The BH3 domain of BAD fused to the Antennapedia peptide induces apoptosis via its alpha helical structure and independent of Bcl-2

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Abstract

Since the over-expression of Bcl-2 is a common cause of multi-drug resistance, cytotoxic peptides that overcome the effects of Bcl-2 may be clinically useful. We harnessed the death-promoting alpha helical properties of the BH3 domain of BAD by fusing it to the Antennapedia (ANT) domain, which allows for cell entry (ANTBH3BAD). Treatment of 32D cells with the ANTBH3BAD peptide results in a 99% inhibition of colony formation. No significant toxicity is observed after treatment with ANT or BH3BAD alone. A mutant fusion peptide unable to bind Bcl-2 induces cell death as effectively as the wild-type ANTBH3BAD. Furthermore, 32D cells over-expressing Bcl-2 show no resistance to the ANTBH3BAD peptide. Therefore, the toxicity of the peptide was independent of the Bcl-2 pathway. We demonstrate that the toxicity of the peptide is due to its alpha helicity that disrupts mitochondrial function. Since this peptide overcomes major forms of drug resistance, it may be therapeutically useful if appropriately targeted to malignant cells. *Cell Death and Differentiation* (2001) **8**, 725 – 733.

Introduction

Since the over-expression of Bcl-2 is a common mechanism by which malignant cells become resistant to multiple chemotherapeutic agents,[1–5] cytotoxic peptides that over-come Bcl-2-mediated drug resistance may be useful in the treatment of malignant disease. One example is the alpha helical BH3 domain of BAD that binds and inhibits Bcl-2 and Bcl-xL.[6,7] Wang *et al.*[8] created a cell-permeable version of the peptide by fusing it to cpm, a decanoic fatty acid internalization sequence. This fusion peptide induced rapid cell death and apoptosis. However, its cytotoxicity was significantly impaired by the over-expression of Bcl-xL, which could limit the usefulness of this compound in treating malignancies with increased levels of Bcl-2 and Bcl-xL. Therefore, enhancing the ability of the BH3 domain of BAD to kill cells over-expressing these survival factors might be therapeutically useful. One strategy to enhance its toxicity is to capitalize on the inherent alpha helical shape of the peptide, since some amphipathic alpha helices are potent naturally occurring antibiotics. These compounds disrupt negatively charged bacterial membranes, but are not toxic to the mammalian cells, because their membranes are neutrally charged and stabilized by cholesterol.[9–11] When internalized into mammalian cells, however, the alpha helical peptide (KLALAK)$_2$ induced cell death and apoptosis by disrupting the negatively charged mitochondria.[12]

Here, we have harnessed the alpha helical properties of the BH3 domain of BAD by fusing it to the Antennapedia internalization sequence. We have created a 37 amino acid fusion peptide corresponding to the 21 amino acids of the human BH3 domain of BAD fused to the C-terminus of the 16 amino acids of the ANT peptide (ANTBH3BAD). ANT is an internalization sequence that successfully translocates across a variety of cell membranes with high efficiency and low toxicity.[13–15] We report that the ANTBH3BAD fusion peptide induces rapid cell death and apoptosis. Its alpha helical secondary structure disrupts mitochondrial function and allows the peptide to overcome caspase inhibitors and Bcl-2 over-expression.

Results

Bcl-2 binds ANTBH3BAD and BH3BAD, but not ANT

To demonstrate that ANTBH3BAD binds Bcl-2, the interaction between Bcl-2 and the peptides was analyzed with real time surface plasmon resonance detection. ANTBH3BAD, BH3BAD and ANT peptides and BSA were coupled to a biosensor chip through their amine groups. GST-Bcl-2 was injected at increasing concentrations onto the sensor chip and binding to the peptides above the BSA control was measured. GST-Bcl-2 bound avidly to ANTBH3BAD and BH3BAD in a dose-dependent manner and did not bind significantly to ANT (Figure 1). Bcl-2 remained tightly bound to the peptides with minimal dissociation. GST alone did not bind to the peptides.

Internalization of the peptides

ANT is an internalization sequence that facilitates the transport of peptides across cell membranes with high efficiency. To study the internalization and localization of our peptides, 32D cells were treated with biotinylated versions of ANT and ANTBH3BAD. In a representative experiment, 89 and 100% of 32D cells treated with biotinyated ANT or ANTBH3BAD, respectively, internalized the peptides as measured by flow cytometry. The peptides were distributed diffusely throughout the nucleus and cytoplasm as determined by confocal fluorescent microscopy (Figure 2).

ANTBH3BAD induces a rapid cell death

To assess the impact of the peptides on cell viability, HeLa cells were treated with 50 μM of ANT, BH3BAD or ANTBH3BAD for 3 h. Cells treated with ANTBH3BAD displayed morphologic evidence of cell death characterized by cell shrinkage, membrane blebbing, and detachment from the tissue culture plate. In contrast, no significant morpholo-

gical changes were seen after treatment with 50 μM ANT or BH3BAD (Figure 3).

To quantitate the effects of the peptides on cell survival, HeLa, 32D and yeast cells were treated with the peptides at a final concentration of 50 μM and cell survival was measured by a colony formation assay. Dramatic decreases in cell survival were seen in all three cell types after treatment with ANTBH3BAD. No significant toxicity was observed in cells treated with ANT or BH3BAD alone (Figure 4). Therefore, ANTBH3BAD induces cell death, but the toxicity is not related to the ANT domain. The BH3BAD peptide alone is not toxic because it is not internalized.

