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Chemical Re-engineering of Chlorotoxin Improves Bioconjugation Properties for Tumor Imaging and Targeted Therapy

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Bioconjugates composed of chlorotoxin and near-infrared fluorescent (NIRF) moieties are being advanced toward human clinical trials as intraoperative imaging agents that will enable surgeons to visualize small foci of cancer. In previous studies, the NIRF molecules were conjugated to chlorotoxin, which results in a mixture of mono-, di-, and trilabeled peptide. Here we report a new chemical entity that bound only a single NIRF molecule. The lysines at positions 15 and 23 were substituted with either alanine or arginine, which resulted in only monolabeled peptide that was functionally equivalent to native chlorotoxin/Cy5.5. We also analyzed the serum stability and serum half-life of cyclized chlorotoxin, which showed an 11 h serum half-life and resulted in a monolabeled product. Based on these data, we propose to advance a monolabeled chlorotoxin to human clinical trials.

Introduction

Neurosurgeons have long sought methods to illuminate brain cancer cells to help them identify cancer foci and distinguish cancer from normal tissue in real time during tumor resection operations. We recently reported a bioconjugate (named "Tumor Paint") composed of chlorotoxin (CTX[a]), a peptide-based toxin derived from scorpion venom, and near-infrared fluorescent (NIRF) molecules such as Cy5.5, which clearly identifies tumor foci with high sensitivity.[1,2] CTX was originally selected for these studies because it preferentially bound to glioma cells compared with normal brain tissue.[3,4] The CTX target appears to be shared by multiple other cancer types, and thus CTX/Cy5.5 effectively illuminated prostate, colon, sarcoma, medulloblastoma, and other types of solid tumors.[1]

CTX is a 36 amino acid peptide with four disulfide bonds that confer a high degree of structural stability to this small peptide.[5−7] It contains three lysines at positions 15, 23, and 27 that were utilized for conjugation to *N*-hydroxysuccinimide-ester (NHS-ester) modified Cy5.5. The resultant bioconjugate contained a mixture of typically 75−85% monolabeled peptide at position 27 and smaller amounts of di- and trilabeled peptide in which Lys15 and Lys23 were also labeled (Olson laboratory, unpublished data). Although it is possible to have mixtures such as this approved by the US Food and Drug Administration (FDA) and similar regulatory agencies elsewhere, commercialization is potentially hindered because it is expensive and difficult to match the ratio of mono-, di-, and trilabeled batches in the future.

In addition to delivering a monolabeled product to the FDA for evaluation, it was our goal to ensure that both the serum stability and half-life of the product were optimized. Conjugation to NIRF probes dramatically increased the serum half-life of CTX; however, we wished to determine whether cyclization of CTX would further improve the stability of CTX bioconjugates. The rationale was that cyclization eliminates the availability of terminal amino acids to serum peptidases. In several other instances, cyclization of peptides increased serum stability and half-life and, in some cases, stabilized peptides in a manner that made them orally bioavailable.[8−12]

Here we report that substitution of Lys15 and Lys23 with either alanine or arginine eliminates mixed bioconjugate species, while retaining functional efficacy. Cyclized CTX exhibited increased serum stability but did not increase *in vivo* half-life compared with linear CTX and unexpectedly yielded only monolabeled Cy5.5 bioconjugates. These findings pave the way for progression of chemically re-engineered chlorotoxin as a tumor imaging agent.

Results

Design and Synthesis of CTX Analogs. The designed alanine- and arginine-substituted K15A_K23A-CTX and

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[a] Abbreviations: BOC, *tert*-butyloxycarbonyl; CTX, chlorotoxin; DIPEA, diisopropylethylamide; ES-MS, electrospray mass spectroscopy; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HF, hydrogen fluoride; LC-MS, liquid chromatography−mass spectroscopy; MMP2, matrix metalloproteinase-2; NHS, *N*-hydroxysuccinimide; NIRF, near-infrared fluorescent; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; RP-HPLC, reversed-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; TOCSY, total correlation spectroscopy.

