Supplementary information

From venoms to BBB-shuttles. MiniCTX3: a molecular vector derived from scorpion venom

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Materials and methods

Solvents and reagents

Solvents and reagents were obtained from commercial suppliers such as Iris Biotech (Marktredwitz, Germany), Aldrich (Milwaukee, WI), GL Biochem Shangai Ltd. (Shangai, China) and Fluka Chemika (Buchs, Switzerland), and were of the highest purity commercially available.

Protected amino acids were supplied by Iris Biotech and SDS (Barcelona, Spain). RinkAmide ChemMatrix® resin was purchased from PCAS BioMatrix Inc. (Quebec, Canada). Syringes, eppendorfs and falcons were acquired from Scharlau (Barcelona, Spain) and Deltalab. Synthetic chlorotoxin was acquired from Smartox Biotechnology (La Tronche, France).

Cell culture-treated plates and flasks were purchased from Corning Costar. Culture medium was acquired from Lonza. XTT cell proliferation kit was purchased from Biological Industries (Cromwell, CT). Pierce® iodination beads were obtained from Pierce. Desalting columns (MiniTrap and MidiTrap G-25) were obtained from GE-Healtcare.

Peptide synthesis and characterisation

Solid-phase peptide synthesis

Peptides were assembled using Fmoc/*t*Bu solid-phase peptide synthesis (SPPS), manually or with an automated microwave-assisted peptide synthesiser (Liberty Blue synthesiser, CEM). The peptides were synthesised in a 250 µmols scale using RinkAmide ChemMatrix® resin (substitution of 0.52 mmol/g) or RinkAmide AM resin (0.74 mmol/g).

Synthesis was performed on polypropylene syringes with a polypropylene porous filter. The stirring was done manually, intermittently, with a Teflon stirring bar and reagents and solvents were eliminated under vacuum filtration. Amino acids were coupled to the growing chain using standard protocols. Briefly, the resin was washed several times and the temporary protecting group was removed using a solution of piperidine. After washing the resin again, the amino acid and coupling reagents were added and left to react for 20-60 min. Finally, after the washings of the resin from the reagents and by-products, the coupling control was carried out by the ninhydrin or chloranil test.

Temporary protecting group removal

The Fmoc group was removed by treating the resin with a solution of 20% piperidine in DMF (1 x 1 min and 2 x 10 min).[1]

Fmoc group quantification/resin loading capacity

In order to know the degree of functionalisation, the Fmoc adduct, formed during removal of the Fmoc group with piperidine, can be quantified with UV-Vis spectroscopy. The Fmoc removal and washing steps solution were collected in a volumetric flask and diluted with DMF. Part of this solution was used to measure the absorption at 300 nm, and knowing the amount of resin and the volume of the flask, the resin functionalisation was calculated using the following formula:

$$f = \frac{A_{300} \cdot V}{\varepsilon \cdot l \cdot m}$$

where A_{300} is the measured absorbance at 300 nm, V the volume of the flask, ε is the molar extinction coefficient of Fmoc at 300 nm (7800 $M^{-1}cm^{-1}$), l is the path length of the cuvette and m the weight of the resin.

Coupling methods

Manual synthesis

Several coupling methods were used depending on the amino acids involved. Method 1 was used as the default method, while Method 2 was used for milder couplings. Method 3 was used for long couplings, cyclisations and for the incorporation of fluorophores.

Method 1 – HBTU/DIEA: 4 eq Fmoc-AA-OH, 4 eq HBTU, 8 eq DIEA, DMF (20-40 min).

Method 2 – DIC/Oxyma: 4 eq Fmoc-AA-OH, 4 eq DIC, 4 eq Oxyma, DMF (30-45 min).

Method 3 – PyBOP/HOAt: 4 eq Fmoc-AA-OH, 4 eq PyBOP, 12 eq HOAt, 12 eq DIEA, DMF (45-60 min or o/n).

Automated microwave-assisted peptide synthesiser – Liberty Blue

The protected amino acid (0.2 M in DMF), DIC (0.5 M in DMF), Oxyma (1 M in DMF) and HOBt (1M in DMF) were added to the reaction vessel. The mixture was allowed to react for 2 min with a microwave potency of 170 V (90°C). The solvents were removed by filtration, and the resin was washed with DMF (3 x 30 s). For His the maximum temperature was reduced to 50°C. The removal of the Fmoc group was performed with piperidine (20% in DMF) or piperazine (10% in NMP/EtOH [9/1]) for 1 min.

Monitoring of coupling and deprotection

The Kaiser colorimetric[2] test assay was used to detect primary amines bond to solid-phase and the chloranil test for the detection of secondary amines.

Mini-cleavage of the resin

Few beads of dried but DCM solvated resin were transferred into an eppendorf. The procedure was the same as for the cleavage described in *Cleavage from the resin/side-chain deprotection*, using 95% of TFA, 2.5% H_2O and 2.5% of TIS, for 15-30 minutes. Then the peptide was washed with diethyl ether and dissolved in H_2O/ACN (1:1).

Lactam bridge formation

The peptides were synthesised with Fmoc/*t*Bu SPPS using Fmoc-Dap(Alloc)-OH and Fmoc-Asp(OAll)-OH (or Fmoc-Glu(OAll)-OH) as the amino acids at the cyclisation points. Before the deprotection of the final Fmoc group, Allyl and Alloc groups were removed using tetrakis(triphenylphosphine)palladium(0) (0.2 eq) and phenylsilane

(10 eq) in DCM, in Ar atmosphere. Three treatments for 15 min were performed and the deprotection was assessed by the kaiser test and mini-cleavage with UPLC-MS analysis.

Cyclisation was achieved with two reactions (2h and overnight) with PyBOP (4 eq), HOAt (12 eq) and DIEA (12 eq). The completion of the reaction was assessed through a mini-cleavage and UPLC-MS analysis. Finally the Fmoc group was removed using 20% piperidine in DMF.

Incorporation of carboxyfluorescein

In some peptides and before the final cleavage, carboxyfluorescein (5(6)-Carboxyfluorescein, CF) was incorporated. CF (3 eq), HOBt (3 eq) and DIC (3 eq) in DMF were added to 100 µmol of resin and left to react for 2-16 h. The resin was washed with DCM (3 x 1 min) and DMF (3 x 1 min) and treated with 20% piperidine in DMF (1h).

Cleavage from the resin/side-chain deprotection

After the deprotection of the *N*-terminus of the last amino acid, the resin was washed several times with DMF and DCM and dried by suction for 15 minutes. The dried resin was transferred to a 50 mL falcon and the peptides were cleaved from the resin using the appropriate cleavage mixture. The peptide was left to react for 2-4 h using enough solvent to allow a good swelling of the resin. All the cocktails incorporated a high percentage of TFA with the presence of scavengers to capture the cleaved protecting groups and avoid side reactions, such as H_2O, TIS, TA and EDT. After cleavage of the peptides, the solvent was evaporated applying a N_2 current. The following are the cleavage cocktails used:

Cocktail A: TFA/H_2O/TIS (95:2.5:2.5) [for MiniCTX2 and MiniCTX3]

Cocktail B: TFA/H_2O/EDT/TIS (94:2.5:2.5:1) [for MiniCTX1]

Cocktail C: TFA/TA/H_2O/EDT/*p*-cresol (92:2.5:2.5:2.5:0.5) [for CTX]

tert-Butyl methyl ether was added at 0°C to precipitate the peptide and eliminate non-peptidic impurities, and the mixture was centrifuged (4°C, 3500 rpm, 10 min). The supernatant liquid was decanted and the process was repeated three times. After that, the cleaved peptides were dissolved in H_2O/MeCN (1:1) and filtered off the resin. Finally, peptides were lyophilised.

