Selected isothiocyanates rapidly induce growth inhibition of cancer cells

Yuesheng Zhang, Li Tang, and Veronica Gonzalez

Arizona Cancer Center and Department of Medicine, College of Medicine, University of Arizona, Tucson, AZ

Abstract

Many plant-derived isothiocyanates (ITCs), which occur in human diet, are potent cancer chemopreventive agents in animals. Among the anticarcinogenic mechanisms that have been revealed for ITCs is the inhibition of cell proliferation. We report that exposure of cancer cells to either allyl-ITC (AITC), benzyl-ITC (BITC), or phenethyl-ITC (PEITC) for only 3 h was long enough for the inhibition of cell growth, based on a comparison of IC_{50} values; regardless of the origin of cancer cells; and even in drug-resistant cells that overexpressed multidrug resistance associated protein-1 (MRP-1) or P-glycoprotein-1 (Pgp-1). In contrast, the inhibitory effect of another ITC, sulforaphane (SF), on these cells was highly time dependent. The finding that some ITCs could inhibit the proliferation of cancer cells in a largely time-independent manner is significant because ITCs that enter the human body are rapidly cleared through urinary excretion. Using human promyelocytic leukemia HL60/S as model cells, and focusing on AITC and BITC, we found that these ITCs modulated multiple cellular targets involved in proliferation, including the disruption of mitochondrial membrane potential, activation of multiple caspases, arrest of cell cycle progression, and induction of differentiation. Again, only a 3-h incubation of the cells with the ITCs was enough to exert their full effect on these targets. Taken together, our findings suggest that selected ITCs can rapidly initiate growth inhibition of cancer cells by simultaneously modulating multiple cellular targets, and their antiproliferative activity may be largely unaffected by their metabolism and disposition *in vivo*. (Mol Cancer Ther. 2003;2:1045–1052)

Introduction

Isothiocyanates (ITCs) are a family of small organic compounds that occur in a wide variety of plants, many of which are consumed by humans on a regular basis (1, 2). For example, mustard, garden cress, water cress, and broccoli (especially broccoli sprouts) are rich sources of allyl-ITC (AITC) (3), benzyl-ITC (BITC) (4), phenethyl-ITC (PEITC) (5), and sulforaphane (SF) (6, 7), respectively. ITCs are synthesized and stored in plant cells as glucosinolates (β-thioglucoside *N*-hydroxysulfates), then released when plant cells are injured. The conversion is catalyzed by myrosinase, which coexists with, but is structurally segregated from, glucosinolates in intact plants (1). However, human enteric microflora also possess myrosinase activity and can convert a significant portion of the ingested unhydrolyzed glucosinolates to ITCs (8–10). Unlike the ITCs which are electrophilic and biologically active, the glucosinolates are, in large part, chemically stable and biologically inert.

To date, the most important biological property discovered about ITCs is their ability to inhibit chemical carcinogenesis. More than 20 ITCs, the majority of which occur in vegetables, have been shown to inhibit tumorigenesis induced by a wide variety of chemical carcinogens in animal models (11, 12), inhibiting tumorigenesis in the lung, stomach, colon, liver, esophagus, bladder, and mammary glands (11–14). Several recent epidemiological studies have suggested that humans who consumed higher levels of ITCs might be less likely to develop lung and colon cancer (15–17). Mechanistic investigations have shown that the remarkable anticarcinogenic property of ITCs stems from their ability to disrupt multiple steps in the carcinogenic process: reducing genetic damage as a result of the inhibition of carcinogen-activating enzymes and induction of carcinogen-detoxifying enzymes, inhibiting proliferation of genetically damaged cells as a result of induction of apoptosis and cell cycle arrest, and inducing the differentiation of malignant cells (11, 14, reported in this paper). Their anticarcinogenic potential is demonstrated further by the finding that their metabolites formed *in vivo* possess similar, if not more potent, anticarcinogenic activity in cultured cells or animal models (18–20). ITCs are rapidly metabolized mainly through the mercapturic acid pathway in both humans and animals, giving rise to various dithiocarbamate metabolites (21–23). Specifically, an initial conjugation with glutathione (GSH) promoted by glutathione transferase gives rise to the corresponding conjugates, which then undergo further enzymatic modification (metabolism of the GSH moiety) to form sequentially the cysteinylglycine, cysteine (Cys), and *N*-acetylcysteine conjugates which are excreted in the urine. Many lines of evidence show that these dithiocarbamates are merely carriers of ITCs, as they are unstable and dissociate readily to ITCs (24–26).

While much focus has been concentrated on their ability to block chemical carcinogenesis, ITCs also may be important cancer therapeutic agents. Several ITCs are known to induce both apoptosis and cell cycle arrest in several types of cultured human cancer cells (18, 27–30).

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Requests for Reprints: Yuesheng Zhang, Department of Chemoprevention, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, New York 14263. E-mail: yuesheng.zhang@roswellpark.org

AITC, given i.p., significantly inhibited the growth of human prostate cancer xenografts in mice (31). We were especially intrigued by the observation of Adesida *et al.* (32) that exposure of human leukemia cells to either PEITC or its Cys conjugate for only 3 h was long enough for a maximum inhibition of cell growth, indicating a very rapid interaction of the drugs with cellular targets. This finding was interesting because it suggested that the anticancer activity of PEITC *in vivo* might be largely unaffected by the rapid disposal kinetics associated with this compound.

We report here that a comparison of four ITCs that have shown potent anticarcinogenic activity, that is, AITC, BITC, PEITC, and SF (see Fig. 1 for chemical structures), showed that their ability to rapidly induce the inhibition of cell growth was not cell-specific, but ITC-specific. Overall, the ITCs appeared to be more potent against blood cancer cells than epithelial cancer cells. Moreover, the ITCs also inhibited the growth of cancer cells that overexpressed multidrug resistance associated protein-1 (MRP-1) or P-glycoprotein-1 (Pgp-1). Focusing on the two most potent ITCs, AITC and BITC, and using human leukemia HL60/S cells as model cells, we found that the ITCs, after only 3 h incubation with the cells, simultaneously modulated multiple cellular targets, including the damage of mitochondria, induction of apoptosis, arrest of cell cycle progression, and induction of differentiation.