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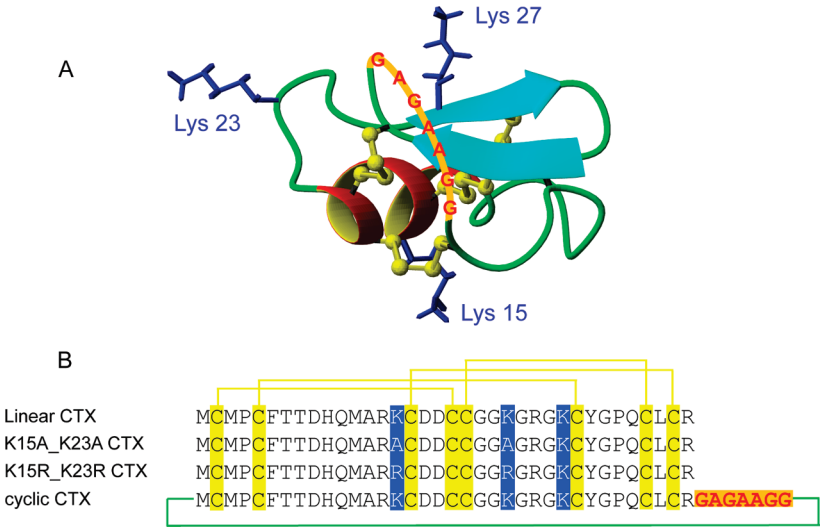

Figure 1. Three-dimensional structure of chlorotoxin and sequences of the synthesized peptides: (A) the structure of linear chlorotoxin has six backbone loops (1−6), and the disulfide bonds are shown in yellow ball and stick format; (B) the amino acid sequences of linear alanine- and arginine-substituted and cyclic chlorotoxin; the disulfide connectivities between cysteine residues are shown by solid yellow lines, substituted residues are highlighted in blue boxes, and the linker residues are shown in red.

K15R_K23R-CTX and the cyclic-CTX sequences are shown in Figure 1. The peptides were synthesized using *tert*-butoxycarbonyl (Boc)/2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) *in situ* neutralization chemistry.[13] From the three-dimensional structure of CTX (1CHL.pdb), the distance between N and C termini was measured as 11.7 ± 1.5 Å. Based on a previous study on a cyclized conotoxin,[14] it was determined that a seven-residue linker, GAGAAGG, would be sufficient to span the distance between the N and C termini of the native peptide. Due to their small side chains, glycine and alanine were selected as linker constituents to minimize side chain interactions that might otherwise perturb the native 3D conformation of the peptide. An intramolecular native chemical ligation strategy[15] was used to join the two ends of CTX, resulting in backbone cyclization.[16−18] A buffer solution of 0.1 M Tris-HCl (pH 7.8), 0.2 M NaCl, and 5 mM reduced glutathione/0.5 mM oxidized glutathione was used both to oxidize the substituted peptides and to cyclize and oxidize CTX at room temperature overnight. Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to purify the peptides, and the purity and the molecular masses of the three CTX analogs, K15A_K23A-CTX, K15R_K23R-CTX, and cyclic-CTX, were confirmed by analytical RP-HPLC and electrospray mass spectroscopy (ES-MS).

Nuclear Magnetic Resonance (NMR) Spectroscopy. The peptides were dissolved in 90% H_2O and 10% D_2O, and one-dimensional, total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) spectra were recorded at 600 MHz at 298 K. The NMR spectra were assigned using well-established techniques.[19] The peaks in the amide region are well dispersed, confirming that the peptides are correctly folded, and the fingerprint region in the NOESY spectrum of each peptide shows a complete cycle of αH−NH sequential connectivities with the exception of the two proline residues (Pro4 and Pro31). However, as expected, NOEs were observed from the δ protons of the proline residues and their preceding residues. A comparison of secondary αH chemical shifts of native CTX and the synthesized analogs are shown in Figure 2. These data unequivocally demonstrate that the chemically modified peptides structurally mimic the native species.

