The role of disulfide bonds in structure and activity of chlorotoxin

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Abstract

Background: Chlorotoxin is a small scorpion peptide that inhibits glioma cell migration. We investigated the importance of a major component of chlorotoxin's chemical structure – four disulfide bonds – to its tertiary structure and biological function.

Results: Five disulfide bond analogues of chlorotoxin were synthesised, with L-α-aminobutyric acid residues replacing each or all of the disulfide bonds. Chemical oxidation and circular dichroism experiments revealed that Cys III-VII and Cys V-VIII were essential for native structure formation. Cys I-IV and Cys II-VI were important for stability to enzymatic proteolysis but not for the inhibition of human umbilical vein endothelial cell migration.

Conclusions: The disulfide bonds of chlorotoxin are important for its structure and stability, and have a minor role in its activity against cell migration.

Introduction

Chlorotoxin (CTX) is a 36-amino acid peptide found in the venom of the giant Israeli scorpion *Leiurus quinquestriatus* [1], which possesses therapeutically interesting bioactivities [2, 3]. CTX displays high binding selectivity for glioma cells and also for cells from other tumours of neuroectodermal origin but not to healthy mammalian cells [2, 4]. This specific activity was initially thought to involve chloride channels expressed on the surface of cancer cell types [5] but recently, matrix metalloproteinase-2 and annexin A2 have been proposed as other possible targets of CTX [6, 7]. CTX also displays anti-angiogenic activity, as demonstrated in *in vitro* and *in vivo* bioassays [3]. For example, CTX inhibits the migration of human umbilical vein endothelial cells (HUVECs) in a dose-dependent fashion and inhibits the growth of blood vessels in a chick chorioallantoic membrane (CAM) assay [3].

The biological activities of CTX have led to several promising applications in cancer treatment or detection [8-14]. The synthetic version of CTX (TM-601) conjugated to a fluorophore, such as Cy5.5 or IRDye 800CW, retains its tumour binding properties and is being developed as an imaging agent to help distinguish cancerous from non-cancerous cells during surgical resection of tumours [9, 10]. A radio-labelled analogue of CTX, ^{131}I-CTX, has been used as a radiotherapy agent to control and detect tumour progression [11, 12]. In a phase I clinical trial, a single-dose study showed that ^{131}I-CTX was well-tolerated by all individuals tested and displayed an encouraging safety profile with no grade 3 or 4 toxicities observed, and was eliminated from the body within 24 to 48 h [12]. ^{131}I-CTX is currently in phase II clinical trials [12]. CTX has also been used as a targeting signal to deliver drugs to

cancer cells [13, 14], adding to the growing number of applications of CTX in cancer drug development.

Because of its small size and proteinaceous nature, CTX is well suited to modern chemistry methods designed to enhance its therapeutic potential. In a recent study, CTX was modified to produce an optimal peptide for bio-conjugation to Cy5.5 for tumour imaging [15]. The native sequence of CTX, which is shown in **Figure 1A**, has three lysine residues, leading to a heterogenous mixture of mono-, di-, and tri-labelled peptides after Cy5.5 conjugation, which is an undesirable characteristic for regulatory approval. Thus, CTX was chemically re-engineered to contain only a single lysine at position 27, which was used to produce a mono-labelled molecule able to be used in clinical trials [15]. CTX has also been engineered to have improved stability against serum peptidases. A seven amino acid linker was introduced to connect the N- and C-termini of the peptide to produce a backbone cyclised analogue, which showed improved serum stability by around 20% compared to that of the native peptide. The cyclic analogue also maintained a similar overall tertiary fold and the ability to bind malignant tissue [15].

Attempts to re-engineer CTX can be guided by structural information. CTX adopts a well-structured tertiary fold, with four disulfide bonds at the protein core **(Figure 1B)** surrounded by an α-helix and three anti-parallel β-strands [16]. Three of the disulfide bonds, Cys II-VI, Cys III-VII and Cys V-VIII, are involved in linking the α-helix to the β-strands, and the fourth disulfide bond, Cys I-IV, connects the N-terminal region with the remainder of the peptide. This structure is similar to that found in members of a scorpion peptide family characterised by an α/β motif and three disulfide bonds

[16]. As shown in **Figure 2A**, comparison of CTX with iberotoxin, a member of the α/β scorpion peptide family, shows that the connectivity of three disulfide bonds are similar, with the additional fourth disulfide bond of CTX, Cys I-IV, being the distinguishing structural feature.

The disulfide bond configuration of CTX incorporates a cystine knot motif. This motif is present in a number of peptide families, including the cyclotides, which are plant-derived cyclic peptides that have emerged as ultra-stable scaffolds in drug design [17-19] due to a combination of their cystine knot and head-to-tail cyclic backbone. In the cyclotide family, the cystine knot is composed of a topological ring formed by Cys I-IV and Cys II-V and their intervening backbone sequences, through which Cys III-VI penetrates, forming a stable and rigid configuration. In CTX, of the four disulfide bonds, Cys I-IV, Cys II-VI and Cys III-VII are involved in forming the knot and Cys V-VIII can be regarded as an 'extra' crosslink **(Figure 2B).** From this discussion it is clear that CTX is an interesting disulfide-bonded peptide that shares common features with diverse examples of plant and animal toxins and hence it is of interest to examine the role of its individual disulfide bonds in defining structure and activity.

Studies on disulfide bonds in proteins and peptides have revealed that they can have a number of roles in structure, stability and activity, and their modification can be useful for modulating the pharmacological properties of peptides [20]. For the cyclotides, the disulfide bonds contribute to the stability of naturally-occurring examples, as well as to engineered drug leads based on the cyclotide framework [21-

23]. For the α/β scorpion family members, leiurotoxin I and charybdotoxin, specific disulfide bonds are critical for maintaining the native-like structure [24, 25].

