

Ingestion of an isothiocyanate metabolite from cruciferous vegetables inhibits growth of human prostate cancer cell xenografts by apoptosis and cell cycle arrest

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Epidemiological surveys indicate that intake of cruciferous vegetables is inversely related to prostate cancer incidence, although the responsible dietary factors have not been identified. Our studies demonstrated that exposure of human prostate cancer cells in culture to the *N*-acetyl-cysteine (NAC) conjugate of phenethyl isothiocyanate (PEITC-NAC), the major metabolite of PEITC that is abundant in watercress, inhibited proliferation and tumorigenesis. The PEITC-NAC is known to mediate cytoprotection at initiation of carcinogenesis. The relevance of PEITC-NAC in diets on the growth of prostate tumor cells has been evaluated in immunodeficient mice with xenografted tumors of human prostate cancer PC-3 cells. The daily PEITC-NAC (8 μ mol/g) supplemented diet group showed a significant reduction in tumor size in 100% of the mice during the 9-week treatment period. Tumor weight at autopsy was reduced by 50% compared with mice on the diet without PEITC-NAC ($P = 0.05$). Mitosis and *in vivo* 5-bromo-2'-deoxyuridine labeled proliferating cells were reduced in these tumors. The PEITC-NAC diet up-regulated the inhibitors of cyclin-dependent kinases p21^{WAF-1/Cip-1} and p27^{Kip1}, and reduced the expression of cyclins D and E, indicating they were potential molecular targets. As a result, phosphorylated Rb was significantly decreased and the G₁- to S-phase transition retarded. The treated tumors also showed a significant increase in apoptosis as determined by *in situ* end-labeling, and by poly ADP-ribose polymerase cleavage. This study demonstrates the first *in vivo* evidence of dietary PEITC-NAC inhibiting tumorigenesis of prostate cancer cells. PEITC-NAC may prevent initiation of carcinogenesis and modulate the post-initiation phase by targeting cell cycle regulators and apoptosis induction.

Introduction

There is epidemiological evidence that the incidence of prostate cancer varies in different regions of the world. As populations move to a high-risk area, they assume the risk of that

Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; cdk, cyclin-dependent kinases; PARP, poly ADP-ribose polymerase; PEITC, phenethyl isothiocyanate; PEITC-NAC, *N*-acetylcysteine conjugate of phenethyl isothiocyanate.

geographic region (1–3). This points to the potential relationship of dietary factors with cancer risk. Recent conclusions from two epidemiological studies show a strong correlation of consumption of diets rich in cruciferous vegetables such as broccoli, cabbage, Brussels sprouts, cauliflower and watercress with a reduced incidence of prostate cancer (4,5). These studies, however, did not identify the responsible dietary factors or the mechanisms of the preventive or therapeutic effects.

The vegetables of the family of *Cruciferae* are distinguished from other vegetables due to their high content of glucosinolates and their hydrolyzed products (6). When the vegetables are cut, crushed or masticated, the enzyme myrosinase is released from the plant cell compartment to hydrolyze glucosinolates to aglucone, glucose and sulfate. The aglucones are unstable and spontaneously convert to isothiocyanates and other compounds. Hydrolysis of the glucosinolate gluconasturtiin, found abundantly in watercress (7,8), yields phenethyl isothiocyanate (PEITC). During metabolism PEITC is processed by the mercapturic acid pathway; ultimately is converted to the *N*-acetylcysteine (NAC) conjugates (PEITC-NAC) as the major metabolite and excreted in the urine (9,10). PEITC-NAC and several other thiol conjugates of isothiocyanates have been reported as potent cancer chemopreventive agents in a number of experimental animal models (11–13). They induce cytoprotection against carcinogenesis by blocking phase 1 enzymes such as cytochrome P450s that metabolize procarcinogens to carcinogens and by inducing phase 2 enzymes such as glutathione *S*-transferases that facilitate the excretion of the electrophilic metabolites generated from carcinogen metabolism (14,15). Recently we have reported that PEITC-NAC significantly inhibited the growth of prostate cancer cells in cultures, with parallel induction of the inhibitors of cyclin-dependent kinases for G₁ arrest (16). In this study the efficacy of PEITC-NAC in the diet was examined on the growth of prostate cancer cells *in vivo* as xenografts in immunodeficient mice. This paper demonstrates growth inhibition and apoptosis of the xenografted tumors, revealing that the regulators of the cell cycle of prostate cancer cells may be potential molecular targets of PEITC-NAC.