Characterization of Substituted and Cyclic CTX Bioconjugates. The native and chemically modified peptides were conjugated to Cy5.5 and purified as described before.[1] Resulting bioconjugates were analyzed by RP-HPLC and mass spectrometry, and in all cases >95% conjugation was observed. In some cases, MS/MS sequencing showed that a small proportion of conjugation (< 5%) occurred at the N-terminus of the linear CTX analogs. Though not anticipated, labeling of the cyclized CTX also resulted in only monolabeled bioconjugate. The latter observation suggests that cyclization modified the peptide structure or reactivity such that only a single lysine was available for Cy5.5 conjugation. Furthermore, the re-engineered cyclic analog had improved biological stability.

Serum Stability of Cyclized and Linear CTX. A serum stability assay was used to determine the extent to which cyclized and linear peptide were susceptible to proteolytic degradation caused by peptidases in serum. The assay involved incubating the peptide in human serum at 37 °C for a period of 24 h and assessing the amount of intact peptide at multiple time points. After 24 h, 90% of the cyclized CTX remained intact, whereas approximately 70% of linear CTX peptides (CTX and A15K_A23K CTX) were intact at the same time point (Figure 3A). Thus, cyclization improved resistance to proteolytic cleavage. The similar degradation rates of the linear CTX peptides suggests that removal of the lysine residues, and consequently removal of proteolytic cleavage sites, does not significantly influence the stability.

Serum Half-Life of Cyclized and Linear CTX/Cy5.5 Bioconjugates. In contrast to the serum stability studies, which focused on proteolytic degradation, serum half-life studies determined the extent to which the bioconjugated peptides remained in the bloodstream of mice that received a single intravenous injection of the bioconjugate. In addition to proteolytic degradation, the other factors that limit serum half-life include tissue distribution, metabolism, and excretion. We knew, in advance of the current studies, that the

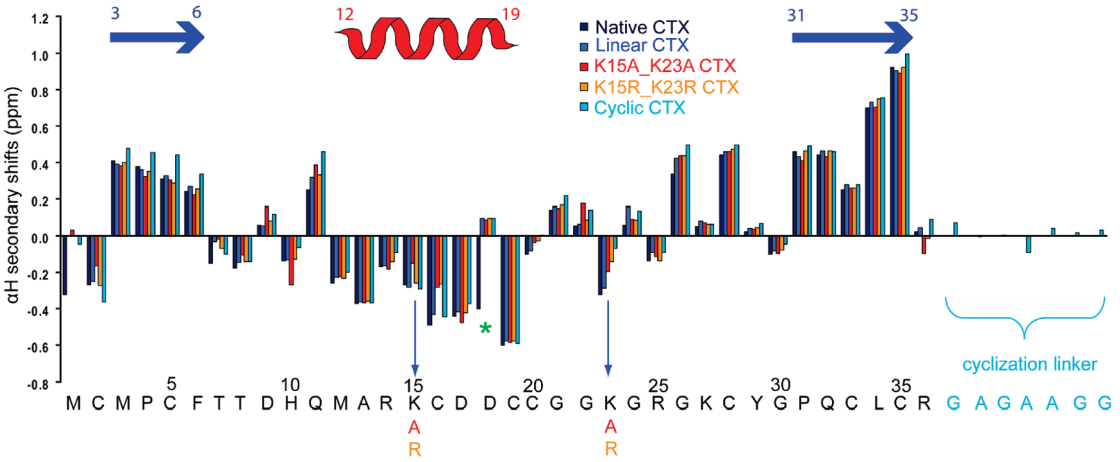

Figure 2. A comparison of the secondary αH chemical shifts of the synthesized analogs and native chlorotoxin. The secondary αH shifts were calculated by subtracting the random coil shifts[31] from the experimental αH shifts. Alanine- and arginine-substituted lysines are shown by blue thin arrows. Asp18 is highlighted with a green asterisk, because it is the only residue that differs between linear and cyclic CTXs by more than 0.15 ppm. We believe that this is due to a typographical error in the reported chemical shift[7] of this residue. Two β-sheets are shown with blue large arrows, and the helical region is indicated by a red helix.

