30). DU 145 cells were seeded at a density of 2×10^5 cells/ml in RPMI-1640 medium with 10% fetal bovine serum for 24 h. Cells were treated with or without 10 μM PEITC for 0, 6, 12, 24 and 48 h. Cells from each treatment were collected and protein was extracted into the PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea), and protein concentrations were determined by a BCA Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) (26). Proteins were resolved on a polyacrylamide gel via electrophoresis (SDS-PAGE). After SDS-PAGE transfer, the Polyvinylidene Fluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, USA) was stained by primary antibody (anti-CDC25C, WEE1, p53, FAS, Fad ligand, BCL-2, BAK, BID, PARP, cytochrome c, AIF, GRP78 and GADD153), washed and then stained by appropriate horseradish peroxidase–conjugated secondary antibodies (GE Healthcare Pewaukee, WI, USA) for enhanced chemiluminescence (ECL) reagent (Millipore) as described previously (29, 32).

Immunofluorescence staining and confocal laser scanning microscopy. DU 145 cells were seeded at a density of 5×10^4 cells/well on 4-well chamber slides before being treated with 10 μM PEITC for 24 h. Cells were then fixed in 3% formaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 1 h with blocking of non-specific binding sites using 2% bovine serum albumin (BSA) as described previously (29, 33). Fixed cells were stained with primary antibodies to GADD153 and endonuclease G (Endo G) (1:200 dilution) overnight and then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody at 1:100 dilution (green fluorescence) followed by mitochondria and nuclei as counterstaining with PI (red fluorescence). Photomicrographs were obtained using a Leica TCS SP2 confocal spectral microscope.

Statistical analysis. The data are presented as the mean±S.D. of three independent experiments. The statistical significance of the mean difference between the control and treated groups was determined by a paired t-test. P<0.05 was considered statistically significant.

Results

PEITC induced morphological changes and reduced the percentage of viable DU 145 cells. To evaluate the effect of PEITC on DU 145 prostate cancer cells, we determined and measured the cell morphological changes and viability using a phase-contrast microscope and the MTT assay, respectively. The
cells were dose-dependently treated with PEITC and morphological changes of DU 145 cells after treatment with PEITC are shown in Figure 1A. Compared with the control (untreated) cells, the majority of the PEITC-treated DU 145 cells became irregularly shaped spinouts and less cell number (Figure 1A). These morphological changes demonstrated cell damage after PEITC treatment. As shown in Figure 1B, PEITC significantly reduced the viability of DU 145 cells in a time- and dose-dependent manner. A sharp decrease in cell viability was present at 5 μM of PEITC treatment time. The cytotoxic effect was more evident at 48 h. The viability of DU 145 cells after exposure to 10 μM of PEITC for 48 h decreased by almost 50%.

**PEITC induced cell cycle arrest and apoptosis in DU 145 cells.** The effects of PEITC on the cell cycle progression in DU 145 cells were determined by flow cytometry and PI staining. In DU 145 cells, treatment with PEITC for 48 h resulted in a dose-dependent increase in the percentage of
Figure 4. PEITC affected the levels of mitochondria membrane potential ($\Delta \Psi_m$), production of reactive oxygen species (ROS) and Ca$^{2+}$ and in DU 145 cells. Cells were treated with 10 $\mu$M of PEITC for various time periods then were collected and stained by DiOC6 for the $\Delta \Psi_m$ levels (A), with 2,7-dichlorodihydrofluorescein diacetate for ROS production (B) and by Fluo-3 for Ca$^{2+}$ level (C) as described in the Materials and Methods. Data represents the mean±S.D. of three experiments. *p<0.05, significantly different from the control.
Figure 5. PEITC induced caspase-3, -8 and -9 activities in DU 145 cells. Cells were treated with 10 μM of PEITC for 12, 24, 48 or 72 h and cells were collected for determination activities of caspase-3 (A), caspase-8 (B) and caspase-9 (C) as described in the Materials and Methods. Data represents the mean±S.D. of three experiments. *p<0.05, significantly different from the control.

