Proteasome-mediated degradation of cell division cycle 25C and cyclin-dependent kinase 1 in phenethyl isothiocyanate-induced G_2-M-phase cell cycle arrest in PC-3 human prostate cancer cells

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Abstract

Phenethyl isothiocyanate (PEITC), a constituent of many cruciferous vegetables, offers significant protection against cancer in animals induced by a variety of carcinogens. The present study demonstrates that PEITC suppresses proliferation of PC-3 cells in a dose-dependent manner by causing G_2-M-phase cell cycle arrest and apoptosis. Interestingly, phenyl isothiocyanate (PITC), which is a structural analogue of PEITC but lacks the -CH_2 spacers that link the aromatic ring to the -N = C = S group, neither inhibited PC-3 cell viability nor caused cell cycle arrest or apoptosis. These results indicated that even a subtle change in isothiocyanate (ITC) structure could have a significant impact on its biological activity. The PEITC-induced cell cycle arrest was associated with a >80% reduction in the protein levels of cyclin-dependent kinase 1 (Cdk1) and cell division cycle 25C (Cdc25C; 24 h after treatment with 10 μM PEITC), which led to an accumulation of Tyr^{15} phosphorylated (inactive) Cdk1. On the other hand, PITC treatment neither reduced protein levels of Cdk1 or Cdc25C nor affected Cdk1 phosphorylation. The PEITC-induced decline in Cdk1 and Cdc25C protein levels and cell cycle arrest were significantly blocked on pretreatment of PC-3 cells with proteasome inhibitor lactacystin. A 24 h exposure of PC-3 cells to 10 μM PEITC, but not PITC, resulted in about 56% and 44% decrease in the levels of antiapoptotic proteins Bcl-2 and Bcl-X_L, respectively. However, ectopic expression of Bcl-2 failed to alter sensitivity of PC-3 cells to growth inhibition or apoptosis induction by PEITC. Treatment of cells with PEITC, but not PITC, also resulted in cleavage of procaspase-3, procaspase-9, and procaspase-8. Moreover, the PEITC-induced apoptosis was significantly attenuated in the presence of general caspase inhibitor and specific inhibitors of caspase-8 and caspase-9. In conclusion, our data indicate that PEITC-induced cell cycle arrest in PC-3 cells is likely due to proteasome-mediated degradation of Cdc25C and Cdk1, and ectopic expression of Bcl-2 fails to confer resistance to PEITC-induced apoptosis. Furthermore, the results of the present study point toward involvement of both caspase-8- and caspase-9-mediated pathways in apoptosis induction by PEITC. [Mol Cancer Ther 2004; 3(5):567–75]

Introduction

Epidemiological studies continue to support the premise that cruciferous vegetables may offer protection against the risk for cancers of several anatomical sites including prostate cancer (1–5). Anticancer effect of cruciferous vegetables is attributable to isothiocyanates (ITCs), which occur as thioglucoside conjugates (glucosinolates) in a variety of edible plants including broccoli, watercress, cabbage, etc. (6–10). Organic ITCs are generated by hydrolysis of corresponding glucosinolates through catalytic mediation of myrosinase, which is released on damage of plant cells during cutting or chewing of cruciferous vegetables (6, 7). Phenethyl ITC (PEITC), a naturally occurring member of the ITC family of chemopreventive agents, has received particular attention because of its potent cancer chemopreventive activity (11–21). PEITC has been shown to inhibit tumorigenesis in animal models induced by a variety of chemical carcinogens, many of which are highly relevant to human health (11–21). Cancer chemoprevention by PEITC has been observed against 7,12-dimethylbenzanthracene-induced mammary tumorigenesis in rats (12), *N*-nitrosobenzylmethylamine-induced esophageal cancer in rats (13, 14), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced pulmonary neoplasia in rats and mice (15, 16), benzo(*a*)pyrene-induced carcinogenesis in mice (17), and *N*-nitrosobis(2-oxopropyl)-amine-induced pancreatic tumorigenesis in hamsters (19). It is interesting to note that the thiol conjugates of ITCs retain cancer chemopreventive activity (19, 20). The mechanism by which ITCs offer protection against chemically induced cancer involves suppression of carcinogen activation due to inhibition of cytochrome *P*450-dependent monooxygenases and enhancement of carcinogen detoxification due to induction of phase II drug metabolizing enzymes, including glutathione transferases (reviewed in Refs. 7–10, 21). More recent studies have

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indicated that inhibition of benzo(*a*)pyrene-induced pulmonary carcinogenesis in mice by dietary administration of *N*-acetylcysteine conjugate of PEITC during the post-initiation phase is associated with activation of mitogen-activated protein kinases and p53 activity and induction of apoptosis (20).

Evidence is mounting to indicate that some naturally occurring ITCs, including PEITC, can suppress proliferation of human cancer cells by causing apoptosis and/or cell cycle arrest (22–36). Chen *et al.* (22) were the first to observe apoptosis induction by PEITC in Jurkat and HeLa cells, which was associated with sustained activation of c-Jun NH_2-terminal kinases. The PEITC-induced apoptosis was suppressed by interfering with c-Jun NH_2-terminal kinase activation (22). In another study, Huang *et al.* (23) provided convincing evidence to indicate an essential role of p53 in apoptosis induction by PEITC. Growth inhibition and apoptosis induction by ITCs, including PEITC and/or its thiol conjugates, have also been demonstrated in human hepatoma, leukemia, myeloma, prostate, and colon cancer cells (27–36). Despite these advances, however, the sequence of events leading to PEITC-induced cell cycle arrest and apoptosis is not fully defined.

The present study demonstrates that suppression of PC-3 human prostate cancer cell growth by PEITC is associated with cell cycle arrest in G_2-M phase due to proteasome-mediated degradation of cyclin-dependent kinase 1 (Cdk1) and cell division cycle 25C (Cdc25C). We also provide experimental evidence to indicate that Bcl-2 overexpression fails to confer resistance to PEITC-induced apoptosis. Instead, the PEITC-induced apoptosis in PC-3 cells is significantly attenuated by pharmacological inhibition of both caspase-9 and caspase-8. In summary, the results of the present study suggest that cell cycle arrest and apoptosis induction by PEITC may contribute to its anticarcinogenic activity against prostate cancer cells.

Materials and Methods

Reagents

PEITC and phenyl ITC (PITC) were purchased from Aldrich Chemical Co. (Milwaukee, WI). F-12K nutrient mixture, penicillin/streptomycin antibiotic mixture, and fetal bovine serum were from Life Technologies, Inc. (Grand Island, NY), propidium iodide was from Sigma Chemical Co. (St. Louis, MO), RNase A was from Promega (Madison, WI), lactacystin was from Calbiochem (La Jolla, CA), and the reagents for electrophoresis were from Bio-Rad (Hercules, CA). Antibodies against Bcl-X_L, Bax, Cdk1, Cdc25C, cyclin B1, and procaspase-8 were from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies specific for cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP) were from Cell Signaling Technology (Beverly, MA), antibodies against Bcl-2 were from DAKO Cytomation (Carpinteria, CA), antibodies against phospho-Cdk1 (Tyr^{15}) were from Sigma Chemical, and antibodies against Cdc25B and procaspase-9 were from BD PharMingen (San Diego, CA). The caspase inhibitors z-VADfmk (general caspase inhibitor), z-IETDfmk (caspase-8), and z-LEHDfmk (caspase-9) were from Enzyme Systems (Dublin, CA).

