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Selective Apoptotic Killing of Solid and Hematologic Tumor Cells by Bombesin-Targeted Delivery of Mitochondria-Disrupting Peptides

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Abstract: Tumor-homing peptides are attractive tools for tumor imaging and targeted therapy due to their ability to specifically bind and enter tumor cells and masses. Bombesin and its analogues show promise for the targeted delivery of radioactive and chemotherapeutic agents to a wide variety of solid tumors. Here, we describe the bombesin-targeted delivery of toxic peptides to solid tumor cells and leukemia cells. We found that bombesin specifically bound to solid tumor cells and leukemia cells with similar affinity. Conjugation to bombesin significantly (5−15 times) enhanced the cytotoxicity of three mitochondria-disrupting peptides (KLA, B27, and B28) in solid tumor cells and leukemia cells through improvement of their binding affinity. The bombesin-directed peptides (KB, BB27, and BB28) contained the same bombesin leader sequence but had different mitochondria-disrupting peptides, which selectively induced caspase-dependent apoptosis in solid tumor cells and leukemia cell lines. The IC_{50} values of these peptides (BB27, 3−5 μmol/L; BB28, 4−6 μmol/L) for solid tumor cells and leukemia cells are approximately 5−10 times lower than the IC_{50} values for normal cells. BB27 and BB28 also displayed cytotoxicity in primary leukemia cells from patients ($n = 4$) with acute myeloid leukemia. Intratumoral (10 mg/kg) and intraperitoneal (20 mg/kg) injection of BB27 and BB28 exerted substantial inhibition on K562 tumor xenograft growth without obvious systematic toxicity. Our results suggest that the bombesin-directed mitochondria-disrupting peptides BB27 and BB28 might be used as therapeutic agents not only for solid tumors but also for hematologic tumors.

Keywords: Targeted therapy; tumor-homing peptide; drug delivery system; bombesin; apoptosis

Introduction

Accumulating evidence shows that small tumor-homing peptides could bind to tumor cells or tumor blood/lymphatic vessels with high affinity and specificity.[1−4] Many of these homing peptides not only targeted the desired location but

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also were internalized by the cancer cells.[5,6] Therefore, these peptides have the potential to be highly efficient drug delivery agents and diagnostic tools for the imaging of tumor masses.[7] Indeed, the radiolabeled somatostatin analogue Sandostatin LAR (octreotide; Novartis) is approved by the U.S. Food and Drug Administration for tumor imaging.[8] Many agents based on tumor-homing peptides are also being investigated in preclinical or clinical trials for their diagnostic and therapeutic potential.[9−11]

Bombesin is a 14-amino acid tumor-homing peptide that was isolated from frog skin.[12] It binds to several receptors including neuromedin B preferring receptor (BB1), gastrin-releasing peptide preferring receptor (BB2), orphan bombesin receptor subtype-3 (BB3), and BB4 receptor.[13,14] These receptors are overexpressed in a variety of common human cancers, such as prostate, kidney, uterine, ovarian, breast, pancreas, gastrointestinal, head and neck, neuroblastomas, esophageal, and small cell lung cancer.[15,16] Over the past few decades, bombesin and its analogues labeled with different radionuclides have been widely used for solid tumor imaging.[17,18] Targeted chemotherapy was also achieved by conjugating bombesin analogues with pyrrolino-doxorubicin[19,20] and camptothecin.[21,22] Therefore, bombesin-based agents show promise for the imaging and treatment of solid tumors expressing bombesin receptors.

Previous works had focused on the application of radioactive and chemotherapeutic conjugates of bombesin and its analogues to treat solid tumors. In addition to the targeted delivery of those agents of small molecular weight, we are also interested in whether bombesin could deliver a toxic peptide to solid tumors cells. If that is the case, another interesting question is whether bombesin could effectively and specifically deliver the toxic peptide to hematologic tumor cells. In this work, we constructed three different chimeric peptides by individually attaching one of three mitochondria-disrupting peptides to bombesin. The *in vitro* and *in vivo* cytotoxicity of these peptides demonstrates that bombesin can effectively and selectively deliver toxic peptide to solid tumor cells and leukemia cells.

Experimental Section

Chimeric Peptide Design and Synthesis. The mitochondria-disrupting peptide KLA can induce considerable mitochondrial swelling at low micromolar concentrations with little random penetration of the cytoplasmic membrane of eukaryotic cells.[23,24] The nontoxic KLA peptide coupled to a carrier specific for a cellular target has carrier-directed cytotoxicity.[23,24] Therefore, we first constructed the chimeric peptides KB and BK by fusing the KLA peptide to the N-terminus and C-terminus of bombesin, respectively. BMAP-27 and BMAP-28 are antimicrobial peptides derived

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Table 1. Peptides and Sequences

peptide	sequence	length (aa[a])	MW (Da)
Bombesin	QRLGNQWAVGHLM	13	1509
KLA	KLAKLAKKLAKLAK	14	1523
KB	KLAKLAKKLAKLAKGGQRLGN QWAVGHLM	29	3129
BK	QRLGNQWAVGHLMGGKLAKL AKKLAKLAK	29	3129
TAT	YGRKKRRQRRR	11	1559
TK	YGRKKRRQRRRGGKLAKLAK KLAKLAK	27	3179
B27	GRFKRFRKKFKKLFKKLS	18	2342
BB27	GRFKRFRKKFKKLFKKLSQR LGNQWAVGHLM	31	3834
TB27	YGRKKRRQRRRGGRFKRFRK KFKKLFKKLS	30	3941
B28	GGLRSLGRKILRAWKKYG	18	2059
BB28	GGLRSLGRKILRAWKKYGQR LGNQWAVGHLM	31	3551
TB28	YGRKKRRQRRRGGLRSLGRK ILRAWKKYG	29	3601
URP	DSHAKRHHGYKRKFHEKH HSHRGY	24	3036

[a] Amino acid.

from bovine myeloid cells.[25] Deletion of the hydrophobic C-terminus of both peptides drastically reduces their cytotoxicity. However, truncated BMAP27 (B27) and BMAP28 (B28), which lack the C-terminal tail, show activity toward the mitochondrial membrane.[25,26] Therefore, we also constructed the chimeric peptides BB27 and BB28 by attaching B27 and B28, respectively, to the N-terminus of bombesin. The nonspecific cell-penetrating peptide TAT_{48-59}[27] replaced bombesin in the construction of another three peptides, TK, TB27, and TB28.

