Phenethyl isothiocyanate-induced apoptosis in PC-3 human prostate cancer cells is mediated by reactive oxygen species-dependent disruption of the mitochondrial membrane potential

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The present study was undertaken to gain insights into the molecular mechanism of apoptosis induction by phenethyl isothiocyanate (PEITC), which is a cancer chemopreventive constituent of cruciferous vegetables, using PC-3 human prostate cancer cells as a model. The PEITC-induced cell death in PC-3 cells was associated with disruption of the mitochondrial membrane potential, release of apoptogenic molecules (cytochrome c and Smac/DIABLO) from mitochondria to the cytosol and generation of reactive oxygen species (ROS), which were blocked in the presence of a combined mimetic of superoxide dismutase and catalase (Euk134). Ectopic expression of Bcl-xL, whose protein level is reduced markedly on treatment of PC-3 cells with PEITC, conferred partial protection against PEITC-induced apoptosis only at higher drug concentrations (>10 μM). Administration of 12 μmol PEITC/day (Monday through Friday) by oral gavage significantly retarded growth of PC-3 xenografts in athymic mice. For instance, 31 days after the initiation of PEITC administration, the average tumor volume in control mice (721 ± 153 mm³) was ~2-fold higher compared with mice receiving 12 μmol PEITC/day. The PEITC-mediated inhibition of PC-3 xenograft growth was associated with induction of Bax and Bid proteins. In conclusion, the present study indicates that the PEITC-induced apoptosis in PC-3 cells is mediated by ROS-dependent disruption of the mitochondrial membrane potential and regulated by Bax and Bid.

Introduction

Epidemiological studies continue to support the premise that dietary intake of cruciferous vegetables may be protective against the risk of various types of malignancies including cancer of the prostate (1,2). Anti-carcinogenic effect of cruciferous vegetables is attributed to organic isothiocyanates (ITCs) that occur naturally as thioglucoside conjugates (glucosinolates) in a variety of edible cruciferous vegetables such as broccoli, watercress, cabbage and so forth (3). Organic ITCs are generated due to hydrolysis of corresponding glucosinolates through catalytic mediation of myrosinase, which is released upon damage of the plant cells during processing (cutting or chewing) of cruciferous vegetables. Phenethyl-ITC (PEITC) is one of the best-studied members of the ITC family of compounds that has generated a great deal of research interest due to its cancer chemopreventive activity (4–9). For example, PEITC administration was shown to significantly inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced pulmonary neoplasia in mice (4), N-nitrosobenzylethylamine-induced esophageal cancer in rats (5), and benz[a]pyrene-induced carcinogenesis in mice (8,9). Reduced carcinogen activation due to inhibition of cytochrome P450-dependent monoxygenases and/or increased detoxification of the carcinogenic intermediates due to induction of Phase II enzymes (e.g. glutathione transferases) is believed to be responsible for PEITC-mediated inhibition of chemically-induced cancers in animal models [reviewed in Ref. (3)].

Recent studies have indicated that PEITC and other naturally occurring ITC analogs can suppress proliferation of cancer cells in culture by causing G2/M phase cell cycle arrest and/or apoptosis induction (10–20). Growth inhibition, apoptosis induction and/or cell cycle arrest by PEITC has been observed in human leukemia, hepatoma and prostate cancer cells (10–17). The PEITC-induced cell cycle arrest in PC-3 human prostate cancer cells is associated with a marked reduction in the protein levels of cyclin-dependent kinase 1 (Cdk1) and Cdc25C leading to accumulation of Tyr15 phosphorylated (inactive) Cdk1 (16). The G2/M phase cell cycle arrest in association with reduction in the protein levels of Cdk1 and/or Cdc25C is not unique to PEITC since similar effects have been reported for other naturally occurring ITC analogs including allyl-ITC, benzyl-ITC and sulforaphane (18–20). Because apoptosis induction is now believed to be an important mechanism by which PEITC may inhibit proliferation of cancer cells, an understanding of the mechanism of PEITC-induced cell death is essential for its further development as a clinically useful chemopreventive or therapeutic agent because this knowledge could lead to identification of mechanism-based biomarkers potentially useful in future clinical trials.

Even though PEITC has been shown to cause apoptosis in different cellular systems (10–17), the sequence of events leading to cell death is not fully defined. The present study provides experimental evidence to indicate that the cell death caused by PEITC is initiated by generation of reactive oxygen species (ROS) leading to disruption of the mitochondrial membrane potential and release of apoptogenic molecules (cytochrome c and Smac/DIABLO) from mitochondria to the cytosol. In addition, we show that oral gavage of PEITC significantly inhibits the growth of PC-3 xenografts...
in athymic mice in association with induction of pro-apoptotic Bcl-2 family members Bax and Bid.

