Review

Chlorotoxin: Structure, Activity, and Potential Uses in Cancer Therapy

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ABSTRACT:

Chlorotoxin is a disulfide-rich stable peptide from the venom of the Israeli scorpion Leiurus quinquestriatus, which has potential therapeutic applications in the treatment of cancer. Its ability to preferentially bind to tumor cells has been harnessed to develop an imaging agent to help visualize tumors during surgical resection. In addition, chlorotoxin has attracted interest as a vehicle to deliver anti-cancer drugs specifically to cancer cells. Given its interesting structural and biological properties, chlorotoxin also has the potential to be used in a variety of other biotechnology and biomedical applications. Here, we review the structure, activity and potential applications of chlorotoxin as a drug design scaffold. © 2015 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 106: 25–36, 2016.

Keywords: *scorpion venom; peptides; cancer; drug delivery; drug development*

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INTRODUCTION

Scorpion venom contains a complex mixture of peptides and other molecules produced by the animal to paralyze prey or protect against predators.[1,2] These peptide toxins can interact with a wide variety of pharmacological targets, causing either inhibition or activation depending on the specific toxin and target.[2–4] In recent years, peptides have attracted interest as promising templates for drug development,[5–7] with scorpion venom peptides gaining a significant following due to their extraordinary specificity and selectivity for therapeutic targets.[8,9] Scorpion toxins are considered to be promising drug candidates for the treatment of cancer;[10] primarily due to the discovery of chlorotoxin, a peptide from the venom of the giant Israeli scorpion *Leiurus quinquestriatus*,[11] which can preferentially bind to cancer cells.[12] In the time since chlorotoxin was first reported, several reviews have been published describing its potential as an imaging agent as well as a platform to specifically target cancer cells, with the focus having been on glioblastoma, a very aggressive type of brain tumor.[13–18] Here, we discuss chlorotoxin with respect to its structure and potential uses in cancer detection and treatment.

Chlorotoxin: From Primary to Tertiary Structure

Scorpion venom peptides are typically small, with a compact and stable structure, and are typically classified according to three criteria: (i) their molecular size (short-chain peptides or long-chain peptides); (ii) the presence or absence of disulfide bonds; and (iii) their activity on ion channels, including sodium, potassium, calcium or chloride channels.[19–21]

The primary structure of chlorotoxin comprises 36 amino acids,[11] including eight cysteines, and it is therefore classified as a short-chain, disulfide-containing peptide. Other scorpion

peptides with a similar primary structure to chlorotoxin have been collectively referred to as chlorotoxin-like peptides. A sequence alignment of selected chlorotoxin-like peptides, shown in Table I, illustrates that the sequence is generally well-conserved across species, with the segments between amino acids 1 to 9 and 30 to 35 being the most conserved. The segment that follows the last cysteine, as well as that between Cys^{V} and Cys^{VI}, are the most variable.

The secondary structure of chlorotoxin was first determined using nuclear magnetic resonance (NMR) spectroscopy in 1995.[22] The NMR data showed that chlorotoxin comprises an α-helix, formed by amino acids 11 to 21, and three β-sheets, formed by amino acids 1 to 4, 26 to 29, and 32 to 36 (Figure 1).[22] Based on the predictive values of proton-proton distances between cysteines, the disulfide bonds were deduced to be Cys5-Cys28, Cys16-Cys33 and Cys20-Cys35, with the disulfide bond Cys2-Cys19 linking the N-terminus with the last segment of the α-helix. A recent study utilizing partial reduction and alkylation of the disulfide bonds of chlorotoxin analogues supports this connectivity.[23] This disulfide bond connectivity produces three loops: the longest contains seven amino acids and connects the first β-sheet with the α-helix; the second loop, which connects the α-helix with the second β-sheet, contains four amino acids; and the last loop connects the second and third β-sheets and contains just two amino acids.

In addition to deducing the disulfide connectivity and secondary structure of chlorotoxin, Lippens and colleagues used the αH NMR chemical shifts of synthetic chlorotoxin to derive information about the backbone topology[22] (Figure 2). As an aside, we note that the αH chemical shifts that our group reported for chlorotoxin[24] are in agreement with those reported by Lippens and colleagues, with the exception of Asp18, which we believe reflects a typographical error for the shift of this residue in the Lippens paper. We studied the αH NMR chemical shifts at two pH values and found no significant differences in shifts recorded at pH 2.8 and 5.5, suggesting that the structure is stable across this range of pH values (Figure 2). In the Lippens et al. study, the solution structure of chlorotoxin was determined by distance geometry and simulated annealing using 2D ^{1}H NMR spectroscopy. Seven of the lowest energy structures, which showed no NOE violations greater than 0.3 Å, were chosen to represent the structure of chlorotoxin (PDB: 1CHL).[22] As expected, the structure confirmed the presence of the α-helix and the three β-sheets as well as the disulfide bond pairing.

