Combination of cecropinXJ and LY294002 induces synergistic cytotoxicity, and apoptosis in human gastric cancer cells via inhibition of the PI3K/Akt signaling pathway

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Abstract. The aim of the present study was to investigate the cytotoxic and apoptotic effects of cecropinXJ against human gastric cancer BGC823 cells, either alone, or in combination with a specific phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002. Cell viability and the apoptosis rate were measured using flow cytometry with Annexin-V staining. Additionally, the expression levels of several RAC-α serine/threonine kinase (Akt) phosphorylation-associated proteins and apoptosis-regulating proteins were evaluated by western blot analysis. It was observed that the combination of cecropinXJ and LY294002 resulted in significant synergistic cytotoxic and apoptosis effects, as compared with any single agent alone, in a dose-dependent manner. Corresponding to enhanced apoptosis, the expression levels of certain apoptosis-regulating proteins were changed, the most notable being the upregulation of caspase-3, B-cell lymphoma-2 (Bcl-2)-associated death promotor, Bcl-2 homologous antagonist killer, Bcl-2 interacting killer, Bcl-2-like protein 11, Bcl-2-like protein 4 and cytochrome c, and the downregulation of phosphorylated-Bad and Bcl-2 proteins. The present study provided a novel therapeutic regimen for the use of the cecropinXJ in combination with LY294002 for the treatment of gastric cancer.

Introduction

Gastric cancer is one of the most common types of malignant tumors, with the highest incidence and mortality rates among all malignant tumors globally, and accounting for ~160,000 mortalities annually in China (1). The proportion of postoperative survival >5 years is usually low (2). In addition, ~84% of patients with gastric cancer will exhibit advanced disease with a median survival time of 3-4 months if they are not treated with chemotherapy (1).

Several previous reports have indicated that the pathogenesis of gastric cancer involves complex molecular mechanisms, and multiple genetic and epigenetic alterations to oncogenes and tumor suppressor genes (3,4). Signaling pathway alterations are currently considered to be important in the development of gastric cancer (5). The aberrant activation of signaling systems may cause cancer (6). Among the signaling pathways associated with cell apoptosis, the phosphatidylinositol 3-kinase-(PI3K)-RAC-α serine/threonine kinase (Akt) signaling pathway is currently considered to be important in cell survival, which is closely associated with the occurrence and development of multiple tumors (7,8). Additionally, it serves an important role in promoting the growth, malignant proliferation and metastasis of tumor cells, inhibiting apoptosis, accelerating angiogenesis, and inducing resistance to chemotherapy and radiotherapy (9). As an essential effector in the PI3K/Akt signaling pathway, activated Akt exerts a range of biological effects by facilitating the phosphorylation of downstream substrates, including glycogen synthase kinase-3β (10). It has been demonstrated that the enhanced activity of Akt kinase is present in gastric cancer, and that Akt overexpression is associated with the poor prognoses and high recurrence rates of patients with gastric cancer (11). Therefore, inhibiting the activated PI3K/Akt signaling pathway may effectively inhibit the growth of gastric cancer cells.

LY294002 is the specific inhibitor of PI3K. A previous study demonstrated that LY294002 administered in combination with certain anti-tumor drugs may enhance curative effects and reduce drug resistance (12). CecropinXJ was isolated from the larvae of *Bombyx mori*, which possesses a 37-amino acid cationic antimicrobial peptide with specific amphipathic α-helices (13). CecropinXJ elicits a broad spectrum of effects against bacteria and fungi (14,15). Our previous studies indicated that cecropinXJ exhibits anti-tumor and anti-proliferation activities, and an apoptosis-promoting effect on human gastric cancer AGS (16), and BGC823 cells (17). In addition, apoptosis is mediated by the

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mitochondrial apoptotic pathway. However, the anti-tumor effect of the CecropinXJ antibacterial peptide involves multiple mechanisms. Therefore, the present study primarily aimed to identify the effect of cecropinXJ alone or in combination with LY294002 on the PI3K/Akt signaling pathway in BGC823 cells, and to demonstrate whether they synergistically facilitate the inhibition and apoptosis-promoting effect on cell proliferation, thus providing an experimental basis for a novel treatment of gastric cancer.

Materials and methods

Chemicals and reagents. LY294002, MTT, Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Ins. (Waltham, MA, USA). All other chemicals used were of analytical grade, available locally.