Disulfide bridge formation

For those peptides containing free thiols from Cys, the oxidation was performed at room temperature under air oxygen for 1 to 5 days. A 1 L round-bottom container with a large aperture was used to allow a better oxidation. High dilution conditions (0.5 – 1 mg/mL) were required to favour the formation of intramolecular disulfide bridges over intermolecular bonds. The peptide was dissolved in NH_4HCO_3 buffer (0.1 M, pH 8.0) or in Tris buffer (0.1 M, pH 8.0). For CTX, which contains more than one disulfide bridge, reduced/oxidised glutathione (1 µM and 0.5 µM) were added to favour the formation of the more stable disulfide bridges. The reactions were monitored by UPLC and UPLC-MS. After the oxidation was completed, the buffer was acidified with TFA to pH 3-4 and the peptide lyophilised.

Purification of peptides

Peptides were dissolved in H_2O/MeCN (the percentage depending on the peptide) and filtered through 0.45 µm filters. Then, they were purified on a Waters system with ChromScope software, a 2707 Autosampler, a Prep Degasser, a 2545 binary gradient module, a 2489 UV/Visible Detector, and a Fraction Collector III. A XBridge C_{18} column (150 x 19 mm x 5 µm, Waters) or a Aeris Peptide XB C_{18} column (250 x 21.1 mm x 5 µm, Phenomenex)

was used, with MeCN (0.1% TFA) and H_2O (0.1% TFA) as solvents and a flow rate of 15-20 mL/min. The fractions containing the peptide were analysed by UPLC or UPLC-MS, pooled together and lyophilised.

Identification of peptides

UPLC-MS

Chromatograms and spectra were recorded on a Waters high class (PDA detector, sample manager FNT and Quaternary solvent manager) coupled to an electrospray ion source ESI-MS Micromass ZQ and using the MassLynx 4.1 software (Waters, Milford, MA). Using a BEH C_{18} column (50 x 2.1 mm x 1.7 μm, Waters). The flow rate was 0.6 mL/min, and MeCN (0.07% formic acid) and H_2O (0.1% formic acid) were used as solvents. Samples were analysed with positive ionisation: the ion spay voltage was 30 V and the capillary temperature was 1 kV.

LTQ-FT MS

A high-resolution mass spectrometer was used to determine the exact mass of the peptides. The samples were diluted in H_2O/MeCN (1:1) with 1% formic acid and analysed with an LTQ-FT Ultra (Thermo Scientific). They were introduced by automated nanoelectrospray. A NanoMate (Advion BioSciences, Ithaca, NY) infused the samples through the nanoESI Chip (which consisted of 400 nozzles in a 20 x 20 array). The spray voltage was 1.70 kV, and the delivery pressure was 0.5 psi. MS conditions were as follows: Nano-ESI, positive ionisation, capillary temperature 200°C, tube lens 100 V, ion spray voltage 2 kV, and *m/z* 200–2000 a.m.u.

UPLC-PDA

UPLC chromatograms were obtained on an Acquity high class (PDA detector, sample manager FNT and Quaternary solvent manager), using an Acquity BEH C_{18} (50 x 2.1 mm x 1.7 μm) column. The flow rate was 0.61 mL/min and MeCN (0.036% TFA) and H_2O (0.045% TFA) were used as solvents. 2-min linear gradients were used in all cases. Detection was performed at 220 nm.

Amino acid analysis

Amino acid analysis was performed to assess the amino acids present and the amount obtained for each peptide. For this purpose, ion exchange chromatographic analysis after acid hydrolysis was performed. The samples were hydrolysed with 6 M HCl at 110°C for 16 h. They were then evaporated to dryness at reduced pressure and dissolved in 20 mM of aqueous HCl. Finally, the amino acids were derivatised using the AccQ Tag protocol from Waters, which uses 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as a derivatisation reagent, and analysed by ion exchange HPLC.

Peptide stability in human serum

Peptides were dissolved in HBSS/human serum (1/9) at a final concentration of 150 μM. The incubation was performed at 37°C for 24 h, taking aliquots of 50 μL at several time points. These samples were treated with 200 μL of MeOH to precipitate the serum proteins and centrifuged for 30 min at 4°C. The supernatant was filtered and analysed by UPLC to calculate the percentage of intact peptide in the sample.

Circular dichroism

Spectra were recorded using a Jasco 810 UV-Vis spectropolarimetric, equipped with a CDF 426S/426L peltier. Peptide samples were dissolved in 10 mM Na_2HPO_4, pH 7.2, with or without 10% trifluoroethanol (TFE) and the

spectra were recorded at a 50 µM concentration. The following parameters were used: sensitivity (standard, 100 mdeg), start (250 nm), end (190 nm), data pitch (0.5 nm), scanning mode (continuous), scanning speed (50 nm/min), response (1s), band width (1.0 nm), and accumulation (3). A blank spectrum of the buffer was subtracted from all recordings, and mean residue molar ellipticity values were calculated from experimental ellipticity using the following formula:

$$\theta = \frac{\theta_{exp} \cdot 10^6}{c \cdot l \cdot n}$$

where θ is molar ellipticity in deg cm^2 dmol^{-1}, θ_{exp} is measured ellipticity in mdeg, c is the concentration of the peptide in µM, l is the optical path in mm and n is the number of residues in the peptide.

After unit conversion, the spectra was smoothed using the Savitzky-Golay method (convolution width = 21) and taken zero at the far-UV region (λ = 250 nm).

Molecular dynamics simulations

To assess the structure of MiniCTX3 in solution, replica exchange molecular dynamics (REMD) were performed. Atom coordinates were extracted from the Protein Data Bank (PDB ID: 1CHL) and the peptide structure was manually modified, performing a short minimization to relieve local clashes.

The peptide was solvated using the SPC explicit water model and 16 REMD replicas were perfomed with the Desmond-2017 software, temperatures ranging from 300 to 500 K. The NPT ensemble was employed and frames collected every 20 ps for a total of 20 ns for each replica. Default settings were used for all other parameters. Then, the 298 K replica was analyzed and representative clustering of all frames was performed in order to calculate the peptide dihedral angles and the most populated structures.

NMR

NMR spectra were recorded at 278 K on a Bruker 800 MHz spectrometer equipped with a cryoprobe. The NMR sample was prepared by dissolving 0.5 mg of peptide in 10 mM sodium phosphate buffer, 25 mM NaCl, 0.01% (w/v) NaN_3, pH=7.0, and 10% (v/v) D_2O. Chemical shifts were referenced to internal DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) at 0.0 ppm.

Complete proton and carbon resonance assignment was obtained by the combined analysis of 2D homo- (TOCSY, ROESY) and hetero-nuclear (^{1}H ^{13}C HSQC) NMR experiments. The TOCSY and ROESY mixing times were 80 and 150 ms, respectively. Suppression of the water signal was achieved by excitation sculpting. C_α chemical shifts were measured from natural abundance ^{1}H-^{13}C HSQC experiments.

C_α and H_α secondary chemical shifts (Δδ) were calculated as the difference between the measured chemical shift ($\delta_{measured}$) and reported random coil values (δ_{RC}).

Coupling constants ($^3J_{NHH\alpha}$) were extracted from the one-dimensional ^{1}H spectrum. Amide proton temperature coefficients were determined from a series of one dimensional ^{1}H spectra acquired between 278 K and 298 K with 5 K increments.

Gold nanoparticle synthesis and functionalisation

12 nm AuNPs were synthesised as previously described.[3] Briefly, 50 mL of 1 mM $HAuCl_4$ in water solution was heated at 110ºC and refluxed for 10 min. 5 mL of 38.8 M sodium citrate buffer was added and was refluxed for another 30 min. The mixture was cooled to room temperature and the pH was adjusted to 8.66 with NaOH. Samples were then filtered and characterised using UV-spectrophotometer (Shimazu) and TEM (Tecnai Spirit). Buffer was exchanged through precipitation and resuspension for more diluted 2.2 mM citrate buffer.