Materials and Methods

Materials

BITC and SF were purchased from LKT Laboratories (St. Paul, MN). AITC, PEITC, and 4-methylesculetin were purchased from Aldrich (Milwaukee, WI). Nitroblue tetrazolium (NBT) and 3,3′-dehexyloxacarbocyanine iodide [$DiOC_6(3)$] were purchased from Sigma Chemical Co. (St. Louis, MO) and Molecular Probes (Eugene, OR), respectively. The GSH or Cys conjugates (dithiocarbamates) of AITC and BITC, including GS-AITC, GS-BITC, Cys-AITC, and Cys-BITC, were prepared in our laboratory, following the procedures described by Brusewitz *et al.* (21). All products were structurally confirmed by mass spectrometric analysis (electrospray) and were found pure as determined by the cyclocondensation assay (33).

Cell Culture

Human promyelocytic acute leukemia HL60/S cells and their doxorubicin-resistant derivative HL60/AR (34), human myeloma 8226/S cells and their doxorubicin-resistant derivative 8226/Dox40 (34), and human colon cancer HT-29 cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). Human breast cancer MCF-7 cells, human hepatoma HepG2 cells, and transformed human epidermal HaCaT keratinocytes were cultured in DMEM with 10% FBS. RPMI 1640 and DMEM were purchased from Life Technologies, Inc. (Rockville, MD). The FBS was purchased from Omega Scientific (Tarzana, CA). All cells were maintained in either 75-cm^2 flasks or in 10-cm plates in a humidified incubator at 37°C with 5% CO_2.

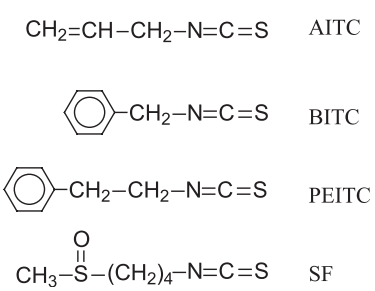

Figure 1. The chemical structure of AITC, BITC, PEITC, and SF.

Except for the cytotoxicity assay described below, in all other analyses, 2×10^6 cells were grown in a 10-cm plate with 10 ml medium for 24 h and then treated with an ITC as indicated. Each ITC was dissolved in DMSO. The final DMSO concentration in the medium was 0.1% (v/v).

Assessment of Cytotoxicity

Cells ($2–5 \times 10^3$) were grown in each well of a 96-well plate with 150 μl medium for 24 h. A test ITC then was added to each well with 50 μl medium. The final concentrations of the compound in each well were 0.39–100 μM (1-fold serial dilutions). All test compounds were originally dissolved in DMSO. The final concentration of DMSO was 0.025% (v/v) or lower. One set of plates was incubated for 72 h without medium replacement, whereas with another set of plates, the test compound was washed off the cells at 3 h incubation time and grown for another 69 h without the test compound. To remove the ITC, the existing medium in each well was removed, the cells washed (adding 200 μl fresh medium per well and then removed), and replaced with 200 μl ITC-free medium. The medium in each well was removed by vacuum aspiration, preceded by centrifugation of the plates at 2000 rpm for 5 min at room temperature for cells growing in suspension. At the end of the incubation, the number of living cells in each well was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (35). The concentration of the test ITC required to inhibit cell growth by 50% (IC_{50}) was determined based on the growth curve of ITC-treated cells.

Analysis of Mitochondrial Transmembrane Potential

Mitochondrial transmembrane potential ($\Delta\Psi_m$) results from the unequal distribution of protons across the mitochondrial membrane, giving rise to negative charges on the inner side of the inner mitochondrial membrane, which is critical for ATP synthesis. Fluorescent dyes, such as $DiOC_6(3)$, which was used in our study (36), are readily and selectively sequestered by actively respiring mitochondria, but are washed out when $\Delta\Psi_m$ is lost. Cells were treated with AITC or BITC at 2 or 10 μM for 3 or 24 h. After the ITC treatment, cells were washed with Dulbecco's PBS (DPBS), resuspended in fresh medium at 5×10^5 cells/ml, and incubated with 15 nM $DiOC_6(3)$ for 30 min at 37°C. The cells were then washed twice with DPBS, resuspended in an equal volume of DPBS, and promptly measured by flow cytometry for $DiOC_6(3)$ content using excitation at 488 nm and emission at 520 nm. Ten thousand cells were analyzed in each sample.

Analysis of Apoptosis and Cell Cycle Arrest

Cells undergoing apoptosis were detected on the basis of phosphatidylserine (PS) externalization on cell membrane, a marker of early apoptosis, using annexin-V, a PS-specific binding dye. A flow cytometry-based apoptosis detection kit (TA4638) from R&D Systems (Minneapolis, MN) was used to measure apoptotic cells. Cells that were stained with propidium iodide (PI, a DNA binding dye), indicative of the loss of cell membrane integrity, were excluded because these cells might either be late apoptotic cells or necrotic cells. As a result, the apoptotic cells reported in our experiments were cells undergoing early apoptosis.

Cell cycle arrest was determined by a flow cytometry-based measurement of DNA content using PI staining. ITC-treated or control cells were suspended at 1.0×10^6 cells in 0.5 ml modified Krishan buffer containing 0.1% sodium citrate, 20 μg/ml RNase, 0.3% NP40, and 50 μg PI/ml (37), and incubated on ice in the dark for at least 30 min before analysis by flow cytometry.

To determine the effect of AITC or BITC on apoptosis and cell cycle progression, cells were treated with AITC or BITC at specified concentrations for 3 h, 3 h followed by 21 h incubation without the ITC (ITC was washed off cells with DPBS and reincubated in ITC-free medium), or 24 h. Cells were harvested at the end of each incubation time and used for the assays described above. Ten thousand cells in each sample were examined by flow cytometry.

