The cathelicidin-BF Lys^{16} mutant Cbf-K$_{16}$ selectively inhibits non-small cell lung cancer proliferation *in vitro*

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Received June 20, 2013; Accepted August 2, 2013

DOI: 10.3892/or.2013.2693

Abstract. The 30-amino acid antimicrobial peptide Cbf-K$_{16}$ is a cathelicidin-BF (BF-30) Lys^{16} mutant derived from the snake venom of *Bungarus fasciatus*. Our previous study found that BF-30 selectively inhibited the proliferation of the metastatic melanoma cell line B16F10 *in vitro* and *in vivo*, but had a negligible effect on human lung cells. In the present study, it was demonstrated for the first time that Cbf-K$_{16}$ selectively inhibits the proliferation of lung carcinoma cells *in vitro*, with low toxicity to normal cells. The half-maximal inhibitory concentrations (IC$_{50}$) of Cbf-K$_{16}$ against H460 human non-small cell lung carcinoma cells and mouse Lewis lung cancer cells were only 16.5 and 10.5 μM, respectively, which were much less compared to that of BF-30 (45 and 40.3 μM). Data using a transmission electron microscope (TEM) assay showed that, at 20 and 40 μM, Cbf-K$_{16}$ induced the rupture of the cytoplasmic membrane, which was consistent with data obtained from lactate dehydrogenase (LDH) release assays. The LDH release increased from 17.8 to 52.9% as the duration and dosage of Cbf-K$_{16}$ increased. Annexin V-fluorescein and propidium iodide staining assays indicated that there were no obvious apoptotic effects at the different dosages and times tested. In H460 cells, the rate of genomic DNA binding increased from 51.9 to 86.8% as the concentration of Cbf-K$_{16}$ increased from 5 to 10 μM. These data indicate that Cbf-K$_{16}$ selectively inhibits the proliferation of lung carcinoma cells via cytoplasmic membrane permeabilization and DNA binding, rather than apoptosis. Although Cbf-K$_{16}$ displayed significant cytotoxic activity (40 μM) against tumor cells, in splenocytes no significant inhibitory effect was observed and hemolysis was only 5.6%. These results suggest that Cbf-K$_{16}$ is a low-toxicity anti-lung cancer drug candidate.

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Key words: Cbf-K$_{16}$, anticancer activity, H460, permeabilization, DNA binding

Introduction

Despite the recent advance in therapeutic methods, human cancer remains a leading cause of mortality worldwide (1,2). Moreover, the incidence of many types of cancers, including melanoma, prostate, breast, liver and lung cancer, continues to increase (3,4). Human lung cancer continues to be the leading cause of cancer-related mortality among males in developing countries (5). There are two types of lung cancer: small cell lung cancer and non-small cell lung cancer (NSCLC). Among these, NSCLC (6,7) is aggressive and accounts for ~80-85% (8,9) of all lung cancer cases. The current 5-year survival rate for NSCLC is <15% (10). Research on cancer chemotherapy has focused on the handicaps of chemotherapy, including multi-drug resistance caused by the extensive use of conventional chemotherapeutic agents (9,10). Another handicap is that conventional chemotherapeutic agents, which typically target rapidly dividing cancer cells, are also associated with deleterious side-effects in healthy cells and tissues (3). Although treatment of NSCLC is guided by disease stage, most patients with lung cancer are typically diagnosed at an advanced stage when patients have limited treatment options (11). Thus, it is necessary to develop novel anticancer drug candidates with lower toxicity than conventional chemotherapeutic agents as new treatment strategies.

Antimicrobial peptides (AMPs) have been isolated from a wide range of organisms (12,13) such as prokaryotes, insects, fish, amphibians and mammals (including humans). Most AMPs are cationic and amphiphilic, but they can differ greatly in regards to other characteristics such as sequence, size, structural motifs and the presence of disulphide bonds (14,15). AMPs possess broad antimicrobial activity against bacteria, fungi and viruses. Certain AMPs also present as the first line of defense in the innate immune system (16-18). In addition to the activities mentioned above, the anticancer activity of AMPs has attracted wide attention in recent years. Recent studies have demonstrated that cationic AMPs could play a promising role in fighting various multi-drug resistant tumors as most types of cancer cells have more anionic phospholipids on their external membranes (19).

Cathelicidin-BF (BF-30), isolated from the snake venom of *Bungarus fasciatus*, is an antimicrobial peptide that consists of 30 amino acids (19-21). BF-30 was found to exert broad antimicrobial activity against bacteria and to exhibit excellent inhibitory activity toward the murine metastatic melanoma cell line B16F10, *in vitro* and *in vivo*, as determined in our previous study (22). However, BF-30 had a negligible effect on H460 and Lewis cells, with IC_{50} values of 45 and 40.3 μM, respectively.

Cbf-K_{16} is a mutant of BF-30 that was generated by a Glu^{16} to Lys^{16} substitution, which increases the positive charge of the molecule. In our previous study, Cbf-K_{16} exhibited stronger antimicrobial activity than BF-30, particularly against drug-resistant bacteria. The minimum inhibitory concentration (MIC) of Cbf-K_{16} against *E. coli* BL21 (DE3)-NDM-1 was only 4 μg/ml while the MBC was 8 μg/ml. Cbf-K_{16} displayed MICs of 32 μg/ml against penicillin-resistant *E. coli* and 16 μg/ml against *S. aureus* (21). Previously, we found that Cbf-K_{16} exhibits selective anticancer activity, particularly against lung cancer. Therefore, in the present study, we investigated the anticancer activity of Cbf-K_{16} against human lung cancer *in vitro* and its molecular mechanisms.

