Phenethyl Isothiocyanate Triggers Apoptosis in Jurkat Cells Made Resistant by the Overexpression of Bcl-2

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Abstract

Isothiocyanates are a class of naturally occurring chemopreventive agents known to be effective at triggering apoptosis. In this study, we show that whereas overexpression of the oncprotein Bcl-2 renders Jurkat T-lymphoma cells resistant to a range of cytotoxic agents, phenethyl isothiocyanate is able to overcome the inhibitory action of Bcl-2 and trigger apoptosis. A 50-fold increase in Bcl-2 expression shifted the dose-response curve, with an increase in the phenethyl isothiocyanate LD50 from 7 to 15 μmol/L, but there was still a complete loss in cell viability at doses in excess of 20 μmol/L. At these concentrations, cytotoxicity was strongly associated with caspase activation, phosphatidylserine exposure, and morphologic changes characteristic of apoptosis. Cytotoxicity was inhibited by treatment of the cells with a broad-spectrum caspase inhibitor. A structure-activity analysis showed that the phenethyl and benzyl isothiocyanates were most effective at triggering apoptosis in cells overexpressing Bcl-2 whereas phenyl isothiocyanate and benzyl thiocyanate had no proapoptotic activity. Allyl isothiocyanate also had limited efficacy despite its ability to trigger apoptosis in the parental Jurkat cell line. From this information, we propose that isothiocyanates modify a key cysteine residue in an apoptosis regulatory protein and that the aromatic side chain facilitates access to the target site. An in-depth investigation of the cellular targets of the aromatic isothiocyanates is warranted. (Cancer Res 2006; 66(13): 6772-7)

Introduction

Isothiocyanates are chemoprotective against a variety of carcinogen-induced cancers in experimental animal models (1). They are derived from glucosinolate precursors in cruciferous vegetables and a number of epidemiologic studies have shown that high intake of cruciferous vegetables is associated with a decreased incidence of various cancers (2–4). The chemopreventive properties of the isothiocyanates are traditionally linked to their ability to inhibit the cytochrome P450 enzymes that activate procarcinogens and to induce phase II detoxification enzymes such as quinone reductase and the glutathione S-transferases. It has also been recognized that isothiocyanates can inhibit cell cycle progression and trigger apoptosis in tumor cells, as well as inhibit NF-κB activation and histone deacetylase activity (5–7). The relative importance of these different mechanisms for chemoprevention is not yet clear.

Defects in the apoptotic pathway play an important role in the pathogenesis and progression of many cancers (8). It is thought that defective apoptosis is necessary for cellular transformation, enabling the survival of damaged and mutated cells and balancing the action of oncopgenes that jointly enhance proliferation and apoptosis (9). A diminished rate of apoptosis promotes resistance to immune cell attack and supports cell survival in the absence of growth factors or on detachment from the extracellular matrix. In addition, defective apoptosis pathways contribute to multidrug resistance (10).

New treatments that promote apoptosis in resistant cells are being explored (11). The induction of apoptosis in premalignant cells is hypothesized to contribute to chemoprevention, and there is growing interest in exploring the therapeutic potential of chemopreventive natural products that display proapoptotic properties (12–14). One of the most actively studied oncongenes is the antiapoptotic protein Bcl-2. Increased expression of Bcl-2 is reported in a number of hematologic malignancies and solid tumors, in which it renders cells resistant to a wide range of apoptotic stimuli and chemotherapeutic agents (15). Tumor regression following Bcl-2 targeting has been observed in mouse models (16, 17), and a variety of antisense, peptide, and small-molecule inhibitors are currently under development or in clinical trial (18). One class of compound being explored is the naturally occurring polyphenols, which bind in a hydrophobic crevice of Bcl-2, preventing it from sequestering proapoptotic family members (19).

Overexpression of Bcl-2 in the Jurkat T-lymphoma cell line is sufficient to make the cells resistant to Fas-mediated apoptosis. We have previously reported that phenethyl isothiocyanate (PEITC) was able to sensitize these cells to Fas-mediated apoptosis, which occurs within 2 to 6 hours (20). The present study was initiated to determine whether PEITC could also sensitize the Bcl-2 overexpressing cells to chemotherapeutic agents. We now show that significant cytotoxicity was observed in the cells treated with PEITC alone at later times than in the Fas study. Further characterization showed that PEITC is a strong inducer of apoptosis in cells expressing Bcl-2 despite Bcl-2 rendering the cells resistant to all of the chemotherapeutic agents tested. A structure-activity relationship was undertaken to provide insight into the chemical features of the isothiocyanates important for overcoming the antiapoptotic properties of Bcl-2. The nature of the side chain and isothiocyanate reactivity both seem to contribute to efficacy, suggesting that these compounds function in a manner different from the polyphenolic inhibitors.