The conjugation was performed by ligand exchange using 2.9 nM AuNPs and 500 equiv. of the peptides with an extra Cys, namely C-MiniCTX-2, C-MiniCTX3 and C-MiniAp-4, in a 1:1 dilution. They were left to react for 2h under mild shaking in an end-over-end rotor. Unconjugated peptide was removed by centrifugation and resuspension in citrate buffer (2.2 mM) until no peptide was detected by UPLC in the supernatant. Conjugated NPs were again characterised by UV-spectrophotometry and TEM. The amount of conjugated peptide was obtained using amino acid analysis after 3-day hydrolysis in 6M HCl. The NPs were quantified spectrophotometrically ($\varepsilon = 3.67 \cdot 10^8\ M^{-1}\ cm^{-1}$).

Inductively coupled plasma – mass spectrometry (ICP-MS) quantification

ICP-MS was used to quantify AuNPs in the cell-based BBB model transport assay. The measurements were performed at Centres Científics i Tecnològics of the University of Barcelona (CCiTUB). The instrument was a Perkin Elmer Nexlon 350D and the only stable isotope of gold, ^{197}Au was quantified. The amount of Au in the initial concentration of AuNPs was quantified by ICP-OES (optical emission spectrometry) on a Perkin Elmer Optima 3200RL.

Previous to quantification, the samples were digested with a mixture of HNO_3/H_2O_2/HCl (5:2.5:2) for 18-24 h at 90ºC. Then, the samples were dissolved in 200 ppm thiourea 1% HCl in H_2O and analysed.

Parallel Artificial Membrane Permeability Assay (PAMPA)

The effective permeability of the compounds was measured at an initial concentration of 200 µM. The PAMPA buffer solution was prepared from a commercially concentrated solution, supplied by pION Inc., and the pH was adjusted to 7.0 with 0.5 M NaOH. 5% DMSO was added as a cosolvent to the buffer solution. The PAMPA sandwich was separated, a magnetic stirrer was placed in each well and he donor well was filled with 195 µL of the peptide solution. Then, 4 µL of a mixture of phospholipids from a porcine polar brain extract (20 mg/mL) in dodecane (composition: 12.6% phosphatidylcholine (PC), 33.1% phosphatidylethanolamine (PE), 18.5% phosphatidylserine (PS), 4.1% phosphatidylinositol (PI), 0.8% phosphatidic acid and 30.9% of other compounds) was added to the filter of each well, followed by 200µL of buffer solution. The acceptor plate was put into the donor plate, ensuring that the underside of the membrane was in contact with the buffer. The plate was then covered and incubated at room temperature in a saturated humidity atmosphere for 4h under orbital agitation at 25 µm of unstirred water layer. After this period, 10 µL of each acceptor and donor wells were injected in the UPLC. The effective permeability, the transport and mass balance were calculated with the following formulas:

$$P_e = \frac{-218.3}{t} \cdot log\left[1 - \frac{2 \cdot C_A(t)}{C_D(t_0)}\right] \cdot 10^{-6} cm/s$$

$$T(\%) = \frac{C_A(t)}{C_D(t_0)} \cdot 100$$

$$MB(\%) = \frac{Q_D(t) + Q_A(t)}{Q_D(t_0)} \cdot 100$$

where *t* is time in h, $C_A(t)$ is the peptide concentration in the acceptor compartment at the time *t* and $C_D(t_0)$ is the peptide concentration in the donor compartment at time 0h. $Q_D(t)$ is the amount of peptide in the donor compartment at time *t*, $Q_A(t)$ is the amount of peptide in the acceptor compartment at time *t* and $Q_D(t_0)$ is the amount of peptide in the donor compartment at time 0h.

Propanolol [(*RS*)-1-(isopropilamino)-3-(naftalen-1-iloxi)propan-2-ol] was used as a control of permeability, with a reported value of $1.6 \pm 0.4 \cdot 10^{-5}$ cm/s.

Cell-based assays

Internalisation experiments

bEnd.3 cells were cultured in DMEM complete medium (glucose 4.5 g/L, 2 mM glutamine, 10% FBS and 0.5% Pen/Strep) (Sigma). Medium was changed 3 times per week and cells were detached using 0.05% trypsin/EDTA.

Two days before the experiment, 100.000 bEnd.3[4] cells/well were seeded in 24-well plates. On the day of the assay, the cells were washed with Ringer HEPES (RH) for 15 min and then treated for another 15 min with selected inhibitors, namely sodium azide (10 mM), filipin III (10 µg/mL), chlorpromazine hydrochloride (10 µg/mL) or vehicle (RH). After this preincubation, enough Rh- or CF-labelled peptide was added to reach a final concentration of 50 µM. After 30 min of incubation at 37ºC or 4ºC, the cells were washed 5 times with Ringer HEPES or Gly buffer at 4ºC, trypsinised and kept on ice. Cells were immediately analysed using a FACSAria Fusion flow cytometer with a 582 nm laser or a Beckman Coulter CyAn flow cytometer with a 488nm laser (Beckman Coulter).

XTT cell proliferation assay

bEnd.3 were seeded on 96-well plates (5.000 cells/well). After 24 h, the peptide was added at several concentrations (100 to 0.4 µM) to the wells and incubated for another 24 h. The Activated-XTT solution was prepared by adding 0.1 mL of the activation reagent (PMS: *N*-methyl dibenzopyrazine methyl sulphate) to 5 mL of the XTT reagent. 50 µL of this solution was then added to each well and incubated for 4 h. Absorbance was measured at a wavelength of 475 nm using a microtiter plate reader.

Cell viability was calculated using the following formula:

$$\%\ viability = 100 - \left(100 \cdot \frac{A_{max} - A}{A_{max} - Blank}\right)$$

where A_{max} is the absorbance of the cells without peptide, *Blank* is the absorbance of the medium alone, and *A* is the absorbance of the sample. Measurements were performed in triplicate. As a positive control, cells were incubated with DMSO.

Permeability assays in the *in vitro* BBB cellular model

These experiments were performed using the model developed in Prof. R. Cecchelli's laboratory.[5] In brief, endothelial cells derived from pluripotent stem cells and bovine pericytes were defrosted in gelatin-coated Petri dishes (Corning). Pericytes were cultured in DMEM pH 6.8 while endothelial cells were cultured in supplemented endothelial cell growth medium (sECM) (Sciencells). After 48 h, endothelial cells were seeded in 12-well Transwell inserts (8000 cell/well) and pericytes were plated in 12-well plates (50000 cells/well) previously coated with

Matrigel and gelatin, respectively. The medium was changed every 2-3 days and the assays were performed 7-8 days after seeding by placing the transwells into new wells without pericytes.

The following concentrations of compounds were used: 25 μM for peptides and 5 nM for AuNPs conjugates. Lucifer Yellow (25 μM) was added as a control of barrier integrity (Papp < $15 \cdot 10^{-6}$ cm/s). To perform the assay, 500 μL of the compound in Ringer HEPES was added to the donor compartment and 1500 μL of Ringer HEPES was introduced into the acceptor compartment. The plates were incubated for 2 h at 37ºC, and the solutions from both compartments were recovered and analysed. The samples were evaluated in triplicates. The amount of peptide was quantified using UPLC or UPLC-MS and the amount of Au in AuNPs was analysed by ICP-OES or ICP-MS.