Materials and methods

Chemicals and culture

The NAC conjugate of PEITC (PEITC-NAC) was synthesized by a modification of a published method (8). The product was crystallized from hexane, and purity established by HPLC, NMR and MS; purity was >98%. A human prostate cancer cell line, androgen-independent PC-3, was seeded at 1.5×10^5 cells/ml in RPMI-1640 containing 15% heat inactivated fetal calf serum and 1% penicillin and streptomycin. PEITC-NAC was supplemented to each culture at various concentrations. Cell cycle phase determination was performed using a BD FACScan cytometer according to published procedures (17,18). The cells were fixed with 80% ethanol at 4°C, and incubated on ice before the DNA was stained with propidium iodide (50 μ g/ml).

Xenograft tumor assays

Five-week-old BALB/c (nu/nu) male mice purchased from Charles River Laboratories (Wilmington, MA) were housed in a barrier facility with 12-h light/dark cycles; tap water and diets provided *ad libitum*. They were randomly divided into two groups of nine mice. One group provided modified AIN-76A diet (5% corn oil) (basal diet), the other with 8 μ mol PEITC-NAC/g in AIN-76A, established as an optimal dose by a separate maximum tolerated dose assay. Freshly prepared diets were replenished twice per week, and the PEITC-NAC was stable for at least one month after preparation (13). The diets began 1 week prior to inoculation of PC-3 cells and continued until termination of experiments.

The xenografted tumors were established by a single s.c. injection in the flank of 0.8×10^6 PC-3 cells suspended in ice-cold matrigel (Sigma, St Louis, MO). The tumor volumes were measured every 7–8 days and calculated by length \times width \times height \times 0.5236. Animal body weights were recorded weekly and at autopsy; food consumption was determined twice per week. Two hours prior to death of mice, 5-bromo-2'-deoxyuridine (BrdU) (10 mg/kg body wt) was injected i.p. for *in vivo* labeling of proliferating cells. At autopsy, xenograft tumors were weighed and frozen in liquid nitrogen or fixed in 10% formalin and embedded in paraffin. The BrdU-labeled cells of paraffin embedded tissues on slides were detected employing a monoclonal anti-BrdU antibody (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer's direction. The labeled cells were calculated from multiple fields of each tumor. Several sections from each tumor were analyzed to obtain the mean of BrdU positive cells. The means of the proliferating cells from tumors of mice (eight in each group) were reported. The spleens and kidneys were also weighed at autopsy but they and other organs were not processed for histological examinations.

Apoptosis and protein expression

The apoptotic cells of xenografted tumors were determined with paraffin embedded tissue slides by the characteristic morphology of the nucleus, and by the presence of DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end-labeling (TUNEL). An *in situ* detection kit from Roche Molecular Biochemicals was employed according to manufacturer's direction (18). For each tumor section, at least six different fields but not necrotic tissues were examined for the presence of apoptotic cells. The means of apoptotic cells of eight tumors from the control or experimental group were reported. The mean values of tumor volume and weight, body and organ weight, apoptotic or proliferating cells from the control or experimental groups were compared with the two-tailed Student's *t*-test using commercial software. A *P* value ≤ 0.05 was considered to be statistically significant.

The protein levels of xenografts were determined by western blot analyses using standard procedures (18). Total proteins were prepared from each group of pooled individual xenografts of equal weight. Eight tumors from the control or the experimental group were used to prepare the lysates. The tissues were homogenized in the presence of a lysis buffer with protease inhibitors (18) and lysates collected after centrifugation at 4°C. Polyclonal antibodies against human p21^{WAF-1/Cip-1}, p27^{Kip1}, cyclin E and an anti-cyclin D1 cross reactive with cyclins D2 and D3, an anti-Rb reactive with both phosphorylated and non-phosphorylated proteins, and an antibody against intact poly ADP-ribose polymerase (PARP), and the 89 kDa fragment were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Three separate experiments were performed with each antibody. The measurements of protein expression were based on densitometry and the values from the control or the experimental tumors were compared.