All peptides (Table 1) with 95% purity were synthesized using fluoren-9-ylmethoxycarbonyl chemistry. FITC labels were linked to the N-terminus of peptides by introducing 5-carboxyfluorescein during the final synthetic cycle. All the peptides were dissolved in isotonic phosphate-buffered saline (PBS: 137 mmol/L NaCl, 2.68 mmol/L KCl, 8.09 mmol/L Na_2HPO_4, 1.76 mmol/L KH_2PO_4, pH 7.4) and stored at −80 °C.

Cells and Culture Conditions. Unless otherwise mentioned, all cell lines were purchased from the American Type Culture Collection. Human peripheral blood mononuclear cells (hPBMCs) were isolated by low-density (Ficoll-Paque PLUS, 1.077 g/mL) gradient centrifugation. Cells were cultured in either Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO-BRL), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere.

Binding and Cellular Uptake of the Peptides. About 1×10^4 cells/well were seeded into 96-well plates and allowed to attach overnight. After being washed with PBS, the cells were further incubated with 100 μL of FITC-labeled peptides for 1 h at 37 °C and then observed under fluorescence microscope. To determine peptide uptake by fluorescence-activated cell sorting (FACS), the adherent cells were first digested by the addition of 0.25% w/v trypsin in serum-free medium containing 0.02% EDTA at 37 °C. After washing and resuspension in serum-free medium, the FITC-labeled peptides were added to 2×10^5 cells (300 μL) and incubated at 37 °C for 1 h. The cells were then washed three times with 1 mL of PBS and analyzed by FACS.

Cytotoxicity of the Peptides in Cell Lines. Cytotoxicity of each peptide was quantitatively determined using the CCK-8 cell counting kit. Adherent cells were plated in a 96-well plate at a density of 1×10^4 cells/well and allowed to attach overnight. The cells in suspension were seeded in a 96-well plate immediately before use. After washing cells once with PBS, increasing concentrations of peptide in 100 μL of serum-free medium supplemented with 2% BSA were added. Following incubation at 37 °C for 1−2 h, 10 μL of CCK-8 solution was added to the wells. Subsequently, the absorbance was measured at 450 nm 4 h later. Cytotoxicity was expressed as the percentage of viable cells after treatment with the peptide, which was calculated by assuming 100% survival with the URP control peptide. Each sample was done in triplicate, and the IC_{50} value was obtained from the respective cell viability curve. The Live/Dead BacLight bacterial viability kit (Molecular Probe) was used as an additional method to evaluate cytotoxicity of the peptides. Based on differences in membrane permeability, the Syto 9 and propidium iodide (PI) mixture stained living cells green and dead cells red.

Apoptosis Assays. The assays were performed according to the description by Rege et al.[23] Briefly, the presence of phosphatidylserine on the outer membranes of cells, which reflects early apoptosis, was detected using the FITC-Annexin V/PI kit (Invitrogen). The Annexin V+/PI− and Annexin V+/PI+ cells were considered apoptotic and necrotic, respectively. Mitochondrial depolarization was examined by JC-1 staining (Invitrogen). The pan-caspase inhibitor z-VAD-Fmk (Invitrogen) was used to probe the possible involvement of the caspase-mediated apoptotic pathway.

Tumor Xenograft Models. Six- to eight-week-old female BALB/c nu/nu mice were obtained from the University Animal Production Center. Pooled K562 leukemia cells (1×10^7 cells in 100 μL of saline) were injected subcutaneously into the right flank of mice. The mice were injected intratumorally (10 mg/kg) or intraperitoneally (20 mg/kg) with peptide or an equal volume of PBS on a daily basis for

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five consecutive days. The tumor volume (cm^3) was calculated as length × width^2 × 0.5. At the end of the experiment, all animals were sacrificed and the tumor masses were measured. To probe the apoptosis of tumor cells *in vivo*, mice bearing a tumor graft (0.3−0.5 cm^3) were intratumorally injected with 0.1 mg of peptide in 100 μL of PBS. About 24 h later, the mice were sacrificed and the tumor tissues were excised, paraffin-embedded, sectioned, and stained with H&E to examine the histological architecture. Terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) staining (Invitrogen) were used to evaluate the apoptosis of tumor cells. Simultaneously, the histological architecture of major organs from mice injected with peptides were examined by H&E staining and compared to mice in the control group.

Statistical Analysis. Results are presented as the mean ± SD of at least three experiments. Multiple comparisons were made using SPSS software.

Results

Bombesin Enhances Binding Affinity and Cytotoxicity of KLA in Solid Tumor Cells and Leukemia Cells. Figure 1A shows that the unconjugated KLA peptide showed low binding ability (positive rate <20%) to all tested solid tumor cells (MCF-7, Du145, SMMC-7721, and HeLa) and leukemia cells (NB4, CEM, K562, Molt4, Jurkat, and Raji). But the unconjugated bombesin and KLA−bombesin conjugate KB exhibit similar high affinity to all tested tumor cells. As determined by FACS, the positive rates of these cells stained with FITC-labeled bombesin or KB varied from 60% to 95%. These results indicate that conjugation of bombesin siginificantly enhances the binding affinity of KLA peptide for tumor cells. Bombesin, bombesin-directed peptide KB and the control URP peptide bound to normal cells, including human umbilical vein endothelial cells (HUVECs), human decidual stromal cells (HDSCs), human lung fibroblasts (MRC5), human hepatocytes (L02), human endothelial cells (ECV304), and hPBMCs, at a low level. The positive rates of these three peptides for all normal cells are less than 5%. These results demonstrate that both bombesin and KB specifically bind to solid tumor and hematologic tumor cells with similar high affinity.

Cytotoxicity assays demonstrate that the KLA−bombesin conjugate KB, in which the KLA peptide was covalently coupled to the N-terminus of bombesin, induced approximately 55−95% cell death in Du145 cells at concentrations ranging from 10 to 50 μmol/L (Figure 1B, left). The IC_{50} of the KB peptide in Du145 cells falls within the range of 10−15 μmol/L. The loss of viability in Du145 cells was accompanied by condensed nuclei, rounding, cell detachment, and clumping (Figure 1B, right). The KB similarly affected leukemia cells and solid tumor cells. KB induced 90% cell death in K562 cells at 50 μmol/L (Figure 1C). The IC_{50} of the KB peptide in K562 cells is within the range of 12−15 μmol/L. In contrast, the Du145 cells and K562 cells were resistant to the untargeted KLA