Cell Culture and Cell Survival Assays

Monolayer cultures of PC-3 cells were maintained in F-12K nutrient mixture (Kaighn's modification) supplemented with 7% (v/v) non-heat-inactivated fetal bovine serum and 10 ml/l of PSN antibiotic mixture at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The PC-3/neo and PC-3/Bcl-2 cells were a generous gift from Dr. Natasha Kyprianou (University of Maryland School of Medicine, Baltimore, MD) and were maintained similarly, except that 500 μg/ml G418 was added to the cultures. Effect of ITCs on proliferation of PC-3 cells was determined by trypan blue dye exclusion assay as described by us previously (34, 35).

Analysis of Cell Cycle Distribution

The effect of PITC or PEITC on cell cycle distribution was determined by flow cytometry following staining of the cells with propidium iodide. Briefly, cells (5×10^5 cells) were seeded and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentrations of PEITC or PITC (or DMSO for controls) and the flasks were incubated for 24 h at 37°C. The cells were washed with PBS and fixed in 70% ethanol. The cells were then treated with 80 μg/ml RNase A and 50 μg/ml propidium iodide for 30 min and analyzed using a Coulter Epics XL flow cytometer (Miami, FL).

Analysis of Apoptosis

Apoptosis induction in PITC- or PEITC-treated cells was assessed by quantitation of cytoplasmic histone-associated DNA fragments and Western blotting for PARP cleavage. The Cell Death Detection ELISA method quantifies apoptotic cell death in cellular systems by measuring cytoplasmic histone-associated DNA fragments. Cytoplasmic histone-associated DNA fragments in control (DMSO-treated) and PITC- or PEITC-treated cells were quantified using a commercial kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. In some experiments, cells were treated with caspase inhibitors for 2 h prior to PEITC treatment and quantification of cytoplasmic histone-associated DNA fragments.

Western Blot Analysis

After treatment with DMSO (control) or desired concentration of PITC or PEITC for specified time intervals, floating and attached cells were collected and lysed as described by us previously (34). Cell lysate was cleared by centrifugation at 14,000 rpm for 15 min. Lysate proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membrane was incubated with Tris-buffered saline containing Tween 20 (0.05%) and 10% (w/v) nonfat dry milk and exposed to the desired primary antibody for 1 h at room temperature. Following treatment with appropriate secondary antibody, the immunoreactive bands were visualized using enhanced chemiluminescence method. The blots were stripped and reprobed with antibodies against actin to correct for differences in protein loading.

Results

PEITC Inhibited Proliferation of PC-3 Cells by Causing G_2-M-Phase Cell Cycle Arrest and Apoptosis Induction

The effect of PEITC against proliferation of PC-3 cells was assessed by trypan blue dye exclusion assay, and the results are shown in Fig. 1. Viability of PC-3 cells was inhibited significantly in the presence of PEITC in a concentration- and time-dependent manner. For example, a 24 h exposure to 10 μM PEITC reduced PC-3 cell viability by about 65% compared with control (Fig. 1A). Growth inhibitory effect of PEITC against PC-3 cells was even more pronounced at 20 μM concentration (Fig. 1B). On the other hand, proliferation of PC-3 cells was not affected in the presence of PITC, which is a close structural analogue of PEITC, except that it lacks the $-CH_2$ spacers that link the aromatic ring to the -N=C=S moiety (Fig. 1, A and B). These results indicated that even minor structural difference could have a significant impact on biological activity of an ITC.

To gain insights into the mechanism of antiproliferative activity of PEITC, its effect on cell cycle distribution was determined and the data are summarized in Table 1. Representative histogram for cell cycle distribution following a 24 h exposure of PC-3 cells to DMSO (control) or 10 μM PEITC is shown in Fig. 2. PITC was included in the analysis as a negative control. A 24 h exposure of PC-3 cells to growth suppressive concentrations of PEITC (5 and 10 μM) resulted in accumulation of cells in G_2-M phase, which was accompanied by a decrease in G_0-G_1-phase cells (Table 1). For example, the fraction of cells with G_2-M-phase DNA content was between 1.6- and 1.9-fold higher in PC-3 cultures exposed for 24 h to 5 and 10 μM PEITC compared with control. In agreement with the results of the trypan blue dye exclusion assay (Fig. 1), PITC treatment did not alter cell cycle distribution (Fig. 2; Table 1). Treatment of PC-3 cells with PEITC, but not PITC, also led to a statistically significant increase in the fraction of cells with sub-G_0-G_1 DNA content, indicating apoptosis induction.

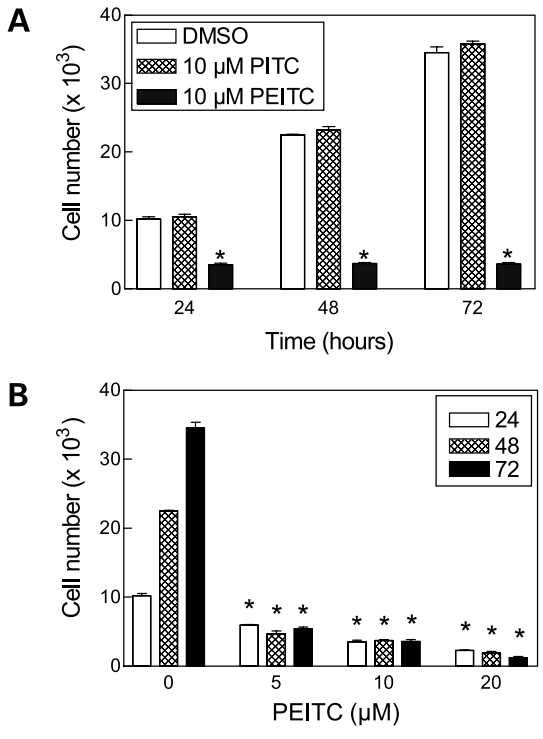

Figure 1. **A,** time course response for effect of PEITC and PITC on proliferation of PC-3 cells as determined by trypan blue dye exclusion assay. **B,** dose response for effect of PEITC on PC-3 cell proliferation as determined by trypan blue dye exclusion assay. PC-3 cells were plated, allowed to attach overnight, and treated with DMSO (control) or desired concentration of the test compound (PITC or PEITC) for specified time intervals. Both floating and adherent cells were collected and used for counting of dead and live cells. *Columns,* mean; *bars,* SE ($n = 3$). *, $P < 0.05$, significantly different compared with DMSO-treated control (one-way ANOVA).