Results*PEITC-NAC diet inhibited growth of xenografts*

Dietary effects of PEITC-NAC on the growth of xenografted tumors of human prostate cancer PC-3 cells in immunodeficient mice were evaluated. Animals were observed throughout the study period and showed no signs of unusual behavior on the PEITC-NAC diet. Mice on control basal diet without PEITC-NAC developed palpable tumors 7–10 days after inoculation of PC-3 cells, with 100% tumor incidence. The tumors in mice subjected to the experimental PEITC-NAC diet took a longer time period, 7–22 days became palpable, with 88% tumor incidence. Figure 1A shows that the PEITC-NAC diets suppressed the tumor growth. The tumor volumes were smaller soon after the tumors became palpable, and

persisted during most of the study period as compared with control tumors (*P* < 0.05). Tumor inhibition was noted in 100% of mice. Both smaller and larger (>100 mm³) tumors from the early and late study period were inhibited. The weight of tumors determined at autopsy confirmed that the tumors after feeding with PEITC-NAC diet were ~50.2% smaller (*P* = 0.05) (Figure 1B).

At termination, the average body weight of experimental mice was 20.93 g compared with control mice 21.89 g, corresponding to ~4.4% less in the experimental mice (*P* = 0.11) (Figure 1C). The average weights of kidneys and spleens from experimental mice were similarly less than the controls, but the difference was not significant (*P* > 0.13) (Figure 1D) indicating no overt toxicity with the PEITC-NAC diet. Measurement of food consumption revealed that mice on PEITC-NAC diets ate ~6.5% less daily as compared with control mice, perhaps due to palatability.

Reduced proliferation and apoptosis induction

Sections of tumors from mice on PEITC-NAC or control diet all had central necrosis surrounded by neoplastic cells that were large, containing a large vesicular nucleus and a prominent nucleolus. Occasional multinucleated giant cells were present. Numerous, focally abnormal mitosis was evident. Tumors from PEITC-NAC fed mice had fewer mitotic figures. They averaged three per high power field as compared with the control tumors that had greater than five mitotic figures per field. Apoptotic cells, with the appearance of condensed cytoplasm and pyknotic hyperchromatic nuclei were numerous. They were more concentrated in the area rimming the central necrosis of the control tumors. Apoptotic cells in the experimental tumors were seen rimming the area of central necrosis and also within areas of viable tumors.

The apoptotic cells with DNA strand breaks in xenografted tumors was determined *in situ* using the TUNEL assay. The average number of apoptotic cells in the experimental tumors was elevated ~2-fold, from 14.4 to 29.29% (*P* = 0.02) as compared with control tumors (Figure 2). Apoptosis was further confirmed by the cleavage of PARP, target of proteolysis of caspases that execute apoptosis with western blot analyses. A significant increase of the cleaved fragments, including a signature 89 kDa apoptotic fragment, was detected in pooled experimental tumors (Figure 2).

Inhibiting cell cycle progression

All mice were injected with BrdU for labeling proliferating cells. The results of immunohistochemical staining of the xenografted tumors for BrdU are presented in Figure 3A. The average proliferating cells was ~75% less in tumors from experimental mice versus control mice, indicating reduced DNA synthesis.

Levels of inhibitors of cyclin-dependent kinases (cdk) p21 and p27 that affect cell cycle progression were examined with the xenografted tumors by western blot analyses. An increased p21 and p27 levels (32 and 40%), along with a reduced expression of cyclins D1 (32%) and E (42%) were determined in the pooled experimental tumors when compared with the controls (Figure 3B). The PEITC-NAC effects were further examined on phosphorylation of Rb, because an induction of cdk inhibitors or decrease in cyclins could lead to decreased cdk activity that affects down-stream phosphorylation of Rb proteins as regulators of G₁- to S-phase transition (19–21). Figure 3B shows a significant reduction of Rb phosphorylation in