Western Blot Analysis of Caspase Activation and Cell Cycle Regulators

Caspase activation and levels of cell cycle regulators were measured by Western blot analysis. For the detection of caspase activation, cells were treated with AITC or BITC at a specified concentration for 3 or 24 h. Cells incubated with the ITC for 3 h either were harvested at the end of the incubation or washed to remove the ITC and incubated in ITC-free medium for another 3 h to detect the delayed activation of a caspase. For detection of cell cycle regulators, the cells were treated with ITC either for 3 h, followed by a wash with DPBS to remove the ITC and further incubation in ITC-free medium for 21 h, or for 24 h without medium change. At the end of each incubation time, cells from each plate were pelletized by centrifugation and lysed in 200 μl cell lysis buffer from Cell Signaling Technology (Beverly, MA) which was supplemented with 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant was used for the analyses. Each sample (50 μg protein) was resolved by SDS-PAGE (12–15%) and blotted onto polyvinylidene difluoride membranes. Each membrane then was probed by an antibody and the band of interest visualized using an ECL chemiluminescence system from Amersham Biosciences (Piscataway, NJ).

The antibodies used for the caspases were those specific for pro- and cleaved caspase-3, -7, -8, -9, or -12. The antibodies used for cyclins and cdks were those specific for cyclin B1, cyclin D1, cyclin E, cdk-2, cdk-4, or cdc-2. These antibodies were purchased from either Cell Signaling Technology or from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against α-actin was used to ensure the comparability of results among samples.

Assay of Cell Differentiation

Cell differentiation was measured by NBT reduction, following the procedure described by Kawaii *et al.* (38). Cells that reduce NBT yield intracellular black-blue formazan deposits, which can be detected microscopically. A minimum of 200 cells from each slide was counted; 4-methylesculetin was used as a positive control (38).

Results

Effect of ITCs on Growth of Cancer Cells

A panel of six cancer cell lines from both epithelial and blood origins was used to evaluate the effect of treatment time on the growth-inhibitory potentials of four ITCs, including AITC, BITC, PEITC, and SF. These cells were selected to represent the major organ sites, including blood (HL60/S and 8226/S), breast (MCF-7), liver (HepG2), colon (HT-29), and skin (HaCaT). Cells were treated with an ITC for either 3 h followed by 69 h incubation without the ITC, or for 72 h without medium replacement. We found that the IC_{50} values for AITC, BITC, and PEITC with only 3 h treatment were very similar (less than 1-fold increase) to those with 72 h treatment in all cases (Table 1). In contrast, while the IC_{50} values for SF with 72 h treatment were similar to those of the three other ITCs, SF's ability to inhibit cell growth was reduced markedly with only a 3-h treatment (IC_{50} values increased by 5.7- to 11.2-fold) (Table 1). These results, therefore, show that the ability of ITCs to rapidly initiate inhibition of cell growth was related to the specific ITCs, but not to cell types.

Among the six cell lines tested, those that originated in blood appeared to be more sensitive to the ITCs than the epithelial cancer cells (Table 1). Overall, AITC and BITC were more potent than PEITC and SF in inhibiting cell growth. Interestingly, HepG2 cells were especially resistant to AITC, and the reason is unknown. Because ITCs are known to be metabolized rapidly through the mercapturic acid pathway *in vivo*, it was important to determine how the metabolism would affect the antiproliferative activity of the ITCs. We tested the GSH and Cys conjugates of AITC and BITC in HL60/S cells. All conjugates showed potency very similar to their parent ITCs in inhibiting the growth of the cells and also were largely schedule independent, that is, the IC_{50} values were very similar whether the cells were treated with the compound for 3 or 72 h (compare the results in Tables 1 and 2). This result, however, was not unexpected because the conjugates are known to readily dissociate to ITCs.

Cancer Cells Overexpressing ABC Drug Transporters Were Not Resistant to ITCs

Two human blood cancer cell lines, which were highly resistant to doxorubicin and overexpressed either MRP-1 (HL60/AR) or Pgp-1 (8226/Dox40) (34), were sensitive to the four ITCs. The IC_{50} values of the four ITCs from these

Table 1. Growth inhibition of cultured cancer cells by ITCs

Cell Line	IC_{50} (μM)							
	3 h ITC + 69 h ITC-Free				72 h ITC			
	AITC	BITC	PEITC	SF	AITC	BITC	PEITC	SF
HL60/S	3.3 ± 1.1	2.0 ± 0.3	4.0 ± 1.3	31.5 ± 1.3	2.5 ± 0.1	1.8 ± 2.3	3.6 ± 0.4	3.4 ± 0.6
8662/S	5.3 ± 0.7	2.1 ± 0.2	5.3 ± 1.2	21.3 ± 1.5	4.0 ± 0.6	2.2 ± 0.6	3.1 ± 0.3	1.9 ± 0.2
MCF-7	12.3 ± 1.3	8.4 ± 2.4	17.5 ± 1.5	>50	6.4 ± 1.1	4.6 ± 0.5	11.0 ± 1.2	6.7 ± 0.5
HepG2	44.8 ± 3.5	8.1 ± 0.8	10.1 ± 0.2	>50	35.5 ± 5.7	7.3 ± 0.6	11.2 ± 2.7	12.8 ± 1.3
HT-29	5.9 ± 0.7	5.4 ± 0.9	10.0 ± 0.5	>50	4.5 ± 0.2	5.1 ± 1.4	9.6 ± 1.1	10.0 ± 0.1
HaCaT	9.5 ± 1.0	6.0 ± 0.7	8.4 ± 1.4	>50	5.3 ± 0.4	4.3 ± 0.7	6.1 ± 1.0	6.7 ± 1.0

Note: Cells were grown in 96-well plates for 24 h and then exposed to a series of concentrations of an ITC, which lasted for either 72 h, or was terminated at 3 h time point (medium removed and cells washed with culture medium) and then grown in ITC-free medium for 69 h. Cell density in each well then was determined with MTT assay and the IC_{50} value was calculated based on cell growth curve. Each value is the mean of four determinations (±SD).

cells were very close to those from the corresponding parent cells (HL60/S and 8226/S) that did not overexpress the drug transporters (compare results in Tables 1 and 3). Furthermore, AITC, BITC, and PEITC also showed little time dependence in inhibiting the growth of these cells, whereas the effect of SF on these cells was again time dependent (Table 3). Because the antiproliferative effects of the ITCs were observed in both drug-resistant cell lines, it seems likely that overexpression of MRP-1 or Pgp-1 may not render cancer cells resistant to the ITCs. In addition, we found that the ITC dithiocarbamate metabolites described in Table 2 also potently inhibited the growth of these drug-resistant cells (results not shown), providing further evidence that the ITCs are promising anticancer agents.