Dose response curves for ANTBH3BAD-induced cell death were constructed. 32D cells were treated with increasing concentrations of ANTBH3BAD peptide for 3 h and cell survival was measured in a colony formation
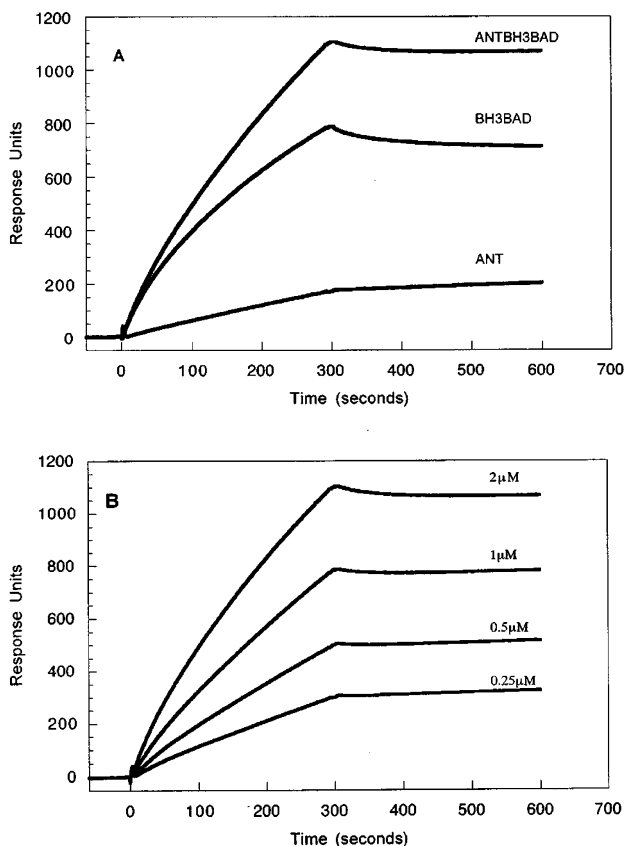

Figure 1 Real time surface plasmon resonance. BH3BAD, ANT, ANTBH3BAD and BSA were coupled to a CM5 BIAcore sensor chip. GST-Bcl-2 was injected onto the chip at increasing concentrations. The sensor surface was regenerated with 1 M NaCl and 50 mM NaOH between each injection. Binding to the peptides above the BSA control was measured as an increase in Response Units above the BSA control. A representative sensogram after the injection of GST-Bcl-2 (2 μM) is shown (**A**). A dose response curve for the binding of GST-Bcl-2 to ANTBH3BAD is presented (**B**)
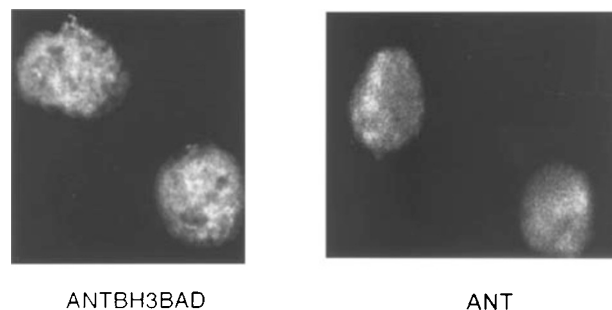
ANTBH3BAD   ANT

Figure 2 Internalization of the peptides. Biotinylated versions of ANT and ANTBH3BAD were obtained. 32D cells (1 × 10⁶ cells) were treated with the peptides at a final concentration of 50 μM for 20 min. The cells were washed, fixed in 4% paraformaldehyde, permeabilized with 1% Triton X-100 and probed with Streptavidin-FITC to detect the biotinylated peptides. Cells were viewed under a fluorescent confocal microscope to determine the distribution of the peptide

ANT   BH3BAD
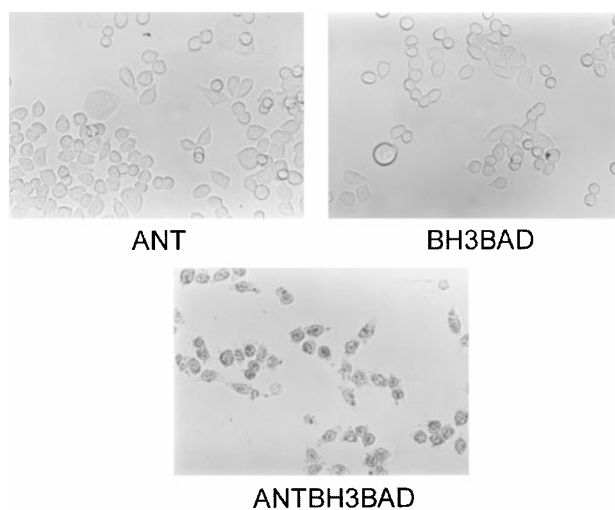
ANTBH3BAD

Figure 3 Morphologic evidence of cell death. HeLa cells (1 × 10⁵ cells) were seeded into 12-well plates in DMEM H21+10% fetal calf serum. Twenty-four hours later, BH3BAD, ANT and ANTBH3BAD were added to the cells in SF-DMEM H21 at a final concentration of 50 μM. Three hours after incubation, morphologic evidence of cell death was observed among the cells treated with ANTBH3BAD. Cells treated with ANT or BH3BAD showed no evidence of toxicity
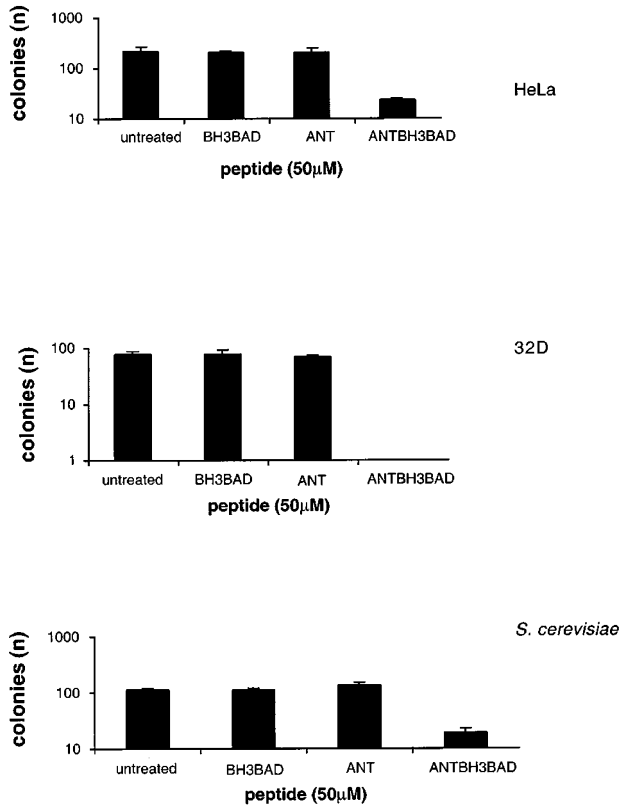
Figure 4 ANTBH3BAD induces cell death. HeLa cells, 32D cells and *S. cerevisiae* were treated with BH3BAD, ANT, or ANTBH3BAD at a final concentration of 50 μM for 3h. After the incubation, equal volumes of cells were plated in a colony formation assay as described in Materials and Methods. The number of colonies formed was counted

