# **BmK CT and <sup>125</sup>I-BmK CT suppress the invasion of** glioma cells *in vitro* via matrix metalloproteinase-2

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Abstract. Chlorotoxin (CTX) is an established blocker of small-conductance Cl channels and has previously been demonstrated to inhibit the invasion of glioma cells. Buthus martensii Karsch chlorotoxin-like toxin (BmK CT) is the first chlorotoxin-like peptide. The present study aimed to determine the inhibitory effect of BmK CT on the invasive ability of glioma cells, using a Transwell assay. BmK CT was subsequently radiolabeled with radionuclide <sup>125</sup>I and its activity was compared with BmK CT. Additionally, the underlying anti-invasive mechanism of BmK CT and <sup>125</sup>I-BmK CT on glioma cells was investigated by ELISA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). It was revealed that BmK CT and <sup>125</sup>I-BmK CT were able to inhibit the invasion of glioma cells and that 125I-BmK CT was superior to BmK CT. Consistent with the results of the Transwell assay, matrix metalloproteinase-2 (MMP-2) secretion by glioma cells was significantly reduced following treatment with BmK CT or <sup>125</sup>I-BmK CT (P<0.05). However, no significant differences in MMP-2 mRNA expression levels were identified by RT-qPCR (P>0.05). In conclusion, the present study demonstrated that BmK CT and 125I-BmK CT reduced the invasion of glioma cells via downregulation of MMP-2 expression. However, inhibition of the invasion of glioma cells was not demonstrated at the mRNA level.

# Introduction

Gliomas are the most common primary intracranial tumors. In the Netherlands, their incidence rate has gradually increased from 4.9 per 100,000 in 1989 to 5.9 in 2010 (1).

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With multiple psychiatric, cognitive and neurological symptoms, gliomas may seriously affect the daily lives of patients. Despite great efforts to improve diagnosis and treatment over the past decades, the prognosis for glioma patients remains poor. According to World Health Organization statistics, the median survival of grade IV glioma patients is 12-14 months (2). Currently, the only effective treatment for patients is neurosurgery followed by chemo- or radiotherapy. However, due to the invasive ability of gliomas, it is unfeasible to remove all glioma cells by surgery, resulting in a high recurrence rate.

Glioma cell invasion is a complex process, involving receptor-mediated matrix adhesion, degradation of extracellular matrix (ECM) and active cell migration into the newly created space. To invade the narrow extracellular space, cells undergo dramatic morphological alterations by reducing their water content and secreting Cl<sup>-</sup>, K<sup>+</sup> or Na<sup>+</sup> (3,4). Therefore, ion channels serve an important role in cell invasion. In China, dried whole scorpion (Quan Xie) has been used to treat apoplexy, epilepsy, migraine and other channelopathies for ~1,000 years. Previous studies suggested that the primary active ingredient of Quan Xie may be associated with its toxicity to ion channels (5,6). Chlorotoxin (CTX) is a neurotoxin isolated from the venom of Leiurus quinquestriatus in 1991 and is an established blocker of small-conductance Clchannels (7,8). It has previously been demonstrated that CTX may specifically bind to glioma Cl<sup>-</sup> channels and thus reduce the invasive ability of glioma cells (9-11). Previous studies have indicated that CTX exerted an anti-invasive effect on glioma cells by reducing the surface expression of matrix metalloproteinase (MMP)-2 and suppressing its enzymatic activity, which was associated with and indirectly regulated the function of the glioma-specific Cl<sup>-</sup> channels (3,12). CTX radiolabeled with radionuclide <sup>125</sup>I or <sup>131</sup>I has been revealed to selectively accumulate in the brains of glioma-bearing mice (13-15). Additionally, <sup>131</sup>I-CTX has been used in patients with recurrent high-grade gliomas to determine its biodistribution, toxicity and therapeutic effect (16).

However, the isolation and purification of CTX remains costly. Therefore, alternatives, and methods for the synthesis of chlorotoxin-like peptides, have been investigated; >70 different toxins have been isolated and identified. *Buthus martensii* Karsch (BmK) chlorotoxin-like toxin (BmK CT) was the first chlorotoxin-like peptide and was isolated from BmK, a

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scorpion widely distributed throughout Asia (17). The amino acid sequence identity of BmK CT reveals ~68% homology with CTX (18,19). Previous studies determined that BmK CT may specifically bind to glioma Cl<sup>-</sup> channels and alter their functional properties, as for CTX (20,21). Fu *et al* (22) demonstrated that BmK CT may additionally suppress the enzymatic activity of MMP-2 in glioma cells.