ITCs Rapidly Disrupted $\Delta\Psi_m$

On the basis that the ITCs showed especially potent antiproliferative activity against the blood cancer cell lines, efforts were made to determine the underlying mechanisms, using HL60/S. We focused on AITC and BITC, which were more potent than the other two ITCs. Exposure of HL60/S cells to either AITC or BITC resulted in dose-dependent damage to mitochondria. Treatment of the cells with 10 μM AITC or BITC for 3 h resulted in a 3.3- or 5.9-fold increase in the number of cells with the loss of $\Delta\Psi_m$, respectively. Disruption of normal $\Delta\Psi_m$ presumably will compromise ATP synthesis in cells, causing cytotoxicity, and may also be responsible for the activation of caspase-9 which is mitochondria mediated, as described below. Mitochondrial damage appeared to occur largely in the first 3 h of ITC exposure, as treatment of the cells with each ITC for 24 h had a lower number of cells with mitochondrial damage (Fig. 2). It is noteworthy that we consistently observed that the ITCs were less toxic to the cells grown in 10-cm dishes (*i.e.*, IC_{50} values were 1- to 2-fold higher) than in 96-well plates (results not shown). This difference was probably related to the higher amount of test agent available to each cell grown in the 96-well plate than in the dishes. There were approximately 25 cells/μl ITC-containing medium in each microtiter plate well and 200 cells/μl ITC-containing medium in the dishes, as described in the ''Materials and Methods.'' As a result, in all experiments where cells were grown in 10-cm dishes, we used 2 μM and/or 10 μM ITC concentrations.

Table 2. Growth inhibition of HL60/S cells by GSH and Cys conjugates of ITCs

Conjugate	IC_{50} (μM)	
	3 h Conjugate + 69 h Conjugate-Free	72 h Conjugate
GS-AITC	2.4 ± 0.1	1.6 ± 0.1
Cys-AITC	1.8 ± 0.1	1.6 ± 0.1
GS-BITC	1.4 ± 0.1	0.9 ± 0.1
Cys-BITC	1.1 ± 0.1	0.7 ± 0.1

Note: Used the same protocol as described in Table 1.

Induction of Apoptosis

AITC and BITC activated several caspases in HL60/S cells in a time-dependent fashion (Fig. 3). Treatment of the cells with 10 μM of either AITC or BITC for only 3 h led to the activation of caspase-3, -8, and -9. While the activation of caspase-8 and -9 was observed at the end of the 3-h exposure, caspase-3 activation was delayed for another 3 h, which is consistent with caspase-3 being activated by activated caspase-8 or -9. Endoplasmic reticulum (ER) stress-mediated activation of caspase-12 was detected only when the cells were incubated with the ITCs for 24 h, suggesting that it might play a minor role in ITC-induced apoptosis. Activation of caspase-7 was not detected in the ITC-treated cells. However, as described below, activation of the caspases did not appear to reliably predict cell death. Transmembrane externalization of PS is a recognized early event of apoptosis, detection of which by a flow cytometry-based annexin-V staining is used widely for identifying early apoptotic cells. There were no increases in the number of apoptotic cells if they were exposed to 2 or 10 μM ITC for only 3 h, with or without an additional 21-h incubation of the cells in ITC-free medium (results not shown). However, there was a significant increase in the number of apoptotic cells when they were treated with ITCs for 24 h. Treatment

Table 3. ITC-induced growth inhibition of cancer cells overexpressing MRP-1 or Pgp-1

Cell Line	IC_{50} (μM)							
	3 h ITC + 69 h ITC-Free				72 h ITC			
	AITC	BITC	PEITC	SF	AITC	BITC	PEITC	SF
HL60/AR	5.2 ± 0.9	4.1 ± 0.4	6.7 ± 0.9	22.6 ± 3.9	3.1 ± 0.2	3.0 ± 0.6	4.9 ± 0.4	4.3 ± 0.5
8226/Dox40	6.3 ± 0.5	2.8 ± 0.2	8.4 ± 0.7	32.3 ± 0.5	3.5 ± 0.3	2.5 ± 0.2	4.6 ± 0.3	2.3 ± 0.3

Note: Used the same protocol as described in Table 1.

of the cells with 2 or 10 μM BITC increased apoptotic cells by 4.9- and 3.2-fold, respectively, whereas the same concentrations of AITC led to 0.8- and 4.4-fold changes (Fig. 4). The lower number of apoptotic cells with 10 μM BITC might be related to the fact that annexin-V staining only detects early apoptotic cells. We found 35.5 ± 2.53% cells in late apoptosis and/or necrosis (cells stained with both annexin-V and PI).

Cell Cycle Arrest

Short treatment of HL60/S by AITC or BITC also blocked cell cycle progression. While neither ITC at 2 μM was effective (results not shown), both significantly arrested cell cycle progression at 10 μM. It appeared that only 3 h exposure to each ITC could efficiently render the cells growth arrested (Table 4). Interestingly, while AITC blocked the cells in G_1 phase (the number of cells in this phase increased 1.7- to 1.8-fold), BITC blocked the cells in G_2-M phase (the number of cells in this phase increased 1.9- to 2.8-fold); although 24 h BITC exposure also resulted in G_1-phase arrest (Table 4). Selected cyclines and cdks involved in regulating G_1 and G_2-M phases were examined. There were no detectable changes in the expression levels of cyclin D1 and cyclin E (important for G_1 progression), nor was there any change in the levels of cdk-2 and -4 (regulating G_1 phase), when HL60/S cells were treated with 2 or 10 μM AITC for 24 h, or for 3 h followed by incubation in ITC-free medium for 21 h (results not shown). Likewise, similar treatment of the cells with BITC did not affect the expression level of cyclin B1 (important for G_2-M phase progression) and cdc-2 (regulating G_2-M phase) (results not shown). Consequently, our survey of cell cycle regulators did not identify the molecular targets of the ITCs.