Table 1. Effects of PEITC and PITC on PC-3 cell cycle distribution

Treatment	% Cells			
	Sub-G_0-G_1	G_0-G_1	S	G_2-M
Control (DMSO)	4 ± 0.5	56 ± 1	15 ± 0.5	19 ± 0.5
5 μM PEITC	14 ± 3*	42 ± 3*	14 ± 0.5	31 ± 1*
10 μM PEITC	16 ± 1*	34 ± 5*	13 ± 1	36 ± 4*
5 μM PITC	4 ± 0.5	59 ± 1	14 ± 0.5	20 ± 1
10 μM PITC	4 ± 0.5	56 ± 0.5	15 ± 0.5	21 ± 0.5

Note: Cells were treated with DMSO (control) or different concentrations of PEITC or PITC for 24 h at 37°C. Both floating and attached cells were collected and processed for analysis of cell cycle distribution by flow cytometry following staining with propidium iodide. Data are means ± SE ($n = 3$).
*, $P < 0.05$, significantly different compared with control (one-way ANOVA).

Apoptosis-inducing effect of PEITC was confirmed by quantitation of cytoplasmic histone-associated DNA fragments (Fig. 3A) and Western blot analysis for PARP cleavage (Fig. 3B). Treatment of PC-3 cells with PEITC, but not PITC, resulted in a statistically significant increase in the levels of cytoplasmic histone-associated DNA fragments when compared with control (Fig. 3A). In time course experiments using 10 μM PEITC, an immunoreactive band corresponding to cleaved PARP (89 kDa) was observed at 16 and 24 h time points (Fig. 3B). PARP cleavage was not observed in PITC-treated cells. These results indicated that, similar to other cellular systems, PEITC induced apoptotic cell death in PC-3 cells.

PEITC Modulated Expression of Proteins That Regulate G_2-M Transition

Eukaryotic cell cycle progression is regulated by sequential activation of cyclin-dependent kinases, the activity of which is dependent on their association with corresponding cyclins (37–39). A complex formed by the association of Cdk1 (also known as p34Cdc2) with cyclin B is important for entry into mitosis in most organisms (37–39). Kinase activity of Cdk1/cyclin B complex is regulated by phosphorylations. The phosphorylation of Cdk1 at Thr^{161} is required for complete activation of the Cdk1/cyclin B kinase

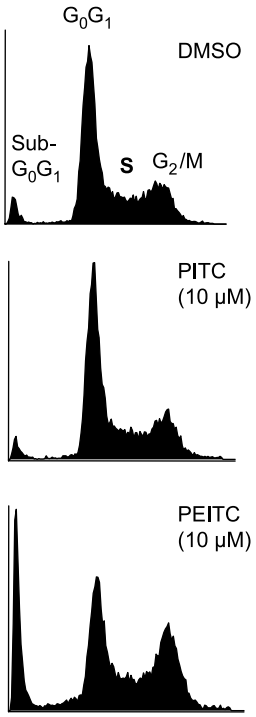

Figure 2. Effect of PEITC and PITC on PC-3 cell cycle distribution. PC-3 cells were treated with DMSO (control) or 10 μM PEITC or PITC for 24 h. The cells were then stained with propidium iodide and analyzed using a Coulter Epics XL flow cytometer.

complex (37–39). On the other hand, the kinase activity of Cdk1/cyclin B is inhibited by reversible phosphorylations on Thr^{14} and Tyr^{15} of Cdk1 (37–39). Dephosphorylation of Thr^{14} and Tyr^{15} of Cdk1, and hence activation of the Cdk1/cyclin B kinase complex, is catalyzed by dual-specificity phosphatases Cdc25B and Cdc25C (40). To elucidate the mechanism of PEITC-induced cell cycle arrest, lysates from control and PEITC-treated (10 μM PEITC for different time intervals) PC-3 cells were subjected to Western blotting using antibodies specific for above-mentioned proteins. Representative Western blots for the effect of PEITC on levels of cyclin B1, Cdk1, Cdc25B, and Cdc25C are shown in Fig. 4A. The expression of cyclin B1 or Cdc25B was comparable in control and PEITC-treated PC-3 cells. On the other hand, a statistically significant decrease (about 85% reduction compared with control) in Cdk1 protein level was observed in cells treated with 10 μM PEITC for 24 h ($P < 0.05$). The level of Cdc25C protein was reduced dramatically (near complete loss) in PEITC-treated cells compared with the control. The PEITC-induced reduction in Cdc25C protein level was evident as early as 4 h after treatment (about 88% reduction compared with the control; $P < 0.05$ by one-way ANOVA).

Because Cdc25C protein level was significantly lower in PEITC-treated cells compared with control, we raised the question whether PEITC treatment led to accumulation of Tyr^{15} phosphorylated (inactive) Cdk1. To explore this possibility, the blot was probed with antibodies specific for phospho-Cdk1 (Tyr^{15}). As can be seen in Fig. 4A (*bottom panel*), PEITC treatment led to an increase in Tyr^{15} phosphorylation of Cdk1 that was evident as early as 4 h after treatment and persisted for the duration of the experiment (24 h post-PEITC treatment).

The effect of PITC on level of cyclin B1, Cdk1, Cdc25B, and Cdc25C was also determined for direct comparison, and the data are shown in Fig. 4A (*right panels*). While PITC treatment did not significantly alter the protein levels of Cdk1, Cdc25B, or Cdc25C, the expression of cyclin B1 was increased by about 167%, 216%, and 270% in cells treated with 10 μM PITC for 1, 4, and 16 h, respectively, compared with control (Fig. 4A). However, Tyr^{15} phosphorylation of Cdk1 was not affected by PITC treatment, which explains its inability to cause cell cycle arrest (Fig. 2; Table 1).

Next, we raised the question whether PEITC-induced reduction in Cdk1 or Cdc25C protein level was mediated