In this study our aim was to determine the role of each of the four disulfide bonds in CTX and delineate their importance to the structure, stability and activity of the peptide. We reasoned that this information might facilitate the use of CTX as a drug design scaffold. We sequentially replaced each disulfide bond with a pair of L-α-aminobutyric acid (Abu) residues and also synthesised an analogue with all disulfide bonds replaced. Herein we report the structural and functional characterisation of these analogues.

Experimental procedures

Synthesis and purification

CTX and the analogues were synthesised on a Symphony Multiplex automated synthesiser using Fmoc solid phase peptide synthesis (SPPS) chemistry. Rink amide resin was used for all synthesis. The Fmoc protecting group was removed using 30% piperidine in DMF, and a 4-fold excess of amino acid was used for coupling of amino acids (1:1:2 ratio of amino acid:HCTU:DIPEA). Completed peptides were washed with dichloromethane and the resin dried under nitrogen. The peptides were cleaved using a mixture of trifluoracetic acid (TFA), triisopropylsilane and water (95:2.5:2.5). The resin was incubated for two hours while being stirred continuously. After the incubation period, TFA was removed by rotary evaporation and the peptides were precipitated by adding approximately 50 mL of cold diethyl ether to the remaining solution and extracted three times with 70 mL of a 50/50 mixture of buffer A (0.05% (v/v) TFA in water) and buffer B (90% (v/v) acetonitrile, 0.045% (v/v) TFA in water) using a separation funnel. The crude peptide from this extraction was lyophilised overnight and followed by RP-HPLC purification using C18 columns with 1% min^{-1} or 0.5% min^{-1} gradient from 0–60% buffer B and the eluant was monitored at 215 and 280 nm. Purity of peptides was confirmed by LC-MS and analytical RP-HPLC.

Folding and purification

Reduced peptides at 0.1 mg/mL were oxidised in a buffer containing 0.1 M Tris-HCl pH 7.8, 0.2 M NaCl, 5 mM reduced glutathione and 0.5 mM oxidised glutathione for 16–20 h at room temperature. The peptides were also oxidised in folding buffer plus 10% (v/v) DMSO or in folding buffer plus 15% (v/v) isopropanol. The oxidised

peptides were purified using C18 columns on RP-HPLC. A gradient of 0.5% min^{-1} from 0–60% buffer B was used, and the eluant was monitored at 215 and 280 nm. Purity of peptides was confirmed by LC-MS and analytical RP-HPLC.

Conformational analysis

The peptides were prepared at 50 µM in 2 mM Tris HCl buffer at pH 7.4 for CD analysis. Spectra were recorded at room temperature with a 0.1 cm path length quartz cell by accumulating three scans, from 180 to 250 nm using a CD spectropolarimeter (Jasco J-810).

Reduction and alkylation of disulfide bonds

From a 0.2 mM oxidised peptide stock in water of CTX2 or CTX3, 50 µL was mixed with 200 µL of 0.2 M citrate buffer pH 3.0 and 50 µL of 20 mM TCEP ((tris(2-carboxyethyl)phosphine). Peptides were partially reduced following incubation at 30°C for 40 min and alkylated by adding 300 µL of 0.1 M N-ethylmaleimide (NEM) and incubating for a further 15 min at 30°C. The reaction was stopped by acidification with 500 µL of 1% formic acid. The peptides were then purified using RP-HPLC with a gradient of 0.5% min^{-1} of 20–40% buffer B. Following lyophilisation the alkylated peptides were redissolved and fully reduced in 40 µL of 0.1 M ammonium bicarbonate pH 8.0 containing 10 mM DTT (dithiothreitol) at 60°C for an hour. A second alkylation was performed by adding 25 mM iodoacetamide (IAM) and incubating for 30 min at room temperature. Sequencing grade trypsin was added to selected peptides at 1:20 (w/w) ratio in 100 mM ammonium bicarbonate buffer pH 8.0 and digestion was allowed to proceed overnight at 37°C. The samples were desalted using Ziptips (Millipore, C18) prior to analysis.

Sequence determination

ESI-MS analysis was conducted for the digested and undigested peptides. The ESI-MS measurements were performed on a QStar Pulsar I QqTOF mass spectrometer (AB/Sciex, Framingham, USA) equipped with an ESI source in positive ion mode. The spray voltage was set to 900 V. Nitrogen was used as the collision gas and the collision energies were adjusted manually in the range from 5 to 50 V. After the precursor ion for MS/MS was selected, the MS/MS spectra were collected and recorded for up to 30 min. Recorded mass spectra were analysed with the Analyst QS 2.0 (AB/Sciex) software and the peptide sequenced using b- and y- ions (N- and C-terminal) as described previously [26].

Serum-stability assay

The stability of native CTX and its analogues was determined as described previously [27, 28]. The incubation time points for all peptides with human serum were 0, 1, 2, 3, 6, 12 and 24 h at 37°C.

Cytotoxicity assay

The cytotoxicity of native CTX and its analogues was performed and analysed as described previously [23]. Briefly, $5x10^3$ HUVECs/well were seeded in a 96-well flat-bottomed plate in a final volume of 100 µL in 10% FBS/EBM-2 media with supplements (Lonza) and incubated in 5% CO_2 overnight at 37°C. Then, the media was removed and 90 µL of fresh media without serum was added to each well. 10 µL of CTX and its analogues were added in triplicates to a final concentrations ranging from 0.1 to 200 µM. 0.1% Triton X-100 was used as a positive control. After incubation for another 2 h, 10 µL of MTT (Sigma) solution in PBS (5 mg/mL) was

added to each well and incubated for 3 h. Media was removed, and formazan crystals were resuspended in 100 µL of DMSO. After 10 min the absorbance was read at 600 nm with BioTek PowerWave XS machine.