Induction of Differentiation

HL60/S cells are known to differentiate when exposed to certain chemical agents, such as 6,7-dihydroxy-4-methylcoumarin and retinoic acid (38). NBT reduction is considered one of the earliest differentiation markers expressed in maturing HL60 cells. This assay evaluates the ability of

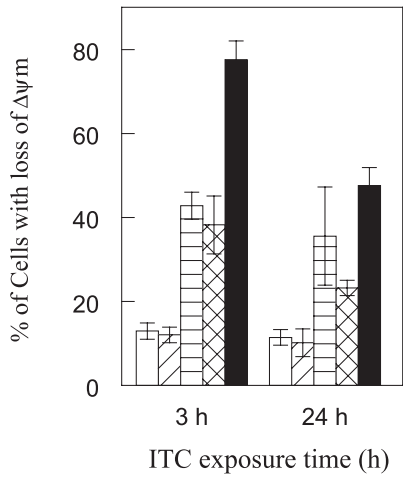

Figure 2. Effect of AITC or BITC on $\Delta\Psi_m$ in HL60/S cells. Cells were exposed to vehicle (□), 2 μM AITC (▨), 10 μM AITC (▤), 2 μM BITC (⊠), or 10 μM BITC (■) for 3 or 24 h. Cells then were harvested for determination of $\Delta\Psi_m$ by flow cytometry. *Columns,* mean of three measurements; *bars,* SD. The values obtained from ITC-treated cells, except for 2 μM AITC, were significantly different from the values in the control cells (*t* test, $P < 0.01$).

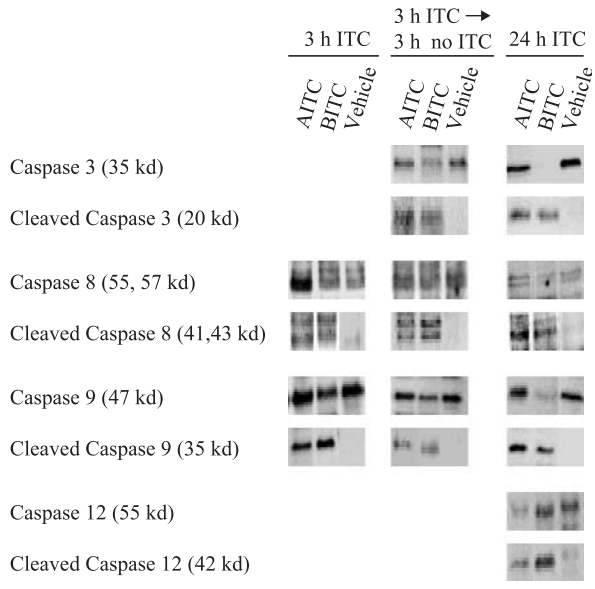

Figure 3. Activation of various caspases by AITC or BITC in HL60/S cells. Cells were treated with 10 μM ITC for 3 h, 3 h followed by incubation of the cells in ITC-free medium for 3 h, or 24 h. The cells then were harvested and lysed in lysis buffer. The lysates were used to determine the activation of various caspases (see ''Materials and Methods'' for details). The molecular weight for each cleaved and uncleaved caspase is shown in parentheses.

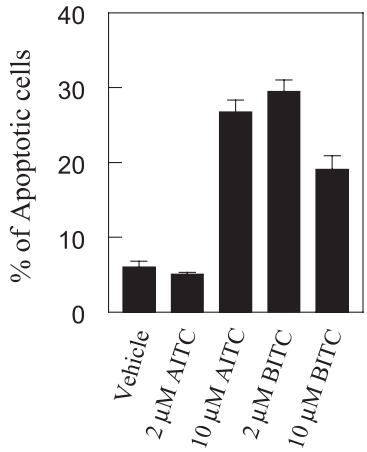

Figure 4. Effect of AITC or BITC on apoptosis in HL60/S cells. Cells were treated with AITC or BITC at the specified concentrations for 24 h. Apoptotic cells then were identified, using flow cytometry-based annexin-V staining. *Columns,* mean of three measurements; *bars,* SD. The values obtained from ITC-treated cells, except for 2 μM AITC, were significantly different from that in control cells (*t* test, $P < 0.01$).

cells to produce superoxide when challenged with 12-*O*-tetradecanoylphorbol 13-acetate. Monocytes, macrophages, and granulocytes are able to reduce NBT, but their immature progenitors cannot (39). The NBT assay is known to give results with a lower variance than a number of other differentiation markers, although it does not show a specific lineage. We found that both AITC and BITC significantly induced the differentiation of HL60/S in a dose-dependent manner. Treatment of the cells with each ITC at 2 or 10 μM for only 3 h (followed by 69 h ITC-free culture) increased NBT-reducing cells from 15.4% to 32.4–72.8%, a 2.1- to 4.7-fold increase (Fig. 5). BITC appeared to be slightly more potent than AITC in the induction of differentiation. Our results also showed that 3 h exposure of cells to the ITC was long enough, because a longer exposure time (*e.g.*, 72 h) led to a lower degree of induction of differentiation (results not shown).