Materials and Methods

Materials. The caspase substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) was purchased from Peptide Institute,
Inc. (Osaka, Japan), the caspase inhibitor benzoxycarbonyl-Val-Ala-Asp(Ome)-CH$_2$F (zVAD) was from Enzyme Systems Products (Livermore, CA), and human anti-Fas immunoglobulin M (clone CH-11) was from Upstate Biotechnology (Lake Placid, NY). Melphalan, etoposide, cytosine arabinoside (ara-C), PEITC, and propidium iodide were from Sigma Chemical Co. (St. Louis, MO); allyl isothiocyanate was generously provided by Dr. Rex Munday (Agriculture Research, Ruakura, New Zealand); and the other isothiocyanates were from LKT Laboratories (St. Paul, MN). Lipofectamine 2000 was obtained from Stratagene (La Jolla, CA), and the pCIneo from Promega (Madison, WI). Mouse anti-Bcl-2 antibody (clone Bcl-2-100) was from Zymed Laboratories, Inc. (San Francisco, CA) and mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase antibody from Research Diagnostics, Inc. (Flanders, NJ). Phosphatidylserine exposure was measured with the ApoTest-FITC kit from Nexins Research (Hoeven, the Netherlands). Cell culture materials were from Invitrogen New Zealand Ltd. (Auckland, New Zealand).

**Cell culture.** The Jurkat T-lymphocyte cell line was obtained from American Type Culture Collection (Rockville, MD). Jurkat cells were maintained in RPMI 1640 containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin whereas the vector control and the Bcl-2-overexpressing lines were maintained in RPMI 1640 containing 10% fetal bovine serum and 350 µg/mL geneticin (G418). Fresh antibiotic-free medium was added to cells 1 hour before treatment. Working solutions of isothiocyanates and etoposide were prepared in DMSO and diluted in growth medium just before the addition to cells. Melphalan was dissolved in a 0.1% (v/v) ethanol/HCl solution. The final concentration of solvents in the medium was kept constant at 0.1%. Cells were always maintained at 37°C in a humidified atmosphere with 5% CO$_2$.

**Generation of Bcl-2-overexpressing Jurkat cells.** Human Bcl-2 cDNA, pClbcl-2, was obtained from Prof. Suzanne Cory (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). The Bcl-2 coding region was subcloned from pClbcl-2 into pCIneo and sequenced. Jurkat cells were transfected with the Bcl-2-overexpressing vector and the empty vector using Lipofectamine 2000. After 24 hours, cells were selected using geneticin at 700 µg/mL. Stable lines were selected by serial dilution. Resistant lines were analyzed for Bcl-2 expression by Western blotting, with GAPDH levels determined as a loading control. Densitometry of scanned images was undertaken using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

**Assessment of cell viability.** Plasma membrane integrity was monitored using propidium iodide staining. Cells were harvested by centrifugation and resuspended in PBS containing 2 µg of propidium iodide. The cells were incubated in the dark for 10 minutes and the cell fluorescence was measured using a bivariate flow cytometer (Becton Dickinson, Mountain View, CA), Propidium iodide–positive cells were expressed as a percentage of the total cells analyzed. In some cases, cells were costained with Annexin V-FITC to detect phosphatidylserine exposure on apoptotic cells according to the instructions of the manufacturer.

To calculate the LD$_{50}$ for each of the isothiocyanates, the number of dead cells in control samples ($b$) was subtracted from the treated samples ($a$), which were then expressed as a percentage according to the formula $[(a - b) / (100 - b)] \times 100$. Data from several experiments were pooled, a sigmoid curve fitted, and the LD$_{50}$ and SE generated from the curve parameters (SigmaPlot, SSI, Richmond, CA).

**Determination of caspase activity.** At selected times, 0.5 × 10$^6$ cells were harvested by centrifugation and stored as cell pellets at −80°C. Immediately before assay, the pellets were resuspended in 10 µL of PBS transferred to a 96-well fluorescent plate, and 95-µL buffer (100 mM HEPES, 10% sucrose, 5 mM DTT, 0.0001% NP40, and 0.1% CHAPS at pH 7.25) containing 50 µmol/L DEVD-AMC was added. The rate of release of fluorescent AMC was monitored at 37°C ($\lambda_{ex}$, 390 nm; $\lambda_{em}$, 460 nm).