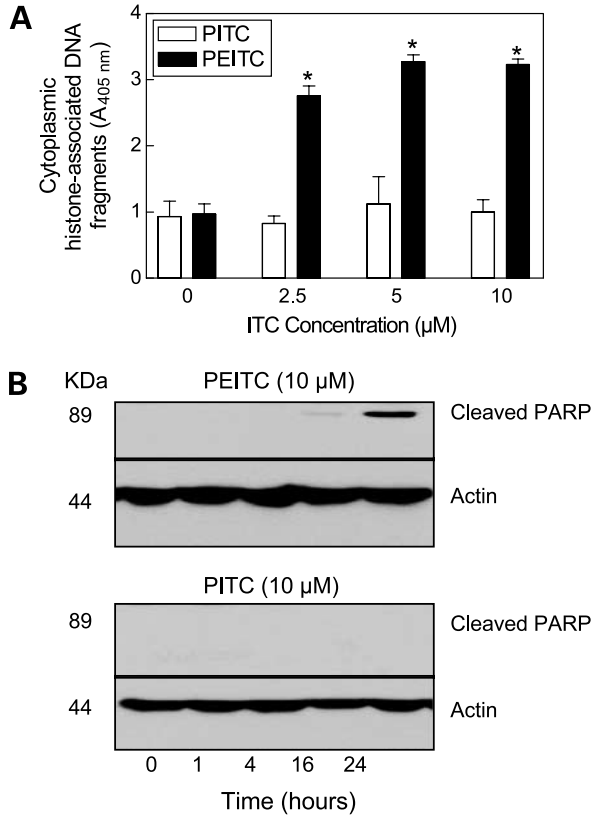

Figure 3. **A,** ELISA-based quantitation of cytoplasmic histone-associated DNA fragments in PC-3 cells following a 24 h exposure to DMSO (control) or different concentrations of PEITC or PITC. Cytoplasmic histone-associated DNA fragments were quantified using Cell Death Detection ELISA kit from Roche Diagnostics according to the manufacturer's instructions. *Columns,* mean; *bars,* SE ($n = 3$). *, $P < 0.05$, significantly different compared with control (one-way ANOVA). **B,** Western blot analysis for the effect of PEITC and PITC on cleavage of PARP. Cells were treated with 10 μM PEITC or PITC for the indicated time intervals and harvested for preparation of cell lysates. The lysate proteins were subjected to Western blotting using antibodies that recognize cleaved PARP. Blots were stripped and reprobed with anti-actin antibodies to ensure equal protein loading. The experiment was repeated twice using independently prepared lysates, and the results were comparable.

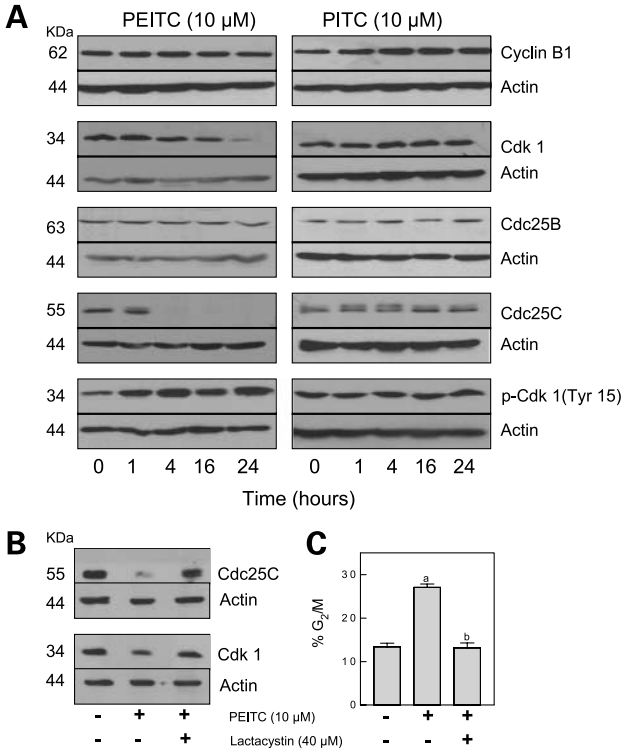

Figure 4. **A,** representative Western blots for the effects of PEITC and PITC on protein levels of cyclin B1, Cdk1, Cdc25B, Cdc25C, and phospho-Cdk1 (Tyr^{15}). Blots were stripped and reprobed with anti-actin antibodies to correct for differences in protein loading. The experiment was repeated two or more times using independently prepared lysates to ensure reproducibility. **B,** effect of lactacystin, a specific inhibitor of proteasome, on PEITC-induced decline in Cdc25C and Cdk1 protein levels. Cells were pretreated with DMSO or 40 μM lactacystin for 2 h at 37°C and exposed to 10 μM PEITC for 24 h. Cell lysates were prepared and subjected to Western blotting using antibodies against Cdc25C or Cdk1. The experiment was repeated twice, and the results were comparable. **C,** effect of lactacystin on PEITC-induced cell cycle arrest. Cells were pretreated with DMSO or 40 μM lactacystin for 2 h at 37°C and exposed to 10 μM PEITC for 24 h. Both floating and attached cells were collected and processed for cell cycle distribution analysis following staining with propidium iodide. *Columns,* mean of three determinations; *bars,* SE. *a*, significantly different compared with DMSO-treated control; *b*, significantly different compared with PEITC alone group.

by the proteasome. We explored this possibility by using lactacystin, which is a specific inhibitor of proteasome. As shown in Fig. 4B, the PEITC-induced degradation of both Cdk1 and Cdc25C was significantly attenuated on treatment of cells with lactacystin prior to and during exposure to PEITC. To determine the functional significance of Cdc25C/Cdk1 down-regulation to PEITC-induced cell cycle arrest, the effect of lactacystin on PEITC-induced G_2-M arrest was examined. As can be seen in Fig. 4C, the PEITC-induced G_2-M block was attenuated in the presence of lactacystin. Collectively, these observations indicated that PEITC-induced cell cycle arrest was mainly due to proteasome-mediated degradation of Cdk1 and Cdc25C.

PEITC Reduced Expression of Bcl-2 and Bcl-X_L

Studies of apoptosis in a variety of cellular systems have indicated that the members of the Bcl-2 family proteins function as gatekeepers of the cell death process (41–44). The proapoptotic multidomain Bcl-2 proteins such as Bax induce release of cytochrome *c* from mitochondria to the cytosol leading to activation of caspase cascade for execution of the death program (44, 45). The antiapoptotic multidomain Bcl-2 proteins such as Bcl-2 and Bcl-X_L interact with and inhibit the function of proapoptotic Bcl-2 proteins (46). To gain insights into the mechanism for apoptosis induction by PEITC, its effect on levels of Bcl-2, Bcl-X_L, and Bax were determined by Western blotting, and representative blots are shown in Fig. 5A. The bar diagram in Fig. 5B summarizes the results of densitometric scanning of the immunoreactive bands for PEITC samples. Lysates from cells treated with 10 μM PITC, which failed to elicit apoptosis, were included in the analysis as negative controls. As can be seen in Fig. 5, Bcl-2 protein level was reduced significantly by PEITC treatment in PC-3 cells at 24 h time point (~56% reduction compared with control; $P < 0.05$ by one-way ANOVA). A statistically significant reduction in the levels of Bcl-X_L protein was also evident in PEITC-treated PC-3 cells at 16 and 24 h time points compared with control (37% and 44% reduction, respectively). The level of Bax protein was not affected by PEITC (Fig. 5). These results indicated that apoptosis induction in PEITC-treated PC-3 cells may be due to reduction in the levels of Bcl-2 and/or Bcl-X_L.

