Peptides Derived from BH3 Domains of Bcl-2 Family Members: A Comparative Analysis of Inhibition of Bcl-2, Bcl-x_L and Bax Oligomerization, Induction of Cytochrome *c* Release, and Activation of Cell Death†

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ABSTRACT: Overexpression of Bcl-2, an anti-apoptotic oncoprotein, is commonly observed in a variety of human malignancies and is associated with resistance to chemotherapy and radiotherapy. Although the precise mechanism of Bcl-2 action remains elusive, current evidence indicates that Bcl-2 inhibits apoptosis by binding and inhibiting pro-apoptotic molecules such as Bax. Therefore, agents that disrupt the ability of Bcl-2, or other anti-apoptotic molecules, to bind to pro-apoptotic molecules may have therapeutic value. Several studies have shown that the BH3 domains of Bcl-2 and Bax are critically important for Bax/Bcl-2 heterodimerization. In this report, we designed and synthesized peptides based on the BH3 domains of three distinct Bcl-2 family members, Bcl-2, Bax and Bad. In vitro interaction assays were used to compare the abilities of the different peptides to inhibit Bax/Bcl-2 and Bax/Bcl-x_L heterodimerization, as well as Bcl-2 and Bax homodimerization. Bax BH3 peptide (20-amino acids) potently inhibited both Bax/Bcl-2 and Bax/Bcl-x_L interactions, exhibiting IC_{50} values of 15 and 9.5 μM, respectively. The Bad BH3 peptide (21 amino acids) was slightly more potent than Bax BH3 at inhibiting Bax/Bcl-x_L but failed to disrupt Bax/Bcl-2. Bcl-2 BH3 peptide (20-amino acids) was inactive toward Bax/Bcl-2 and had only a weak inhibitory effect on Bax/Bcl-x_L heterodimerization. All three BH3 peptides failed to significantly inhibit homodimerization of Bcl-2 or Bax. Consistent with its ability to disrupt Bax/Bcl-2 heterodimerization, Bax BH3 peptide was able to overcome Bcl-2 overexpression and induce cytochrome *c* release from mitochondria of Bcl-2-overexpressing Jurkat T leukemic cells. Bad BH3 peptide, while potently inducing cytochrome *c* release in wild-type Jurkat cells, only partially overcame the effects of Bcl-2 overexpression. Bcl-2 BH3 failed to induce cytochrome *c* release, even in wild-type cells. Delivery of the Bax BH3 and Bad BH3 peptides into wild-type Jurkat cells induced comparable levels of cell death. In cells overexpressing Bcl-2, the potency of Bax BH3 peptide was similar to that seen in wild-type cells, while the efficacy of Bad BH3 peptide was reduced. By contrast, in Bcl-x_L-overexpressing cells, Bad BH3 exhibited greater cell-killing activity than Bax BH3. The Bcl-2 BH3 peptide and a mutant Bax BH3 peptide had no appreciable effect on Jurkat cells. Together, our data suggest that agents based on the Bax BH3 domain may have therapeutic value in cancers overexpressing Bcl-2, while agents based on the BH3 domain of Bad may be more useful for tumors overexpressing Bcl-x_L.

Apoptosis is an orderly and synchronized physiological process utilized by multicellular organisms to eliminate excess or damaged cells (*1*). This process is regulated by pro-apoptotic members of the Bcl-2 protein family that promote cell death and anti-apoptotic members that promote survival. Each member of the Bcl-2 protein family contains at least one of four conserved protein sequences termed Bcl-2 homology (BH) domains, namely, BH1, BH2, BH3, and BH4. Anti-apoptotic Bcl-2 family members such as Bcl-2 (*2*, *3*), Bcl-x_L (*4*), Bcl-w (*5*), and Mcl-1 (*6*) contain all the four BH-domains (*7*). However, either pro-apoptotic molecules from this family are multidomain like Bax (*8*), Bak (*9*, *10*), and Bok/Mtd (*11*), which contain all but the BH4 domain, or they contain only the BH3 domain, as with Bad (*12*), Bid (*13*), and Bik/Nbk (*14*).

Since apoptosis is critically important for maintaining homeostasis of cell numbers in adult organisms, dysfunction in this process can lead to various disorders (*15*, *16*). For example, excessive cell death has been observed in neuro-

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[1] Abbreviations: aa, amino acid; AIF, apoptosis-inducing factor; BH, Bcl-2 homology; Fmoc, fluoren-9-ylmethoxycarbonyl; GST, glutathione-S-transferase; IC_{50}, concentration required to inhibit 50% of protein−protein interaction; IPTG, isopropyl β-D-thiogalactoside; PMSF, phenylmethylsulfonyl fluoride.

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degenerative disorders, AIDS, and stroke (*17*−*19*), while reduced apoptosis is associated with autoimmune diseases and cancer (*20*−*22*). Altered levels of Bcl-2 family members, especially Bcl-2, have been reported and are considered to contribute toward these disorders. The *bcl-2* proto-oncogene was first identified at the chromosomal breakpoint of t(*14*; *18*)-bearing human follicular B-cell lymphomas (*3*). Increased expression of Bcl-2 has been observed in a wide variety of cancers, including malignancies of both hematologic and solid tissue origin (*20*, *23*). When overexpressed in cells, Bcl-2 confers resistance to a wide variety of apoptotic stimuli (*24*). Thus, Bcl-2 overexpression is associated with chemoresistance and radioresistance (*25*, *23*). In this context, Bcl-2 has become an attractive target for cancer therapy, and efforts have been made to either inhibit its function or reduce its cellular levels (*26*−*29*).

Although the precise mechanisms of action used by Bcl-2 family members remain unclear, studies have established that these proteins are key regulators of the mitochondrial-mediated apoptosis pathway (*15*, *30*). The mitochondrial pathway is characterized by organelle dysfunction and the release of mitochondrial proteins, including cytochrome *c*, Smac/Diablo, and AIF (*31*, *32*). This pathway is governed by protein−protein interactions between anti- and pro-apoptotic Bcl-2 family members (*7*, *8*, *12*, *30*, *31*). It has been reported that Bcl-2 exerts its anti-apoptotic effects, in part, by heterodimerizing with pro-apoptotic Bax (*7*, *8*, *32*); Bcl-2 homodimerization may not be required for the anti-apoptotic action of this molecule (*33*). Bcl-2 and Bcl-x_L are mainly localized on the outer mitochondrial membrane and have been shown to inhibit cytochrome *c* release (*34*, *35*). Bax, which exists as an inactive monomer in the cytoplasm, homodimerizes in response to apoptotic signals and is recruited to the mitochondrial membrane (*36*, *37*). The addition of recombinant Bax to purified mitochondria has been shown to promote cytochrome *c* release (*38*, *39*). By heterodimerizing with Bax or Bak, the anti-apoptotic proteins Bcl-2 and Bcl-x_L essentially neutralize the pro-apoptotic or cytochrome *c*-releasing effects of these molecules.

Mutational analyses have determined that Bax/Bcl-2 heterodimerization requires the BH1, BH2, and BH3 domains of Bcl-2 but only the BH3 domain of Bax (*40*−*44*). Yin et al. (*43*) demonstrated that mutations in the BH1 and BH2 domains of Bcl-2 which disrupt Bax/Bcl-2 interaction also disrupt the anti-apoptotic activity of Bcl-2. At the same time, these mutants retained the ability to homodimerize. Similar findings have been reported in the case of Bcl-x_L (*45*). However, in the case of Bax, only the BH3 domain was shown to be important for the ability to bind to Bcl-2 and for the death promoting activity (*46*−*48*, *44*). Mutation of residues along the hydrophobic surface of the Bax BH3 domain reduced or abrogated the pro-apoptotic and heterodimerization functions of this molecule (*48*).

In view of the critical importance of BH3 domains in protein−protein interactions, peptides derived from the BH3 domains of pro-apoptotic proteins are likely to disrupt Bax/Bcl-2 and Bax/Bcl-x_L heterodimerization. Since resistance to conventional chemotherapy drugs is frequently observed in cancers exhibiting overexpression of Bcl-2 (*20*) or Bcl-x_L (*22*), agents which can disrupt Bax/Bcl-2 or Bax/Bcl-x_L interactions may have considerable therapeutic potential. There have been a few reports on the use of Bax, Bak, and Bad peptides to inhibit heterodimerization interactions (*49*, *50*). For example, Diaz et al. (*49*) employed a plate binding assay to show that human Bax and Bak peptides disrupted Bax/Bcl-2 and Bax/Bcl-x_L heterodimerizations. In this same study, Bad BH3 peptide only weakly inhibited these interactions (*49*). By contrast, Ottilie et al. (*50*) demonstrated significant inhibition of Bax/Bcl-x_L interaction by Bad BH3 peptide. Still other studies have shown that BH3 peptides from Bak (*51*, *52*), Bax (*51*), and Bad (*53*, *54*) can induce apoptosis in various cell types. Moreover, a Bax BH3 peptide was found to induce cytochrome *c* release from mitochondria of a neural cell line but failed to fully overcome the protective effects of Bcl-2 overexpression (*55*).