Migration assay

The *in vitro* cell migration assay provides measurable antiangiogenic activity of molecules. To determine the activity of native CTX and its analogues, the peptides were evaluated for their ability to inhibit the cell migration of human umbilical vein endothelial cells (HUVEC). HUVECs were grown in 10% FBS/EBM-2 media with supplements (Lonza). Cells were serum-starved overnight, harvested and resuspended in media containing 0.1% FBS. Inserts were precoated with attachment factor (Gibco) prior to the assay and were removed before the assay. 100 µL of $1x10^6$ cells/mL were added to the upper chamber of the 8 µm pore transwell plate (Corning) and 600 µL of 0.1% FBS/EBM-2 without supplements or EBM-2 with 0.3 nM vascular endothelial growth factor (VEGF) were added in the lower chamber. Cells were incubated with peptides (20 µM of CTX or analogues) for 30 min prior adding to the inserts. Next, cells were incubated with peptides for 4 h at 37°C. The assay was stopped by removing media from upper and lower chambers, before being fixed with 4% formaldehyde/PBS for 15 min and stained with 0.05% crystal violet for 10 min. Cells were destained with 200 µL of 10% acetic acid/H_2O for 10 min and absorbance was measured at 590 nm with BioTek PowerWave XS machine. Each peptide was tested in triplicates. Statistical analyses using one way ANOVA were performed to analyse the activities of CTX1, CTX2 and CTX3 and compared with that of the native parent peptide.

Results

Synthesis, Folding and Purification

The sequences of CTX and the five disulfide bond analogues used in this study, as well as their predicted disulfide bond connectivities are shown in **Figure 3**. In CTX1, all cysteine residues were replaced with Abu, whereas for the remaining peptides, only one pair of cysteine residues was replaced. Abu was chosen to be the cysteine substitute because it is isosteric to cysteine but is unable to form a disulfide bond. All peptides were synthesised using solid-phase Fmoc chemistry and yielded reduced peptides that were purified using RP-HPLC and showed a defined peak on analytical HPLC **(Figure 4, black traces)**.

To examine the effect of the disulfide bonds on the oxidative folding of CTX, the reduced peptides were oxidised at a concentration of 0.1 mg/mL in folding buffer (0.1 M Tris-HCl pH 7.8, 0.2 M NaCl, 5 mM reduced glutathione and 0.5 mM oxidised glutathione), which was the same in composition as that used previously to chemically produce native-like CTX [15]. The addition of organic solvent or detergents has been reported to improve the oxidative yield of disulfide-rich cyclotides [29]. The peptides were therefore also oxidised in folding buffer containing 10% (v/v) DMSO or 15% (v/v) isopropanol to explore the effect of additives on the folding of CTX. The ratio of oxidised (GSSG)/reduced (GSH) glutathione in the folding buffer was kept constant because this ratio has previously been used to obtain the native disulfide bond connectivity for CTX [15] and two related toxins [24, 25].

The folding mixtures were analysed using analytical HPLC to characterise the yield of the oxidised peptides bearing the native-like disulfide bond connectivity. As illustrated in **Figures 4A** and **4F**, oxidation of reduced CTX resulted in a defined

early-eluting peak that corresponded to a folded molecule with the correct disulfide bond connectivity, and this was confirmed by a proton chemical shift analysis using NMR (data not shown). The correctly folded CTX had an earlier elution time than its reduced form, consistent with the correct disulfide bond connectivity promoting the greater exposure of hydrophilic and polar residues. This result was found to be a general phenomenon, with the presence of an early-eluting peak being an indicator of a native-like structure having been formed. The area of the early-eluting peak formed after oxidation of CTX in the folding buffer, the folding buffer plus 10% (v/v) DMSO, and the folding buffer plus 15% (v/v) isopropanol was measured to be 60%, 80% and 100%, respectively. The improved yield of the native-like peptide with the addition of DMSO or isopropanol suggests that organic solvents may encourage the exposure of some hydrophobic residues of CTX, thereby facilitating the formation of the correct disulfide bond connectivity.

Oxidation of reduced CTX2 and CTX3 also produced a defined early-eluting peak in the folding buffer but the intensity of the peak was lower than that obtained for CTX in the same buffer. The area of the peak formed for CTX2 and CTX3, after oxidation, corresponded to 40% and 30%, respectively, compared to that of the corresponding reduced peptide, indicating that the folding process was affected by the removal of the associated disulfide bond **(Figures 4B and 4C)**. Similar to what was observed for CTX, the presence of 10% (v/v) DMSO or 15% (v/v) isopropanol resulted in an improved yield of the early-eluting peak **(Figures 4G and 4H)**. The area of the peak formed for CTX2 and CTX3, after oxidation, increased to 60% and 40%, respectively, in the presence of DMSO, and to approximately 60% and 80%, respectively, in the presence of isopropanol. CTX4 did not form a distinctive and high

yielding early-eluting peak, suggesting that non-native-like structures were favoured during folding **(Figure 4D)**. Similar results were obtained for CTX5 in the folding buffer **(Figure 4E)**, and for CTX4 and CTX5 in the folding buffer with 10% (v/v) DMSO or 15% (v/v) isopropanol **(Figures 4I and 4J)**.

Oxidised peptides were purified and their masses determined using mass spectrometry. The observed masses were consistent with the calculated masses of CTX and its analogues, which were 3993.6 Da and 3959.6 Da, respectively, confirming that the correct number of disulfide bonds had formed. After the oxidation of CTX3 in the presence of DMSO, an additional early-eluting peak with a mass of 4572.2 Da was observed **(Figure 4H, marked with an asterisk)**, which probably represents CTX3 attached to two glutathione molecules (MW = 612.6 Da). Covalent linkage of glutathione during oxidation was previously reported for charybdotoxin [25]. A mass spectrometry analysis of the chromatogram of the oxidation of CTX4 and CTX5 identified the presence of an oxidised peptide mass at several different retention times, suggesting that multiple non-native-like disulfide bond connectivities had formed. CTX analogues contain six cysteine residues that can adopt 15 different oxidised disulfide species. The presence of multiple peaks during the folding process suggests that the native-like peptide is not the most favoured end product. Since the oxidation trials indicated that CTX4 and CTX5 did not efficiently oxidise into the native-like fold, we focussed on CTX2 and CTX3 in the subsequent conformational and stability studies.