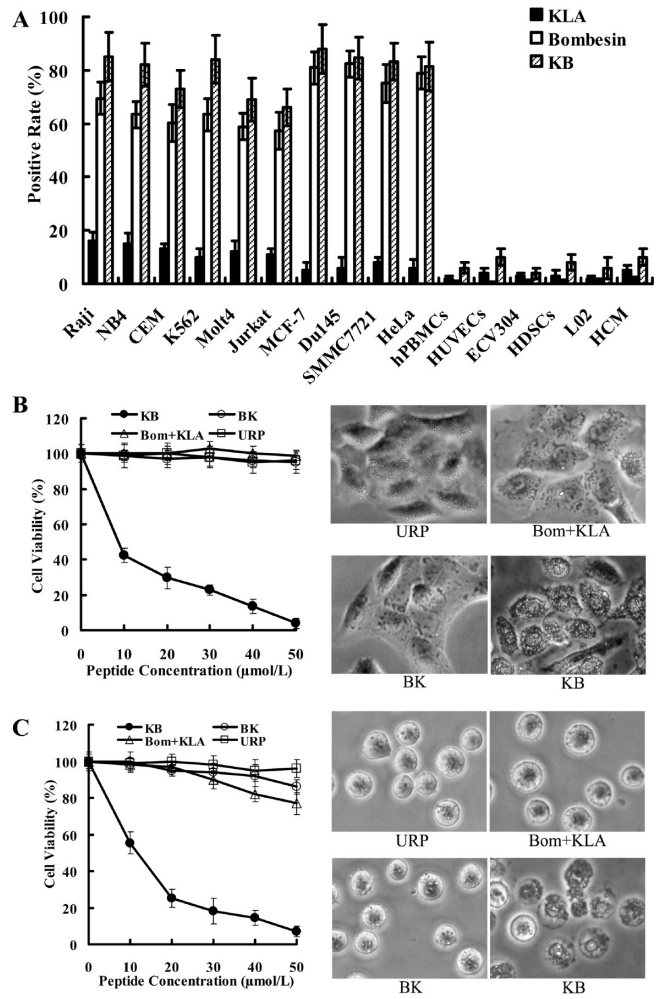

Figure 1. Bombesin enhances binding and cytotoxicity of KLA peptide in tumor cells. A: Binding of KLA peptide, bombesin and KLA−bombesin conjugate KB to cells. The peptide (20 μmol/L) was incubated with cells at 37 °C for 1 h. After washing with PBS, cells were analyzed using FACS. The positive cell rates were calculated by using URP peptide as a negative control. The data are expressed as mean ± SD. B, C: Enhancement of KLA-induced cytotoxicity in solid tumor Du145 cells (B) and leukemia K562 cells (C). Cytotoxicity was measured using the cell counting reagent CCK-8. Morphological changes associated with death were observed under a contrast microscope after treatment with 50 μmol/L control peptide (URP), unconjugated bombesin (Bom) plus KLA (Bom + KLA), the chimeric peptide BK, and the chimeric peptide KB (original magnification: ×320).

(Figure 1B,C). The IC_{50} values of untargeted KLA for Du145 and K562 cells are within the range of 100−150 μmol/L. These results demonstrate that the cytotoxicity of KLA in tumor cells was increased 6−15 times by conjugation to bombesin at its N-terminus. However, the bombesin−KLA conjugate BK, in which the KLA peptide was attached to the C-terminus of bombesin, was not cytotoxic in either Du145 or K562 cells at 50 μmol/L

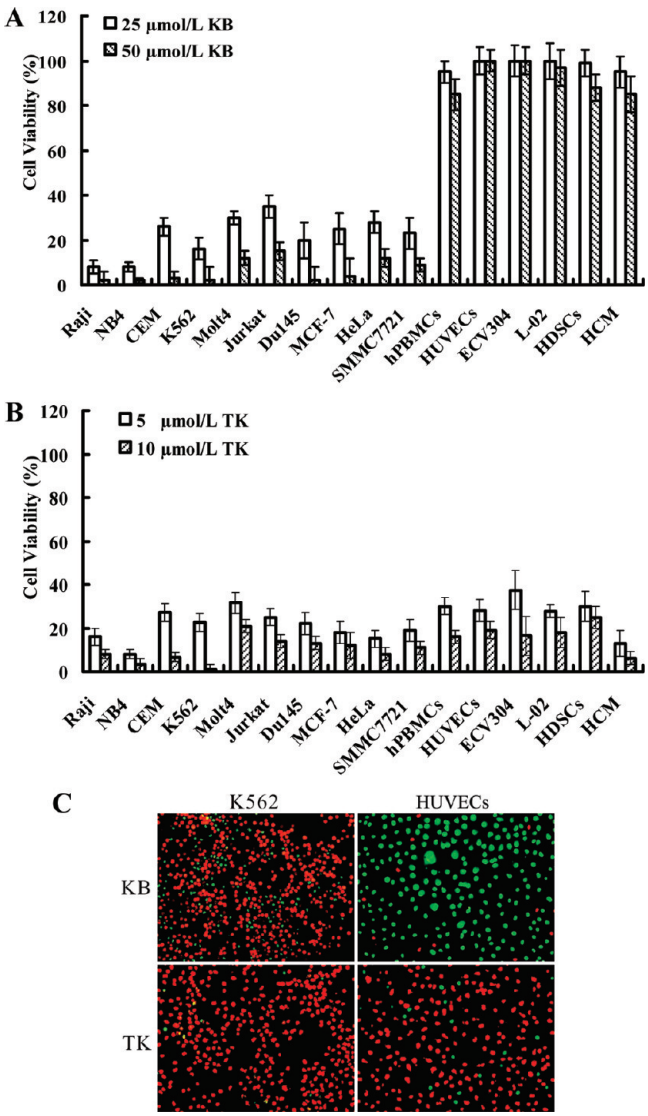

Figure 2. KB selectively induced cell death in tumor cells. A, B: Cytotoxicity of KB (A) and TK (B) in tumor cells and normal cells. Cells were treated with peptide for 2 h, and cell viability was determined using CCK-8 and compared with the control (cells treated with an equal volume of PBS). C: Live/dead assays for K562 and HUVECs treated with KB (50 μmol/L) or TK (10 μmol/L). After treatment with the peptide, the cells were double stained with SYTO 9 and PI to simultaneously show live (green) and dead (red) cells (original magnification: $\times$200). D: FACS analysis for the cellular uptake of KB by K562 and HUVECs. The cells were incubated with FITC-labeled KB (20 μmol/L) at 37 °C for 10 min and then analyzed by FACS. The percentage of positive cells is indicated.

(Figure 1B,C). These data indicate that a KLA−bombesin conjugate, attached at the N-terminus of bombesin, enhanced cytotoxicity in solid tumor and leukemia cells.

