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Advances in Brief

Cell Permeable Bcl-2 Binding Peptides: A Chemical Approach to Apoptosis Induction in Tumor Cells[1]

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Abstract

Bcl-2 is a potent suppressor of apoptosis, and its overexpression contributes to tumorigenesis in many types of human cancers. To test the possibility of modulating Bcl-2 function as an anticancer strategy, a cell permeable Bcl-2 binding peptide, cell permeable moiety (cpm)-1285, was designed by chemically attaching a fatty acid to a peptide derived from the proapoptotic protein Bad. cpm-1285 entered HL-60 tumor cells, bound Bcl-2 protein, and induced apoptosis *in vitro*. In contrast, cpm-1285 had little effect on normal human peripheral blood lymphocytes. Furthermore, cpm-1285 had *in vivo* activity in slowing human myeloid leukemia growth in severe combined immunodeficient mice. These results demonstrate a novel approach for therapeutic intervention of tumor growth *in vivo* with small molecule inhibitors of Bcl-2.

Introduction

Apoptosis is the prevalent form of programmed cell death that, when altered, contributes to a number of human diseases, including cancer, autoimmune disease, and neurodegenerative disorders (1). Bcl-2 family proteins are key regulators of apoptosis and play an essential role in cancer and chemoresistance (2, 3). Bcl-2 contributes to neoplastic cell expansion by preventing normal cell turnover caused by physiological cell death mechanisms. High levels of *Bcl-2* gene expression are found in a wide variety of human cancers and correlate with relative resistance to current chemotherapeutic drugs and γ-irradiation (4). The members of the Bcl-2 family include both death antagonists such as Bcl-2 and Bcl-X_L and death agonists such as Bax, Bak, Bid, and Bad. They share at least one of four homologous regions termed BH[4] domains (BH1 to BH4). The BH3 domain of death agonists is required for these proteins to heterodimerize with Bcl-2 and to promote apoptosis (2, 3). The three-dimensional structure of a death antagonist Bcl-X_L, a close homologue of Bcl-2, as determined by X-ray crystallography and nuclear magnetic resonance spectroscopy, has revealed a surface pocket for the interaction with the BH3 domain of death agonists (5, 6). BH3 domain-derived peptides are shown to have *in vitro* activity in inducing apoptosis in cell-free systems (7) and in HeLa cells (8). However, their use for *in vivo* control of the Bcl-2-regulated apoptotic process remains a challenge. In addition, the therapeutic potential of using such small molecules to intervene in tumor growth in mouse models has yet to be demonstrated.

Materials and Methods

Cell Culture. HL-60 cells were provided by K. Bhalla (University of Miami School of Medicine, Miami, FL). The cells had been transfected with pZip-Bcl-2 and overexpress Bcl-2 protein. The cells were maintained in suspension culture in RPMI 1640 supplemented with 10% fetal bovine serum and 750 μg/ml G418 (Life Technologies, Inc.) and split at 1:10 dilution every 3–4 days.

Peptide Synthesis and Modification. Peptides were prepared by solid phase synthesis using Fmoc-strategy on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perseptive Biosystems, Cambridge, MA), as described previously (9). The purity of the peptides was >99%, as assessed by analytical reverse phase high performance liquid chromatography, capillary electrophoresis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The decanoid acid (Aldrich) was incorporated into the NH_2 terminus of deprotected peptide in DCM at room temperature for 2 h. Biotinylated peptides were chemically synthesized with Biotin (Sigma)/TBTU/HoBt at room temperature after removal of Dde deprotected Nε-Lys with 2% hydrazine in *N,N*-dimethylformamide or 1-methyl-2-pyrrolidinone. A fluorescence-labeled peptide, Flu-BakBH3, used in the Bcl-2 competitive binding assay, was prepared by acylation of the resin-linked peptide with 5-carboxyfluorescein succinimidyl ester (4 equivalents; Sigma) and triethylamine (4 equivalents in dimethyl formamide). The coupling was carried out overnight and followed by simultaneous side-chain deprotection and resin cleavage with a mixture of trifluoroacetic acid, thioanisole, H_2O, and phenol.