Conformational analysis

Circular dichroism (CD) spectra for proteins or peptides have particular signatures that reflect the secondary structure [30]. As shown in **Figure 5**, the CD spectrum of CTX showed both a positive maximum at around 190 nm and two negative minima at approximately 209 nm and 220 nm, indicating the presence of an α-helical secondary structure element. This result is consistent with the three dimensional structure of CTX where there is an α-helix spanning residues 12 to 19 **(Figure 1)**. CTX2 and CTX3 displayed a similar spectrum to that of CTX, strongly suggesting that they adopted the native-like fold. For a peptide with a random coil structure, the CD spectrum should contain a positive maximum at around 212 nm and a negative minimum at 195 nm. CTX1 presents this signature **(Figure 5, open rhombus)**, indicating that the disulfide bonds are important for stabilising the structure of CTX. Thus, we conclude that the overall structure of the analogues CTX2 and CTX3 is comparable to that of CTX but different to that of CTX1. It should be noted that although the CD spectra indicated that CTX2 and CTX3 had similar overall conformations compared to the native peptide, the ^{1}H 1D spectra of both peptides indicated that there was significant conformational flexibility (Supplementary Figure 1). Based on this observation, we postulate that removal of both Cys I-IV and II-VI bonds will have a significant destructive effect on conformation.

Disulfide bond connectivity

To confirm that the correct disulfide bond pairing had formed for CTX2 and CTX3, these peptides were partially reduced to generate a mixture of isoforms with some disulfide bonds reduced and the remainder oxidised. The mixtures were subjected to alkylation with NEM and subsequently analysed using mass spectrometry, identifying the presence of peptides with either two or four NEM-alkylated cysteines. Peptides

with a differing number of NEM modifications were separated using RP-HPLC and completely reduced using DTT and further subjected to a second alkylation with IAM. To facilitate their MS/MS characterization, the alkylated peptides were digested with trypsin to obtain smaller peptide fragments. The peptides that were analyzed by MS/MS are shown in **Table 1** and Supplementary Figure 2. MS/MS sequencing of the CTX2 isoform containing two NEM-alkylated cysteines reveled that Cys II and Cys VI were alkylated with NEM, confirming that the disulfide bond Cys II-VI had formed correctly. Similarly, MS/MS analysis of CTX3 containing two NEM-alkylated cysteines indicated that Cys I-IV had also formed correctly. For the CTX3 derivative containing four NEM-alkylated and two carbamidomethyl cysteines, the NEM-alkylated cysteines were identified to be Cys I, Cys IV, Cys V, and Cys VIII, which is consistent with a Cys I-IV and Cys V-VIII connectivity, and the carbamidomethyl cysteines were Cys III and Cys VII. We were unable to isolate the CTX2 derivative containing four NEM-alkylated cysteines because it did not produce a defined peak during the partial reduction procedure (Supplementary Figure 3). Nonetheless, we concluded that CTX2 formed the correct disulfide bond connectivity based on the obtained MS data and the excellent agreement between the CD spectra of CTX, CTX2 and CTX3 (Figure 5). Thus, sequencing of the differentially alkylated derivatives of CTX2 and CTX3 indicated that the correct disulfide bond connectivity had formed.

Stability of peptides

CTX is a very stable peptide that can resist enzymatic proteolysis *in vit*ro and *in vivo* [15]. To compare the relative stabilities of the analogues, CTX, CTX1, CTX2 and CTX3 were incubated in human serum (containing proteolytic enzymes), and in PBS

at 37°C and monitored over 24 h. As expected, PBS did not have an effect on peptide stability (data not shown). Consistent with a previous study [15], CTX displayed high stability, with around 80% of intact CTX remaining after 24 h incubation **(Figure 6A)**. Compared to CTX, CTX2 and CTX3 degraded rapidly, with less than 15% of the intact peptides remaining after 12 h. **Figure 6A** shows that CTX1 had the lowest stability of all peptides tested, as it had completely degraded after 3 h.

Biological activity

CTX can inhibit cell migration of HUVECs through a mechanism that may involve multiple pathways [3]. The biological activity of native CTX and its analogues were studied by measuring their ability to inhibit HUVEC migration. Firstly, we confirmed that CTX, CTX1, CTX2 and CTX3 were not toxic to HUVECs; CTX and CTX1 showed no toxic effects up to a tested concentration of 200 µM, whereas CTX2 and CTX3 were not toxic up to a tested concentration of 75 µM (Supplementary Figure 4). All peptides were then examined for their inhibitory effect on cell migration at 20 µM in the presence of 0.3 nM vascular endothelial growth factor (VEGF), which acted as a chemoattractant. This peptide concentration was chosen based on a dose-response curve that was performed for CTX (Supplementary Figure 5). CTX and its analogues were able to inhibit approximately 50% of cell migration induced by VEGF (Figure 6B). Surprisingly, CTX1, which is analogous to the completely reduced form of CTX, had inhibitory activity that was similar to the native peptide, suggesting that the three dimensional structure of CTX formed by the disulfide bonds is not required for the inhibitory effect on cell migration.

Discussion

CTX forms a compact tertiary structure containing a highly cross-linked core composed of four disulfide bonds. In this study, we aimed to gain insights into the role of individual disulfide bonds on the structure and function of CTX, which would facilitate the design and development of optimised cancer drug leads based on the CTX scaffold.