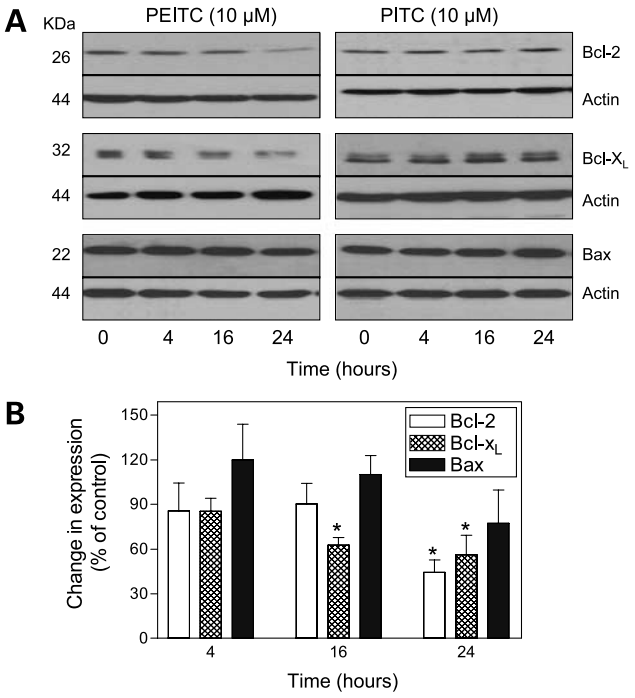

Figure 5. **A,** representative Western blots for the effects of PEITC and PITC on levels of Bcl-2 family proteins. PC-3 cells were cultured in the presence of 10 μM PEITC or PITC for the indicated time intervals and processed for preparation of cell lysate and Western blotting. Blots were stripped and reprobed with antibodies against actin to correct for differences in protein loading. **B,** results of densitometric scanning of the immunoreactive bands corresponding to Bcl-2, Bcl-X_L, and Bax for PEITC samples. *Columns,* mean; *bars,* SE ($n = 3$). *, $P < 0.05$, significantly different compared with control (one-way ANOVA).

Effect of Bcl-2 Overexpression on PEITC-Induced Apoptosis

To further investigate the role of Bcl-2 in PEITC-induced apoptosis, the effect of PEITC on cell proliferation and apoptosis induction was determined in PC-3 cells stably transfected with Bcl-2 (PC-3/Bcl-2). The cells transfected with the empty vector (PC-3/neo) were included as a control. As can be seen in Fig. 6A, Bcl-2 was overexpressed in PC-3/Bcl-2 cells. The proliferation of both PC-3/neo and PC-3/Bcl-2 cells was reduced significantly in the presence of 5 or 10 μM PEITC compared with DMSO-treated control at both 24 and 48 h time points (Fig. 6B). To our surprise, however, Bcl-2 overexpression failed to confer resistance to PEITC-induced cell killing. In agreement with the results of cell proliferation assay, apoptosis induction by PEITC, as determined by quantitation of sub-G_0-G_1 cells, was comparable in PC-3/neo and PC-3/Bcl-2 cells following a 24 h exposure to 5 or 10 μM PEITC (Fig. 6C). These results indicated that level of Bcl-2 protein did not affect sensitivity of PC-3 cells to either growth inhibition or apoptosis induction by PEITC.

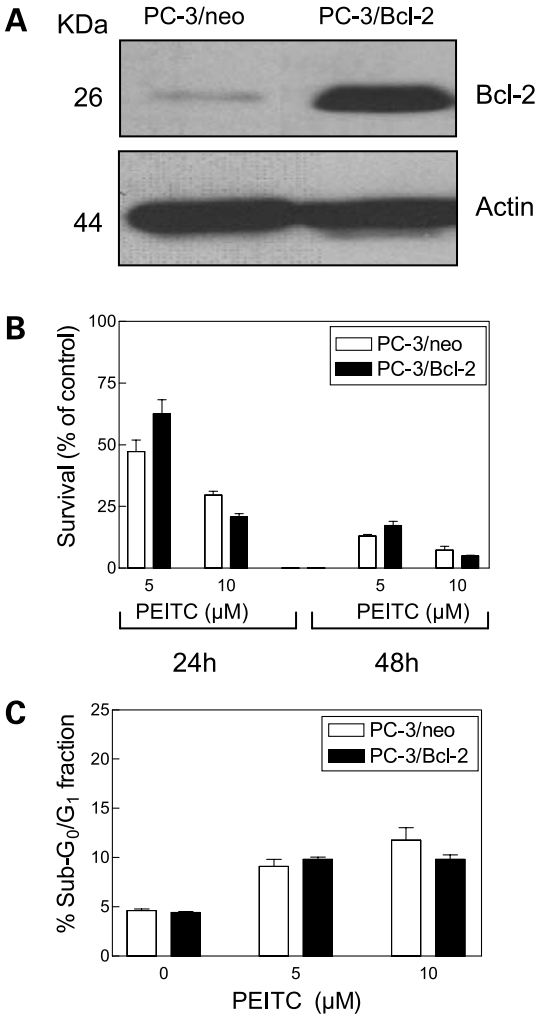

Figure 6. **A,** Western blot analysis for Bcl-2 protein expression using lysates from PC-3 cells stably transfected with empty vector (*PC-3/neo*) and Bcl-2 (*PC-3/Bcl-2*). **B,** effect of PEITC on proliferation of PC-3/neo and PC-3/Bcl-2 cells as determined by trypan blue dye exclusion assay. PC-3/neo or PC-3/Bcl-2 cells were exposed to DMSO or PEITC (5 or 10 μM) for 24 or 48 h. Both floating and attached cells were collected and used for trypan blue dye exclusion assay. *Columns,* mean; *bars,* SE (n = 3). The percentage of surviving cells did not differ significantly between PC-3/neo and PC-3/Bcl-2 cells at either concentration of PEITC at both time points. **C,** analysis of sub-G_0-G_1 cells in cultures of PC-3/neo and PC-3/Bcl-2 cells following a 24 h exposure to DMSO or PEITC (5 or 10 μM PEITC). Cells with sub-G_0-G_1 DNA content were quantified by flow cytometry following staining with propidium iodide. *Columns,* mean; *bars,* SE (n = 3). The fraction of sub-G_0-G_1 cells did not differ significantly between PC-3/neo and PC-3/Bcl-2 cells either at 5 or 10 μM PEITC.

Involvement of Caspases in PEITC-Induced Apoptosis

Caspases are aspartate-specific cysteine proteases that play critical roles in apoptosis processes (47–50). Activation of caspases results in cleavage and inactivation of key cellular proteins, including the DNA repair enzyme PARP. Because PARP cleavage was observed in PEITC-treated PC-3 cells (Fig. 3B), we reasoned that PEITC-induced apoptosis might involve caspases. We explored this possibility by determining the effect of PEITC treatment on activation of caspases by Western blotting using antibodies that recognize either full-length (procaspase-8 and procaspase-9) or cleaved (caspase-3) caspases, and representative blots are shown in Fig. 7. Treatment of PC-3 cells with 10 μM PEITC resulted in cleavage of caspase-3 as evidenced by appearance of 19 kDa intermediate at 16 and 24 h time points (Fig. 7A). Caspase-3 is an executioner caspase that can be activated by (*a*) mitochondrial pathway involving caspase-9 or (*b*) death receptor pathway involving caspase-8 (47–50). Treatment of PC-3 cells with an apoptosis-inducing concentration of PEITC (10 μM) resulted in a significant decrease in the levels of both procaspase-9 and procaspase-8 (Fig. 7B). Role of caspase-9 and caspase-8 in PEITC-induced apoptosis was experimentally verified by using caspase inhibitors. As can be seen in Fig. 7C, the PEITC-induced apoptosis, as determined by quantitation of cytoplasmic histone-associated DNA fragments, was significantly attenuated on treatment of cells with general caspase inhibitor z-VADfmk and specific inhibitors of caspase-8 (z-IETDfmk) and caspase-9 (z-LEHDfmk). Taken together, these results pointed toward involvement of both caspase-8 and caspase-9 pathways in execution of PEITC-induced apoptosis.