Previous studies indicated that BmK CT may induce apoptosis of cultured malignant glioma cells *in vitro* (19,20,23). As CTX had previously been radiolabeled with <sup>131</sup>I or <sup>125</sup>I for glioma Single-Photon Emission Computed Tomography (SPECT) imaging and therapy, our previous study radiolabeled BmK CT with <sup>131</sup>I, which revealed that it was superior to BmK CT in inhibiting the growth of glioma cells (24). The present study used various experiments to further evaluate the inhibitory ability of BmK CT and <sup>125</sup>I-BmK CT on the invasion of glioma cells.

## Materials and methods

Cell cultures and reagents. C6 rat glioma cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in Ham's F12K medium (Applichem GmbH, Darmstadt, Germany) supplemented with 15% horse serum and 2.5% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Logarithmic phase cells were used in the experiments. BmK CT was synthesized by the Institute of Protein Research, Shanghai Tongji University (Shanghai, China).

Radiolabeling of BmK CT. BmK CT was labeled with <sup>125</sup>I using the indirect labeling method according to the protocols described in our previous study (24). Following the complicated radiolabeling process, the initial mass concentration of <sup>125</sup>I-BmK CT was hard to calculate, and it was  $<2 \mu g/ml$ . The initial radioactivity concentration of it was 50  $\mu$ Ci/ml. Briefly, 5-10 mCi (0.1-0.2 ml) sterile Na<sup>125</sup>I solution (Shanghai GMS Pharmaceutical Co., Ltd., Shanghai, China) was mixed with 100  $\mu$ g chloramine-T (J&K Scientific Ltd., Beijing, China; dissolved in 200 µl PBS) and 200 µg Bolton-Hunter reagent (J&K Scientific Ltd.; dissolved in benzene) and stirred constantly. Subsequently, the reaction mixture was incubated for 1 min at room temperature. Next, 100  $\mu$ g Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (J&K Scientific Ltd.) and 100  $\mu$ g KI (J&K Scientific Ltd.) were dissolved in PBS, and added to terminate the aforementioned reaction and obtain 125I-labeled Bolton-Hunter reagent (125I-BH). Subsequently, 5 ml N, N-dimethylformamide (J&K Scientific Ltd.) and 200 ml benzene (J&K Scientific Ltd.) were added to extract the <sup>125</sup>I-BH from the reaction mixture. Next, 200  $\mu$ g BmK CT was added to the extracted dry <sup>125</sup>I-BH in a glass tube and incubated in an ice-bath for 30 min with constant stirring. Following this, 50  $\mu$ g glycine (J&K Scientific Ltd.) was added to the aforementioned mixture dropwise to combine with the remaining <sup>125</sup>I-BH. The final reaction mixture was eluted with a PD-10 desalting column (GE Healthcare Life Sciences, Shanghai, China) and 1 ml liquid was collected in each tube. The radioactivity of 30 tubes was determined with a CRC-15R radioisotope dose calibrator (Capintec, Inc., Ramsey, NJ, USA).

Table I. Nucleotide sequences of primers and product size.

Gene	Primer sequence (5'-3')	Product size (bp)
MMP-2	F: ACCGGGATAAGAAGTATGGATT	182
	R: GTCATCATCGTAGTTGGTTGTG	
GAPDH	F: ACAGCAACAGGGTGGTGGAC	252
	R: TTTGAGGGTGCAGCGAACTT	
MMP-2, m	atrix metalloproteinase 2; F, forward; R, reverse	

Cell invasion assays. Cell invasion assays were performed using Transwell inserts (Corning, Inc., Corning, NY, USA) with polycarbonate membrane filters containing  $8-\mu m$  pores, according to the manufacturer's protocol. The upper side of the membrane was coated with Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA). Cell culture media was replaced with serum-free Ham's F12K medium and logarithmic phase C6 cells were treated with 0, 0.15, 0.3 or  $1.5 \mu mol/l$  BmK CT. The cells were incubated at 37°C for 24 h and seeded into the upper chambers at a density of  $4 \times 10^5$  cells/filter in 200  $\mu$ l serum-free medium. The lower chambers were filled with Ham's F12K medium supplemented with 30% horse serum and 10% fetal calf serum as the chemoattractant. Following a 24 h incubation at 37°C, the cells that remained in the upper chamber were scraped off the inserts and the invaded cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. The entire membrane was observed under a light microscope (Olympus Corporation, Tokyo, Japan). The relative inhibition of invasion was expressed as the number of invaded cells/well in the presence of different concentrations of BmK CT. The experiments were performed in triplicate and repeated three times.