Materials and methods

Reagents

Propyl-ITC and butyl-ITC were purchased from Aldrich (Milwaukee, WI, USA), whereas benzyl-ITC, PEITC, phenylpropyl-ITC and phenylhexyl-ITC were procured from LKT Laboratories (St Paul, MN, USA). Tissue culture media, antibiotic mixture and fetal bovine serum (FBS) were from GIBCO (Grand Island, NY, USA), propidium iodide was from Sigma (St Louis, MO, USA), RNase A was from Promega (Madison, WI, USA), and the ELISA kit for quantification of cytoplasmic histone associated DNA fragmentation, a measure of apoptotic cell death, was from Roche Diagnostics (Mannheim, Germany). JC-1 Mitochondrial Membrane Potential Detection Kit was from Cell Technology (Mountain View, CA, USA). Hydroethidine (HE), 6-carboxy-2',7'- dichlorodihydrofluorescein diacetate (H$_2$DCFDA), and anti-cytochrome c oxidase (complex IV) antibody (COX IV; catalog no. A21348) were from Molecular Probes (Eugene, OR, USA). The combined superoxide dismutase/catalase mimetic Euk134 was a gift from Eukarion (Bedford, MA, USA). Antibodies against Bak (clone G-23; catalog no. sc-832), Bax (clone N-20; catalog no. sc-493), Bcl-xL (clone H-5; catalog no. sc-8392), Bok (catalog no. sc-11424), Smac/DIABLO (clone V-17; catalog no. 12683) and Bid (catalog no. sc-6538) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), antibodies specific for detection of cleaved caspase-3 (catalog no. 9661) and cleaved poly(ADP-ribose)polymerase (PARP; catalog no. 9548) were from Cell Signaling Technology (Beverly, MA, USA), anti-cytochrome c antibody (catalog no. 556433) was from BD Pharmingen (San Diego, CA, USA) and anti-actin antibody (catalog no. CP01) was from Oncogene Research Products (Boston, MA, USA). Male athymic mice (6–8 weeks old) were purchased from Taconic (Germantown, NY) and housed in accordance with the Institutional Animal Care and Use Committee guidelines.

Cell culture and cell survival assay

Monolayer cultures of PC-3 cells were maintained in F-12K Nutrient Mixture (Kaighn’s Modification) supplemented with 7% (v/v) non-heat inactivated FBS and antibiotics. Cells were maintained in an atmosphere of 95% air and 5% CO$_2$ at 37°C.

Measurement of mitochondrial membrane potential

Effect of PEITC treatment on mitochondrial membrane potential was measured using potential-sensitive dye JC-1 (5,5',6,6'-tetrachloro-1',3',3'-tetraethylrhodaminecarbocyanine iodide) according to the manufacturer’s instructions. The JC-1 dye bearing a deoxylized positive charge enters the mitochondrial matrix due to the negative charge established by the intact mitochondrial membrane potential (22). In apoptotic cells, JC-1 dye accumulates in the cytoplasm in monomeric form and stains the mitochondria red due to formation of J-aggregates (22). In intracellular ROS generation was measured by flow cytometry following staining with HE and H$_2$DCFDA, which have been shown to be somewhat specific for detection of superoxide anion and peroxides, respectively (23,24). In some experiments, cells were pretreated for 2 h with 30 μM HE and 5 μM H$_2$DCFDA for 30 min at 37°C. The cells were collected, and the fluorescence was measured using a Coulter Epics XL Flow Cytometer. In some experiments, cells were pretreated with 30 μM Euk134 prior to PEITC exposure and analysis of ROS generation.

Detection of apoptosis

Stable transfection of PC-3 cells with Bcl-xL

PC-3 cells were stably transfected with pSSFF-6-Bcl-xL and pSSFF-neo plasmids (a gift from Dr Stanley J. Korsmeyer) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells were grown in medium containing 800 μg G418/ml for 3 weeks. Several G418 resistant clones were expanded and screened for the Bcl-xL protein level by immunoblotting. A clone with overexpression of Bcl-xL was selected for functional studies, and maintained in the presence of 500 μg G418/ml. PC-3 cells transfected with the empty vector were used as control for direct comparison.

Xenograft assay

PC-3 cells were mixed in a 1:1 ratio with Matrigel (Becton Dickinson, Bedford, MA, USA), and a 0.1 ml suspension containing 1 × 10$^6$ cells was injected subcutaneously on both left and right flank of each mouse. Mice were randomized into three groups of 5 mice/group. Experimental animals were treated orally with 9 or 12 mg PEITC/day in 0.1 ml PBS (Monday through Friday) beginning the day of tumor cell implantation. The concentrations of PEITC were selected from previous studies examining cancer chemoprevention by PEITC (8). Control mice received an equal volume of the vehicle. Tumor size was measured as described previously by us (26). Body weights of the control and PEITC-treated mice were recorded once weekly. Mice of each group were also monitored for other symptoms of side effects such as food and water withdrawal and movement. The xenograft assay was repeated at least twice using independently prepared lysates and the results were comparable.