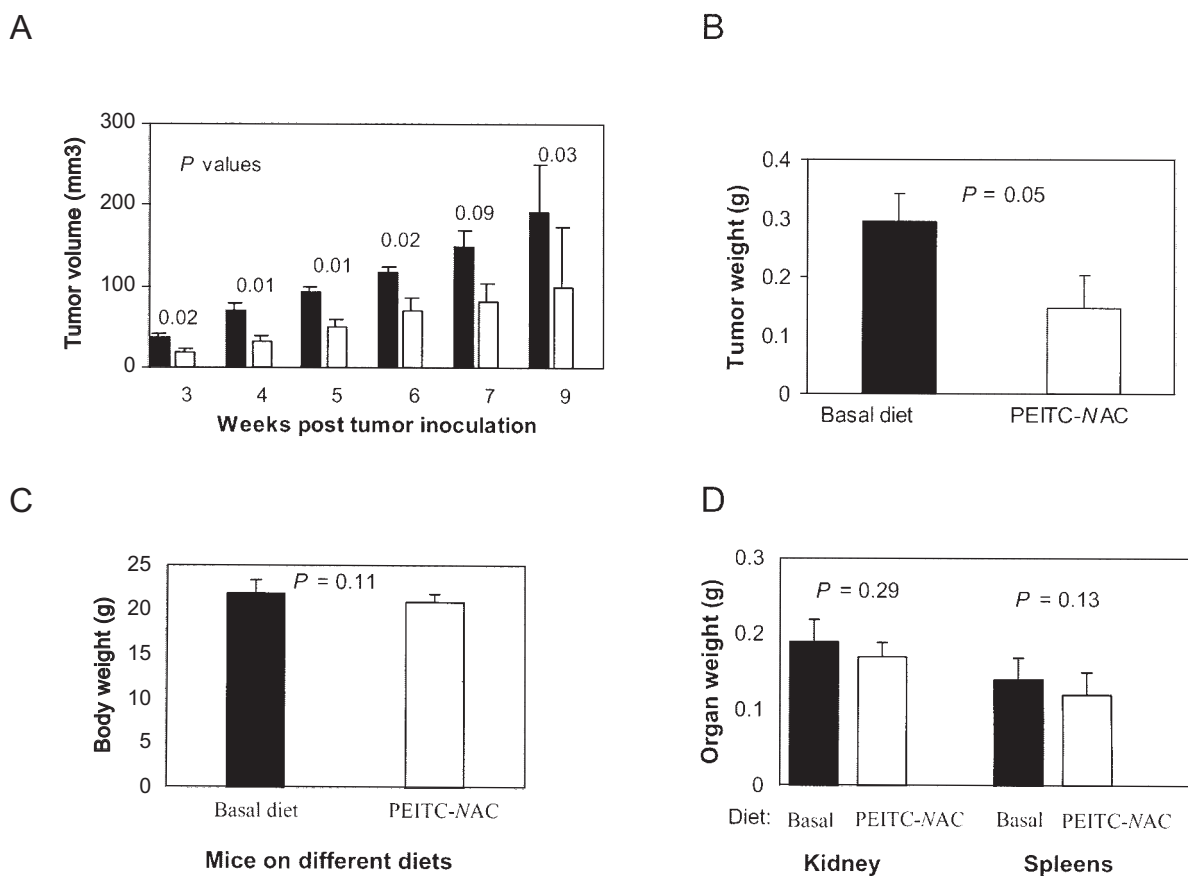


Fig. 1. PEITC-NAC diet inhibited growth of xenografted tumors of PC-3 prostate cancer cells in immunodeficient mice. (A) The volumes of xenografted tumors were measured once a week. Basal diet AIN 76A supplemented with PEITC-NAC (8 μ mol/g of basal diet) was started with nine mice 1 week before PC-3 cell injection, and the empty columns indicate the mean volumes of tumors during the study period. The filled columns indicate the mean volumes of control tumors on nine mice fed with basal diet AIN 76A without PEITC-NAC. The numbers above vertical bars are *P* values comparing the tumor volumes between PEITC-NAC and control diets. (B) Mean weights of tumors at autopsy; after feeding mice for 9 weeks with basal diet (filled column) or basal diet supplemented with PEITC-NAC (empty column). (C) Mean body weights of mice on PEITC-NAC diet (empty column) or control basal diet (filled column) at autopsy. (D) Mean weights of kidneys and spleens from mice on PEITC-NAC (empty column) or basal diet (filled column) at autopsy. Vertical bars in figures are means \pm SD and Student's *t* test used for comparison of two groups.

experimental tumors compared with controls. Equal protein loading was confirmed by probing the western blots with an anti- β actin antibody (Figure 3B).