Figure 6. Caspase inhibitors protected against PEITC-induced cytotoxicity in DU 145 cells. Cells were pretreated with the caspase-9 (Z-LEHD-FMK) (A), caspase-8 (Z-IETD-FMK) (B) and caspase-3 (Z-DEVD-FMK) (C) inhibitors, and then were treated with 10 μM PEITC for 24 h before determination of viable cells as described in the Materials and Methods. Data represent the mean±S.D. of three experiments. *p<0.05, significantly different from the control.
cells in the G2/M phase and a concomitant reduction of cell numbers in the S phase (Figure 2A). Higher concentrations of PEITC (15 and 20 μM) returned the proportion of G2/M cells back to control levels. However, the proportion of G0/G1 phase cells increased at treatment of PEITC at 15 and 20 μM. The sub-G1 phase was clearly to increase when the treatment of PEITC was increased and in a dose-dependent manner (Figure 2A). This was further verified by microscopic examination that showed a decrease of viable cell number in treated samples. Results from Western blotting shown in Figure 2B also show that PEITC increased the protein level of p53, but it inhibited the protein levels of CDC25C and WEE1. Taken together, these data indicated that PEITC induced apoptosis and arrested cell cycle progression at the G2/M stage in DU 145 cells.

**PEITC induced apoptosis and DNA damage in DU 145 cells.** We found that PEITC induced apoptosis in a dose-dependent manner in DU 145 cells (Figure 3A). PEITC-induced DNA damage was examined by Comet assay in DU 145 cells (Figure 3B). Using this assay, we found that PEITC-induced DNA damage in a dose-dependent manner in DU 145 cells.

**PEITC reduced the level of ΔΨm and increased the production of ROS and Ca2+ in DU 145 cells.** To investigate whether PEITC induced cytotoxic effects involved in ΔΨm, ROS and Ca2+ in DU 145 cells, the levels of ΔΨm and ROS and Ca2+ were measured by flow cytometry and the results are shown in Figure 4. The data indicate that PEITC significantly reduced the level of ΔΨm within 48 and 72 h treatment of PEITC (Figure 4A) and promoted ROS production at 6 and 12 h treatment of PEITC (Figure 4B). However, treatments for 24 h led to a decrease the ROS levels when compared to the control group, but not significantly so. Furthermore, PEITC increased the intracellular Ca2+ level at 0.5-6 h treatment (Figure 4C) in DU 145 cells.

**PEITC promoted the activities of caspase-3, -8 and -9 in DU 145 cells.** To determine whether PEITC-induced apoptosis was associated with caspase activation in DU 145 cells, the activities of caspase-3, -8 and -9 in DU 145 cells after exposure to PEITC were assayed by flow cytometry and the results are shown in Figure 5. The results indicate that PEITC promoted the activities of caspase-3 at 24-72 h in a time-dependent manner (Figure 5A), increased the caspase-8.
activity at 48 h, (Figure 5B), and that of caspase-9 at 24 h (Figure 5C). Based on these observations, we suggest that caspases might be involved in the induction of apoptosis in DU 145 cells after exposure to PEITC.

Caspase-specific inhibitors protected against PEITC-induced cell death in DU 145 cells. DU 145 cells were pretreated with the caspase-3 inhibitor (Z-DEVE-FMK), caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK),

Figure 8. PEITC altered the GADD153 and Endo-G distributions in DU 145 cells. Cells were incubated with 10 μM of PEITC for 24 h, and then were fixed and stained with primary antibodies to GADD153 (A) and Endo G (B) which were stained by FITC-labeled secondary antibodies (green fluorescence) and the proteins were detected by a confocal laser microscopic system. The nuclei were stained by PI (red fluorescence). Areas of colocalization of GADD153 and Endo G expressions in the merged panels are yellow. Scale bar, 20 μm.
respectively, and then cells were harvested for measuring the percentage of viability and the results are shown in Figure 6. These data indicate that caspase-8 inhibitor (Z-IETD-FMK) significantly increased the percentage of viable cells (Figure 6B), while the other two inhibitors did not (Figure 6A and C) after exposure to PEITC.

