Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G$_2$/M arrest and inducing apoptosis

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Dietary isothiocyanates (ITCs) are highly effective in affording protection against chemically induced cancers in laboratory animals. In the present study, we demonstrate that allyl isothiocyanate (AITC), a constituent of cruciferous vegetables, significantly inhibits proliferation of cultured PC-3 (androgen-independent) and LNCaP (androgen-dependent) human prostate cancer cells in a dose-dependent manner with an IC$_{50}$ of ~15–17 μM. On the other hand, survival of a normal prostate epithelial cell line (PrEC) was minimally affected by AITC even at concentrations that were highly cytotoxic to the prostate cancer cells. Reduced proliferation of PC-3 as well as LNCaP cells in the presence of AITC correlated with accumulation of cells in G$_2$/M phase and induction of apoptosis. In contrast, AITC treatment failed to induce apoptosis or cause G$_2$/M phase arrest in PrEC cells. A 24 h treatment of PC-3 and LNCaP cells with 20 μM AITC caused a significant decrease in the levels of proteins that regulate G$_2$/M progression, including Cdk1 (32–50% reduction), Cdc25B (44–48% reduction) and Cdc25C (>90% reduction). A significant reduction in the expression of cyclin B1 protein (~45%) was observed only in LNCaP cells. A 24 h exposure of PC-3 and LNCaP cells to an apoptosis-inducing concentration of AITC (20 μM) resulted in a significant decrease (31–68%) in the levels of anti-apoptotic protein Bcl-2 in both cell lines, and ~58% reduction in Bcl-X$_L$ protein expression in LNCaP cells. In conclusion, it seems reasonable to hypothesize that AITC, and possibly other ITCs, may find use in the treatment of human prostate cancers.

Introduction

Isothiocyanates (ITCs) are present as glucosinolates in various cruciferous vegetables, such as broccoli, cabbage, watercress, etc., and are generated upon cutting or chewing of these vegetables (1). ITCs are highly effective in affording protection against chemically induced cancers in animal models (2–4). Chemopreventive activity for ITCs has been observed against diethylnitosamine or benzo[a]pyrene-induced pulmonary and forestomach cancers in mice, N-nitrosobenzyl-methylamine-induced esophageal cancer in rats, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butane-induced pulmonary neoplasia in rats, and azoxymethane-induced colonic aberrant crypt foci in rats (2–8). In addition, thiol-conjugates of ITCs can also prevent pulmonary tumorigenesis in experimental animals induced by tobacco-derived carcinogens (9,10). Inhibition of carcinogen activation and/or an increase in detoxification of the activated carcinogenic metabolites through induction of Phase II drug metabolizing enzymes, including glutathione transferases and quinone reductase, is believed to be responsible for the chemopreventive effects of ITCs (2–4).

More recent studies have indicated that some naturally occurring ITCs can inhibit proliferation of cultured cancer cells by inducing apoptosis (11–15). For example, treatment of HeLa cells with phenethyl isothiocyanate (PEITC) resulted in apoptosis induction in a time- and concentration-dependent manner that was associated with a rapid and transient induction of caspase-3-like activity (11). Involvement of caspases in ITC-induced apoptosis has also been observed in other cell lines (13,16). In another study, Huang et al. (14) found that PEITC effectively blocked tumor promoter (12-O-tetradecanoylphorbol-13-acetate or epidermal growth factor)-induced cell transformation in mouse epidermal JB6 cells, which correlated with apoptosis induction.

Recent epidemiological studies have suggested that increased consumption of cruciferous vegetables may be protective against prostate cancer risk (17,18). In spite of compelling epidemiological correlation, however, the activity of ITCs against prostate cancer cells has not been systematically assessed. In the present study, we report that allyl isothiocyanate (AITC), a constituent of cruciferous vegetables, is a potent inhibitor of proliferation of PC-3 and LNCaP human prostate cancer cells. Interestingly, survival of a normal prostate epithelial cell line (PrEC) is minimally affected by AITC treatment. Furthermore, our studies indicated that the antiproliferative activity of AITC against human prostate cancer cells was due to its ability to arrest cells in G$_2$/M phase and induce apoptosis. Thus, it seems reasonable to speculate that AITC may find use in the treatment of human prostate cancers.