In this report, we synthesized and systematically evaluated peptides that are based on BH3 domains of molecules representing three distinct subclasses of the Bcl-2 superfamily. Bax BH3 peptide (20 aa) was from "multidomain" pro-apoptotic Bax, Bad BH3 peptide (21 aa) was from 'BH3 domain-only' pro-apoptotic Bad, while Bcl-2 BH3 peptide (20 aa) was from anti-apoptotic Bcl-2. Using in vitro protein interaction assays, Bax BH3 was found to efficiently disrupt both Bax/Bcl-2 and Bax/Bcl-x_L heterodimerization. Additionally, our Bax BH3 peptide was able to overcome the effects of Bcl-2 overexpression and induce cytochrome *c* release from mitochondria of Bcl-2-overexpressing Jurkat T leukemic cells. In contrast, Bad BH3 had no effect on Bax/Bcl-2 heterodimerization but very efficiently disrupted Bax/Bcl-x_L. Bad BH3 peptide only partially overcame Bcl-2 overexpression in the cytochrome *c* release assay. Bcl-2 BH3 peptide had no effect on Bax/Bcl-2, only a small inhibitory effect on Bax/Bcl-x_L, and failed to induce release of cytochrome *c* from mitochondria of either wild-type Jurkat or Bcl-2-overexpressing cells. Delivery of Bax BH3 into Jurkat cells resulted in cell death that was only slightly diminished by Bcl-2 overexpression. By contrast, Bax BH3 was less effective at overcoming the effects of Bcl-x_L overexpression following peptide delivery into cells. The Bad BH3 peptide, on the other hand, was more effective at inducing cell death in Bcl-x_L-overexpressing cells than in cells overexpressing Bcl-2. These findings have important implications for the potential clinical utility of peptides derived from different BH3 domains.

MATERIALS AND METHODS

Cells and Reagents. Jurkat T leukemic cells (American Type Culture Collection) overexpressing Bcl-2 were generated as described elsewhere (*56*). Jurkat cells overexpressing Bcl-x_L were kindly provided by Dr. Craig Thompson (University of Pennsylvania, Philadelphia). Jurkat cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine 1% penicillin/streptomycin, and 0.2% fungizone at 37 °C in a humidified 5% CO_2 atmosphere. Additionally, media for transfected Jurkat cells always contained G418 at a concentration of 0.5 mg/mL. Media and supplements were obtained from Life Technology Inc. (GibcoBRL, Gaithersburg, MD). ^{35}S-methionine was obtained from Amersham Pharmacia Biotechnology (1000 Ci/mmol; Piscataway, NJ). BioPorter peptide delivery reagent was from Gene Therapy Systems (San Diego, CA). Monoclonal antihuman Bcl-2 antibody was from DAKO corporation (Clone 124; Carpinteria, CA), and anti-Bax rabbit polyclonal was from Santa Cruz

Table 1: BH3-Domain Peptides from Bcl-2 Family Members

peptide	sequence[a]	length (amino acids)
Bax (wt)	^{55}STKKLSECLKRIGDELDSNM^{74}	20
Bax (L63E)	^{55}STKKLSECEKRIGDELDSNM^{74}	20
Bad	^{103}NLWAAQRYGRELRRMSDEFVD^{123}	21
Bcl-2	^{89}VPPVVHLTLRQAGDDFSRRY^{108}	20

[a] Numbering based on human Bax, Bad, and Bcl-2 protein sequences (refs *8*, *50*, *59*, *60*). The Bcl-2 BH3 peptide was selected by aligning amino acid residues in the BH3 domains of Bcl-2 and Bax.

Biotechnology (P-19; Santa Cruz, CA). Monoclonal anti-cytochrome *c* was purchased from BD Pharmingen (7H8.2C12; San Diego, CA), and anti-cytochrome *c* oxidase IV antibody was purchased from Molecular Probes (2OE8-C12; Eugene, Oregon). Monoclonal anti-PARP was purchased from BioMol Research Laboratories Inc. (Plymouth Meeting, PA). All other chemicals were of molecular biology grade and obtained from Sigma unless otherwise stated.

DNA Constructs. Bluescript vector (Stratagene, La Jolla, CA) constructs containing full-length murine Bcl-2 cDNA or full-length human Bax cDNA were utilized for in vitro translation reactions and are described elsewhere (*57*). For production of GST fusion proteins, full-length Bcl-2 or Bax cDNAs (beginning at amino acid number 2) were subcloned downstream from GST coding sequences in the pGEX-2T vector (Pharmacia, Peapack, NJ), as previously described (*57*). pCDNA3-HA-Bcl-x_L, encoding full-length human Bcl-x_L protein, was kindly provided by Dr. Gabriel Nunez and Dr. Naohiro Inohara (University of Michigan, Ann Arbor) (*58*).

Peptides. Short peptides of varying length derived from the Bcl-2 homology domain-3 (BH3) of human Bax, Bad, and Bcl-2 (*59*, *8*, *50*, *60*) were synthesized commercially by SynPep Corporation (Dublin, CA) (Table 1). Peptides were synthesized by fluoren-9-ylmethoxycarbonyl (Fmoc)-based solid-phase chemistry and purified to greater than 90% purity by high-pressure liquid chromatography. Wild-type Bax BH3 and L63E Bax BH3 peptides were solubilized in deaerated HBS (10 mM Hepes, pH 7.2, 150 mM NaCl) containing 50 mM DTT to increase the shelf life of these cysteine/methionine-containing peptides. Bad BH3 and Bcl-2 BH3 peptides were solubilized in DMSO. All peptides were prepared as 10 mM stock solutions, except Bad BH3, which was prepared as a 5 mM stock. The dissolved peptides were stored as aliquots at −80 °C.

Protein Purification. pGEX-2T constructs encoding GST-Bcl-2, GST-Bax, and GST alone were transformed into DH5-α *Escherichia coli* strain (Life Technologies Inc., Gaithersburg, MD) for expression of the GST-fusion proteins. For protein production, single bacterial colonies were inoculated into 25 mL of Terrific Broth (TB) medium and grown overnight at 37 °C. The overnight culture was further inoculated into 500 mL of TB and grown until the OD_{600} was between 0.4 and 0.6. Protein expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for 3 h at 37 °C. The induced bacteria were harvested by centrifugation and resuspended in 25 mL of lysis/HKMEN buffer (10 mM Hepes, pH 7.2, 140 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, and 0.5% NP40) containing 1 mM DTT, 5 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM PMSF. This was followed by the addition of SDS to 0.03% and lysozyme (Sigma) to 100 μg/mL and incubation on ice for 1 h with intermittent gentle mixing. Cells were further lysed by sonication (Cell Disruptor; Heat Systems-Ultrasonics Incorporation, Plainview, NY), and clarified cell lysate was obtained by centrifuging the mixture for 15 min at 10 000*g* and 4 °C. Proteins were purified by mixing the 25 mL of lysate with 200 μL of a 50% slurry of glutathione-agarose beads (Sigma) for 1 h at 4 °C. Purified proteins were eluted in three batches of 300 μL using 20 mM reduced glutathione in 50 mM Tris, pH 8.0. The eluted, purified proteins were dialyzed against PBS (5.4 mM Na_2HP04, 1.8 mM KH_2PO4, 15 mM NaCl, pH 7.2) and quantified using a Bio-Rad protein assay kit (Hercules, CA).

In Vitro Translation. ^{35}S-methionine-labeled Bcl-2, Bcl-x_L, and Bax were generated from full-length cDNA templates subcloned into Bluescript KS vector (for Bcl-2 and Bax; Stratagene, La Jolla, CA) or pCDNA3 vector (for Bcl-x_L; Invitrogen, Carlsbad, CA). Reactions were performed in 50 μL volumes using the TNT T7/T3 Coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's instructions.

Peptide Inhibition. To test the ability of peptides to inhibit protein−protein interactions, we performed peptide inhibition assays. In brief, 5 μg of purified GST-fusion protein was incubated with 10 μL of ^{35}S-methionine-labeled, in vitro translated protein in the absence or presence of increasing concentrations of peptide. Reactions were carried out for 3 h at 4 °C in a final volume of 400 μL HKMEN buffer containing 1 mM DTT, 5 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM PMSF. The mixtures were then incubated with 50 μL of glutathione−agarose beads for another 1 h at 4 °C. Finally, the beads were washed twice with 0.75 mL of HKMEN buffer and boiled in 100 μL of 2 × SDS−PAGE sample buffer. Samples were resolved on 12.5% SDS−PAGE gels, transferred to nitrocellulose membranes, and stained with 0.1% naphthol blue black to verify an equal amount of GST or GST-fusion protein in each reaction. The membranes were exposed to X-ray film (Kodak Biomax MR film; Eastman Kodak Company, Rochester, NY), and the autoradiogram was analyzed to quantify the radioactive protein bound to the GST-fusion protein. Quantification of the autoradiogram was performed using a Bio-Rad GS-710 calibrated imaging densitometer and Quantity One software. The densitometric value in the absence of peptide was set as 100%, and values obtained in the presence of peptide were compared to this figure. Each protein−protein interaction assay also included a negative control containing GST alone incubated with in vitro translated protein in the absence of peptide.

Whole Cell Lysate Preparation and GST Pull-Down Assay. Jurkat cells were lysed for 20 min in ice-cold lysis buffer (10 mM Hepes, pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.25% NP40) containing 1 mM DTT, 5 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM PMSF. The cell lysates were then clarified by centrifugation for 10 min at 10 000*g* and 4 °C. To test the ability of the peptides to inhibit interaction between cellular Bcl-2 and GST-Bax fusion protein, we mixed 5 μg of purified GST-Bax protein with 500 μg of Jurkat whole cell lysate in the absence or presence of increasing concentrations of peptides. After 3 h of incubation, 50 μL of glutathione-agarose beads was added, and incubation proceeded for an additional 1 h at 4 °C. The

beads were then washed 3 times with 1 mL of lysis buffer, and the bound proteins were eluted by boiling in 100 μL of 2 × SDS−PAGE sample buffer. As a control for specificity of the GST pull-down assay, 5 μg of purified GST alone was incubated with 500 μg of total protein lysate in the absence of peptides. Samples (30 μL/lane) were loaded on 12.5% SDS−PAGE gels and subjected to immunoblotting using anti-Bcl-2 antibody. The same blot was reprobed with anti-Bax antibody to ensure equal levels of GST-Bax in each reaction.