Disulfide bonds often occur within well-defined structural motifs (such as the CSα/β or ICK motif),[25] and typically have a role in stabilizing peptides by reducing their conformational flexibility.[26] Disulfide-rich peptide cores can also play a role in activity by forming a framework that is able to display critical amino acid residues.[27] The disulfide bond configuration of chlorotoxin has some parallels to both the cystine-stabilized α/β motif (CSα/β motif)[22] and the inhibitor cystine knot motif (ICK motif). The CSα/β motif is common to short-chain scorpion toxins, including the chlorotoxin-like peptides (Table I and Figure 3). It is defined by an α-helix and an antiparallel triple-stranded β-sheet connected by two disulfide bonds, Cys^{II}-Cys^{V} and Cys^{III}-Cys^{VI}.[28] A third disulfide bond, Cys^{I}-Cys^{IV}, is present among members of the CSα/β family, although this bond is not thought to be necessary for maintaining the structure.[29–31] In chlorotoxin, Cys5-Cys28, Cys16-Cys33 and Cys20-Cys35 (Cys^{II}-Cys^{VI}, Cys^{III}-Cys^{VII} and Cys^{V}-Cys^{VIII}) form the CSα/β motif and Cys2-Cys19 (Cys^{I}-Cys^{IV}) is considered to be an "extra" disulfide bond.[22] The ICK motif incorporates an antiparallel β-sheet stabilized by a cystine knot.[32,33] The cystine knot is formed by two disulfides (Cys^{I}-Cys^{IV}, Cys^{II}-Cys^{V}) that form a ring, with a third disulfide (Cys^{III}-Cys^{VI}) penetrating the ring to form the knot (Figure 3). In chlorotoxin, the bonds Cys2-Cys19, Cys5-Cys28 and Cys16-Cys33 (Cys^{I}-Cys^{IV}, Cys^{II}-Cys^{VI} and Cys^{III}-Cys^{VII}) follow this configuration, and Cys20-Cys35 (Cys^{V}-Cys^{VIII}) is considered to be an "extra" connection. ICK peptides have very good chemical, thermal and biological stability, as they are resistant to extremes of pH and temperature as well as enzyme digestion, making them promising templates in drug design.[34,35] Several reviews have been published describing ICK peptides and their potential applications in more detail.[36,37]

Despite some parallels to both motifs, a recent study showed that the disulfide bond connectivity in chlorotoxin behaves more like a CSα/β motif than an ICK motif.[23] In this study, four analogues, each lacking one disulfide bond, were chemically synthesized by replacing each bridge by a pair of L-α-aminobutyric acid residues. The results showed that the disulfide bonds Cys^{I}-Cys^{IV} and Cys^{II}-Cys^{VI} (Cys2-Cys19 and Cys5-Cys28, respectively) were not essential for the formation of its tertiary structure; whereas, the absence of Cys^{III}-Cys^{VII} or Cys^{V}-Cys^{VIII} (Cys16-Cys33 and Cys20-Cys35, respectively) led to difficulties in obtaining a native-like product (the process of oxidative folding is described below). This finding has been reported for other members of the CSα/β family,[30] supporting the conclusion that the configuration of chlorotoxin is more closely related to the CSα/β motif than the ICK motif. Interestingly, the data showed that despite being unimportant for the overall structure, chlorotoxin analogues lacking either Cys^{I}-Cys^{IV} or Cys^{II}-Cys^{VI} had higher flexibility and lower stability in human serum, when compared with the native peptide.[23]

Chlorotoxin: Synthesis and Folding

The amount of chlorotoxin present in venom is limited, and so an alternative source of the peptide is necessary for biochemical

Table I Amino Acid Sequences of Selected Chlorotoxin-Like Peptides

Peptide	Amino acid sequence	UniProt #	Organism
	I *II* *III* *IV* *V* *VI* *VII* *VIII*		
CTX	M C M P C F T T D H Q M A R K C D D C C G G K G R G K C Y G P Q C L C R - - -	P45639	*Leiurus quinquestriatus quinquestriatus*
Bs8	R C K P C F T T D P Q M S K K C A D C C G G K G K G K C Y G P Q C L C - - - -	P15229	*Hottentotta tamulus sindicus*
I4	M C M P C F T T D H N M A K K C R D C C G G N - - G K C F G P Q C L C N R - -	P60269	*Mesobuthus eupeus*
Lqh 8/6	R C S P C F T T D Q Q M T K K C Y D C C G G K G K G K C Y G P Q C I C A P Y -	P55966	*Leiurus quinquestriatus hebraeus*
I3	M C M P C F T T D H Q T A R R C R D C C G G R - - G R C C F G Q C L C G Y D -	P60268	*Mesobuthus eupeus*
I5A	M C M P C F T T D P N M A K K C R D C C G G N - - G K C F G P Q C L C N R - -	P15222	*Mesobuthus eupeus*
MeuClTx	M C M P C F T T D H N M A K K C R D C C G G N - - G K C F G Y Q C L C N R G -	P86401	*Mesobuthus eupeus*
GaTx1	- C G P C F T T D H Q M E Q K C A E C C G G I - - G K C Y G P Q C L C N R - -	P85066	*Leiurus quinquestriatus hebraeus*
I5	M C M P C F T T D P N M A N K C R D C C G G G - - K K C F G P Q C L C N R - -	P60270	*Mesobuthus eupeus*
I1	M C M P C F T T R P D M A Q Q C R A C C K G R - - G K C F G P Q C L C G Y D -	P15220	*Mesobuthus eupeus*
Bm12-b	G C G P C F T T D A N M A R K C R E C C G G N - - G K C F G P Q C L C N R E -	Q9BJW4	*Mesobuthus martensii*
BmK CT	G C G P C F T T D A N M A R K C R E C C G G I - - G K C F G P Q C L C N R I -	Q9UAD0	*Mesobuthus martensii*
AaCtx	M C I P C F T T N P N M A A K C N A C C G S R - R G S C R G P Q C I C - - - -	P86436	*Androctonus australis*
MeuClTx-1	M C M P C F T T R P D M A Q Q C R D C C G G N - - G K C F G Y Q C L C N R - -	P86402	*Mesobuthus eupeus*
Bs14	- C G P C F T K D P E T E K K C A T C C G G I - - G R C F G P Q C L C N R G Y	P59887	*Hottentotta tamulus sindicus*
AmmP2	- C G P C F T T D P Y T E S K C A T C C G G R - - G K C V G P Q C L C N R I -	P01498	*Androctonus mauretanicus mauretanicus*
BtlTx3	R C G P C F T T D P Q T Q A K C S E C C G R K G - G V C K G P Q C I C G I Q Y	P81761	*Mesobuthus tamulus*

Cys residues are highlighted in yellow and are numberd I to VIII. The sequence that comprises the α-helix is shown in a red box and the β-strands in black boxes. The disulfide conectivities are shown as solid lines at the bottom of the table. Chlorotoxin is abbreviated as CTX.