**PEITC affected the apoptosis-associated protein levels in DU 145 cells.** The effects of PEITC on the apoptotic associated protein levels in DU 145 cells were assayed by Western blotting and the results are shown in Figure 7. The results indicate that PEITC increased the protein levels of FAS ligand, FAS (Figure 7A), BAK (Figure 7B), AIF, cytochrome c (Figure 7C) and GRP78 (Figure 7D), but reduced the levels of BCL-2 and BID (Figure 7B), PARP (Figure 7C) and did not affect GADD153 (Figure 7D) level in DU 145 cells.

**PEITC altered the distribution of GADD153 and Endo G in DU 145 cells.** The effects of PEITC on the levels and distribution of apoptosis-associated proteins GADD153 and Endo G in DU 145 cells were examined by confocal laser microscope and the results are shown in Figure 8. PEITC increased the protein levels of GADD153 (Figure 8A) and Endo G (Figure 8B) and increased their translocation to nuclei.

**Discussion**

PEITC has been isolated from various common vegetables, especially cruciferous, and has anticancer activities against many human cancer cell types *in vitro* and *in vivo* (34-39). In this study, we found that PEITC exhibited antitumor activity as it significantly inhibited prostate cancer cell growth and induced apoptosis, and more importantly, through mitochondrial-dependent and -independent pathways.

It is well known that cell proliferation and viability are one of major observations for the assessment of cytotoxicity and screening of anticancer drugs. Our results also show that PEITC induced morphological changes and reduced the percentage of viable of DU 145 cells (Figure 1A and B). Apoptosis is one form of programmed cell death and is characterized by morphological changes, chromatin condensation and extensive DNA fragmentation. The frequency and time of appearance of apoptosis depend on the apoptosis-inducing signal and cell types (40, 41). It was reported that failure of apoptosis in cancer cells may promote survival and accumulation of cells to form tumors (42). Therefore, the induction of apoptosis in cancer cells has become a strategy for their elimination (43, 44).
In the present study, we investigated the effect of PEITC on apoptosis in DU 145 human prostate cancer cells in vitro. Flow cytometric assay for sub-G1 phase examination, DAPI staining and the comet assay were used to study the morphological changes of apoptosis and DNA damage, respectively. Results showed that PEITC induced sub-G1 phase in DU 145 cells and the data from DAPI staining also confirmed that PEITC induced DU 145 cells apoptosis and that the DNA damage occurred in the cell apoptotic process. To further verify the DNA damage induced by PEITC, the single-cell gel electrophoresis assay (Comet assay) was performed and the results showed that PEITC induced DNA damages dose dependently. We suggest that DNA damage might be one of the means by which apoptosis is induced by PEITC in DU 145 cells.

It is also reported that ROS are involved in apoptosis in cancer cells (45). Thus, we used flow cytometric assay and found that PEITC promoted the release of ROS in DU 145 cells (Figure 4B) at 6-12 h treatment. The results also showed that PEITC reduced the levels of ΔΨm and resulted in Ca2+ increased in DU 145 cells (Figure 4A and C). These observations indicate that PEITC-induced apoptosis also involves mitochondria. It is well known that caspases also play important roles in apoptosis, and we investigated the activities of caspases and found that PEITC significantly activated of caspase-3, -8 and -9 (Figure 6A, B and C). PEITC-induced apoptosis involves a caspase-dependent pathway. Caspase-3 is a critical enzyme in the execution phase of apoptosis and a hallmark of apoptosis (46). Our findings are in agreement with this showing a significant increase in caspase-3 activity in DU 145 cells treated with 10 μM PEITC for 24 h, but no change in caspase-8 protein expression (Figure 5B). This shows that the treatment time was insufficient for an increase in protein expression but sufficient for enhancement of activity.

The BCL-2 family of proteins are the most important regulators of apoptosis. BAX is an apoptotic agonist that promotes cells’ apoptosis. BCL-2 is an intracellular suppressor of apoptosis, which prolongs cell survival. Therefore, the ratio of BCL-2/BAX of the cells will determine whether or not apoptosis occurs (47).

In conclusion, our results clearly indicate that PEITC promoted the expression of BAX but inhibited the expression of BCL-2 in DU 145 cells thus contributing to the activation of caspase-3 and the induction of apoptosis via the mitochondrial apoptosis pathway (Figure 9).

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References