***In Vitro* Bcl-2 Binding Assay.** The *in vitro* binding of peptides to the Bcl-2 protein was determined by a competitive binding assay based on fluorescence polarization. For this assay, 5-carboxyfluorescein was coupled to the NH_2 terminus of a peptide GQVGRQLAIIGDDINR, derived from the BH3 domain of Bak (Flu-BakBH3), which has been shown to have high-affinity binding (dissociation constant K_D of ~0.34 μM) to a surface pocket of the Bcl-X_L protein that is essential for its death antagonist function (6). According to our molecular modeling studies and binding measurement using fluorescence polarization, the Flu-BakBH3 peptide also binds the surface pocket of Bcl-2 with a similar affinity (dissociation constant K_D of ~0.20 μM).[5] Bcl-2 used in this assay was a recombinant glutathione *S*-transferase-fused soluble protein (Santa Cruz Biotechnology). Flu-BakBH3 and Bcl-2 protein were mixed in the presence or absence of peptide inhibitors under standard buffer conditions, using near-physiological salt concentrations, neutral pH, and a small amount of reductant. After 30 min incubation, Bcl-2 binding of Flu-BakBH3 was measured by a LS-50 luminescence spectrometer equipped with polarizers using a dual path length quartz cell (500 μl; Perkin-Elmer). The binding affinity of each modified or unmodified peptide for the Bcl-2 protein was assessed by determining the ability of different concentrations of the peptides to inhibit Flu-BakBH3 binding.

Immunofluorescence and Confocal Microscopy. To determine the intake of peptides into cells, HL-60 Bcl-2 cells were incubated in the presence or absence of biotinylated peptides at 50 μM for different times. Cells were washed with PBS and fixed with 4% formaldehyde solution. An aliquot of 50 μl of cell suspension was smeared on slides and dried at room temperature. The slides were further

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[4] The abbreviations used are: BH, Bcl homology; cpm, cell permeable moiety; PARP, poly(ADP-ribose) polymerase; SCID, severe combined immunodeficient; PBL, peripheral blood lymphocyte; PHA, phytohemagglutinin; MST, median survival time.

[5] S. Li, S. Shan, and Z. Huang, unpublished results and manuscript in preparation.

incubated with 100 μl of 20 μg/ml Streptavidin Fluorescein (Boehringer Mannheim) for 20 min and counterstained with propidium iodide. To localize the biotinylated peptides in the cells, 12 images were produced through top to bottom edges of the scanned cells on each section under Zeiss Axiovert 100 microscope with Bio-Rad MRC 600 confocal facility. This provided detailed information of the whole intact cell from the membrane through nucleus. The images were photographed and stored in the same facility.

DNA Fragmentation. Cells (1×10^6) were incubated with peptides for the time indicated. Cells were washed once with PBS and pelleted by centrifugation. The cell pellets were fixed in 70% ethanol for 1 h and then resuspended in PC buffer (192 mM Na_2HPO_4 and 4 mM citric acid, pH 7.8) to extract DNA. After centrifugation for 5 min at 13,000 RPM, the supernatants were collected and incubated with 10 μg/ml RNase A for 1 h at 37°C, followed by digestion with 20 μg/ml proteinase K at 50°C for 3 h. The DNA fragments were separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining.

Cell Viability Assay. The effect of the peptides on the viability of tumor cells was tested using the CellTiter 96AQ kit (Promega Corp., Madison, WI). Briefly, the cell suspension containing 1×10^5 cells in 100 μl of medium was plated into 96-well plates and incubated with peptides at different concentrations. The numbers of apoptotic cells were determined by measuring the absorbance on a Wallac Victor (2) counter (EG&G, Gaithersburg, MD) at 490 nm.

Western Blot Analysis. Cells were harvested and washed once with cold PBS. The cell pellets were immediately lysed in 100 μl of ice-cold RIPA buffer with fresh added protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, and 10 μg/ml aprotinin) and incubated on ice for 30 min. The samples were transferred to microcentrifuge tubes and centrifuged at 10,000 × *g* for 10 min at 4°C. The total cell lysates from supernatant were measured using the Bradford dye-binding procedure (Bio-Rad). Fifty μg of total protein lysate were separated on 12% SDS/polyacrylamide gels followed by blotting to pure nitrocellulose membrane (Bio-Rad). Membranes were blocked in Tris buffered saline [10 mM Tris-HCl (pH 8.0), 150 mM NaCl) with 5% dry milk and 0.05% Tween 20 on 4°C overnight. Antibodies to detect caspase-3 activation (PharMingen; 65906E, diluted to 1:1000) and PARP activation (Boehringer Mannheim; 1835238, diluted to 1:2000) were used for 1 h of incubation at room temperature. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibody and visualized using the ECL system (Amersham).

