Therapeutic potential of chlorotoxin-like neurotoxin from the Chinese scorpion for human gliomas

Yue-Jun Fu, Li-Tian Yin, Ai-Hua Liang, Chao-Feng Zhang, Wei Wang, Bao-Feng Chai, Jian-Yi Yang, Xiao-Jun Fan

Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Biotechnology, Shanxi University, Taiyuan 030006, PR China

Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Molecular Science, Shanxi University, Taiyuan 030006, PR China

Department of Biology, Shanxi Medical University, Taiyuan 030001, PR China

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Abstract

Chlorotoxin, one of the key toxins in scorpion Leiurus quinquestriatus venom, has been shown to bind specifically to glioma cell surface as a specific chloride channel blocker. In this study, a purified, recombinant chlorotoxin-like peptide from the scorpion Buthus martensii Karsch (named rBmK CTa) was characterized by in vivo and in vitro studies. The results from cell proliferation assay with human glioma (SHG-44) cells showed that rBmK CTa inhibits the growth of glioma cells in a dose-dependent manner, with an IC50 value of approximately 0.28 μM. Under the same conditions, the IC50 value for normal astrocytes increased to 8 μM. This clearly indicated that rBmK CTa had specific toxicity against glioma cells but not astrocytes. Results from whole-cell patch-clamp recording showed that chloride current in SHG-44 was inhibited by rBmK CTa in a voltage-dependent manner and percent inhibitions for the blocking action of rBmK CTa (0.07 and 0.14 μM) on ICl was 17.64 ± 3.06% and 55.86 ± 2.83%, respectively. Histological analysis of rBmK CTa treated mice showed that brain, leg muscle and cardiac muscle were the target organs of this toxin. These results suggest that rBmK CTa may have potential therapeutic application in clinical treatment of human glioma. It represents an approach for developing a novel therapeutic agent.

Keywords: Chlorotoxin-like peptide; Human glioma cells (SHG-44); Chloride current; Scorpion; Buthus martensii Karsch; Therapeutic agent

Buthus martensii Karsch is a widely distributed scorpion species in Asia. A variety of studies have been conducted to identify and purify toxins from this particular species [5]. A chlorotoxin-like peptide gene, BmK CT, was cloned and sequenced from the venom of B. martensii Karsch by Wu et al. and Zeng et al. [14,16]. This 36-mer peptide, cross-linked by four disulfide bridges, shares 68% of amino acid sequence identity to that of chlorotoxin purified from the scorpion Leiurus quinquestriatus [1] (Fig. 1).

Glioma is a highly invasive, rapidly spreading form of brain cancer that is resistant to surgical and medical treatment. In US alone, there are about 36,000 primary brain tumors reported each year, and almost half of these patients have high-grade gliomas. An earlier study indicated that chlorotoxin from the scorpion L. quinquestriatus binds specifically to the surface of glioma cells and inhibits their ability to invade [2]. We previously reported the successful expression and purification of recombinant BmK CTa in Escherichia coli by modifying the BmK CT gene sequence according to the codon usage in E. coli and subcloning into an expression vector pExSecI, in which the IgG-binding domain-ZZ of Protein A is fused to the N-terminal of rBmK CTa [4]. The fusion protein, ZZ-rBmK CTa, expressed in E. coli as a soluble form, was purified to a single band on SDS-PAGE. The rBmK CTa was then separated from the domain-ZZ by cleaving the ZZ-rBmK CTa fusion at an Asn-Gly peptide bond with hydroxylamine, followed by the specific removal of the IgG-binding moiety (domain-ZZ) by passing the cleaved product through a human IgG affinity column. In this study, the property of rBmK CTa was investigated using human glioma cell line to explore the potential application of this chlorotoxin-like neurotoxin in diagnosis and clinical treatment of gliomas.

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Fig. 1. Sequence alignment of BmKCT peptide with the similar short-chain chlorotoxin by matching cysteine residues. NH2-terminus is on the left. Amino acids common to all peptides are in the same panes; gaps are indicated by, chlorotoxin is from *Leiurus quinquestriatus* [16].

DMEM was from Gibco BRL, calf serum was from Hangzhou Evergreen Corp., MTT was purchased from Beijing Xiasi Biotechnology Co. Ltd. All other reagents were of highest grades. Human glioma cell line SHG-44 (grades II and III glioma cell line) was obtained from the cell bank of Shanghai Institute of Life Sciences, and grown in DMEM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 15 mM HEPES and 10% heat-inactivated newborn calf serum. This glioma cell line has been passaged for five times. The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO2. Astrocytes were isolated from rat pups at postnatal day and cultured as described before [2]. All experiments conformed to local and international guidelines on ethical use of animals and all efforts were made to minimize the number of animal used and their suffering.