Discussion

Cruciferous vegetables, including broccoli, cabbage, watercress, etc., are a rich source of ITCs that are generated on enzymatic hydrolysis of corresponding glucosinolates (6). Animal studies have indicated that ITCs are highly effective in affording protection against chemically induced cancers of various organ sites, including lung, esophagus, mammary gland, colon, and pancreas (11–21). Moreover, epidemiological studies have concluded that increased consumption of cruciferous vegetables may be protective against the risk for prostate cancer (4, 5), which prompted us to hypothesize that ITCs may affect proliferation of prostate cancer cells. The present study was undertaken to

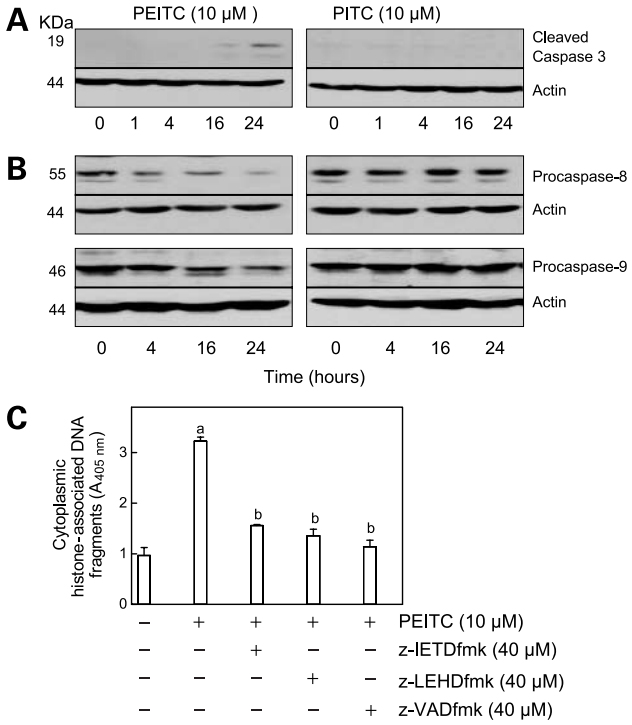

Figure 7. Western blot analysis for (**A**) cleaved caspase-3 and (**B**) procaspase-8 and procaspase-9 using lysates from PC-3 cells exposed to 10 μM PEITC or PITC for different time intervals. Data are representative of at least two independent experiments with similar results. **C,** effect of general caspase inhibitor z-VADfmk, caspase-8 specific inhibitor z-IETDfmk, and caspase-9 specific inhibitor z-LEHDfmk on PEITC-induced apoptosis as determined by quantitation of cytoplasmic histone-associated DNA fragments. *Columns,* mean; *bars,* SE (n = 3). *a*, $P < 0.05$, significantly different compared with DMSO-treated control; *b*, $P < 0.05$, significantly different compared with PEITC alone group (ANOVA followed by Bonferroni's multiple comparison test).

systematically explore this possibility using PEITC, which is one of the best-studied members of the ITC family of cancer chemopreventive agents. We found that PC-3 cells are highly sensitive to growth inhibition by PEITC. Interestingly, proliferation of PC-3 cells was not affected by PITC, which is a close structural analogue of PEITC, except that it lacks the -CH_2 spacers that link the aromatic ring to the -N=C=S moiety. These results are similar to those in Jurkat cells and human head and neck squamous carcinoma cells, which were found to be insensitive to growth inhibition by PITC (22, 51). Collectively, these observations suggest that minor structural alterations in ITCs could have a significant impact on their antiproliferative activity.

Suppression of PC-3 cell viability in the presence of PEITC correlated with a net increase in the fraction of cells with G_2-M-phase DNA content. In agreement with the results of cell proliferation assay, cell cycle distribution was not affected when the PC-3 cells were cultured in the presence of PITC even at 10 μM concentration. PEITC-induced cell cycle arrest was associated with a statistically significant reduction in the protein levels of Cdk1 and Cdc25C, which was prevented in the presence of proteasome inhibitor lactacystin. A reduction in the levels of these proteins is likely to affect the kinase activity of Cdk1/cyclin B1 complex in two ways. First, PEITC treatment may reduce the level of Cdk1/cyclin B1 complex due to down-regulation of Cdk1. Second, PEITC treatment is expected to cause accumulation of phospho-Cdk1 (Tyr^{15}) due to reduction in Cdc25C expression. Although we have not yet examined the effect of PEITC on the level of Cdk1/cyclin B1 complex, an increase in Tyr^{15} phosphorylated (inactive) Cdk1 was observed in PEITC-treated cells when compared with control. Down-regulation of Cdk1 and Cdc25C seems to be a common feature in ITC-induced cell cycle arrest because previous studies from our laboratory indicated that G_2-M-phase arrest in PC-3 cells in the presence of allyl ITC, another naturally occurring ITC analogue, was associated with a reduction in the expression of both these proteins (34). Unlike the results of the present study, however, expression of Cdc25B was also reduced significantly in cells treated with allyl ITC (34). Taken together, these findings suggest that while G_2-M-phase arrest may be a common feature in ITC-treated cells, the mechanism for the cell cycle arrest may be different for structurally divergent ITC analogues.

The PEITC-induced decline in protein levels of Cdk1 and Cdc25C was significantly blocked on treatment of cells with lactacystin, indicating that PEITC treatment promoted proteasome-mediated degradation of both these proteins. Involvement of proteasome system in degradation of Cdc25C has been suggested previously, but the results are inconsistent. For example, Chen *et al.* (52) have provided convincing evidence to indicate that G_2-M-phase arrest in a human bronchial epithelial cell line on treatment with arsenite was associated with proteasome-mediated degradation of Cdc25C. The arsenite-induced Cdc25C degradation was prevented in the presence of lactacystin (52). On the other hand, Savitsky and Finkel (53) have shown that hydrogen peroxide-induced degradation of Cdc25C in HeLa cells could not be rescued by treatment of cells with either proteasomal inhibitors or inhibitors of lysosomal degradation. Instead, these investigators proposed a different mechanism for Cdc25C destruction in hydrogen peroxide-treated cells involving formation of intramolecular disulfide bond between Cys^{377} and Cys^{330} (53). The *in vivo* stability of Cdc25C was substantially reduced by the mutation of either of these two cysteines (53). Because generation of reactive oxygen species in PEITC-treated cells has been reported (32), PEITC-induced destruction of Cdc25C in our model may apparently be mediated by the oxidation of cysteines. However, the results of the present study argue against this possibility because PEITC-induced degradation of Cdc25C was nearly fully blocked by treatment of cells with lactacystin.