Materials and methods

Peptide synthesis. Cbf-K_{16} (KFFRKLKKSVKKRAKKFFK KPRVIGVSIPF) was synthesized by GL Biochem (Shanghai, China) via a stepwise solid phase methodology. The resulting peptide was purified by a Sephadex gel column and HPLC, and the homogeneity of the purified peptide was >98.12%. The synthetic polypeptide was reconstituted in phosphate-buffered saline (PBS, pH 7.4) for subsequent experiments.

Cell lines and reagents. A series of human cancer cell lines [human lung non-small cell carcinoma cell line (H460), human prostate cancer cell line (PC-3), human breast cancer cell line (MCF-7), human hepatocellular carcinoma cell line (HepG2) and human melanoma cell line (A375)], mouse cancer cell lines [mouse lung cancer cell line (Lewis), mouse melanoma cell line (B16) and mouse malignant melanoma cell line (B16F10)] and Madin-Daby canine kidney (MDCK) cells were used to investigate anticancer activity. All of the cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were cultured in either RPMI-1640 medium, DMEM or F12 medium supplemented with 10% fetal bovine serum (FBS) provided by Gibco (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium pyruvate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The DNA extraction kit was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The lactate dehydrogenase kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Annexin V-fluorescein (AV) and propidium iodide (PI) were purchased from Invitrogen (Shanghai, China). Male ICR mice between 6 and 8 weeks of age (weight, 18-22 g) were purchased from the Laboratory Animal Center of Yangzhou University (Yangzhou, China) and acclimatized for 1 week prior to use in the experiment. Animals were provided with continuous standard rodent chow and water and were housed in a rodent facility at 22±1˚C with a 12-h light-dark cycle. All procedures involving animals and their care in the present study were in strict accordance with the protocols approved by the Ethics Committee of the China Pharmaceutical University.

Assay of cell viability. To evaluate the effects of Cbf-K_{16} on cell proliferation, MTT assays were conducted as previously described (22-24). Spleens were collected from the ICR mice under aseptic conditions in 0.1 M PBS, gently homogenized and passed through a 200-mesh sieve to obtain single-cell suspensions that were then treated with erythrocyte lysis buffer and washed three times in PBS to remove the erythrocytes. The resulting splenocytes were resuspended in RPMI-1640 medium containing 10% FBS for further research.

Normal splenocyte and MDCK cells, as well as the tumor cell lines, were collected at the logarithmic growth phase (adherent cells were digested with trypsin) and centrifuged. Tumor cells were seeded into 96-well plastic plates at a density of $5x10^5$ cells/ml 24 h prior to peptide treatments. Cells were then challenged with different doses of Cbf-K_{16} (0, 5, 10, 20, 40 and 80 μM) and cultured for 48 h at 37˚C in a humidified 5% CO_2 atmosphere. An additional 4-h incubation was carried out with 5 mg/ml MTT solution (15 μl/well). The supernatant was then discarded, and 150 μl DMSO was added to each well to dissolve the formazan precipitate by gently shaking, and the optical density at 570 nm was determined by spectrophotometry using a microtiter plate reader. The cell viability was calculated using the following formula: Cell viability (%) = OD_1/OD_2 x 100%, where OD_1 is the absorbance at 570 nm of the experimental group and OD_2 is that of the control group.

Morphological changes in the human lung non-small cell carcinoma cell line H460 as detected by transmission electron microscopy (TEM). Transmission electron microscopy (TEM) was conducted to confirm changes in cellular and mitochondrial morphology, as previously described (25-27). H460 cells were harvested after exposure to Cbf-K_{16} (0, 20 and 40 μM) for 24 h. Glutaraldehyde (2.5%) was added to pre-fix the H460 cells and preserve morphological structure. The samples were washed twice with PBS and post-fixed in 1% osmium tetroxide for 2 h. The cells were then stained with 2% uranyl acetate and dehydrated with ethanol before being embedded in LR White resin. After overnight polymerization at 60˚C, embedded specimens were sectioned and stained with uranyl acetate and lead citrate before examination with a JEM-1011 electron microscope (Jeol, Tokyo, Japan). Experiments were repeated three times.

Cytoplasmic membrane permeability assay based on lactate dehydrogenase release. Increased release of lactate dehydrogenase (LDH) into the medium supernatant occurs when plasma membranes are injured in necrotic cells (28,29). Based on this theory, the effect of Cbf-K_{16} on the membrane integrity of H460 cells, splenocytes and MDCK cells were evaluated using an LDH release assay. H460 and MDCK cells were seeded in 96-well plastic plates at a density of $5x10^4$ cells/well, while splenocytes were seeded at a density of $5x10^5$ cells/well 24 h prior to Cbf-K_{16} treatment. The cells were cultured at 37˚C in the absence or presence of different concentrations of Cbf-K_{16} (0, 20 and 40 μM) for 12, 24 or 48 h. Supernatants

were collected at the indicated times, and LDH activities were assessed according to the kit protocols. Cells that had been ultrasonically disrupted were used as a positive control. The reported results represent 3 independent repeats.

Cell apoptosis assay. Cell apoptosis assays (30-32) were conducted by double staining with Annexin V-fluorescein (AV) and propidium iodide (PI) to investigate whether Cbf-K_{16} induces apoptosis in H460 cells. H460 cells were seeded in 6-well plastic plates ($5x10^5$ cells/well) 24 h prior to Cbf-K_{16} treatments. The medium supernatant in the plates was then replaced, and various concentrations of Cbf-K_{16} (0, 20 and 40 μM) diluted in PBS were added to the plates. After incubation at 37˚C in a humidified atmosphere with 5% CO_2 for 24 or 48 h, the cells were harvested by trypsinization and collected by centrifugation according to the manufacturer's specifications. Briefly, cells were washed three times and then diluted in 100 μl reaction buffer containing 5 μl AV and 1 μl PI, followed by a 15-min incubation. Binding buffer (400 μl) was added to each sample prior to the flow cytometric analysis.