Apparent permeability:

$$P_{app} = \frac{Q_A(t) \cdot V_D}{t \cdot A \cdot Q_D(t_0)}$$

where P_{app} is obtained in cm/s, $Q_A(t)$ is the amount of compound at the time t in the acceptor well, V_D is the volume in the donor well, t is time in seconds, A is the area of the membrane in cm and $Q_D(t_0)$ is the amount of compound in the donor compartment at the beginning of the experiment.

Processing of the transwells for TEM

After the assay, the cells on the transwells were fixed with a solution of 2.5% glutanaldehide in phosphate buffer for 1h at room temperature and for 24 h at 4ºC. Then, they were treated with osmium tetroxide in the same buffer to provide contrast to the image. After that, they were dehydrated in acetone, infiltrated and embedded with epoxy resin and polymerised. Finally, ultrathin sections were obtained and mounted on Formvar-coated copper grids. Sections of the cells were observed by TEM (Tecnai™ Spirit)

Supplementary figures and tables

Table S1 Sequence, molecular formula, high-resolution molecular weight (MW), UPLC characterisation, purity and yield after synthesis and purification of CTX and MiniCTXs. CF = carboxyfluorescein. t_R = retention time; *t_R HPLC, min.

Peptide	Molecular formula	Calc MW, Da	Found MW, Da	t_R UPLC, min	Purity, %	Yield, %	Sequence and cargoes
CTX	$C_{158}H_{249}N_{53}O_{47}S_{11}$	3993.57295	3993.57527	1.17	>95	3	H-MC(&$_1$)MPC(&$_2$)FTTDHQMARK C(&$_3$)DDC(&$_1$)C(&$_4$)GGKGRGKC(&2)Y GPQC(&3)LC(&4)R-NH_2
CF-CTX	$C_{179}H_{259}N_{53}O_{53}S_{11}$	4351.6207	4351.6005	1.32	>95	2	CF-MC(&$_1$)MPC(&$_2$)FTTDHQMARK C(&$_3$)DDC(&$_1$)C(&$_4$)GGKGRGKC(&2)Y GPQC(&3)LC(&4)R-NH_2
MiniCTX1	$C_{50}H_{81}N_{17}O_{13}S_2$	1192.5714	1192.5684	1.18	>99	60	H-GKC(&)YGPQC(&)LAR-NH_2
CF-MiniCTX1	$C_{71}H_{91}N_{17}O_{19}S_2$	1550.6191	1550.6165	1.32	>90	50	CF-GKC(&)YGPQC(&)LAR-NH_2
MiniCTX2	$C_{52}H_{84}N_{18}O_{14}$	1184.64144	1184.63949	1.14	>97	25	H-Gk[Dap](&)YGPQE(&)LAr-NH_2
CF-MInCTX2	$C_{73}H_{94}N_{18}O_{20}$	1542.68918	1542.68818	1.32	>95	6	CF-Gk[Dap](&)YGPQE(&)LAr-NH_2
C-MiniCTX2	$C_{55}H_{89}N_{19}O_{15}S$	1287.65062	1287.65024	1.13	>90	45	H-CGk[Dap](&)YGPQE(&)LAr-NH_2
MiniCTX3	$C_{28}H_{39}N_9O_9$	646.2944	646.2940	1.03	>95	30	H-[Dap](&)YGPQD(&)-NH_2
CF-MiniCTX3	$C_{49}H_{49}N_9O_{15}$	1004.3421	1004.3410	1.36	>99	15	CF-[Dap](&)YGPQD(&)-NH_2
C-MiniCTX3	$C_{31}H_{44}N_{10}O_{10}S$	748.30354	748.30199	1.06	>95	60	H-C[Dap](&)YGPQD(&)-NH_2
MiniAp-4	$C_{39}H_{66}N_{12}O_{13}$	910.487	910.487	3.7*	>99	12	H-[Dap](&)KAPETALD(&)-NH_2
CF-MiniAp-4	$C_{60}H_{76}N_{12}O_{19}$	1325.556	1325.561	4.6*	>98	7	CF-[Dap](&)KAPETALD(&)-NH_2
C-MiniAp-4	$C_{42}H_{71}N_{13}O_{14}S$	1013.496	1013.498	3.9*	>99	4	H-C[Dap](&)KAPETALD(&)-NH_2

Table S2. PAMPA results after 4h of incubation in a transwell coated with a mixture of phospholipids from a porcine polar brain extract

Peptide	Pe (cm/s)	Transport (%)	Mass Balance (%)
CTX	0	0	100
MiniCTX2	0	0	100
MiniCTX3	0	0	97
Propranolol	$16 \cdot 10^{-6}$	24.7	68

Table S3. Permeability of peptides assayed in the human BBB-cellular model.

Peptide	Papp (cm/s)	Normalised Papp*
CTX	$3.0 \pm 0.2 \cdot 10^{-6}$	1.00
MiniCTX1	$4.1 \pm 0.6 \cdot 10^{-6}$	1.37
MiniCTX2	$7.3 \pm 0.8 \cdot 10^{-6}$	2.43
MiniCTX3	$9.3 \pm 1.5 \cdot 10^{-6}$	3.10
MiniAp-4	$9.9 \pm 0.3 \cdot 10^{-6}$	3.28
CF-CTX	$3.2 \pm 0.4 \cdot 10^{-6}$	1.00
CF-MiniCTX2	$6.6 \pm 0.6 \cdot 10^{-6}$	2.06
CF-MiniCTX3	$7.0 \pm 0.1 \cdot 10^{-6}$	2.19
AuNP	$2.1 \pm 0.6 \cdot 10^{-9}$	1.00
AuNP-MiniCTX2	$2.7 \pm 0.1 \cdot 10^{-9}$	1.33
AuNP-MiniCTX3	$6.5 \pm 1.8 \cdot 10^{-9}$	3.05
AuNP-MiniAp-4	$7.1 \pm 7.0 \cdot 10^{-9}$	2.97

*The Papp of all the peptides was normalised by the Papp of CTX, CF-CTX or the AuNP alone.

Table S4. Chemical shifts (δ, ppm), $^3J_{NHH}$ coupling constants (Hz) and temperature dependence of amide proton chemical shifts (dδ/ΔT, ppb/K) of MiniCTX3 peptide in sodium phosphate buffer (pH= 7.0).

Residue	NH	Hα	Hβ	Cα	Cβ	Others	Δδ/ΔT	$^3J_{NHH}$
Dap 1	8.24	3.91	3.81 – 3.24	51.03	39.52	-	-8.2[a]	6.32[c]
Tyr 2	8.64	4.75	3.02 – 2.95	53.45	35.91	H_{ar}= 7.14 – 6.85 C_{ar}= 114.10 – 129.29	-10.8[b]	6.64
Gly 3	8.50	3.91 – 4.18	-	41,19		-	-6.5[b]	5.60
Pro 4	-	4.39	1.98 – 2.30	60.22	28.02	H_{γ}= 2.02 H_{δ}= 3.54 C_{γ}= 23.17 C_{δ}= 45.87	-	-
Gln 5	8.45	4.34	2.02 – 2.19	52.22	24.36	H_{γ}= 2.36 δNH_2= 7.68 – 6.98 C_{γ}= 30,10	-6.2[b]	7.68
Asp 6	7.77	4.66	2.70 – 2.60	48.77	35.71	$NH_{2\ (S.C)}$= 7.51 – 7.23	-4.3[b]	7.84