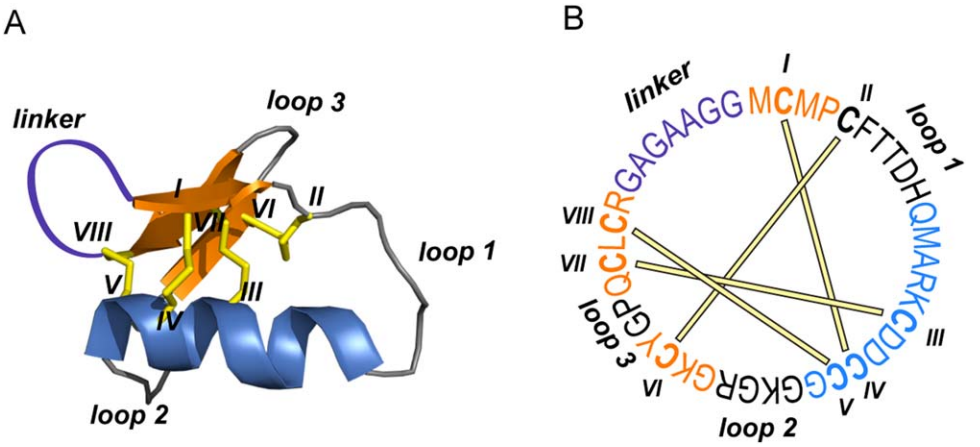

FIGURE 1 A: Three-dimensional structure of chlorotoxin (PDB: 1CHL). The disulfide bonds are shown in stick format (yellow), the β-sheet in arrow format (orange), and the α-helix in blue. B: Sequence representation of cyclic chlorotoxin. The disulfide bond connectivities are represented by yellow lines, and the cysteines are labeled using Roman numerals (I−VIII). The extra sequence (linker) for the cyclic form is shown in violet.

and biophysical studies, as well as for potential therapeutic applications. Chlorotoxin has been successfully synthesized and folded *in vitro* using solid-phase peptide synthesis (SPPS). SPPS involves the attachment of one amino acid to a solid polymer support or "resin",[38] before sequential coupling of the subsequent amino acids to complete the sequence. Following synthesis, the peptide is cleaved from the resin to produce a linear reduced form and purified using reverse-phase high performance liquid chromatography (RP-HPLC).[24] The reduced peptide is then oxidized overnight at room temperature in a folding buffer before being purified using RP-HPLC.[24] For analogues of chlorotoxin, variations to the standard folding buffer, such as the addition of isopropanol or dimethylsulfoxide, have been used to improve the yield.[23]

Chlorotoxin can also be recombinantly expressed. This method is particularly useful for large-scale production, which can become costly if using SPPS.[39] Chlorotoxin with either a His-tag (His-CTX) or His-tag plus a glutathione transferase (GST)-tag (His-GST-CTX) has been expressed in the *E. coli* strain BL21 Star™ (DE3), and purified using ion affinity chromatography.[39] The GST-tag was used to reduce peptide degradation and improve solubility. Whereas most of the expressed His-CTX was found in inclusion bodies, about 60% of the expressed His-GST-CTX was found in the supernatant of lysed bacteria. In that study, chlorotoxin was initially S-sulfonated before oxidative refolding. The yield of purified chlorotoxin was ~200 μg per liter and ~2 mg per liter for the His-CTX and His-GST-CTX constructs, respectively.[39] His-CTX was also expressed in *E. coli* by Deshane and coworkers, who showed that both native chlorotoxin and His-CTX displayed similar biological activities.[40]

Chlorotoxin: Biological Activities

Insecticidal Activity. Chlorotoxin belongs to the peptide family of insectotoxins, named for their selective paralytic activity on insects and other invertebrates.[11] For example, in crayfish, chlorotoxin produced a failure of motor control, which progressed to rigid paralysis.[11] When administered to American cockroaches, similar results were observed.[11] These paralytic effects have been also described for I1, I3, I4, I5 and ButaIT, which are all members of the chlorotoxin-like peptide family (Table I). Although the molecular target responsible for these effects has not yet been reported, the degree of paralysis has been shown to depend on the amount of peptide administered.

Tumor Binding Activity. The tumor binding activity of chlorotoxin was first studied using a ^{125}I-labeled peptide (^{125}I-

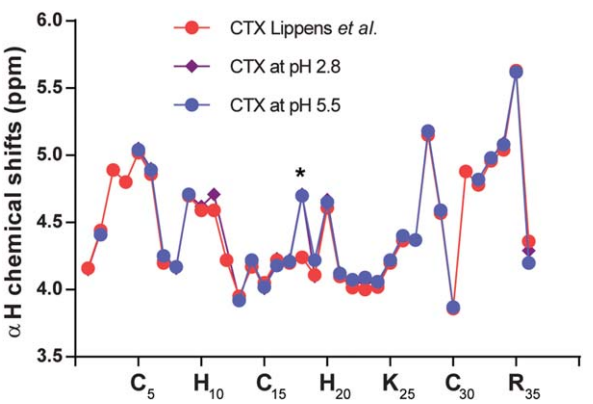

FIGURE 2 A comparison of the αH chemical shifts of chlorotoxin at pH 2.8 and 5.5 with those given by Lippens et al.[22] Asp18 is marked with an asterisk.