Preparation of antimicrobial peptide cecropinXJ. CecropinXJ of *B. mori* was prepared through the Saccharomyces cerevisiae eukaryotic expression system and purified to homogeneity by a nickel-chelating Sepharose column as described previously (14). The concentration of purified recombinant cecropinXJ protein was detected using the Bradford protein assay method. Prior to use, the peptide was dissolved in DMEM at a concentration of 1 mg/ml and sterilized by filtration through a 0.22 μm filter.

Cell Culture. The human gastric cancer cell line BGC823 was provided by Professor Youyong Lv (Beijing Cancer Hospital, Beijing, China). The BGC823 cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C under a humidified atmosphere in a 5% CO_2 incubator. Cells in mid-logarithmic growth were used for the following experiments.

Cell viability assay. The viability of cells treated with cecropinXJ and LY294002 were measured using an MTT assay. The experiments divided into four groups: Group I, control group (only medium); Group II, cecropinXJ alone treatment (20, 50 or 100 μg/ml); Group III, LY294002 alone treatment (25 μmol/l); Group IV, cecropinXJ in combination with LY294002 treatment. During the logarithmic growth phase, cells were collected and seeded in 96-well plates at a density of $5x10^4$ cells/well, and cultured. Following 12 h of incubation, the cells were treated with medium only, cecropinXJ (20, 50 and 100 μg/ml), LY294002 (25 μmol/l), or cecropinXJ (20, 50 and 100 μg/ml) and LY294002 (25 μmol/l) for 12, 24 and 48 h. Subsequent to treatment, 20 μl MTT solution (5 mg/ml) was added to each well and the cells were then incubated at 37˚C for 4 h. The culture medium was then replaced with 100 μl dimethyl sulfoxide. The absorbance of the solution at 490 nm was measured with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The cell inhibitory rate (%) was calculated as follows: (A490 control-A490 sample)/(A490 control-A490 blank) x100%.

Annexin V/PI staining assay for apoptosis. The cells of the four groups were treated for 24 h. Subsequently, cells were collected following digestion with 0.25% trypsin (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), washed with cold PBS (pH 7.4; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) twice and suspended in 400 μl binding buffer from the Annexin V-FITC/PI apoptosis assay kit (BestBio Biotechnologies, Shanghai, China). Cell suspensions were stained with 5 μl Annexin V-FITC and 10 μl PI and incubated at 4˚C for 30 min in the dark according to the manufacturer's protocol (BestBio Biotechnologies). Cells were analyzed using a FACScan flow cytometer with CellQuest software version 3.0 (BD Biosciences, Franklin Lakes, NJ, USA).

Protein extraction and western blot analysis. Proteins from the cells were extracted using CytoBuster™ protein extraction reagent (Merck Millipore, Darmstadt, Germany) with a cocktail of proteinase inhibitors (Roche Applied Science, Rotkreuz, Switzerland) and a cocktail of phosphatase inhibitors (Roche Applied Science) according to the manufacturer's protocol. The concentration of protein was determined by BCA assay. For western blot analysis, the protein lysates (40 μg/lane) were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% skimmed milk at room temperature for 1 h, incubated with specific primary antibodies against Akt (cat. no., 4691), p-Akt (cat. nos., 4046, 13038), Bad (cat. no., 9239), p-Bad (cat. no., 5284), Bak (cat. no., 12105), Bid (cat. no., 8762), Bik (cat. no., 4592), Puma (cat. no., 12450), Bim (cat. no., 2933), Bax (cat. no., 14796), Bcl-2 (cat. no., 15071), caspase-3 (cat. no., 9664), cytochrome c (cat. no., 11940) and GAPDH (cat. no., 2118; all dilutions, 1:1,000; all from Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight and followed by incubation with horseradish peroxidase-conjugated goat-anti-rabbit (cat. no., 7074) or goat-anti-mouse (cat. no., 7076) antibodies (dilutions, 1:2,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. Subsequent to washing three times with TBST buffer, the protein signals were visualized with an enhanced chemiluminescence immunoblotting detection kit (Invitrogen; Thermo Fisher Scientific, Inc.). Equal loading of samples was confirmed using probes for GAPDH.