Following evaluation of the inhibitory effect of BmK CT on the invasive ability of C6 cells, the effect of <sup>125</sup>I-radiolabeled BmK CT on C6 cells was investigated. The initial radioactivity concentration of <sup>125</sup>I-BmK CT was 50  $\mu$ Ci/ml (<2  $\mu$ g/ml). The cell culture media was replaced with Ham's F12 K medium and C6 cells in the logarithmic phase were treated with <sup>125</sup>I-NaI (0.5, 5 or 50  $\mu$ Ci/ml), BmK CT (0.02, 0.2 or 2  $\mu$ g/ml) or <sup>125</sup>I-BmK CT (0.5, 5 or 50  $\mu$ Ci/ml). The control group was treated with the same volume of Ham's F12K medium. Following a 24 h incubation, the cells were seeded, further incubated, fixed and stained as aforementioned. The experiments were performed in triplicate and repeated three times.

*ELISA analysis.* Logarithmic phase C6 cells were cultured in serum-free Ham's F12K medium and treated with <sup>125</sup>I-NaI (0.5, 5 or 50  $\mu$ Ci/ml), BmK CT (0.02, 0.2 or 2  $\mu$ g/ml) or <sup>125</sup>I-BmK CT (0.5, 5 or 50  $\mu$ Ci/ml). Following a 24 h incubation at 37°C, the supernatants were collected and centrifuged (1,006 x g, 4°C, 15 min) for ELISA analysis. The levels of MMP-2 in the culture supernatants were detected using Rat MMP-2 ELISA kit (cat. no. KB18320; Shanghai Pucheng Biotechnology Company, Shanghai, China) according to the manufacturer's protocol. Each assay was performed in triplicate and repeated three times.



Figure 1. A Transwell assay was used to evaluate the inhibitory effect of BmK CT on the invasive ability of C6 cells. Transwell membranes were observed under a light microscope (magnification: x100) and images captured. For each Transwell membrane, 4 different fields were selected to calculate the invaded cells and the mean number was set as thr final value. (A) Control group ( $125\pm8.9$ ). (B)  $0.15 \mu$ mol/l BmK CT ( $91\pm7.7$ ). (C)  $0.3 \mu$ mol/l BmK CT ( $67\pm5.9$ ). (D)  $1.5 \mu$ mol/l BmK CT ( $35\pm7.9$ ). \*P<0.05 vs. control group. BmK CT, *Buthus martensii* Karsch chlorotoxin-like toxin.

RNA isolation, cDNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In a previous study, BmK CT ~0.2  $\mu$ g/ml demonstrated obvious inhibition of the invasion of glioma cells (22). To elucidate the underlying mechanism, logarithmic phase C6 cells were treated with BmK CT (0.2 µg/ml), <sup>125</sup>I-NaI (5 µCi/ml) or <sup>125</sup>I-BmK CT (5  $\mu$ Ci/ml) and incubated at 37°C for 24 h. The control group was treated with the same volume of Ham's F12K medium. Subsequently, total RNA was extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The purity of the RNA was examined by spectrophotometry (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and had an optical density value (A260/A280) between 1.8 and 2.0. The extracted RNA was reverse-transcribed into cDNA using Prime Script<sup>™</sup> RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). GAPDH served as an internal control. The sequences of the primers for MMP-2 and GAPDH (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China) are presented in Table I. The cDNA products were amplified using Taq polymerase (Takara Biotechnology Co., Ltd.) under the following thermocycling conditions: Denaturation at 95°C for 2 min, annealing at 95°C for 15 sec and extension at 58°C for 1 min, for 38 cycles. All samples were run in triplicate in each experiment. Data were analyzed using the  $2^{-\Delta\Delta Cq}$  method (25).