Table 4. Effect of AITC and BITC on HL60/S cell cycle progression

ITC Exposure	ITC	Cell Cycle Distribution, %		
		G_1	S	G_2-M
3 h ITC + 21 h ITC-free	vehicle	32.2 ± 2.9	51.1 ± 1.8	16.6 ± 2.0
	AITC	53.6 ± 18.6*	33.1 ± 17.5	13.4 ± 1.3
	BITC	39.0 ± 13.0	15.9 ± 9.5	45.8 ± 20.1*
24 h ITC	vehicle	32.0 ± 0.5	57.9 ± 2.3	10.1 ± 2.6
	AITC	57.9 ± 6.0*	38.5 ± 8.2	3.6 ± 2.4
	BITC	43.8 ± 1.1*	37.2 ± 1.2	18.9 ± 1.4*

Note: Cells were grown in 10-cm plates for 24 h and then exposed to 10 μM AITC or BITC. The exposure lasted for either 24 h, or for 3 h (followed by growing the cells in ITC-free medium for 21 h). The cells then were harvested and analyzed for cell cycle distribution by flow cytometry. Each value is a mean of three measurements (±SD). Each value labeled with an asterisk is significantly different from the control (*t* test, $P < 0.01$).

Discussion

Studies in both humans and animals have shown that on ingestion, ITCs are rapidly absorbed and eliminated (3, 40, 41). ITCs are metabolized principally *in vivo* through the mercapturic acid pathway and excreted in urine as an *N*-acetylcysteine conjugate (21, 23, 42). Although it has been shown that lipophilic ITCs may be retained in blood and tissue for longer periods of time (43), the elimination of these compounds is generally very fast (half plasma life around 1 h) (3, 40). Therefore, it is highly intriguing that three of the four ITCs tested were able to almost fully display their antiproliferative activities after only 3 h incubation with the tested cancer cells. Furthermore, the dithiocarbamates derived from ITCs, known to form as major metabolites *in vivo*, also displayed similar antiproliferative activity, suggesting that the metabolism of ITCs may not significantly affect their anticancer activity. The efficacy of ITCs in the elimination of cancer cells was further documented by our finding that they could potently inhibit the growth of cells that were highly resistant to doxorubicin and overexpressed either MRP-1 or Pgp-1. Overall, BITC appeared to be the most potent antiproliferative agent in our study, followed in decreasing order by AITC, PEITC, and SF. While AITC, BITC, and to larger extent PEITC, required only 3 h incubation with the cells to exert nearly full growth-inhibitory impact on the cells, the effect of SF was time dependent in all cell lines tested. Although the underlying reason is unknown at the present time, ITCs that can rapidly initiate the inhibition of cell growth are undoubtedly more promising anticancer agents than those that do not. Our study also revealed that leukemia cells and myeloma cells might be more sensitive to the ITCs than the epithelial cancer cell lines tested.

Focusing on AITC and BITC, using HL60/S as model cells, we investigated the molecular mechanisms responsible

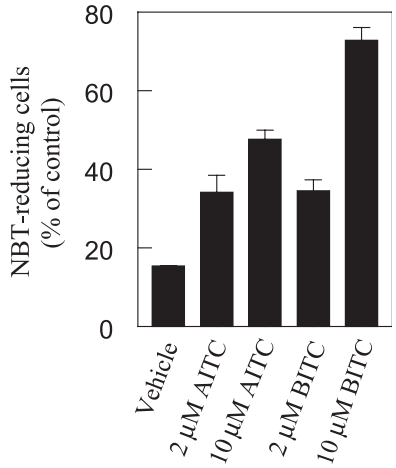

Figure 5. Induction of differentiation in HL60/S cells by AITC and BITC. Cells were treated with AITC or BITC at the specified concentrations for 3 h and then grown in ITC-free medium for 3 days. The cells then were harvested and processed as described in ''Materials and Methods.'' *Columns,* mean of three measurements; *bars,* SD. Each value of treated cells is significantly different from the control cells (*t* test, $P < 0.01$).

for the antiproliferative activity of the ITCs, emphasizing the effect of incubation time of each ITC with the cells on these mechanisms. Exposure of the cells to either AITC or BITC resulted in the rapid loss of $\Delta\Psi_m$, likely disrupting energy production in the cells. The effect of the ITCs was dose dependent. The maximal effect appeared to occur within the first 3 h of exposure. Exposure to either AITC or BITC also resulted in the activation of several caspases, including caspase-3, -8, -9, and -12 in HL60/S cells. Caspase-8 (Fas receptor mediated), -9 (mitochondria mediated), and -12 (ER mediated) are involved in three apoptotic pathways, activation of which in turn activates the so-called executioner caspases (44–46). Although ITC-induced activation of caspase-9, -8, and -3 has been observed before (27), the time course of activation of these caspases by an ITC has not been examined previously. Also, the activation of caspase-12 by an ITC is previously unreported. Our results show that ITCs are capable of stimulating all three apoptotic pathways in HL60/S cells, although how the ITCs activated these caspases in these cells is largely unknown. However, it appears that 3 h ITC treatment may not be long enough to induce apoptotic cell death, even though certain caspases were induced. As described in the ''Results,'' there were no increase in the number of apoptotic cells, when the cells were exposed to either AITC or BITC for only 3 h, with or without an additional 21-h incubation, while there was a significant increase in the number of apoptotic cells when the cells were treated with the ITCs for 24 h (Fig. 4).

Both AITC and BITC also potently blocked cell cycle progression in HL60/S cells, but in different phases. AITC arrested cells at the G_1 phase, whereas BITC mainly arrested cells in the G_2-M phase. It is noteworthy that the effect of AITC on cell cycle progression in these cells was different from that in human prostate PC-3 and LNCaP cancer cells where the cell cycle was arrested in the G_2-M phase (47). Induction of cell cycle arrest by the ITCs also was rapid, as incubation of the cells with each compound for only 3 h had a very similar impact on cell cycle as did 24 h incubation. However, the examination of selected cyclins and cdks that regulate the G_1 and G_2-M phases by Western blot analysis failed to identify a target(s) for the ITCs. It remains to be investigated whether other cell cycle regulators that were not examined in our study were targeted by the ITCs. However, because Western blot analysis only detects protein expression levels, it is unclear if the activity of some of the cell cycle regulators might have been altered by the ITCs. Our study also revealed that both AITC and BITC were potent inducers of differentiation in HL60/S cells, inducing differentiation in a dose-dependent manner. Again, only a short time exposure (3 h) of cells to the compounds was required for full inducing activity. AITC was shown previously to induce histone acetylation in mouse erythroleukemia cells (48), thereby suggesting a potential mechanism for the above-described observation in HL60 cells.