Isolation of Mitochondria and Cytochrome c Release Assay. Mitochondria from Jurkat cells and Bcl-2/Jurkat cells were isolated on a discontinuous gradient of Percoll (Amersham Pharmacia, Piscataway, NJ) as previously described (*61*). In brief, cells were cultured at a density of less than 5 × 10^5 cells/mL, and 5 × 10^8 cells were harvested by centrifugation at 600 g for 5 min at 4 °C and then washed with ice-cold RPMI 1640 medium. The cell pellets were resuspended in 2 mL of isotonic mitochondrial resuspension buffer (MIB Buffer, pH 7.4; 10 mM MOPS, 4 mM KH_2PO_4, 300 mM sucrose) containing 1 mM DTT, 5 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM PMSF and kept on ice for 20 min. The cell suspensions were then homogenized with 120 strokes in a tissue homogenizer (Potter-Elvehjem Tissue Grinder; Wheaton, Millville, NJ) and centrifuged at 650 g for 10 min at 4 °C. Supernatants were stored on ice, and the pellets were resuspended and homogenized with an additional 120 strokes on ice. After centrifuging at 650*g*, the supernatant was collected and pooled with the first supernatant. The supernatants were again centrifuged in a Corex II centrifuge tube (Corning Incorporated, Corning, NY) at 10 000*g* for 12 min at 4 °C. The pellets, containing the mitochondria, were resuspended in 5 mL of MIB buffer, centrifuged at 10 000*g* for 12 min at 4 °C, and then resuspended again in 3 mL of MIB buffer. A discontinuous gradient of Percoll was prepared by carefully layering 2.5 mL each of 70%, 30%, 18%, and 10% Percoll (prepared using MIB buffer), with 70% Percoll at the bottom. The resuspended mitochondrial suspension was layered gently on top of the Percoll gradient and centrifuged at 15 000*g* for 30 min at 4 °C. Mitochondria were carefully aspirated from the interface of the 70% and 30% Percoll and the 30% and 18% Percoll layers. The collected mitochondria were resuspended in a minimum of 5 volumes of MIB containing 1% BSA and sedimented at 20 000*g* for 40 min at 4 °C. The pelleted mitochondria were then washed with MIB buffer, centrifuged at 10 000*g* for 10 min at 4 °C, and resuspended in MIB buffer. Protein concentrations were determined, and mitochondria were finally resuspended in MIB buffer at a concentration of 1 mg/mL.

Cytochrome *c* release assays were carried out by incubating 40 μg of mitochondria in the absence or presence of peptides or GST-Bax protein for 1 h at 37 °C. During incubation, the isotonicity of MIB buffer was carefully maintained, particularly when treating with larger volumes of the GST-Bax protein. At the end of the incubation, mitochondria were sedimented at 10 000*g* for 10 min at 4 °C, and the supernatants containing released proteins were recentrifuged. Finally, the supernatant was carefully collected and heated with 2 × SDS−PAGE sample buffer. Mitochondrial pellets were washed once with ice-cold MIB buffer and finally heated with 2 × SDS−PAGE sample buffer. The mitochondrial pellet samples and supernatant samples were subjected to immunoblotting against anti-cytochrome *c* antibody. Mitochondrial pellet samples were also probed with cytochrome *c* oxidase IV antibody to ensure equal loading and as a control for mitochondrial membrane integrity.

Peptide Delivery and Cell Viability. Peptides were delivered into cells at a concentration of 200 μM using the BioPorter peptide delivery system according to slight modifications of manufacturer's instructions (Gene Therapy Systems, San Diego, CA). In brief, 2.5 μL of BioPorter reagent was dried for 4 h at room temperature and then mixed with peptide in a total volume of 20 μL of Hepes-buffered saline (HBS, pH 7.2; 10 mM Hepes, 150 mM NaCl) for 5 min at room temperature. As controls, peptide in the absence of BioPorter reagent and BioPorter reagent in the absence of peptide were also prepared. The peptide/BioPorter mixture or peptide alone was vortexed for 15 s, gently mixed with 10^5 cells in a 225 μL volume of serum-free medium, and the resulting mixture was plated in a 24-well plate. Following 4 h of incubation at 37 °C, 250 μL of RPMI media containing 20% heat-inactivated serum was added, and the incubation was continued for another 6 h. The effect of peptides on cell viability was assessed by trypan blue exclusion assay.

RESULTS

Bax BH3 Peptide Inhibits the Interaction Between Bcl-2 and Bax. Peptides belonging to the BH3 domains of human Bax, Bcl-2, and Bad (Table 1) were tested for their ability to inhibit Bax/Bcl-2 interaction using in vitro assays. L63E Bax BH3, a mutant Bax peptide, wherein leucine 63 is changed to glutamic acid, was also included in this assay. Mutation of Leu 63 has been shown to abrogate the ability of full-length Bax to homodimerize as well as heterodimerize with Bcl-2 and Bcl-x_L, and the mutation also markedly reduced the pro-apoptotic activity of Bax protein (*48*). For the in vitro interaction assays, GST alone or GST-Bax was incubated with in vitro translated ^{35}S-Bcl-2 protein in the absence or presence of increasing concentrations of peptides. ^{35}S-Bcl-2 that bound to GST-Bax was captured on glutathione-agarose beads and analyzed by SDS−PAGE, followed by autoradiography and densitometric scanning of the autoradiogram. Binding of ^{35}S-Bcl-2 to GST-Bax was found to be strong and specific since GST alone was unable to pull down the in vitro translated ^{35}S-Bcl-2 (Figure 1A, compare lanes 1−3). As demonstrated in Figure 1A, there was a dose-dependent inhibition of Bax/Bcl-2 interaction in the presence of 10−100 μM of Bax BH3 peptide. Relative to the control (absence of peptide), the interaction between Bcl-2 and Bax decreased to 60% and 12% in the presence of 10 and 100 μM Bax BH3 peptide, respectively (Figure 1A, lanes 3−6). However, L63E Bax BH3 peptide had no effect on Bax/Bcl-2 interaction (lane 12). In addition, BH3 peptides from Bad and Bcl-2 were also ineffective at disrupting Bax/Bcl-2 binding, even at concentrations as high as 100 μM (Figure 1A, lanes 7−11).

We also assessed the ability of the peptides to inhibit interactions between endogenous cellular Bcl-2 and GST-Bax. For these experiments, purified GST-Bax was incubated with 500 μg of Jurkat whole cell lysate, and GST-Bax/Bcl-2 complexes were pulled down by glutathione−agarose beads. Cellular Bcl-2 protein that bound to GST-Bax was detected

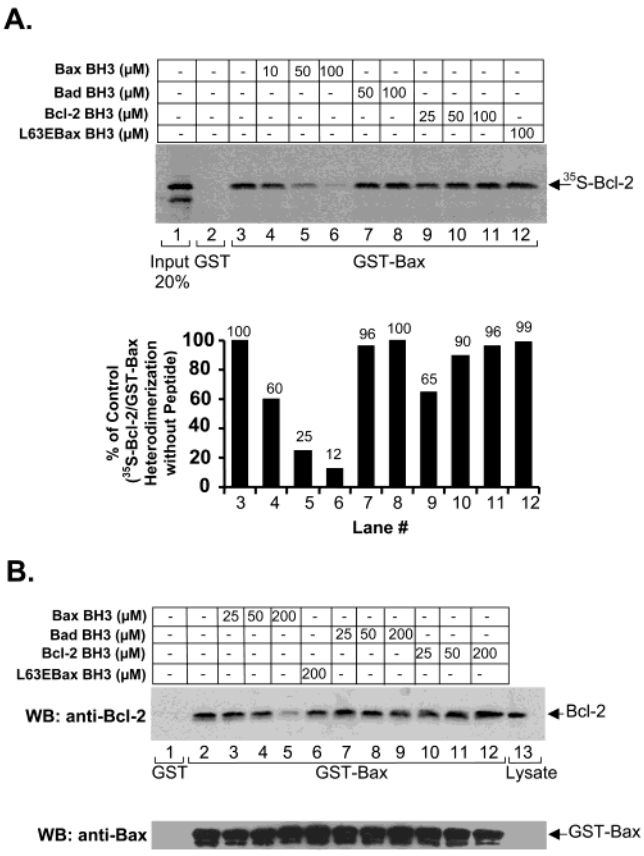

FIGURE 1: Bax BH3 peptide disrupts heterodimerization of Bax and Bcl-2. (A) In vitro translated ^{35}S-Bcl-2 was incubated with GST-Bax in the absence (lane 3) or presence of varying concentrations of the BH3 peptides (lanes 4−12). The ^{35}S-Bcl-2 protein bound to GST-Bax was captured on glutathione−agarose beads, washed with HKMEN lysis buffer, and boiled with 2 × SDS−PAGE sample buffer. Samples were resolved by SDS−PAGE, transferred to a nitrocellulose membrane, and subjected to autoradiography. The membrane was also stained with 0.1% naphthol blue black to confirm equal amounts of GST proteins in each reaction. In lane 1, ^{35}S-Bcl-2 representing 20% of the input used in lanes 2−12 was electrophoresed. As a negative control, purified GST alone was incubated with ^{35}S-Bcl-2 in lane 2. To quantify the disruption of GST-Bax/^{35}S-Bcl-2 heterodimerization by the peptides, we performed densitometric scanning of the autoradiogram and plotted the values as a bar diagram (lower panel). Values obtained in the absence of peptide were taken as 100%. (B) GST-Bax was incubated with Jurkat whole cell lysates in the absence or presence of increasing concentrations of the indicated peptides (lanes 3−12). The complexes of GST-Bax/cellular-Bcl-2 were captured on glutathione-agarose beads, and Bcl-2 bound to GST-Bax was detected by immunoblotting with anti-Bcl-2 antibody. The blot was reprobed with anti-Bax polyclonal antibody to ensure equivalent amounts of GST-Bax in each reaction. In lane 1, GST alone was incubated with whole cell lysate as a negative control, and in lane 13, whole cell lysate alone was loaded on the gel.