and cell viability was measured by trypan blue extrusion. Over-expression of Bcl-2 did not abrogate the toxicity of our peptide (Figure 6). In contrast, 32D cells over-expressing Bcl-2 were protected from IL-3 withdrawal, indicating that Bcl-2 can protect this cell line from other apoptotic stimuli.

To confirm the lack of impact of Bcl-2 on the toxicity of ANTBH3BAD, a mutant fusion peptide was obtained in which residues L114 and D119 in the BH3 domain of BAD were substituted with A114 and R119. In a BIAcore binding assay, GST-Bcl-2 did not bind to this mutant peptide. Although incapable of binding Bcl-2, the mutant peptide was as toxic as the wild type indicating that the toxicity of ANTBH3BAD is independent of the Bcl-2 pathway.

Toxicity of the fusion peptide is due to its alpha helical shape

Certain alpha helical peptides are potent antimicrobial agents that induce cell death by disrupting the negatively charged bacterial cell membrane.[9,10] When internalized into eukaryotic
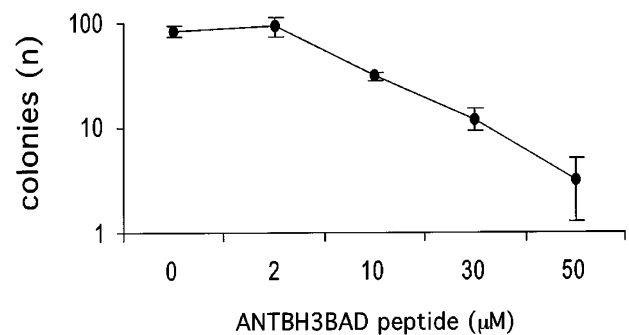
Figure 5 Dose response curve. A dose response curve for ANTBH3BAD-induced cell death was constructed. 32D cells were incubated with increasing concentrations of ANTBH3BAD for 3h. After incubation, equal volumes of cells were plated in a colony formation assay as described in Materials and Methods. One week later, the number of colonies formed was counted

assay. As the peptide concentration increased, there was a corresponding decrease in cell survival observed (Figure 5). The dose response curve was steep, as minimal toxicity was observed below a peptide concentration of 10 μM and nearly complete growth inhibition was seen at a concentration of 50 μM.

ANTBH3BAD induces apoptosis

Next, we determined whether the cell death induced by ANTBH3BAD occurred via apoptosis. 32D cells were treated with the peptides for 30 min and stained with FITC labeled anti-Annexin V and PI. The percentage of apoptotic cells was determined by flow cytometric analysis. After treatment with ANTBH3BAD 97±1%, of cells were apoptotic based on Annexin V staining. In contrast, only 15±2% of cells treated with ANT and 13±2% of cells treated with BH3BAD were apoptotic by Annexin V staining.
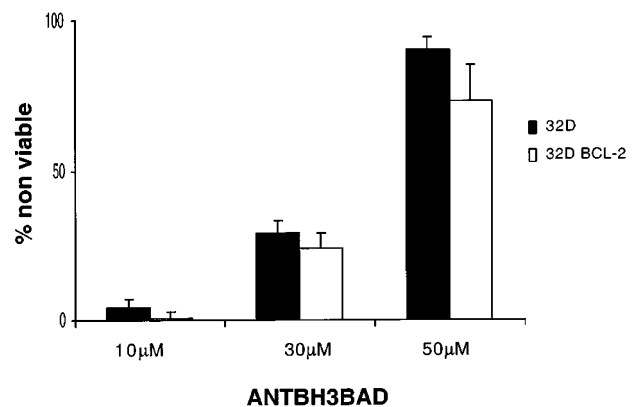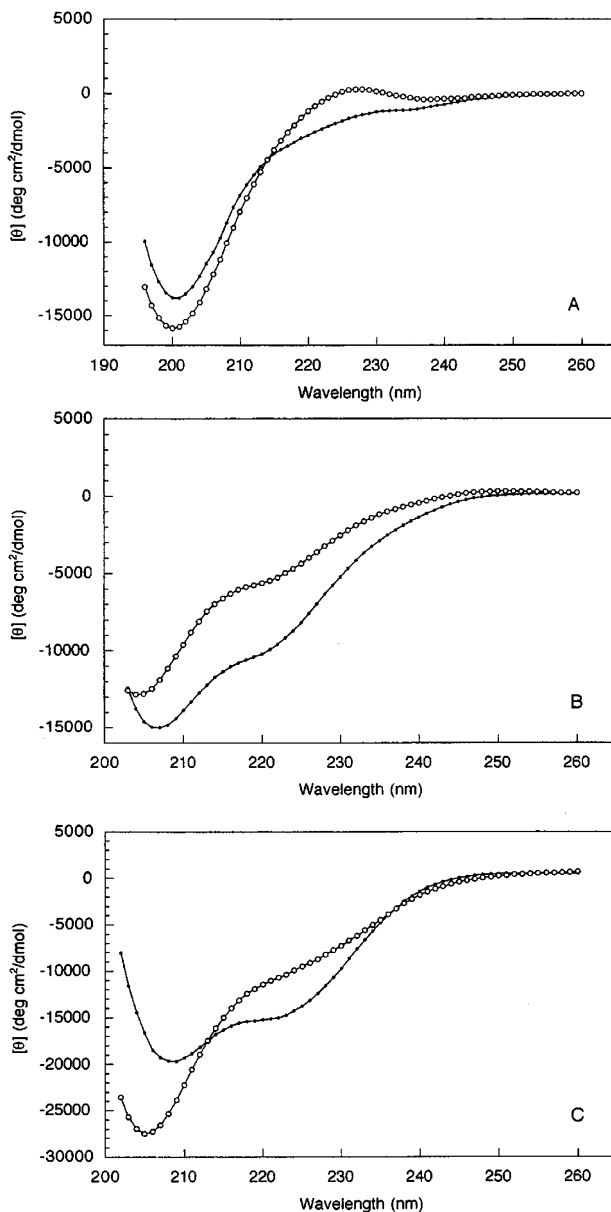
ANTBH3BAD induces cell death independent of Bcl-2