**Results**

**Bcl-2 overexpression renders Jurkat cells resistant to chemotherapeutic agents.** An apoptosis-sensitive Jurkat T-lymphoma cell line was genetically modified to overexpress the antiapoptotic protein Bcl-2. A number of stably transfected cell lines were generated. We selected several lines with increased levels of Bcl-2 for use in this study, including a control line in which an empty vector was inserted (N2). B1, B38, and B9 had Bcl-2 levels ranging from 10 to 50 times that of the parental Jurkat cells (Fig. 1A).

Treatment of the overexpressing cells with the chemotherapeutic agents etoposide, ara-C, and melphalan confirmed that Bcl-2 provides resistance to the drug-mediated apoptosis (Fig. 1B-D). Incubation of the apoptosis-sensitive Jurkat line and the vector control with these agents resulted in almost 100% killing (Fig. 1B-D). However, only partial killing was achieved in the B1 and B38 cell lines that overexpress 10-fold and 20-fold Bcl-2, respectively. With melphalan, in particular, the dose-response curve reached a plateau in these two cell lines (Fig. 1D). The B9 strongly overexpressing line was completely resistant to all three of the drugs at the concentrations tested over a 48-hour period.

**PEITC kills Bcl-2-overexpressing cells.** The initial goal was to assess whether treatment of the cells with PEITC could sensitize them to the cytotoxic effects of the drugs in Fig. 1. A dose-response curve was undertaken to enable determination of sublethal doses. We were surprised to see that PEITC was cytotoxic to all of the lines tested and that there was a complete loss in cell viability, even in B9 cells (Fig. 2). The LD$_{50}$ of these cells increased from 7.4 µmol/L to a maximum of 15 µmol/L; however, this was small in comparison with the complete protection against the chemotherapeutic agents provided by high Bcl-2 expression.

**PEITC triggers caspase activation and phosphatidylserine exposure in Bcl-2-overexpressing cells.** The Bcl-2 cell lines treated with PEITC all showed signs of apoptosis, with the “popcorn-like” membrane blebbing characteristic of apoptotic Jurkat cells strongly evident. Caspase-3-like DEVDase activity was measured 6 hours after treatment with PEITC. The parental Jurkat cells, N2, B1, and B38 all showed a 15- to 20-fold increase in caspase

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**Figure 1.** Characterization of Jurkat cells overexpressing Bcl-2. A, stable Bcl-2 transfectants were selected and Bcl-2 expression levels were measured by densitometry of Western blots. Columns, mean of three experiments; bars, SE. The effect of etoposide (B), ara-C (C), and melphalan (D) on cell viability was assessed 48 hours after treatment of N2 (○), Jurkat (●), B1 (▲), B38 (■), and B9 (▲) cells. Points, mean cytotoxicity [% propidium iodide (PI)–positive cells] from three experiments; bars, SE.
activity at 10 μmol/L PEITC (Fig. 3A). Caspase activation was not as elevated at the high concentrations of PEITC, indicating a shift from apoptotic to necrotic cell death. A 10-fold increase in caspase activity was observed in B9 cells, confirming that apoptosis was occurring (Fig. 3A). It required 20 μmol/L PEITC to maximize caspase activity in these cells. No caspase activity could be detected in this line following treatment with melphalan (Fig. 3B) or any of the other chemotherapeutic drugs tested (not shown). To determine whether caspase activation was contributing to cell death, we treated B9 cells with a general caspase inhibitor before addition of PEITC. Significant protection of cell viability was observed (Fig. 3C).

To further confirm that PEITC triggers apoptosis in cells overexpressing Bcl-2, phosphatidylserine exposure was measured. PEITC induced phosphatidylserine exposure in both control-transfected and B9 cells (Fig. 4). The numbers of phosphatidylserine-positive cells corresponded closely to the loss in viability measured with 15 μmol/L PEITC after 24 hours (Fig. 4 versus Fig. 2). Addition of the caspase inhibitor before incubation with PEITC resulted in complete inhibition of phosphatidylserine exposure (Fig. 4B).