[a] This value correspond to $^3J_{N\gamma HH\beta}$

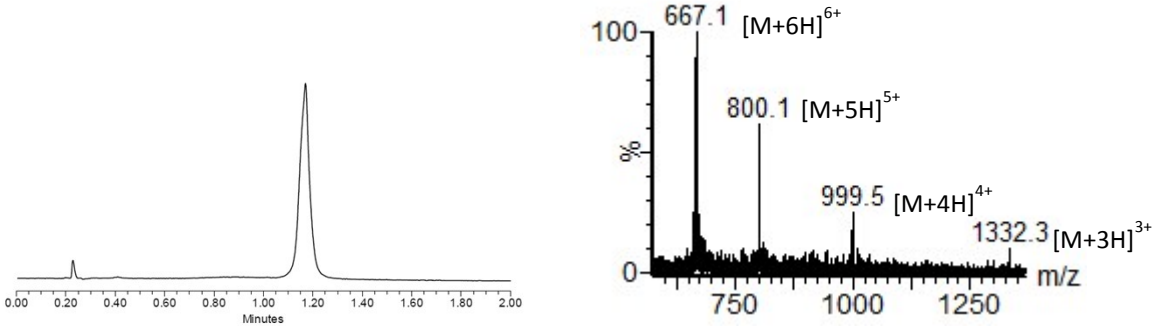
[b] These values correspond to $^3J_{NHH\alpha}$

[c] Temperature coefficient value of $N_{\gamma}H$

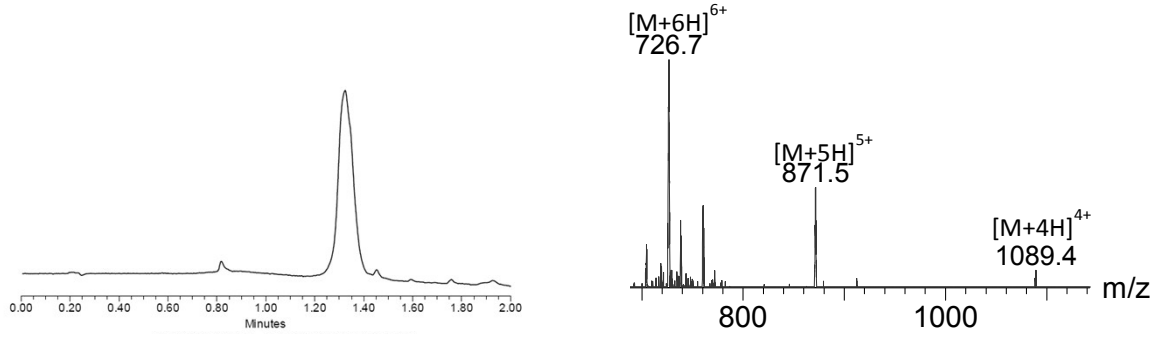
Table S5. Characterisation of the AuNPs of this work.

Nanoparticle	UV_{max} (nm)	[NP] final (nM)	Peptide copies by AAA
AuNP	521	2.9	
AuNP-MiniCTX2	526	5.6	192
AuNP-MiniCTX3	524	6.3	226
AuNP-MiniAp-4	525	6.5	132

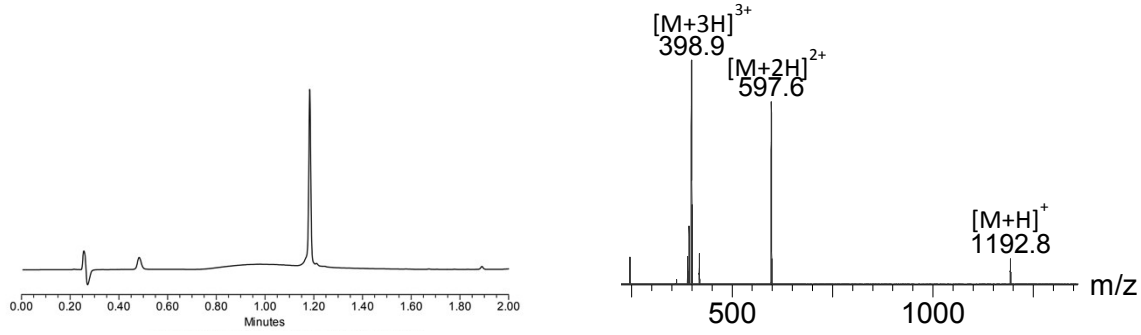
Chlorotoxin (CTX) $[M+6H]^{6+}$=667 Da, $[M+5H]^{5+}$=800 Da, $[M+4H]^{4+}$=999 Da, $[M+3H]^{3+}$=1332 Da



CF-CTX $[M+6H]^{6+}$=726 Da, $[M+5H]^{5+}$=871 Da, $[M+4H]^{4+}$=1089 Da



MiniCTX1 $[M+3H]^{3+}$=398 Da, $[M+2H]^{2+}$=597 Da, $[M+H]^{+}$=1193 Da



CF-MiniCTX1 $[M+3H]^{3+}$=518 Da, $[M+2H]^{2+}$=776 Da

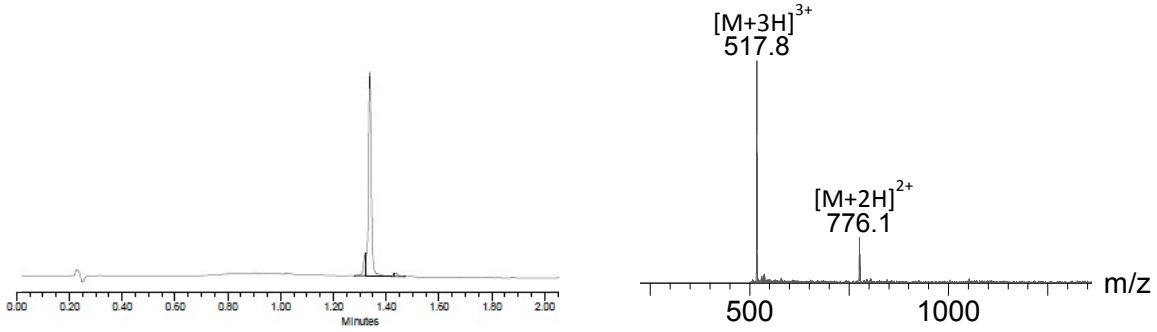
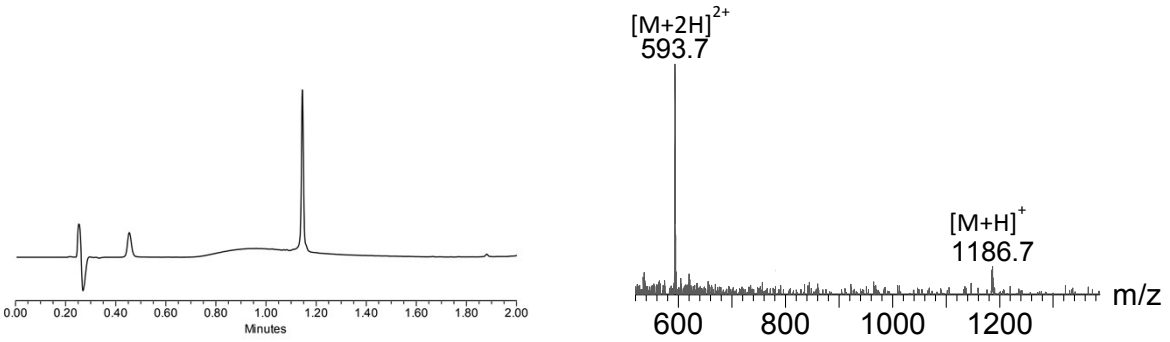
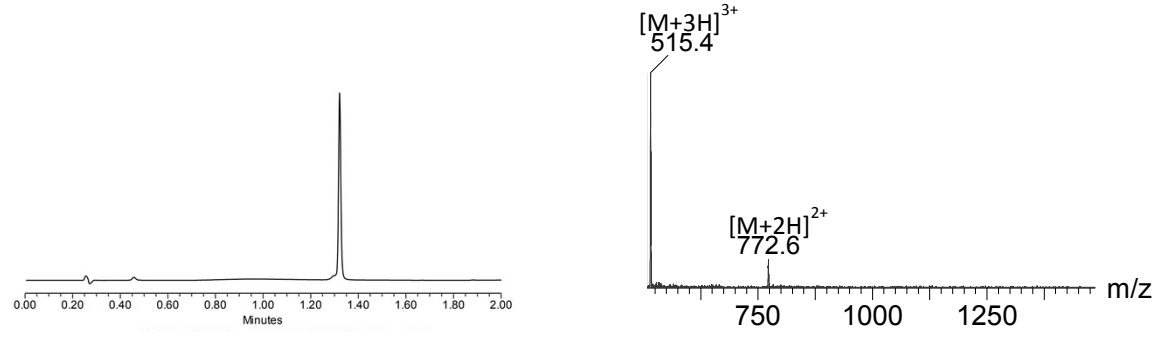