Bombesin-Directed Peptide KB Selectively Induces Apoptosis of Solid Tumor Cells and Leukemia Cells. Figure 2A shows that KB was toxic to the solid tumor cells MCF7, Du145, HeLa, and SMMC-7721. The viability of these cells was less than 30% after treatment with 25 μmol/L KB, and more than 90% of the cells died when the concentration was increased to 50 μmol/L. KB showed similar cytotoxicity in the Raji, NB4, CEM, K562, Molt4, and Jurkat leukemia cells. The IC_{50} values of KB for these tumor cells fall within the range of 10−15 μmol/L. The normal cells, including HUVECs, ECV304, L02, HDSCs, hPBMCs, and HCM (human cardiac myocytes), were relatively resistant to KB, and the IC_{50} values varied from 120−150 μmol/L. These data demonstrate that the KB peptide is selectively cytotoxic in tumor cells, and the sensitivity of leukemia cells to KB is similar to that of solid tumor cells. In contrast to the selectivity of KB, TK-containing nonspecific cell-penetrating peptides TAT and KLA induced over 80% cell death in both tumor cells and normal cells at 10 μmol/L (Figure 2B). This indicated that the leader sequence bombesin in the KB contributed to its specificity.

The live/dead assay using SYTO 9 and PI also demonstrated that KB affected cancer cells differently than normal cells. As shown in Figure 2C, after treatment with 50 μmol/L KB, more than 90% of 1×10^4 K562 cells were stained red (indicating dead cells), while most HUVECs (>90%) were stained green (indicating live cells). However, the effect of TK on K562 leukemia cells and normal HUVECs was similar. Treatment with 10 μmol/L TK resulted in 90% loss of cell viability in both K562 cells and HUVECs. The selective cytotoxicity of KB in other tumor cells and normal cells was also proved by live/dead assay (not shown). These data demonstrate that the cytotoxicity of KB in tumor cells and normal cells is positively related to its binding affinity for these cells. In contrast, the nonspecific TAT-directed peptide TK induced over 70−90% cell death in all tested cells at 5 μmol/L (Figure 2B). Further analysis demonstrates that TK binds to both tumor cells and normal cells at similar high affinity. TK enters 80−90% of these cells at 5 μmol/L (not shown). These results indicated that bombesin-directed, preferential cellular entry contributed to the selective cytotoxicity of KB in tumor cells.

Phosphatidylserine assays demonstrated that KB induces the apoptosis of K562 cells. After treatment with KB, apoptotic (Annexin V+/PI−) and necrotic (Annexin V+/PI+) cells were seen under the fluorescence microscope (Figure 3A). After treatment with 0, 20, 30, and 50 μmol/L KB, the percentage ratios of apoptotic/necrotic cells were 5.2/2.8, 16.4/3.7, 22.7/6.2, and 34.3/25.0, respectively (Figure 3B). JC-1 staining revealed a concentration-dependent loss of mitochondrial membrane potential (Figure 3C left), which was accompanied by the disappearance of the healthy mitochondria in the cytoplasm of K562 cells after treatment with KB (not shown). A concentration-dependent increase in cytochrome C release from KB-treated cells was also detected by Western blot (Figure 3C right). Furthermore, about 20−40% of K562 cells were rescued by preincubating the cells with 100 μmol/L pan-caspase inhibitor z-VAD-Fmk for 2 h before treatment with 15−30 μmol/L KB (Figure 3d). These results demonstrate that KB induces caspase-dependent apoptosis in K562 cells.