We studied the effects of Bcl-2 over-expression on the toxicity of ANTBH3BAD. 32D cells with and without over-expression of murine Bcl-2 (5×10^5 cells) were treated with ANTBH3BAD

Figure 6 Bcl-2 does not abrogate the toxicity of the fusion peptide. 32D cells and 32D cells over-expressing Bcl-2 were treated with ANTBH3BAD at finalconcentrations of 10, 30 and 50 μM. Six hours after treatment, an equal volume of trypan blue was added. The percentage of cells staining positive for trypan blue was determined. The results represent the mean of three experiments

cells, alpha helical peptides induce apoptosis by disrupting the negatively charged mitochondria.[12] ANTBH3BAD is predicted to have an alpha helical conformation. To evaluate the contribution of the helical shape to its toxicity, we created, a non-helical mutant of ANTBH3BAD by substituting A106, A107, and L114 in BH3BAD with prolines. We tested the impact of this mutation on helical structure by circular dichroism (CD) (Figure 7). In 100 mM phosphate buffer (pH 7), both the wild type and non-helical mutant ANTBH3BAD peptides displayed a CD spectrum consistent with a random coil. However, the wild-type peptide adopted an alpha helical
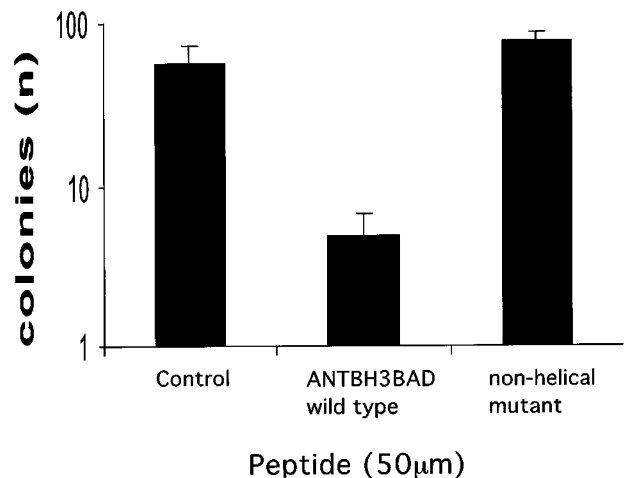
conformation in 1% SDS and 40% (v/v) TFE, but the non-helical mutant mainly remained as a random coil in these solutions. In addition, the ANT and BH3BAD peptides alone adopted alpha helical secondary structure in these solutions.

A colony formation assay was performed with the non-helical mutant to determine the contribution of the alpha helicity to the toxicity of ANTBH3BAD (Figure 8). The non-helical mutant was not toxic to 32D cells demonstrating that the toxicity of ANTBH3BAD depends on its helical configuration.

ANTBH3BAD disrupts mitochondrial function

Alpha helical peptides induce swelling of isolated mitochondria. Therefore, to confirm that the cytotoxcity of ANTBH3BAD was related to its helical configuration, we investigated the effects of the peptides on intracellular mitochondrial function. Hallmarks of mitochondrial dysfunction include loss of mitochondrial membrane potential ($\Delta\Psi_M$) and increased production of reactive oxygen intermediates (ROI). Changes in $\Delta\Psi_M$ and ROI production were measured over time by flow cytometry after treating 32D cells with the peptides. ANTBH3BAD rapidly decreased $\Delta\Psi_M$, whereas no significant change was observed after treatment with ANT or BH3BAD (Figure 9). The non-helical mutant also did not induce loss of $\Delta\Psi_M$. The population with decreased $\Delta\Psi_M$ also showed increased ROI production. The increased ROI and decreased $\Delta\Psi_M$ were not accompanied by significant increases in cytosolic calcium. Therefore, the loss of $\Delta\Psi_M$ is not secondary to large increases in cytosolic calcium from disruption of the endoplasmic reticulum.