Role of disulfide bonds in the structure of chlorotoxin

Our conformational analysis suggested that Cys I-IV and Cys II-VI can be removed without affecting the overall topology of CTX, highlighting the structural similarity between CTX and the α/β family of scorpion peptides. As mentioned in the introduction, the disulfide bonds Cys II-VI, Cys III-VII and Cys V-VIII of CTX are topologically similar to the disulfide bond configuration of the α/β family of scorpion toxins. Deletion of Cys I-IV resulted in a disulfide bond connectivity identical to that of the α/β family, which require only three disulfide bonds to form their native structures. Moreover, deletion of Cys II-VI of CTX, which corresponds to the first disulfide bond in the α/β motif, did not affect the overall structure of CTX. This behaviour has been reported for other α/β toxin family members, namely leiurotoxin I and charybdotoxin, where deletion of the first disulfide bond did not affect the overall structure [24, 25, 31]. In studies on leiurotoxin I and charybdotoxin, deletion of the second or third disulfide bonds was important for folding into a native structure. We observed a similar behaviour for CTX analogues; deletion of either Cys III-VII or Cys V-VIII in CTX, which correspond to the second and third disulfide bonds, respectively, in leiurotoxin I and charybdotoxin, had a significant impact on the ability of the peptides to fold into their native structure.

Analysis of the disulfide bond connectivity of native CTX indicates that it contains a cystine knot motif, with Cys V-VIII being a fourth and additional disulfide bond. However, the oxidative folding of CTX was significantly affected by the removal of Cys V-VIII. Thus, even though CTX may contain features of the cystine knot family, structurally it is more closely related to the α/β motif family.

Role of disulfide bonds in stability

CTX contains four disulfide bonds, and, as is typical for disulfide-rich peptides [20], is very stable against chemical and biological degradation both *in vivo* and *in vitro* [15]. When discussing the potential of CTX in drug design, its stability is one of the most attractive pharmacological features, as poor stability is a major limitation facing the development of peptides as drugs or drug scaffolds [32]. The high serum stability of CTX may be due to its highly compact fold, which is stabilised by the presence of four disulfide bonds, reducing exposure of its sequence to proteolytic attack. Our finding that CTX1, which contains no disulfide bonds, was rapidly degraded provides evidence to support the idea that a well-defined conformation is important for stability. Interestingly, CTX2 and CTX3 were both less stable than CTX, though they were able to maintain the native-like fold. This might be due to the fact that both lacked one disulfide bond compared to CTX, which could potentially introduce increased conformational flexibility and therefore increased accessibility to enzymatic proteolysis. This is consistent with reports of other disulfide rich peptides, where modification of the disulfide bond conformation affected stability [21, 28].

Role of disulfide bonds in activity

The biological activity of native CTX and its analogues was tested by measuring their ability to inhibit the migration of HUVECs. As shown in **Figure 6B**, all peptides were able to inhibit HUVEC migration. In the case of CTX2 and CTX3, the deletion of one disulfide bond does not result in any changes in the ability to inhibit angiogenesis, which was expected because CTX2 and CTX3 adopted a native-like conformation and we initially assumed that the activity of CTX was dependent on its tertiary structure. However, the reduced form, CTX1, which adopted a random coil structure, was also found to inhibit the migration of HUVECs with a similar potency as CTX, suggesting that a well-defined native-like fold is not required for the inhibitory activity and that it is, in fact, the primary structure which is important. It is therefore tempting to speculate that the activity of CTX against cell migration does not involve a specific cell receptor.

Our work contrasts with some aspects of a study on charybdotoxin, which demonstrated that its disulfide bond arrangement is important to maintain activity of the peptide [25]. The observed uncoupling between structure and activity for CTX with respect to cell migration inhibitory activity might not apply to other activities: in nature, the function of CTX is to subdue a target prey or organism and in that role, the disulfide bonds might be vital. On the other hand, our results are consistent with a recent study on a θ-defensin, a three disulfide-bond peptide from leukocytes of rhesus macaques, which showed that its disulfide bonds are not important for its anti-bacterial activity [28]. From a drug design perspective, it is worth noting that the migration assay was performed in a serum-free environment, i.e., no peptidases were present to degrade the peptides. Therefore, in an *in vivo* setting, the higher stability of

CTX (compared to that of CTX1) would probably translate to higher effective inhibitory activity.

Cyclisation to improve stability

Our study showed that for CTX, three disulfide bonds are sufficient to produce a native-like structure. Cys III-VII and Cys V-VIII are critical for the formation of a folded peptide, whereas Cys I-IV and Cys II-VI are not required for maintaining the overall fold. This is a significant finding because the reduced chemical complexity of a CTX analogue containing only three disulfide bonds, compared to the four-disulfide bond CTX, may be more favourable for large-scale industrial production. However, it is important to note that the deletion of one disulfide bond does significantly affect the stability of the peptide. We postulate that this reduced stability might be reversed through backbone cyclisation, based on the fact that cyclisation can reduce the accessibility of terminal amino acids to serum peptidases and enhance conformational rigidity [33, 34]. Although cyclisation might potentially complicate production of CTX analogues, modern chemical and biochemical approaches (e.g. enzyme-mediated methods [35, 36] facilitate efficient synthesis of cyclic peptide derivatives. Backbone cyclisation has been applied to the native CTX to improve serum stability by around 20%, [15] as well as other disulfide-rich peptides [23, 37, 38]. In some cases, cyclisation can also improve the potency of the peptide; for example, cyclisation of gomesin, an 18-residue cysteine-rich peptide from the spider *Acanthoscurria gomesiana*, improved its toxicity against cancer cells [23] as well as its antimalarial activity [23].

CTX as a scaffold for drug design

Primary structure comparison of CTX with other small scorpion toxins containing the α/β structural motif indicates that the only conserved amino acids are the six cysteines that form the motif [25]. The α/β motif can tolerate multiple sequence combinations, indicating that we should be able to modify the sequences between the cysteines of the α/β motif without losing the overall structure. Thus, CTX is potentially a valuable scaffold for incorporation of biologically-active peptide epitopes, such as those with antitumor or anti-angiogenesis activity into its sequence using an approach called molecular grafting to combine the favourable pharmacological features of CTX (i.e. stability and binding specificity to cancer cells) with the activity of the peptide epitope. This approach has been successfully used to design stable disulfide-rich peptides that have potent activity in animal models of chronic diseases [19, 27, 39, 40].