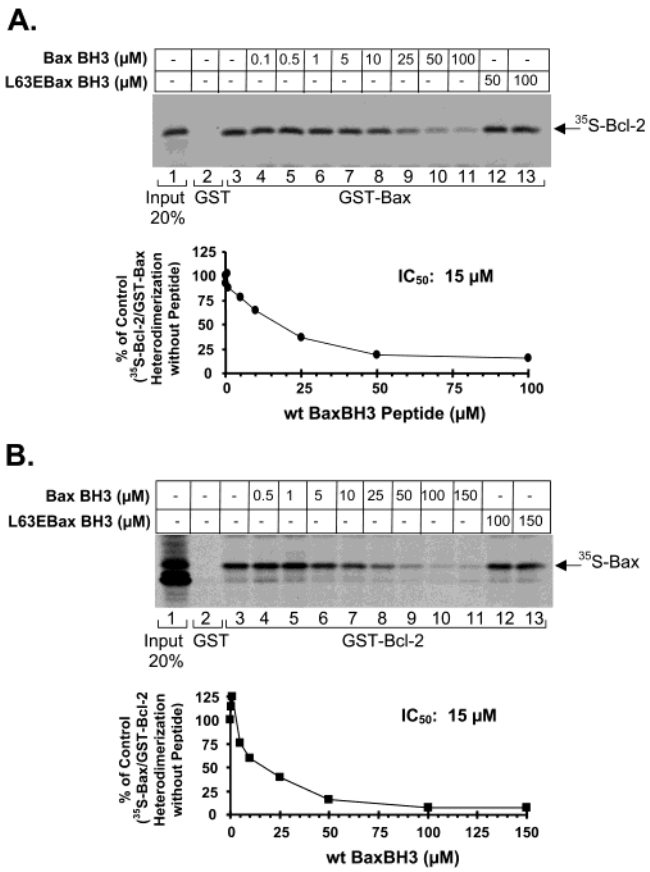

FIGURE 2: Bax BH3 disrupts GST-Bax/^{35}S-Bcl-2 and GST-Bcl-2/^{35}S-Bax interactions with an IC_{50} of 15 μM. (A) In vitro interaction assays using ^{35}S-Bcl-2 and GST-Bax were performed in the absence (lane 3) or presence (lanes 4−11) of a range of Bax BH3 peptide concentrations as described in Figure 1. Samples were analyzed by SDS−PAGE and autoradiography, and the amount of ^{35}S-Bcl-2 bound to GST-Bax was quantified by densitometry. The values obtained in the presence of peptide were compared to those obtained in the absence of peptide (taken as 100%) to calculate the IC_{50} (lower panel). The L63E mutant Bax BH3 peptide was included in two reactions (lanes 12 and 13), and incubation of ^{35}S-Bcl-2 with GST alone served as a negative control (lane 2). (B) In reciprocal assays, ^{35}S-Bax and GST-Bcl-2 were incubated in the absence (lane 3) or presence (lanes 4−11) of increasing concentrations of Bax BH3 peptide. The IC_{50} was obtained by plotting values obtained from densitometric scanning of the autoradiogram (lower panel).

by probing the blot with an anti-Bcl-2 antibody. No binding of cellular Bcl-2 was seen with GST alone (Figure 1B, lane 1). In agreement with our previous observations, Bax BH3 peptide inhibited the interaction between cellular Bcl-2 and GST-Bax in a dose-dependent manner, with a marked inhibition of this interaction observed at 200 μM peptide (Figure 1B, lanes 2−5). The L63E Bax BH3 peptide exhibited no effect on this interaction (Figure 1B, lane 6). Moreover, both Bad BH3 and Bcl-2 BH3 had no detectable effect on GST-Bax/Bcl-2 interactions at the concentrations used in this assay, further strengthening the observations obtained in Figure 1A. Taken together, experiments using either ^{35}S-Bcl-2 or cellular Bcl-2 demonstrate that Bax BH3, but not Bad BH3 or Bcl-2 BH3, are inhibitory to Bax/Bcl-2 interaction. Furthermore, these results also underscore the importance of leucine 63 in the Bax BH3 peptide, since mutation of this amino acid completely abrogated the ability of the peptide to inhibit Bax/Bcl-2 interaction.

Bax BH3 Inhibits Bax/Bcl-2 Interaction with an IC_{50} of 15 μM. To evaluate the potency of the Bax BH3 peptide, we performed experiments to determine the IC_{50} value of the peptide for disrupting Bax/Bcl-2 interaction. Peptide inhibition assays were carried out using GST-Bax and in vitro translated ^{35}S-Bcl-2 in the absence or presence of Bax BH3 peptide (0.1−100 μM), and values obtained from quantification of the bound ^{35}S-Bcl-2 were plotted in the form of a graph (Figure 2A). L63E Bax BH3, at higher concentrations, was also included in the assay as a negative control. The results shown in Figure 2A reveal a gradual decrease in Bax/

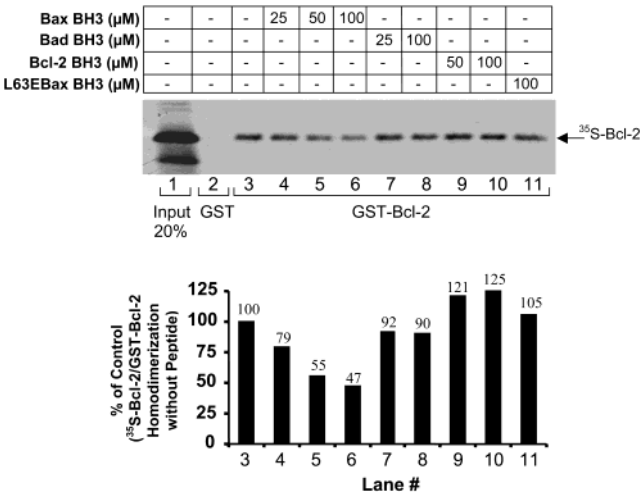

FIGURE 3: Bax BH3 peptide moderately inhibits Bcl-2 homodimerization. Inhibition of Bcl-2 homodimerization by the indicated BH3 peptides was analyzed in in vitro interaction assays using purified GST-Bcl-2 and in vitro translated ^{35}S-Bcl-2, as described in Materials and Methods. Incubation of GST alone with ^{35}S-Bcl-2 served as a negative control (lane 2). Computation of inhibition of Bcl-2 homodimerization was determined following densitometry of the autoradiogram. The levels of Bcl-2 homodimerization seen in the presence of peptide were compared to that seen in the absence of peptide, which was set as 100% (lower panel).

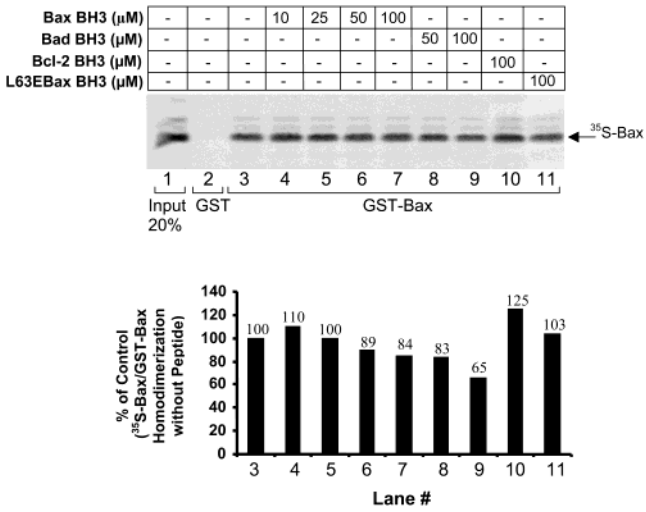

FIGURE 4: Effect of BH3 peptides on Bax homodimerization. The effect of Bax, Bad, and Bcl-2 BH3 peptides on Bax homodimerization was tested in in vitro interaction assays using in vitro translated ^{35}S-Bax and purified GST-Bax. Assays were performed as described for Figure 3, and the effect of peptides on Bax homodimerization was determined following densitometric scanning of the autoradiogram (lower panel). As a negative control, ^{35}S-Bax was incubated with GST alone (lane 2).

Bcl-2 heterodimerization in the presence of increasing concentrations of Bax BH3 peptide. The concentration of Bax BH3 which yielded 50% inhibition (IC_{50}) of Bax/Bcl-2 interaction was calculated to be 15 μM.

Similar peptide inhibition experiments were performed in a reverse orientation where GST-Bcl-2 was used as bait to pull down radiolabeled ^{35}S-Bax in the absence or presence of 0.5−150 μM of Bax BH3 peptide (Figure 2B). In these studies, the IC_{50} for Bax BH3 was also determined to be 15 μM, corroborating the value obtained in the earlier experiments. As expected, L63E peptide was completely inactive at inhibiting the interaction between GST-Bcl-2 and ^{35}S-Bax (lanes 12 and 13).

Bax BH3 Moderately Inhibits Bcl-2 Homodimerization. Mutagenesis studies on full-length Bcl-2 and Bax have shown that the BH3 domain is important not only in Bax/Bcl-2 interaction, but in homodimerization of these proteins as well (*33*, *42*, *44*, *62*). Therefore, we examined the impact of all three BH3 peptides (Bax, Bad, Bcl-2) on Bcl-2 homodimerization. Homodimerization interactions were studied using purified GST-Bcl-2 and radiolabeled ^{35}S-Bcl-2 in pull-down assays. As illustrated in Figure 3, Bax BH3 moderately inhibited Bcl-2 homodimerization, exhibiting 21% inhibition at 25 μM and approximately 53% inhibition at 100 μM (lanes 4−6). However, Bad BH3 and L63E Bax BH3 showed insignificant reduction of this interaction (lanes 7, 8, and 11). Surprisingly, Bcl-2 BH3, even at 100 μM, failed to inhibit Bcl-2 homodimerization (lanes 9 and 10). Taken together, these studies reveal a moderate capacity of Bax BH3 to inhibit Bcl-2 homodimerization while BH3 peptides from Bcl-2 and Bad failed to disrupt this interaction.