Statistical analyses. At least three replicates were performed for each experiment. All data were presented as mean ± SD deviation. The differences between experimental groups were compared using one-way analysis of variance followed by Student-Newman-Keuls. $P<0.05$ were considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Inhibiting effect of cecropinXJ on Akt phosphorylation. To determine whether cecropinXJ reduced Akt activation in BGC823 cells, the expression level of total Akt protein and p-Akt in BGC823 cells 24 h after intervention with different drugs was detected by western blot analysis (Fig. 1). The results demonstrated that the p-Akt protein band was marked

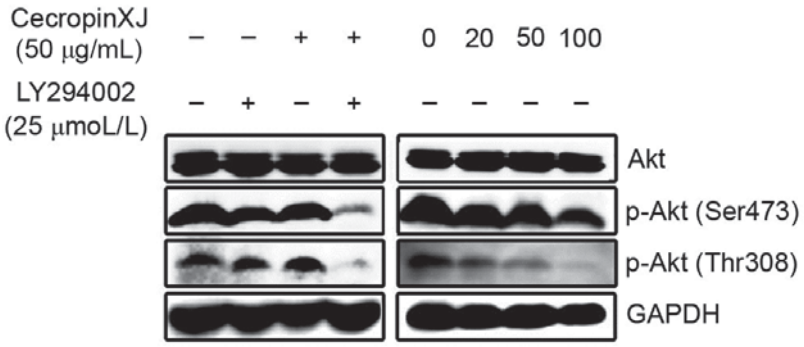

Figure 1. Specific phosphatase activity of cecropinXJ and LY294002 in BGC823 cells. The expression levels of Akt and p-Akt were measured by western blot analysis, and GAPDH was used as a loading control. Akt, RAC-alpha serine/threonine protein kinase; p-Akt, phosphorylated Akt; Ser, serine; Thr, threonine.

in untreated BGC823 cells, while the expression of the p-Akt protein bands in the cecropinXJ treatment and LY294002 treatment groups were downregulated by different concentrations. In addition, the concentration of p-Akt protein in the cecropinXJ treatment group was significantly downregulated in a concentration-dependent manner. Only a faint protein band was observed in the cecropinXJ and LY294002 combined treatment group. The phosphorylation of Akt protein at the Ser473 and Thr308 sites was inhibited, while total Akt protein in each group did not demonstrated any marked changes, indicating that cecropinXJ inhibited Akt activation and consequently the PI3K/Akt signaling pathway. In combination with LY294002, the results suggest that the inhibitory effect of cecropinXJ on the PI3K/Akt signaling pathway was enhanced (Fig. 1).

Inhibiting effect of alone and combined cecropinXJ at different concentrations and LY294002 on cell viability. As summarized in Table I, 12, 24 and 48 h after the treatment of BGC823 cells with cecropinXJ at different concentrations, the viability of cells were significantly inhibited. LY294002 alone exhibited a weak inhibitory effect on cell viability. However, cecropinXJ in combination with LY294002 exhibited a significantly higher inhibitory effect on cell viability compared with cecropinXJ alone. Additionally, the cell survival rate was decreased with the increase of cecropinXJ concentration in a dose- and time-dependent manner. It indicated that the toxic effect of cecropinXJ in combination with LY294002 was greater compared with cecropinXJ alone, which may inhibit the *in vitro* growth of gastric cancer BGC823 cells more effectively.

Enhancement of LY294002 on cecropinXJ-mediated apoptosis. The results of the flow cytometry analysis demonstrated that the apoptosis rate of BGC823 cells in each treatment group was significantly increased in comparison with the control group (Fig. 2; Table II): The early apoptosis and total apoptosis rates of the combined treatment group were significantly higher compared with the two single-treatment groups (Table II), indicating that LY294002 in combination with cecropinXJ exhibited a synergistic apoptosis-promoting effect.

Inhibiting effect of cecropinXJ on Bad phosphorylation. The results demonstrated that p-Akt may phosphorylate Bad. Bad phosphorylation leads to the decomposition of protein complex consisting of anti-apoptotic proteins in the Bad and Bcl-2 protein families, and promotes cell survival (18). The expression level of p-Bad and Bad in the BGC823 cells that were treated with different interventions was detected by western blot analysis (Fig. 3). The results indicated that the p-Bad protein band in the single-compound treatment groups of cecropinXJ and LY294002 was downregulated by varying degrees, and Bad protein expression was markedly upregulated. In the combined treatment group of cecropinXJ and LY294002, no p-Bad protein band was observed and Bad protein expression was slightly upregulated in comparison with the two single-treatment groups. These results indicate that cecropinXJ inhibited the growth of BGC823 cells, mediated by the PI3K/Akt signaling pathway by downregulating Bad phosphorylation.