DNA retardation assay. A DNA retardation assay was used for quantitative and qualitative evaluation of the degree of DNA binding by Cbf-K_{16} in the H460 tumor cell line as previously reported, with slight modifications (33). H460 cells were trypsinized and collected at the logarithmic growth phase. According to the manufacturer's protocol, total DNA was isolated using a DNA extraction kit, and the DNA concentration was measured using an ELISA reader at 280 nm. Genomic DNA was mixed with different concentrations of Cbf-K_{16} (0, 5, 10 and 20 μM) at a 1:1 (vol:vol) ratio for 30 min before electrophoretic analysis of DNA ladder formation using a 0.8% agarose gel containing 0.1 mg/ml ethidium bromide and visualized under UV light. DNA levels were quantified based on density analysis using the ImageJ software, and the DNA-binding rate (%) was calculated using the following formula: DNA-binding rate (%) = [1 - (A/B)] x 100%, where A is the average density of the electrophoretic band and B is the average density of the total genomic DNA band.

Hemolysis assay. Hemolytic activity was investigated according to previously reported methods, which were slightly modified (34). The sheep erythrocyte (SRBC) pellet was gently washed three times with cold PBS buffer (pH 7.4), and the erythrocytes were then resuspended in 10 volumes of the same buffer (stock cell suspensions). The cell stock suspensions were diluted 25-fold with the same buffer for a final erythrocyte concentration of 0.4% (v/v). The SRBC suspension was then added to a 96-well microtiter plate (100 μl/well), and increasing amounts of the test samples (from 0 to 40 μM) were added to the erythrocyte solution. After incubation for 1 h at 37˚C, samples were centrifuged at 4,000 x g for 5 min and the absorbance of the supernatant at 540 nm was determined.

Statistical analysis. All of the experiments described above were performed in triplicate. The results were presented as the means ± SD. The Student's t-test was used for two-group comparisons, and a one-way ANOVA was used for multiple comparisons to determine the level of significance between the control and treated groups. A P-value of <0.05 was considered to indicate a statistically significant result.

Table I. IC_{50} values of Cbf-K_{16} against tumor cell lines.

Sources	Tumor cell lines	IC_{50} (μM)
Human	H460	16.5
	PC-3	12.5
	HepG2	35.7
	MCF-7	50.8
	A375	70.3
Mouse	Lewis	10.5
	B16	0.4
	B16F10	7.3

Results

Cbf-K_{16} selectively inhibits growth of the human lung non-small cell carcinoma cell line H460 and the mouse Lewis cell line in a dose- and time-dependent manner in vitro. The effect of Cbf-K_{16} at various concentrations on the growth of different human cancer cell lines (H460, PC-3, MCF-7, HepG2 and A375) and mouse cancer cell lines (B16F10, B16 and Lewis) was examined using an MTT assay. After being exposed to Cbf-K_{16} for 48 h, the cell viability of these tumor cell lines was determined, and the resulting IC_{50} values are reported in Table I. These tumor cells exhibited differing sensitivities to Cbf-K_{16}. Among them, the human non-small cell lung carcinoma cell line H460 and mouse lung cancer Lewis cells were more sensitive, with IC_{50} values of 16.5 and 10.5 μM, respectively. Although IC_{50} values for Cbf-K_{16} against the mouse melanoma B16 and mouse malignant melanoma B16F10 cell lines (0.4 and 7.3 μM, respectively) were less than those against the Lewis mouse lung cancer cell line (10.5 μM), the IC_{50} value for Cbf-K_{16} against the human melanoma cell line A375 (70.3 μM) was much greater than the value against the human non-small cell lung carcinoma cell line H460 (16.5 μM). As shown in Fig. 1A, the viability of these tumor cell lines decreased in a dose-dependent manner as the concentration of Cbf-K_{16} increased. Cbf-K_{16} significantly suppressed the proliferation of all the tested cell lines. We observed that certain cell lines, such as human melanoma cell A375, human breast cell line MCF-7 and human hepatocellular carcinoma cell line HepG2, showed a slowly descending tendency while the others such as human non-small cell lung carcinoma H460 and the Lewis mouse lung cancer cell line and the mouse melanoma B16 cell line showed the opposite trend. In addition, there were significant changes in the morphology and total number of H460 cells treated with Cbf-K_{16} at doses of 20 and 40 μM in comparison to the controls (Fig. 1B). The untreated H460 cells showed a smooth, flattened morphology and a typical growth pattern under phase contrast microscopy. In contrast, the number of H460 cells decreased significantly and the morphology became abnormal following Cbf-K_{16} treatment. The cells displayed shrinkage, abnormal boundaries