Bax Homodimerization Remains Unaffected by BH3-Domain Peptides. To test the ability of the synthetic BH3 peptides to disrupt Bax homodimerization, we performed in vitro binding assays using GST-Bax and ^{35}S-Bax in the presence of varying concentrations of peptides. As shown in Figure 4, Bax BH3 peptide did not significantly inhibit Bax homodimerization. Even at 100 μM Bax BH3, only 16% inhibition was observed (Figure 4, lanes 3−7). Similarly, BH3 peptides belonging to Bad, Bcl-2, and L63E Bax were largely ineffective at inhibiting Bax homodimerization (lanes 8−11). These results demonstrate the inability of the synthetic BH3 peptides to inhibit Bax homodimerization.

Bax and Bad BH3 Peptides Efficiently Inhibit Bax/Bcl-x_L Heterodimerization. Bcl-x_L, a potent anti-apoptotic member of the Bcl-2 protein family, has been shown to bind and inactivate Bax and Bad (*30*, *45*, *63*). Furthermore, Bad exhibits preferential binding to Bcl-x_L over Bcl-2 (*12*, *50*). Thus, we also examined the ability of the BH3 peptides to inhibit Bax/Bcl-x_L interactions (Figure 5A). At a concentration of 50 μM, Bax BH3 reduced the heterodimerization between Bcl-x_L and Bax by as much as 90% (Figure 5A, lanes 3 and 4). Bad BH3 at the same concentration was even more potent than Bax BH3 and inhibited this heterodimerization by as much as 94% (lane 5). By contrast, Bcl-2 BH3 peptide showed much less inhibitory potential, with a maximum of 46% reduction at 100 μM (Figure 5A, lanes 6−8). In comparison, L63E Bax BH3 peptide, even at 100 μM, had only a minor effect on Bax/Bcl-x_L interaction (lane 9).

In Figure 5B,C, IC_{50} values for Bax BH3 and Bad BH3 peptides were determined in the Bax/Bcl-x_L interaction assays. Bax BH3 inhibited Bax/Bcl-x_L heterodimerization in a dose-dependent manner with an IC_{50} of 9.5 μM (Figure 5B). Bad BH3 was even more potent, exhibiting an IC_{50} of 3.7 μM (Figure 5C). These results and those obtained with Bax/Bcl-2 heterodimerization show that Bax BH3 is able to inhibit both Bax/Bcl-2 and Bax/Bcl-x_L heterodimerization. By contrast, Bad BH3 peptide causes a dramatic reduction in Bax/Bcl-x_L interaction but is inactive toward Bax/Bcl-2.

Bax BH3 and Bad BH3 Induce the Release of Cytochrome c from Isolated Mitochondria of Bcl-2-Overexpressing Cells. Mitochondria are critical sites of action for Bax and Bad

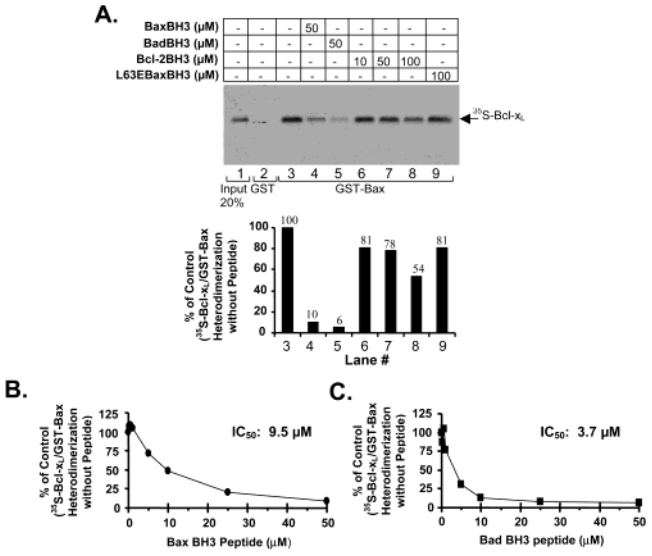

FIGURE 5: Bax BH3 and Bad BH3 peptides are potent inhibitors of Bax/Bcl-x_L interaction. (A) In vitro interaction assays were performed by incubating GST-Bax and ^{35}S-Bcl-x_L in the absence (lane 3) or presence of the indicated concentrations of Bax BH3 (lane 4), Bad BH3 (lane 5), and Bcl-2 BH3 (lanes 6−8) peptides. The L63E Bax BH3 peptide was also tested (lane 9). Incubation of ^{35}S-Bcl-x_L with GST alone served as a negative control (lane 2). ^{35}S- Bcl-x_L alone, representing 20% of the input used in lanes 2−9, was electrophoresed in lane 1. Calculation of the degree of Bax/Bcl-x_L disruption by the different peptides was performed following densitometry of the autoradiogram (Bar graph). For panels B and C, a range of Bax BH3 and Bad BH3 concentrations were employed to determine the IC_{50} of each peptide for disrupting Bax/Bcl-x_L heterodimerization.

molecules. Upon translocation to the mitochondria, Bax and Bad promote the release of cytochrome *c* (*30*, *64*). It is also known that both recombinant Bax protein (*38*, *39*) and Bax BH3 peptides (*38*, *39*, *55*, *65*) can induce cytochrome *c* release from isolated mitochondria. For example, a 34-amino-acid Bax BH3 peptide induced release of cytochrome *c* from mitochondria derived from neural cells but not from rat liver or rat forebrain (*55*). These studies also found that overexpression of Bcl-2 conferred protection against cytochrome *c* release (*55*). To investigate the ability of our BH3 peptides to induce cytochrome *c* release and their potential to overcome the effects of Bcl-2 overexpression, we performed cytochrome *c* release assays using mitochondria from Jurkat T leukemic cells and transfected Jurkat cells overexpressing Bcl-2. As shown in Figure 6, we found that both Bax BH3 and Bad BH3 induced cytochrome *c* release from wild-type as well as Bcl-2-overexpressing Jurkat cells. Substitution of leucine with glutamic acid in the L63E Bax BH3 peptide led to a loss of cytochrome *c*-releasing ability (lanes 4 and 10). Likewise, Bcl-2 BH3 at 100 μM was unable to induce cytochrome *c* release from mitochondria of either cell line (lanes 6 and 12). Interestingly, the parental Bax BH3 peptide, at concentrations of 50 and 100 μM, induced a comparable amount of cytochrome *c* release from mitochondria of wild-type Jurkat and Bcl-2/Jurkat cells (lanes 2 and 3 and lanes 8 and 9). Thus, Bax BH3 was able to overcome the inhibitory effect of Bcl-2 overexpression. By contrast, although Bad BH3 peptide caused cytochrome *c* release in wild-type mitochondria, overexpression of Bcl-2 markedly decreased the ability of Bad BH3 to promote such release (lanes 5 and 11). This is consistent with our previous observations that

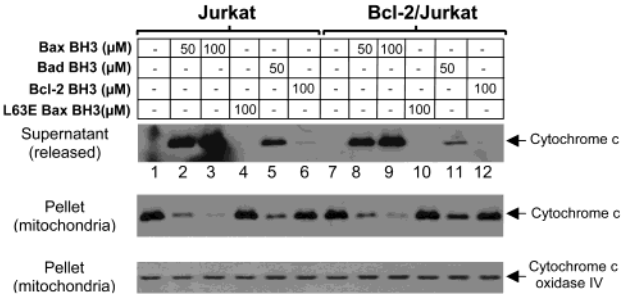

FIGURE 6: Bax BH3 and Bad BH3, but not Bcl-2 BH3, induce cytochrome *c* release from Bcl-2-overexpressing mitochondria. Purified mitochondria from wild-type or Bcl-2-overexpressing Jurkat cells were incubated with (lanes 2−6 and 8−12) or without (lanes 1 and 7) the indicated peptides for 1 h at 37 °C. Following incubation, supernatants containing released proteins were separated from mitochondrial pellets, and both fractions were subjected to immunoblotting. The resulting blots were probed with anti-cytochrome *c* monoclonal antibody (top and middle panels). As a control for mitochondrial integrity and equal loading, the mitochondrial pellets were reprobed with anti-cytochrome *c* oxidase IV antibody (bottom panel). Unlike cytochrome *c*, cytochrome *c* oxidase IV is not released from mitochondria during apoptosis.

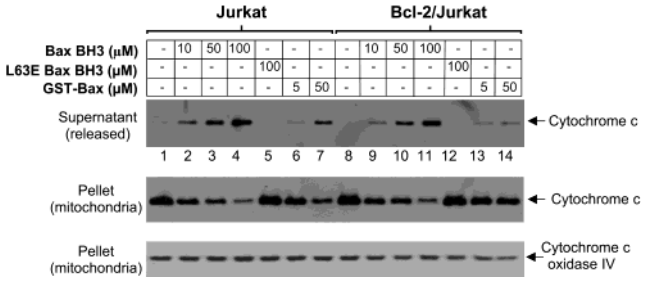

FIGURE 7: Bax BH3 Peptide, but not full-length GST-Bax protein, induces cytochrome *c* release from Bcl-2-overexpressing Jurkat mitochondria. Isolated mitochondria from wild-type and Bcl-2-overexpressing Jurkat cells were incubated with equivalent concentrations of Bax BH3 peptide (lanes 2−4 and 9−11) or GST-Bax protein (lanes 6 and 7 and lanes 13 and 14) as described in Materials and Methods. The ability of the L63E Bax BH3 peptide to induce cytochrome *c* release was also examined (lanes 5 and 12). Cytochrome *c* release (top panel) and depletion from mitochondria (middle panel) were assessed by subjecting the mitochondrial supernatant and pellet samples to SDS−PAGE, followed by immunoblotting with anti-cytochrome *c* antibody. As a control, the pellet blot was reprobed with anti-cytochrome *c* oxidase IV (bottom blot).

Bad BH3, while capable of disrupting Bax/Bcl-x_L interactions, is ineffective at disrupting Bax/Bcl-2.