Xenogeneic Tumor Mouse Model. Female CB.17-SCID/NCr (SCID) mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). At 6 weeks of age, the mice were challenged with human HL-60 cells (2×10^7; i.p.), and either left untreated or treated with cpm-1285 and cpm-1285m peptides (0.5 mg in 0.2 ml H_2O i.p.; daily, days 0–3). The mice were monitored for signs of tumor growth, such as abdominal distension and mortality. The data were analyzed using a multi-ANOVA two-way test, and a probability score of $P < 0.05$ was considered statistically significant.

Results and Discussion

To explore the feasibility of using small molecular inhibitors of Bcl-2 function in cancer treatment, we designed cell permeable Bcl-2 binding peptides in which a functional peptide sequence was attached

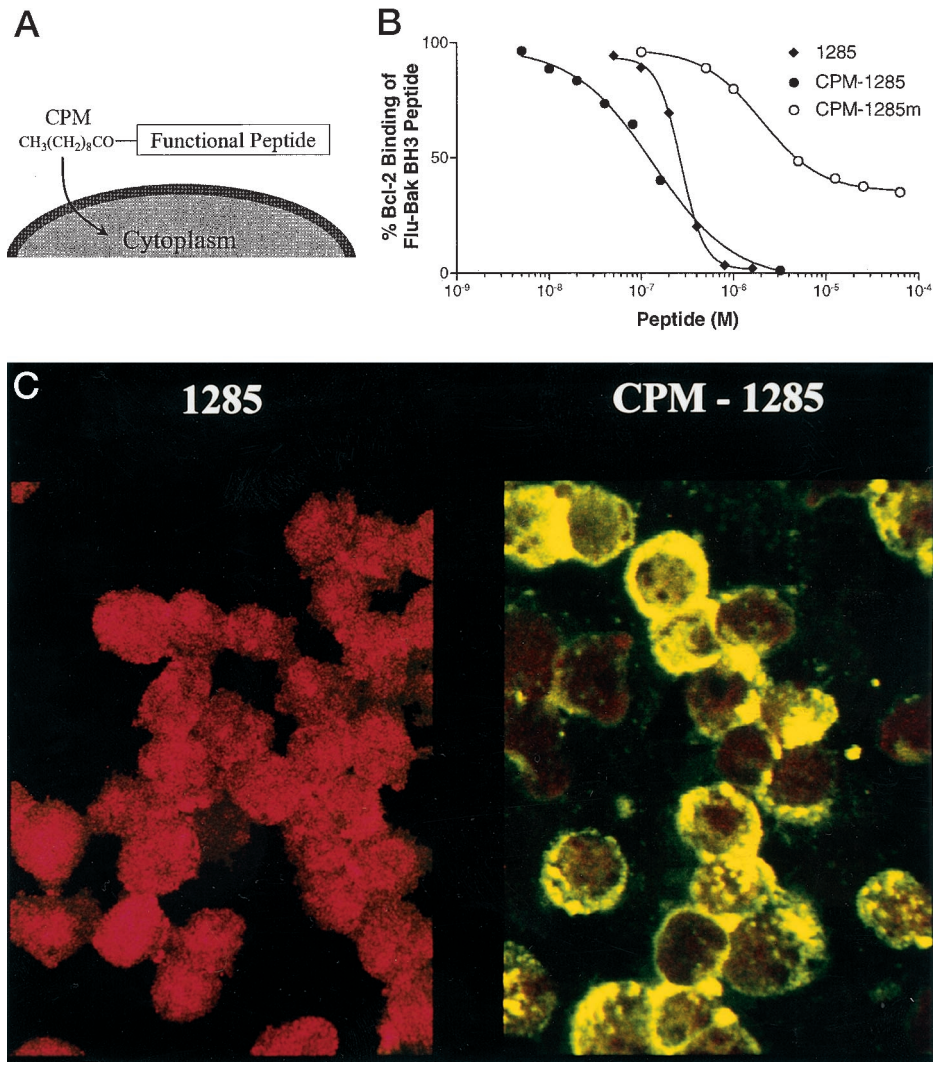

Fig. 1. Bcl-2 binding affinity and cell permeability of cpm-1285. *A*, schematic representation of a cpm. With membrane penetrating capability, the cpm serves as a carrier to deliver a functional peptide into the cell. *B, in vitro* binding affinity of cpm-1285 to Bcl-2. The competition of the binding of the labeled Flu-BakBH3 peptide (30 nM) to GST-Bcl-2 protein (0.5 μM) was measured in the presence of increasing concentrations of cpm-1285 or other peptides. The sequences of peptides are as following: 1285, KNLWAAQRYGRELRRMSDEFEGSFKGL; cpm-1285, $CH_3(CH_2)_8$CONH-KNLWAAQRYGRELRRMSDEFEGSFKGL; and cpm-1285m, $CH_3(CH_2)_8$CONH-KNLWAAQRYGRE-ARRMSDEFEGSFKGL (a single replacement of Leu-151 by Ala is *underlined*). Note that in all of these peptides, a lysine was added to the NH_2 terminus of the peptide