Testing the ability of different isothiocyanates to trigger apoptosis in cells overexpressing Bcl-2. There are more than 120 naturally occurring isothiocyanates that are broadly classified according to the structure of their side chains (21). We selected some of the commonly studied isothiocyanates to determine their ability to kill cells overexpressing Bcl-2 (Fig. 5A). Phenyl isothiocyanate was unable to trigger apoptosis in either the sensitive or resistant cell lines (Fig. 5B). The addition of an alkyl bridge between the aromatic ring and the isothiocyanate moiety restored activity. Benzyl isothiocyanate was the most effective at killing Jurkat cells, with an LD50 of 6.1 μmol/L (Fig. 5C). However, this compound was less effective than PEITC at killing the Bcl-2-overexpressing cells (LD50 22 μmol/L). Benzyl thiocyanate was a weak apoptosis inducer in the Jurkat cells and it had no effect on the cells overexpressing Bcl-2 (Fig. 5D), indicating that isothiocyanate reactivity is a key factor in efficacy. Extending the alkyl chain length to six decreased cytotoxicity (Fig. 5E). Sulforaphane was able to kill both parental Jurkat and Bcl-2-overexpressing cell lines with similar efficacy but at higher concentrations than that required for the aromatic isothiocyanates (Fig. 5F). In contrast, allyl isothiocyanate, although showing cytotoxicity to the Jurkat cells, was extremely ineffectual at killing the resistant cells (Fig. 5G).

Discussion

Attempts are being made to develop therapeutically useful inhibitors of Bcl-2 and Bcl-xL, for use either alone or in combination, to kill apoptosis-resistant cancer cells (18). In one approach, screening of natural compounds yielded a range of polyphenolic compounds that bind the BH3 domain of these proteins and interfere with their ability to sequester proapoptotic members of the Bcl-2 family (19). The authors proposed that other natural products...
Isothiocyanates and Bcl-2

A

![Diagram showing PEITC triggering phosphatidylserine exposure in Jurkat cells overexpressing Bcl-2.]

B

![Graph showing percentage of PS-positive cells.]

Figure 4. PEITC triggers phosphatidylserine exposure in Jurkat cells overexpressing Bcl-2. N2 and B9 cells were treated with 15 μmol/L PEITC for 2 and 4 hours (N2) or 4 and 8 hours (B9) before phosphatidylserine exposure was measured. A, data from a representative experiment with B9 cells. B, columns, mean of three experiments of the percentage of cells appearing in the bottom right quadrant [phosphatidylserine (PS) positive, propidium iodide negative]; bars, SE. The caspase inhibitor (CI) zVAD-fmk was used at 10 μmol/L.

with chemopreventive activity might also act via inhibition of the Bcl-2 family and included PEITC in their list. We can now partially substantiate this prediction, with the results of this study clearly indicating that isothiocyanates are strong inducers of apoptosis in cells overexpressing Bcl-2. It is not yet apparent whether the isothiocyanates act to inhibit Bcl-2 function or whether Bcl-2 remains functional but is bypassed by an alternate pathway.

Apoptosis in the Bcl-2-positive cells proceeded by a conventional caspase-dependent mechanism. Caspase activation, phosphatidylserine exposure, membrane blebbing, and an eventual loss in cell viability were all apparent in Jurkat cells expressing ~50 times more Bcl-2 than the parental cell line. A caspase inhibitor was able to block the apoptotic events and provide protection against cytotoxicity, indicating that PEITC was triggering a conventional apoptosis program in the cells. In contrast, and consistent with a wealth of published literature, there was no induction of apoptosis or loss in cell viability when the same cells were treated with a range of cytotoxic drugs. Chen et al. (22) previously reported that Bcl-2 overexpression could inhibit PEITC-mediated apoptosis. This was done in embryonic kidney 293 cells, and whereas the disparity with our conclusion could reflect a difference between cell types, the authors only reported on use of a single concentration of PEITC. At this concentration, there was a 40% loss in cell viability in the control cells and no killing in the Bcl-2-expressing cells. However, our results show that Bcl-2 shifts the dose-response curve rather than providing substantial protection from apoptosis. If we had restricted our experiments to 10 μmol/L PEITC, which showed 40% cytotoxicity in the Jurkat cells, then we would have incorrectly concluded that Bcl-2 prevents PEITC-mediated apoptosis (see Fig. 2). Interestingly, the shift in the dose-response curve we observed with PEITC was almost identical to that observed with gossypol, one of the newly identified polyphenolic inhibitors of Bcl-2 (23).

A number of studies have explored the induction of apoptosis by isothiocyanates in sensitive cell lines. These have focused on mitochondria but differ in the hypothesized mechanisms for isothiocyanates to trigger the release of proapoptotic mitochondrial factors. Thornalley proposed that isothiocyanates modify proteins associated with the death receptor–induced signaling complex to promote caspase-8 activation, followed by Bid cleavage and mitochondrial changes (24, 25). Studies with isolated mitochondria suggest that aromatic isothiocyanates can act directly on mitochondria to facilitate the release of cytochrome c (26, 27). However, Rose et al. (28) obtained results implicating an essential role for proapoptotic members of the Bcl-2 family in induction of apoptosis. This is supported by Singh and colleagues who showed that isothiocyanate-mediated apoptosis is impaired in embryonic fibroblasts from Bak and Bax knockout mice (29, 30).