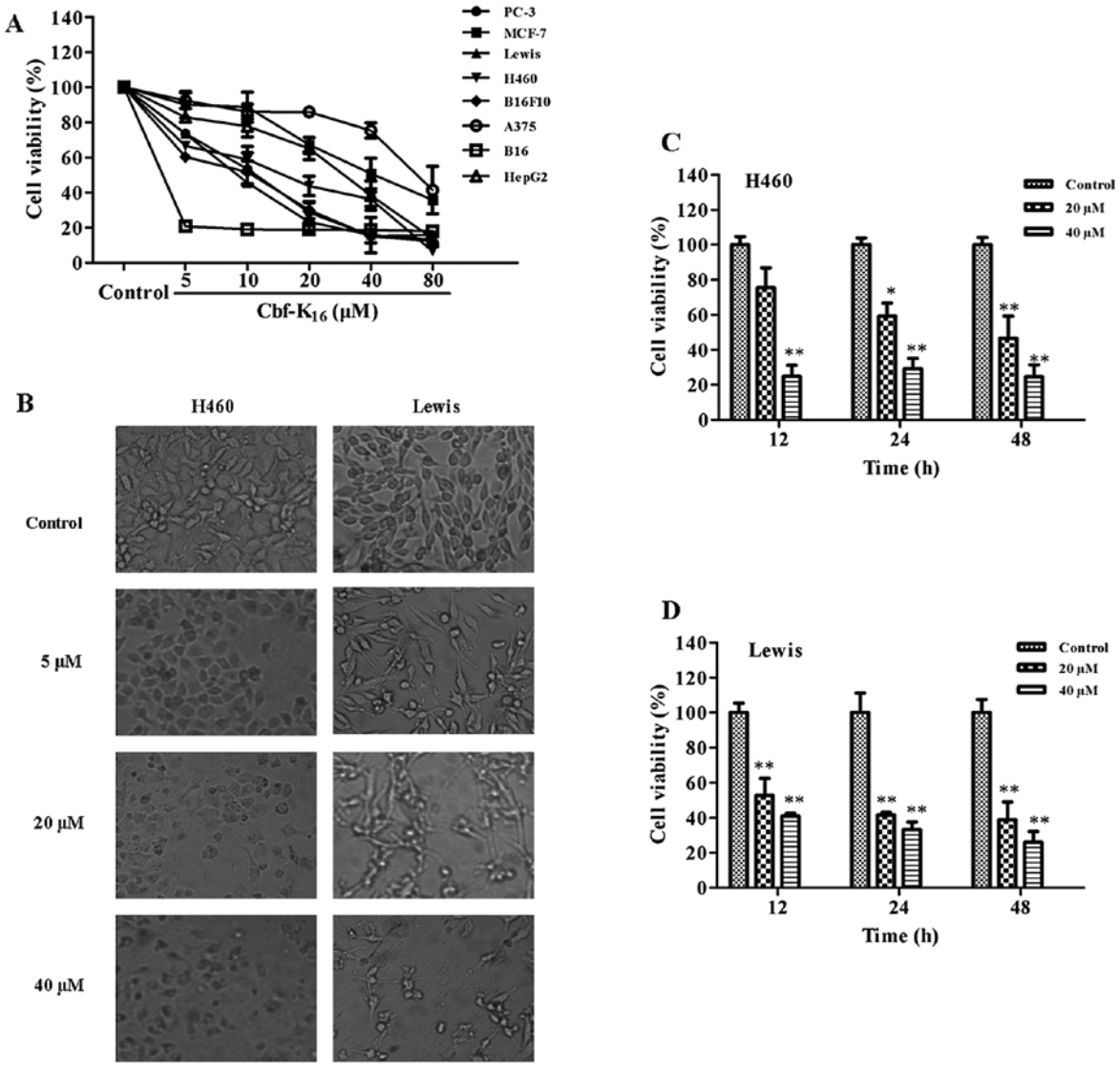

Figure 1. Effects of Cbf-K_{16} on the proliferation of tumor cell lines. (A) The 8 tumor cell lines (H460, PC-3, HepG2, MCF-7, A375, Lewis, B16 and B16F10) were treated with different doses of Cbf-K_{16} (ranging from 0 to 80 μM) for 48 h, followed by an MTT assay. Cbf-K_{16} treatment affected cell viability in a dose-dependent manner. (B) Optical micrographs of the treated H460 and Lewis cells. H460 and Lewis cells treated with Cbf-K_{16} at different dosages (0, 5, 20 and 40 μM) for 48 h were observed and photographed by microscopy. The morphology of H460 and Lewis cells was altered, and cell numbers were significantly reduced, following treatment with Cbf-K_{16}. Representative micrographs are shown (magnification, x200). Inhibition of proliferation of (C) H460 and (D) Lewis cells by Cbf-K_{16} in a dose- and time-dependent manner. H460 cells were treated with Cbf-K_{16} (0, 20 and 40 μM) for 12, 24 and 48 h. Cell viability was measured by an MTT assay following Cbf-K_{16} treatment. Cbf-K_{16} suppressed the proliferation of H460 and Lewis *in vitro* in a dose- and time-dependent manner (*P<0.05, **P<0.01, compared with the untreated control at each time point).

and cellular lysis as the concentration of Cbf-K_{16} increased to 20 μM. As shown in Fig. 1C, the cell viability of the human non-small cell lung carcinoma H460 cells treated with 20 μM Cbf-K_{16} for 12, 24 and 48 h was 75.4, 59.1 and 46.6%, respectively. These results indicated that the effects of Cbf-K_{16} on H460 cells were time-dependent. The same phenomena were noted for Lewis cells (Fig. 1D). Cell viability decreased when cells were treated with 40 μM Cbf-K_{16} for 12, 24 and 48 h, indicating that 40 μM Cbf-K_{16} could kill >80% of the tumor cells. However, cell viability varied significantly, from 46.6 to 24.5%, when cells were treated with dosages of 20 and 40 μM, respectively. These data demonstrated that the antiproliferative effects of Cbf-K_{16} on the lung cancer cell lines H460 and Lewis were dose- and time-dependent and suggest that Cbf-K_{16} selectively inhibits the proliferation of cancer cells.