sequence. In experiments using confocal microscopy, this lysine was used for labeling with a biotin. *C*, cpm-1285 enters HL-60 cells shown as a total image by confocal microscopy. Control 1285 peptide and cpm-1285 peptide were both labeled with biotin and added to the cells for 15 min at 37°C. The *green* fluorescein around the cell (light ring) indicates the presence of cpm-1285 inside the cells.

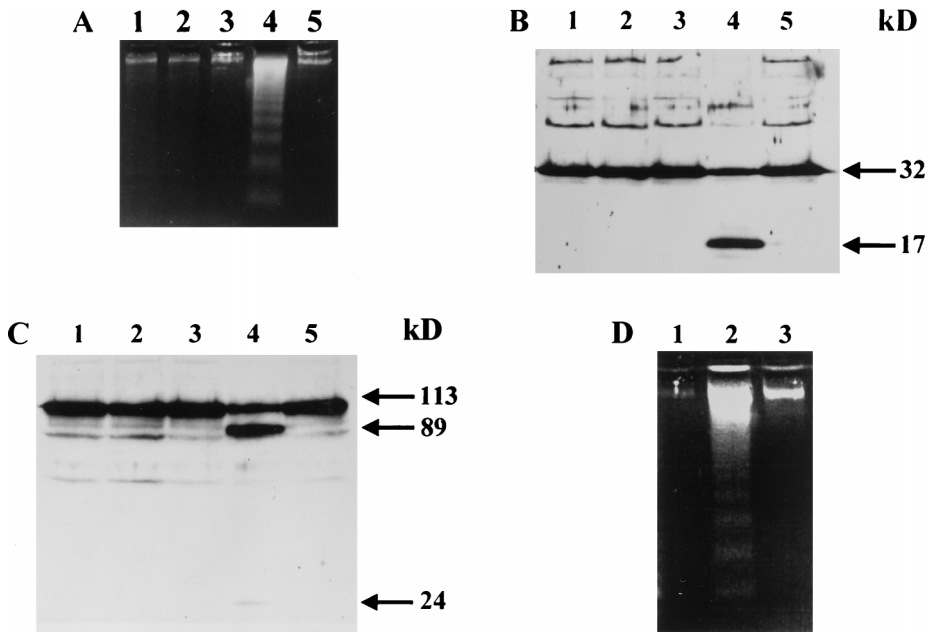

Fig. 2. *A*, cpm-1285 induces apoptosis in HL-60 cells as indicated by DNA fragmentation. *B* and *C*, cpm-1285 induces the cleavage of caspase-3 and PARP, respectively, by Western blot analysis. *Lane 1*, untreated; *Lane 2*, cpm; *Lane 3*, 1285 peptide; *Lane 4*, cpm-1285 peptide; *Lane 5*, mutant cpm-1285m peptide. HL-60 cells were treated with various peptides at 50 μM for 2 h and analyzed. *D*, inhibition by zVAD-fmk (100 μM) of the DNA fragmentation induced by cpm-1285. *Lane 1*, untreated; *Lane 2*, cpm-1285; *Lane 3*, zVAD-fmk + cpm-1285.

to a fatty acid as the cpm (Fig. 1*A*). We found that decanoic acid could effectively assist peptides to pass through the cell membrane. As compared with other peptide delivery methods based on large internalization peptide sequence (8), a potential advantage of using such a small molecule as the cpm is that it is more amenable to chemical synthesis and less likely to affect the structure of the functional peptide sequence. Other larger fatty acids, such as myristic acid and stearic acid, have also been used for the intracellular delivery of peptide inhibitors of protein kinase C (10) and protein-tyrosine phosphatase (11), respectively.