Evidence is mounting to indicate that antiproliferative effect of ITCs involves apoptosis induction (22–35). Similar to the results in other cellular systems or with other ITC analogues (22–35), PEITC was able to induce apoptosis in PC-3 cells at growth inhibitory concentrations (present study). The PEITC-induced apoptosis in PC-3 cells was associated with down-regulation of Bcl-2 and Bcl-X_L. To

our surprise, however, overexpression of Bcl-2 did not influence sensitivity of PC-3 cells to PEITC-induced growth inhibition or apoptosis. Further studies are needed to determine if overexpression of Bcl-X_L in PC-3 cells provides protection against PEITC-induced apoptosis. It is important to point out, however, that overexpression of Bcl-2 and Bcl-X_L through transient transfection in human embryonic kidney 293 cells was shown to significantly attenuate PEITC-induced apoptosis (22). The reasons for this discrepancy are not clear, but the contribution of Bcl-2 to apoptosis induction by PEITC, and possibly other ITC analogues, is apparently cell line specific. This premise is supported by the observations that p53 is required for PEITC-induced apoptosis in some but not all cells (23, 24, 28).

Activation of caspases leads to cleavage and inactivation of key cellular proteins such as PARP (47–50). In our model, PARP cleavage was observed within 16 h of PEITC treatment (Fig. 3B), which was accompanied by cleavage of caspase-3 (Fig. 7A). Caspase-3 is an executioner caspase that can be activated by a mitochondrial pathway involving caspase-9 or a death receptor pathway involving caspase-8 (47–50). In the present study, PEITC treatment caused cleavage of both procaspase-9 and procaspase-8. Moreover, the PEITC-induced apoptosis was significantly attenuated in the presence of pan caspase inhibitor and specific inhibitors of caspase-8 and caspase-9. These results suggested involvement of both mitochondrial and death receptor pathways in PEITC-induced apoptosis. Similar conclusions regarding involvement of caspase-9 and/or caspase-8 pathways in apoptosis induction by PEITC has been made in other cellular systems (24, 26, 31, 33). Further characterization of the mitochondrial and death receptor pathways in PEITC-treated cells would establish relative contribution of caspase-9 and caspase-8 cascades to PEITC-induced apoptosis. Nonetheless, involvement of caspases in apoptosis induction by PEITC is clearly not cell line specific.

Growth inhibition, cell cycle arrest, and/or apoptosis induction by PEITC in PC-3 cells was observed at 2.5–10 μM concentrations (present study), which raises the question whether micromolar concentrations of PEITC are achievable in humans to be therapeutically beneficial. An equally important question is whether micromolar levels of PEITC can be achieved through dietary intake of cruciferous vegetables or a pharmacological intervention using pure PEITC may be needed to achieve the therapeutic concentration of this agent. Carefully designed pharmacokinetic studies are needed to systematically address these questions. However, a recent pharmacokinetic study involving four healthy volunteers showed that the maximal plasma concentration of PEITC (C_{max}) following ingestion of 100 g watercress (a rich source of PEITC) ranged between 673 and 1155 nM (mean 928 ± 250 nM) with t_{max} (time to reach C_{max}) of about 2.1 ± 1.1 h (54). A C_{max} between 0.64 and 1.4 μM (mean 1.04 ± 0.22 μM) of total ITC in three subjects taking a single dose PEITC (40 mg) was reported in another study (55).

In conclusion, the present study indicated that (*a*) PEITC effectively inhibited proliferation of PC-3 cells by causing cell cycle arrest in G_2-M-phase and apoptosis induction; (*b*) growth inhibition, cell cycle arrest, or apoptosis induction was not observed in PC-3 cells cultured in the presence of PITC, suggesting that even a subtle change in ITC structure could have a significant impact on its biological activity; (*c*) PEITC-induced cell cycle arrest was associated with proteasome-mediated degradation of Cdk1 and Cdc25C leading to accumulation of Tyr^{15} phosphorylated (inactive) Cdk1; (*d*) sensitivity of PC-3 cells to growth inhibition or apoptosis induction by PEITC was not influenced by overexpression of Bcl-2; and (*e*) PEITC-induced apoptosis was mediated by activation of both caspase-8 and caspase-9 pathways.

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References

1. Verhoeven DT, Goldbohm RA, van Poppel G, Verhagen H, van den Brandt PA. Epidemiological studies on *Brassica* vegetables and cancer risk. Cancer Epidemiol Biomarkers & Prev 1996;5:733–48.

2. Kohlmeier L, Su L. Cruciferous vegetables consumption and colorectal cancer risk: meta-analysis of the epidemiological evidence [abstract]. FASEB J 1997;11:A369.

3. Zhang SM, Hunter DJ, Rosner BA, et al. Intakes of fruits, vegetables, and related nutrients and the risk of non-Hodgkin's lymphoma among women. Cancer Epidemiol Biomarkers & Prev 2000;9:477–85.

4. Cohen JH, Kristal AR, Stanford JL. Fruit and vegetable intakes and prostate cancer risk. J Natl Cancer Inst 2000;92:61–8.

5. Kolonel LN, Hankin JH, Whittemore AS, et al. Vegetables, fruits, legumes and prostate cancer: a multiethnic case-control study. Cancer Epidemiol Biomarkers & Prev 2000;9:795–804.

6. Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 2001;56:5–51.

7. Zhang Y, Talalay P. Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. Cancer Res 1994;54:1976s–81s.

8. Hecht SS. Chemoprevention by isothiocyanates. J Cell Biochem Suppl 1995;22:195–209.

9. Hecht SS. Inhibition of carcinogenesis by isothiocyanates. Drug Metab Rev 2000;32:395–411.

10. Conaway CC, Yang YM, Chung FL. Isothiocyanates as cancer chemopreventive agents: their biological activities and metabolism in rodents and humans. Curr Drug Metab 2002;3:233–55.

11. Wattenberg LW. Inhibition of carcinogenic effects of polycyclic hydrocarbons by benzyl isothiocyanate and related compounds. J Natl Cancer Inst 1977;58:395–8.

12. Futakuchi M, Hirose M, Miki T, Tanaka H, Ozaki M, Shirai T. Inhibition of DMBA-initiated rat mammary tumor development by 1-*O*-hexyl-2,3,5-trimethylhydroquinone, phenethyl isothiocyanate, and novel synthetic ascorbic acid derivatives. Eur J Cancer Prev 1998;7:153–9.

13. Stoner GD, Morrissey DT, Heur YH, Daniel EM, Galati AJ, Wagner SA. Inhibitory effects of phenethyl isothiocyanate on *N*-nitrosobenzylmethylamine carcinogenesis in the rat esophagus. Cancer Res 1991;51:2063–8.

14. Siglin JC, Barch DH, Stoner GD. Effects of dietary phenethyl isothiocyanate, ellagic acid, sulindac and calcium on the induction and progression of *N*-nitrosomethylbenzylamine-induced esophageal carcinogenesis in rats. Carcinogenesis 1995;16:1101–6.