Materials and methods

Materials

AITC was purchased from Aldrich Chemical Company (Milwaukee, WI). Tissue culture media, fetal bovine serum and trypan blue were procured from Gibco (Grand Island, NY). Sulforhodamine B and propidium iodide were from Sigma (St Louis, MO). RNase A was from Promega (Madison, WI), ApoAlert Annexin V-FITC and ApoAlert Caspase Colorimetric Assay kits were from Clontech (Palo Alto, CA), and Cell Death Detection ELISA kit was from Roche Diagnostics GmbH (Mannheim, Germany). The antibodies against cyclin B1 and actin were from Oncogene Research Products (Boston, MA), antibodies against Bcl-X$_L$, Bax, cyclin-dependent kinase 1 (Cdk1), Cdc25C and BID were from Santa Cruz Biotecnology (Santa Cruz, CA), antibodies against Bcl-2 were from DAKO (Carpenteria, CA), and antibodies against Cdc25B were from BD PharMingen (San Diego, CA).

Abbreviations: AITC, allyl isothiocyanate; Cdk1, cyclin-dependent kinase 1; ITCs, isothiocyanates; PEITC, phenethyl isothiocyanate; PrEC, prostate epithelial cell line.

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**Cell culture**

Human prostate cancer cell lines PC-3 and LNCaP were obtained from the American Type Culture Collection (Rockville, MD). Monolayer cultures of PC-3 cells were maintained in F-12K Nutrient Mixture (Gibco BRL, Grand Island, NY). Monolayer cultures of LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, PSN Antibiotic Mixture (10 ml/l), 2 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 20% glucose. PrEC was purchased from Clonetics (Walkersville, MD). Cultures of PrEC cells were maintained in PrEGM Singlequots medium (Clonetics). Cultures of all three cell lines were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**Cell survival/proliferation assays**

The effect of AITC treatment on survival/proliferation of PC-3, LNCaP or PrEC cells was determined by sulforhodamine B or trypan blue dye exclusion assay. Sulforhodamine B assay was performed as described by us previously (19). The IC₅₀ value was determined from a plot of percentage of survival versus AITC concentrations. For trypan blue dye exclusion assay, 5 × 10⁵ cells were plated in 6-well plates, and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentrations of AITC or DMSO (control), and the plates were incubated for 24, 48 or 72 h at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Both floating and adherent cells were collected and pelleted by centrifugation at 700 g for 5 min. The cells were re-suspended in 25 μl phosphate buffered saline (PBS), mixed with 5 μl of 0.4% trypan blue solution and counted using a hemocytometer.

**Cell cycle analysis**

The effect of AITC treatment on cell cycle distribution was determined by flow cytometric analysis of DNA content of nuclei of cells following staining with propidium iodide. PC-3, LNCaP or PrEC cells (10⁶ cells) were seeded in T75 flasks, and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentrations of AITC or DMSO (control), and the cells were incubated for 24 h at 37°C. The cells were washed with PBS and fixed in 70% ethanol. The cells were then treated with 100 μg/ml RNase A and 50 μg/ml propidium iodide for 30 min, and analyzed using a Coulter Epics XL Flow Cytometer.