The decanoic acid was attached to a synthetic peptide derived from the BH3 domain (residues 140–165) of Bad to generate a cell permeable ligand of the Bcl-2 surface functional pocket. Unlike other death agonists that have multiple functions (12), Bad acts as a selective death ligand of Bcl-2 and Bcl-X_L (13). Molecular biological studies of Bad mutants with deletion of residues 142–165 of the BH3 domain have shown that this "death domain" is responsible for the functional blockade of Bcl-2 and Bcl-X_L and induction of apoptosis (14). A peptide derived from the same region has also been shown to possess much higher *in vitro* binding affinity for Bcl-X_L than peptides derived from the BH3 domain of other Bcl-2 family proteins (15). In the present study, this Bad BH3 peptide is designated as 1285, whereas the cell permeable peptide with a decanoic acid linked to the NH_2 terminus of 1285 is designated as cpm-1285. As a control, a mutated peptide analogue of cpm-1285 containing a single replacement of Leu-151 by alanine was also synthesized and designated as cpm-1285m. In the native Bad protein, this substitution of Leu-151 by alanine (L151A) has been shown to reduce Bad binding to either Bcl-2 or Bcl-X_L (14).

Using an *in vitro* binding assay based on fluorescence polarization, we confirmed the binding interaction of cpm-1285 with the surface pocket of Bcl-2 protein. For this assay, a Bak BH3 peptide that is known to bind the surface pocket of Bcl-2 and Bcl-X_L (6) was labeled with fluorescein (designated as Flu-Bak BH3) and used as a competitive binding probe. cpm-1285 displayed strong binding potency for Bcl-2 with an IC_{50} of 130 nM and was even ~2-fold higher than that of wild-type 1285 (Fig. 1*B*). The cpm itself did not show any interaction with Bcl-2, even in high concentration of 100 μM (data not shown). The increase in Bcl-2 binding of cpm-1285 could be contributed to the formation of additional interaction of the hydrophobic cpm with Bcl-2 surface sites. As a control, the mutant cpm-1285m peptide exhibited a decrease in Bcl-2 binding affinity with a reduction in IC_{50} of ~15-fold.

The biological effect of cpm-1285 was studied mostly in human myeloid leukemia HL-60 cells overexpressing Bcl-2 protein. This cell line was used as a model system to analyze the inhibition of Bcl-2 function because Bcl-2 has been shown in this cell line to regulate apoptosis and resistance to chemotherapeutic drugs (16). To verify the design concept that the cpm facilitates the cellular entry of peptides, the cells were incubated with cpm-1285 or 1285, both of which were labeled with biotin. After 15 min of incubation, a significant intake of cpm-1285 was observed by confocal microscopy in >90% of the cells (Fig. 1*C*). cpm-1285 peptides were detected inside cells as early as 5 min after exposure and mostly localized in the cytoplasm. In contrast, the control 1285 peptide did not show any cell intake, even after a prolonged 24-h incubation. The mutant cpm-1285m peptide was also found capable of entering HL-60 cells (data not shown).

To determine whether cpm-1285 mimicking the natural Bad death domain could induce apoptosis after entering the cell, HL-60 cells were incubated with cpm-1285 or various controls at 50 μM for 2 h. Cells treated with cpm-1285 displayed morphological changes characteristic of apoptotic cell death, *i.e.*, chromatin condensation, margination, cellular shrinkage, and blebbing, whereas no such changes were observed in untreated cells or cells treated with cpm alone, or with the 1285 and mutant cpm-1285m peptides (data not shown). Because a characteristic feature of apoptotic cells is the presence of DNA strand breaks, HL-60 cells were further analyzed for DNA fragmentation in 2% agarose gel under electrophoresis. The characteristic DNA ladders were found only in cells treated with the cpm-1285 peptide for 2 h (Fig. 2*A*). Neither 1285 peptide, which could not enter the cell, nor cpm alone, even in higher concentrations (100 μM), induced DNA ladders during the same period (Fig. 2*A*) or after a prolonged time (24 h, not shown). These results suggest that the apoptosis-inducing effect of cpm-1285 depends on the 1285 peptide sequence and its efficient delivery into the tumor cells. In the case of the mutant cpm-1285m peptide, no apoptosis was detectable during the 2-h incubation, correlating with its decreased Bcl-2 binding capability. These data are consistent with the mechanism by which cpm-