Bax BH3 Peptide is More Potent than Full-Length Bax Protein at Inducing Cytochrome c Release from Mitochondria of Bcl-2-overexpressing Cells. In additional studies, we compared the abilities of Bax BH3 peptide and recombinant full-length Bax protein to induce cytochrome *c* release from mitochondria of wild-type Jurkat and Bcl-2/Jurkat cells (Figure 7). Confirming our earlier observations, Bax BH3 induced a concentration-dependent release of cytochrome *c* from mitochondria of wild-type Jurkat cells. Furthermore, Bax BH3 induced similar levels of cytochrome *c* release from Bcl-2-overexpressing mitochondria (Figure 7, lanes 2−4 and 9−11). Full-length GST-Bax protein also promoted efficient release of cytochrome *c* from mitochondria of wild-type Jurkat cells. However, while Bax BH3 peptide was able to overcome Bcl-2 overexpression, GST-Bax, at equivalent concentrations, was markedly less efficient at inducing cytochrome *c* release from the mitochondria of Bcl-2/Jurkat

Table 2: Comparison of the Ability of Peptides to Inhibit Protein−Protein Interactions and Induce Cell Death

	IC_{50} for disruption of interaction[a,b]				induction of cell death[c] (% Viability)		
peptide	Bax/Bcl-2	Bax/Bcl-xL	Bax/Bax	Bcl-2/Bcl-2	Vector/Jurkat	Bcl-2/Jurkat	Bcl-x_L/Jurkat
Bax BH3	15 μM	9.5 μM	±	±	51 ± 2.52	59 ± 3.52	74 ± 7.57
L63E Bax BH3	−	−	−	−	88 ± 3.79	90 ± 6.02	92 ± 2.08
Bad BH3	−	3.7 μM	−	−	55 ± 5.86	77 ± 7.02	64 ± 4.51
Bcl-2 BH3	−	±	−	−	91 ± 1.73	88 ± 3.61	84 ± 3.21

[a] The values shown represent the IC_{50} of the indicated peptide for disruption of the indicated protein−protein interaction. [b] "±" denotes an insignificant effect, and "−" denotes no effect of the peptide on the indicated protein−protein interaction. [c] Cell death was measured by trypan blue exclusion assay 10 h after peptide delivery.

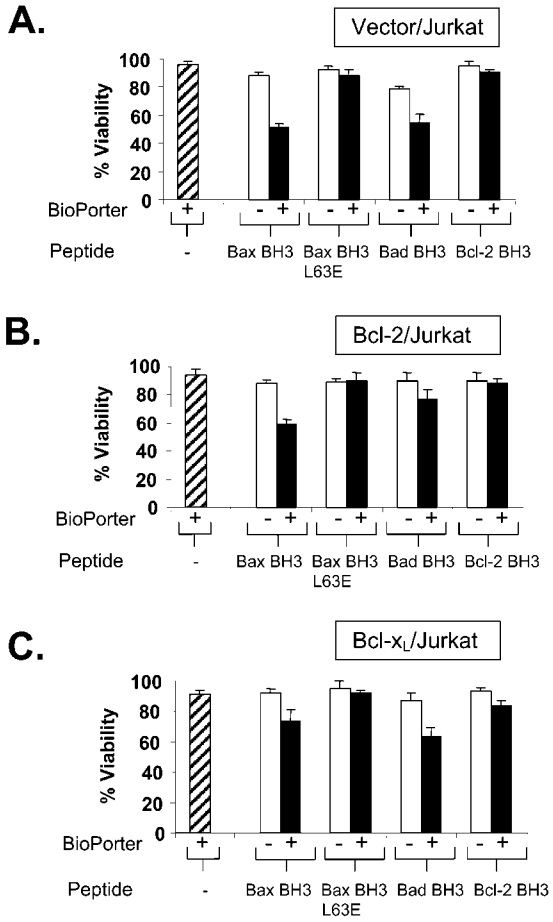

FIGURE 8: Effect of BH3-domain peptides on the viability of Bcl-2- or Bcl-x_L- overexpressing cells. Peptides (200 μM) were delivered into vector-transfected cells, or cells engineered to overexpress Bcl-2 or Bcl-x_L using BioPorter delivery reagent. As controls, cells were also incubated with BioPorter reagent alone, or peptide alone. The effect of each peptide on cell viability was determined 10 h after delivery using a trypan blue exclusion assay. Cell viability was measured in triplicate and is represented by the mean value. Standard deviations are shown by the error bars.

cells (lanes 6 and 7 and lanes 13 and 14). These results point to a remarkable potency of Bax BH3 peptide at inducing cytochrome *c* release, even in the presence of Bcl-2 overexpression.

Bax BH3 and Bad BH3 Peptides Induce Cell Death. Examination of cytochrome *c*-release from isolated mitochondria of wild-type Jurkat and Bcl-2/Jurkat cells revealed the apoptotic potential of the Bax BH3 and Bad BH3 peptides. To investigate their effects on intact cells, peptides were delivered into cells using the BioPorter peptide delivery system (Gene Therapy Systems, San Diego, CA). Ten hours after intracellular delivery of the peptides, cell viability was

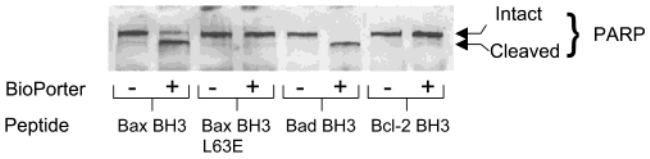

FIGURE 9: Bax BH3 and Bad BH3 peptides induce PARP cleavage. Peptides (200 μM) were delivered into vector-transfected Jurkat cells using BioPorter reagent (+) or the cells were treated with peptides alone (−). After lysing the cells, we subjected the whole cell lysate containing 20 μg of protein to Western blotting and assessed the cleavage of PARP by probing the blot with anti-PARP antibody.

determined by trypan blue exclusion assay. Both Bax BH3 and Bad BH3 had comparable cytotoxic effects on vector-transfected Jurkat cells, decreasing the viability of cells to 51% and 55%, respectively (Figure 8A and Table 2). In comparison, mutant Bax BH3 peptide as well as Bcl-2 BH3 had no significant effect. Similarly, cells incubated with peptide in the absence of BioPorter or with BioPorter alone were not affected. The cell death induced by Bax BH3 and Bad BH3 was accompanied by cleavage of cellular PARP protein, indicating that the peptides were invoking an apoptotic cell death (Figure 9).

A comparison of the cytotoxic effects of the peptides toward Bcl-2/Jurkat cells revealed only a small reduction in the cell-killing effect of Bax BH3 (59% viability) as compared to its effect on vector-transfected cells (51% viability), as shown in Figure 8B and Table 2. Bad BH3 peptide, on the other hand, exhibited an appreciable loss of cell-killing toward Bcl-2/Jurkat cells (77% viability) compared to vector/Jurkat cells (59% viability). These data are consistent with the reduced ability of Bad BH3, relative to Bax BH3, to disrupt Bax/Bcl-2 interaction (Figures 1A,B and 2A,B) and the reduced ability of Bad BH3 to induce cytochrome *c* release in Bcl-2-overexpressing mitochondria. However, when peptides were delivered into Bcl-x_L/Jurkat cells, Bad BH3 peptide was more potent than Bax BH3 at inducing cell death (Figure 8C). This again was consistent with our earlier data, showing that Bad BH3 is more potent than Bax BH3 at disrupting Bax/Bcl-x_L interaction (IC_{50} values of 3.7 and 9.5 μM, respectively; Figure 5B,C). In all cell lines tested, Bcl-2 BH3 peptide and L63E Bax BH3 peptide did not appreciably impact cell viability.

DISCUSSION

Protein−protein interactions and pore-formation are considered to play critical roles in the regulation of apoptosis by Bcl-2 family members. The X-ray crystallographic and NMR structures of Bcl-x_L (*66*) and Bcl-2 (*67*) reveal the presence in these molecules of two central, largely hydro-

phobic α helices (α5 and α6), surrounded by five amphipathic α helices (α1, α2, α3, α4, and α7). A similar overall helical structure has been described for Bax (*47*). On the basis of structural similarities between α5/α6-containing Bcl-2 family members and the pore-forming bacterial toxins colicin A1, colicin E1, and diphtheria toxin, certain Bcl-2 family proteins are postulated to form ion channels that regulate ion and/or small-molecule traffic across the mitochondrial outer membrane (*68*). Indeed, Bcl-2 (*69*), Bcl-x_L (*70*), and Bax (*71*) have been reported to possess channel-forming properties in synthetic lipid membranes. It remains unclear, however, how pore formation may contribute to the apoptosis regulatory activities of Bcl-2 family members.

The amphipathic α2 helix, which constitutes the BH3 domain of Bax, is involved in both homodimerization and heterodimerization and is essential for the death-promoting function of this protein (*7*, *42*, *47*, *46*, *68*). Interestingly, BH3 is the only BH domain retained by pro-apoptotic "BH3-domain only" molecules such as Bad and Bid, further emphasizing the functional significance of this domain (*31*). Because the BH3 domain is important for protein−protein interactions among Bcl-2 family members, we and others have hypothesized that peptides derived from this domain should competitively inhibit the oligomerization of these proteins. To test this hypothesis, we designed and synthesized peptides based on the BH3 domain of Bax, Bad, and Bcl-2. We compared the potency of these peptides at inhibiting the oligomerization of Bcl-2, Bcl-x_L and Bax using in vitro protein binding assays. We extended our studies to compare the ability of the different BH3 peptides to induce cytochrome *c* release and activate cell death in Jurkat cells overexpressing Bcl-2 or Bcl-x_L.