Figure S1 (continued in the following page) UPLC traces and MS spectra of all peptides described in this paper, except for MiniAp-4 and analogues that are HPLC traces. UPLC chromatograms are recorded at 220 nm in a 2-min linear gradient from 0 to 100% of MeCN (0.036% TFA) in H_2O (0.045% TFA), 8-min for HPLC chromatograms.

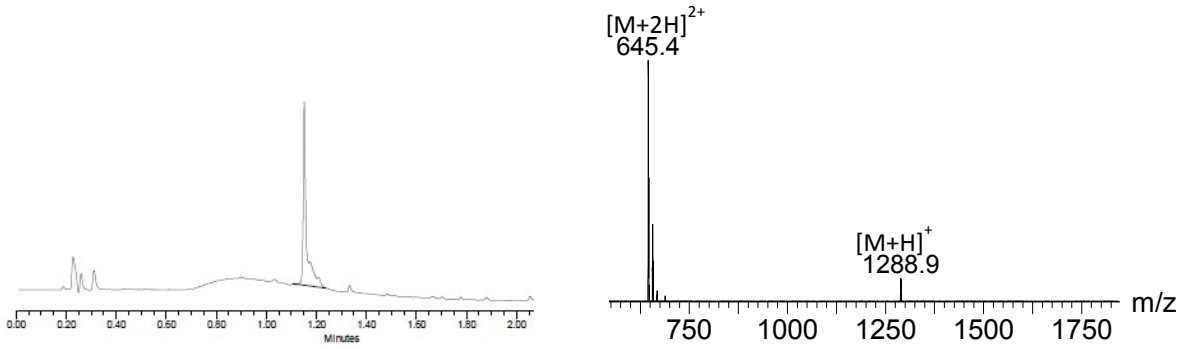
MiniCTX2 $[M+2H]^{2+}$=594 Da, $[M+H]^{+}$=1186 Da



CF-MiniCTX2 $[M+3H]^{3+}$=515 Da, $[M+2H]^{2+}$=773 Da



C-MiniCTX2 $[M+2H]^{2+}$=645 Da, $[M+H]$=1289 Da



MiniCTX3 $[M+H]^{+}$=647 Da

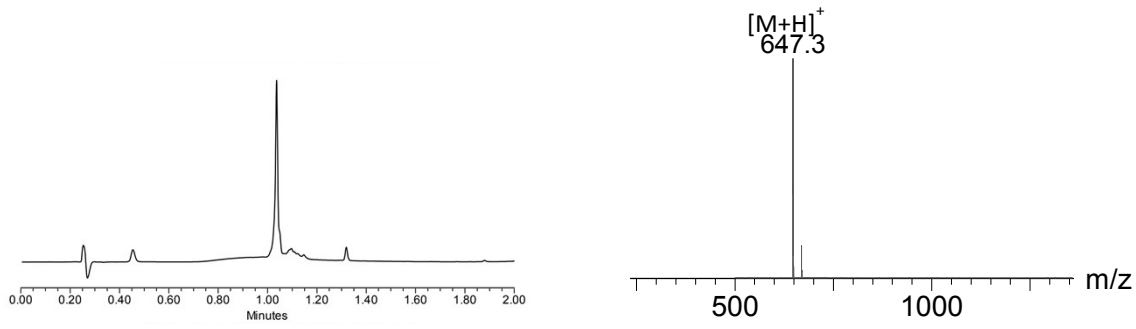
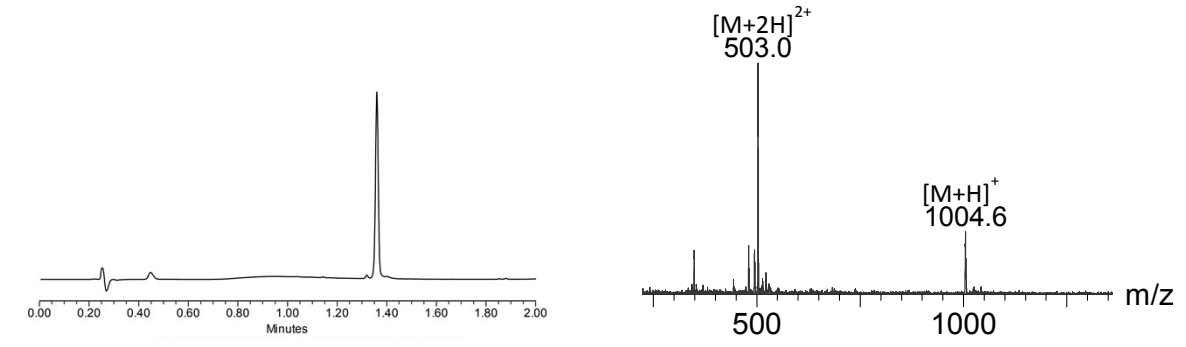
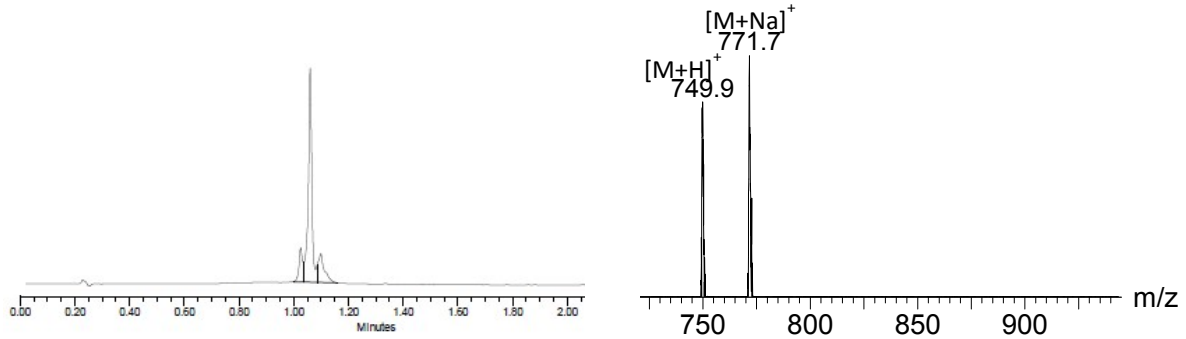


Figure S1 (continued from the previous page and in the following page) UPLC traces and MS spectra of all peptides described in this paper, except for MiniAp-4 and analogues that are HPLC traces. UPLC chromatograms are recorded at 220 nm in a 2-min linear gradient from 0 to 100% of MeCN (0.036% TFA) in H_2O (0.045% TFA), 8-min for HPLC chromatograms.