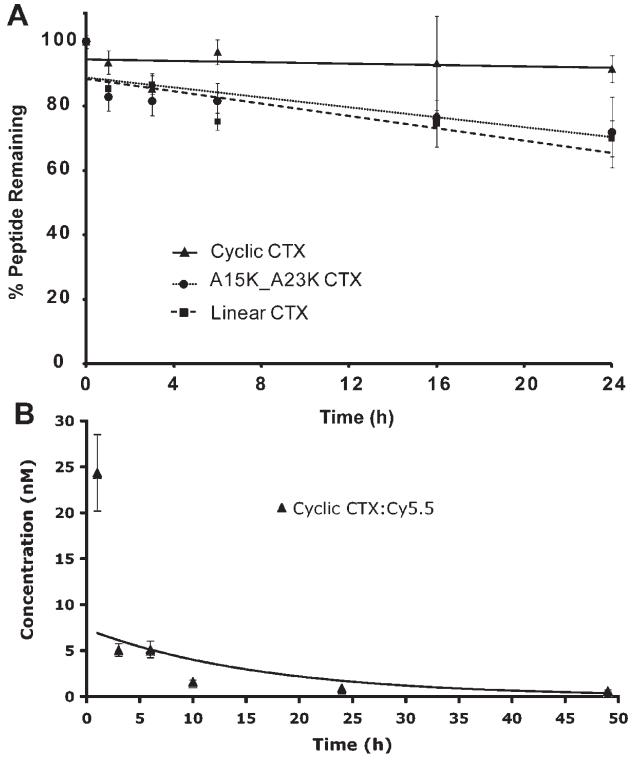

Figure 3. The stability and the serum half-life of linear and cyclic chlorotoxin: (A) The stability of linear and cyclic chlorotoxin in human blood plasma. The stability of peptides in human serum was determined over 24 h. The stability of the cyclic chlorotoxin is 15−20% higher than that of the linear version. (B) Serum half-life of cyclic CTX/Cy5.5 (n = 3, error bars represent SD). Blood was collected 1, 3, 6, 10, 24, and 49 h after injection of cyclic CTX/Cy5.5, and fluorescent intensity in the serum was measured using the Odyssey Imaging System. Serum half-life was calculated to be 11 h using the formula $t_{1/2} = \ln(2)/\lambda$, where λ is the decay constant.

serum half-life of linear CTX/Cy5.5 was approximately 14 h, which is dramatically longer than that of linear CTX alone. This suggested that conjugation of Cy5.5 protected the peptide from rapid degradation or elimination. Cyclized CTX/Cy5.5 had a serum half-life of 11 h, showing that cyclization does not extend the *in vivo* serum half-life compared with linear CTX/Cy5.5 (Figure 3B).

Functional Assessment of Cyclized and Substituted CTX/Cy5.5. The potential benefits of cyclization or substitution depend on whether the functional targeting activity of the new peptides is comparable to native CTX bioconjugates. The capacity of each peptide to target Cy5.5 signal to medulloblastoma cells preferentially over normal brain was assayed by biophotonic imaging. In each case, 50 μL of 40 μM bioconjugate was injected into the tail vein of mice that showed clinical signs consistent with advanced brain tumors. After three days, the mice were sacrificed, and their brains were imaged using the Caliper/Xenogen Spectrum biophotonic imaging system. All of the modified peptide conjugates preferentially illuminated medulloblastoma cancer tissue compared with normal brain (Figure 4). In all cases, signal, measured as fluorescent efficiency (cm^2) on the Caliper/Xenogen Spectrum, in the tumor was compared with signal in the cerebellum of injected control animals that did not have medulloblastoma. The signal in the tumor compared with normal was 1.96 ± 0.47 units for native CTX/Cy5.5 (n = 10), 2.49 ± 1 for cyclized CTX/Cy5.5 (n = 5), 3.3 ± 1.8 for alanine-substituted (n = 8), and 2.6 ± 0.85 for arginine-substituted CTX/Cy5.5 (n = 5). Statistically, all of the modified peptide bioconjugates were indistinguishable from native CTX/Cy5.5, indicating that cyclization and lysine substitutions did not interfere with CTX binding to its target.

Discussion and Conclusions

When advancing a new therapeutic or imaging agent toward human clinical trials, teams must consider not only efficacy, pharmacokinetics, pharmacodynamics, and toxicity but also practical issues that could compromise regulatory approval or increase manufacturing costs.[20,21] In the case of Tumor Paint, the original bioconjugate posed a manufacturing challenge, because it was a mixture of mono-, di-, and trilabeled CTX. In this manuscript, we report three new chemical entities that are functionally equivalent to CTX for targeting NIRF molecules to cancer yet conjugate to only a single NIRF molecule.