Our studies revealed that a 20-amino-acid Bax BH3 peptide (amino acid 55−74) inhibited heterodimerization of ^{35}S-Bcl-2 with GST-Bax (or ^{35}S-Bax with GST-Bcl-2) in a dose-dependent manner with an IC_{50} of 15 μM. Similar findings have been reported with a 21-amino-acid Bax BH3 peptide (amino acid 52−72) using a plate binding assay to measure Bax/Bcl-2 interaction (*49*). We also discovered that our Bax BH3 peptide was somewhat less efficient at inhibiting the interaction of GST-Bax with cellular Bcl-2 in whole cell lysates. This reduced efficiency could be due to binding of the Bax BH3 peptide to proteins other than Bcl-2 in the cell lysate, thereby decreasing the availability of the peptide to bind to Bcl-2. Nevertheless, both protein−protein interaction assays showed dose-dependent inhibition of Bax/Bcl-2 interaction. As compared to Bax BH3, the Bad BH3 peptide failed to prevent interaction between Bcl-2 and Bax, an observation confirmed using both types of interaction assays. By contrast, Bad BH3 potently inhibited Bax/Bcl-x_L heterodimerization, with an IC_{50} of 3.7 μM. These results are consistent with earlier reports showing that both Bad BH3 peptide and intact Bad protein bind with higher affinity to Bcl-x_L than to Bcl-2 (*12*, *63*). The Bax BH3 peptide also inhibited Bax/Bcl-x_L interaction, exhibiting an IC_{50} of 9.5 μM.

Mutational analyses of Bcl-2 have revealed a role for the BH1, BH2, and BH3 domains in binding to Bax (*33*, *40*, *43*, *68*). However, in our studies, synthetic peptides derived from the BH3 domain of Bcl-2 failed to significantly inhibit Bax/Bcl-2 or Bax/Bcl-x_L interactions. On the basis of three-dimensional structures, it has been proposed that anti-apoptotic molecules such as Bcl-2 and Bcl-x_L are receptor-like molecules possessing a hydrophobic binding cleft, whereas pro-apoptotic molecules such as Bax, Bad, and Bak bind as ligands through their BH3 domain (*7*, *33*). Consistent with this model, it is not surprising that the Bcl-2 BH3 peptide, which is derived from the binding pocket of a receptor-like molecule, is unable, by itself, to competitively inhibit interactions between Bcl-2-family members.

Our experiments also showed that the L63E mutant Bax BH3 peptide failed to exert any inhibitory influence on protein−protein interactions. Comparison of amino acid sequences of Bcl-2 family members reveals that Leu 63 and Asp 68 of Bax are highly conserved residues with maximum consensus value, followed by Gly 67 of Bax (*63*). Substitution of Leu 78 in Bak peptide, which corresponds to Leu 63 of Bax, with an alanine residue results in an 800-fold decrease in binding to Bcl-x_L due to loss of hydrophobic interactions (*72*). Similarly, Asp 83 of Bak peptide, corresponding to Asp 68 of Bax, is important for electrostatic interactions with Arg 139 of Bcl-x_L, and substitution of this aspartic acid with alanine significantly disrupts heterodimerization (*72*). The importance of Gly 67 in Bax is evidenced by the natural occurrence of a G67R missense mutation found in HPB-ALL cancer. Interestingly, G67R Bax fails to interact with Bcl-2 and Bcl-x_L and does not exhibit killing activity (*73*). Moreover, three-dimensional computer modeling and site-directed mutagenesis of Bax protein have clearly demonstrated the importance of Leu 63, Gly 67, Leu 70, and Met 74 residing on the hydrophobic surface of the amphipathic Bax BH3 domain (*48*). Substitution of Leu 63 with Glu in the full-length protein, for example, abrogated the ability of Bax to heterodimerize with Bcl-2, accompanied by partial to complete loss of pro-apoptotic activity. The failure of L63E Bax BH3 to disrupt any of the tested protein−protein interactions supports the critical nature of this residue.

Our studies also revealed that homodimerization of Bcl-2 was unaffected by Bcl-2 BH3 and Bad BH3 peptides in the in vitro assays. Previously, deletion mutagenesis and yeast two-hybrid analyses have implicated a role for BH4, BH1, BH2, and BH3 in Bcl-2 homodimerization (*33*, *40*, *43*). A modest inhibition of Bcl-2 homodimerization by Bax BH3 was observed, which may be due to the high affinity of Bax BH3 peptide for the Bcl-2 protein. None of the tested BH3 domain peptides, however, significantly inhibited Bax homodimerization. This is consistent with the notion that Bax functions as a ligand and would, therefore, lack a receptor-like binding pocket for BH3 domain peptides (*7*, *33*).

An overall objective in designing the BH3 peptides is to develop agents capable of inducing apoptosis in Bcl-2-overexpressing tumor cells. The release of cytochrome *c* from mitochondria is a hallmark feature of the intrinsic apoptosis pathway (*31*, *32*) and is widely used as a marker of apoptosis induction. Having demonstrated the ability of Bax BH3 peptide to disrupt both Bax/Bcl-2 and Bax/Bcl-x_L, and Bad BH3 peptide to disrupt Bax/Bcl-x_L, we investigated the ability of these peptides to induce cytochrome *c* release from mitochondria isolated from wild-type and Bcl-2-overexpressing Jurkat cells. Both peptides were found to efficiently induce cytochrome *c* release from mitochondria of wild-type Jurkat cells. Strikingly, the Bax BH3 peptide was also able to overcome the protective effect of Bcl-2 overexpression.

Previous studies have examined the ability of full-length pro-apoptotic proteins such as Bax (*38*, *39*) and synthetic peptides derived from the BH3 domains of Bax and Bak (*38*, *39*, *55*, *65*) to induce cytochrome *c* release from isolated mitochondria or in a cell-free system. Recombinant full-length Bax was shown to induce the release of cytochrome *c* from mitochondria isolated from rat liver, although a 16-amino-acid Bax BH3 peptide (amino acid 61−76) was found inactive (*38*). Cosulich et al. (*65*) demonstrated a pro-apoptotic activity of Bax and Bak BH3 peptides in a cell-free system based on extracts from Xenopus oocytes. Similarly, a 20-amino-acid Bax peptide (amino acid 55−74) and a 15-amino-acid Bak peptide (amino acid 73−87) have been shown to induce cytochrome *c* release from rat liver mitochondria (*39*). In addition, a 34-amino-acid Bax BH3 peptide (amino acid 53−86) induced cytochrome *c* release from isolated mitochondria of wild-type GT1−7 neural cells (*55*). However, this 34-amino-acid peptide was unable to fully overcome the effects of Bcl-2-overexpression (*55*). By contrast, we demonstrated a potent ability of our 20-amino-acid Bax BH3 peptide to induce cytochrome *c* release from mitochondria of Bcl-2-overexpressing Jurkat cells.

In contrast to Bax BH3 peptide, full-length GST-Bax was inefficient at inducing cytochrome *c* release in the Bcl-2-overexpressing mitochondria. This could be due to the increased accessibility, and therefore activity, of the small BH3 domain peptide compared to full-length Bax. In addition, the α helicity of the Bax BH3 peptide may also contribute, in part, to the cytochrome *c*-releasing activity. The ability of a 21-amino-acid Bad BH3 peptide to induce cytochrome *c* release has been attributed to its alpha helical structure (*53*). However, we failed to observe any cytochrome *c*-releasing activity with Bcl-2 BH3 peptide, which contains the α2 helix, suggesting that peptide helicity alone is not sufficient to cause cytochrome *c* release.

Similar to the Bax BH3 peptide, our Bad BH3 peptide was able to induce cytochrome *c* release from wild-type Jurkat cells. However, in contrast to Bax BH3, Bad BH3 failed to fully overcome the protective effect of Bcl-2. This was not unexpected, given the preference Bad BH3 exhibited for disrupting Bax/Bcl-x_L interactions over Bax/Bcl-2 interactions. To verify the different results obtained with the Bax BH3 and Bad BH3 peptides in protein−protein interaction and cytochrome *c* release experiments, we examined the abilities of the peptides to induce cell death in Jurkat cells overexpressing either Bcl-2 or Bcl-x_L. Following delivery into cells, BH3 peptides from Bax and Bad were equally toxic toward vector-transfected Jurkat cells. In cells overexpressing Bcl-2, Bax BH3 retained strong killing activity, while the activity of Bad BH3 was attenuated. This was consistent with the ability of Bax BH3, but not Bad BH3, to potently overcome the effects of Bcl-2 overexpression in cytochrome *c* release assays. In cells overexpressing Bcl-x_L, the potencies of Bax BH3 and Bad BH3 were reversed, again consistent with the preference of Bad BH3 for disrupting Bax/Bcl-x_L over Bax/Bcl-2. The abilities of the Bax BH3 and Bad BH3 peptides to preferentially kill either Bcl-2/Jurkat or Bcl-x_L/Jurkat cells also argue that killing by these peptides is due to selective binding to either Bcl-2 or Bcl-x_L and not simply due to the alpha-helical content of the peptides. This argument is further supported by the fact that Bcl-2 BH3, although α helical, failed to induce cytochrome *c* release or cell killing in any of the cell types tested.

In summary, our findings indicate that the clinical utility of agents based on Bax or Bad BH3 domains will depend, in part, on the expression pattern of the target tumor cells. In cells overexpressing Bcl-x_L, agents based on Bad BH3 may prove more useful, while in Bcl-2-overexpressing cells, agents based on Bax BH3 may be more efficacious. In view of the frequency of Bcl-2 and Bcl-x_L overexpression in human malignancies, further refinement and evaluation of molecules that mimic BH3 domains appears warranted.