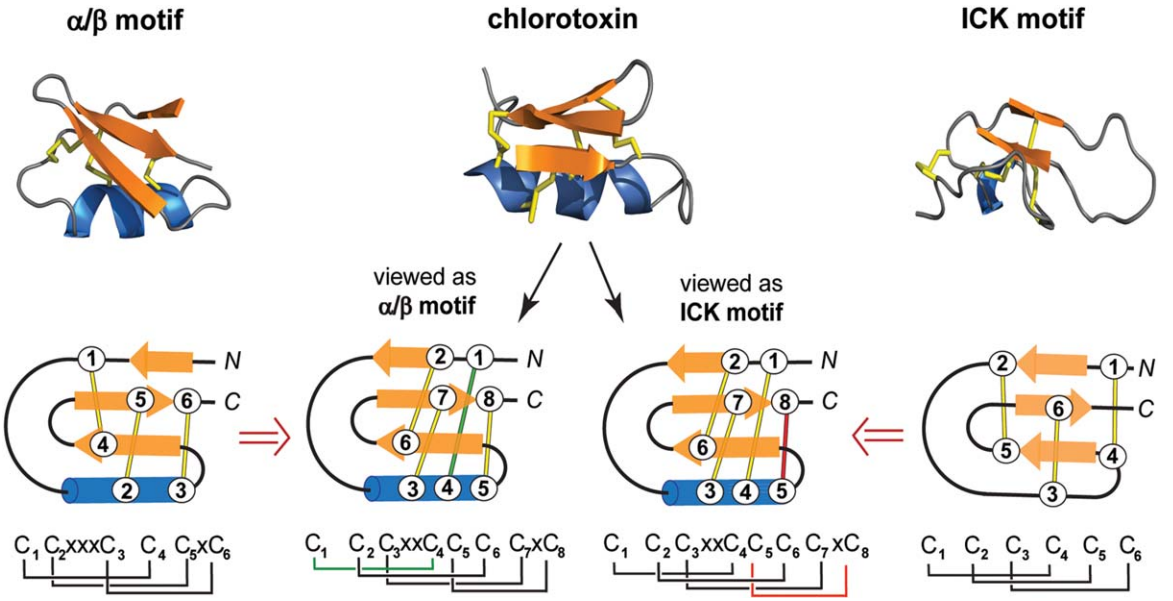

FIGURE 3 3D structure and graphical representation of the CSα/β motif, chlorotoxin and the inhibitor cystine knot (ICK) motif. The disulfide bonds are shown in stick format or lines (yellow), the β-sheet in arrow format (orange), and the α-helix in blue. Top: 3D structure of *charybdotoxin* (PDB: 2CRD), chlorotoxin (PDB: 1CHL) and maurocalcine (PDB: 1C6W). Bottom: Schematic representation of the CSα/β motif, chlorotoxin and the ICK motif. The disulfide bond connectivities for each motif are shown at the bottom of the panel, where "C" means cysteine and "x" shows the conserved spacing between cysteines. The cysteines are labeled 1 − 8. The extra disulfide bond for the CSα/β and ICK motifs is represented by a green and a red line, respectively.

CTX).[41] The study showed that, after the administration of ^{125}I-CTX to tumor-bearing mice, the peptides accumulated within the tumor. In the same study, ^{125}I-CTX was shown to bind glioma cells, but was unable to bind normal rat astrocytes and Te671, a human rhabdomyosarcoma cell line.[41] In another study, chlorotoxin was found to bind tumors of neuroectodermal origin (tumors that share a common embryonic origin with cells from the central nervous system).[12] Chlorotoxin conjugated with biotin bound specifically to >200 biopsy samples of malignant glioma and other tumors of varying stages, including melanomas, neuroblasotomas, meduloblastomas, and small cell lung carcinomas, but was unable to bind to normal tissues from brain, skin, kidney and lung. In the same study, chlorotoxin was unable to bind samples that presented other, nontumorigenic, neurological diseases such as Parkinson's disease and Alzheimer's disease.[12] In vivo studies, using chlorotoxin conjugated to a fluorescent dye (Cy5.5, BLZ-100, 800CW), have further confirmed the specific binding of chlorotoxin to tumors.[42–44]

Antiangiogenic Activity. Chlorotoxin also displays antiangiogenic properties; *in vitro* it has been shown to inhibit the migration and invasion of glioma cells and human umbilical vein endothelial cell (HUVEC) migration.[45,46] Using a Transwell migration assay, chlorotoxin inhibited the migration of U251MG (glioma) cells, with an IC_{50} of 600 n*M*.[45] In another study using the same approach, chlorotoxin inhibited the migration of HUVECs, the only normal cell line chlorotoxin was found to affect, but only when the cells were stimulated with VEGF and bFGF[46]—growth factors implicated in the regulation of proliferation, migration and differentiation of endothelial cells. In the same study, using the chick chorioallantoic membrane (CAM) assay, chlorotoxin inhibited vessel formation in a dose-dependent manner induced by a range of stimuli, including VEGF, bFBF, LPS, EGF, IL-6, and TNFα. Chlorotoxin (100 μM) administration did not show any toxic effects, suggesting that inhibition of migration of HUVECs was due to a mechanism other than cell death.[46]