Stable transfection of PC-3 cells with Bcl-xL

PC-3 cells were stably transfected with pSSFF-6-Bcl-xL and pSSFF-neo plasmids (a gift from Dr Stanley J. Korsmeyer) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells were grown in medium containing 800 μg G418/ml for 3 weeks. Several G418 resistant clones were expanded and screened for the Bcl-xL protein level by immunoblotting. A clone with overexpression of Bcl-xL was selected for functional studies, and maintained in the presence of 500 μg G418/ml. PC-3 cells transfected with the empty vector were used as control for direct comparison.
Results

Structure activity relationship

We have shown previously that PEITC inhibits proliferation of PC-3 human prostate cancer cells by causing G2/M phase cell cycle arrest and apoptosis induction (13,16) at concentrations that can be generated through dietary intake of cruciferous vegetables (28,29). Interestingly, we also found that phenyl-ITC, which is a close structural analog of PEITC but lacks the –CH2 spacers that link the aromatic group to the –N=C=S moiety, neither inhibits PC-3 cell viability nor causes cell cycle arrest or apoptosis induction (16). These results suggested that even a subtle change in the ITC structure could have a significant impact on its antiproliferative activity. In the present study, we used ITC analogs with varying alkyl chain length (compare Figure 1A for structures of the ITC analogs) to gain further insights into the structure–activity relationship. The effects of ITC analogs on viability of PC-3 cells were assessed by sulforhodamine B assay following a 48 h drug exposure. The IC50 value (the drug concentration causing 50% inhibition of PC-3 cell viability) was calculated for each ITC compound from a plot of percent cell survival versus ITC concentration, and the results are summarized in Figure 1B. Butyl-ITC and propyl-ITC were relatively less effective against proliferation of PC-3 cells compared with aromatic ITCs. Among the ITCs examined in the present study, maximal growth inhibitory effect was exhibited by PEITC with an IC50 of ~3.3 μM. Moreover, the alkyl chain length markedly affected the activity of aromatic ITCs. For instance, the IC50 for benzyl-ITC, phenylpropyl-ITC and phenylhexyl-ITC was between 1.7- and 3.8-fold higher than that for PEITC (Figure 1B). These results supported our previous conclusion that even a subtle change in ITC structure affects its growth suppressive activity.

PEITC treatment disrupted mitochondrial membrane potential

We have shown previously that PEITC-induced apoptosis in PC-3 cells is associated with activation of caspase-3 and caspase-9 (16). Activation of caspase-9 in response to different stimuli is often associated with disruption of the mitochondrial membrane potential leading to release of cytochrome c and other apoptogenic molecules from mitochondria to the cytosol (30). Once in the cytosol, cytochrome c binds to Apaf-1, and recruits and activates caspase-9 in the apoptosome (30). Active caspase-9 cleaves and activates executioner caspases including caspase-3 (31). Because PEITC treatment caused activation of caspase-9 in PC-3 cells (16), we sought to determine whether PEITC-induced apoptosis was associated with disruption of the mitochondrial membrane potential. The effect of PEITC treatment on mitochondrial membrane potential was determined by flow cytometry following staining with potential sensitive dye JC-1. Representative histograms for red fluorescence (indicator of intact mitochondrial membrane potential) and green fluorescence (indicator of mitochondrial membrane potential collapse) in PC-3 cells following a 6 h treatment with DMSO (control), PEITC or FCCP (positive control) are shown in Figure 2A. In DMSO-treated control PC-3 cells, mitochondria predominantly exhibited red fluorescence due to accumulation of J-aggregates in mitochondria indicating intact mitochondrial membrane potential. PEITC treatment disrupted mitochondrial membrane potential in a concentration-dependent manner as revealed by an increase in green fluorescence resulting from cytosolic accumulation of monomeric JC-1. In time course experiments using 5 μM PEITC, disruption of PC-3 mitochondrial membrane potential was evident as early as 4 h after treatment (data not shown). To determine if PEITC-mediated disruption of the mitochondrial membrane potential was accompanied by cytosolic release of apoptogenic molecules, mitochondrial and cytosolic fractions were prepared from control and PEITC-treated (5 μM) PC-3 cells for immunoblotting of cytochrome c and Smac/DIABLO. As can be seen in Figure 2B, majority of cytochrome c and Smac/DIABLO was accompanied by a reduction in their mitochondrial levels (Figure 2B). The blots were stripped and re-probed with anti-COX IV antibody to ensure equal protein loading as well as to rule out contamination of mitochondrial preparation in cytosolic fraction. We also performed immunoblotting for cytochrome c and...
Smac/DIABLO using mitochondria-free cytosolic fractions prepared from PC-3 cells treated for 4 or 8 h with either DMSO or 10 μM PEITC. As can be seen in Figure 2C, the cytosolic level of cytochrome c as well as Smac/DIABLO was markedly higher in PEITC-treated cells compared with corresponding DMSO-treated controls at both time points. These results indicated that PEITC treatment caused disruption of the mitochondrial membrane potential and cytosolic release of apoptogenic molecules.