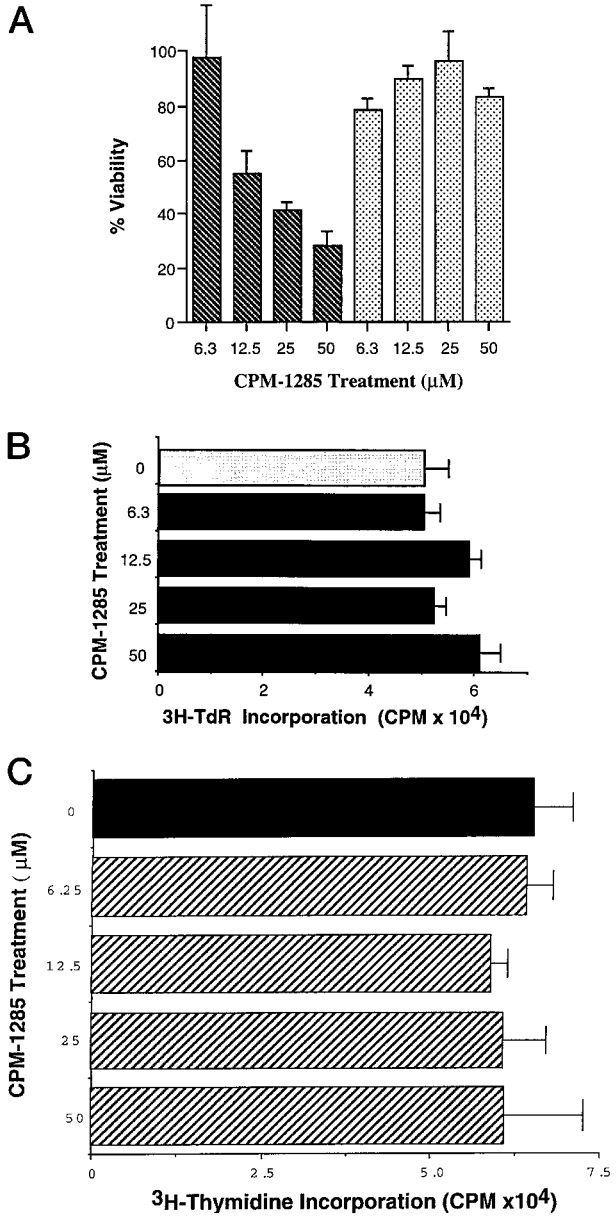

Fig. 3. *In vitro* effect of cpm-1285 on viability of HL-60 cells and normal human PBL. *A*, HL-60 cells (5×10^3) and PBL cells (1×10^4) were incubated for 1 h with varying concentrations of cpm-1285 peptide in microtiter plates ($n = 6$) and evaluated for viability by trypan blue exclusion and morphological appearance. The data are expressed as percentage of viability relative to the cells incubated without treatment. ▧, HL-60 cells; ▨, PBL cells. *Bars*, SE. *B*, PBL cells (2×10^4) after the 1-h treatment, as above, were stimulated in microtiter plates with PHA (1 μg/ml) for 48 h, the last 6 h in the presence of [^{3}H]thymidine (1 μCi/ml). Proliferation was indicated by the level of [^{3}H]thymidine incorporation (background without stimulation was 250 cpm). *Bars*, SE. *C*, PBL cells (5×10^5) were stimulated in microtiter plates with PHA (1 μg/ml) for 48 h in the presence of varying concentrations of cpm-1285 peptide. Proliferation was indicated by the level of [^{3}H]thymidine (1 μCi/ml) incorporated in the last 6 h of incubation (background without PHA stimulation was 475 cpm). *Bars*, SE.

1285 induces apoptosis through functional blockade of intracellular Bcl-2 and related death antagonists.