In summary, among the four known dietary anticarcinogenic ITCs tested, AITC, BITC, and PEITC required only 3 h incubation with cancer cells to display their antiproliferative activity, whereas the effect of SF was time dependent. Both AITC and BITC, which are more potent inhibitors of cell proliferation than the other two ITCs, displayed a multifaceted antiproliferative mechanism in HL60/S cells, including disruption of $\Delta\Psi_m$, induction of apoptosis, arrest of cell cycle progression, and induction of cell differentiation.

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References

1. Fenwick, G. R., Heaney, R. K., and Mullin, W. J. Glucosinolates and their breakdown products in food and food plants. Crit. Rev. Food Sci. Nutr., *18:* 123–201, 1983.

2. Fahey, J. W., Zalcmann, A. T., and Talalay, P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry, *56:* 5–51, 2001.

3. Ioannou, Y. M., Burka, L. T., and Matthews, H. B. Allyl isothiocyanate: comparative disposition in rats and mice. Toxicol. Appl. Pharmacol., *75:* 173–181, 1984.

4. Gorler, K., Krumbiegel, G., Mennicke, W. H., and Siehl, H. U. The metabolism of benzyl isothiocyanate and its cysteine conjugate in guinea-pigs and rabbits. Xenobiotica, *12:* 535–542, 1982.

5. Chung, F. L., Morse, M. A., Eklind, K. I., and Lewis J. Quantitation of human uptake of the anticarcinogen phenethyl isothiocyanate after a watercress meal. Cancer Epidemiol., Biomarkers & Prev., *1:* 383–388, 1992.

6. Zhang, Y., Talalay, P., Cho, C. G., and Posner, G. H. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc. Natl. Acad. Sci. USA, *89:* 2399–2403, 1992.

7. Fahey, J. W., Zhang, Y., and Talalay, P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes from broccoli: isolation and elucidation of structure. Proc. Natl. Acad. Sci. USA, *94:* 10367–10372, 1997.

8. Shapiro, T. A., Fahey, J. W., Wade, K. L., Stephenson, K. K., and Talalay, P. Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. Cancer Epidemiol., Biomarkers & Prev., *7:* 1091–1100, 1998.

9. Shapiro, T. A., Fahey, J. W., Wade, K. L., Stephenson, K. K., and Talalay, P. Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. Cancer Epidemiol., Biomarkers & Prev., *10:* 501–508, 2001.

10. Getahun, S. M. and Chung, F. L. Conversion of glucosinolates to isothiocyanates in humans after ingestion of cooked watercress. Cancer Epidemiol., Biomarkers & Prev., *8:* 447–451, 1999.

11. Zhang, Y. and Talalay, P. Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. Cancer Res., *54:* 1976s–1981s, 1994.

12. Hecht, S. S. Chemoprevention by isothiocyanates. J. Cell. Biochem., Suppl., *22:* 195–209, 1995.

13. Hecht, S. S. Chemoprevention of cancer by isothiocyanates, modifiers of carcinogen metabolism. J. Nutr., *129:* 769s–774s, 1999.

14. Conaway, C. C., Yang, Y. M., and Chung, F. L. Isothiocyanates as cancer chemopreventive agents: their biological activities and metabolism in rodents and humans. Curr. Drug Metab., *3:* 233–255, 2002.

15. London, S. J., Yuan, J. M., Chung, F. L., Gao, Y. T., Coetzee, G. A., Ross, R. K., and Yu, M. C. Isothiocyanates, glutathione *S*-transferase M1 and T1 polymorphisms, and lung-cancer risk: a prospective study of men in Shanghai, China. Lancet, *356:* 724–729, 2000.

16. Spitz, M. R., Duphorne, C. M., Detry, M. A., Pillow, P. C., Amos, C. I., Lei, L., de Andrade, M., Gu, X., Hong, W. K., and Wu, X. Dietary intake of isothiocyanates: evidence of a joint effect with glutathione *S*-transferase polymorphisms in lung cancer risk. Cancer Epidemiol., Biomarkers & Prev., *9:* 1017 – 1020, 2000.

17. Lin, H. J., Zhou, H., Dai, A., Huang, H. F., Lin, J. H., Frankl, H. D., Lee, E. R., and Haile, R. W. Glutathione transferase GSTT1, broccoli, and prevalence of colorectal adenomas. Pharmacogenetics, *12:* 175 – 179, 2002.

18. Chiao, J. W., Chung, F. L., Kancherla, R., Ahmed, T., Mittelman, A., and Conaway, C. C. Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. Int. J. Oncol., *20:* 631 – 636, 2002.

19. Yang, Y. M., Conaway, C. C., Chiao, J. W., Wang, C. X., Amin, S., Whysner, J., Dai, W., Reinhardt, J., and Chung, F. L. Inhibition of benzo(*a*)pyrene-induced lung tumorigenesis in A/J mice by dietary *N*-acetylcysteine conjugates of benzyl and phenethyl isothiocyanates during the postinitiation phase is associated with activation of mitogen-activated protein kinases and p53 activity and induction of apoptosis. Cancer Res., *62:* 2 – 7, 2002.

20. Jiao, D., Smith, T. J., Yang, C. S., Pittman, B., Desai, D., Amin, S., and Chung, F. L. Chemopreventive activity of thiol conjugates of isothiocyanates for lung tumorigenesis. Carcinogenesis, *18:* 2143 – 2147, 1997.

21. Brusewitz, G., Cameron, B. D., Chasseaud, L. F., Gorler, K., Hawkins, D. R., Koch, H., and Mennicke, W. H. The metabolism of benzyl isothiocyanate and its cysteine conjugate. Biochem. J., *162:* 99 – 107, 1977.

22. Mennicke, W. H., Gorler, K., and Krumbiegel, G. Metabolism of some naturally occurring isothiocyanates in the rat. Xenobiotica, *13:* 203 – 207, 1983.