Cbf-K_{16} inhibits the human lung non-small cell carcinoma cell line H460 by rupturing the cytoplasmic membrane rather than by inducing cellular apoptosis. To investigate the effect of Cbf-K_{16} on the membrane integrity of H460 cells, a transmission electron microscope assay was conducted. As shown in Fig. 2A, when treated with Cbf-K_{16} at 20 and 40 μM doses, H460 cells exhibited condensed and almost ruptured membranes, which resulted in the leakage of the intracellular contents. Furthermore, the mitochondria of H460 cells treated with Cbf-K_{16} were swollen when compared with the control cells. These results indicated that Cbf-K_{16} induced drastic changes in cellular morphology and killed tumor cells via cytoplasmic membrane permeabilization. Furthermore, the LDH activity of the H460 cells increased from 42.4 to 52.9% following a 48-h Cbf-K_{16} treatment at 20 and 40 μM,

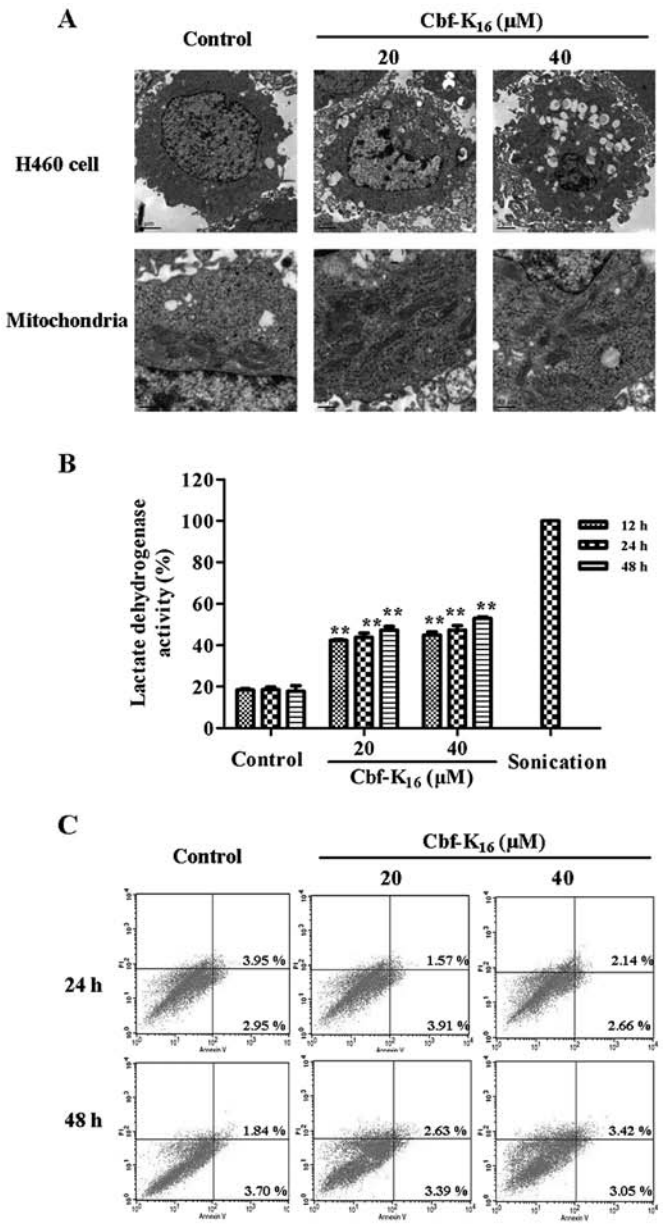

Figure 2. Effects of Cbf-K_{16} on cell membrane permeabilization of human lung non-small cell carcinoma H460 cells. (A) Untreated cells (control group) showed a normal smooth surface, while cells treated with Cbf-K_{16} (20 and 40 μM) for 24 h revealed a disrupted cell membrane by transmission electron microscopy (TEM). (B) The lactate dehydrogenase (LDH) release following Cbf-K_{16} treatment of H460 cells using an LDH assay. Cbf-K_{16} (20 and 40 μM) increased LDH release in a dose-dependent manner at 12, 24 and 48 h. (**P<0.01, compared with untreated control group). (C) Analysis of cell apoptosis of H460 cells by flow cytometry using Annexin V-fluorescein (AV) and propidium iodide (PI) staining. Late apoptotic cells that bind Annexin V and PI are in the upper right quadrant, while early apoptotic cells that bind AV are in the lower right quadrant. There were no significant differences between the control and Cbf-K_{16}-treated H460 cells, which indicated that Cbf-K_{16} does not induce cell apoptosis.

respectively, which was significantly different from the control (17.8%). These data indicate that the H460 membrane was ruptured by Cbf-K_{16} treatment (Fig. 2B) and suggest that the anticancer mechanism of Cbf-K_{16} is partially due to impaired cytoplasmic membrane integrity. AV/PI staining was further conducted to investigate whether apoptosis plays a role in the

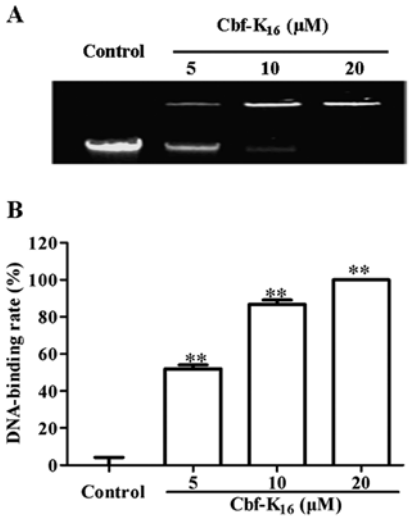

Figure 3. Retarded electrophoretic migration of Cbf-K_{16}-treated genomic DNA from H460 cells. (A) Image of gel retardation assay of genomic DNA from H460 cells treated with Cbf-K_{16}. Genomic DNA extracted from H460 cells was treated with Cbf-K_{16} (0, 5, 10 and 20 μM) for 30 min and analyzed by electrophoresis for 30 min. These results demonstrated that Cbf-K_{16} was able to bind genomic DNA in H460 cells. (B) Densitometry analysis of the relative ratios of genomic DNA levels in the gel retardation assay. The electrophoresis bands were photographed and analyzed using ImageJ software. Cbf-K_{16} blocked the electrophoresis of genomic DNA from H460 cells in a dose-dependent manner (**P<0.01, compared with control group).