**PEITC treatment caused ROS generation**

Next, we tested the hypothesis whether initial signal for PEITC-induced cell death was derived from ROS generation, which is implicated in apoptosis induction by different stimuli including ionizing radiation and hyperoxia (32,33). This possibility was highly likely since PEITC is an electrophilic molecule capable of reacting with cellular nucleophiles including glutathione (34), which could lead to oxidative stress. Intracellular ROS generation in control and
PEITC-treated PC-3 cells was assessed by flow cytometry following staining with HE and H$_2$DCFDA. Representative histograms for ethidium bromide and DCF fluorescence in control and PEITC-treated (5 μM PEITC for 1 or 6 h) PC-3 cells are depicted in Figure 3A. As can be seen in Figure 3B and C, PEITC-treated PC-3 cells exhibited a dose- and time-dependent increase in mean DCF fluorescence compared with control cells. For instance, the mean DCF fluorescence in PC-3 cells treated for 6 h with 1, 2.5 and 5 μM PEITC was increased by about 1.9- to 2.5-fold compared with vehicle-treated control (Figure 3B). In time course experiments using 5 μM PEITC, statistically significant increase in mean DCF fluorescence relative to DMSO-treated control was evident as early as 1 h after treatment ($P < 0.05$ by one-way ANOVA followed by Dunnett’s test), and increased gradually with increasing exposure time (Figure 3C). These results clearly indicated that PEITC treatment resulted in ROS generation in PC-3 cells.

Effect of Bcl-xL overexpression on PEITC-induced cell death
We have shown previously that PEITC treatment causes a marked reduction in the protein levels of Bcl-2 as well as Bcl-xL in PC-3 cells (16), which function as inhibitors of the cell death process (35). We also found that ectopic expression of Bcl-2 in PC-3 cells failed to confer significant protection against PEITC-induced cytoplasmic histone-associated DNA fragmentation (Figure 4B). In addition, Euk134 conferred statistically significant protection against PEITC-induced cytoplasmic histone-associated DNA fragmentation (Figure 4C), which has emerged as a sensitive method for quantification of apoptotic cells. The Euk134 treatment alone neither disrupted mitochondrial membrane potential nor caused cytoplasmic histone-associated DNA fragmentation (Figure 4B and C). Collectively, these results indicated that PEITC-induced apoptosis in PC-3 cells was initiated by ROS, which caused disruption of the mitochondrial membrane potential leading to release of apoptogenic molecules to the cytosol.
present study, we addressed this question by determining the effect of ectopic expression of Bcl-xL, through stable transfection in PC-3 cells, on cell death caused by PEITC. Figure 5A depicts immunoblotting for Bcl-xL protein in vector-transfected control cells (hereafter abbreviated as PC-3/neo cells; lane 1) and in Bcl-xL-transfected PC-3 cells (PC-3/Bcl-xL cells; lane 2) confirming Bcl-xL overexpression in PC-3/Bcl-xL cells. Next, we compared sensitivities of PC-3/neo and PC-3/Bcl-xL cells toward PEITC-induced apoptosis by determining cytoplasmic histone-associated DNA fragmentation. As can be seen in Figure 5B, relative to DMSO-treated control, the PEITC treatment resulted in a concentration-dependent increase in cytoplasmic histone-associated DNA fragmentation in both vector-transfected control cells and PC-3/Bcl-xL cells. The PEITC-mediated DNA fragmentation did not differ significantly between PC-3/neo and Bcl-xL cells at 2.5 or 5 μM concentrations. On the other hand, the Bcl-xL overexpressing PC-3 cells were significantly more resistant to DNA fragmentation caused by 10 and 20 μM PEITC compared with PC-3/neo. For example,
treatment of PC-3/neo cells with 10 and 20 μM PEITC for 24 h resulted in ~3.6- and 4.8-fold increase in cytoplasmic histone-associated DNA fragmentation, respectively, compared with DMSO-treated control. The PEITC-mediated increase in DNA fragmentation at similar concentrations relative to DMSO-treated control in PC-3/Bcl-xL cells ranged between 2.6- and 3.2-fold (Figure 5B). The extent of PEITC-induced DNA fragmentation at 10 and 20 μM concentrations was statistically significantly lower in PC-3/Bcl-xL cells than in PC-3/neo (Figure 5B).