23. Mennicke, W. H., Gorler, K., Krumbiegel, G., Lorenz, D., and Rittmann, N. Studies on the metabolism and excretion of benzyl isothiocyanate in man. Xenobiotica, *18:* 441 – 447, 1988.

24. Baillie, T. A. and Slatter, J. G. Glutathione: a vehicle for the transport of chemically reactive metabolites *in vivo*. Acc. Chem. Res., *24:* 264 – 270, 1991.

25. Bruggeman, I. M., Temmink, J. H. M., and van Bladeren, P. J. Glutathione- and cysteine-mediated cytotoxicity of allyl and benzyl isothiocyanate. Toxicol. Appl. Pharmacol., *83:* 349 – 359, 1986.

26. Conaway, C. C., Krzeminski, J., Amin, S., and Chung, F. L. Decomposition rates of isothiocyanate conjugates determine their activity as inhibitors of cytochrome p450 enzymes. Chem. Res. Toxicol., *14:* 1170 – 1176, 2001.

27. Xu, K. and Thornalley, P. J. Studies on the mechanism of the inhibition of human leukemia cell growth by dietary isothiocyanates and their cysteine adducts *in vitro*. Biochem. Pharmacol., *60:* 221 – 231, 2000.

28. Gamet-Payrastre, L., Li, P., Lumeau, S., Cassar, G., Dupont, M. A., Chevolleau, S., Gasc, N., Tulliez, J., and Terce, F. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res., *60:* 1426 – 1433, 2000.

29. Musk, S. R. and Johnson, I. T. Allyl isothiocyanate is selectively toxic to transformed cells of the human colorectal tumour line HT29. Carcinogenesis, *14:* 2079 – 2083, 1993.

30. Fimognari, C., Nusse, M., Cesari, R., Iori, R., Cantelli-Forti, G., and Hrelia, P. Growth inhibition, cell-cycle arrest and apoptosis in human T-cell leukemia by the isothiocyanate sulforaphane. Carcinogenesis, *23:* 581 – 586, 2002.

31. Srivastava, S. K., Xiao, D., Lew, K. L., Kokkinakis, D. M., Hershberger, P. A., Johnson, C. S., and Trump, D. L. Allyl isothiocyanate inhibits growth of PC-3 human prostate cancer xenografts *in vivo* in nude mice. Proc. Am. Assoc. Cancer Res. (93rd AACR Annual Meeting), *43:* 1108, 2002.

32. Adesida, A., Edwards, L. G., and Thornalley, P. J. Inhibition of human leukaemia 60 cell growth by mercapturic acid metabolites of phenylethyl isothiocyanate. Food Chem. Toxicol., *34:* 385 – 392, 1996.

33. Zhang, Y., Wade, K. L., Prestera, T., and Talalay, P. Quantitative determination of isothiocyanates, dithiocarbamates, carbon disulfide and related thiocarbonyl compounds by cyclocondensation assay with 1,2-benzenedithiol. Anal. Biochem., *239:* 160 – 167, 1996.

34. Zhang, Y. and Callaway, E. C. High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate. Biochem. J., *364:* 301 – 307, 2002.

35. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, *65:* 55 – 63, 1983.

36. Finucane, D. M., Waterhouse, N. J., Amarante-Mendes, G. P., Cotter, T. G., and Green, D. R. Collapse of the inner mitochondrial transmembrane potential is not required for apoptosis of HL60 cells. Exp. Cell Res., *251:* 166 – 174, 1999.

37. Dressler, L. G. and Bartow, S. A. DNA flow cytometry in solid tumors, practical aspects and clinical application. Semin. Diagn. Pathol., *6:* 55 – 82, 1989.

38. Kawaii, S., Tomono, Y., Katase, E., Ogawa, K., and Yano, M. Effect of coumarins on HL-60 cell differentiation. Anticancer Res., *20:* 2505 – 2512, 2000.

39. Suh, N., Luyengi, L., Fong, H. H. S., Kinghorn, D., and Pezzuto, J. M. Discovery of natural product chemopreventive agents utilizing HL-60 cell differentiation as a model. Anticancer Res., *13:* 233 – 240, 1995.

40. Ye, L., Dinkova-Kostova, A., Wade, K. L., Zhang, Y., Shapiro, T. A., and Talalay, P. Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. Clin. Chim. Acta, *316:* 43 – 53, 2002.

41. Eklind, K. I., Morse, M. A., and Chung, F. L. Distribution and metabolism of the natural anticarcinogen phenethyl isothiocyanate in A/J mice. Carcinogenesis, *11:* 2033 – 2036, 1990.

42. Kassahun, K., Davis, M., Hu, P., Martin, B., and Baillie, T. Biotransformation of the naturally occurring isothiocyanate sulforaphane in the rat: identification of phase 1 metabolites and glutathione conjugates. Chem. Res. Toxicol., *10:* 1228 – 1233, 1997.

43. Conaway, C. C., Jiao, D., Kohri, T., Liebes, L., and Chung, F. L. Disposition and pharmacokinetics of phenethyl isothiocyanate and 6-phenylhexyl isothiocyanate in F344 rats. Drug Metab. Dispos., *27:* 13 – 20, 1999.

44. Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol., *15:* 269 – 290, 1999.

45. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., and Yuan, J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. Nature, *403:* 98 – 103, 2000.

46. Baker, S. J. and Reddy, E. P. Modulation of life and death by the TNF receptor superfamily. Oncogene, *17:* 3261 – 3270, 1998.

47. Xiao, D., Srivastava, S. K., Zeng, Y., Hershberger, P. A., Johnson, C. S., Trump, D. L., and Singh, S. V. Allyl isothiocyanate suppresses *in vitro* proliferation of PC-3 and LNCaP human prostate cancer cells by inducing apoptosis and mediating G2/M arrest. Proc. Am. Assoc. Cancer Res. (93rd AACR Annual Meeting), *43:* 641, 2002.

48. Lea, M. A., Randolph, V. M., Lee, J. E., and DesBordes, C. Induction of histone acetylation in mouse erythroleukemia cells by some organosulfur compounds including allyl isothiocyanate. Int. J. Cancer, *92:* 784 – 789, 2001.

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