anti-H460 activity of Cbf-K_{16}. As shown in Fig. 2C, there was no significant apoptosis of H460 cells following Cbf-K_{16} treatment at 20 μM for 24 or 48 h; cells exhibited late apoptosis rates of 1.6 and 2.6%, respectively. These results indicate that the molecular mechanism of Cbf-K_{16} inhibition of H460 cell proliferation is a loss of cytoplasmic membrane integrity rather than apoptosis.

Cbf-K_{16} bound genomic DNA of the human lung non-small cell carcinoma cell line H460. Given that Cbf-K_{16} impairs the integrity of the cytoplasmic membrane in H460 cells, we next sought to determine whether Cbf-K_{16} interacts with genomic DNA after the rupture of the cytoplasmic membrane. Using a gel retardation assay with an ethidium bromide-stained agarose gel, the electrophoretic mobility of genomic DNA was determined for a series of Cbf-K_{16} concentrations. As shown in Fig. 3A, the forward motion of genomic DNA extracted from H460 cells was inhibited in a dose-dependent manner by Cbf-K_{16}. The quantification of DNA levels by density analysis using ImageJ software is depicted in Fig. 3B. The genomic DNA-binding rate in H460 cells increased from 51.9 to 86.8% as the concentration of Cbf-K_{16} increased from 5 to 10 μM. Additionally, the genomic DNA-binding rate in H460 cells increased to 100% at 20 μM Cbf-K_{16}. This result was consistent with the IC_{50} values listed in Table I. The results detailed above indicate that Cbf-K_{16} selectively inhibits the proliferation of lung cancer cells by rupturing the cytoplasmic membrane and by binding to genomic DNA rather than by inducing cellular apoptosis.

Cbf-K_{16} exhibits hypotoxicity against splenocytes and MDCK cells and limited hemolytic activity. A series of assays were

