Caspase-Dependent Apoptosis Induction by Phenethyl Isothiocyanate, a Cruciferous Vegetable–Derived Cancer Chemopreventive Agent, Is Mediated by Bak and Bax

Dong Xiao,[1] Yan Zeng,[1] Sunga Choi,[1] Karen L. Lew,[1] Joel B. Nelson,[2] and Shivendra V. Singh[1,2]

Abstract

Purpose: The present study was undertaken to gain insights into the molecular mechanism of apoptosis induction by phenethyl isothiocyanate (PEITC) using prostate cancer cell lines derived from transgenic adenocarcinoma mouse prostate (TRAMP) mice (TRAMP-C1 and TRAMP-C2).

Experimental Design and Results: The viability of TRAMP-C1 and TRAMP-C2 cells was reduced significantly in the presence of PEITC in a concentration-dependent manner as determined by sulforhodamine B and trypan blue dye exclusion assays. Treatment of TRAMP-derived cells with PEITC revealed features characteristic of apoptosis induction, including appearance of subdiploid cells (determined by flow cytometry), cytoplasmic histone-associated DNA fragmentation (determined by an ELISA assay), and cleavage of caspase-3 (determined by immunoblotting). The PEITC-induced apoptosis in TRAMP-derived cells was associated with a marked increase in the level of proapoptotic protein Bak and/or a decrease in the levels of antiapoptotic protein Mcl-1 or Bcl-x_L and disruption of mitochondrial membrane potential. The SV40 immortalized mouse embryonic fibroblasts derived from Bak and Bax double knockout mice were significantly more resistant to PEITC-induced DNA fragmentation compared with wild-type or Bak$^{-/-}$ mouse embryonic fibroblasts. The PEITC-induced apoptosis in both cell lines was significantly attenuated in the presence of caspase inhibitors zVAD-fmk, zLEHD-fmk, and zIETD-fmk. Oral administration of PEITC (9 or 12 μmol PEITC/d, Monday-Friday) significantly retarded growth of TRAMP-C1 xenografts in nude mice without causing weight loss or any other side effects.

Conclusion: The results of the present study indicate that caspase-dependent apoptosis by PEITC is mediated by Bak and Bax proteins.

Epidemiologic data continue to support the contention that dietary intake of cruciferous vegetables may be protective against the risk of various types of malignancies, including cancer of the prostate (1–5). Antineoplastic effect of cruciferous vegetables is attributed to isothiocyanates, which occur as thioglucoside conjugates (glucosinolates) in a variety of edible plants, including broccoli, watercress, cabbage, etc. (6–10). Organic isothiocyanates are generated due to hydrolysis of corresponding glucosinolates through catalytic mediation of myrosinase, which is released on damage of the plant cells during processing (cutting or chewing) of cruciferous vegetables (6, 9, 10). Phenethyl isothiocyanate (PEITC) is one such naturally occurring isothiocyanate compound that has attracted a great deal of attention due to its remarkable cancer chemopreventive activity (11–20). For example, PEITC administration has been shown to significantly inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced pulmonary neoplasia in rats and mice (12, 13), *N*-nitrosobenzylmethylamine-induced esophageal cancer in rats (14, 15), and benzo[*a*]pyrene-induced carcinogenesis in mice (19). The mechanism by which PEITC inhibits chemically induced cancer involves inhibition of carcinogen activation due to inhibition of cytochrome *P*450–dependent monooxygenases and increased carcinogen inactivation due to induction of phase II enzymes, including glutathione transferases (reviewed in refs. 7, 8, 10, 21).

Evidence is accumulating to indicate that PEITC can suppress proliferation of cancer cells in culture by causing apoptosis and/or cell cycle arrest (22–32). Growth inhibition, apoptosis induction, and/or cell cycle arrest by PEITC has been noted in human leukemia, prostate, myeloma, hepatoma, and colon cancer cells (22–32). Chen et al. (22) were the first to show apoptosis induction in association with sustained activation of c-Jun NH_2-terminal kinase in PEITC-treated Jurkat cells. Subsequently, Huang et al. (23) provided convincing evidence to suggest a critical role for p53 in apoptosis induction by PEITC, but this hypothesis was not supported in other cellular systems (24, 27, 29). Instead, the PEITC-induced apoptosis in p53-deficient PC-3 human prostate cancer cell line was associated with a rapid and sustained activation of extracellular

Authors' Affiliations: Departments of [1]Pharmacology, and [2]Urology, and University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

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Requests for reprints: Shivendra V. Singh, Hillman Cancer Center Research Pavilion, Suite 2.32A, University of Pittsburgh Cancer Institute, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-623-3263; Fax: 412-623-7828; E-mail: singhs@upmc.edu.

signal-regulated kinases (27). More recent studies have indicated that PEITC-induced cell death involves mitochondria-mediated activation of caspase cascade (30–32). A role for the extrinsic caspase pathway in cell death caused by PEITC has also been suggested in some systems (25, 31, 32). Despite these advances, however, not much is known about the upstream pathways or molecules involved in regulation of PEITC-induced caspase activation and apoptosis.

Members of the Bcl-2 family proteins have emerged as important regulators of caspase activation and apoptotic death in response to various stimuli (33–36). Previous studies from our laboratory indicated that although exposure of PC-3 human prostate cancer cell line to growth suppressive concentrations of PEITC resulted in a marked decline in Bcl-2 protein level, ectopic expression of Bcl-2 failed to confer significant protection against cell death caused by PEITC (32). Here, we provide experimental evidence to implicate Bak and Bax proteins in PEITC-mediated apoptosis. Furthermore, the present study shows that the growth of a prostate tumor cell line implanted in nude mice is inhibited significantly on oral administration of PEITC.

Materials and Methods

Reagents. PEITC was purchased from Sigma-Aldrich (Milwaukee, WI). Tissue culture medium and fetal bovine serum were from Life Technologies (Grand Island, NY), propidium iodide was from Sigma (St. Louis, MO), RNase A was from Promega (Madison, WI), tetramethyl rhodaminemethyl ester (TMRME) was from Molecular Probes (Eugene, OR), and ApoTag Plus Peroxidase *In situ* Apoptosis detection kit for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was from Intergen (Purchase, NY). Antibodies against Bak (clone G-23), Bax (clone N-20), Bcl-x_L (clone H-5), and Mcl-1 (clone S-19) were from Santa Cruz Biotechnology (Santa Cruz, CA); antibody specific for caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) was from Cell Signaling Technology (Beverly, MA); antibody against cytochrome *c* was from BD PharMingen (San Diego, CA); and anti-actin antibody was from Oncogene Research Products (Boston, MA). The caspase inhibitors zVAD-fmk (general caspase inhibitor), zIETD-fmk (caspase-8), and zLEHD-fmk (caspase-9) were from Enzyme Systems (Dublin, CA).

Cell culture and cell survival assays. Monolayer cultures of TRAMP-C1 and TRAMP-C2 cells (37), a generous gift from Dr. Barbara Foster (Roswell Park Cancer Institute, Buffalo, NY), were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 10^{-8} mol/L 5α-androstan-17β-ol-3-one, and antibiotics. Mouse embryonic fibroblasts (MEF) from wild-type, Bak knockout ($Bak^{-/-}$), and Bak-Bax double knockout mice ($Bak^{-/-}Bax^{-/-}$) and immortalized by transfection with a plasmid containing SV40 genomic DNA were generously provided by Dr. Stanley J. Korsmeyer (Dana-Farber Cancer Institute, Boston, MA) and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mmol/L nonessential amino acids, 0.1 μmol/L 2-mercaptoethanol, and antibiotics (38). The PrEC normal prostate epithelial cell line (Clonetics, San Diego, CA) was maintained in PrEBM (Cambrex, Walkersville, MD). Each cell line was maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The effect of PEITC on cell viability was determined by sulforhodamine B and trypan blue dye exclusion assays as described previously (27, 39).