In this study, normal astrocytes and glioma cells were used for examining the effects of rBmK CTa on cell growth by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. This assay is based on the cellular conversion of a tetrazolium salt (MTT) into a formazan product that is easily detected using a 96-well plate reader. Therefore, MTT assay is simple and sensitive in reflecting cellular survival and cellular growth, useful in cytotoxicity, cell attachment and apoptosis assays. rBmK CTa was initially diluted in PBS and then serial dilutions were prepared in cell culture medium in 96-well microtiter plates (Costar). Cells (5 × 10^4 cells well) were added into wells containing various concentrations of rBmK CTa, and grown for 24 h. At 24 h, cell culture medium in each well was replaced with 200 μl of medium containing 0.5 mg/ml MTT, followed by incubation at 37°C for 3 h. DMSO was added into each well and the optical density for at 570 nm, was measured by a microplate reader (Bio-Rad 550). Percent inhibition (II%) was calculated according to the following equation: \[ II(\%) = (1 - T/C) \times 100\% \], where T and C represents the mean optical density of the treated group and the control group, respectively. Experiments were performed three times and the average was reported. Regression analysis of dose–response curves was used to determine 50% inhibition concentration (IC50) value. In addition, the total DNA electrophoresis analysis of cells SHG-44 after treatment with rBmK CTa was performed. After incubations with the designated concentrations and schedules of rBmK CTa, 5 × 10^6 cells were pelleted. The genomic DNA was extracted, electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining. The gel was photographed under UV light (ultra-violet products Gel Documentation System, Upland, CA, USA).

Membrane potential and currents of chloride, potassium and sodium were recorded with the whole-cell patch-clamp technique in current- and voltage-clamp modes. IC\textsubscript{50}: Previous studies have demonstrated the existence of a glioma chloride channel in human astrocytoma cells that is sensitive to chlorotoxin (e.g., ∼80% inhibition at 600 nM) [10–13]. These results suggest that CITx may serve as glioma-specific markers with diagnostic and therapeutic potential. To identify the nature of this type current, the bath solution contained (in mM): NaCl 125, KCl 5, MgCl\textsubscript{2} 1.2, CaCl\textsubscript{2} 1.0, Na\textsubscript{2}HPO\textsubscript{4} 1.6, NaH\textsubscript{2}PO\textsubscript{4} 0.4, glucose 10.5 and HEPES 32.5, pH 7.4. The pipette solution contained (in mM): KC\textsubscript{1} 145, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 0.2, EGTA 10, HEPES 10 and Na\textsubscript{2}ATP 3, pH 7.4 [7].

![Fig. 2. Effects of rBmK CTa on normal astrocytes (A) and human glioma cell line SHG-44 (B) proliferation. Proliferation assay was performed by MTT test as described in the text. Each point represents the mean of three independent experiments. Curve 1, control group; Curve 2, test group (rBmK CTa). This figure showed that rBmK CTa inhibits the survival of glioma cells in a dose-dependent manner, and the 50% inhibitory concentration (IC\textsubscript{50}) was approximately 0.28 μM (B) while as much as 8 μM on normal astrocytes (A), which demonstrated that the cytotoxicity of this toxin to normal astrocytes must have been low. Internucleosomal DNA fragmentation in SHG-44 cells was readily detected 24 h after treatment with rBmK CTa (0.28 μM).](image-url)
Fig. 3. Effects of rBmK CTa on chloride (A–C), potassium (D–F) and sodium (G–I) current. (A), (D) and (G): Recording prior to the addition of rBmK CTa; (B), (E) and (H): recording 5 min after the addition of rBmK CTa (0.07 M); (C), (F) and (I): recording 5 min after the addition of rBmK CTa (0.14 M).

$I_K$ and $I_{Na}$: The standard extracellular solution was a modified Krebs solution containing (in mM): NaCl 150, KCl 5, MgCl$_2$ 1.1, CaCl$_2$ 2.6, HEPES 10, Glucose 10, titrated with NaOH to pH 7.4. The solution inside the patch pipette ($I_K$) contained (in mM): KCl 65, KOH 5, KF 80, MgCl$_2$ 2, HEPES 10, EGTA 10 and Na$_2$ATP 2, pH 7.5. The standard pipette solution ($I_{Na}$) contained (in mM): CsCl 75, CsF 75, MgCl$_2$ 2, HEPES 10, EGTA 2.5 and Na$_2$ATP 3, pH 7.5.