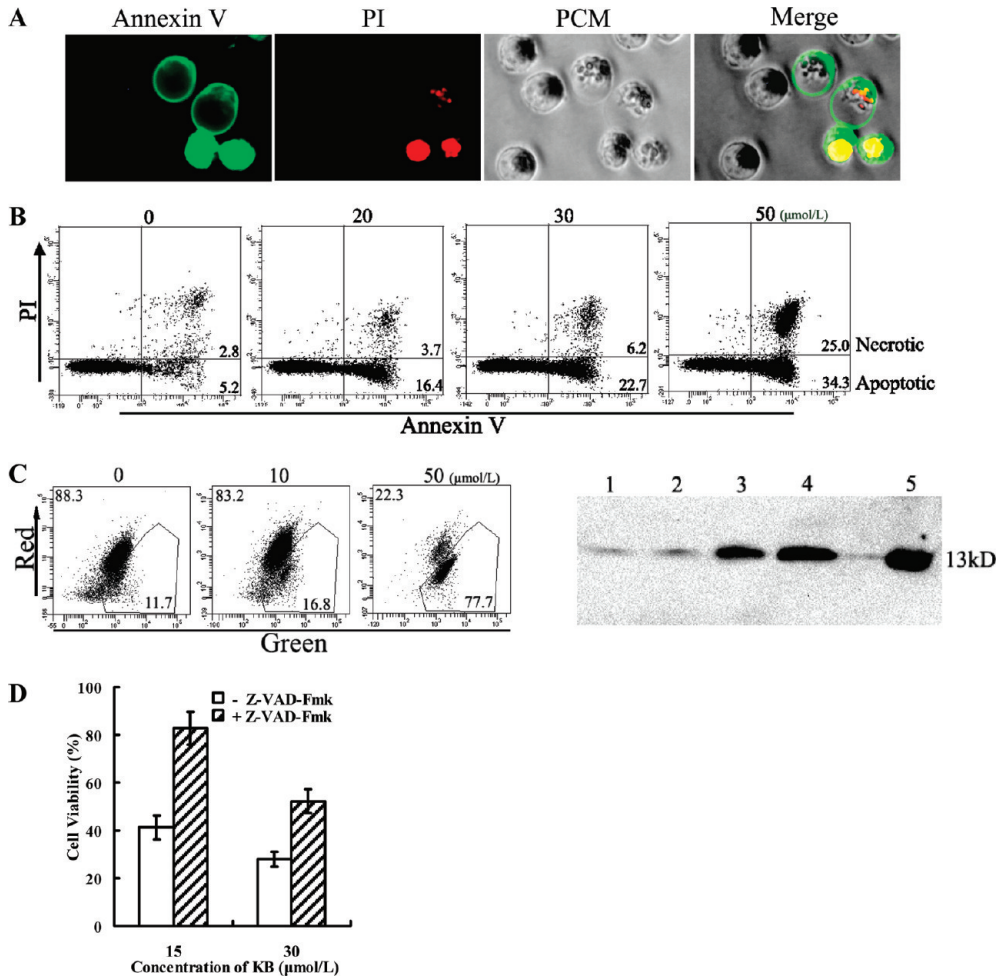

Figure 3. KB-induced caspase-dependent apoptosis of leukemia cells. A: Microscope view of KB-induced apoptotic cells. After treatment with KB, the cells were double stained with FITC-Annexin V (green) and PI (red) and then observed under a fluorescence microscope and phase contrast microscope (PCM). Necrotic, apoptotic, and live cells appear as Annexin V+/PI+, Annexin V+/PI−, and Annexin V−/PI−, respectively. B: FACS analysis for KB-induced apoptotic cells. After treatment with peptide at indicated concentration, the K562 cells were then double stained with FITC-Annexin V and PI and assayed by FACS. The percentage of apoptotic and necrotic cells is indicated. C: The loss of mitochondrial membrane potential (left) and release of cytochrome C from the mitochondria (right) in K562 cells treated with peptide. After treatment with KB at indicated concentration, the cells were stained with JC-1 and then analyzed by FACS. The percentage of red and green fluorescent cells is indicated. K562 cells were treated with KB at concentrations of 0 (lane 1), 20 (lane 2), 100 (lane 3), or 200 μmol/L (lane 4) for 2 h at 37 °C, and then the cytochome C in the supernatant was detected by Western blotting. An equal amount of cells treated without peptide was lysed and used as a control (lane 5). D: Inhibiton of the cytotoxicity of KB by the pan-caspase inhibitor.

Bombesin Enhances the Cytotoxicity of Truncated BMAP28 and BMAP27 in Solid Tumor Cells and Leukemia Cells. Supplementary Figure 1A in the Supporting Information shows that the truncated BMAP28 (B28) with unconjugated bombesin induced an approximately 10% loss in viability in MCF-7 cells at 15 μmol/L. However, the B28−bombesin conjugate BB28 caused ∼70% cell death in MCF-7 cells at 5 μmol/L. Over 90% of the cells died when the concentration of BB28 was increased to 10 μmol/L. Condensed nuclei, cell rounding, detachment, and clumping were all typical of BB28-treated cells (Supplementary Figure 1B in the Supporting Information). BB28 induced 70−90% cell death in CEM cells at concentrations of 7.5−15 μmol/L (Supplementary Figure 1C in the Supporting Information). The BB28-caused cell death in CEM cells was accompanied by the typical condensed nuclei (Supplementary Figure 1D in the Supporting Information). The IC_{50} values of BB28 for MCF-7 and CEM cells are within the range of 4−6 μmol/L. Nevertheless, untargeted B28 alone displayed only mild cytotoxicity (<20% loss of cell viability) in MCF-7 and CEM cells at 15 μmol/L (Supplementary Figure 1 in the Supporting Information). Compared to the IC_{50} values (25−30 μmol/L) of untargeted B28 for these cells, conjugation of B28 to bombesin at its N-terminus increases its cytotoxicity by 5−8 times. Conjugation of the truncated BMAP27 (B27) to bombesin at its N-terminus also increased its potency in MCF-7 cells

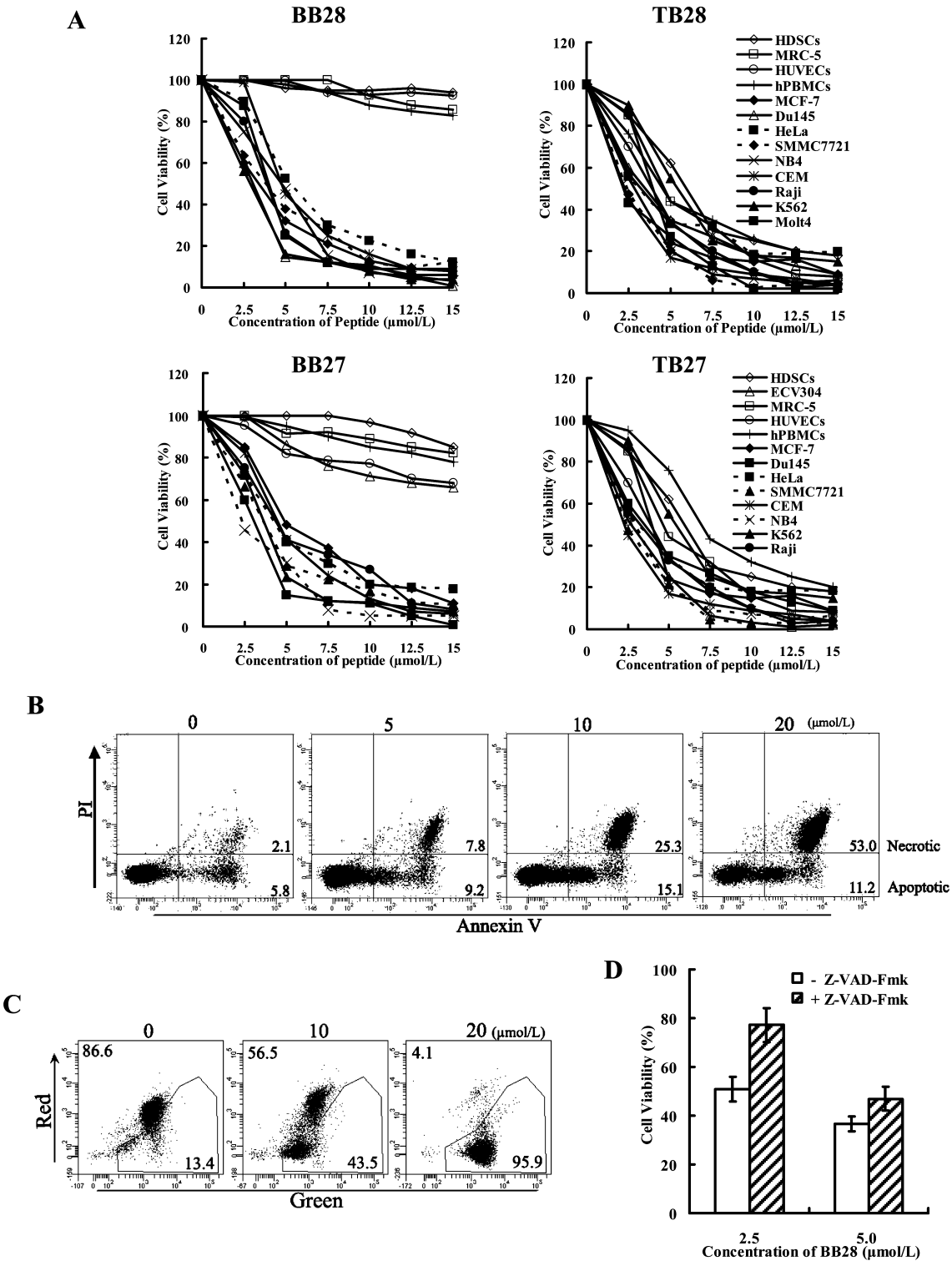

Figure 4. BB28- and BB27-induced caspase-dependent apoptosis of leukemia cells. A: Cytotoxicity of BB28, BB27, TB28, and TB27 in tumor cells and normal cells. The results are means of three experiments, SD values were <10% (not shown). B: FACS analysis for BB28-induced apoptotic CEM cells. The percentage of apoptotic and necrotic cells is indicated. C: BB28-caused loss of mitochondrial membrane potential in CEM cells. The percentage of red and green fluorescent cells is indicated. D: Inhibiton of the cytotoxicity of BB28 by the pan-caspase inhibitor. All methods are described in Figure 3.

and CEM cells (not shown). These results demonstrate that conjugation with bombesin could enhance the cytotoxicity of truncated BMAP28 and BMAP27 in tumor cells.