Analysis of cell cycle distribution and apoptosis induction. Effect of PEITC treatment on cell cycle distribution was determined by flow cytometry following staining with propidium iodide as described previously (40). Briefly, the desired cells (5×10^5) were seeded in T25 flasks and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentrations of PEITC. Stock solution of PEITC was prepared in DMSO and diluted with complete medium. An equal volume of DMSO (final concentration, 0.2%) was added to the controls. Following incubation at 37°C for 24 hours, floating and attached cells were collected, washed with PBS, and fixed with 70% ethanol. Fixed cells were then treated with 80 μg/mL RNase A and 50 μg/mL propidium iodide for 30 minutes, and stained cells were analyzed using a Coulter Epics XL Flow Cytometer (Miami, FL). Cells in different phases of the cell cycle, including apoptotic (sub-G_0-G_1) cells, were computed for control (DMSO-treated) and PEITC-treated cultures. Apoptosis induction by PEITC was confirmed by analysis of cytoplasmic histone-associated DNA fragmentation as described previously (39). In some experiments, cells were pretreated with 40 μmol/L pan-caspase inhibitor zVAD-fmk, 40 μmol/L caspase-9 specific inhibitor zLEHD-fmk, or 40 μmol/L caspase-8 specific inhibitor zIETD-fmk for 2 hours before PEITC treatment and assessment of apoptosis.

Immunoblotting. Control and PEITC-treated cells were lysed as described previously (27, 39, 40). The cell lysate was cleared by centrifugation at 19,000 × *g* for 15 minutes, and the supernatant fraction was used for immunoblotting. For immunoblotting of cytochrome *c*, mitochondria-free cytosolic fraction from control and PEITC-treated TRAMP-C1 cells was prepared using a kit from Oncogene Research Products. Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk in TBS containing 0.05% Tween 20, the membrane was incubated with the desired primary antibody for 1 hour. The membrane was then treated with appropriate secondary antibody, and the immunoreactive bands were visualized by enhanced chemiluminescence method. Each membrane was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was measured using fluorescent lipophilic cationic dye TMRME, which accumulates within mitochondria in a potential-dependent manner. Briefly, TRAMP-C1 cells (5×10^5) were plated in T25 flasks, allowed to attach overnight, exposed to desired concentrations of PEITC for desired time period, and collected by trypsinization. The cells were resuspended in growth medium (1 mL) and stained with 0.2 μmol/L TMRME for 15 minutes at 37°C in the dark. The cells were washed twice with ice-cold PBS, and fluorescence was measured using a Coulter Epics XL Flow Cytometer. Mitochondrial decoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (25 μmol/L) was included as a positive control.

Xenograft assay. Male athymic mice (6 weeks old) were purchased from Taconic (Germantown, NY). TRAMP-C1 cells were mixed in a 1:1 ratio with Matrigel (Becton Dickinson, Bedford, MA), and a 0.1 mL suspension containing 2×10^6 cells was injected s.c. on both left and right flanks of each mouse. Mice were randomized into four groups (five mice per group). Experimental animals were treated orally with 6, 9, or 12 μmol PEITC/d in 0.1 mL PBS from Monday to Friday beginning the day of tumor cell implantation. The concentrations of PEITC were selected based on previous studies examining cancer chemoprevention by PEITC (19). Control mice received an equal volume of the vehicle. Tumor size was measured as described by us previously (39). 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 intake and movement. Data were analyzed by one-way ANOVA followed by Bonferroni's test for multiple comparisons to assess statistical significance of difference in tumor volume. At the termination of the experiment, the tumor tissues from control and PEITC-treated mice were harvested and processed for immunohistochemical analysis of apoptotic bodies (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay) as described by us previously (39).

Results

Phenethyl isothiocyanate reduced viability of TRAMP-derived cells by causing apoptosis. We have shown previously that the viability of PC-3 human prostate cancer cell line is reduced

significantly in the presence of PEITC (27, 32) at concentrations that can be generated through dietary intake of cruciferous vegetables (41, 42). In the present study, we extended these findings and determined the effect of PEITC treatment on survival of TRAMP-C1 and TRAMP-C2 cell lines to rule out a possibility that inhibitory effect of PEITC against proliferation of PC-3 was cell line specific. TRAMP-C1 and TRAMP-C2 cell lines were established from spontaneously developing prostate tumor of a 32-week-old transgenic adenocarcinoma of the mouse prostate (TRAMP) model (37). Both cell lines were shown to be epithelial in origin (cytokeratin positive) and positive for androgen receptor. In addition, TRAMP-C1 and TRAMP-C2 cells have a similar doubling time of ~24 hours (37). The effect of PEITC treatment on viability of TRAMP-C1 and TRAMP-C2 cells was assessed by sulforhodamine B (Fig. 1A) and trypan blue dye exclusion assays (Fig. 1B). As can be seen in Fig. 1A, the viability of TRAMP-C1 and TRAMP-C2 cells was reduced significantly on a 24-hour exposure to PEITC in a concentration-dependent manner with an IC_{50} of ~5 μmol/L. Consistent with the results of sulforhodamine B assay (Fig. 1A), trypan blue dye exclusion assay revealed a dose-dependent inhibition of TRAMP-C1 and TRAMP-C2 cell proliferation in the presence of PEITC (Fig. 1B). We also determined the sensitivity of a normal prostate epithelial cell line (PrEC) to growth inhibition by PEITC, and the results are shown in Fig. 1C. The PrEC cells were relatively more resistant to cell killing by PEITC compared with TRAMP-derived cell lines. For instance, the survival of TRAMP-derived cells was reduced by ~50% on a 24-hour exposure to 5 μmol/L PEITC as judged by sulforhodamine B assay (Fig. 1A). On the other hand, the viability of PrEC cells was minimally affected by a similar treatment with PEITC (24-hour treatment with 5 μmol/L PEITC; Fig. 1C). Even at 20 μmol/L PEITC concentration (24-hour treatment), which reduced survival of TRAMP-derived cells by >90% (data not shown), a reduction of only ~43% in survival of PrEC cells was observed. Collectively, these results indicated that, similar to PC-3 cells (27), the TRAMP-derived prostate tumor cells were sensitive to growth inhibition by PEITC, whereas a normal prostate epithelial cell line was relatively more resistant to cell killing by PEITC compared with cancer cells.

We showed previously that a 24-hour exposure of PC-3 cells to growth suppressive concentrations of PEITC (5 and 10 μmol/L) resulted in G_2-M-phase cell cycle arrest due to proteasome-mediated degradation of cyclin-dependent kinase 1 and cell division cycle 25C leading to accumulation of Tyr^{15} phosphorylated (inactive) cyclin-dependent kinase 1 (32). To determine if PEITC inhibited proliferation of TRAMP-derived cells by perturbing cell cycle progression, its effect on cell cycle distribution was determined (Table 1). Interestingly, unlike PC-3 cells (32), PEITC treatment did not cause G_2-M-phase arrest in either TRAMP-C1 or TRAMP-C2 cells even at 10 μmol/L, a concentration that inhibited cell viability by >80%. Instead, as shown in Fig. 2A, the PEITC treatment resulted in a dose-dependent and statistically significant increase in the fraction of cells with sub-G_0-G_1 DNA content, which is a characteristic feature of cells undergoing apoptosis. These results indicated that PEITC-mediated inhibition of TRAMP-C1 and TRAMP-C2 cell proliferation was due to apoptosis induction.