Discussion

This study presents evidence that dietary PEITC-NAC, a major metabolite of PEITC from cruciferous vegetables, inhibited xenografts of human prostate cancer cells in immunodeficient mice. In mice on the PEITC-NAC diet, the tumor volume was consistently smaller, and tumor weight was less throughout the study period. Despite the relative small number of mice in each condition, the cellular and molecular responses of the tumors to PEITC-NAC diet, including decreased mitotic index and replicating cells, indicated that the growth of the tumor cells was inhibited. The presence of DNA strand breaks, cleavage of PARP and the abundant necrotic regions in the treated tumors further indicated that apoptotic cell death had been induced. These *in vivo* effects substantiated our earlier observations in culture, that isothiocyanates and their metabolites such as PEITC-NAC modulate the growth of prostate cancer cells (16).

Our lab published the first report of growth suppressive activity of isothiocyanates and their thiol compounds on human prostate cancer cells in 2000 (16). Other reports

regarding the potential chemopreventive effects of isothiocyanates and cruciferous vegetables for prostate cancer have appeared since that time. Sulforaphane, a dominant isothiocyanate in broccoli (18,22), and allyl isothiocyanate (23,24) both suppress the growth of prostate cancer cells in culture. Sulforaphane and PEITC are also potent inducers of phase 2 enzymes such as quinone reductase activity in prostate cancer cells, indicating their chemopreventive potential (25,26). The current study, to determine the dietary effects of the major metabolite of an isothiocyanate, PEITC, *in vivo*, represent an extension from our earlier observations using cell cultures. In the present work, the mechanism of effects of PEITC-NAC was similar to those observed with allyl isothiocyanate. Although we administered PEITC-NAC as a dietary component, allyl isothiocyanate was injected as an i.p. bolus of 1 mg (10 μ mol) three times per week to nude mice (24). Both studies demonstrated that isothiocyanates inhibit growth of the human prostate cancer cell xenografts and induced tumor cell apoptosis. The dietary effects of PEITC-NAC to inhibit the xenografts were similar to that mediated by sulforaphane, which was administered to nude mice by gavages (27). A >50% reduction in xenograft volume could be achieved after being treated with sulforaphane for 10 days. While apoptosis induction was determined as a mechanism of sulforaphane to