To ensure that the toxicity of ANTBH3BAD is due to the alpha helical properties of BH3BAD and not an artifact of the fusion peptide, we investigated the effects of BH3BAD on $\Delta\Psi_M$ in permeablized cells. 32D cells were suspended in respiratory buffer to mimic the environment of the

Figure 7 Circular dichoism. The secondary structure of ANTBH3BAD and the non-helical mutant were determined by circular dichroism. The peptides were dissolved in 0.1 M NaH$_2$PO$_4$ buffer (A), 1% SDS (B), or 40% (v/v) TFE (C). CD spectra were recorded at room temperature. Each point represents the averaged ellipticity value recorded over 1 nm spectral intervals. ●●●●=ANTBH3BAD, ○○○○=non-helical mutant

Figure 8 The non-helical mutant is non toxic. 32D cells (5×10^4 cells) were treated with ANTBH3BAD or the non-helical mutant at a final concentration of 50 μM. After a 3-h incubation, equal volumes of cells were plated into complete medium with 0.85% methyl cellulose. One week after seeding, the number of colonies formed was counted
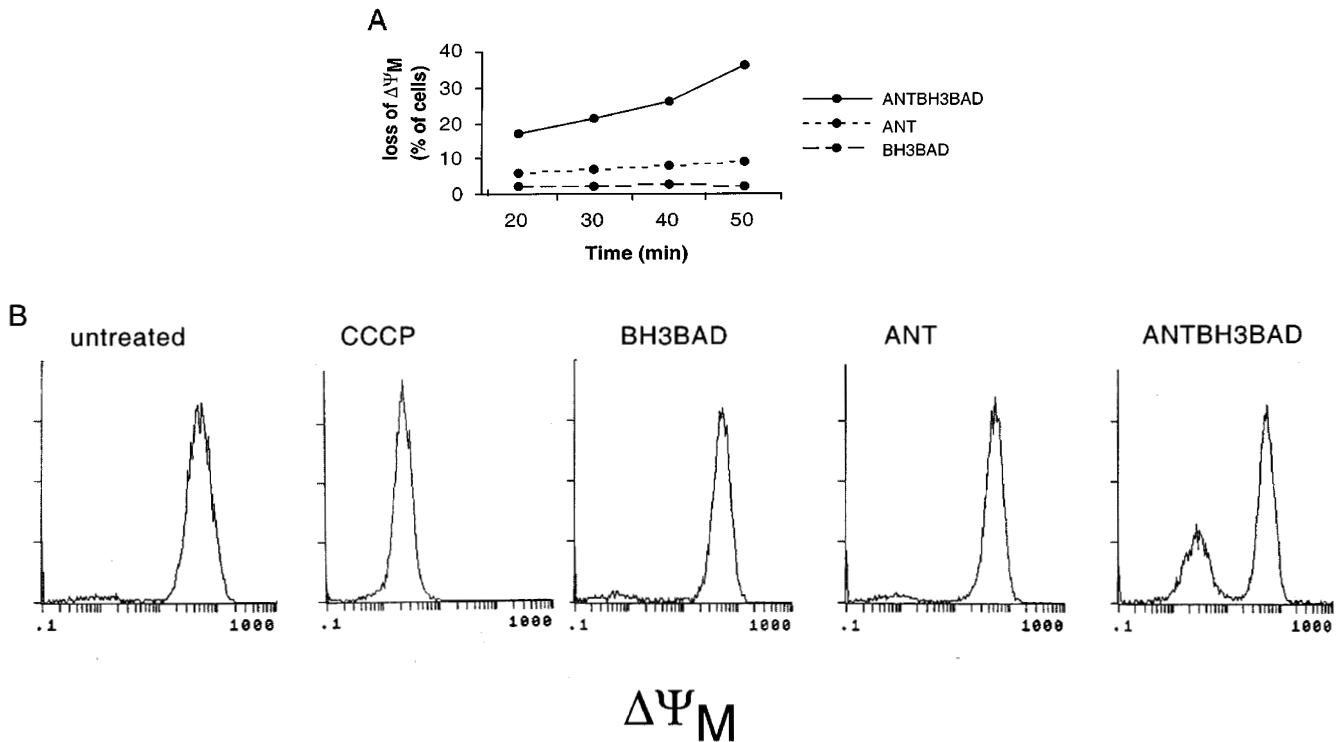
Figure 9 ANTBH3BAD induces loss of $\Delta\Psi c_M$. 32D cells (5×10^5 cells) were treated with BH3BAD, ANT, ANTBH3BAD, at a final concentration of 50 μM. Cells were also treated with the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) (final concentration 100 μM) to induce complete loss of $\Delta\Psi c_M$ and thereby serve as a positive control. $\Delta\Psi c_M$ was detected by staining with DiIC1 (40 nm). Changes in $\Delta\Psi c_M$ were measured at various times after theaddition of the peptides by flow cytometry. A representative time course for the change in $\Delta\Psi c_M$ is shown (**A**). A representative flow cytometric experiment after the addition of the peptides is presented (**B**)

cytoplasm. The plasma membrane was permeablized with digitonin and mitochondrial activity was driven with succinate. BH3BAD (50 μM) was added to the permeablized cells for 15 min and changes in $\Delta\Psi_M$ were measured by flow cytometry. BH3BAD induced a 4.6 ± 0.3-fold loss of $\Delta\Psi_M$ compared to treatment with digitonin and succinate alone. Therefore, when internalized into cells and exposed to the mitochondria, BH3BAD displays an ability to induce loss of $\Delta\Psi_M$ like the fusion peptide.

ANTBH3BAD induces caspase-3 activation, but caspase inhibitors do not protect against cell death

Mitochondrial damage can activate caspases. To determine if the cytotoxicity of ANTBH3BAD is caspase-dependent, we studied the effect of ANTBH3BAD on caspase activation. Treatment with ANTBH3BAD induced a 5 ± 0.9-fold increase in caspase-3 activity compared to untreated cells. In contrast, no increase in caspase-3 activity was seen after treatment with ANT or BH3BAD. Next, we investigated the effects of the general caspase inhibitor zVAD on ANTBH3BAD-induced caspase activation and cytotoxicity. Pretreatment with 100 μM zVAD for 1 h prevented ANTBH3BAD-induced caspase-3 activation. Pretreatment with zVAD also prevented the death of 32D cells after withdrawal of IL-3 indicating that zVAD is

capable of inhibiting apoptotic stimuli in this cell line. Interestingly, pretreatment with zVAD did not inhibit ANTBH3BAD-induced cell death (Figure 10). Therefore, ANTBH3BAD induces caspase-dependent cell death, but in the presence of caspase inhibitors, it remains cytotoxic due to caspase-independent mechanisms.