Apoptosis-inducing effect of PEITC was confirmed by analysis of cytoplasmic histone-associated DNA fragmentation (Fig. 2B) and caspase-3 cleavage (Fig. 2C). As can be seen in Fig. 2B, treatment of TRAMP-C1 and TRAMP-C2 cells with 2.5 or 5 μmol/L PEITC (24-hour exposure) resulted in a statistically significant increase in cytoplasmic histone-associated DNA fragmentation compared with vehicle-treated control. In time course experiments using 10 μmol/L PEITC, an immunoreactive band corresponding to cleaved caspase-3 (19 kDa) was evident

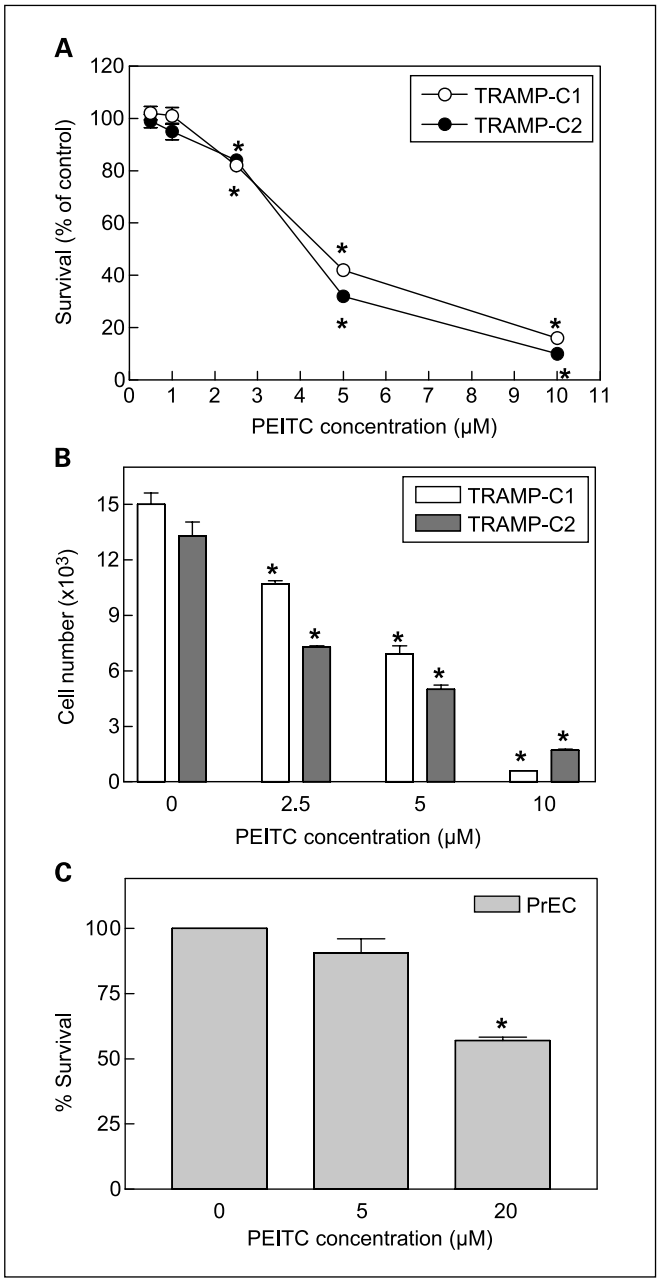

Fig. 1. Effect of PEITC treatment on survival of TRAMP-derived cells determined by (*A*) sulforhodamine B assay and (*B*) trypan blue dye exclusion assay. TRAMP-C1 or TRAMP-C2 cells were treated with different concentrations of PEITC for 24 hours. Stock solution of PEITC was prepared in DMSO, and an equal volume of DMSO (final concentration, 0.2%) was added to the controls. *C*, effect of PEITC treatment on survival of PrEC normal prostate epithelial cell line determined by sulforhodamine B assay. Columns, mean ($n = 3$); *bars*, SE. *, $P < 0.05$, significantly different compared with DMSO-treated control (one-way ANOVA).

Table 1. Effect of PEITC treatment on cell cycle distribution in TRAMP-C1 and TRAMP-C2 cells

Treatment	% Cells in		
	G_0-G_1	S	G_2-M
TRAMP-C1			
Control (DMSO)	50 ± 1	16 ± 1	21 ± 1
5 μmol/L PEITC	13 ± 1*	12 ± 1*	12 ± 1*
10 μmol/L PEITC	10 ± 1*	8 ± 1*	6 ± 1*
TRAMP-C2			
Control (DMSO)	54 ± 1	14 ± 1	26 ± 1
5 μmol/L PEITC	44 ± 1*	13 ± 1	28 ± 1
10 μmol/L PEITC	18 ± 1*	8 ± 1*	15 ± 1*

NOTE: TRAMP-C1 or TRAMP-C2 cells were treated with DMSO (control) or different concentrations of PEITC for 24 hours 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. Mean (n = 3) ± SE.
*P < 0.05, significantly different compared with corresponding DMSO-treated control (one-way ANOVA).

in both cell lines (Fig. 2C). These results indicated that PEITC treatment caused caspase-3-mediated apoptosis in TRAMP-derived cell lines.

Growth inhibition and apoptosis induction by PEITC in TRAMP-derived cells following a 24-hour drug treatment was observed between 2.5 and 10 μmol/L concentrations (Figs. 1 and 2). Previous studies have shown that the maximal plasma concentration of PEITC (C_{max}) following ingestion of 100 g watercress ranges between 673 and 1,155 nmol/L (mean −928 ± 250 nmol/L) with t_{max} (time to reach C_{max}) of ~2.1 ± 1.1 hours (43). A C_{max} between 0.64 and 1.4 μmol/L (mean −1.04 ± 0.22 μmol/L) of total isothiocyanate in three subjects taking a single dose PEITC (40 mg) was reported in another study (44). Because the 24-hour drug exposure experiments did not reveal significant effect of PEITC on cell survival at concentrations <2.5 μmol/L (Fig. 1A), we tested the possibility that longer incubation time may be necessary to observe growth inhibition and/or apoptosis induction at pharmacologically relevant concentrations of PEITC. We therefore determined the effect of 1 μmol/L PEITC on cell survival (trypan blue dye exclusion assay) and apoptosis induction (analysis of cytoplasmic histone-associated DNA fragmentation and immunoblotting for cleaved PARP and procaspase-3) using TRAMP-C1 cells following a 24-, 48-, or 72-hour drug exposure. As can be seen in Fig. 3A, the viability of TRAMP-C1 cells was reduced significantly in the presence of 1 μmol/L PEITC following a 48- or 72-hour drug treatment (Fig. 3A). Consistent with these results, a 48- or 72-hour exposure of TRAMP-C1 cells to 1 μmol/L PEITC resulted in a statistically significant increase in cytoplasmic histone-associated DNA fragmentation compared with corresponding DMSO-treated controls (Fig. 3B). In addition, TRAMP-C1 cells treated for 48 or 72 hours with 1 μmol/L PEITC exhibited cleavage of PARP and procaspase-3 (Fig. 3C). These results indicated that PEITC was able to inhibit cell survival and cause apoptosis induction even at clinically achievable concentrations, although a longer incubation time was required to observe these effects.