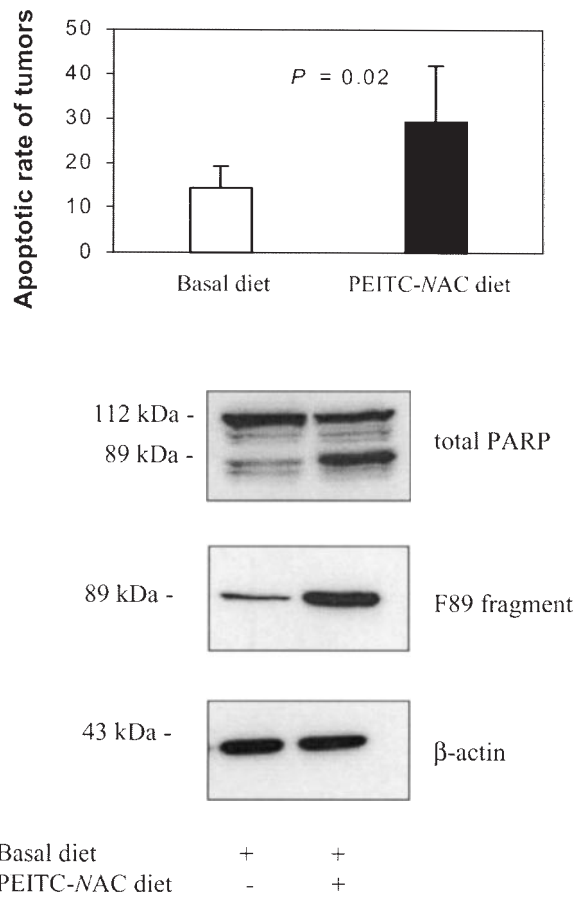


Fig. 2. Evaluation of apoptosis in the xenografted tumors from immunodeficient mice at autopsy. The upper graph shows increase of apoptotic cells in the tumors from mice fed for 9 weeks with PEITC-NAC supplemented diet (filled column) as compared with that of mice on control basal diet (empty column). The apoptotic cells were determined *in situ* by the presence of DNA strand breaks with TUNEL assay. Vertical bars represent means \pm SD of apoptotic cells of tumors from eight mice in each diet group, and Student's *t* test used for comparison of the two groups. Western blots were performed with pooled total proteins from tumor tissues of each diet group obtained at autopsy as described in the Materials and methods. Representative blots from three separate experiments showed the cleavage of total PARP, and the 89-kDa fragment of PARP. The antibody to β -actin was used as a loading control.

inhibit PC-3 cells in culture and in xenografts (27), our analyses indicated that the mediators and the mechanism of cell cycle progression and apoptosis were targets of PEITC-NAC to inhibit the xenografts.

Two recent epidemiological studies reported a strong association of cruciferous vegetable consumption with reduced prostate cancer risk (4,5); studies from earlier years also provide modest support for this conclusion (28). These investigations and the analyses in culture and animals support the hypothesis that constituents of cruciferous vegetables, including isothiocyanates and their metabolites, may be some of the dietary factors associated with a lowered risk for prostate cancer.

The mice on the PEITC-NAC diet weighed marginally less, ~4%, than mice on control diets without PEITC-NAC. The extent of the body weight disparity is probably too small to account for a 50% reduction in the tumor weight. The differences of body weight were probably caused by a reduced intake of the food with PEITC-NAC, as supported by the food consumption data. The reduced food intake could be

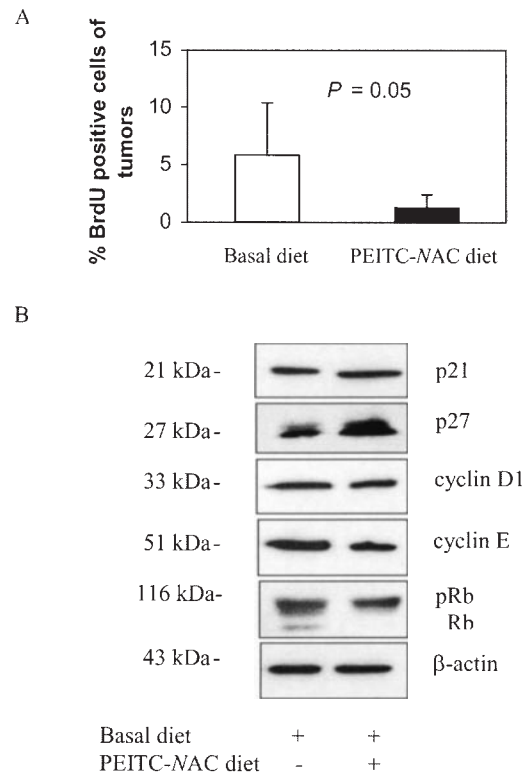


Fig. 3. Evaluation of the dietary effects of PEITC-NAC on proliferation of xenografted tumor cells. (A) Mice were injected with BrdU for labeling of proliferating cells that were identified in tumors obtained at autopsy by immunohistochemical staining as described in the Materials and methods. Vertical bars represent means \pm SD of proliferating cells of tumors from mice (eight in each diet group) on PEITC-NAC diet (filled column) or on control basal diet (empty column). (B) Western blots were performed with pooled total proteins from tumor tissues of each diet group obtained at autopsy as described in the Materials and methods. Representative blots from three separate experiments showed altered expression of cell cycle regulators. Antibodies against p21, p27, cyclin D1 (cross reactive with D2 and D3), cyclin E, Rb related proteins or β -actin (loading control) were used.

due to palatability. It was observed previously that A/J mice also ate less diet supplemented with PEITC-NAC and that was ruled out as a factor to change the size of carcinogen-induced lung tumor (13).