Chlorotoxin: Molecular Targets

Chloride Channels. As its name suggests, chlorotoxin was first described as a chloride channel inhibitor.[11] In this report, the inhibitory activity was observed only when it was applied to the intracellular face of reconstituted small-conductance chloride ion channels from rat epithelia and embryonic rat brain (with a K_d of 1.15 μ*M* at −20 mV).[11] In later reports, the

inhibitory effect of chlorotoxin on chloride channels present in human brain cancer cells was described.[47,48] Another study, performed to evaluate a new type of astrocytes found in injured adult rat brains, showed that chlorotoxin could inhibit calcium-activated chloride currents with an EC_{50} of ~50 n*M*.[49] Contrary to these studies, Maertens and colleagues showed that chlorotoxin does not inhibit volume-regulated, calcium-activated and cyclic AMP-activated chloride channels expressed in various human, bovine and monkey cells using concentrations of up to 1.2 μ*M*.[50] Given the discrepancies between the studies, we cannot clearly conclude that chlorotoxin is a potent inhibitor of chloride channels. It may be that chlorotoxin is not a general inhibitor, and instead acts on a specific sub-type of chloride channel. This possibility is supported by a report of a glioma-specific chloride channel found to be sensitive to chlorotoxin, and could be responsible for the specific binding of chlorotoxin to tumor cells.[51]

Matrix Metalloproteinase-2. Matrix metalloproteinase-2 (MMP-2), a zinc-dependent enzyme able to degrade structural proteins of the extracellular matrix during cancer invasion of normal tissue,[52,53] has been proposed to be the molecular receptor of chlorotoxin on glioma cells.[40] The authors of this study showed that recombinant His_6-CTX co-purified with MMP-2 from glioma cells using affinity purification (MMP-2 was not detected in normal brain tissue).[40] Chlorotoxin also inhibited the enzymatic activity of MMP-2 in a dose-dependent fashion and reduced MMP-2 surface expression. Moreover, chlorotoxin did not bind to MMP-1, MMP-3, or MMP-9, which are also expressed by glioma cells.[40] In addition, when the purified proteins (MMP-1, MMP-2, MMP-3, and MMP-9) were probed against His_6-CTX, only MMP-2 was able to react with chlorotoxin.[40] In another study, the binding of chlorotoxin to cancer cells was reduced in the presence of MMP-2 inhibitors, and when MMP-2 was overexpressed in MCF7 cells the binding of chlorotoxin was higher to transfected cells than non-transfected cells.[44]

Annexin A2. Recently, annexin A2, a member of the annexin superfamily of calcium-dependent phospholipid-binding proteins,[54] was proposed as a molecular target for chlorotoxin in tumor and vascular endothelial cells.[55] The A2 complex, which is comprised of annexin A2 and the protein p11,[56,57] is overexpressed at the cell surface in many human cancers and is correlated with poor prognosis.[58,59] Using siRNA knockdown for annexin A2 in Panc-1 cells, a pancreatic tumor cell line, the binding of chlorotoxin to the surface of the cells was abolished.[55]

Chlorotoxin: Cell-Penetrating Properties

Cell-penetrating peptides (CPPs) are typically short, positively-charged peptides of less than 30 amino acids, which can cross the cell membrane. CPPs have attracted significant interest because they can deliver a range of cargoes, including therapeutics, into cells to target intracellular targets.[60–62] The mechanisms of cellular internalization of CPPs can be roughly divided into energy-independent mechanisms, such as translocation (temperature-independent, passive and direct penetration), and endocytic pathways, such as micropinocytocis[63,64] or clathrin-mediated endocytosis (temperature-dependent and active transport).[65]

Soroceanu and coworkers were the first to demonstrate cellular internalization of chlorotoxin.[41] The authors observed that ~60% of the peptide was internalized after a 1-h incubation at 37°C, compared to only ~10% after a 3-h incubation at 4°C.[41] This finding suggests that the main mechanism by which chlorotoxin is internalized is likely to be dependent upon endocytic mechanisms.

Wiranowska et al. investigated the uptake of chlorotoxin,[66] and found, using fluorescent confocal microscopy and immunocytochemistry, that U373 human glioma cells could internalize chlorotoxin in a dose-dependent manner.[66] The study found that internalization of chlorotoxin was unaffected by inhibitors such as filipin, an inhibitor of cholesterol-dependent caveolar cell transport, or amiloride, an inhibitor of nonselective transport by macropinocytosis; however, it was affected by chlorpromazine, an inhibitor of clathrin-mediated intracellular transport. Wiranowska et al. found that internalization of chlorotoxin by normal human dermal fibroblasts (NHDF) cells was affected by amiloride, suggesting that, in this case, macropinocytosis was the mechanism of entry. The authors concluded that chlorotoxin has different cell entry mechanisms: clathrin-mediated entry for glioma cells and macropinocytosis for normal cells, and both mechanisms require membrane receptors to internalize chlorotoxin.[66]

Interestingly, chlorotoxin is not the only scorpion toxin to have cell-penetrating properties. For example, maurocalcine from the venom of *Scorpio maurus palmatus*,[67] which is an agonist of the type-1 ryanodine receptor,[68] has been reported as a CPP. Esteve and colleagues reported that, in contrast to chlorotoxin, maurocalcine penetrates cells through an energy-independent mechanism.[69] They showed that neither lower temperature (4°C) nor the presence of specific inhibitors of pinocytosis and endocytosis (amiloride, nystatin) affected the uptake of maurocalcine into HEK293 cells.[69] Recently, the cell-penetrating properties of maurocalcine were confirmed using an iodinated analog of the toxin.[70] These results suggest that