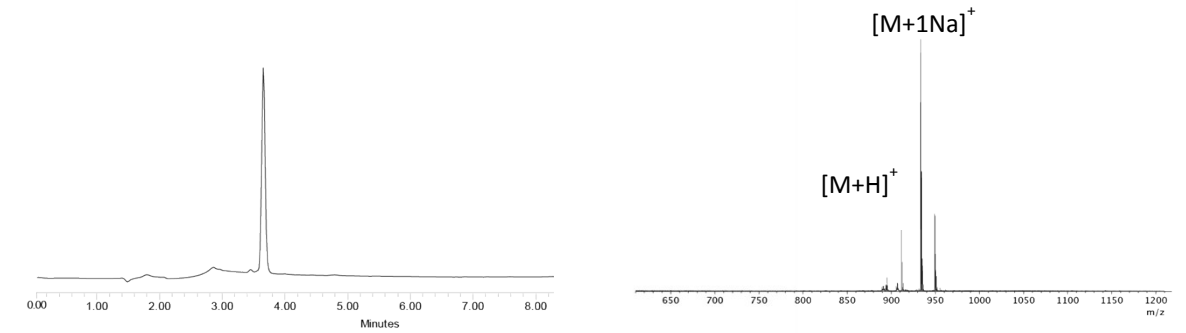
CF-MiniCTX3 $[M+2H]^{2+}$=503 Da, $[M+H]^{+}$=1005 Da



C-MiniCTX3 [M+H]⁺=749 Da, [M+Na]⁺=772 Da



MiniAp-4 [M+H]⁺=911.5 Da, [M+Na]⁺=934.5 Da



CF-MiniAp-4 [M+1H]⁺=1326.7 Da

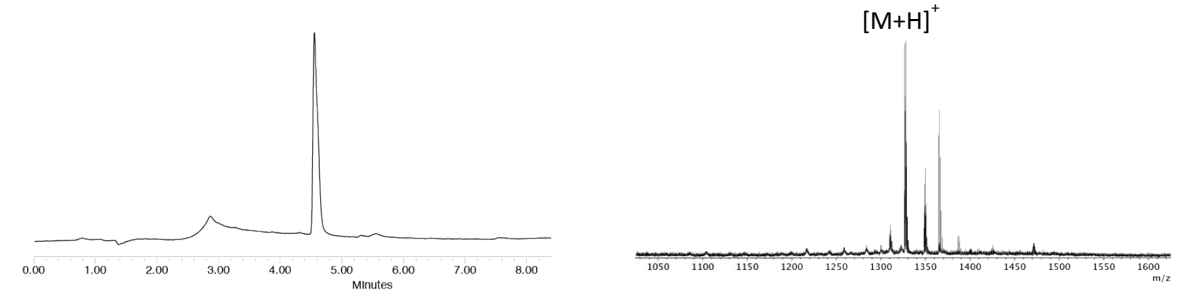


Figure S1 (continued from the previous page and in the following page) UPLC traces and MS spectra of all peptides described in this paper, except for MiniAp-4 and analogues that are HPLC traces. UPLC chromatograms are recorded at 220 nm in a 2-min linear gradient from 0 to 100% of MeCN (0.036% TFA) in H_2O (0.045% TFA), 8-min for HPLC chromatograms.

C-MiniAp-4 $[M+H]^+$=1014.6 Da

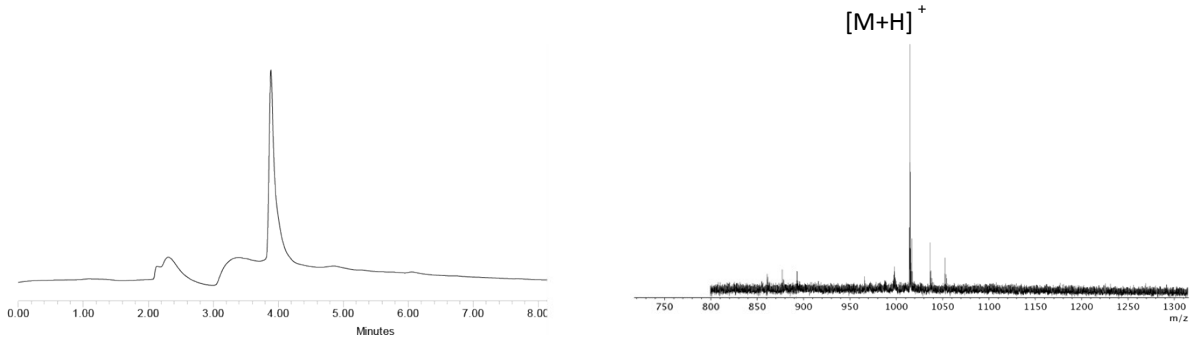


Figure S1 (continued from the previous page) UPLC traces and MS spectra of all peptides described in this paper, except for MiniAp-4 and analogues that are HPLC traces. UPLC chromatograms are recorded at 220 nm in a 2-min linear gradient from 0 to 100% of MeCN (0.036% TFA) in H_2O (0.045% TFA), 8-min for HPLC chromatograms.

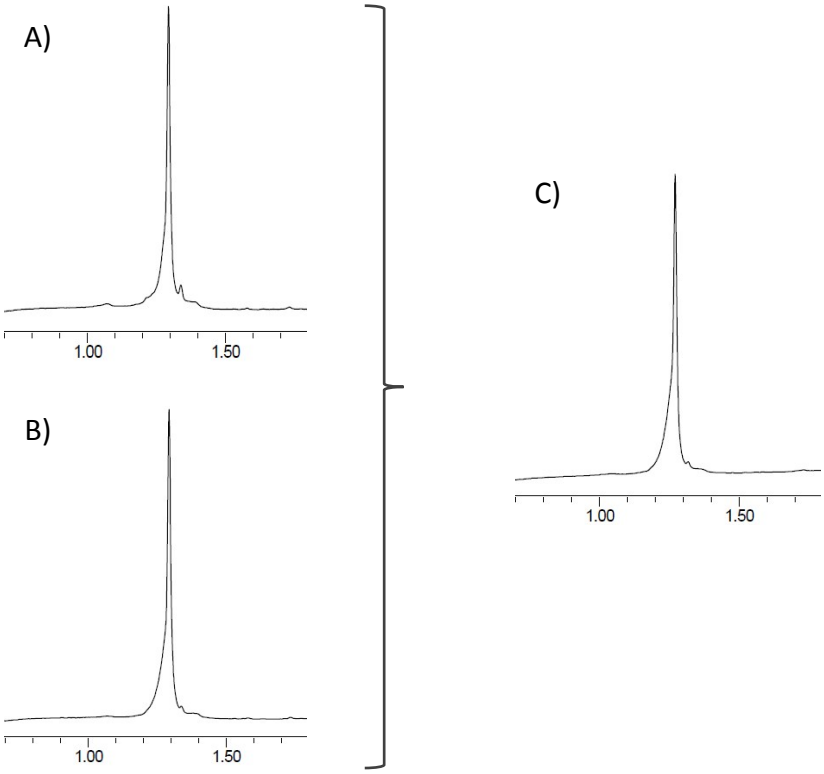


Figure S2. HPLC profile of A) natural standard, B) synthetic CTX and C) coelution of the two products. The chromatograms were obtained in a 2-min linear gradient from 10 to 40% of MeCN (0.036% TFA) in H_2O (0.045% TFA), in an Acquity BEH C_{18} column at 40°C.