The experimental results indicate that PEITC-NAC may modulate the expression and function of cell cycle regulators, and the resulting cell cycle arrest may represent a relevant molecular mechanism. As revealed in the xenografts at autopsy, PEITC-NAC induced signals that up-regulate cdk inhibitors p21 and p27, and reduced the expression of cyclins D and E. This would effectively block the G₁-phase progression, because the progression is mediated by the combined activity of cyclin D1/cdk4, 6 and cyclin E/cdk 2 (21). As a result, phosphorylated Rb proteins, which activate the cell cycle progression, were decreased. The transition from G₁- to S-phase was most probably blocked (21). The reduction of mitosis and BrdU labeled proliferating cells confirmed that proliferation and DNA synthesis were decreased. The presence of apoptotic cells was increased in the same tumors, suggesting that the cells may undergo apoptosis after growth arrest. A similar effect on the cell cycle was observed with prostate cancer cell lines LNCaP and DU-145 after exposure to PEITC-NAC (16). The PEITC-NAC effects on cell cycle and the checkpoints are complex, especially if different growth stages

of the xenografts are considered. Further experiments will be required to obtain xenografts from different growth period for the analyses. Exposure of prostate cancer PC-3 cells to allyl isothiocyanate for 24 h was correlated with accumulation of cells at G₂/M phase (23). While the reasons for seeing different checkpoint effects are not yet entirely clear, preliminary experiments have indicated that it may be related to the dosage and time of exposure, the specific isothiocyanates involved, and the culture conditions. Since tumorigenesis is characterized by chronic loss of proliferation control (29), the action of PEITC-NAC by blocking cell cycle phase progression could represent a targeted mechanism overcoming the tumorigenesis of prostate cancer cells.

Mice were fed 8 μ mol PEITC-NAC/g of diet that is close to the maximum tolerated dosage. Assuming dietary consumption of 5 g diet/day by a mouse and 60% absorption of PEITC-NAC, the mouse would absorb 24 μ mol/day. The plasma concentration would however be considerably less than that as the initial half-life for PEITC-NAC is 4–8 h in rodents, and the mice eat sporadically during the night. There is no information about human consumption of cruciferous vegetables at maximum dosage. In one study, volunteers ingested about one bowl of fresh watercress in the morning and the total isothiocyanate absorption would reach 17.2–77% based on urinary analyses (30). As PEITC constitutes ~30% of total isothiocyanates in watercress and considering the metabolic conversion between PEITC and PEITC-NAC (31), the level of PEITC-NAC from one bowl of watercress would be considerably lower than those given to mice in the present experiments.

Isothiocyanates and their thiol conjugates are known as potent chemopreventives to affect carcinogen metabolism and facilitate their excretion with the phase 2-detoxification enzymes (11–15). The cytoprotection occurs at the levels of initiation of carcinogenesis. The demonstration of inhibition of xenografts from rapidly growing human prostate cancer cells by PEITC-NAC diet is significant, especially as it is the first time such effects are reported *in vivo* using a conjugate of an isothiocyanate. The results strongly suggest that the potential chemoprevention of prostate cancer by PEITC-NAC could occur at two levels. One is inhibition of post-initiation progression of prostate cancer cells, and the other is cytoprotection at the initiation levels of carcinogenesis. After the initiation of carcinogenesis, the cells with genetic changes may gain a growth advantage and undergo clonogenesis and progress eventually to cancer. The multiple mechanisms of isothiocyanates, including down-regulation of the androgen receptor (18), induction of cell cycle arrest and apoptosis, activation of mitogen-activated protein kinases and oxidative stress (13), could block the clonogenesis and the growth of emerging prostate cancer cells. This would delay the onset of clinically significant prostate tumors. The hypothesis for the post-initiation effects is corroborated by the recent epidemiological studies on prostate cancer. The study by Kolonel *et al.* (4) concluded that the intake of cruciferous vegetables was inversely related to especially those advanced cases of prostate cancer, indicating that the development of prostate cancer beyond initiation could be affected by the dietary factors from the vegetables.

Acknowledgements

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