Table II Timeline for Development/Study of Chlorotoxin A9nalogues

Year	Analogue	Aim of study	Ref
1998	CTX^{125}/CTX^{131}	To study the binding properties and biodistribution of CTX	41
2002	CTX N-terminal biotin	To study binding properties of CTX on PNET and gliomas	12
2005	CTX^{131}	A dosimetry analysis of radiolabeled CTX	85
	CTX:Cy5.5:PEG	To visualize tumors by MRI or FM	72
2007	CTX:Cy5.5 (Tumor paint)	To visualize tumors	44
	CTX:FITC:SPIO	To visualize tumors by MRI	98
2008	CTX:PEG:methotrexate	To deliver therapeutics to cancer cells	74
2009	CTX:PEI:DNA	Gene delivery	79
	CTX:PEG:NIRdye	To visualize tumors	99
2010	CTX:PEI:siRNA	To delivery short siRNA to cancer cells	78
	CTX:PEI:siRNA	To delivery short siRNA to cancer cells with pH sensitive activity	80
	CTX:NP:DNA	Gene delivery	81
2011	Cyclic CTX	To improve stability	24
	PAMAM/PEG/CTX:DNA	Gene delivery	82
2012	CTX:LS:levodopa	To deliver therapeutics to the brain	88
	CTX:cisplatin	To target selectively tumor cells	75
	CTX:IgG-Fc	Targeting glioblastoma cells	100
2013	CTX:NO	Nitric oxide delivery to improve chemotherapy	101
	CTX:800CW	To visualize tumors	42
	CTX:NP:DNA	DNA delivery	83
2014	CTX:NP:alisertib	Delivery of therapeutics	76
	CTX:BLZ100	To optimize the NIR dye for imaging	43
	CTX:GO:doxorubicin	Delivery of therapeutics	102
	Ox26/CTX-PL/pDNA	Gene delivery	84
	CTX:NP:temozolomide	Delivery of therapeutics	103
	CTX:NP:BG	Delivery of therapeutics	104
2015	CTX:SNALP:anti-miR-21	Oligonuleotide delivery	105
	CTX:NP	Drug delivery	106
	CTX:NP	Drug delivery	107
	CTX:onconase	Delivery of therapeutics	108

PNET: peripheral neuroectodermal tumors; PEG: polyethylene glycol; MRI: magnetic resonance imaging; FM: fluorescence microscopy; LS: stealth liposomes; NO: nitric oxide; NP: nanoparticles; PAMAM: polyamidoamine; SPIO: super paramagnetic iron oxide; FITC: fluorescein isothiocyanate; PMAM: polyamidoamine; PEI: polyethyleneimine; SNALP: stable nucleic acid lipid particle; BG: O(6)-benzylguanine.

although many more scorpion toxins may be able to internalize cells their mechanism of entry may differ.

Applications

Imaging. Many applications of chlorotoxin, which can be classified into three categories – imaging, nanotechnology and radiotherapy, have been proposed (Table II). One of the most promising applications is in optical imaging (see Stroud et al. for a review).[16] An example of this is "tumor paint"—a fluorescent molecular probe, whereby chlorotoxin is conjugated to Cy5.5 (CTX:Cy5.5).[44] Cy5.5 absorbs light in the near infrared spectrum, and because light at this wavelength is poorly absorbed by water or hemoglobin, Cy5.5 is compatible with intra-operative imaging. In the ND2:Smo1 mouse model of medulloblastoma, CTX:Cy5.5 was used to delineate tumorous from non-tumorous cells.[44] In this study, CTX:Cy5.5 was shown to enter the brain and no damage to the blood-brain barrier (BBB) was observed. Furthermore, CTX:Cy5.5 did not cause damage to other major organs, and no major differences in hematocrit, platelet count, white blood cell count, electrolytes, liver function and kidney function was reported. Therefore, at the concentration tested (0.1 mL of 20 μmol/L bioconjugate), CTX:Cy5.5 is nontoxic and therefore a potentially safe therapeutic agent.[44] The binding of CTX:Cy5.5 to tumors of other origins was also evaluated, and was found to bind to human and mouse prostate cancer as well as lung metastases.[44] This study provides evidence that the conjugated structure preserves the tumor-binding features of chlorotoxin.

To improve the pharmaceutical properties of CTX:Cy5.5, chlorotoxin was engineered to produce a mono-labeled and cyclic peptide.[24] As the native sequence of chlorotoxin (Figure 1) contains three lysine residues that can react to the Cy5.5 N-hydroxysuccinimide ester, a mixture of mono-, di-, and tri-labeled peptides is produced during the labeling

procedure, complicating production and isolation of the mono-labeled form. To simplify the manufacture of CTX:Cy5.5, lysines at position 15 and 23 were replaced by alanines or arginines and only a single lysine at position 27 was left to enable production of a mono-labeled molecule.[24] To further improve its proteolytic stability, chlorotoxin was also cyclized by using a seven amino acid residue linker to join the N- and C-termini (Figure 1B), as cyclization of peptides has been shown to enhance their stability and half-life in serum.[71] As expected, the cyclic analogue showed increased stability compared to that of the native peptide and retained the ability to bind malignant tissue.[24]

The fluorescent molecular probes IRDye 800CW and indocyanine green have also been conjugated to chlorotoxin (to produce CTX:800CW and BLZ-100, respectively) and tested for their intra-operative potential. CTX:800CW retained the ability to specifically bind to brain tumors as well as the ability to be internalized by cells in the ND2:SmoA1 mouse model of medulloblastoma.[42] In contrast with the aforementioned study on CTX:Cy5.5 showing that the BBB was intact in ND2:Smo1 mice,[44] in this study the integrity of the BBB was compromised even at the early stages of the tumor.[42] The leakage of Evan's blue, the dye used to indicate membrane integrity, was localized near the tumor, suggesting that localization of CTX:800CW to the tumor site was not restricted by an intact BBB.[42] Regardless of whether tumor growth results in BBB disruption or whether chlorotoxin can cross the BBB to reach tumors in the brain, the results on the activity of CTX:800CW support its use as an alternative imaging agent to delineate tumor cells.[42] BLZ-100 was also found to have the same tumor-binding properties as chlorotoxin and was shown to localize to human glioblastoma cells implanted in mice.[43] The Food and Drug Administration recently approved BLZ-100 for Phase I human clinical trials to help surgeons during tumor resection (Clinical trial identifier number: NCT02234297).