Resistance of PC-3/Bcl-xL cells to cell death caused by higher concentrations of PEITC was confirmed by analysis of sub-diploid cells, which is another widely used technique for detection of apoptotic cells. The sub-diploid fraction in cultures of PC-3/neo and PC-3/Bcl-xL cells following a 24 h exposure to 5 or 10 μM PEITC was determined by flow cytometry, and data are summarized in Figure 5C. The PEITC treatment resulted in a concentration-dependent increase in percentage of sub-diploid fraction in both PC-3/neo and PC-3/Bcl-xL cultures. Consistent with the results of cytoplasmic histone-associated DNA fragmentation assay (Figure 5B), the PEITC-mediated increase in sub-diploid fraction (apoptotic cells) was comparable in PC-3/neo and PC-3/Bcl-xL cells at 5 μM concentration. On the other hand, the PEITC-mediated accumulation of sub-diploid cells at 10 μM concentration was ~1.8-fold higher (P < 0.05 by paired t-test) in PC-3/neo cells than in the PC-3/Bcl-xL (Figure 5C). These results indicated that Bcl-xL overexpression protected against PEITC-induced apoptosis only at higher drug concentrations.

We have shown previously that the PEITC-induced apoptosis in PC-3 cells is associated with cleavage of caspase-3 and PARP (16). We therefore explored the possibility whether Bcl-xL overexpression affected PEITC-mediated cleavage of caspase-3 and PARP. We addressed this possibility by determining the effect of PEITC treatment on cleavage of caspase-3 and PARP by immunoblotting, and representative data are shown in Figure 6. The PEITC-mediated cleavage of caspase-3 and PARP at both 5 μM (Figure 6A) and 10 μM concentrations (Figure 6B) was relatively more pronounced in PC-3/neo cells than in Bcl-xL overexpressing PC-3 cells. For instance, the cleavage of PARP following treatment with 10 μM PEITC for 4, 8, 16 and 24 h was statistically significantly higher in PC-3/neo cells (P < 0.05 by one-way ANOVA followed by Tukey’s multiple comparison test) than in PC-3/Bcl-xL as judged by densitometric scanning of the immunoreactive bands after correction for actin loading control. The difference in PEITC-mediated cleavage of caspase-3 between PC-3/neo and PC-3/Bcl-xL cells, however, did not reach statistical significance due to inter-experimental data scatter. Nonetheless, these results indicated that Bcl-xL overexpression conferred partial protection against PEITC-induced cleavage of caspase-3 and PARP especially at 10 μM concentration.

**PEITC administration inhibited PC-3 xenograft growth**

Cellular systems are valuable in obtaining mechanistic insights not accessible otherwise. We already know that the viability of PC-3 cells in culture is reduced significantly in the presence of PEITC (Figure 1B). The observations made in cells, however, need to be confirmed using appropriate animal models to determine in vivo relevance of the cellular findings. Moreover, in vivo efficacy testing of potential anticancer agents is a prerequisite for their further clinical development. We therefore determined the effect of PEITC administration by oral gavage on growth of PC-3 xenografts in male athymic mice using concentrations employed previously in cancer chemoprevention studies (8), and the results are shown in Figure 7A. Even though the average tumor volume in mice treated with 9 μmol PEITC/day (Monday through Friday) was generally lower compared with control mice on each day of tumor measurement, the difference was statistically significant only on day 16 after tumor cell implantation (P < 0.05 by one-way ANOVA followed by Dunnett’s test). On the other hand, the average tumor volume in mice treated with 12 μmol PEITC/day was statistically significantly lower compared with control mice on each day of tumor measurement except on day 25 after tumor cell implantation. For example, on day 21, the average tumor volume in mice treated with 12 μmol PEITC was 57% lower compared with control mice (412 ± 99 mm3; P < 0.05 by one-way ANOVA followed by Dunnett’s test). Similarly, 31 days after the initiation of PEITC administration, the average tumor volume in mice treated with 12 μmol PEITC was ~57% lower compared with control mice (412 ± 99 mm3; P < 0.05 by one-way ANOVA followed by Dunnett’s test).
even on day 31. The body weights of the control and PEITC-treated mice did not differ significantly at the start of the experiment as well as immediately prior to the termination of the experiment (data not shown). Moreover, the mice of PEITC group did not exhibit any other signs of toxicity such as impaired movement and posture, indigestion or diarrhea and areas of redness or swelling. These results indicated that PEITC administration significantly inhibited PC-3 xenograft growth without causing any side effects to the mice.