**Determination of apoptosis**

Apoptosis induction in control (DMSO treated) or AITC-treated PC-3, LNCaP or PrEC cells was assessed by using (i) Annexin V-FITC kit, (ii) Cell Death Detection ELISA kit or (iii) ApoAlert Caspase Colorimetric Assay kit according to the manufacturer’s instructions. For Annexin V assay, cells were exposed to desired concentrations of AITC or DMSO for 24 h, and the floating and adherent cells were collected. Pooled cells were washed with the manufacturer supplied binding buffer. Approximately 5 × 10⁵ cells were suspended in 200 μl of binding buffer, and mixed with 5 μl of Annexin V-FITC and 10 μl of propidium iodide. After 15 min of incubation in the dark, cells were analyzed using a Coulter Epics XL flow cytometer. The Cell Death Detection ELISA method detects apoptotic cell death in cellular systems by measuring cytoplasmic histone-associated DNA fragments. Briefly, 1 × 10⁵ cells (PC-3, LNCaP or PrEC) were seeded in T25 flasks, allowed to attach, and treated with desired concentrations of AITC or DMSO for 24 h. Both floating and adherent cells were collected, and processed for quantification of cytoplasmic histone-associated DNA fragments according to the manufacturer’s instructions. For caspase-3 activity assay, PC-3 and LNCaP cells were treated with DMSO (control) or 20 μM AITC for different time intervals. Both floating and adherent cells were collected and suspended in 50 μl of manufacturer supplied lysis buffer. After 10 min of incubation on ice, the cell lysate was cleared by centrifugation at 20 000 g for 3 min. Caspase-3-likelike activity was determined according to the manufacturer’s instructions.

**Western blot analysis**

Cells were exposed to 20 μM AITC for different time intervals as described above. The cells were washed twice with ice-cold PBS, lysed on ice with a solution containing 50 mM Tris, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 2 mM Na₂VO₃, 2 mM EGTA, 12 mM β-glycerol phosphate, 10 mM NaF, 16 μg/ml benzamidine hydrochloride, 10 μg/ml phenanthrolin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM phenyl methyl sulfonyl fluoride. The cell lysate was cleared by centrifugation at 14 000 g for 15 min. Protein content in 14 000 g supernatant fraction was determined by the method of Bradford (20). Lysate containing 20-60 μg protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (21), and the proteins were transferred onto polyvinylidene fluoride membrane (22). After blocking with 10% non-fat dry milk in Tris-buffered saline containing Tween-20, the membrane was incubated with the desired primary antibody for 1 h at the following dilutions: cyclin B1 (1:100 dilution), Cdc25B (1:1000 dilution), Cdc25C (1:200 dilution), Cdk1 (1:100 dilution), Bcl-2 (1:100 dilution), Bcl-Xl (1:100 dilution), Bax (1:500 dilution), Bid (1:100 dilution). Subsequently, the membrane was incubated with appropriate secondary antibody, and the immunoreactive protein bands were visualized using enhanced chemiluminescence kit (NEL Life Science Products, Boston, MA) according to the manufacturer’s instructions. Each membrane was stripped and re-probed with antibodies against actin (1:15 000 dilution) to correct for differences in protein loading.

**Results**

The effect of AITC treatment on survival of PC-3 and LNCaP cells was assessed by sulforhodamine B assay, and the results are shown in Figure 1A. Survival of both cancer cell lines was...
reduced significantly upon a 24 h exposure to AITC in a concentration-dependent manner with an IC₅₀ of ~15–17 µM (Figure 1A). On the other hand, survival of PrEC was minimally affected by AITC treatment even at concentrations that were highly cytotoxic to the prostate cancer cells (Figure 1A). For example, ~83% of PrEC cells were viable following a 24 h exposure to 40 µM AITC, whereas only ~36–38% of PC-3/LNCaP cells survived under similar conditions of AITC treatment (Figure 1A). Antiproliferative activity of AITC was further confirmed by trypan blue dye exclusion assay, and the results are shown in Figure 1B (PC-3 cells) and C (LNCaP cells). Proliferation of both cancer cells was inhibited significantly upon treatment with AITC in a concentration- and time-dependent manner. The viability of PC-3 and LNCaP cells was reduced by ~90–92% upon a 72 h exposure to 20 µM AITC.