15. Morse MA, Wang CX, Stoner GD, et al. Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced DNA adduct formation and tumorigenicity in the lung of F344 rats by dietary phenethyl isothiocyanate. Cancer Res 1989;49:549–53.

16. Morse MA, Amin SG, Hecht SS, Chung FL. Effects of aromatic isothiocyanates on tumorigenicity, O^6-methylguanine formation, and metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mouse lung. Cancer Res 1989;49:2894–7.

17. Hecht SS, Kenney PM, Wang M, Trushin N, Upadhyaya P. Effects of phenethyl isothiocyanate and benzyl isothiocyanate, individually and in combination, on lung tumorigenesis induced in A/J mice by benzo(*a*)pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Cancer Lett 2000;150:49 – 56.

18. Nishikawa A, Furukawa F, Uneyama C, et al. Chemopreventive effects of phenethyl isothiocyanate on lung and pancreatic tumorigenesis in *N*-nitrosobis(2-oxopropyl)amine-treated hamsters. Carcinogenesis 1996;17:1381 – 4.

19. Jiao D, Smith TJ, Yang CS, et al. Chemopreventive activity of thiol conjugates of isothiocyanates for lung tumorigenesis. Carcinogenesis 1997;18:2143 – 7.

20. Yang YM, Conaway CC, Chiao JW, et al. Inhibition of benzo(*a*)-pyrene-induced lung tumorigenesis in A/J mice by dietary *N*-acetylcysteine conjugates of benzyl and phenethyl isothiocyanates during the postinitiation phase is associated with activation of mitogen-activated protein kinases and p53 activity and induction of apoptosis. Cancer Res 2002; 62:2 – 7.

21. Talalay P, Fahey JW. Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J Nutr 2001;131: 3027s – 33s.

22. Chen YR, Wang W, Kong AN, Tan TH. Molecular mechanisms of c-Jun N-terminal kinase-mediated apoptosis induced by anticarcinogenic isothiocyanates. J Biol Chem 1998;273:1769 – 75.

23. Huang C, Ma WY, Li J, Hecht SS, Dong Z. Essential role of p53 in phenethyl isothiocyanate-induced apoptosis. Cancer Res 1998;58: 4102 – 6.

24. Xu K, Thornalley PJ. Studies on the mechanism of the inhibition of human leukemia cell growth by dietary isothiocyanates and their cysteine adducts *in vitro.* Biochem Pharmacol 2000;60:221 – 31.

25. Gamet-Payrastre L, Li P, Lumeau S, et al. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res 2000;60:1426 – 33.

26. Xu K, Thornalley PJ. Signal transduction activated by the cancer chemopreventive isothiocyanates: cleavage of BID protein, tyrosine phosphorylation and activation of JNK. Br J Cancer 2001;84:670 – 3.

27. Chiao JW, Chung FL, Kancherla R, Ahmed T, Mittelman A, Conaway CC. Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. Int J Oncol 2002;20:631 – 6.

28. Xiao D, Singh SV. Phenethyl isothiocyanate-induced apoptosis in p53-deficient PC-3 human prostate cancer cell line is mediated by extracellular signal-regulated kinases. Cancer Res 2002;62:3615 – 9.

29. Fimognari C, Nusse M, Cesari R, Iori R, Cantelli-Forti G, Hrelia P. Growth inhibition, cell-cycle arrest and apoptosis in human T-cell leukemia by the isothiocyanate sulforaphane. Carcinogenesis 2002;23:581 – 6.

30. Chen YR, Han J, Kori R, Kong AN, Tan TH. Phenylethyl isothiocyanate induces apoptotic signaling via suppressing phosphatase activity against c-Jun N-terminal kinase. J Biol Chem 2002;277:39334 – 42.

31. Hu R, Kim BR, Chen C, Hebbar V, Kong AN. The roles of JNK and apoptotic signaling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells. Carcinogenesis 2003;24:1361 – 7.

32. Rose P, Whiteman M, Huang SH, Halliwell B, Ong CN. β-phenylethyl isothiocyanate-mediated apoptosis in hepatoma HepG2 cells. Cell Mol Life Sci 2003;60:1489 – 503.

33. Zhang Y, Tang L, Gonzalez V. Selected isothiocyanates rapidly induce growth inhibition of cancer cells. Mol Cancer Ther 2003;2:1045 – 52.

34. Xiao D, Srivastava SK, Lew KL, et al. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G_2-M arrest and inducing apoptosis. Carcinogenesis 2003;24:891 – 7.

35. Singh AV, Xiao D, Lew KL, Dhir R, Singh SV. Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts *in vivo.* Carcinogenesis 2004; 25:83 – 90.

36. Srivastava SK, Xiao D, Lew KL, et al. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits growth of PC-3 human prostate cancer xenografts *in vivo.* Carcinogenesis 2003;24:1665 – 70.

37. Morgan DO. Cell cycle control in normal and neoplastic cells. Curr Opin Genet Dev 1992;2:33 – 7.

38. Molinari M. Cell cycle checkpoints and their inactivation in human cancer. Cell Prolif 2000;33:261 – 74.

39. Hartwell LH, Kastan MB. Cell cycle control and cancer. Science 1994;266:1821 – 8.

40. Draetta G, Eckstein J. Cdc25 protein phosphatases in cell proliferation. Biochim Biophys Acta 1997;1332:M53 – 63.

41. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990;348:334 – 6.

42. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456 – 62.

43. Reed JC. Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. Semin Hematol 1997;34:9 – 19.

44. Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. Annu Rev Immunol 1998;16:395 – 419.

45. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281: 1309 – 12.

46. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281:1322 – 6.

47. Thornberry N, Lazebnick Y. Caspases: enemies within. Science 1998; 281:1312 – 6.

48. Wolf BB, Green DR. Suicidal tendencies: apoptotic cell death by caspase family proteinases. J Biol Chem 1999;274:20049 – 52.

49. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999;15:269 – 90.

50. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305 – 8.

51. Lui VWY, Wentzel AL, Xiao D, Lew KL, Singh SV, Grandis JR. Requirement of a carbon spacer in benzyl isothiocyanate-mediated cytotoxicity and MAPK activation in head and neck squamous cell carcinoma. Carcinogenesis 2003;24:1705 – 12.

52. Chen F, Zhang Z, Bower J, et al. Arsenite-induced Cdc25C degradation is through the KEN-box and ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 2002;99:1990 – 5.

53. Savitsky PA, Finkel T. Redox regulation of Cdc25C. J Biol Chem 2002;277:20535 – 40.

54. Ji Y, Morris ME. Determination of phenethyl isothiocyanate in human plasma and urine by ammonia derivatization and liquid chromatography-tandem mass spectrometry. Anal Biochem 2003;323:39 – 47.

55. Liebes L, Conaway CC, Hochster H, et al. High-performance liquid chromatography-based determination of total isothiocyanate levels in human plasma: application to studies with 2-phenethyl isothiocyanate. Anal Biochem 2001;291:279 – 89.