The Bcl-2 family proteins have emerged as critical regulators of apoptosis by diverse stimuli (35). To gain insights into the mechanism of PEITC-mediated suppression of PC-3 xenograft growth, we compared the levels of Bcl-2 family of pro-apoptotic and anti-apoptotic proteins in tumors harvested from the control and PEITC-treated mice at the time of killing. Representative blots for Bak, Bax, Bcl-xL, Bok and Bid are shown in Figure 7B. Change in protein level was determined by densitometric scanning of the immunoreactive bands and corrected for actin loading control. As can be seen from Figure 7B and C, the level of Bak protein was statistically significantly lower in tumors of PEITC-treated mice compared with control. On the other hand, Bax protein level was significantly higher in tumors of mice treated with 12 μmol PEITC, but not 9 μmol group, compared with control (Figure 7C). The tumors of PEITC-treated mice revealed an increasing trend in the protein levels of Bcl-xL.

![Image](http://carcin.oxfordjournals.org/)

**Fig. 7.** (A) Average tumor volume in vehicle-treated control mice and mice treated daily (Monday through Friday) with 9 μmol PEITC or 12 μmol PEITC. Data are mean ± SE (n = 10; 5 mice/group with tumors implanted on both left and right flank of each mouse). *P < 0.05, significantly different compared with control by one-way ANOVA followed by Dunnett’s test. The tumor volume did not differ significantly between 9 and 12 μmol PEITC-treated groups at any time point. (B) Immunoblotting for Bcl-2 family proteins using lysates from tumor tissues harvested from control and PEITC-treated mice at the time of killing. The blots were stripped and re-probed with anti-actin antibody to correct for differences in protein loading. (C) Densitometric scanning data for Bcl-2 family protein levels in tumors of control and PEITC-treated mice. Tumor tissues from four mice of each group were used for immunoblotting. Data are mean ± SE (n = 4). *P < 0.05, significantly different compared with control by one-way ANOVA followed by Dunnett’s test.
and a decreasing trend in the level of Bok protein compared with control tumor, although the differences were statistically insignificant at \( P = 0.05 \). However, the tumors of mice treated with 12 \( \mu \)mol PEITC exhibited a >3-fold increase in Bid protein level compared with control tumors (Figure 7C).

**Discussion**

Cruciferous vegetables such as watercress and broccoli are rich sources of ITCs that are highly effective in affording protection against cancer in animal models induced by structurally different chemical carcinogens (4-9). Our interest in ITCs stemmed from epidemiological data documenting an inverse correlation between dietary intake of cruciferous vegetables and the risk of prostate cancer (2), which prompted us to determine the sensitivity of prostate cancer cells toward PEITC. Indeed, we found that PEITC suppresses proliferation of PC-3 human prostate cancer cells as well as cell lines (TRAMP-C1 and TRAMP-C2) derived from spontaneously developing prostate tumor of a transgenic mouse (transgenic adenocarcinoma of the mouse prostate; commonly abbreviated as TRAMP) (13,16,17). In the present study, we extended these findings and determined the effects of several ITC analogs differing in alkyl chain length on proliferation of PC-3 cells. We found that a subtle change in the ITC structure could have a significant impact on its anti-proliferative activity. For instance, the IC\(_{50}\) for butyl-ITC and propyl-ITC is between 7.8- and 14.8-fold higher compared with that for PEITC (Figure 1B). In addition, the alkyl chain length has a marked effect on the activity of aromatic ITCs against proliferation of PC-3 cells. Thus, the IC\(_{50}\) for PEITC is between 43 and 74% lower compared with those of other aromatic ITCs examined in the present study.

Cellular effects of PEITC (e.g. cell killing, ROS generation, disruption of the mitochondrial membrane potential and apoptosis induction) in PC-3 cells are evident at 1-5 \( \mu \)M concentrations. The maximal plasma concentration of PEITC (C\(_{\text{max}}\)) following ingestion of 100 g watercress ranges between 673 and 1155 nM (mean 928 ± 250 nM) with \( t_{\text{max}}\) (time to reach C\(_{\text{max}}\)) of ~2.1 ± 1.1 h (36). A C\(_{\text{max}}\) between 0.64 and 1.4 \( \mu \)M (mean 1.04 ± 0.22 \( \mu \)M) of total ITC in three subjects taking a single dose PEITC (40 mg) was reported in another study (37). Previous studies from our laboratory have revealed that even clinically achievable concentration of PEITC (1 \( \mu \)M) causes a statistically significant reduction in cell viability due to apoptosis induction in TRAMP-derived prostate cancer cells, although a longer exposure time (48 and 72 h) is necessary to observe these effects (17). Further studies are needed to determine whether clinically achievable concentration of PEITC is effective in causing apoptosis in PC-3 cells, which is likely because cell growth inhibition (results not shown), disruption of the mitochondrial membrane potential (Figure 2A) and ROS generation (Figure 3B) are evident in PC-3 cells treated with 1 \( \mu \)M PEITC.