We recently mapped CTX conjugation sites in CTX/Cy5.5 using arginase cleavage coupled with proteomic analyses

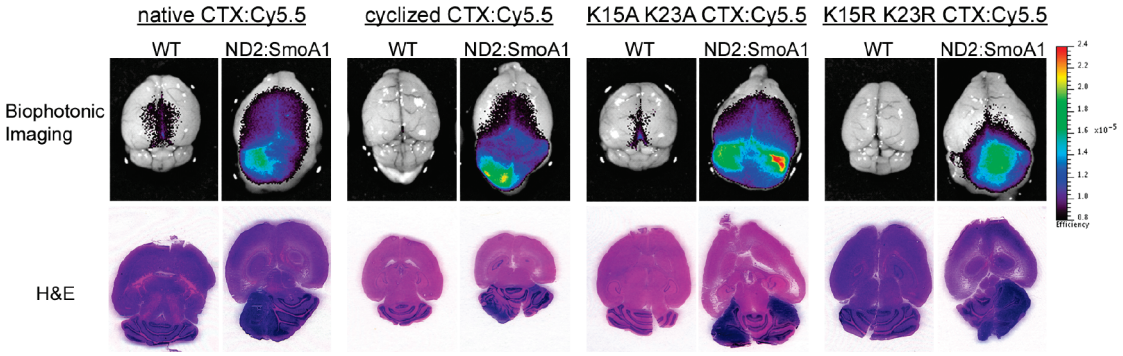

Figure 4. Functional imaging with modified CTX/Cy5.5 bioconjugates. Wild-type and ND2:SmoA1 tumor-bearing mice were injected with 50 μL of 40 μM of native, cyclized, K15A_K23A, or K15R_K23R chlorotoxin/Cy5.5 bioconjugate through the tail vein. Biophotonic images were taken three days after the injection using the Xenogen Spectrum. The brains were then frozen in Tissue-Tek Optimal Cutting Temperature Compound, cut in 12 μm sections, and stained with hematoxylin and eosin to determine tumor burden.

(M. Veiseh, B. Bahrami, J. Olson, unpublished data). This analysis showed that typically >80% of product was monolabeled at Lys27 and smaller amounts were also conjugated at Lys15 or Lys23. In an analysis of CTX conjugated to four other NIRF dyes, similar patterns of predominantly monolabeled peptide with smaller amounts of di- and trilabeled peptide were observed, with the exception of Dylight 750, a NIRF dye that creates only monolabeled species when bound to CTX (S. Hansen, M. Stroud, J. Olson, unpublished data). The fact that Dylight 750 binds in a monomeric fashion to unmodified CTX suggests that access to the other two lysines is limited in a way that is dependent on the nature of the dye. Here we show that cyclization is also able to moderate the reactivity because cyclized CTX binds only one Cy5.5 molecule per peptide despite the fact that all three lysine residues remain intact in the cyclized version.

Other examples where macrocyclic disulfide-rich peptides have been labeled include the studies of Greenwood et al. on biotin-labeled cyclotide MCoTI-II and that of Herrmann et al. on the chemical modification of lysine/arginine residues in cycloviolacin O2 to probe structure−activity relationships.[22,23] Both of these peptides have multiple labeling sites that are accessible even in the cyclic form, in contrast to the current study. Combined with the results reported here for cyclic chlorotoxin, these studies highlight the practicality of labeling cyclic peptides despite the lack of an N-terminus.