P-glycoprotein protects against ANTBH3BAD toxicity

Over-expression of p-glycoprotein is another important mechanism of multidrug resistance and is a poor prognostic marker in patients with leukemia.[20,21] Peptides and proteins can act as substrates of p-glycoprotein.[22–24] Therefore, we studied the effects of p-glycoprotein over-expression on the toxicity of our peptides. CEM cells and CEM VBL cells over-expressing p-glycoprotein were treated with varying concentrations of ANTBH3BAD. Compared to the CEM parent cell line, CEM VBL cells displayed resistance to ANTBH3BAD that was most apparent at a final concentration of 10 μM ANTBH3BAD. Pretreatment of CEM VBL with the p-glycoprotein inhibitor cyclosporine (final concentration 10 μM) for one hour restored the toxicity of ANTBH3BAD to levels seen in the wild type CEM line (Figure 11). Therefore, p-glycoprotein limits the toxicity of this peptide, but the effect can be reversed with p-glycoprotein inhibitors.
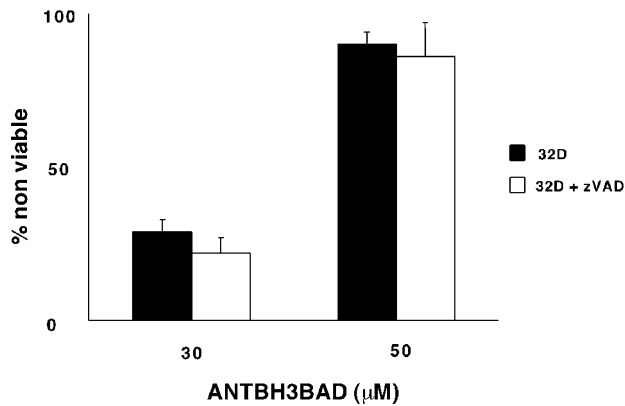
Figure 10 zVAD fails to prevent cell death. 32D cells (5×10^5 cells) were pretreated with 100 μM zVAD for 1 h followed by the addition of ANTBH3BAD (30 and 50 μM). After 3 h, cell viability was determined by trypan blue extrusion. The results represent the mean of three experiments
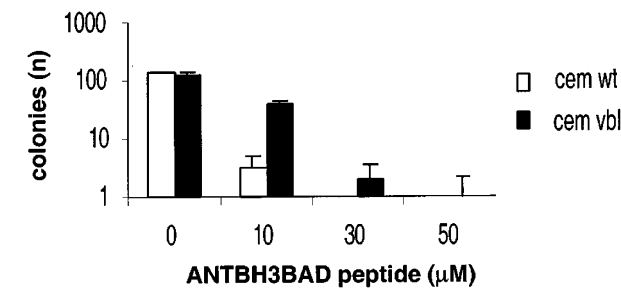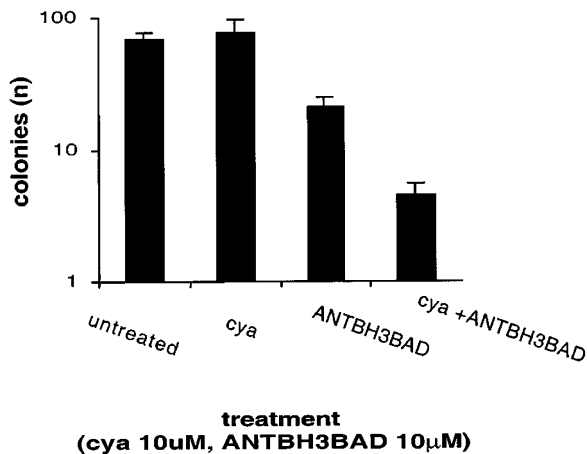
Figure 11 p-glyocprotein protects against ANTBH3BAD toxicity. (**A**) CEM and the p-glycoprotein over-expressing cell line CEM VBL were treated with ANT, BH3BAD, or ANTBH3BAD for 3 h at various concentrations. Cells were then seeded in complete medium with 0.85% methylcellulose. One week after seeding the number of colonies formed was counted. (**B**) CEM VBL cells were pretreated with cyclosporine (cya) at a final concentration of 10 μM for 1 h. ANTBH3BAD was added at a final concentration of 10 μM. Three hours after incubation, the cells were seeded in complete medium with 0.85% methylcellulose. One week after seeding the number of colonies formed was counted

Discussion

As a prototype for a novel therapeutic agent, we studied the effects of the BH3 domain of human BAD fused to the ANT internalization sequence. This fusion peptide induced a rapid cell death in all cells tested including yeast. We demonstrated that this peptide was toxic because of its alpha helical shape and that it acted by disrupting mitochondrial function.

In a cell free system, the ANTBH3BAD peptide bound Bcl-2 with higher affinity than the BH3BAD peptide alone. A similar finding was reported with BH3BAD fused to the fatty acid moiety cpm.[8] The increased binding likely reflects the additional interaction of the positively charged ANT sequence with Bcl-2.

Despite being capable of binding Bcl-2, the cytotoxicity of ANTBH3BAD was independent of the Bcl-2 pathway. Cells over-expressing Bcl-2 showed no resistance to the peptide and a mutant fusion peptide unable to bind Bcl-2 was as toxic as the wild-type. We could not demonstrate Bcl-2-dependence because the alpha helical properties of the peptide overcame the protective role of Bcl-2.

The cytotoxicity of ANTBH3BAD was not an artifact of the fusion peptide, but rather related to the helical properties of BH3BAD. As evidence to this point, BH3BAD was capable of inducing loss of $\Delta\Psi_M$ in permeabilized cells.