Regardless of the exact mechanism of isothiocyanate action in apoptosis-sensitive cells, Bcl-2 overexpression would be expected to inhibit all of the pathways described above. We therefore propose that isothiocyanates act to inhibit Bcl-2 action. This could be a direct effect on Bcl-2 itself or, alternatively, on proteins involved in the regulation of Bcl-2 activity. There are two reports showing that treatment of cells with benzyl isothiocyanate and PEITC leads to Bcl-2 phosphorylation and interference in the binding of Bcl-2 to proapoptotic family members (27, 31). However, in at least one case, it was a late event and, as such, it is not clear if these events are essential for driving apoptosis or if they are a consequence of the cells undergoing G2-M arrest and apoptosis.

Determination of isothiocyanate targets will be the key to elucidating the mechanism of action. At physiologic pH, the major reaction of isothiocyanates is with the sulfhydryl group of cysteine residues in proteins or the tripeptide glutathione (32, 33). The reaction with glutathione is catalyzed by glutathione S-transferases whereas the reaction with protein cysteinyl residues will be restricted to a subset of cysteines that exist as thiolate anions within the cell. The ability of isothiocyanates to trigger phase II enzyme induction has been pinpointed to cysteines 273 and 288 in the protein Keap1, which controls localization of the transcription factor Nrf2 (34, 35). It is not clear if Keap1 modification occurs by indirect oxidation or via direct thiocarbamoylation; however, there is a precedent for the latter reaction. Papain is directly inhibited by aromatic isothiocyanates at its active site cysteine (36). We hypothesize that isothiocyanates promote apoptosis by modifying key cysteine residues in a protein involved in the regulation of apoptosis. This protein might be Bcl-2 itself, which has one
accessible cysteine residue in its normal conformation (37), or
proteins that act upstream of Bcl-2 to inhibit or bypass its
antiapoptotic activity. The difficulty in assessing the primary target
of the isothiocyanates is that the dithiocarbamate product is
unstable, thereby hindering the use of conventional proteomic
techniques.

Both electronic and steric affects seem to influence isothiocyanate
activity in our system. The aromatic isothiocyanates were the

**Figure 5.** Assessment of the cytotoxicity of different isothiocyanates in Jurkat cells
overexpressing Bcl-2. A, structures of the various isothiocyanates used in this study. B to G, dose response of various isothiocyanates 24 hours after treatment of Jurkat and B9 cells. Points, mean cytotoxicity (% propidium iodide–positive cells) from at least three experiments; bars, SE.
most effective at triggering apoptosis in the Jurkat cells whereas the two aliphatic compounds used in this study had LD<sub>50</sub> of up to five times higher. Sulforaphane is of interest, however, because it seemed to kill the Jurkat and B9 cells with similar efficacies. Alkyl chain length had a strong influence on the efficacy of the aromatic isothiocyanates. PEITC was more effective against the Bcl-2-expressing cells than benzyl isothiocyanate despite benzyl isothiocyanate being a better inducer of apoptosis in the parental Jurkat cells. This may be due to the different metabolism of these compounds in the two different cell lines or that the site of action for apoptosis induction is different from that involved in inhibiting Bcl-2 function. Phenyl isothiocyanate was unable to trigger apoptosis in either the sensitive or resistant Jurkat cells, consistent with other reports of its lack of biological activity in cell culture (38). This is despite the only difference with benzyl isothiocyanate being a single alkyl group between the aromatic ring and the isothiocyanate moiety. The absence of a bridge will promote electron withdrawal by the ring and increased electrophilicity of the isothiocyanate carbon. Increased reactivity of phenyl isothiocyanate means that it could react with constituents in the culture media and other cellular proteins before reaching the intracellular site(s) critical for promotion of apoptosis.3 Alternatively, it was shown that phenyl isothiocyanate could not inhibit purified papain, and the authors proposed a structural model in which the distance between the aromatic binding site and the target cysteine was too large for the electrophilic carbon of phenyl isothiocyanate (36).

In summary, our study shows that aromatic isothiocyanates can overcome the antiapoptotic action of Bcl-2 and promote apoptosis in cells that overexpress this important oncoprotein. The detailed mechanism of action is not clear but it will be different from that of the polyphenolic compounds that bind to a hydrophobic crevice of Bcl-2 and prevent interaction with proapoptotic family members. Instead, the reactive isothiocyanate moiety seems to be critical, and we propose that the isothiocyanates target a cysteine residue in an apoptosis regulatory protein. An in-depth investigation of the key cellular targets of the aromatic isothiocyanates is warranted.

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