Effect of cecropinXJ on expression of Bcl-2 family, caspase-3 and cytochrome C. The expression of pro-apoptotic and anti-apoptotic proteins in BGC823 cells was detected by western blot analysis 24 h following different treatments (Fig. 3). The results indicated that the expression levels of pro-apoptotic proteins were upregulated following single treatments with cecropinXJ and LY294002, and that the upregulated expression of pro-apoptotic protein expression levels in the cecropinXJ treatment group was concentration-dependent. In the combined treatment group of cecropinXJ and LY294002, the expression of pro-apoptotic proteins was markedly upregulated in comparison with the two single-treatment groups. Concurrently, cecropinXJ induced apoptosis by activating the mitochondrial pathway, while LY294002, as the inhibitor of PI3K, synergistically increased the proportion of cecropinXJ-induced Bax/Bcl-2, but did not synergistically promote the shearing of caspase-3 and the release of cytochrome C.

Discussion

The PI3K/Akt signaling pathway serves an important in regulating cell proliferation, growth and apoptosis. Due to its important role in cancer, there is great interest in the development of inhibitors able to target on the signaling pathways in preclinical trials. Sukawa *et al* (19) detected the expression of p-Akt in the tissue specimens of 231 patients with gastric

Table I. Inhibitory effects of different concentrations of cecropinXJ in combination with LY294002 on the viability of BGC823 cells.

Group	Dose	Cell viability, %		
		12 h	24 h	48 h
Control	Dulbecco's modified Eagle's medium	100	100	100
CecropinXJ	20 μg/ml	88.96±9.67	75.20±3.27[a]	52.58±6.04[a]
	50 μg/ml	78.80±7.01[a]	68.02±6.39[a]	44.81±2.84[a]
	100 μg/ml	40.46±6.95[a]	35.98±7.38[a]	16.30±1.42[a]
LY294002	25 μmol/l	91.01±11.18	83.51±9.74[a]	57.90±14.33[a]
CecropinXJ and LY294002	20 μg/ml cecropinXJ +25 μmol/l LY294002	70.56±7.10[a-c]	59.36±8.48[a-c]	32.14±10.64[a-c]
	50 μg/ml cecropinXJ +25 μmol/l LY294002	62.23±12.26[a-c]	49.34±9.86[a-c]	19.31±9.30[a-c]
	100 μg/ml cecropinXJ +25 μmol/l LY294002	23.90±11.71[a-c]	16.23±6.95[a-c]	2.64±1.88[a-c]

[a]P<0.01 vs. control; [b]P<0.05 vs. cecropinXJ; [c]P<0.01 vs. LY294002. Data are presented as the mean ± SD, and are representative of an average of three independent experiments.

Table II. Apoptotic effects of cecropinXJ alone and in combination with LY294002 in BGC823 cells.

Group	Apoptosis rate, %		
	Early	Late	Total
Control	3.03±0.78	5.32±1.82	8.35±2.30
LY294002	11.11±2.96	12.14±0.81	23.25±2.42[a]
CecropinXJ	18.03±3.42	32.51±2.55	50.54±5.97[a]
CecropinXJ and LY294002	9.44±0.69	60.91±1.46	70.34±2.15[a,b]

[a]P<0.05 vs. control, [b]P<0.05 cecropinXJ and LY294002 vs. cecropinXJ and LY294002 alone. Data are presented as the mean ± SD, and are representative of an average of three independent experiments.

cancer, and identified that 53% gastric cancer tissue specimens exhibit p-Akt expression and that patients with p-Akt expression demonstrate poor prognoses. An additional previous study suggested that the expression of p-Akt in 45 gastric cancer tissue specimens was up to 82.2%, and associated with tumor growth and metastasis (20).