In conclusion, our study has shown that for CTX, three disulfide bonds are necessary and sufficient to produce a native-like structure. The bonds formed by Cys III-VII and Cys V-VIII are essential for correct peptide folding; whereas, those formed by Cys I-IV and Cys II-VI can be removed without affecting the structure, though their removal significantly alters the stability of the peptide. Finally, we have shown that the disulfide bonds are not necessary for CTX to exert its inhibitory action on cell migration.

Future perspective

In recent years there has been increased interest in the development of peptides as therapeutics. In general, this is because peptides offer better selectivity and potency compared to small molecules, as well as potentially fewer side effects [20]. Despite these advantages, peptides still face the significant challenges of poor stability and bioavailability. CTX could be an excellent scaffold peptide to try to overcome these challenges, as it is highly stable. Our data suggest that significant changes to the sequence of CTX, without affecting the overall conformation, might be possible. CTX has a natural ability to preferentially bind to cancer cells and therefore has significant potential in cancer drug development.

Executive summary

Role of disulfide bonds in the structure of chlorotoxin

Two specific pairings are important to form the native-like structure of CTX. The cystine pairs Cys I-IV and Cys II-VI can be removed without significantly affecting the structure of CTX. However, cystine pairs Cys III-VII and Cys V-VIII are important for forming the native structure.

Role of disulfide bonds in stability

Although CTX is a very stable peptide, it needs all four disulfide bonds to prevent it from being susceptible to protease degradation. When a single disulfide bond is lacking, stability is reduced.

Role of disulfide bond in activity

The ability of CTX to inhibit HUVEC migration does not depend on the native disulfide bond connectivity stabilising the tertiary structure because the reduced form is able to inhibit HUVEC migration with a similar potency to native CTX.

Key terms

Peptide: A short chain of amino acids (usually fewer than 50 residues).

Glioma: A type of aggressive malignant brain tumour.

Anti-angiogenesis: The physiological ability to inhibit the growth of new blood vessels from pre-existing vessels.

Disulfide bond: A covalent bond, derived by the coupling of two thiol groups.

α/β motif: A structural motif characterised by an α-helix and a triple-stranded antiparallel β-sheet stabilised by three or four disulfide bonds.

Cyclotides: Plant-derived disulfide-rich peptides characterised by a head-to-tail cyclised backbone together with a cystine knot motif.

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Figure captions

Figure 1. Three-dimensional structure and amino acid sequence of CTX. A. The amino acid sequence of CTX. The disulfide connectivities are shown using solid lines and the cysteine residues are labelled using Roman numerals from I to VIII. **B.** Schematic representation of CTX (PDB code 1CHL). The disulfide bonds are shown in stick format and the cysteine residues are labelled using Roman numerals from I to VIII.

Figure 2. Structure and sequences of selected peptides containing an α/β motif or a cystine knot motif. A. Sequence alignment of CTX with iberotoxin, a small scorpion toxin characterised by three disulfide bonds that belongs to the α/β family. The disulfide bond connectivities are shown as solid black lines and the cysteine residues are labelled using Roman numerals from I to VIII. The 'outlier' disulfide connectivity that differs between the two molecules is shown on the CTX sequence. **B.** Sequence alignment of CTX with kalata B1, which comprises a cystine knot motif. The disulfide bond connectives are shown as solid black lines and the cysteine residues are labelled using Roman numerals from I to VIII. The 'outlier' disulfide connectivity that differs between the two molecules is shown on the CTX sequence. The cyclic backbone of kalata B1 is shown at the bottom of the sequence by a dashed black line that joins both ends of the molecule.

Figure 3. Amino acid sequences of CTX and analogues. The cystine pairings are shown using solid black lines. Cysteine residues that were replaced by Abu (α-aminobutyrate) are represented with an **X**.

Figure 4. Analytical RP-HPLC profiles of reduced and oxidised native CTX and its analogues. Panels A to E (CTX, CTX2, CTX3, CTX4, and CTX5) illustrate CTX and its analogues folded in 0.1 M Tris pH 7.8/0.2 M NaCl, 5 mM/0.5 mM GSH/GSSG). Black and pink traces correspond to reduced and oxidised peptides, respectively. Panels F to J (CTX, CTX2, CTX3, CTX4 and CTX5)) illustrate CTX and its analogues folded in the same condition as panels A to E, with the addition of 15% (v/v) isopropanol (brown trace) and 10% (v/v) DMSO (blue trace). The asterisk indicates a disulfide bond isomer mixed with glutathione.

Figure 5. Circular dichroism spectra of CTX and its analogues. Spectra were recorded at room temperature in 2 mM Tris buffer, pH 7.4. [θ] corresponds to molar ellipticity.

Figure 6. Serum stability and migration activity of native CTX and its analogues. A. Degradation of CTX and analogues peptides in human serum after 24 h. B. Effect of native CTX and its analogues on HUVEC migration. VEGF acts as a positive control with 100% HUVECs migration. All peptides were tested at 20 µM. The data is shown as mean and standard deviation of triplicate measurements from a combination of three independent experiments. Statistical analyses using one way

ANOVA showed that the activities of CTX1, CTX2 and CTX3 were not significantly different to that of the native parent peptide. However when compared to the positive control (VEGF), these analogues were significant different with *p*-value < 0.05.

Figure 1.