All experiments were performed at room temperature. Currents were monitored with an EPC-7 patch clamp amplifier. Patch electrodes had a resistance of 2–5 MΩ, while the input resistance of the cells was 1.0 GΩ. An Ag–AgCl wire was used as reference electrode. A holding potential of 0, −80 and −100 mV were chosen, respectively, according to the characterizations of $I_{Cl}$, $I_K$ and $I_{Na}$. Data were analyzed by Origin software (MicroCal Software, Inc.).

Histological analysis was conducted in three adult mice with a body weight of 20 ± 2 g. One mouse received PBS as control, the second mouse received rBmK CTa toxin at a dose of 1.4 μmol/kg, and the third one received rBmK CTa toxin at a dose of 2.8 μmol/kg body weight. PBS and toxin were injected i.p. Kidney, brain, leg muscle and cardiac muscle were dissected 24 h post injection, and histological analysis was performed using standard hematoxylin and eosin (H&E) procedure.

Fig. 4. Current–voltage ($I$–$V$) curve of chloride current. (By SigmaPlot software) $I$–$V$ curve of peak currents obtained for voltage ranges between −105 and +170 mV in 25 mV increments. Chloride currents of glioma cells are sensitive to rBmK CTa. Recordings from five cells were averaged and normalized to whole-cell capacitance prior to (A) and after the addition of rBmK CTa (B and C, 0.07 and 0.14 μM rBmK CTa, respectively).
Several recent studies have suggested that chlorotoxin is a highly specific ligand for malignant human gliomas, without significant binding to normal brain cells [2,6,9]. However, the cytotoxicity and therapeutic characterization of the chlorotoxin-like peptide from the Chinese scorpion _B. martensii_ Karsch have not been studied. In this report, normal astrocytes and glioma cells (SHG-44) were used to examine the effects of rBmK CTa on cell proliferation by a colorimetric MTT assay. The results (see Fig. 2) showed that rBmK CTa inhibited the growth of glioma cells in a dose-dependent manner. The 50% inhibitory concentration (IC50) value was approximately 0.28 µM for glioma cells, but increased significantly (~28-folds) to 8 µM for astrocytes. These inhibition data clearly indicated that rBmK CTa, at very low and potentially safe dose, had specific toxicity effect against glioma cells without significant effect on normal astrocytes. In addition, the DNA electrophoresis of cells SHG-44 after treatment with 0.28 µM rBmK CTa was performed and DNA fragmentation was readily detected 24 h after treatment,

Fig. 5. Representative H&E-stained histochemical analysis results of organs collected from mice treated with toxin rBmK CTa. All figures were 400× original magnification. (A–C) Kidney samples collected from mice treated with 0, 1.4, 2.8 µmol/kg body weight. No damage was observed. (D–F) Brain samples collected from mice with 0, 1.4, 2.8 µmol/kg body weight. The arrows showed the clear signs of apoptosis including cell shrinkage, chromatin condensation, DNA fragmentation, membrane bleeding and formation of apoptotic bodies; (G–I) Leg muscle samples (vertical) collected from mice treated with 0, 1.4, 2.8 µmol/kg body weight. (J–L) Leg muscles (horizontal) collected from mice treated with 0, 1.4, 2.8 µmol/kg body weight. (M–O) Cardiac muscle samples collected from mice treated with 0, 1.4, 2.8 µmol/kg body weight. The boxes showed some break of membrane in sarcous fibres. The harms of rBmK CTa in cardiac muscle and leg muscle was most likely due to the up-regulation of voltage-gated chloride channels (CLC family) in the muscle tissues, which had higher affinity for rBmK CTa.
which resulted in a ladder pattern—a sign of apoptosis (Fig. 2B). This is a novel aspect in the biological properties of the Chinese scorpion neurotoxin and an important step in elucidating the underlying molecular mechanisms of its antitumor action.

In order to explore the mechanism of rBmK CTA, whole-cell patch-clamp recording analysis was performed with gliomas cells. As shown in Fig. 3, chloride current of gliomas cells (SHG-44) was inhibited by rBmK CTA. On the other hand, rBmK CTA did not cause any inhibition for potassium and sodium currents. Effects of rBmK CTA at 0.07 and 0.14 μM (corresponding to 1/4 IC50 and 1/2 IC50, respectively) on chloride current—voltage (I–V) relationship was examined between −105 and +170 mV in 25 mV increments. As shown in Fig. 4, the chloride current was inhibited by 17.64 ± 3.06% at 0.07 μM rBmK CTA and 55.86 ± 2.83% (n = 5) at 0.14 μM rBmK CTA.

