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Induction of apoptosis by phenethyl isothiocyanate in cells overexpressing Bcl-X_L

Sarah L. Cuddihy *, Kristin K. Brown, Susan J. Thomson, Mark B. Hampton

Free Radical Research Group, Department of Pathology, University of Otago, P.O. Box 4345, Christchurch, New Zealand

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Abstract

Isothiocyanates are a class of phytochemicals able to induce apoptosis in numerous cells including Jurkat T-lymphoma cells overexpressing the oncoprotein Bcl-2. To test if isothiocyanates are also effective against other anti-apoptotic members of the Bcl-2 family we generated Jurkat cells stably overexpressing Bcl-X_L. Phenethyl isothiocyanate (PEITC) was cytotoxic to these cells, with an LD_{50} ranging from 9 to 18 μM depending on the level of Bcl-X_L expression. Apoptosis induction in response to PEITC was confirmed by caspase activation and phosphatidylserine exposure. Isothiocyanates specifically target cysteine residues, therefore we tested the hypothesis that PEITC directly impairs Bcl-2 and Bcl-X_L activity by interacting with their conserved cysteine residues. Jurkat cells overexpressing double cysteine mutants of Bcl-2 were generated, but they remained sensitive to PEITC. We conclude that PEITC antagonizes the action of anti-apoptotic Bcl-2 family members via an indirect mechanism.

Keywords: Apoptosis; Bcl-2; Bcl-X_L; Phenethyl isothiocyanate; Caspases

1. Introduction

Overexpression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L negates the action of pro-death stimuli. A screen of sixty cell lines derived from a wide range of human tumours indicated that elevated Bcl-X_L expression correlated most strongly with drug resistance [1], and several studies have associated poor prognostic outcome with increased Bcl-X_L expression [2]. Compounds that inhibit Bcl-2 and Bcl-X_L activity sensitize cancer cells to apoptosis and several small molecule inhibitors are being explored as anti-cancer agents [3,4].

Isothiocyanates are a family of phytochemicals responsible for the pungent odors and peppery taste of many cruciferous vegetables, with anti-cancer properties observed in epidemiological and animal studies [5,6]. Isothiocyanates have a range of biochemical properties, including the ability to trigger apoptosis [7,8]. We reported that phenethyl isothiocyanate (PEITC) can sensitize or trigger apoptosis in Jurkat T-lymphoma cells made resistant to several cytotoxic drugs by the overexpression of Bcl-2 [9,10]. In this study we have explored whether a second member of the anti-apoptotic Bcl-2 protein family, Bcl-X_L, is able

* Corresponding author. Tel.: +64 3 378 6223; fax: +64 3 364 1083.
E-mail address: sarah.cuddihy@otago.ac.nz (S.L. Cuddihy).

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to protect against PEITC-induced cell death. The mechanism by which PEITC overcomes the anti-apoptotic action of Bcl-2 is currently unclear. The highly electrophilic isothiocyanate moiety can react with both amines and thiols, although at physiological pH the primary reaction is with cysteine residues of proteins and the tripeptide glutathione [11–13]. Bcl-2 has two cysteine residues, cys158 in alpha helix 5 and cys229 in the membrane insertion domain, which are conserved in Bcl-X_L. Mutation of these cysteines has been shown to have an effect on protein homodimerization, but does not significantly alter anti-apoptotic activity [14]. These cysteines have also been shown to be susceptible to S-nitrosylation and subsequent degradation under certain conditions [15]. Therefore, a direct mechanism by which isothiocyanates antagonize Bcl-2 action could be through formation of a dithiocarbamate adduct with either of their conserved cysteine residues. To test this mechanism we expressed double cysteine mutants of Bcl-2 and assessed if this afforded protection to PEITC.

2. Materials and methods

2.1. Materials

zVAD-fmk was obtained from Calbiochem (La Jolla, CA). Melphalan, phenethyl isothiocyanate, propidium iodide, and mouse anti-β-actin antibody (clones AC74) were from Sigma Chemical Company (St. Louis, MO). Lipofectamine 2000 was from Stratagene (La Jolla, CA) and pCI-Neo from Promega (Madison, WI). Mouse anti-Bcl-X_L antibody (clone AM05) was from Calbiochem (San Diego, CA). The ApoTarget™ Annexin-V FITC Apoptosis Kit, cell culture materials, and anti-Bcl-2 antibody (clone Bcl-2-100) were from Invitrogen (Carlsbad, CA). Maleimide-polyethylene glycol (MAL-PEG) was from Nektar (San Carlos, CA).

2.2. Cell culture

The Jurkat cell line was obtained from the American Type Culture Collection (Rockville, MD) and was maintained in RPMI-1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells overexpressing Bcl-X_L, Bcl-2 or Bcl-2 double cysteine mutants were maintained in the presence of 350 μg/mL geneticin (G418).

2.3. Generation of Bcl-X_L-overexpressing Jurkat cells and Bcl-2 double cysteine mutant-overexpressing Jurkat cells

Human Bcl-X_L cDNA in pEF FLAG-puro was the generous gift of Prof. Suzanne Cory (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). The FLAG-Bcl-X_L coding region was subcloned into pCIneo (Promega, Madison, WI) and sequenced. Human Bcl-2 cDNA (NM_000633) containing either wild-type sequence or the C158S and C229S mutations was the generous gift of Dr Stephen Lin (Korsmeyer Laboratory, Harvard, MA, USA). Jurkat cells were transfected with wild-type Bcl-X_L, wild-type Bcl-2 or double cysteine mutant Bcl-2 expressing vectors using Lipofectamine 2000. After 24 h stable lines were generated using geneticin at 700 g/mL and by serial dilution. Resistant lines were analyzed for Bcl-2/Bcl X_L expression by Western blotting with β-actin levels as the loading control. Western blots were quantified using chemiluminescence detection (Amersham Biosciences) with the ChemiDoc XRS gel documentation system (Bio-Rad Laboratories, Hercules, CA) and analyzed using Quantity One software. Functional cysteines were labeled with 1 mM MAL-PEG for 30 min, separated by SDS–PAGE, and Western blotted with anti-Bcl-2.

2.4. Assessment of cell viability

Plasma membrane integrity was monitored using propidium iodide staining (5 μg/10^6 cells/mL) analyzed by flow cytometry (FC 500 MPL, Beckman Coulter, Fullerton, CA). In some cases cells were co-stained with Annexin V-FITC to detect phosphatidylserine exposure on apoptotic cells, according to manufacturer's instructions.

To calculate the LD_{50}, the number of dead cells in control samples *(b)* was subtracted from the number present in treated samples *(a)*, which were then expressed as a percentage according to the formula $[(a\text{-}b)/(100\text{-}b)] \times 100$. Data from several experiments were pooled, a sigmoidal curve fitted, and the LD_{50} and SE generated from the curve parameters (SigmaPlot, Version 7, Systat Software, Inc., San Jose, CA).

2.5. Determination of caspase activity

Six hours after treatment 0.5×10^6 cells were harvested and stored as cell pellets at −80 °C. Cell pellets