Phenethyl isothiocyanate treatment differentially affected levels of Bcl-2 family proteins in TRAMP-C1 and TRAMP-C2 cells. The Bcl-2 family proteins play critical roles in regulation of apoptosis by functioning as either promoters (e.g., Bax and Bak) or inhibitors (Bcl-2, Bcl-x_L, or Mcl-1) of the cell death

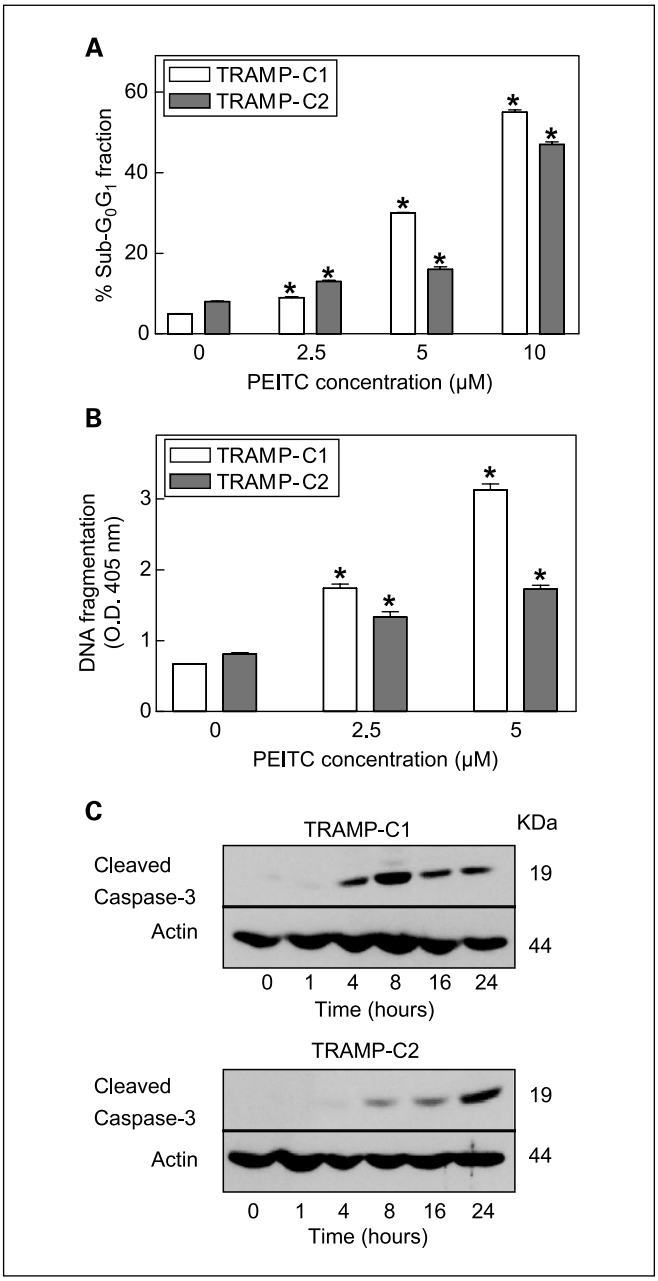

Fig. 2. *A*, flow cytometric analysis of subdiploid fraction in TRAMP-derived cells following a 24-hour treatment with DMSO (control) or different concentrations of PEITC. Following PEITC treatment, both floating and attached cells were collected, stained with propidium iodide, and analyzed using a Coulter Epics XL Flow Cytometer. *B*, ELISA-based quantitation of cytoplasmic histone-associated DNA fragmentation in TRAMP-derived cells following a 24-hour exposure to DMSO (control) or different concentrations of PEITC. Cytoplasmic histone-associated DNA fragments were quantified using Cell Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Columns, mean (n = 3); bars, SE. *, P < 0.05, significantly different compared with control (one-way ANOVA). *C*, immunoblotting for cleaved caspase-3 using lysates from TRAMP-derived cells following treatment with 10 μmol/L PEITC for the indicated times. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading.

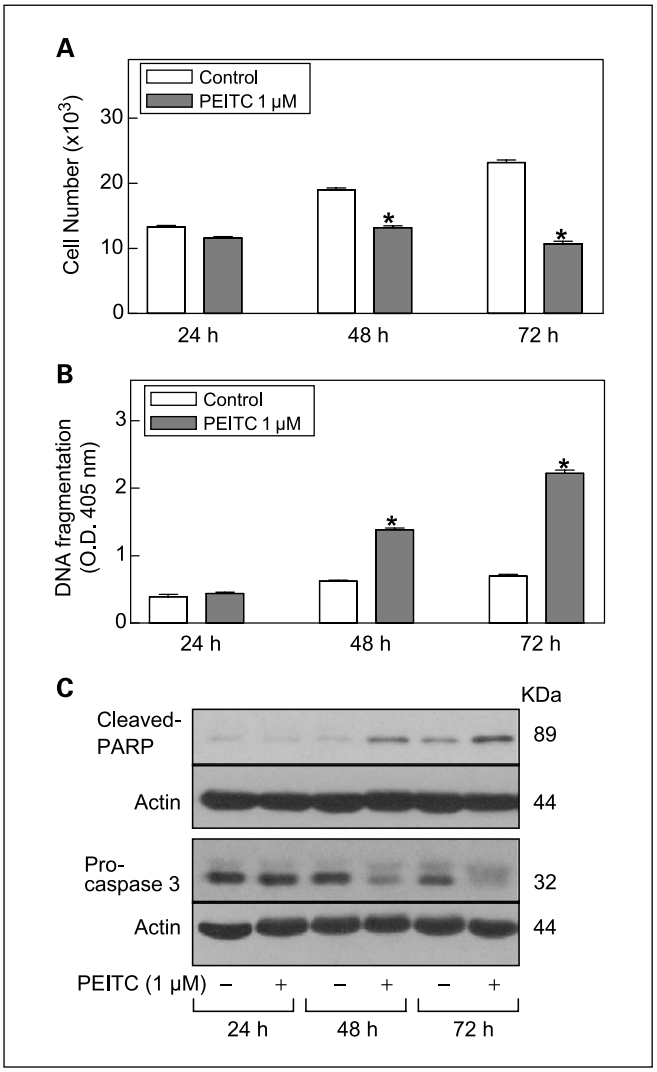

Fig. 3. *A*, survival of TRAMP-C1 cells following a 24-, 48-, or 72-hour exposure to 1 μmol/L PEITC or DMSO (control) determined by trypan blue dye exclusion assay. *B*, analysis of cytoplasmic histone-associated DNA fragmentation in TRAMP-C1 cells following a 24-, 48-, or 72-hour exposure to 1 μmol/L PEITC or DMSO (control). Columns, mean ($n = 3$); bars, SE. *, $P < 0.05$, significantly different compared with control (one-way ANOVA). *C*, immunoblotting for cleaved PARP and procaspase-3 using lysates from TRAMP-C1 cells following a 24-, 48-, or 72-hour treatment with DMSO (control) or 1 μmol/L PEITC. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading.

process (33–36). Initially, we compared endogenous levels of Bcl-2 family proteins between TRAMP-C1 and TRAMP-C2 cells by immunoblotting using equal amounts of lysate proteins from untreated cells. As can be seen in Fig. 4, endogenous levels of Bax, Bcl-x_L, and Mcl-1 proteins were higher by ~45%, 50%, and 550% (based on densitometric scanning of the immunoreactive bands normalized to actin loading control), respectively, in TRAMP-C2 cells than in the TRAMP-C1 cell line. On the other hand, Bak protein level was ~40% higher in TRAMP-C1 cells than in TRAMP-C2 cells (Fig. 4).