None of the lysine residues in CTX seem to be involved in the active binding of CTX to its target on cancer cells. This conclusion is based on the observations that target binding is preserved despite substitution of Lys15 or Lys23 with alanine or arginine and that addition of bulky Cy5.5 or other NIRF dyes to Lys27 does not preclude binding to the active site. The target of CTX was earlier reported to be matrix metalloproteinase-2 (MMP2).[24] Recently, it was reported that a protein called annexin A2, which is expressed on the cell surface of human tumor cells,[25] is a novel target of TM601, a synthetic version of CTX.[26]

Moving toward human clinical trials, we now have three new chemical entities that could be used for targeting purposes. Since Tumor Paint is intended to be administered through a single intravenous injection, it is not necessary to develop an orally available version of the active molecule, and hence this potential advantage of the cyclization is less important in this imaging application than it is in drug development. Also, cyclization did not improve the serum half-life of the peptide. The serum stability was improved by cyclization from 70% intact peptide to 90% intact peptide following 24 h of incubation in human plasma at 37 °C, suggesting proteolytic cleavage was reduced following cyclization, but *in vivo* excretion and metabolism was not improved. Thus, in the presence of Cy5.5 conjugation, which is known to markedly increase the serum half-life of CTX, the additional benefit gained by cyclization does not outweigh the potential added manufacture costs associated with using cyclic versus linear peptides in the clinical setting. Nevertheless, the benefits of cyclization might be useful in future analogs of chlorotoxin or other imaging agents where stability and bioavailability considerations are more important. Both chemical[27] and biological[28] approaches have been developed in recent years for the synthesis of macrocyclic disulfide-rich peptides.

Of the linear peptides we synthesized, the signal in tumors from bioconjugates made with linear alanine substitutions was slightly higher than that of arginine substitutions. Both molecules will be studied further, and one will be selected to move toward human clinical trials. Thus, the work presented in this manuscript successfully identified two monolabeled peptide conjugates that retain the targeting activity of native CTX, while circumventing the regulatory and manufacturing problems of multiply conjugated CTX. These data provide a clear path forward for translating Tumor Paint from mouse trials to human clinical practice.

Experimental Procedures

Solid-Phase Peptide Synthesis (SPPS). Manual Boc SPPS was used to synthesize the peptides with standard amino acid side-chain protection [e.g., Asn(Xan), Asp(OcHex), Arg(TOS), Cys(MeBzl), Lys(ClZ), Ser(Bzl), Thr(Bzl), and Tyr(BrZ)]. The cyclic analog was assembled onto the phenylacetamidomethyl-glycine (PAM-Gly) resin using *in situ* neutralization/2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) protocol for Boc (ter-butoxycarbonyl) chemistry via a thioester moiety generated by coupling of *S*-trityl-β-mercaptopropionic acid to the resin, deprotection with trifluoroacetic acid/tri-isopropylsilane/water (90:5:5), and the addition of the C-terminal amino acid using standard coupling chemistry. GAGAAGG residues were used as a linker to join the N and C termini of the peptide. The alanine- and arginine-substituted linear CTX were assembled onto PAM-Arg resin without the thioester functionality. Cleavage of the peptides from the resin was achieved using hydrogen fluoride (HF) with *p*-cresol and *p*-thiocresol as scavengers [9:0.8:0.2 (v/v) HF/*p*-cresol/*p*-thiocresol] at −5 to 0 °C for 1.5 h. RP-HPLC with a C_{18} column was used to purify the peptides using a gradient of 0−80% solution B (solution A = H_2O/0.05% trifluoroacetic

acid; solution B = 90% CH_3CN/10%H_2O/0.045% trifluoroacetic acid) by monitoring the absorbance at 215 nm. ES-MS confirmed the purity and molecular mass of the synthesized peptides.

Cyclization and Folding. The intramolecular native chemical ligation reaction[15] was used to synthesize a cyclic CTX analog. The cyclic CTX and linear alanine- and arginine-substituted analogs were oxidized in an aqueous buffer solution consisting of 0.1 M Tris-HCl, 0.2 M NaCl, 5 mM reduced glutathione/0.5 mM oxidized glutathione at pH 7.8 and incubated overnight at room temperature. RP-HPLC was used to purify the peptides, and the purity and the molecular masses were confirmed by analytical RP-HPLC and ES-MS.

NMR Spectroscopy. NMR spectroscopy (600 MHz) was used to analyze the three-dimensional structures of the cyclized peptide analogs. The peptide samples were dissolved in 90% H_2O and 10% D_2O (v/v). D_2O (99.99%) was obtained from Cambridge Isotope Laboratories, Woburn, MA, USA. Two-dimensional NMR experiments included TOCSY and NOESY spectra, which were recorded at 298 K.