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REFERENCES

1. Jacobson, M. D., Weil, M., and Raff, M. C. (1997) *Science 88*, 347−354.
2. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. (1990) *Nature 348*, 334−336.
3. Vaux, D. L., Cory, S., and Adams, J. M. (1988) *Nature 335*, 440−442.
4. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. (1993) *Cell 74*, 597−608.
5. Gibson, L., Holmgreen, S. P., Huang, D. C., Bernard, O., Copeland, N. G., Jenkins, N. A., Sutherland, G. R., Baker, E., Adams, J. M., and Cory, S. (1996) *Oncogene 96*, 665−675.
6. Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., and Craig, R. W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3516−3520.
7. Adams, J. M., and Cory, S. (1998) *Science 281*, 1322−1326.
8. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993) *Cell 74*, 609−619.
9. Chittenden, T., Flemington, C., Houghton, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G., and Lutz, R. J. (1995) *Nature 374*, 733−736.
10. Kiefer, M. C., Brauer, M. J., Powers, V. C., Wu, J. J., Umansky, S. R., Tomei, L. D., and Barr, P. J. (1995) *Nature 374*, 736−739.
11. Hsu, S. Y., Kaipia, A., McGee, E., Lomeli, M., and Hsueh, A. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12401−12406.
12. Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) *Cell 80*, 285−91.
13. Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L., and Korsmeyer, S. J. (1996) *Genes Dev. 10*, 2859.
14. Boyd, J. M., Gallo, G. J., Elangovan, B., Houghton, A. B., Malstrom, S., Avery, B. J., Ebb, R. G., Subramanian, T., Chittenden, T., Lutz, R. J., and Chinnadurai, G. (1995) *Oncogene 11*, 1921−28.
15. Antonsson, B. (2001) *Cell Tissue Res. 306*, 347−361.
16. Mullauer, L, Gruber, P., Sebinger, D., Buch, J., Wohlfart, S., and Chott, A. (2001) *Mutat. Res. 488*, 211−231.
17. DeVries, A. C., Joh, H. D., Bernard, O., Hattori, K., Hurn, P. D., Traystman, R. J., and Alkayed, N. J. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 11824−11828.
18. Ferri, K. F., Jacotot, E., Blanco, J., Este, J. A., Zamzami, N., Susin, S. A., Xie, Z., Brothers, G., Reed, J. C., Penninger, J. M., and Kroemer. G. (2000) *J. Exp. Med. 192*, 1081−1092.
19. MacGibbon, G. A., Lawlor, P. A., Sirimanne, E. S., Walton, M. R., Connor, B., Young, D., Williams, C., Gluckman, P., Faull, R. L., Hughes, P., and Dragunow, M. (1997) *Brain Res. 750*, 223−34.
20. Campos, L., Sabido, O., Rouault, J. P., and Guyotat, D. (1994) *Blood 84*, 595−600.
21. Eguchi, K. (2001) *Intern. Med. 40*, 275−284.

22. Lebedeva, I., Raffo, A., Rando, R., Ojwang, J., Cossum, P., and Stein, C. A. (2001) *J. Urol. 166*, 461−469.
23. Scherr, D. S., Vaughan, E. D., Jr., Wei, J., Chung, M., Felsen, D., Allbright, R., and Knudsen, B. S. (1999) *J. Urol. 162*, 12−16.
24. Simonian, P. L., Grillot, D. A., and Nunez, G. (1997) *Blood 90*, 1208−1216.
25. Miyashita, T., and Reed, J. C. (1993) *Blood 81*, 151−157.
26. Cotter, F. E., Waters, J., and Cunningham, D. (1999) *Biochim. Biophys. Acta 489*, 97−106.
27. Gautschi, O., Tschopp, S., Olie, R. A., Leech, S. H., Simoes-Wust, A. P., Ziegler, A., Baumann, B., Odermatt, B., Hall, J., Stahel, R. A., and Zangemeister-Wittke, U. (2001) *J. Natl. Cancer Inst. 93*, 463−471.
28. Huang, Z. (2000) *Oncogene 19*, 6627−6631.
29. Tzung, S. P., Kim, K. M., Basanez, G., Giedt, C. D., Simon, J., Zimmerberg, J., Zhang, K. Y., and Hockenbery, D. M. (2001) *Nature Cell Biol. 3*, 183−191.
30. Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001) *Mol. Cell 8*, 705−711.
31. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) *Genes Dev. 13*, 1899−1911.
32. Shi, Y. (2001) *Nat. Struct. Biol. 8*, 394−401.
33. Conus, S., Kaufmann, T., Fellay, I., Otter, I., Rosse, T., and Borner, C. (2000) *EMBO J. 19*, 1534−1544.
34. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997) *Science 275*, 1129−1132.
35. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999) *J. Cell Biol. 144*, 891−901.
36. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. (1998) *EMBO J. 17*, 3878−3885.
37. Hsu, Y. T., Wolter, K. G., and Youle, R. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3668−3672.
38. Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J. C. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 4997−5002.
39. Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H., and Tsujimoto, Y. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 14681−14686.
40. Hanada, M., Aime-Sempe, C., Sato, T., and Reed, J. C. (1995) *J. Biol. Chem. 270*, 11962−11969.
41. Kelekar, A., and Thompson, C. B. (1998) *Trends Cell Biol. 8*, 324−330.
42. Simonen, M., Keller, H., and Heim, J. (1997) *Eur. J. Biochem. 249*, 85−91.
43. Yin, X.-M., Oltvai, Z. N., and Korsmeyer, S. J. (1994) *Nature 369*, 321−323.
44. Zha, H., Aime-Sempe, C., Sato, T., and Reed, J. C. (1996) *J. Biol. Chem. 271*, 7440−7444.
45. Sedlak, T. W., Oltvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7834−7838.
46. Hunter, J. J., and Parslow, T. G. (1996) *J. Biol. Chem. 271*, 8521−8524.
47. Suzuki, M., Youle, R. J., and Tjandra, N. (2000) *Cell 103*, 645−654.
48. Wang, K., Gross, A., Waksman, G., and Korsmeyer, S. J. (1998) *Mol. Cell. Biol. 18*, 6083−6089.
49. Diaz, J. L., Oltersdorf, T., Horne, W., McConnell, M., Wilson, G., Weeks, S., Garcia, T., and Fritz, L. C. (1997) *J. Biol. Chem. 272*, 11350−11355.
50. Ottilie, S., Diaz, J. L., Horne, W., Chang, J., Wang, Y., Wilson, G., Chang, S., Weeks, S., Fritz, L. C., and Oltersdorf, T. (1997) *J. Biol. Chem. 272*, 30866−30872.
51. Finnegan, N. M., Curtin, J. F., Prevost, G., Morgan, B., and Cotter, T. G. (2001) *Br. J. Cancer 85*, 115−121.
52. Holinger, E. P., Chittenden, T., and Lutz, R. J. (1999) *J. Biol. Chem. 274*, 13298−13304.
53. Schimmer, A. D., Hedley, D. W., Chow, S., Pham, N. A., Chakrabartty, A., Bouchard, D., Mak, T. W., Trus, M. R., and Minden, M. D. (2001) *Cell Death Differ. 8*, 725−733.
54. Wang, J. L., Zhang, Z. J., Choksi, S., Shan, S., Lu, Z., Croce, C. M., Alnemri, E. S., Korngold, R., and Huang, Z. (2000) *Cancer Res. 60*, 1498−1502.
55. Polster, B. M., Kinnally, K. W., and Fiskum, G. (2001) *J. Biol. Chem. 276*, 37887−37894.
56. Dou, Q. P., An, B., Antoku, K., and Johnson D. E. (1997) *J. Cell. Biochem. 64*, 586−594.
57. Maser, R. S., Antoku, K., Scully, W. J., Jr., Cho, R. L., and Johnson, D. E. (2000) *Biochem. Biophys. Res. Commun. 277*, 171−178.
58. Inohara, N., Gourley, T. S., Carrio, R., Munizm M., Merinom J., Garcia, I., Koseki, T., Hu, Y., Chen, S., and Nunez, G. (1998) *J. Biol. Chem. 273*, 32479−32286.
59. Eguchi, Y., Ewert, D. L., and Tsujimoto, Y. (1992) *Nucleic Acids Res. 20*, 4187−4192.
60. Tsujimoto, Y., and Croce, C. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5214−5218.
61. Petit, P. X., O'Connor, J. E., Grunwald, D., and Brown, S. C. (1990) *Eur. J. Biochem. 194*, 389−397.
62. Sato, T., Hanada, M., Bodrug, S., Irie, S., Iwama, N., Boise, L. H., Thompson, C. B., Golemis, E., Fong, L., Wang, H. G., and Reed, J. C. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9238−9248.
63. Kelekar, A., Chang, B. S., Harlan, J. E., Fesik, S. W., and Thompson, C. B. (1997) *Mol. Cell. Biol. 17*, 7040−7046.
64. Huang, D. C., and Strasser, A. (2000) *Cell 103*, 839−842.
65. Cosulich, S. C., Worrall, V., Hedge, P. J., Green, S., and Clarke, P. R. (1997) *Current Biol. 7*, 913−920.
66. Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L., Ng, S. L., and Fesik, S. W. (1996) *Nature 381*, 335−341.
67. Petros, A. M., Medek, A., Nettesheim, D. G., Kim, D. H., Yoon, H. S., Swift, K., Matayoshi, E. D., Oltersdorf, T., and Fesik, S. W. (2001) *Proc. Natl. Acad. Sci U.S.A. 98*, 3012−3017.
68. Kelekar, A., and Thompson, C. B. (1998) *Trends Cell Biol. 8*, 324−330.
69. Schendel, S. L., Xie, Z., Montal, M. O., Matsuyama, S., Montal, M., and Reed, J. C. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 5113−5118.
70. Minn, A. J., Velez, P., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M., and Thompson, C. B. (1997) *Nature 385*, 353−357.
71. Antonsson, B., Montessuit, S., Lauper, S., Eskes, R., and Martinou, J. C. (2000) *Biochem. J. 345*, 271−278.
72. Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S. W. (1997) *Science 275*, 983−986.
73. Meijerink, J. P., Mensink, E. J., Wang, K., Sedlak, T. W., Sloetjes, A. W., de Witte, T., Waksman, G., and Korsmeyer, S. J. (1998) *Blood 91*, 2991−2997.

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