Bombesin-Directed Peptides BB28 and BB27 Selectively Induce Apoptosis of Solid Tumor Cells and Leukemia Cells. Figure 4A demonstrates that BB28 was toxic in the solid tumor cells MCF-7, Du145, HeLa, and

SMMC-7721. BB28 (7.5 μmol/L) reduced the viability of these cells by 70−90%. BB28 was also highly cytotoxic in the Raji, NB4, CEM, K562, and Molt4 leukemia cells, and was relatively nontoxic to the normal hPBMCs, HDSCs, MRC5, and Vero E6 cells (Monkey kidney cells). The IC_{50} values of BB28 for these tumor cells were within the range of ∼4−6 μmol/L, compared to 30−40 μmol/L for tested normal cells. In contrast, there was no obvious difference in cytotoxicity of the chimeric peptide TB28 (in which bombesin is replaced with the nonspecific cell-penetrating peptide TAT) in both tumor cells and normal cells. The effect of BB27 on tumor cells and normal cells was similar to that of BB28. Figure 4A shows that BB27 was more cytotoxic in tumor cells than it was in normal cells. The IC_{50} values of BB27 for the tested tumor cells were within the range of 3−5 μmol/L, compared to 20−30 μmol/L for tested noncancerous cells. However, TB27 was toxic in both tumor cells and normal cells (Figure 4A). Furthermore, the live/dead assay with SYTO 9 and PI also showed that 10 μmol/L BB28 induced 80−90% cell death in CEM cells and ∼10% cell death in HDSCs. However, TB28 was toxic in both cell types (not shown). Cellular translocation assays demonstrated that 10 μmol/L BB28 bound and entered 90% of the CEM cells. But the internalization rate of BB28 by normal HDSCs was only about 10% under the same conditions (not shown). The positive association between cytotoxicity and cell entry of BB28 and BB27 was also observed in other tumor cells and normal cells (not shown). These data indicate that both BB28 and BB27 are selectively cytotoxic in tumor cells due to their preferential binding.

Apoptotic (Annexin V+/PI−) cells were also observed under fluorescence microscope after the treatment of CEM cells with BB28 (not shown). The increased number of apoptotic and necrotic cells (Figure 4B), the loss of mitochondrial membrane potential (Figure 4C), and the release of cytochrome C (not shown) were also concentration-dependent. The cell death in CEM cells induced by 2.5−5 μmol/L BB28 was reduced by 10−30% in the presence of pan-caspase inhibitor z-VAD-Fmk (Figure 4D). Loss of mitochondrial membrane potential and involvement of the caspase pathway in cell death induced by BB27 was also detected (not shown). Taken together, these data indicate the involvement of the caspase-dependent apoptotic pathway in the cell death induced by these bombesin-directed peptides.

Bombesin-Directed Peptides KB, BB28, and BB27 Are Cytotoxic in Blood Cells Freshly Isolated from Patients with AML. Human PBMCs freshly isolated from four patients with AML were treated with increasing concentrations of KB, BB28, and BB27 with the daunomycin hydrochloride (DNR) as a positive control. Cell viability assays demonstrated that these three peptides induced dose-dependent cell death of PBMCs from all four patients (Figure 5A). KB caused 30−60% cell death at 50 μmol/L, whereas BB28 and BB27 caused similar cell death at 20 μmol/L. The cytotoxicity of these three peptides in AML cells from patients 2 (Figure 5B) and 3 (Figure 5C) was verified by apoptotic/necrotic analysis. These results indicate that bombesin-directed peptides are cytotoxic not only in cultured cell lines, but also in primary leukemia cells.

Bombesin-Directed Peptides BB28 and BB27 Suppress Tumor Growth *in Vivo*. At the onset of a palpable tumor (0.03−0.05 cm^3), the mice were intraperitoneally injected with 20 mg/kg peptide (treatment group, $n = 4$) or an equivalent volume of PBS (control group, $n = 4$) on a daily basis for five consecutive days. As shown in Figure 6A, intraperitoneally injected BB28 and BB27 also exerted an obvious tumor-suppressing effect, and the mean tumor volume of peptide-treated mice was significantly different (BB27, $P = 0.013$; BB28, $P = 0.009$) from the volume observed in the control group beginning on day 11 (Figure 6A). At the end of this experiment, the weights of the tumor tissues from PBS-, BB27-, and BB28-treated mice were 2.68 ± 0.53 g, 0.81 ± 0.45 g, and 0.76 ± 0.29 g, respectively.

The antitumor effect of bombesin-directed peptides was further examined using an intratumoral injection model. When the average volume of the tumor xenografts reached 0.1 cm^3, mice ($n = 12$) were randomly divided into three groups and intratumorally injected with BB28 or BB27 (10 mg/kg, treatment group), or an equal volume of PBS (control group) on a daily basis for a total of 5 days. As shown in Figure 6B (left), intratumoral injection of BB28 and BB27 exhibited substantial suppression of the growing tumor xenografts. From day 13 through completion of the study, the mean tumor volume in BB28- and BB27-treated mice was significantly different (BB28, $P = 0.030$; BB27, $P = 0.047$) from the control group. At the end (day 20) of the experiment, the weights of the tumor tissues from PBS-, BB27-, and BB28-treated mice were 2.49 ± 0.78 g, 0.28 ± 0.23 g, and 0.1 ± 0.09 g, respectively.

As shown in Figure 6C, the intratumorally injected BB28 caused extensive necrosis in tumor cells at the injection site. Rich TUNEL-positive staining cells (green) in the residue demonstrate that BB28 induce apoptosis of tumor cells *in vivo* (Figure 6D). However, no obvious histological damage was observed at the end of this experiment in the liver, kidney, and heart of mice both intraperitoneally and intratumorally injected with peptides (not shown).