To gain insights into the mechanism of antiproliferative activity of AITC, its effect on cell cycle distribution was determined and the results are summarized in Figure 2. A 24 h exposure of PC-3 as well as LNCaP cells to 10 and 20 µM AITC resulted in accumulation of cells in G2/M phase. For example, treatment of PC-3 cells with 10 µM AITC caused ~2.8-fold enrichment of cells in G2/M phase that was accompanied by a 46% decrease in G0/G1 phase cells. The G2/M arrest was not observed in PrEC cells treated with 20 µM AITC (Figure 2C). Treatment of PC-3 and LNCaP cells, but not PrEC cells, with AITC also resulted in appearance of cells with sub-diploid DNA content (sub-G₁ peak) suggesting that AITC may induce apoptosis. Proportion of the sub-G₁ peak was negligible in control PC-3 or LNCaP cells but increased considerably upon AITC treatment in both cells (data not shown).

The apoptosis-inducing effect of AITC was further investigated by (i) flow cytometric analysis of cells stained with Annexin V and propidium iodide (Figure 3A), (ii) quantification of cytoplasmic histone-associated DNA fragments (Figure 3B) and (iii) determination of caspase-3-like activity (Figure 3C). Annexin V is a calcium-dependent phospholipid binding protein with a high affinity for phosphatidylserine, which is a negatively charged membrane phospholipid located on the outer cytoplasmic surface of the plasma membrane of living cells (23). However, phosphatidylserine is translocated to the outer side of the plasma membrane in cells undergoing apoptosis (24). Staining with propidium iodide is indicative of necrotic cells. As can be seen in Figure 3A, AITC treatment increased the percentage of apoptotic cells in a concentration-dependent manner in both PC-3 and LNCaP cells. In comparison with DMSO-treated control PC-3 cells where roughly 7% cells were positive for Annexin V, the percentage of apoptotic cells was increased by ~2.2–3.0-fold upon 24 h treatment with 5, 10 and 20 µM AITC (Figure 3A). In LNCaP cells, AITC treatment (5, 10 and 20 µM) resulted in ~1.8–5.3-fold increase in apoptotic cells compared with DMSO-treated control cells (Figure 3A). As shown in Figure 3B, AITC treatment of PC-3 and LNCaP cells resulted in a concentration-dependent increase in the levels of cytoplasmic histone-associated DNA fragments compared with DMSO-treated controls. In contrast, relative to control, an increase in the levels of cytoplasmic histone-associated DNA fragments was not observed in AITC-treated (20 µM for 24 h) PrEC cells (Figure 3B). Apoptosis induction by AITC was further established by determining its effect on caspase-3-like activity in PC-3 and LNCaP cells, and the results are shown in Figure 3C. Caspase-3 is an active cell-death protease involved in the execution of apoptosis and is activated in response to various apoptotic stimuli (25). Caspase-3-like activity rose rapidly in AITC-treated PC-3 cells, reached a maximum between 1 and 4 h, and declined thereafter. In AITC-treated LNCaP cells, the caspase-3-like activity remained elevated for the duration of the experiment.