Serum Stability Assay. Serum stability assays were carried out in 100% human male serum (Sigma) using a peptide concentration of 20 μM. The serum was centrifuged at 14 000*g* for 10 min to remove the lipid component, and the supernatant was incubated at 37 °C for 15 min before the assay. Each peptide was incubated in serum at 37 °C, and 40 μL triplicate aliquots were removed at 0, 1, 3, 6, 10, 16, and 24 h. Each serum aliquot was quenched with 40 μL of 6 M urea and incubated for 10 min at 4 °C. Then, each serum aliquot was quenched with 40 μL of 20% trichloroacetic acid and incubated for another 10 min at 4 °C to precipitate serum proteins. The samples were centrifuged at 14 000*g* for 10 min, and 100 μL of the supernatant was analyzed on RP-HPLC using a linear gradient of solvent B (0.3 mL/min flow rate). The control samples contained equivalent amounts of peptides in phosphate-buffered saline (PBS) subjected to the same treatment procedure. The percentage recovery of peptides was detected by integration at 215 nm.

Bioconjugation of Substituted and Cyclized CTX. Bioconjugation of substituted and cyclized CTX was performed using a mixture of CTX [2 mg/mL in bicarbonate buffer (pH 8.5)] and Cy5.5-NHS-ester (Invitrogen, 10 mg/mL in anhydrous dimethyl formamide) at the molar ratio of 3:1 (dye/CTX). Conjugation was done in the dark at room temperature for 1 h. Unconjugated dye was removed by dialysis against PBS using Slide-A-Lyzer (Pierce Biotechnology) membrane (M_r cutoff, 3500) up to 18 h at 4 °C. Samples were diluted with PBS to produce 40 μM of CTX solution and filtered with a 0.2 μm syringe filter before use. The purity of all bioconjugates was quantified by analytical RP-HPLC as >95%. All batches were further evaluated by liquid chromatography−mass spectroscopy (LC-MS) and Xenogen IVIS-100 to ensure the quality.

Animal Models. All animals were handled in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal studies were conducted in accordance with Fred Hutchinson Cancer Research Center's Institute of Animal Care and Use Committee approved protocols. An autochthonous mouse model of medulloblastoma, ND2:SmoA1,[29,30] on a C57BL/6 background was used to evaluate the specificity of cyclized CTX/Cy5.5, K15A_K23A CTX/Cy5.5, and K15R_K23R CTX/Cy5.5. Hemizygous or homozygous (referred as ND2:SmoA1) mice with symptomatic medullablastoma were selected for enrollment in these studies. Symptoms were detected using an open field cage evaluation. Symptoms include head tilt, hunched posture, ataxia, protruding skull, and weight loss.

***Ex Vivo* Imaging.** ND2:SmoA1 animals exhibiting symptoms of medulloblastoma were injected with 50 μL of 40 μM cyclized CTX/Cy5.5, K15A_K23A CTX/Cy5.5, or K15R_K23R CTX/Cy5.5 through the tail vein. Mice were euthanized using CO_2 inhalation three days after injection, and *ex vivo* biophotonic images of their brain were obtained using the Xenogen Spectrum Imaging System (Caliper). The brains were then frozen in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura), sliced in 12 μm sections, and hematoxylin and eosin (HE) stained according to standard procedures.

Serum Half-Life. Two month old wild-type C57BL/6 mice (Charles River) were intravenously injected with 50 μL of 40 μM cyclized CTX/Cy5.5 through the tail vein. Blood was collected at 1, 3, 6, 10, 24, and 49 h postinjection by terminal heard puncture. Blood was collected for three animals at each time point. The blood was snap frozen immediately after collection and stored at −80 °C. Whole blood was thawed and centrifuged for 5 min to separate the plasma. Plasma was collected and added to clear bottom black 96 well optical plates. A standard curve was generated with a serial dilution of cyclized CTX/Cy5.5 in wild-type C57BL/6 plasma. The plates were scanned on the Odyssey Imaging System (Li-Cor Bioscience) using the 700 nm channel. Serum half-life was calculated using the formula $t_{1/2} = \ln(2)/\lambda$, where λ is the decay constant.

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