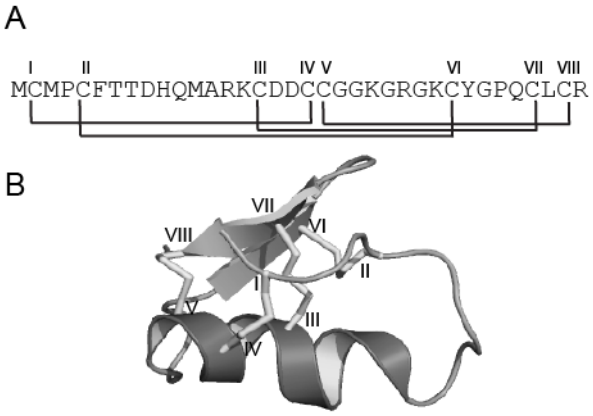

A
I II III IV V VI VII VIII
MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR
B
VII
VI
VIII
II
I
V
III
IV

Figure 2

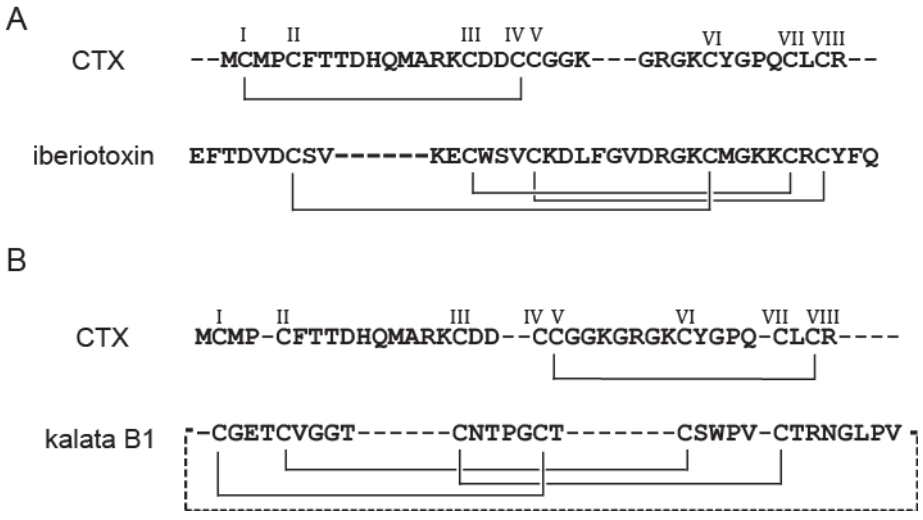
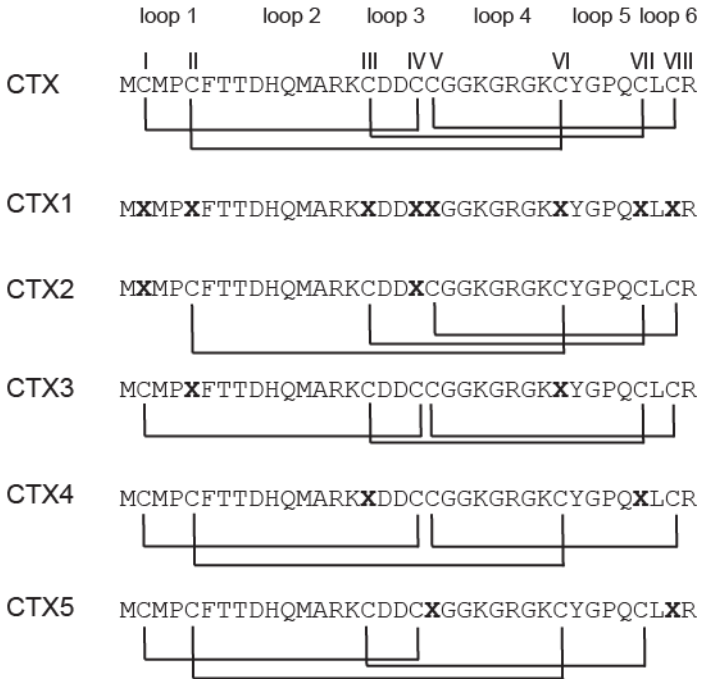

A
I II III IV V VI VII VIII
CTX
--MCMPCFTTDHQMARKCDDCCGGK---GRGKCYGPQCLCR--
iberiotoxin
EFTDVDCSV------KECWSVCKDLFGVDRGKCMGKKCRCYFQ
B
I II III IV V VI VII VIII
CTX
MCMP-CFTTDHQMARKCDD--CCGGKGRGKCYGPQ-CLCR----
kalata B1
-CGETCVGGT------CNTPGCT-------CSWPV-CTRNGLPV

Figure 3


loop 1
loop 2
loop 3
loop 4
loop 5
loop 6
I
II
III
IV
V
VI
VII
VIII
CTX
MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR
CTX1
MXMPXFTTDHQMARKXDDXXGGKGRGKXYGPQXLXR
CTX2
MXMPCFTTDHQMARKCDDXCGGKGRGKCYGPQCLCR
CTX3
MCMPXFTTDHQMARKCDDCCGGKGRGKXYGPQCLCR
CTX4
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CTX5
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Figure 4