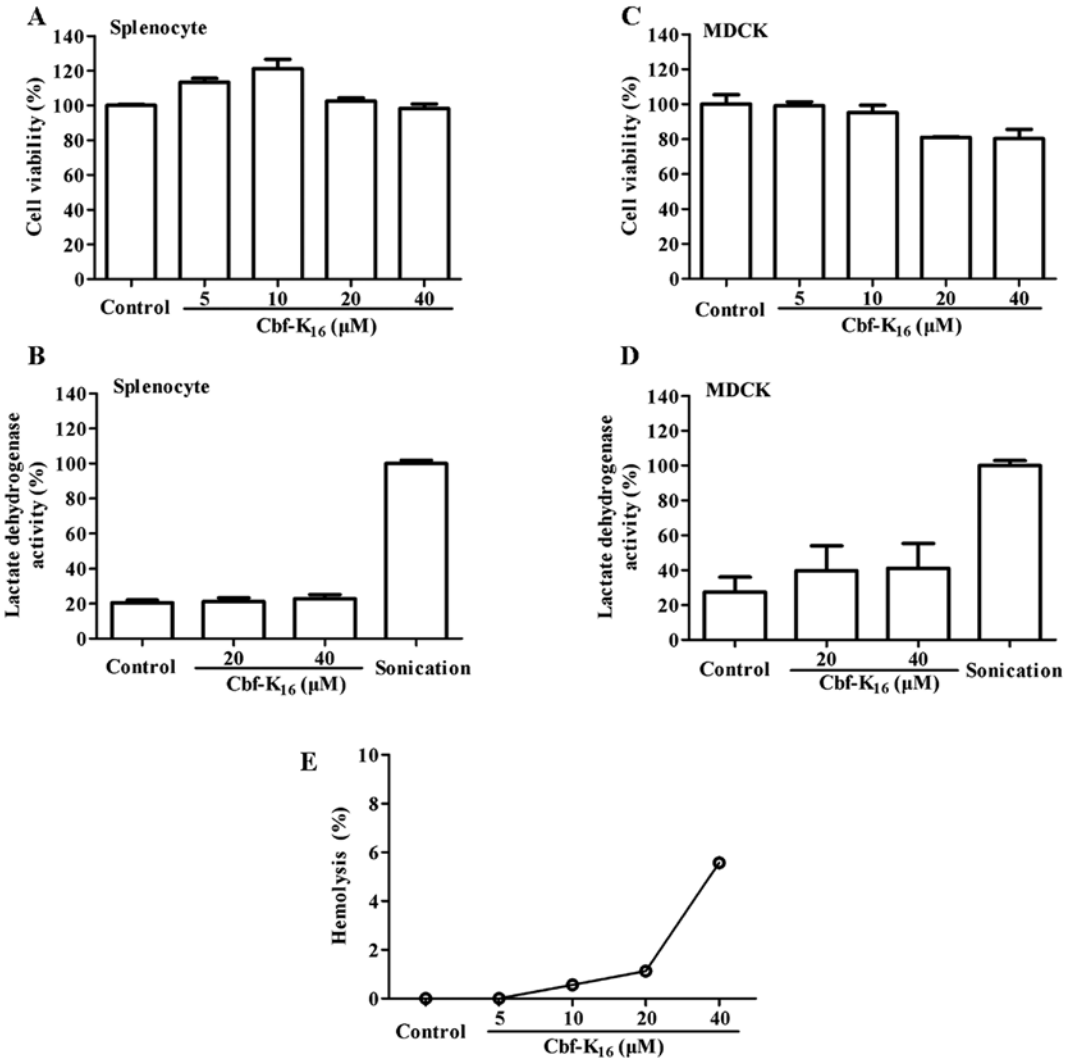

Figure 4. Hypotoxicity of Cbf-K_{16} against splenocytes and MDCK cells and modest hemolysis activity. (A) Splenocytes were treated with different doses of Cbf-K_{16} (ranging from 0 to 40 μM) for 48 h, and cell viability was measured by an MTT assay. (B) No significant increase in the release of lactate dehydrogenase (LDH) from Cbf-K_{16}-treated splenocytes was observed. Splenocytes were treated with different concentrations of Cbf-K_{16} (0, 20 and 40 μM) and LDH activity was measured 48 h after treatment. (C) MDCK cells were treated with different doses of Cbf-K_{16} (ranging from 0 to 40 μM) for 48 h, followed by an MTT assay. (D) Cbf-K_{16}-treated MDCK cells were analyzed by a lactate dehydrogenase (LDH) release assay. Cbf-K_{16} modestly increased LDH release at a 40 μM dose. (E) The hemolysis activities of synthesized peptides were measured by the release of hemoglobin from the pellet of sheep erythrocytes (SRBC). The erythrocytes were incubated with different doses (ranging from 0 to 40 μM) of Cbf-K_{16} for 60 min at 37˚C. There was no significant increase in hemolysis as the doses increased.

implemented to determine whether Cbf-K_{16} induces cellular toxicity in normal cells. As shown in Fig. 4A, Cbf-K_{16} increased the proliferation of splenocytes to 113.3, 121.1 and 102.5% at doses of 5, 10 and 20 μM, respectively; this may have been due to the immunoregulatory activity of Cbf-K_{16}. Compared with the significant cytotoxic activity observed against tumor cells, 40 μM Cbf-K_{16} showed only a modest growth inhibition (<5%) of splenocytes. Moreover, the LDH activities were 21.1 and 22.8% at 20 and 40 μM Cbf-K_{16}, respectively, compared to sonicated cells that were used as a positive control. It should be noted that the LDH activity in the untreated group was 20.5% (Fig. 4B). As shown in Fig. 4C, 20 and 40 μM Cbf-K_{16} resulted in only a modest inhibition of MDCK cells (<20%), while these concentrations showed significant anticancer activity toward lung cancer cells. These data were consistent with the results of the LDH release assay shown in Fig. 4D. These results indicate that the Cbf-K_{16} polypeptide does not induce significant splenic or renal injury at concentrations <40 μM. As shown in Fig. 4E, Cbf-K_{16} exhibited no hemolytic activity at 5 to 20 μM, while 5.6% hemolysis was observed with 40 μM Cbf-K_{16}. In summary, the results above indicate that the Cbf-K_{16} polypeptide (from 5 to 40 μM) selectively inhibits the proliferation of lung cancer cells without harming normal cells.

Discussion

Lung cancer remains the leading cause of cancer-related mortality worldwide. Moreover, non-small cell lung cancer (NSCLC) is a very lethal disease responsible for 80% of all lung cancers. More than a million deaths worldwide are contributed to NSCLC each year, and the 5-year survival rate for NSCLC patients is <15% (35-37). Few treatments, including chemotherapy and radiotherapy, are effective (38,39). Additionally, conventional chemotherapy and radiotherapy are often asso-

ciated with severe side-effects to healthy cells and tissues. Therefore, the most promising drugs are thought to be those with better toxicity profiles, target selectivity and availability for chronic treatment. Cbf-K_{16}, a cationic amphiphilic peptide, is a mutant of BF-30 that was generated by the substitution of Glu^{16} with Lys^{16}, which results in an increase in net positive charge. In our recent study, Cbf-K_{16} was also shown to possess broad-spectrum antimicrobial activity, particularly against drug-resistant bacteria (21); however, its putative anticancer activity had not been elucidated.

In the search for new anticancer agents, antimicrobial peptides and synthetic antimicrobials have recently attracted significant attention owing to their novel mechanisms, decreased likelihood of drug resistance, and low intrinsic cytotoxicity (40,41). Our previous study indicated that BF-30 could selectively inhibit the proliferation of the metastatic melanoma cell line B16F10 without harming normal cells *in vitro* or *in vivo*. Our results indicated that BF-30 had a negative effect on human lung cells (22).

In the present study, we investigated the anticancer activity and mechanism, as well as the toxicity of Cbf-K_{16}. Cbf-K_{16} demonstrated broad-spectrum anticancer activity *in vitro* (Fig. 1). The viability of several tumor cell lines gradually decreased as the concentration of Cbf-K_{16} increased. Furthermore, different tumor cells showed different sensitivities to Cbf-K_{16}, and differences in the membranes of these cancer cells may have contributed to this selective permeability and toxicity, as previously reported by Schweizer (41). Notably, human lung non-small cell carcinoma H460 and Lewis cells were more sensitive to Cbf-K_{16}, with IC_{50} values of 16.5 and 10.5 μM, respectively. Cbf-K_{16} significantly suppressed the proliferation of H460 and Lewis cells in a dose- and time-dependent manner. Furthermore, H460 and Lewis cells displayed cell shrinkage, abnormal boundaries and cell lysis after treatment with Cbf-K_{16} at 20 μM. Thus, Cbf-K_{16} exhibited enhanced cytotoxicity against H460 and Lewis cells compared with BF-30 (45 and 40.3 μM, respectively) (22). Although the IC_{50} values for Cbf-K_{16} against mouse melanoma B16 and mouse malignant melanoma B16F10 cells (0.4 and 7.3 μM, respectively) were less than those against mouse lung cancer Lewis cells (10.5 μM), the IC_{50} value for Cbf-K_{16} against human melanoma A375 cells (70.3 μM) was far greater than that for human lung non-small cell carcinoma H460 cells (16.5 μM). Therefore, considering a practical application in human cancer treatment, we choose lung carcinoma H460 and Lewis cells for further study.