Nanotechnology. Chlorotoxin has been used as a targeting ligand in nanotechnology to deliver magnetic resonance imaging (MRI) contrast agents or nanoprobes to tumorigenic tissue, as shown in Table II and reviewed recently.[14] Chlorotoxin nanoparticles are iron oxide nanoparticles conjugated to both a therapeutic molecule/imaging agent and chlorotoxin through a polyethylene glycol (PEG) linker. The first published example was in 2005, where nanoparticles were produced using chlorotoxin and Cy5.5.[72] The nanoprobe showed specific binding to glioma cells in vitro compared with those produced without chlorotoxin.[72] In a later study, the chlorotoxin-Cy5.5 nanoprobe was shown to be internalized by glioma cells *in vitro*, and no toxicities were observed *in vivo* in a mouse model of cancer.[73]

In addition to the imaging applications, chlorotoxin nanoprobes have been used to deliver other drugs or biologics to tumorigenic cells. For example, a nanoparticle bioconjugated to methotrexate, a chemotherapeutic agent, was shown to have increased cytotoxicity towards cancer cells when compared to the effect of the drug alone.[74] A similar approach was reported for cisplatin, an anti-cancer cytotoxic drug used for chemotherapy,[75] and for alisertib, which is currently in Phase II clinical trials for relapsed and refractory aggressive B- and T-cell non-Hodgkin lymphomas.[76,77] In both studies, the cytotoxicity of the nanoprobe on cancer cells increased compared to the drug alone. This approach was also used to deliver siRNA or DNA to cancer cells as a targeting gene delivery system.[78–84]

Radiotherapy. Chlorotoxin has been radiolabeled with radioactive iodine (^{131}I) attached to its single tyrosine residue to produce ^{131}I-CTX, which has been used as a radiotherapy agent to control tumor progression as well as detect tumor size and localization.[85,86] In a phase I clinical trial, a single dose of ^{131}I-CTX, delivered directly to the brain through a catheter, was shown to be well tolerated and was eliminated from the body within 24 to 48 h.[86] Testing of ^{131}I-CTX has now moved into phase II trials.

Perspectives

Does Chlorotoxin Have Promise in Drug Design? Chlorotoxin has many promising features that could potentially be harnessed for drug design. First, although classified as a toxin, chlorotoxin analogues have displayed no obvious toxicities when administered to humans, which is important for drug development. Moreover, the ability of chlorotoxin to bind selectively to cancer cells may allow one of the most problematic issues of oncological treatment to be overcome, i.e., the lack of specificity that translates into damage of normal tissue and also toxicities and side-effects. There has already been some effort to develop chlorotoxin for use as a vehicle for the delivery of drugs, and to improve their cytotoxic profiles.[75] Most effort has been directed to the treatment of glioblastoma multiforme, for which there is currently no effective treatment, aside from complete resection.[87] Although chlorotoxin has shown much promise in drug design, there are still many questions relating to its activity, highlighted below, that have not yet been fully addressed.

What Is/Are the True Molecular Target(s) of Chlorotoxin? As discussed above, the tumor binding property of chlorotoxin has been linked to three molecular targets: chloride channels, MMP-2, and annexin A2;[11,40,55] however, it is still unclear whether chlorotoxin binds to one or multiple targets.

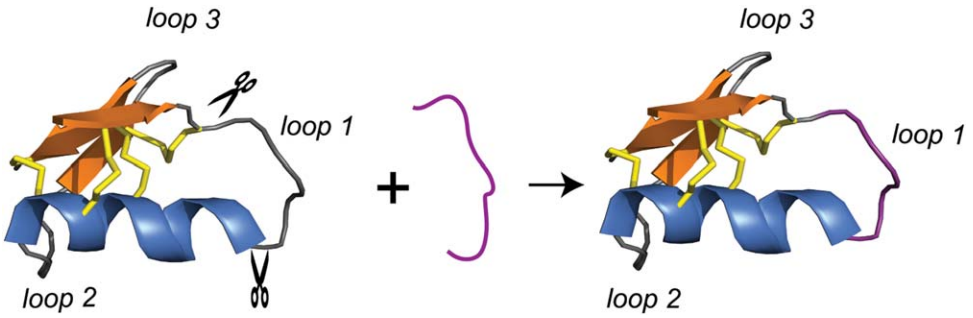

FIGURE 4 Schematic showing the concept of molecular grafting for chlorotoxin. The putative epitope is shown as a purple structure, while the final structure shows a grafted chlorotoxin, including the foreign bioactive epitope.

Additional experiments that provide evidence of a direct interaction between chlorotoxin and its target will be necessary to answer this question. It is also worth noting that the target responsible for the insecticidal activity of chlorotoxin is also unclear, as studies have so far focused on identifying its mammalian tumor-cell target. Considering that other insectotoxins are active on ion channels aside from chloride channels, it may be informative to screen chlorotoxin on other ion channels, which might also provide further insight into its true molecular target in mammals. Confirming the molecular target/s of chlorotoxin is crucial for understanding the mechanism by which it is able to bind to cancer cells but not healthy cells. Moreover, it might give us clues for developing more specific cancer-targeting drugs with fewer side effects.