Previous studies of full length BAD[25,26] or the BH3 domain of BAD[6-8] have not reported toxicity attributable to the alpha helical configuration. These studies demonstrated that over-expression of Bcl-2 and Bcl-xL inhibits apoptosis induced by the full-length protein and peptide. Furthermore, mutants that do not bind Bcl-2 are not toxic.[6-8,25,26] A number of explanations may account for our ability to detect toxicity due to the alpha helicity of BH3BAD. First, fusing the BH3 domain of BAD to the ANT internalization sequence may have targeted the peptide more effectively to the mitochondria where its alpha helical shape could disrupt the inner membrane. BH3BAD peptides coupled to the cpm fatty acid moiety were confined to the cytoplasm. In contrast, our peptide was distributed diffusely throughout the cell. Second, the concentration of BAD used in the previous studies may have been too low to detect the effects of the alpha helix. If BH3 BAD-mutants unable to bind Bcl-2 or Bcl-xL were used at higher concentrations, the cytotoxicity from the alpha helical shape might have become apparent. Finally, the full-length protein BAD may constrain the death promoting attributes of the alpha helical BH3 domain.

In a previous report by Ellerby *et al.*,[12] the 14 amino acid alpha helix (KLALAK)$_2$ induced cell death and apoptosis. The peptide induced mitochondrial swelling and caspase-3 activation in isolated mitochondria but the effect on $\Delta\Psi_M$ was not reported. In addition, the effects of Bcl-2 and p-glycoprotein on the toxicity of (KLALAK)$_2$ were not studied. Here, we have demonstrated that ANTBH3BAD disrupts mitochondria and induces loss of $\Delta\Psi_M$ in intact and permeablized cells. Unlike the (KLALAK)$_2$ peptide, the toxicity of ANTBH3BAD was not inhibited by the general caspase inhibitor zVAD. zVAD can inhibit mitochondrial induced caspase activation, but it does not prevent the

peptide from damaging the mitochondria. As mitochondrial damage continues, the ability of the mitochondria to synthesize ATP is lost, and, as a consequence, cells undergoes a metabolic and caspase independent death.[27–29]

The finding of toxicity not affected by Bcl-2 suggests that alpha helical molecules such as ANTBH3BAD can be potent anti-cancer agents capable of overcoming important mechanisms of multidrug resistance. To see if other mechanisms of multidrug resistance impact the toxicity of the peptide, we studied the effects of p-glycoprotein. Over-expression of p-glycoprotein protected against the toxicity of ANTBH3BAD and this protection was reversed by adding the p-glycoprotein inhibitor cyclosporine. P-glycoprotein is best known for its role in extruding chemotherapeutic agents, but it also exports peptides and large proteins such as IL-2 and gamma interferon.[22–24,30,31] Therefore, p-glycoprotein may protect the cells from the toxicity of ANTBH3BAD by exporting it out of the cells. Alternatively, it may be abrogating the peptide's toxicity through its role in inhibiting apoptosis. p-glycoprotein inhibits apoptosis in response to Fas ligand, ultraviolet irradiation, and serum starvation, and the addition of verapamil can restore the apoptotic signals of these stimuli.[32,33] Currently, it is unknown how it directly protects cells from these apoptotic stimuli.

In conclusion, we describe a cytotoxic effect of the BH3 domain of BAD due to its alpha helical properties and have harnessed this cytotoxic action by fusing BH3BAD to the ANT sequence. This fusion creates a cytotoxic peptide that overcomes two major forms of drug resistance – Bcl-2 and caspase inhibitors. As such, this compound may have clinical utility if it can be selectively targeted. This work also furthers the understanding of how alpha helical peptides induce apoptosis in mammalian cells and investigates factors that impact their toxicity.

Materials and Methods

Peptides

Peptides (Table 1) were synthesized commercially to our specifications (Amgen, Colorado) and were purified to greater than 95% purity by reverse phase HPLC. Peptides were resuspended in DMSO and stored at −20°C.

Real time surface plasmon resonance

Binding of Bcl-2 to the peptides was assessed using a BIAcore sensor system(BIAcore, Uppsala, Sweden). ANTBH3BAD, ANT, BH3BAD, the L114A D119R mutant peptide, and BSA were diluted in 10 mM Na acetate pH 5 and coupled to a CM5 sensor surface activated with

37.5 mg/ml N-Ethyl-N′-(3′dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 7.5 mg/ml N-hydroxysuccinimide (NHS). Peptides were added to saturate the chip. Remaining reactive groups on the chip were then inactivated with 1 M ethanolamine pH 8.5. GST-Bcl-2 (Santa Cruz) was diluted in running buffer (0.01 M HEPES pH 7.4, 0.15 NaCl, 3 mM EDTA, and 0.005% polysorbate 20 (v/v)) and injected onto the sensor chip at varying concentrations. All experiments were performed at 25°C. Between injections of GST-BCL-2, the chip was washed with running buffer and regenerated with 1 M NaCl and 50 mM NaOH. Data were analyzed with the BIAevaluation software.

Cell lines

HeLa cells were grown in Dulbeco's Modified Eagle Medium with high glucose (DMEM H21). CEM and the vinblastine resistant CEM cell line over-expressing p-glyocoprotein (CEM VBL) were grown in RPMI 1640. A 32D cell line over-expressing Bcl-2 (a gift from S Berger) was created by infecting 32D cells with a retrovirus containing murine Bcl-2. Over-expression of Bcl-2 was confirmed by Western blot. 32D cells were maintained in RPMI 1640 and 2% WeHi-3B conditioned medium as a source of IL-3. All cell lines were supplemented with 10% fetal calf serum, penicillin and streptomycin. Cells were grown at 37°C with 5% CO_2 in a humid atmosphere.

Internalization of the peptides

Biotinylated ANT or ANTBH3BAD peptides were added to 1×10^6 32D cells at a final concentration of 50 μM in serum free DMEM H21 (SF-DMEM). After 20 min, the cells were washed twice in PBS and fixed in 4% paraformaldehyde in PBS (pH 7.0) for 10 min at room temperature. After fixation, cells were washed in PBS and permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. Cells were again washed in PBS and incubated with 3% bovine serum albumin in PBS for 1 h. Streptavidin-FITC (5 μl) (Pierce) in 3% bovine serum albumin in PBS was added to the cells in a total volume of 500 μl and incubated at room temperature for 30 min. Cells were washed in PBS and analyzed by flow cytometry (FACScan; Becton Dickinson, Immunocytometry system, CA, USA) or mounted on glass slides and viewed with a fluorescent (Leica DMLB) or a confocal fluorescent (Zeiss LSM 510) microscope.