In an earlier study, the inhibitory effect of glioma chloride current was reversible after eluting the chamber with external solution for 3 min. This was identical with the predicted function (a short-chain glioma chloride channels blocker) based on its high sequence homology with chlorotoxin [15]. In this paper, whole-cell patch-clamp recording analysis showed that chloride current of gliomas cells (SHG-44) was observably inhibited under control conditions in the presence of rBmK CTA, but this inhibition was not presented in potassium current and sodium current, which demonstrates that it was a glioma chloride channels blocker, but not potassium and sodium channel blocker.

To evaluate the safety of rBmK CTA, mice were given to two doses of toxin by intraperitoneal injection. Twenty-four post injection, organs were examined for damages. Fig. 5 shows the histological analysis results of four organs collected from mice treated with PBS (control), 1.4 and 2.8 μmol/kg of rBmK CTA. Voltage-gated chloride channels have been implicated as being important for cell proliferation and migration. Hence, those voltage-gated chloride channels in normal cells may be inhibited by rBmK CTA (a short-chain voltage-gated chloride channel blocker) in regulating cell volume in the context of cell proliferation and migration. Moreover, hematoxylin–eosin stain is a useful method for general histology. Basophilic nuclei are stained “blue” with hematoxylin. And some signs of apoptosis in nuclei can be detected in this assay. Therefore, the following three parameters were measured for each organ: cell membrane, nucleus and cell interval (Table 1). Firstly, the results indicated there are different degrees of damage for brain, leg muscle and cardiac muscle in toxin treated mice. On the other hand, there is no detectable damage for kidney. Thirdly, although penetration of extrinsic proteins through intact blood–brain barrier is very difficult, observed responses (or damage for brain) using this toxin were promising. Two logic important features were presumed [2]: (i) rBmK CTA is a small, 36 amino acid, peptide that can cross blood and tissue barriers, the effects of rBmK CTA were observed within 1 day post injection. (ii) It can binds to brain glioma cells by the high affinity action power with voltage-gated chloride channels. Fourthly, the damage in cardiac muscle and leg muscle caused by rBmK CTA was most likely due to the up-regulation of voltage-gated chloride channels (CLC family) in these muscle tissues, which have higher affinity for rBmK CTA.

The voltage-gated channels are specifically upregulated in glioma membranes and endow glioma cells with an enhanced ability to transport Cl−. This may in turn facilitate rapid changes in cell size and shape as cells divide or invade through tortuous extracellular brain spaces [8]. Based on the findings presented in this study, it is clear that the chlorotoxin-like peptide rBmK CTA has specific affinity for binding to glioma cells and is capable of inhibiting the chloride channel and proliferation. The inhibitory effect of rBmK CTA on glioma cell proliferation is potentially due to the mechanism of apoptosis.

It is interesting to note that the toxin rBmK CTA, alone or conjugated with radioisotope, could be a potential agent for treating human gliomas. Along this line, the recent study [2] had identified matrix metalloproteinase-2 (MMP-2) as a specific receptor for chlorotoxin. MMP-2, a proteinase involved in tumor invasion, is specifically up-regulated in gliomas and related cancers, but is not expressed in normal brain cells. Recent reports from TransMolecular, Inc. indicated that 131I-chlorotoxin is safe in a phase 2 human clinical trial study (see news release on June 05, 2006 from www.transmolecular.com). Currently, we are working on the generation antibodies against rBmK CTA and the identification of specific receptors for toxin rBmK CTA on glioma cells [3]. In our study, polyclonal antibodies to the purified toxin were raised in rats. Overlay assay and pull-down assay showed that this toxin specially binds to two proteins in the glioma cells with corresponding molecular weights of about 80 and 35 kDa. They may serve as candidate receptors or alternative cellular component for interaction with rBmK CTA.

Table 1

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<tr>
<th>Tissues</th>
<th>rBmK CTA concentrations (μmol/kg body weight)</th>
<th>Histological parameters</th>
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<td></td>
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<td>Cell membrane</td>
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<tr>
<td>Kidney</td>
<td>1.4</td>
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<td>2.8</td>
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<tr>
<td>Brain</td>
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<td>Cardiac muscle</td>
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* The following three parameters were measured: cell membrane, nucleus and cell interval. Degree of damage: –, non-significant; +, minor; ++, significant.
In conclusion, the findings presented in this study are essential for the further exploration of this peptide. It represents an approach for developing a novel therapeutic agent and a potential clinical treatment of human gliomas cancer.

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