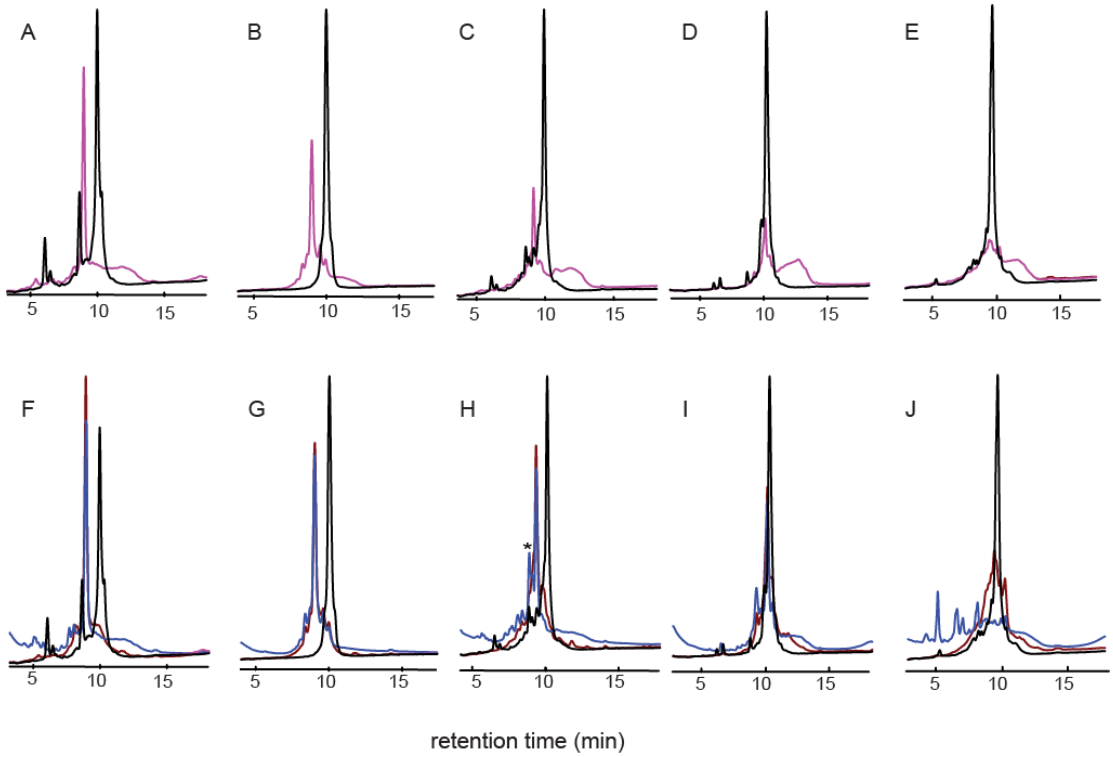

Figure 5

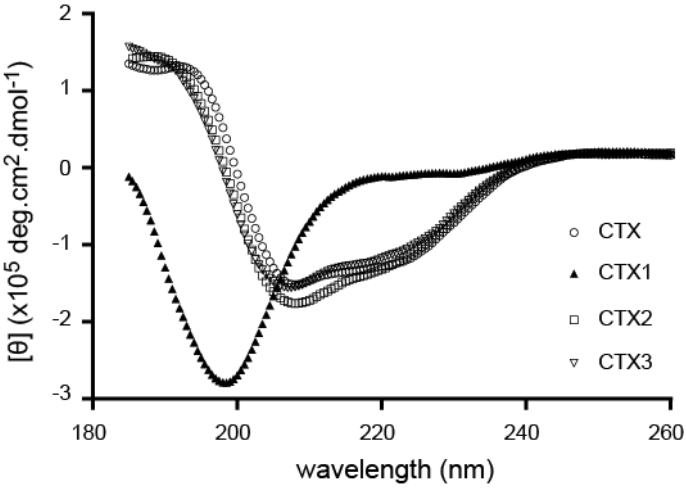
2
1
0
-1
-2
-3
180
200
220
240
260
wavelength (nm)
[θ] (x10^5 deg.cm^2.dmol^{-1})
CTX
CTX1
CTX2
CTX3

Figure 6

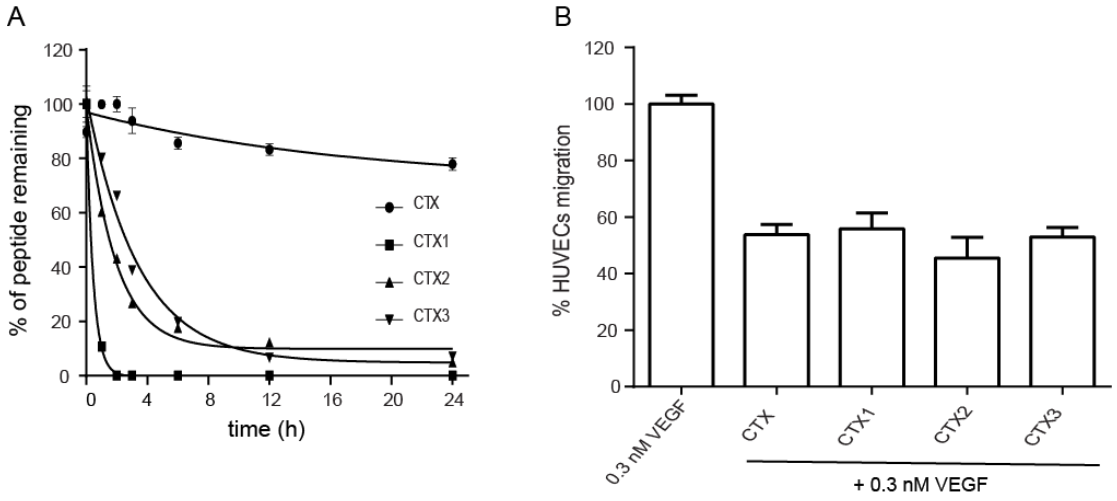
A
% of peptide remaining
time (h)
CTX
CTX1
CTX2
CTX3
B
% HUVECs migration
0.3 nM VEGF
CTX
CTX1
CTX2
CTX3
+ 0.3 nM VEGF

Table 1. Sequences and masses of alkylated peptides of CTX2 and CTX3 that were analysed by MS/MS.

Peptide	Sequence	M (Da)	*m/z* values observed
CTX2+2NEM	MXMPC$_N$FTTDHQMARKCDDXCGGKGRGKC$_N$YGPQCLCR	4215.6	1054.9^{4+}, 843.5^{5+}, 703.7^{6+}
CTX3+2NEM	MC$_N$MPXFTTDHQMARKCDDC$_N$CGGKGRGKXYGPQCLCR	4215.6	1054.9^{4+}, 843.5^{5+}, 703.7^{6+}
CTX3+4NEM	MC$_N$MPXFTTDHQMARKCDDC$_N$C$_N$GGKGRGKXYGPQCLC$_N$R	4443.6	-
CTX3+4NEM+ 2CAM	MC$_N$MPXFTTDHQMARKC$_C$DDC$_N$C$_N$GGKGRGKXYGPQC$_C$LC$_N$R	4579.6	-
CTX3+4NEM+ 2CAM/digest	XYGPQC$_C$LC$_N$R	1205.4	603.7^{2+}
CTX3+4NEM+ 2CAM/digest	KC$_C$DDC$_N$C$_N$GGK	1234.3	618.1^{2+}

C$_C$:Carbamidomethylcysteine. C$_N$: Cysteine alkylated with NEM. X:α-aminobutyric acid.

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