As previously reported, cationic antimicrobial peptides exert their cytolytic activity by folding into an amphipathic helix and inserting into the target membrane, leading to the breakdown of membrane structure, leakage of cell contents, and cell death (15). In the present study, Cbf-K_{16} disrupted the integrity of the cytoplasmic and mitochondrial membranes of lung non-small cell carcinoma H460 cells. This disruption was corroborated by transmission electron microscope images of H460 cells treated with Cbf-K_{16} and by an LDH activity assay of the cell culture supernatant following Cbf-K_{16} treatment (Fig. 2A and B). Cbf-K_{16} may damage H460 cells by binding to the anionic cytoplasmic membrane and spatially separating polar and hydrophobic residues. This conformation facilitates the interaction of Cbf-K_{16} with the membranes of lung non-small cell carcinoma H460 cells and Cbf-K_{16} insertion into the cells, leading to membrane rupture and release of LDH. However, it remains unclear whether Cbf-K_{16} directly or indirectly penetrates the cell membrane of H460 cells. Further experiments are in progress to clarify this issue.

AV and PI staining were used to test whether Cbf-K_{16} induces the apoptosis of lung non-small cell carcinoma H460 cells. As shown in Fig. 2C, there was no significant apoptosis observed when the H460 cells were treated with either 20 or 40 μM (higher than IC_{50}) Cbf-K_{16} for 24 or 48 h. Therefore, the molecular mechanism by which Cbf-K_{16} inhibits the proliferation of H460 cells was not due to apoptosis. Instead, membrane permeabilization and subsequent structural disruption may be one of the main causes by which the Cbf-K_{16} polypeptide kills lung non-small cell carcinoma H460 cells.

Cbf-K_{16} also targeted additional anionic constituents of lung non-small cell carcinoma H460 cells, specifically genomic DNA. The results of our DNA retardation experiment demonstrated that Cbf-K_{16} could bind to genomic DNA from the H460 cells and suppress its electrophoretic mobility in a dose-dependent manner (Fig. 3A). The genomic DNA-binding rate increased to 86.8% as the concentration of Cbf-K16 increased to 10 μM, which was less than the IC_{50}. The genomic DNA-binding rate in H460 cells increased to 100% as the concentration increased to 20 μM, which was higher than the IC_{50}. These data indicated that, at IC_{50}, Cbf-K_{16} significantly binds to genomic DNA. Therefore, Cbf-K_{16} may exert its inhibitory effect on lung non-small cell carcinoma H460 cells by binding to genomic DNA and blocking gene expression. An overall positive charge favors the binding of this antimicrobial peptide to negatively charged membranes through electrostatic attraction, thereby functioning selectivity. Although the precise mechanism of the selective anticancer effect of Cbf-K_{16} has not yet been thoroughly elucidated, our present study demonstrated that Cbf-K_{16} inhibits the proliferation of H460 cells by rupturing the plasma membrane and binding to genomic DNA.

Unlike some antimicrobial peptides with greater intrinsic cytotoxicity, for example NK-18 (11), Cbf-K_{16} exhibited no significant inhibitory effect on the growth of normal cells, including splenocytes and MDCK cells (Fig. 4A and C). As shown in Fig. 4A, Cbf-K_{16} increased the proliferation of splenocytes at concentrations of 5, 10 and 20 μM to 113.3, 121.1 and 102.5%, respectively. This may be due to immunoregulatory activity, as previously reported (42), although the precise mechanism is unknown. At 20 and 40 μM, Cbf-K_{16} showed significant anticancer activity, but exhibited limited inhibition (<20%) against splenocytes and MDCK cells. These data were supported by the results of our LDH activity assays, which indicated that there was no significant toxicity as the concentration of Cbf-K_{16} increased from 0 to 40 μM. LDH activity data at 12 and 24 h are not shown. In addition, Cbf-K_{16} (from 0 to 40 μM) also exhibited no hemolytic activity (Fig. 4E). Taken together, these results indicate that Cbf-K_{16} is not harmful to normal cells.

The selective anticancer effect of Cbf-K_{16} against human non-small cell lung carcinoma H460 cells that ultimately leads to H460 cell death is based on both the formation of channels in the cell membrane and binding to genomic DNA. Although further *in vivo* studies are required to confirm the efficacy of

Cbf-K_{16}, our present research initially suggests that Cbf-K_{16} may be a potential candidate for the treatment of human NSCLC.

Acknowledgements

This research was financially supported by the Scientific and Technological Support and Social Development Plan of Jiangsu Province (SBE201270855), by the Six High Level Talent Project from Jiangsu Province (no. 2011-WSN-048), the '111 Project' from the Ministry of Education of China and the State Administration of Foreign Expert Affairs of China (no. 111-2-07), and the Project Program of the State Key Laboratory of Natural Medicines, China Pharmaceutical University (no. SKLNMZZ201216).

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