The present study reveals that PEITC treatment causes ROS production leading to mitochondrial membrane potential disruption. Because PEITC-mediated disruption of the mitochondrial membrane potential as well as cytoplasmic histone-associated DNA fragmentation are significantly attenuated in the presence of a superoxide dismutase and catalase mimetic (Euk134), it is reasonable to conclude that the cell death caused by PEITC is probably triggered by ROS generation. We have shown previously that ROS generation is a critical event in the initiation of cell death by sulforaphane, which is another naturally occurring ITC compound (38). However, the precise mechanism of ROS generation by ITCs remains to be elucidated.

Because ROS generation is implicated in pathogenesis of many chronic diseases including cancer, the potential side effects of PEITC-mediated ROS production cannot be ignored. Based on the results of the present study and previous findings from our laboratory and by other investigators, we are tempted to speculate that ROS generation by PEITC may not be harmful because (i) PEITC is a dietary agent abundant in many cruciferous vegetables consumed by humans on a daily basis yet epidemiological studies continue to support the premise that dietary intake of ITC-containing cruciferous vegetables may reduce the risk of different types of malignancies including cancer of the prostate (1,2); (ii) the PEITC-treated athymic mice do not exhibit weight loss or any other signs of toxicity (present study); and (iii) We have reported previously that a normal prostate epithelial cell line (PrEC) is significantly more resistant to apoptosis induction by PEITC as well as sulforaphane compared with prostate cancer cells (17,39). It is possible that the PEITC-mediated ROS generation in cancer cells is transient and serves to trigger the apoptosis signaling cascade. Likewise, the possibility that normal epithelial cells are relatively more resistant to PEITC-mediated ROS generation compared with malignant cells cannot be ruled out, which may explain resistance of normal epithelial cells toward cell death caused by PEITC (17,39). However, further studies are needed to systematically explore these possibilities.

We have shown previously that apoptosis induction by PEITC in PC-3 and TRAMP-derived cell lines correlates with changes in the protein levels of Bcl-2 family proteins (16,17). The Bcl-2 family proteins have emerged as critical regulators of apoptosis by functioning as either promoters (e.g. Bax and Bak) or inhibitors (e.g. Bcl-2 and Bcl-xL) of the cell death process (35). Differential interaction among Bcl-2 protein family members as well as their association with other cellular proteins regulates the cell death process. For example, Bcl-2 normally blocks apoptosis by forming heterodimer complex with pro-apoptotic proteins such as Bax (35). The PEITC-induced apoptosis in PC-3 and TRAMP-derived prostate cancer cells correlates with induction of Bak (TRAMP derived cells) and/or downregulation of Bcl-2 (PC-3) or Bcl-xL (PC-3 and TRAMP-derived cells) (16,17). We showed further that the SV40 immortalized mouse embryonic fibroblasts (MEFs) derived from Bax and Bak double-knockout mice are statistically significantly more resistant to PEITC-induced cell death compared with wild-type MEFs (17). Interestingly, we also found that Bcl-2 overexpression fails to confer protection against PEITC-induced apoptosis in PC-3 cells (16). These observations prompted us to determine whether Bcl-xL expression affects PEITC-induced apoptosis. The present study indicates that Bcl-xL overexpression confers partial yet statistically significant protection against PEITC-induced cell death only at higher drug concentrations. Interestingly, transient transfection of both Bcl-2 and Bcl-xL in 293 cells has been shown to significantly attenuate PEITC-induced apoptosis (10). Thus, it is reasonable to postulate that the contribution of Bcl-2 and Bcl-xL in regulation of PEITC-induced apoptosis is probably cell line specific.
Because pre-clinical in vivo efficacy testing of potential anticancer agents in appropriate animal models is a prerequisite for their clinical development, we proceeded to determine whether PEITC administration affects growth of PC-3 xenografts in athymic mice. The concentrations of the PEITC used in the present study (9 and 12 μmol) were selected from published studies examining the effect of PEITC on chemically induced cancers in animal models (8). The present study reveals that oral gavage of 12 μmol PEITC/day (Monday through Friday) significantly inhibits the growth of PC-3 xenografts in athymic mice without causing weight loss or any other side effects. During the preparation of our manuscript, it was shown that i.p. injection of even 5 μmol PEITC significantly retarded growth of PC-3 xenografts in athymic mice (40). It is important to point out that the concentrations of PEITC effective against PC-3 xenograft growth [present study and Ref. (40)] are within the range that can be generated through dietary intake of cruciferous vegetables (28,29). For instance, consumption of one ounce of watercress is estimated to yield ~60 μmol PEITC (28).