Discussion

In addition to an increased understanding of cancer pathogenesis, more and more targeted therapies have been developed as strategies against the malignancy in recent years. These include therapeutic antibodies, radioimmunoconjugates, and immunotoxins.[28−31] Here, we describe a

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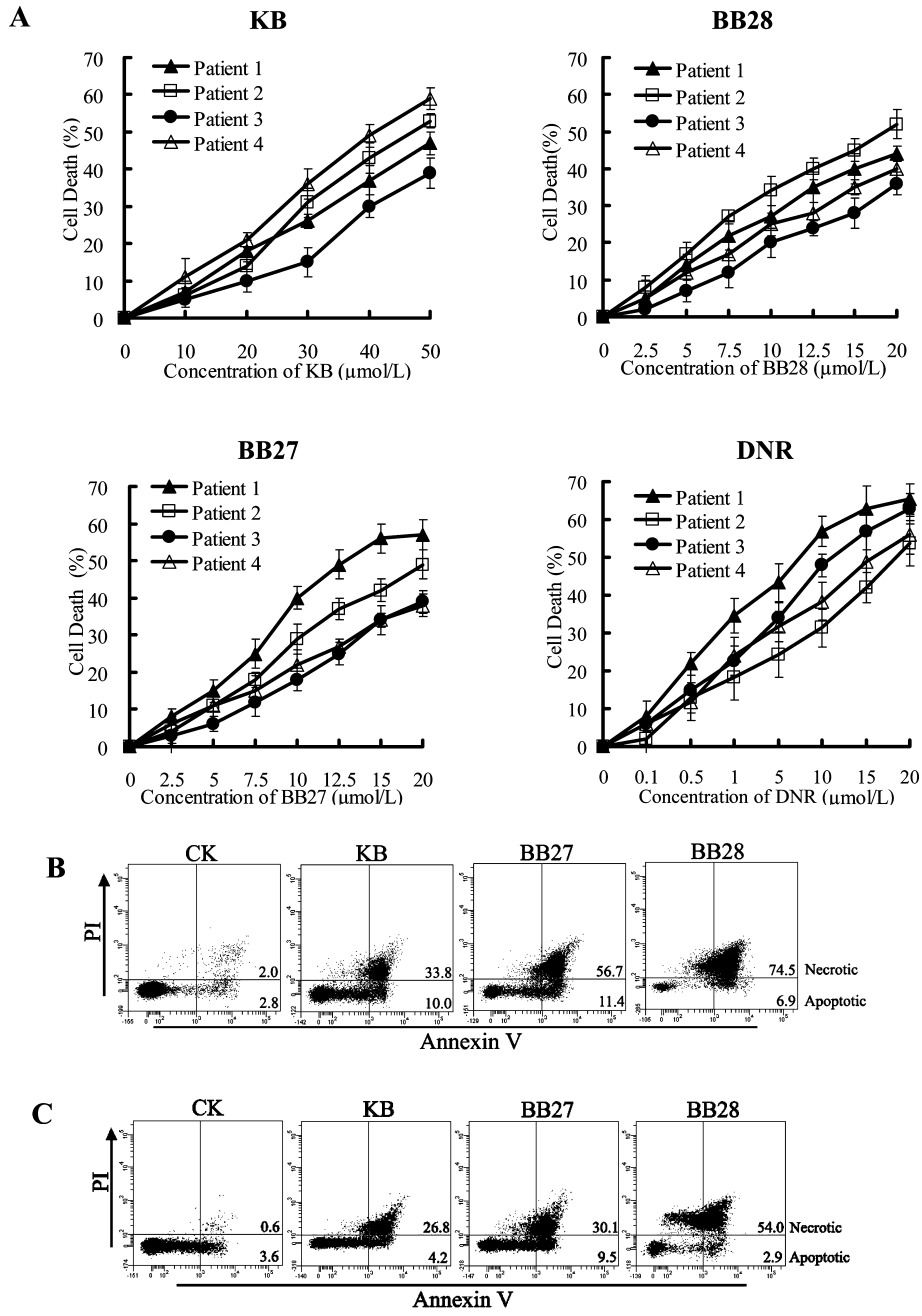

Figure 5. Cytotoxicity of bombesin-directed peptides in primary leukemia cells. A: Cell death induced by KB, BB28, BB27, and DNR. After treatment with peptide, cell viability was determined by using the CCK-8 kit. B, C: FACS analysis of the death in leukemia cells from patient 2 (B) and patient 3 (C). The cells were treated individually with 50 μmol/L KB, 20 μmol/L BB28, and 20 μmol/L BB27, followed by double staining with FITC-Annexin V and PI. The percentage of apoptotic and necrotic cells is indicated.

potential novel approach to the treatment of cancer by induction of caspase-dependent apoptosis using chimeric peptides containing the tumor-homing peptide bombesin and mitochondria-disrupting peptides.

Bombesin receptors are highly expressed in a wide variety of solid tumors,[15,16] and bombesin exhibits ability to deliver radioactive imaging agents[17,18] and chemotherapeutic drugs[19−22] to many solid tumor cells. This prompted our interest in the ability of bombesin to deliver toxic peptides to solid tumor cells. It was reported that KLA, truncated BMAP28 (B28), and truncated BMAP27 (B27) are mitochondria-disrupting peptides.[23−26] These peptides do not randomly penetrate the cytoplasmic membrane of mammalian cells at low concentration. Once internalized by carrier-mediated translocation, they preferentially accumulate in the mitochondria and ultimately induce apoptosis by depolarizing the membrane,[23] suggesting the potential of these peptides as toxic domain. When these peptides were conjugated to bombesin, the cytotoxicity of conjugated KLA, B28, and B27 was