To elucidate the mechanism for G2/M arrest in AITC-treated cells, we determined its effect on expression of proteins that are pivotal for G2/M transition, including cyclin B1, Cdk1, Cdc25B and Cdc25C (26,27). Representative western blots for the time course response effect of 20 µM AITC treatment on cyclin B1, Cdk1, Cdc25B and Cdc25C expression in LNCaP cells are depicted in Figure 4. Changes in the expression of G2/M regulating proteins in AITC-treated cultures of PC-3 and LNCaP cells relative to control are summarized in Figure 5. A statistically significant decrease (~45% reduction compared
with control) in the levels of cyclin B1 protein was observed in LNCaP cells, but not in PC-3, at 24 h time point. On the other hand, a 24 h exposure to 20 μM AITC resulted in a significant down-regulation of Cdk1 protein expression in both cell lines, albeit to a greater extent in PC-3 cells (~50% reduction compared with control) than in the LNCaP cells (~32% reduction compared with control). The level of Cdc25B protein was reduced significantly by AITC treatment at 4, 16 and 24 h time points in PC-3 cells, and at 24 h time point in LNCaP cells. A statistically significant reduction in expression of Cdc25C protein was evident at 4, 16 and 24 h time points in AITC-treated PC-3 cells, and at 16 and 24 h time points in LNCaP cells. These results suggest that changes in expression of multiple G2/M regulating proteins may contribute to AITC mediated cell cycle arrest in PC-3 and LNCaP cells.

Western blot analyses using antibodies against Bcl-2 family of anti- (Bcl-2 and Bcl-XL) and pro-apoptotic proteins (Bax and BID), which play critical roles in apoptosis regulation (28), were performed to elucidate the mechanism of AITC-induced apoptosis. Representative western blots for expression of Bcl-2, Bcl-XL, Bax and BID using lysates from control and AITC-treated LNCaP cells are shown in Figure 6. Data on the effect of AITC treatment on expression of Bcl-2 family of apoptosis regulating proteins are summarized in Figure 7. As can be seen in Figures 6 and 7, the expression of Bcl-2 was reduced significantly by AITC treatment in both cell lines at 24 h time point. A 24 h treatment with 20 μM AITC resulted in 31 and 68% reduction in expression of Bcl-2 in PC-3 and LNCaP cell lines, respectively. The expression of Bcl-XL was reduced by ~58% at 24 h time point in LNCaP cells. On the other hand, expression of Bcl-XL was not affected by AITC treatment in PC-3 cells. AITC treatment did not alter the expression of pro-apoptotic proteins Bax and BID in either cell line (Figure 7).

Discussion

Cruciferous vegetables are a rich source of ITCs that are highly effective in affording protection against cancers in experimental animals induced by a variety of chemical carcinogens (2–8). In the present study, we tested the hypothesis that ITCs may inhibit proliferation of human prostate cancer cells. We found that AITC significantly inhibited proliferation of PC-3 and LNCaP human prostate cancer cells in a concentration-dependent manner. In contrast, viability of a normal PrEC was minimally affected by AITC treatment suggesting that this ITC compound may exhibit minimal toxicity, if any, to normal cells. It is important to point out that AITC was more or less equally effective in suppressing proliferation of PC-3 and LNCaP cells. These observations
have clinical implications since the majority of the human prostate cancers at the time of diagnosis represent androgen-dependent as well as androgen-independent cells.

Eukaryotic cell cycle progression involves sequential activation of Cdns, whose activation is dependent upon their association with cyclins (26,27,29). A complex formed by the association of Cdk1 (also known as p34Cdc2) and cyclin B1 plays a major role at entry into mitosis (26,27,29). Whereas phosphorylation of Thr161 of Cdk1 is required for complete activation of the complex, reversible phosphorylations at Thr14 and Tyr15 suppress activity of Cdk1/cyclin B1 kinase complex (26,27,29). Dephosphorylation of Thr14 and Tyr15 of Cdk1 is catalyzed by dual-specificity phosphatases Cdc25B and Cdc25C, and this reaction is believed to be the rate-limiting step for entry into mitosis (26,27,30). We found that AITC treatment causes a statistically significant reduction in the expression of Cdk1, Cdc25B and Cdc25C in both cells, whereas the level of cyclin B1 is reduced only in LNCaP cells. Thus, it is reasonable to postulate that AITC treatment may cause cell cycle arrest by reducing the activity of Cdk1/cyclin B kinase complex due to down-regulation of multiple G2/M regulating proteins.