Phosphorylated Akt exhibits a range of biological effects, including preventing apoptosis and promoting cell survival by phosphorylating substrates containing Ser/Thr residues. At present, several studies have indicated that chemotherapy drugs, including 5-fluorouracil, adriamycin and cis-platinum may increase p-Akt expression levels, and induce gastric cancer cells to generate chemotherapy resistance (21,22). Therefore, a decrease in p-Akt expression may effectively inhibit tumor cell proliferation, promote tumor cell apoptosis and reduce levels of chemoresistance. The present study identified that the phosphorylation level of Akt in BGC823 cells was high. Individual treatment with cecropinXJ or the inhibitor LY294002 inhibited the expression of p-Akt in BGC823 cells to varying degrees, and the expression levels exhibited a dose-dependent decrease while the expression of total Akt protein was not affected. The combined treatment significantly decreased the expression of p-Akt. It was suggested that protein kinases are the target of antibacterial peptides, and certain antibacterial peptides, including defensins, such as human neutrophil peptide (HNP)-1, HNP-2 and HNP-3, are potential inhibitors of protein kinase C (23). Antibacterial protein PR-39 is an antibacterial peptide rich in proline and arginine. It participates in different cell activities, including cell adhesion and migration by combining with adaptor protein p130Cas. In addition, it may also inhibit the activity of PI3K by combining with a subunit of PI3K (24). As Akt is the downstream target of PI3K, the inhibiting effect of cecropinXJ on Akt phosphorylation demonstrates that cecropinXJ may also inhibit the activity of PI3K.

Previous studies have indicated that antibacterial peptides may significantly inhibit gastric cancer development and promote apoptosis (25,26). The mechanism of antibacterial peptides inducing cell apoptosis is complex. Certain studies have suggested that the antibacterial peptides may lyse tumor cells to directly kill cells (27) or induce tumor cell apoptosis through Fas death receptor (28) and the mitochondrial pathway (29). In addition, antibacterial peptides may inhibit the proliferation and growth of tumor cells by regulating multiple signaling pathways, including the PI3K/Akt signaling (30,31),

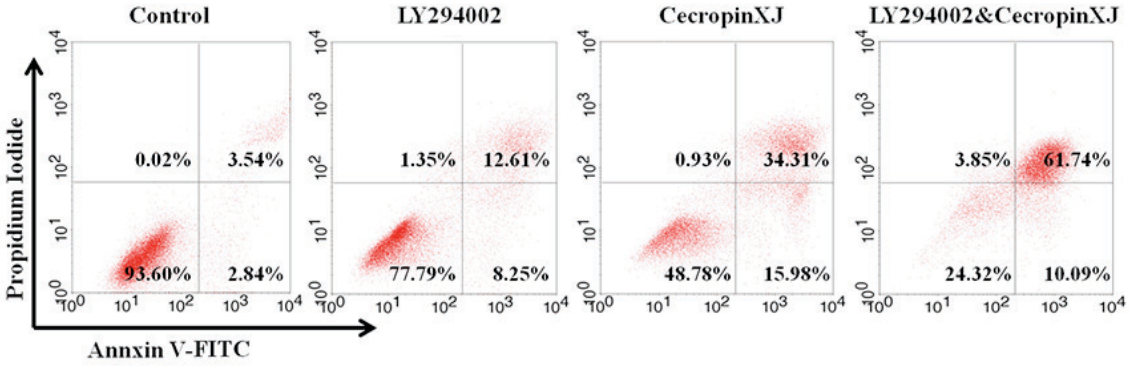

Figure 2. Apoptotic effects of cecropinXJ in combination with LY294002 in BGC823 cells measured by flow cytometry. BGC823 cells were treated with cecropinXJ, LY294002 and cecropinXJ in combination with LY294002. Following treatment, cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry. The percentage (mean ± SD) of apoptotic cells is presented. Data are from 3 independent experiments. FITC, fluorescein isothiocyanate; PI, propidium iodide.