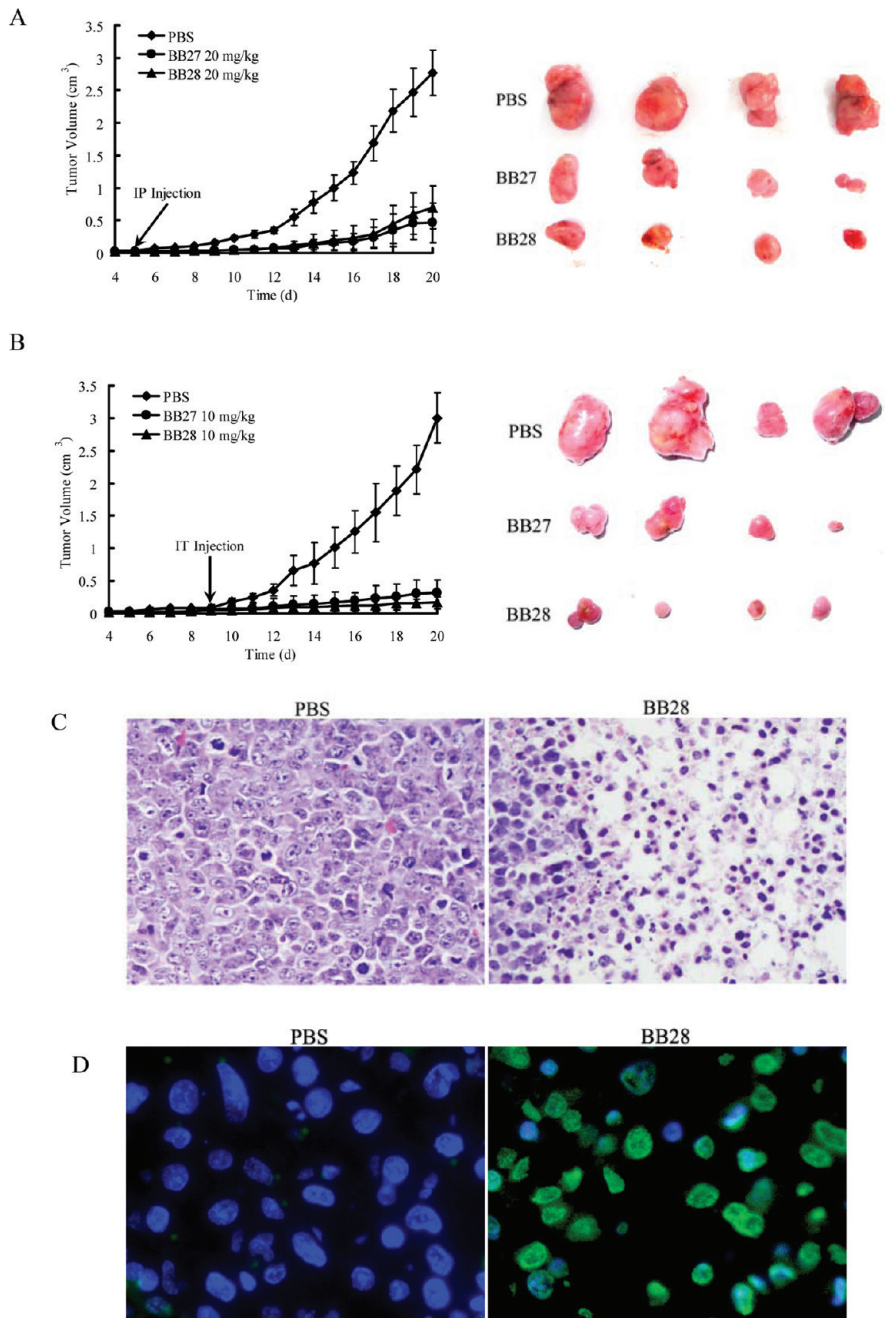

Figure 6. Suppression of tumor growth by BB28 and BB27 *in vivo*. Tumors were established in BALB/c nude mice by subcutaneous injection of K562 cells. Peptides BB28 and BB27 at the indicated concentrations were intraperitoneally (IP) (A) or intratumorally (IT) (B) injected into mice bearing tumor graft for a total of 5 doses (1 dose per day). Equivalent volumes of PBS were injected into the mice in the control group. Tumor volume (cm^3) was calculated as length $\times$ width^2 $\times$ 0.5. To probe the peptide-caused apoptosis *in vivo*, mice bearing K562 xenografts (0.3−0.5 cm^3) were administered 100 μg of BB28. After 24 h, the mice were sacrificed and the paraffin-embedded tumor tissues were stained with H&E (C) and TUNEL (D). The nucleus of cells was stained as blue using DAPI and the TUNEL-positive signals were indicated as green. Original magnification: $\times$400.

increased in solid tumor cells. This indicates that bombesin could deliver these mitochondria-disrupting peptides to solid tumor cells. Moreover, binding analysis showed that bombesin bound to leukemia cells and solid tumor cells with similar high affinity (Figure 1A), suggesting that bombesin could also deliver toxic peptide to hematologic tumor cells. In support of this, leukemia cells were sensitive to the chimeric peptides KB and BB28, but were resistant to the unconjugated KLA (Figure 1C) and B28 (Supplementary Figure 1C in the Supporting Information). It seems that exposure of the C-terminus of bombesin is very important for the cytotoxicity of the chimeric peptide because the chimeric peptide BK, which

contains a blocked C-terminus, failed to kill the cells (Figure 1B,C and Supplementary Figure 1 in the Supporting Information). This coincides with the finding that the C-terminus of bombesin is critical for its receptor binding and activity.[32]

Further investigation on specificity demonstrated that these bombesin-directed peptides (KB, BB28, and BB27) effectively induced apoptosis in tumor cells, but that normal cells were relatively resistant to all three peptides (Figure 2, and Figure 4). The IC_{50} values of these peptides (KB, 10−15 μmol/L; BB27, 3−5 μmol/L; BB28, 4−6 μmol/L) in tumor cells are approximately 5−10 times lower than in normal cells. The cytotoxicity of the chimeric peptides in tumor cells and normal cells is closely related to the specific binding and translocation of bombesin into cancer cells, because replacement of the bombesin sequence with the nonspecific cell-penetrating peptide TAT equalized the cytotoxicity in tumor and normal cells (Figures 2 and Figure 4). The bombesin sequence therefore contributes to the selective cytotoxicity of these peptides.

In addition to the cultured solid tumor cells, these bombesin-directed peptides were also highly cytotoxic in cultured and primary leukemia cells from patients with AML (Figure 5), and both intratumorally and intraperitoneally injected peptides exerted promising therapeutic efficacy on K562 tumor xenografts (Figure 6). These results suggest that the bombesin-targeted delivery of toxic peptides might be potential treatments not only for solid tumors but also for hematologic tumors. Recently, more and more novel peptides that selectively bind to some surface structures of cancer cells have been identified.[33−36] An increasing number of chimeric peptides might be constructed by using these tumor-homing peptides and be applied in cancer targeted therapy.[37,38]

Conclusions

Bombesin binds to solid tumor cells and leukemia cells with high affinity. Bombesin can deliver mitochondria-disrupting peptides to both solid tumor and hematologic tumor cells with similar efficacy. The bombesin-directed peptides described here selectively induce cell death in cultured tumor cell lines and primary leukemia cells. Intratumorally and intraperitoneally injected peptides exert promising antitumor effects *in vivo* without obvious systematic toxicity. Thus, these bombesin-directed peptides might be potentially developed as novel antitumor agents.

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Supporting Information Available: Figure depicting enhancement of B28-induced cytotoxicity in solid tumor cells and leukemia cells after conjugation with bombesin. This material is available free of charge via the Internet at http://pubs.acs.org.

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