The results of the present study indicate that AITC treatment induces apoptotic cell death in both PC-3 and LNCaP cells but not in normal prostate epithelial cells. These observations suggest that AITC-mediated apoptosis induction may be specific to the prostate cancer cells. On one hand, our results are consistent with those of previous studies demonstrating apoptotic induction by structurally related ITCs in other cell types (11,14,15,31). On the other hand, the mechanism for AITC-induced apoptosis in prostate cancer cells appeared to be different than those suggested previously for other ITCs. For example, Gamet-Payrastre et al. (15) showed that apoptosis induction by sulforaphane, a naturally occurring ITC compound abundant in broccoli, in HT29 human colon cancer cells was associated with increased expression of pro-apoptotic protein Bax. The expression of anti-apoptotic protein Bcl-2 could not be detected in HT29 cells. Our results indicate that AITC-induced apoptosis in PC-3 and LNCaP cells is not due to change in Bax expression but likely due to down-regulation of Bcl-2 in both cells and a reduction in Bcl-Xl expression in LNCaP cells. In another study, Huang et al. (14) have concluded that apoptosis induction by PEITC, a structural analogue of AITC, occurs through a p53-dependent pathway. Our results suggest that p53 may not be essential for AITC-induced apoptosis in human prostate cancer cells because PC-3 cell line is p53 deficient. More recently, Chiao et al. (31) have reported that sulforaphane and its N-acetylcysteine conjugate can mediate growth arrest and induce apoptosis in LNCaP cell line. Sulforaphane-mediated growth arrest in LNCaP cells was associated with an attenuation of the expression of phosphorylated and dephosphorylated androgen receptor (31). Interestingly,
these investigators also showed that sulforaphane treatment caused G2/M block that was associated with a down-regulation of cyclin D1. These results are in contrast to those of previous studies with sulforaphane in HT29 human colon cancer cells (15). Similar to our findings, sulforaphane treatment of HT29 cells was associated with G2/M arrest (15). Even though the reasons for these discrepancies are not clear, it is possible that the mechanism for ITC-mediated growth inhibition may be cell specific. This notion is partially supported by the results of the present study indicating that AITC treatment causes a down-regulation of Bcl-2 expression in PC-3 as well as in LNCaP cells, whereas expression of Bcl-XL is reduced only in the LNCaP cell line.

Data presented herein indicated that 10 and 20 μM concentrations of AITC effectively inhibited proliferation of PC-3 and LNCaP cells by inducing apoptosis and causing cell cycle arrest. Our data are consistent with the results of previous cellular studies using other ITC analogues, such as sulforaphane and PEITC, where apoptosis induction, cell cycle arrest and/or molecular changes associated with growth inhibition were observed at ITC concentrations of 50 μM or lower (11,14,15,19,31). Since pharmacokinetics of AITC in humans has not been investigated, it is difficult to predict whether micromolar concentrations of AITC are achievable in humans. However, previous studies have shown that consumption of one ounce of fresh watercress can yield ~6 mg of PEITC suggesting that micromolar concentrations might be achieved in vivo (32,33). Another pharmacokinetics study involving four human volunteers fed with a single dose of myrosinase-hydrolyzed extract of 3-day-old broccoli sprouts (containing about 200 μmol of total ITC) indicated that ITCs were absorbed rapidly and reached peak concentrations of 0.943–2.27 μmol/l in plasma, serum and erythrocytes at 1 h after broccoli-extract ingestion (34). Clearly, additional pre-clinical studies using appropriate in vivo animal models as well as carefully designed pharmacokinetics studies are needed before clinical testing of AITC as a cancer preventive or therapeutic agent.

In conclusion, the results of the present study indicate that AITC effectively inhibits proliferation of both androgen-dependent and androgen-independent human prostate cancer cells by causing G2/M phase arrest and inducing apoptosis. It is reasonable to hypothesize that AITC, and possibly other ITCs, may be useful for delaying onset and/or progression of human prostate cancers.

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References