Statistical analysis. All data were analyzed by one-way analysis of variance with Scheffe's Method using SPSS

software version 18.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean  $\pm$  standard error. P<0.05 was considered to indicate a statistically significant difference. Three independent replicates of each experiment were conducted.

# Results

BmK CT inhibits the migration and invasion of glioma cells. A Transwell assay was conducted to evaluate the inhibitory ability of BmK CT on the invasion of C6 cells. Following staining with crystal violet, 4 different fields (magnification, x100) were examined. The numbers of invaded cells in the four groups were as follows: Control group, 125±8.9; 0.15  $\mu$ mol/l BmK CT, 91±7.7; 0.3  $\mu$ mol/l BmK CT, 67±5.9; 1.5  $\mu$ mol/l BmK CT, 35±7.9. The total numbers of invaded C6 rat glioma cells were significantly reduced following treatment with all concentrations of BmK CT compared with the control group (Fig. 1; P<0.05). These data suggested that BmK CT may inhibit the invasion of glioma cells (P<0.05).

<sup>125</sup>*I-BmK CT is superior to BmK CT in inhibiting the migration and invasion of glioma cells.* Following the validation of the inhibitory ability of BmK CT, Transwell assays were conducted following <sup>125</sup>*I-BmK CT* treatment. As presented in Table II, the number of invaded cells in the BmK CT, <sup>125</sup>*I-NaI* and <sup>125</sup>*I-BmK CT* groups was significantly reduced compared with the control group (P<0.05). Additionally,

Group	Concentration	No. of invaded cells/well	MMP-2 levels (ng/ml)
Control	0	132.0±9.0	59.4±7.2
<sup>125</sup> I-NaI	$0.50 \mu \text{Ci/ml}$	125.0±8.0ª	53.5±4.8°
	$5.00 \mu \text{Ci/ml}$	$117.0\pm10.0^{a}$	51.1±7.2°
	50.00 µCi/ml	$99.0 \pm 7.0^{a}$	48.4±6.8°
BmK CT	$0.02 \mu \mathrm{g/ml}$	119.0±9.0 <sup>a,b</sup>	50.3±8.2 <sup>c,d</sup>
	$0.20 \mu \text{g/ml}$	$86.0 \pm 6.0^{a,b}$	45.0±6.7 <sup>c,d</sup>
	$2.00 \mu \text{g/ml}$	$67.0 \pm 10.0^{a,b}$	39.5±4.8 <sup>c,d</sup>
<sup>125</sup> I-BmK CT	$0.50 \mu \text{Ci/ml}$	$114.0 \pm 11.0^{a,b}$	49.2±5.4 <sup>c,d</sup>
	$5.00 \mu \text{Ci/ml}$	$79.0{\pm}11.0^{a,b}$	33.2±3.5 <sup>c,d</sup>
	50.00 µCi/ml	$56.0\pm 6.0^{a,b}$	27.8±2.7 <sup>c,d</sup>

Table II. Transwell and ELISA assa	ays were performed	l to determine the effect	of <sup>125</sup> I-NaI, BmK C	T and <sup>125</sup> I-BmK CT on C6 cells.
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Data are expressed as the mean  $\pm$  standard error. The initial radioactivity concentration of <sup>125</sup>I-BmK CT was 50  $\mu$ Ci/ml (<2  $\mu$ g/ml). For Transwell assay, <sup>a</sup>P<0.05 vs. control, <sup>b</sup>P<0.05 vs. BmK CT group. For ELISA, <sup>c</sup>P<0.05 vs. control, <sup>d</sup>P<0.05 vs. BmK CT group. BmK CT, *Buthus martensii* Karsch chlorotoxin-like toxin; MMP-2, matrix metalloproteinase 2.

the inhibition of invasion was greater in the <sup>125</sup>I-Bmk CT group compared with the BmK CT group (P<0.05; Table II, Fig. 2A). These findings revealed that <sup>125</sup>I-BmK CT may inhibit the invasion of glioma cells to a greater extent than BmK CT.

*BmK CT and* <sup>125</sup>*I-BmK CT downregulate MMP-2 expression*. MMP-2 protein levels in cell culture supernatants of control, BmK CT, <sup>125</sup>*I-*NaI and <sup>125</sup>*I-*BmK CT groups were quantified. ELISA revealed that MMP-2 secretion in the BmK CT, <sup>125</sup>*I-*NaI and <sup>125</sup>*I-*BmK CT groups was significantly reduced compared with the control group (P<0.05). Additionally, MMP-2 levels were decreased to a greater extent in the <sup>125</sup>*I-*Bmk CT group compared with the BmK CT group (P<0.05; Table II, Fig. 2B).