Can Chlorotoxin Cross the Blood-Brain Barrier? In several of the studies mentioned above, chlorotoxin was demonstrated to be able to enter the brain and bind specifically to malignant tissue, suggesting that it might be able to cross the BBB. In the first *in vivo* binding study of ^{125}I-labeled-chlorotoxin to brain tumor cells in mice, it is probable that intracranial injection was used because it was assumed that chlorotoxin could not enter the brain from the circulatory system due to the BBB.[41] In later studies, chlorotoxin analogues (i.e., conjugated to Cy5.5, 800CW and BLZ-100) were shown to bind to brain tumors in mice when delivered via tail injection.[42,44] Furthermore, when chlorotoxin was used as a carrier for delivering levodopa for the treatment of Parkinson's disease, an increased distribution of dopamine was found in the brain of Parkinson's disease mice model and a reduction in the behavioral disorders was observed.[88] These findings support the conclusion that chlorotoxin is able to enter the brain; however, the important distinction is that they do not confirm whether chlorotoxin can cross the BBB, as the results examining the integrity of the BBB have not been conclusive in these studies.[42,44] Disruption of the BBB observed by Kovar et al.[42] is certainly consistent with other studies reporting BBB disruption associated with brain tumours.[89] It is important to confirm whether chlorotoxin can get into the brain by crossing the BBB without compromising its structure, as this would mean that chlorotoxin could be a very valuable drug delivery tool for a range of neurological disorders.

Is Chlorotoxin a cell-Penetrating Peptide? Although chlorotoxin can enter both cancer and healthy cells,[66] there is still not enough information to definitively classify it as a CPP. The main reason for this is that the uptake of chlorotoxin has been studied using fluorescent dyes such as Alexa Fluor® 488 and Cy5.5,[44,66] and the cargo or the fluorescent dye used to study the features of CPPs have been previously shown to play a role in the mechanism of uptake.[63,64,90,91] It will therefore be necessary to determine whether the fluorescent dyes are playing a role in the cellular uptake of chlorotoxin. Thus, although the potential of chlorotoxin as a CPP is clear, more studies need to be conducted to truly understand its mechanism of internalization. It will be important to confirm the cell-penetrating properties of chlorotoxin because CPPs can be used to deliver drugs to intracellular targets. It is worth noting that chlorotoxin is a very stable peptide that is able to resist proteolytic degradation both in vitro and in vivo,[24] potentially making it a better CPP that many well-studied linear CCPs which have poor metabolic stability.[92]

How Else Can We Use Chlorotoxin in Drug Design? So far, applications of chlorotoxin have focused on conjugating chlorotoxin to visualize tumors or conjugating it to drugs for delivery to the brain or to cancer cells. A novel approach to using chlorotoxin is as a scaffold in molecular grafting, an emerging chemical design principle that describes the transplant of an active peptide epitope onto a stable framework or scaffold.[93] This design approach would help improve the stability of the peptide epitope, which would otherwise degrade rapidly when administered. Chlorotoxin has three loops amenable for engineering, the most promising is the first loop, which is the

longest and may be able to tolerate foreign sequences without its tertiary structure and/or stability being affected (Figure 4). Furthermore, there is evidence that CSα/β toxins can be reengineered without affecting their overall structure,[94,95] suggesting that chlorotoxin, which contains an CSα/β motif, should be able to tolerate changes to its sequence. However, we need a better understanding of the relationship between the structure and tumor-binding properties of chlorotoxin to be able to modify and/or introduce new sequences without introducing adverse effects.

Can We Learn More From Nature? Chlorotoxin shares biological activities with other members of the chlorotoxin-like peptide family; therefore, chlorotoxin-like peptide sequences provide a starting point to better understand the biological properties of chlorotoxin. This naturally occurring set of peptides can also provide information on structure-function relationships, amino acid residues, or domains that may be important for activity. BmK CT from the venom of the Asian scorpion *Buthus martensis* Karsch, for example, shares 68% sequence similarity with chlorotoxin, can inhibit glioma cell growth, and bind specifically to glioma tumors in a rat model.[96] Another peptide, AaCtx, recently identified from the venom of the scorpion *Androctonus australis* shares 70% sequence similarity with chlorotoxin and its synthetic version also inhibits glioma cell migration and invasion, albeit in a weaker fashion.[97] Based on these results, we speculate that the ability to bind glioma cells might be a common feature among chlorotoxin-like peptides and differences in their sequence composition may account for differences in potency. Thus, a comparative study of chlorotoxin-like peptides could provide new insights on sequence-activity relationships and their mode of action.

CONCLUSIONS

Chlorotoxin has several properties that make it a good starting point for drug design: (i) it is able to bind preferentially to tumor cells, making the design of therapeutics using chlorotoxin as a scaffold to treat glioma and other types of cancer of neuroectodermal origin such as melanoma, neuroblastoma and medullobastoma possible; (ii) to date, no obvious toxicities nor immunogenic responses after being administered to humans have been reported; and (iii) chlorotoxin is reportedly able to enter the brain and be internalized by cells, suggesting that it could be used to deliver drugs to the brain and/or intracellular targets. Despite these positives, several questions regarding these properties as well as the function and mechanism of action of chlorotoxin still remain. Therefore, a thorough understanding of the activity and pharmacological properties of chlorotoxin is still needed to realize the full potential of chlorotoxin in diagnosis and treatment of disease.

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