Clonogenic colony assay

Suspension cells (5×10^4) in SF-DMEM H21 were treated with the peptides for 3 h. Equal volumes of cells were plated in quadruplicate into 96-well plates in complete medium, with 0.85% methylcellulose. One week after seeding, the number of colonies formed were counted. HeLa cells (2×10^5) in DMEM H21 with 10% FCS were seeded into 6-well plates. Twenty-four hours later, they were treated with the peptides for 3 h in SF-DMEM H21. Following treatment with the peptides, equal volumes of cells were plated in triplicate into 100 mm^3 tissue culture plates. One week after seeding, the medium was removed, the plates stained with methylene blue, and the number of

Table 1 Peptides

Peptide	Internalization sequence	BH3 BAD sequence
ANT	RQIKIWFQNRRMKWKK	
BH3 BAD		NLWAAQRYGRELRRMSDEFVD
ANTBH3BAD	RQIKIWFQNRRMKWKK	NLWAAQRYGRELRRMSDEFVD
ANTBH3BAD-L114A D119R	RQIKIWFQNRRMKWKK	NLWAAQRYGRE**A**RRMS**R**EFVD
ANTBH3BAD Non-helix mutant	RQIKIWFQNRRMKWKK	NLW**PP**QRYGRE**P**RRMSDEFVD

colonies formed were counted. Yeast strain *S. cerevisiae* was grown overnight in YPD broth at 30°C. The next morning, 3 ml of culture was added to 7 ml YPD broth and grown at 30°C to an OD 595 of 0.3 – 0.35. One ml of culture was diluted in PBS. Peptides were added and incubated with the yeast for 2 h at room temperature. Equal volumes of cells were plated in triplicate onto YPD plates and incubated at 30°C. Four days later, the number of colonies formed were counted.

Annexin V assay

Peptides were added to 1×10^6 32D cells in SF-DMEM at a final concentration of 50 μM for 30 min. FITC conjugated Annexin V (5 μl) (Pharmingen) and propidium iodide (PI) (Sigma) (final concentration 5 μg/ml) were added. The cells were incubated for 20 min at room temperature in the dark and fluorescence was measured by flow cytometry (FACScan; Becton, Dickinson, Immunocytometry system, CA, USA). The Lysis II program was used to analyze these data. Cells staining positive for Annexin V and negative for PI were deemed apoptotic.

Circular dichroism

ANT and ANTBH3BAD peptides were dissolved in 0.1 M Na_2HPO_4 buffer, 0.1 M Na_2HPO_4 buffer and 1% SDS or 0.1 M Na_2HPO_4 buffer and 40% (v/v) TFE at approximately 1 mg/ml. Concentrations of the peptides were determined by tryptophan absorbance.[30] Peptides were aliquoted into a 1 cm path length cuvette. CD spectra were recorded at room temperature on an AVIV CD spectrometer model 62A DS. Spectra were collected with an averaging time of 10 s per point. Each point represents the averaged ellipticity value recorded over a 1 nm spectral interval.

Measurement of mitochondrial membrane potential, reactive oxygen intermediates and cytosolic calcium

Changes in mitochondrial membrane potential ($\Delta\Psi_M$) were detected by staining the cells with DiIC1(5) (1,1'3,3,3',3'-hexamethylindodicar-bocyanine) at a final concentration of 40 nM and propidium iodide (final concentration 5 μg/ml) as described previously.[31–33] Cells stained with DiIC1(5) were incubated at 37°C for 20 min and then analyzed by flow cytometry (Coulter Epics Elite, Coulter, FL, USA) by exciting at 633 nm and measuring through a 675 ± 20 nm bandpass filter. While measuring changes in $\Delta\Psi_M$ over time, stained cells were maintained at 37°C.

Changes reactive oxygen intermediate production, and cytosolic calcium were detected by staining the cells with 5 μM carboxy-DCFDA (carboxy-dichlorofluroescin diacetate), and 3 μM indo-1 AM, respectively. Carboxy-DCF excitation was at 488 nm and fluorescence was measured using a 525 ± 10 nm bandpass filter. Ionized calcium was obtained by electronic ratio measurement of indo-1 AM emission at 405 and 525 nm at an excitation of 360 nm.

To assess changes in $\Delta\Psi_M$ in the presence of a permeabilized cell membrane, 32D cells were treated according to the method of Pham *et al.*[31] Briefly, cells were suspended in respiratory buffer (0.25 M sucrose, 2 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% fatty acid free BSA, 1 mM ADP, and 20 mM MOPS (pH 7.4)). Digitonin (4 μg/ml final concentration) was added to the cells for 5 min. Successful permeabilization was documented by detecting entry of propidium iodine into the cells. Mitochondrial function was driven by adding 5 mM succinate, a complex II substrate. BH3BAD peptides were added at a final concentration of 50 μM and cells were incubated at 37°C for 15 min. $\Delta\Psi_M$ was then measured by flow cytometric analysis after staining with 40 nM DiIC1(5).

Caspase-3 activation

Caspase-3 activation was measured with the Apo-Alert Caspase-3 Fluorescent assay kit (Clontech, CA, USA) according to the manufacturers instructions. Cells (1×10^6) were treated with the peptides (50 μM) for 2 h and lysed. The caspase-3 substrate DEVD-AMC was added to the lysate and incubated for 1 h at 37°C. Release of free AMC was measured in a fluorometer (Photon Techonology International) with a 400 nm excitation filter and a 505 nm emission filter.

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