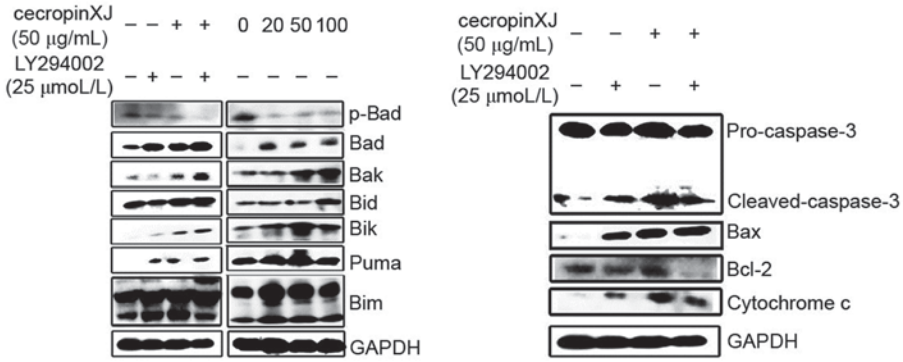

Figure 3. Western blot analyses of BAD, p-BAD, Bak, Bid, Bik, Puma, Bim, caspase-3, Bax, Bcl-2 and cytochrome c expressions following treatment with cecropinXJ alone and in combination with LY294002 in BGC823 cells. Representative data from several independent experiments are presented. Bcl-2, B-cell lymphoma 2; Bad, Bcl-2-associated death promotor; p, phosphorylated; Bak, Bcl-2 homologous antagonist killer, Bid, BH3 interacting-domain death agonist; Bik, Bcl-2 interacting killer; Puma, p53 upregulated modulator of apoptosis; Bim, Bcl-2-like protein 11; Bax, Bcl-2-like protein 4.

mitogen-activated protein kinase signaling (32) and endoplasmic reticulum stress-mediated apoptotic pathways (33). PI3K is a proto-oncogene and LY294002 is its specific inhibitor. *In vitro* and *in vivo* experiments have indicated that LY294002 may inhibit PI3K from phosphorylating Akt in order to inhibit the downstream pathways and increase apoptosis rate (34). In combination with routine chemotherapy and radiotherapy, LY294002 exhibits a synergistic effect on chemosensitization, reduces cytotoxicity, and effectively inhibits tumor cell growth (35,36). Additional studies have revealed that single anti-tumor drugs exhibit lower efficiencies in inhibiting tumor growth and inducing apoptosis compared with multi-targeted inhibition (37,38). In previous years, the combination of inhibitors targeting signaling pathways and anti-tumor drugs has suggested a novel method of tumor treatment, and has gained attention. The present study indicated that cecropinXJ exhibited a significant inhibitory effect on the viability of BGC823 cells, while the inhibitory effect of PI3K-specific inhibitor LY294002 is less significant compared with cecropinXJ. In comparison with cecropinXJ alone, the combination of cecropinXJ and LY294002 demonstrated a significant increased inhibitory effect on cell viability, which was concentration and time-dependent. It was also observed that at the same dose of cecropinXJ, the rate of apoptosis of BGC823 cells was significantly increased following treatment with LY94002, suggesting that LY294002 may promote the inhibitory effect and apoptosis-inducing effect of cecropinXJ on BGC823 cells. This indicates that LY294002 may enhance the sensitivity of BGC823 cells to cecropinXJ and increase cecropinXJ-induced apoptosis of BGC823 cells.

Apoptosis involves the activation and regulation of the expression of a series of genes, including the regulation of the Bcl-2 protein and caspase protein families. Over-activated Akt achieves its anti-apoptotic effects by phosphorylating various substrates such as Bad (39) and caspase-9 (40). Previous studies have revealed that cecropinXJ may inhibit Bad phosphorylation (16,17). p-Bad may combine with Bcl-2 or Bcl-XL on the mitochondrial membrane to prevent the release of cytochrome c from the mitochondria and the activation of caspase-9 (41). A previous study indicated that p-Akt downregulates the pro-apoptotic proteins in the Bcl-2 family, including Bax and Bak, and upregulates the anti-apoptotic proteins in Bcl-2 family, including Bcl-2 and Bcl-XL (42). The inhibition of Akt phosphorylation may significantly downregulate the expression of Bcl-2 and Bcl-XL (43). The results of the present study demonstrated that in BGC823 cells treated with cecropinXJ in combination with LY294002, the pro-apoptotic proteins in the Bcl-2 family, including Bax, were markedly upregulated and

the anti-apoptotic proteins, including Bcl-2, were downregulated. This resulted in an increase in the Bax/Bcl-2 ratio, an increase in mitochondrial membrane permeability, promotion of cytochrome c release and activation of caspase-3 (44). The inhibitory effect on Akt activation of PI3K-specific inhibitors may additionally increase cecropinXJ-induced Bax/Bcl-2 ratio.

In conclusion, the present study provided a novel therapeutic regimen for the use of the cecropinXJ in combination with LY294002 for the treatment of gastric cancer.

Acknowledgements

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