We examined whether the apoptosis occurring in HL-60 cells relies upon the constant presence of cpm-1285 in the cell mixture. After the treatment of cpm-1285 at 50 μm for 15 min, the inhibitor was washed away with PBS. By 2 h, the cells were found undergoing apoptosis that was similar to that by constant incubation of cpm-1285 for the entire period (data not shown). This result correlated with the observation by confocal microscopy that significant uptake of cpm-1285 into cells occurred within 15 min (Fig. 1*C*). Taken together, these data reveal that cpm-1285 internalized during the early stage of incubation was sufficient for the induction of apoptosis in intact HL-60 cells.

During apoptosis, the activation of a group of caspases, such as caspase-3, and subsequent cleavage of cellular substrates, such as PARP, are crucial components of cell death pathways (17). To assess caspase-3 and PARP activation, Western blot analysis was performed with total protein lysate from HL-60 cells after 2 h treatment with the peptides. The appearance of the active M_r 17,000 subunit of caspase-3, corresponding with the decrease of its M_r 32,000 precursor form, was detected only in cells treated with cpm-1285, but not with 1285, cpm, or cpm-1285m (Fig. 2*B*). Consistent with this finding, the M_r 113,000 PARP was found to be cleaved into M_r 89,000 and M_r 24,000 fragments in cells treated with cpm-1285 but not with the other controls (Fig. 2*C*). The role of caspase activation in apoptosis initiated by cpm-1285 was further demonstrated by the complete inhibition of DNA ladder formation by the addition of zVAD-fmk, a broad-spectrum caspase inhibitor (18), to the cells prior to cpm-1285 treatment (Fig. 2*D*).

It has been suggested that oncogenic changes in certain tumor cells render them more susceptible than normal cells to apoptosis (19), which may allow for the selective induction of apoptosis in these tumors by Bcl-2 inhibitors. In this regard, we noted that the cpm-1285 peptide had only a minimal effect on the viability (marked by trypan blue exclusion and lack of blebbing) of normal human PBLs after a 1-h incubation at concentrations of between 6.3–50 μM (Fig. 3*A*). In addition, the remaining viable PBL cells exhibited equivalent proliferative responses as untreated cells after PHA mitogen stimulation (Fig. 3*B*). In contrast, cpm-1285 concentrations of between 12.5–50 μM resulted in increasing loss of viability of HL-60 cells, reaching a 72% loss at the 50 μM level (Fig. 3*A*). Furthermore, to test whether cpm-1285 would have an effect on activated lymphocytes, PBL cells were stimulated with PHA mitogen for 48 h in the presence of peptide at titrated concentrations. Proliferative responses were measured at the end of the incubation period and were found to be equivalent between all concentrations of cpm-1285 and the untreated PBL cells (Fig. 3*C*). Taken together, these data suggest that the cell permeable peptide at these concentrations could be effective against tumor cells without endangering normal cells, such as lymphocytes.

We further tested the effect of cpm-1285 peptide on additional tumor cell lines, including 697 pre-B leukemia and HL-60 overexpressed with Bcl-X_L (provided by Dr. K. Bhalla, University of Miami School of Medicine, Miami, FL). These cells were incubated with 50

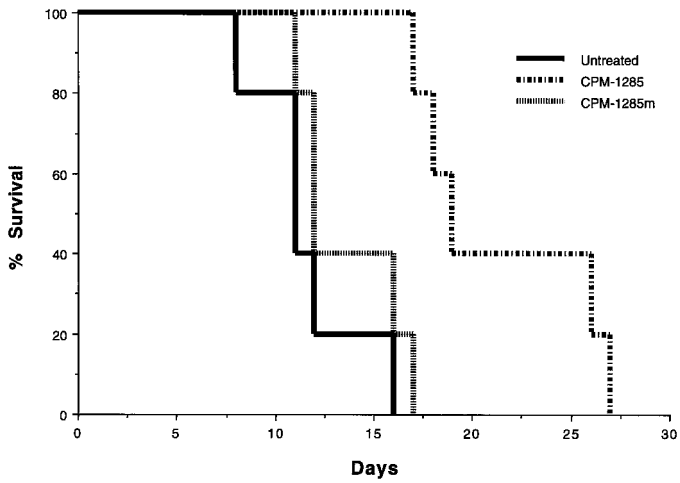

Fig. 4. cpm-1285 suppresses tumor growth in a SCID mouse model. SCID mice were challenged with HL-60 cells and left untreated or treated with cpm-1285 and cpm-1285m peptides ($n = 5$).