To gain insights into the mechanism of PEITC-induced apoptosis, immunoblotting was done for Bax, Bak, Mcl-1, and Bcl-x_L using lysates from TRAMP-derived cells treated with 10 μmol/L PEITC for different time points, and the results are shown in Fig. 5A. The 10 μmol/L PEITC concentration was selected to maximize chances of observing effects. Whereas PEITC treatment did not alter Bax protein level in TRAMP-C1 cells, a marked decrease in its level was evident in PEITC-treated TRAMP-C2 cells at 24-hour time point compared with the control (Fig. 5A). On the other hand, a marked increase in Bak protein level on PEITC treatment was evident in both cell lines. In TRAMP-C1 cells, the PEITC-mediated induction of Bak protein expression increased gradually with exposure time (between 1.4- and 2.1-fold over control) and was maintained for the duration of the treatment (24-hour post-treatment). In TRAMP-C2 cells, the PEITC-mediated Bak protein induction peaked between 8 and 16 hours (up to 2.1-fold increase over control) and returned to the basal level by 24 hours. PEITC treatment also caused a slight increase (between 30% and 78% over control) in the level of Bcl-x_L protein in TRAMP-C1 cells, whereas its level was reduced significantly in PEITC-treated TRAMP-C2 cells. The PEITC-mediated decrease in Bcl-x_L protein level in TRAMP-C2 cells was evident as early as 4 hours after treatment and persisted for the duration of the experiment. In addition, the PEITC treatment caused a decrease of ~35% to 75% in the level of Mcl-1 protein in both cell lines at 16- and 24-hour time points (Fig. 5A).

The effect of pharmacologically relevant concentration of PEITC (1 μmol/L) on protein levels of Mcl-1 and Bak was also determined using TRAMP-C1 cells, and the results are shown in Fig. 5B. The protein level of Mcl-1 was reduced by ~60% to 68% on a 48- and 72-hour exposure of TRAMP-C1 cells to 1 μmol/L PEITC compared with DMSO-treated controls. Similarly, the level of Bak protein was increased by ~3.1- and 2.6-fold, respectively, following a 48- and 72-hour exposure of TRAMP-C1 cells to 1 μmol/L PEITC compared with corresponding vehicle-treated controls (Fig. 5B). These results indicated that clinically achievable concentrations of PEITC could alter protein levels of Bcl-2 family members.

Phenethyl isothiocyanate treatment disrupted mitochondrial membrane potential in TRAMP-C1 cells. Disruption of the mitochondrial membrane potential is an early event in

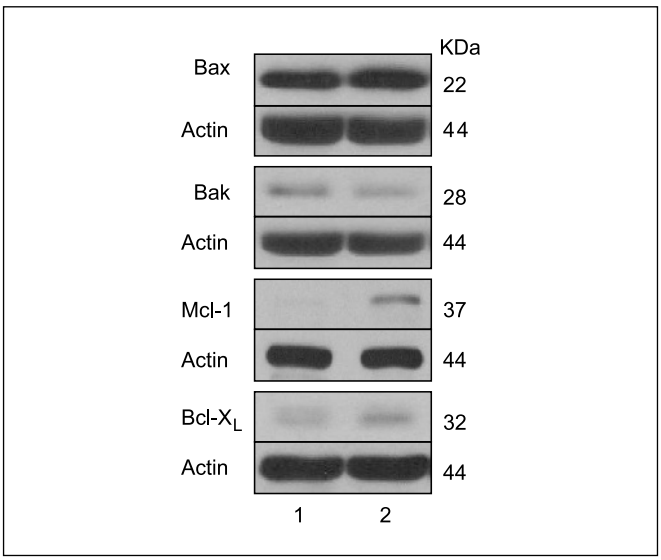

Fig. 4. Immunoblotting for endogenous levels of Bcl-2 family proteins using equal amounts of lysate protein (20 μg) from untreated TRAMP-C1 (*lane 1*) and TRAMP-C2 (*lane 2*) cells. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading.

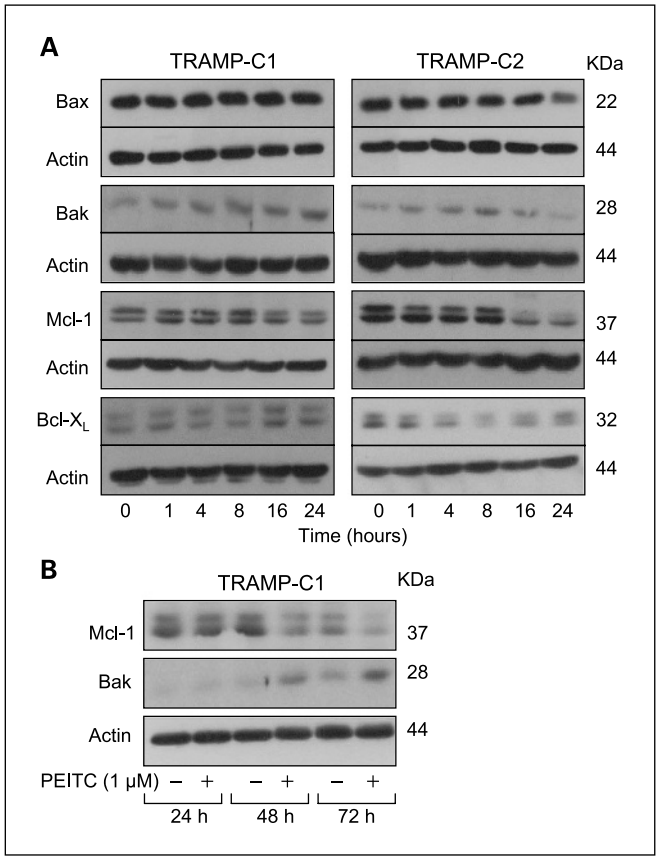

Fig. 5. Immunoblotting for Bcl-2 family proteins using lysates from TRAMP-C1 and/or TRAMP-C2 cells treated with (*A*) 10 μmol/L PEITC or (*B*) 1 μmol/L PEITC for the indicated time periods. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading.

apoptosis induction by a variety of stimuli (45, 46), which triggers release of cytochrome *c* and other apoptogenic molecules from mitochondria to the cytosol (47, 48). Once in the cytosol, cytochrome *c* binds to Apaf-1 and recruits and activates procaspase-9 in the apoptosome (45, 46, 49, 50). Active caspase-9 cleaves and activates executioner caspases, including caspase-3 (49, 50). Because PEITC treatment caused cleavage of caspase-3 in TRAMP-derived cells (Fig. 2C), we sought to determine whether PEITC-induced apoptosis was mediated by disruption of the mitochondrial membrane potential. The effect of PEITC treatment on mitochondrial membrane potential was determined by flow cytometry using cationic lipophilic dye TMRME, which accumulates within mitochondria in a potential-dependent manner. Representative histograms for TMRME retention in TRAMP-C1 cells following a 6-hour treatment with DMSO (control) or 10 μmol/L PEITC are shown in Fig. 6A. The mitochondrial decoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone was included in the analysis as a positive control (data not shown). As can be seen in Fig. 6A, PEITC treatment caused a decrease in TMRME retention compared with vehicle-treated control. A decrease in TMRME retention was also observed in cells treated with the positive control carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (data not shown). In time course experiments using 10 μmol/L PEITC, disruption of TRAMP-C1 mitochondrial membrane potential was evident as early as 4 hours post-exposure (Fig. 6B). In dose-response studies, disruption of the mitochondrial membrane potential was also observed following a 6-hour treatment of TRAMP-C1 cells with 2.5 and 5 μmol/L PEITC (data not shown). In time course experiments using 10 μmol/L PEITC, release of cytochrome *c* to the cytosol was observed as early as 2.5 hours after drug exposure and peaked between 5 and 10 hours post-treatment (Fig. 6C).