Analysis of Bcl-2 family protein levels in tumors of control and PEITC-treated mice harvested at the termination of the experiment (31 days after tumor cell implantation) reveals that the PEITC-mediated changes observed in cells may not be reflected in vivo. For example, unlike cellular data (16,17), tumors of PEITC-treated mice exhibit an increase in the protein levels of Bcl-xL compared with control tumors. Similarly, the tumors of PEITC-treated mice exhibit a decrease, not an increase which is observed in cells (17), in the protein levels of Bak compared with control tumors. Several possibilities need to be systematically investigated to explain inconsistencies in in vitro and in vivo results concerning effect of PEITC on Bcl-2 family protein expression. One possibility is that the concentration of PEITC required to down-regulate Bcl-xL expression or to increase the level of Bak protein is not achieved in vivo. It is also possible that lower concentrations of PEITC may have different effects on Bcl-xL and Bak protein levels than those observed at higher concentrations. Recent unpublished studies from our laboratory have revealed that PEITC analog sulforaphane exhibits a biphasic response on Bcl-xL protein expression in LNCaP human prostate cancer cells; induction of Bcl-xL followed by a decline in its protein level which correlates with a biphasic response on transcriptional activity of transcription factor NF-κB (S. V. Singh, unpublished data). Exposure of cultured cancer cells to ITCs can lead to very high intracellular accumulation of ITCs in mM range (41), which may not be possible in vivo due to rapid excretion of the thiol-conjugates of ITCs. Glutathione transferase catalyzed conjugation with glutathione followed by generation of N-acetylcycteine conjugates via the mercapturic acid pathway is believed to be the primary route of ITC metabolism in vivo (28,42,43). Recent studies have revealed that the N-acetylcycteine conjugates of ITCs retain their cell growth inhibitory and apoptosis-inducing ability presumably due to de-conjugation of the ITC-thiol conjugates (41,44). The N-acetylcycteine conjugates of certain ITCs including PEITC also display chemopreventive activity against chemically induced cancers in animal models (45–47). Thus, the difference in the metabolism of ITCs between cultured cancer cells versus in vivo or differential effect of free ITCs versus their thiol conjugates on expression of Bcl-2 family proteins are other likely possibilities that requires further investigation.

The PEITC-mediated suppression of PC-3 xenograft growth in athymic mice is associated with induction of Bax, which is a multidomain pro-apoptotic Bcl-2 family member that functions to promote cell death by neutralizing anti-apoptotic effect of Bcl-2 (48). The MEFs lacking Bak and Bak proteins are significantly more resistant to apoptosis induction by different agents compared with wild-type MEFs (49). We have shown previously that SV40 immortalized MEFs derived from Bax and Bak double-knockout mice are also resistant to apoptosis induction by PEITC and sulforaphane (17,39). Thus, it is reasonable to propose that Bax induction may be an important event in PEITC-mediated suppression of PC-3 xenograft growth.

The tumors of 12 μmol PEITC-treated mice also exhibit statistically significant induction of Bid when compared with control tumors. Bid is a BH-3 only (Bcl-2 homology domain-3) pro-apoptotic Bcl-2 family member that is predominantly localized in the cytoplasm (50). Caspase-8 activation can cause cleavage of Bid and cleaved Bid translocates to the mitochondria to trigger cytochrome c release and activation of caspase-9. We have shown previously that PEITC treatment causes cleavage of procaspase-8 suggesting activation in PC-3 cells, and that the PEITC-induced cell death is significantly inhibited in the presence of caspase-8 specific inhibitor (16). Cleavage of Bid on treatment with PEITC has been reported in HL-60 cells (12). Thus, induction of Bid in tumors of PEITC-treated mice is likely to contribute to its anticancer effect against PC-3 xenograft. Even though we have not yet determined the effect of Bid deficiency on PEITC-induced cell death, the SV40 immortalized MEFs derived from Bid knockout mice are statistically significantly more resistant to the cell death caused by sulforaphane compared with wild-type MEFs (38).

In conclusion, the results of the present study indicate that PEITC inhibits proliferation of PC-3 cells in culture as well as in vivo. In addition, we provide experimental evidence to indicate that the PEITC-induced apoptosis is initiated by generation of ROS.

Acknowledgements

The authors thank Dr Stanley J. Korsmeyer for the gift of pSFFV-neo and pSFFV-Bcl-xL plasmids. This investigation was supported in part by USPHS grants CA101753 and CA115498 awarded by the National Cancer Institute.

Conflict of Interest Statement: None declared.

References

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Received January 26, 2006; revised April 13, 2006; accepted May 19, 2006