μM of cpm-1285 for 2 h. The cell viability was determined by using the CellTiter 96 AQ kit. It was found that cpm-1285 induced up to ~30% loss of viability in both cell lines (data not shown). The activity of cpm-1285 in inducing the death of HL-60 cells overexpressed with Bcl-X_L suggests that cpm-1285 can also target other Bcl-2-related antiapoptotic proteins such as Bcl-X_L.

Finally, we investigated the effects of the cpm-1285 peptide on tumor growth *in vivo*. SCID mice were challenged with HL-60 cells (2×10^7; i.p.) and either left untreated or administered cpm-1285 and cpm-1285m peptides (0.5 mg in 0.2 ml H_2O i.p.; daily, days 0–3). Mice in the untreated control group all developed ascites and succumbed to tumor growth by day 16 (MST of 11 days; Fig. 4). Mice treated with the mutant cpm-1285 peptide followed a similar fatal pattern ($P > 0.65$) with a MST of 12 days. In contrast, mice treated with the cpm-1285 peptide survived until day 27, with a significantly prolonged MST of 19 days ($P < 0.01$). This result suggested that the cpm-1285 peptide had *in vivo* efficacy in reduction of the tumor burden and thereby slowed the progression of tumor growth. In addition, the effect of the peptide was dependent on its proper sequence, consistent with the *in vitro* findings of diminished effects with the mutant form of the peptide. In a toxicity test with normal C57BL/6J mice that were administered i.p. four daily 1.0-mg dosages (twice that used for the experiment in Fig. 4) of the cpm-1285 peptide on days 0–3, there was no obvious gross signs of organ toxicity upon autopsy performed on day 4, compared with untreated normal mice. In addition, cell yields (mean ± SD) from spleen and bone marrow samples were equivalent, *i.e.*, spleen–$91.8 \pm 10.4 \times 10^6$ (normal) *versus* $94.9 \pm 14.1 \times 10^6$ (cpm-1285), $n = 3$, $P \geq 0.58$; bone marrow–$40.1 \pm 1.2 \times 10^6$ (normal) *versus* $34.7 \pm 2.4 \times 10^6$ (cpm-1285), $n = 3$, $P \geq 0.10$. Flow cytometric analysis of the splenocytes also indicated that there were no significant changes in the subset constituency of the cpm-1285-treated population. It should be pointed out that cpm-1285 may have a short biological half-life *in vivo*, as assessed by the *in vitro* human serum stability test (data not shown), which could, at least in part, explain the partial tumor suppression observed under the current experimental conditions. Further synthetic modifications of this peptide to increase its proteolytic stability and the optimization of peptide treatment conditions may hopefully lead to complete abolishment of tumors. Nevertheless, the data shown here provide an important indication of the *in vivo* activity of the Bcl-2 peptide inhibitor.

The *in vitro* and *in vivo* biological activities of cpm-1285 suggest a promising approach to inhibit the function of Bcl-2 or related antiapoptotic proteins. Further biological and preclinical studies of cpm-1285 are necessary to evaluate its clinical potential. Previous studies using other approaches have suggested that Bcl-2 is an attractive target for the development of antitumor agents. For example, antisense oligonucleotides targeted against *Bcl-2* gene have been shown to inhibit non-Hodgkin's lymphoma in humans (20) and improve the treatment response of human cancers when combined with anticancer drugs (21). Cell permeable peptide inhibitors of Bcl-2 function as shown here represent an alternative strategy for potential therapeutic intervention. cpm-1285 should be useful as a prototype molecule for more chemical modifications to optimize its potency and stability. In addition, nonpeptidic organic compounds may be sought to mimic the activity of the cpm-1285 peptide. In this regard, we discovered recently low molecular weight, organic inhibitors targeted to the Bcl-2 surface pocket by using a structure-based computer screening technique.[6] These cell permeable small molecules, in either peptide or nonpeptidic organic form, can also be used as chemical probes to study the *in vivo* mechanism and signaling pathway of the Bcl-2 family proteins. Despite intensive efforts in the past, the way in which Bcl-2 and other family members control apoptosis remains controversial because different models have been suggested (12). Unlike other peptides that are active only *in vitro* or in the cell-free system, the cell-permeable peptide approach described here provides a new tool to analyze the function of the Bcl-2 family in living cells and animals.

Acknowledgments

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