Bak-Bax double knockout mouse embryonic fibroblasts were resistant to phenethyl isothiocyanate–induced apoptosis. Because PEITC treatment caused a marked increase in Bak protein level in both cell lines, we hypothesized that Bak induction might contribute to PEITC-induced apoptosis. We tested this hypothesis by comparing sensitivities of SV40 immortalized MEFs derived from wild-type and Bak knockout mice towards PEITC-induced cytoplasmic histone-associated DNA fragmentation (apoptosis), and the results are shown in Fig. 7A. The PEITC treatment (5 or 10 μmol/L for 24 hours) caused a dose-dependent increase in cytoplasmic histone-associated DNA fragmentation in both wild-type and Bak knockout MEFs, but the DNA fragmentation was relatively more pronounced in

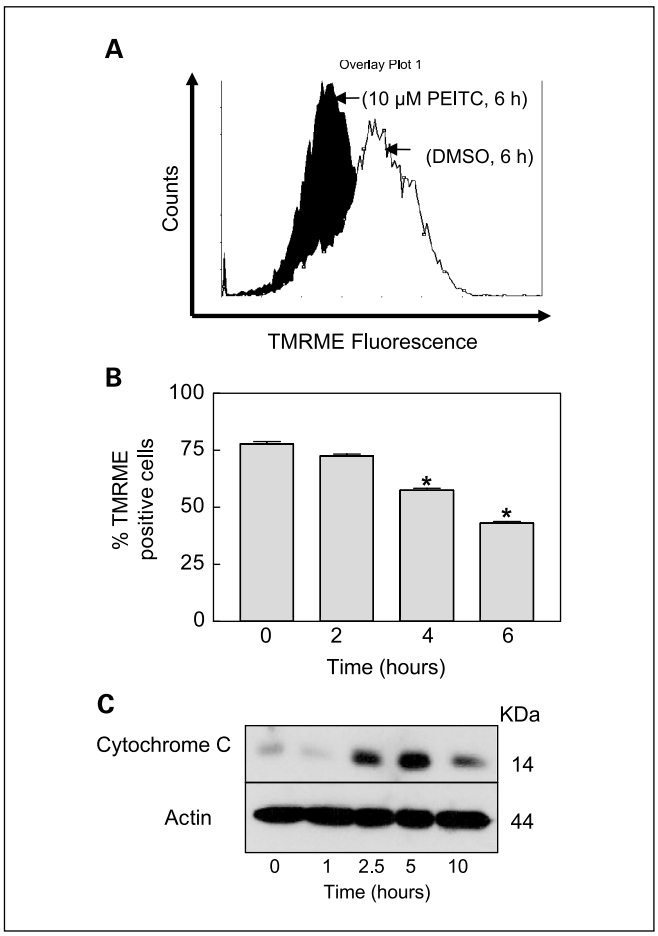

Fig. 6. *A*, representative flow histograms depicting TMRME retention in TRAMP-C1 cells following a 6-hour treatment with DMSO (*white histogram*) or 10 μmol/L PEITC (*black histogram*). *B*, analysis of TMRME fluorescence in TRAMP-C1 cells treated with 10 μmol/L PEITC for the indicated times. *Columns*, mean ($n = 3$); *bars*, SE. *, $P < 0.05$, significantly different compared with control (one-way ANOVA). *C*, immunoblotting for cytochrome *c* using mitochondria-free cytosolic fraction from TRAMP-C1 cells treated with 10 μmol/L PEITC for the indicated times. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading.

wild-type MEFs than in the Bak knockout MEFs. For example, the DNA fragmentation following a 24-hour exposure to 10 μmol/L PEITC was moderately but statistically significantly higher in wild-type MEFs compared with Bak knockout MEFs.

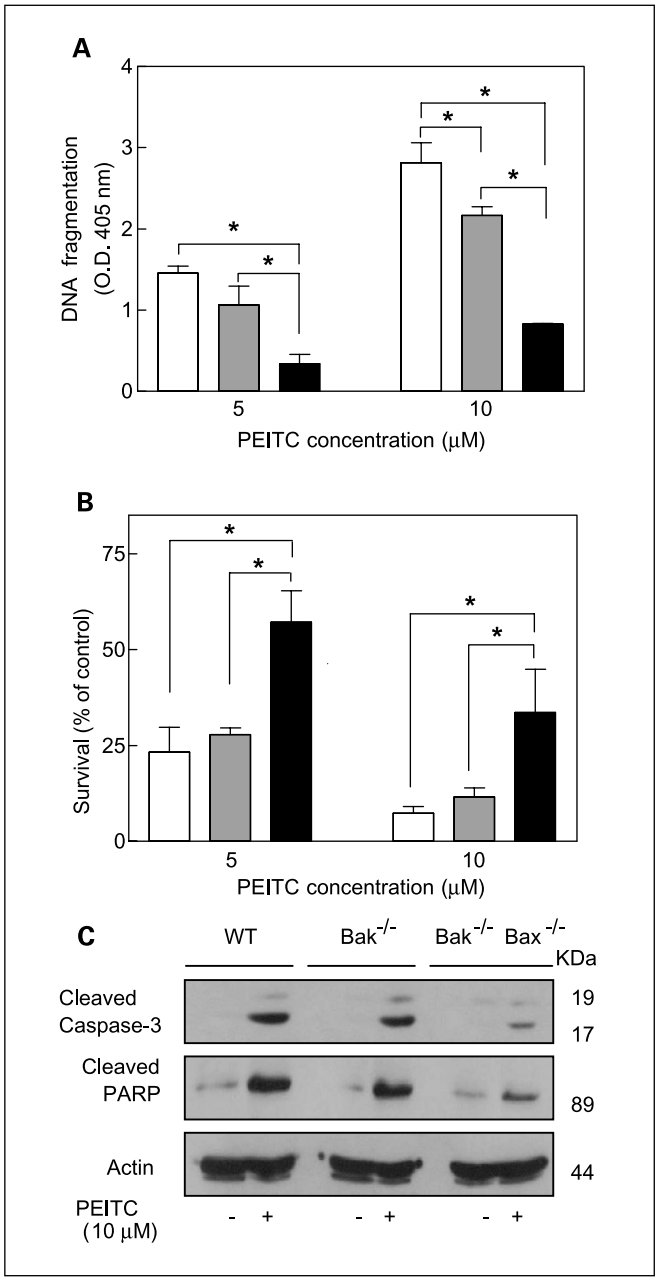

Fig. 7. *A*, PEITC-induced cytoplasmic histone-associated DNA fragmentation in SV40 immortalized MEFs derived from wild-type mice (*white columns*), Bak knockout mice (*shaded columns*), and Bak-Bax double knockout mice (*black columns*). MEFs were treated with 5 or 10 μmol/L PEITC for 24 hours at 37°C and processed for analysis of cytoplasmic histone-associated DNA fragmentation. *B*, effect of PEITC on viability of wild-type MEFs (*white columns*), Bak knockout MEFs (*shaded columns*), and Bak-Bax double knockout MEFs (*black columns*) determined by trypan blue dye exclusion assay. MEFs were treated with DMSO (control) or different concentrations of PEITC for 24 hours and stained with trypan blue, and live and dead cells were counted under an inverted microscope. Columns, mean ($n = 3$); bars, SE. *, $P < 0.05$, significantly different between the indicated groups (one-way ANOVA followed by Bonferroni's test for multiple comparisons). *C*, immunoblotting for cleaved caspase-3 and cleaved PARP using lysates from MEFs treated with DMSO (control) or 10 μmol/L PEITC for 24 hours. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading.