BmK CT and <sup>125</sup>I-BmK CT have no effect on MMP-2 mRNA expression levels. RT-qPCR was performed to identify the effect of BmK CT and <sup>125</sup>I-BmK CT on MMP-2 mRNA expression levels in C6 cells. The  $2^{-\Delta\Delta Cq}$  value of the control group was set as 1.0, and the values of the <sup>125</sup>I-NaI, BmK CT and <sup>125</sup>I-BmK CT groups were 0.85±0.14, 1.21±0.13 and 0.79±0.05, respectively. No statistically significant differences were identified between the groups.

## Discussion

Gliomas comprise the majority of primary intrinsic brain tumors. As gliomas are fast-growing and highly invasive, they preclude traditional treatments and are associated with high mortality. Therefore, inhibiting proliferation and invasion of glioma cells has become the key target of glioma therapy.

Our previous study determined that BmK CT and <sup>131</sup>I-BmK CT may inhibit the growth of glioma cells *in vitro* (24). The present study used Matrigel to simulate the ECM and a Transwell assay to detect the ability of BmK CT and <sup>125</sup>I-BmK CT to inhibit the invasion of glioma cells. It was determined

that BmK CT and <sup>125</sup>I-BmK CT may inhibit the invasion of glioma cells. Additionally, <sup>125</sup>I-BmK CT was more effective compared with BmK CT.

The mechanism underlying glioma cell invasion is complex and the degradation of the ECM is a key step. MMPs are a group of zinc-dependent proteolytic enzymes that degrade the majority of ECM and promote tumor invasion (26). MMP-2 is a core member of the MMP protease family. It has been established that abnormally elevated MMP-2 expression and activity may be directly associated with cell invasion (27,28). The present study used ELISA to evaluate the effect of BmK CT and <sup>125</sup>I-BmK CT on the secretion of MMP-2 by C6 cells. It was determined that the secretion of MMP-2 by C6 cells was significantly reduced following treatment with BmK CT or <sup>125</sup>I-BmK CT. Additionally, the inhibitory effect of I<sup>125</sup>I-BmK CT on C6 cells was greater compared with that of BmK CT.

Previous studies have focused on the inhibitory effect of BmK CT on the protein expression levels of MMP-2 (22,29). The present study used RT-qPCR to investigate whether BmK CT affected the invasive ability of glioma cells at the mRNA level. However, no statistically significant differences were identified between the groups.

The present study additionally aimed to identify novel therapeutic agents for patients with gliomas. As aforementioned, BmK CT was able to inhibit the growth and invasion of glioma cells. Therefore, BmK CT may be a potential novel therapeutic agent for the treatment of glioma. For the clinical application of BmK CT, Fu *et al* (30) combined BmK CT with LiCl and demonstrated synergistic inhibition of proliferation of high-grade glioma cells. Our previous study suggested that BmK CT radiolabeled with the <sup>131</sup>I radionuclide may be used as a radiotracer for glioma SPECT imaging to localize the lesion range. The present study radiolabeled BmK CT with the <sup>125</sup>I radionuclide, which should kill tumor cells by issuing  $\beta$  rays and thereby strengthen the therapeutic effect of BmK CT on glioma cells.



Figure 2. Effect of BmK CT and <sup>125</sup>I-BmK CT on the invasive ability of C6 cells. The initial radioactivity concentration of <sup>125</sup>I-BmK CT was 50  $\mu$ Ci/ml (<2 $\mu$ g/ml). (A) Results of the Transwell assay. (B) Results of the ELISA assay for matrix metalloproteinase-2. \*P<0.05 vs. control, \*P<0.05 vs. BmK CT group. BmK CT, *Buthus martensii* Karsch chlorotoxin-like toxin.

In conclusion, BmK CT and <sup>125</sup>I-BmK CT may suppress the invasion of glioma cells via downregulation of MMP-2 secretion; <sup>125</sup>I-BmK CT was superior compared with BmK CT. However, no alterations were observed in the mRNA expression levels of MMP-2 in glioma cells.

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