At 5 μmol/L PEITC concentration, however, the difference in DNA fragmentation between wild-type and Bak knockout MEFs did not reach statistical significance. On the other hand, Bak-Bax double knockout MEFs exhibited even greater resistance to PEITC-induced apoptosis in comparison with either wild-type or Bak knockout MEFs (Fig. 7A). The effect of PEITC treatment on viability of wild-type and knockout MEFs was determined by trypan blue dye exclusion assay, and the results are shown in Fig. 7B. In agreement with the results of DNA fragmentation assay, Bak-Bax double knockout MEFs were significantly more resistant to cell killing by PEITC compared with either wild-type or Bak knockout MEFs at both 5 and 10 μmol/L PEITC concentrations (Fig. 7B). Relative resistance of double knockout MEFs to PEITC-induced cell death was confirmed by examining the effect of PEITC treatment (10 μmol/L for 24 hours) on cleavage of PARP and caspase-3. As can be seen in Fig. 7C, the PEITC-mediated cleavage of PARP and caspase-3 was evident in wild-type and Bak knockout MEFs but was barely detectable in Bak-Bax double knockout MEFs. Taken together, these results clearly indicated that Bak and Bax proteins play an important role in apoptosis induction by PEITC.

Caspase inhibitors attenuated phenethyl isothiocyanate–induced apoptosis in TRAMP-derived cells. We showed previously that PEITC-induced apoptosis in PC-3 human prostate cancer cell line was significantly attenuated in the presence of specific inhibitors of caspase-9 and caspase-8 (32). We therefore investigated caspase dependence of PEITC-induced apoptosis in TRAMP-derived cells to exclude cell line–specific effects. As can be seen in Fig. 8, the PEITC-induced cytoplasmic histone-associated DNA fragmentation in TRAMP-C2 cells was nearly fully blocked in the presence of zVAD-fmk (general caspase inhibitor), zLEHD-fmk (caspase-9 specific inhibitor), and zIETD-fmk (caspase-8 specific inhibitor). The PEITC-induced apoptosis in TRAMP-C1 cells was also significantly attenuated on pharmacologic inhibition of caspases, although the effect was relatively less pronounced than in TRAMP-C2 cells (Fig. 8). Nonetheless, these results clearly indicated that, similar to PC-3 cells (32), the PEITC-induced apoptosis in TRAMP-derived cell lines was mediated by caspases.

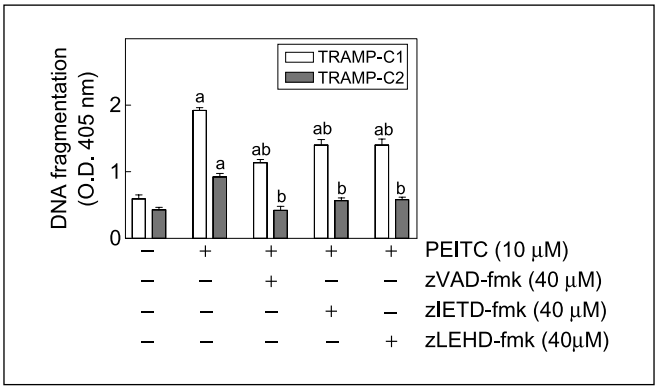

Fig. 8. Effect of pretreatment with general caspase inhibitor zVAD-fmk, caspase-8 specific inhibitor zIETD-fmk, and caspase-9 specific inhibitor zLEHD-fmk on PEITC-induced cytoplasmic histone-associated DNA fragmentation in TRAMP-derived cells. Columns, mean ($n = 3$); *bars*, SE. [a], $P < 0.05$, significantly different compared with DMSO-treated control; [b], $P < 0.05$, significantly different compared with PEITC alone treatment group (one-way ANOVA followed by Bonferroni's test for multiple comparisons).

Phenethyl isothiocyanate administration inhibited TRAMP-C1 xenograft growth in nude mice. *In vivo* efficacy testing of potential anticancer agents is a prerequisite for their clinical development. We therefore determined the effect of PEITC administration on growth of TRAMP-C1 xenografts in male athymic mice, and the results are shown in Fig. 9A. We selected TRAMP-C1 cells for these studies mainly because this cell line was relatively more sensitive to apoptosis induction by PEITC compared with TRAMP-C2 (Fig. 2). As can be seen in Fig. 9A, the growth of TRAMP-C1 xenograft was inhibited significantly on oral feeding of 9 and 12 μmol PEITC/d (Monday-Friday). For instance, 50 days after starting therapy, the average tumor volume in control mice (567 ± 184 mm^3) was ~3- and 3.5-fold higher compared with mice receiving 9 and 12 μmol PEITC, respectively, reflecting a 67% to 71% reduction in tumor volume (Fig. 9A). The growth of TRAMP-C1 xenografts was not significantly affected in mice receiving 6 μmol PEITC/d (data not shown in Fig. 9A). The body weights of the mice of each group were recorded to determine if inhibition of tumor xenograft growth was due to weight loss. As can be seen in Fig. 9B, the average body weights of the control and PEITC-treated mice did not differ significantly throughout the treatment protocol. In addition, the tumors from PEITC-treated mice harvested at the termination of the experiment exhibited significantly higher count of apoptotic bodies compared with control tumors as determined by immunohistochemical terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (data not shown).

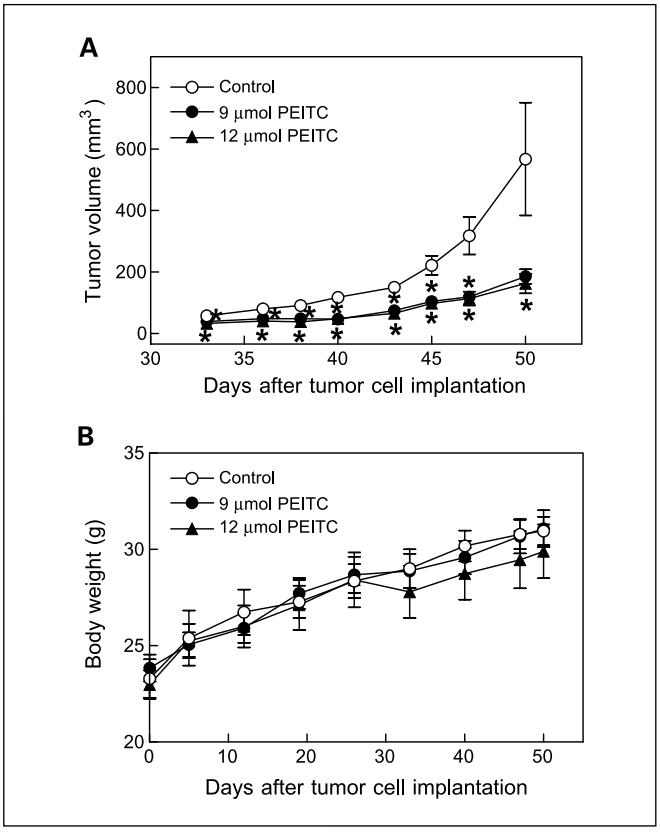

Fig. 9. *A*, average tumor volume in control mice (○), 9 μmol PEITC-treated mice (●), and 12 μmol PEITC-treated mice (▲) as a function of time. *Points*, mean ($n = 9$); *bars*, SE. *B*, average body weights of control mice (○), 9 μmol PEITC-treated mice (●), and 12 μmol PEITC-treated mice (▲) as a function of time. *Points*, mean ($n = 5$); *bars*, SE. *, $P < 0.05$, significantly different compared with control (one-way ANOVA).

Discussion

Cruciferous vegetables, such as watercress and broccoli, are a rich source of isothiocyanates that are highly effective in affording protection against cancer in animals induced by structurally divergent chemical carcinogens (11–21). Our interest in isothiocyanates stemmed from recent epidemiologic data documenting an inverse correlation between dietary intake of cruciferous vegetables and the risk of prostate cancer (4, 5), which prompted us to determine the sensitivity of prostate cancer cells to PEITC. Indeed, we found that PEITC, which is one of the best studied members of the isothiocyanate family of chemopreventive agents (reviewed in refs. 6–8), suppressed proliferation of PC-3 cells by causing apoptosis (27, 32). The PEITC-mediated growth inhibition and apoptosis induction in the PC-3 cell line was observed at concentrations (between 2.5 and 10 μmol/L) that may be generated through dietary intake of cruciferous vegetables. For instance, consumption of 1 ounce of watercress is estimated to yield ~60 μmol/L PEITC (41, 42). In the present study, we extended these findings and determined the effect of PEITC on proliferation of tumor cells derived from the prostate of a 32-week-old transgenic TRAMP mouse. TRAMP-C1 and TRAMP-C2 cells were selected for the present study for the following reasons: (*a*) It was of interest to us to determine whether PEITC-mediated growth inhibition and apoptosis induction observed in PC-3 was cell line specific. (*b*) TRAMP-C1 and TRAMP-C2 cells are derived from the tumor of the same mouse, which made it possible to determine the effect of cellular heterogeneity on activity of PEITC. In addition, we felt that determination of the sensitivity of TRAMP-derived cells to growth inhibition and apoptosis induction by PEITC and elucidation of the mechanism of its action could justify future *in vivo* efficacy studies using TRAMP mice. The results of the present study indicate that TRAMP-derived cell lines are indeed sensitive to growth inhibition and apoptosis induction by PEITC even at clinically achievable concentrations, although a longer exposure time is necessary to observe these effects at clinically relevant concentration. We also found that PEITC administration significantly retards growth of TRAMP-C1 xenografts in nude mice without causing weight loss or any other side effects.

We showed previously that a 24-hour exposure of PC-3 cells to 5 or 10 μmol/L PEITC resulted in a net increase in G_2-M-phase cells, which correlated with accumulation of Tyr^{15} phosphorylated (inactive) cyclin-dependent kinase 1 (32). The G_2-M-phase cell cycle arrest on treatment with benzyl isothiocyanate, a close structural analogue of PEITC, has also been reported in HL60 and human pancreatic cancer cells (25, 51). It is interesting to note that PEITC-mediated cell cycle arrest was not observed in either TRAMP-C1 or TRAMP-C2 cells even at a concentration that significantly inhibited cell proliferation (e.g., 10 μmol/L PEITC). The reasons for this discrepancy are not yet clear but could be attributed to difference in p53 status between PC-3 and TRAMP-derived cells. The PC-3 cell line is p53 deficient, whereas TRAMP-derived cells seem to have wild-type p53 (37). Further studies are needed to test the possibility that p53 status influences cell cycle arrest by PEITC.

One of the objectives of the present study was to determine the contribution of Bcl-2 family proteins in PEITC-induced apoptosis. We showed previously that PEITC-induced apoptosis in PC-3 cells was not affected by overexpression of Bcl-2 (32). Several other proteins with sequence homology to Bcl-2 are implicated in regulation of apoptosis in response to various stimuli (33–36). Bak is one such protein that functions to promote cell death by neutralizing the antiapoptotic effect of Bcl-2 (52, 53). Bax is another key protein that heterodimerizes with Bcl-2 to counteract its antiapoptotic function (54). Mutations in *Bak* and *Bax* genes have been shown to cause resistance to apoptosis induction by certain stimuli (55–57). The present study revealed that PEITC treatment caused a marked increase in the level of Bak protein in both TRAMP-C1 and TRAMP-C2 cells (Fig. 5A). It is interesting to note, however, that the Bak knockout MEFs were only slightly more resistant to PEITC-induced cell death compared with the wild-type MEFs. On the other hand, the MEFs derived from Bak and Bax double knockout mice were significantly more resistant to cell death by PEITC in comparison with either wild-type or Bak knockout MEFs. In agreement with these results, PEITC treatment resulted in disruption of mitochondrial membrane potential in TRAMP-C1 cells, which triggered release of cytochrome *c* and activation of caspase-3. Thus, it seems reasonable to postulate that Bak and Bax proteins play an important role in regulation of PEITC-induced apoptosis.

We found that PEITC treatment caused a marked decrease in the level of antiapoptotic protein Mcl-1 in both TRAMP-C1 and TRAMP-C2 cells. The PEITC-mediated decrease in the protein level of Mcl-1 was not observed until 16 to 24 hours, whereas cleavage of caspase-3 was evident as early as 4 to 8 hours after PEITC treatment. These results suggest that a decrease in Mcl-1 protein level may have an amplifying role rather than an initiating role in PEITC-induced apoptosis in TRAMP-derived cells. PEITC treatment also caused a marked decrease in Bcl-x_L protein level in TRAMP-C2 but not in TRAMP-C1 cells. Thus, it is possible that the PEITC-induced cell death in TRAMP-C2 cells may at least in part be due to a decrease in Bcl-x_L level, especially because Bcl-x_L down-regulation preceded caspase-3 cleavage. It is important to point out that overexpression of Bcl-x_L through transient transfection in 293 cells has been shown to confer resistance to PEITC-induced apoptosis (22).

Activation of caspases leads to cleavage and inactivation of key cellular proteins, such as PARP (49, 50). In TRAMP-derived cells, caspase-3 cleavage was observed within 4 to 8 hours of PEITC treatment (Fig. 2C). 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 (49, 50). The results of the present study indicated that PEITC-induced apoptosis in TRAMP-derived cells was probably mediated by both caspase-9 and caspase-8 because specific inhibitors of these caspases significantly abrogated the cell death caused by PEITC. Involvement of both caspase-9 and caspase-8 pathways in PEITC-induced apoptosis has also been suggested in other cellular systems (32).

In conclusion, the results of the present study indicate that PEITC inhibits proliferation of TRAMP-derived cell lines in culture as well as *in vivo* at concentrations that may be generated through dietary intake of cruciferous vegetables. In addition, we provide experimental evidence to implicate Bak and Bax in PEITC-induced apoptosis.

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