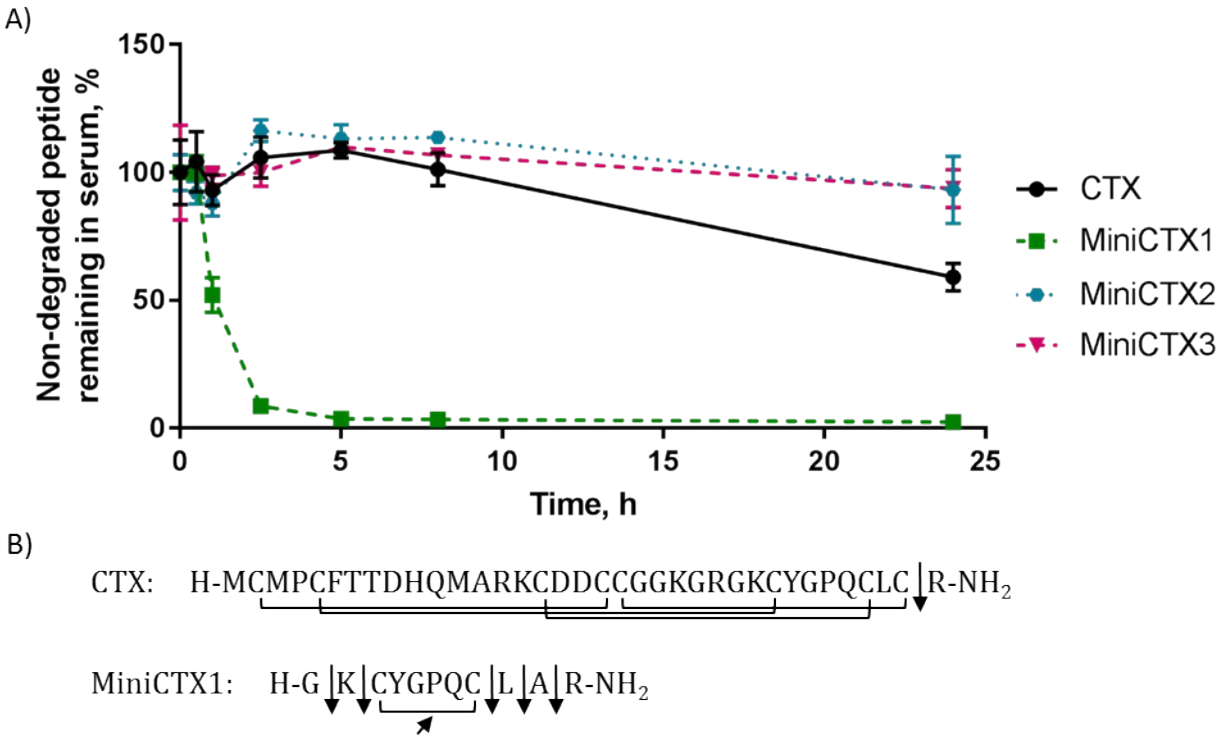


Figure S3. A) Graphic representation of the percentage of intact peptide vs. time incubating with human serum at 37ºC. Data are expressed as the mean ± SD. B) Cleavage points of CTX and MiniCTXs (arrows representing the cleavable sites).

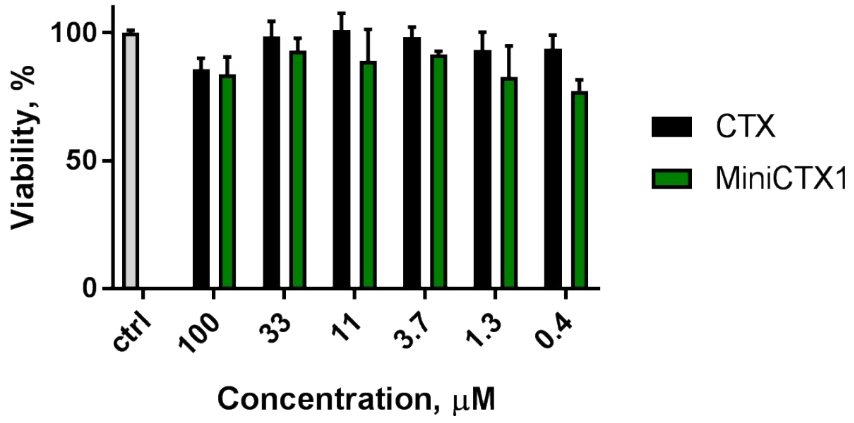
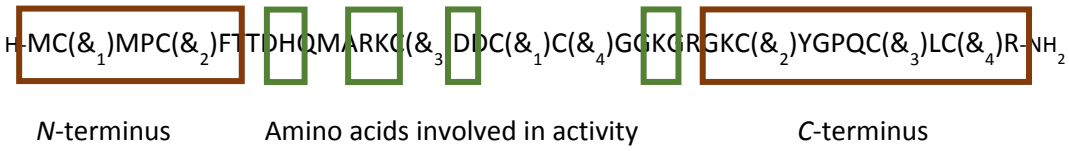


Figure S4. Cell viability in bEnd.3 cells in the presence of CTX or MiniCTX1, assessed by XTT assay after 24 h of peptide incubation.

A)



B)

```
CTX              MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR                    36
Angiopep-2       ---TFF-----------YGGSRGKRNNFKTEEY--                    19
                      *             *.:*: : :  :

CTX              MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR------------------- 36
ApoB             ---SSV----------IDA----LQYKLEGTTRLTRKRGLKLATALSLSNKFVEGS 39
                     ..          *.     : *  *   * *

CTX              MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR-------             36
ApoE             ------------------------LRKLRKRLLLRKLRKRLL             18
                                          :    : * *

CTX              MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR                    36
TGN              ----------------------TGNYKALHPHNG--                   12
                                      .*. *.  *:

CTX              MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR---                 36
TAT              ----------------------------YGRKKRRQRRR                11
                                           ** :   :

CTX              MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR                    36
Peptide-22       -CMPRLRGC---------------------------                    8
                  *** :

CTX              -----MCMPCFT-----TDHQMARKCDDCCGGKGRGKCYGPQCLCR          36
Leptin30         YQQILTSMPSRNVIQISNDLENLRDLLHVL----------------          30
                       .**. .     .* :  *.  .

CTX              --MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR                  36
RVG29            YTIWMPE---NPRPGTPCDIFTNSRGKRASNG------                  29
                   : **    : : .  **   ..:*:  . *
```

Figure S5. A) Chlorotoxin sequence highlighting the *N*- and *C*-terminus and the amino acids involved in its activity. B) Sequence alignment of CTX with known BBB-shuttles, highlighting the amino acids that are common.

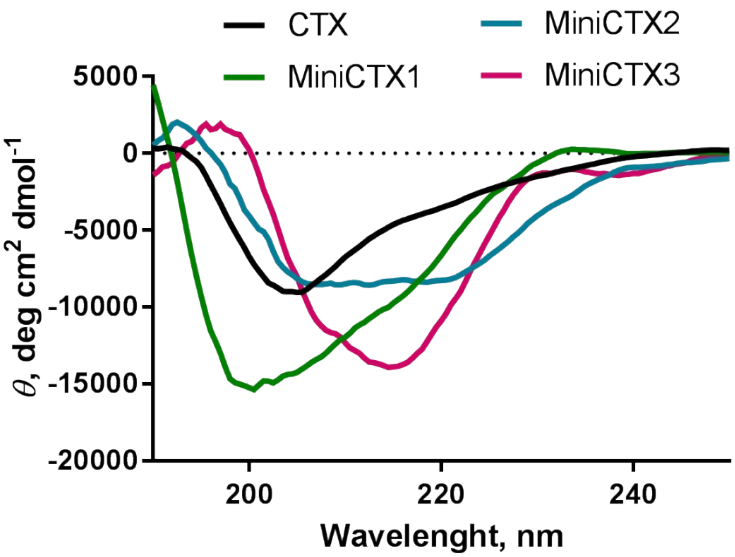


Figure S6. Circular dichroism of CTX and MiniCTXs. Peptides in 10 mM Na_2HPO_4 buffer (pH 7.2).

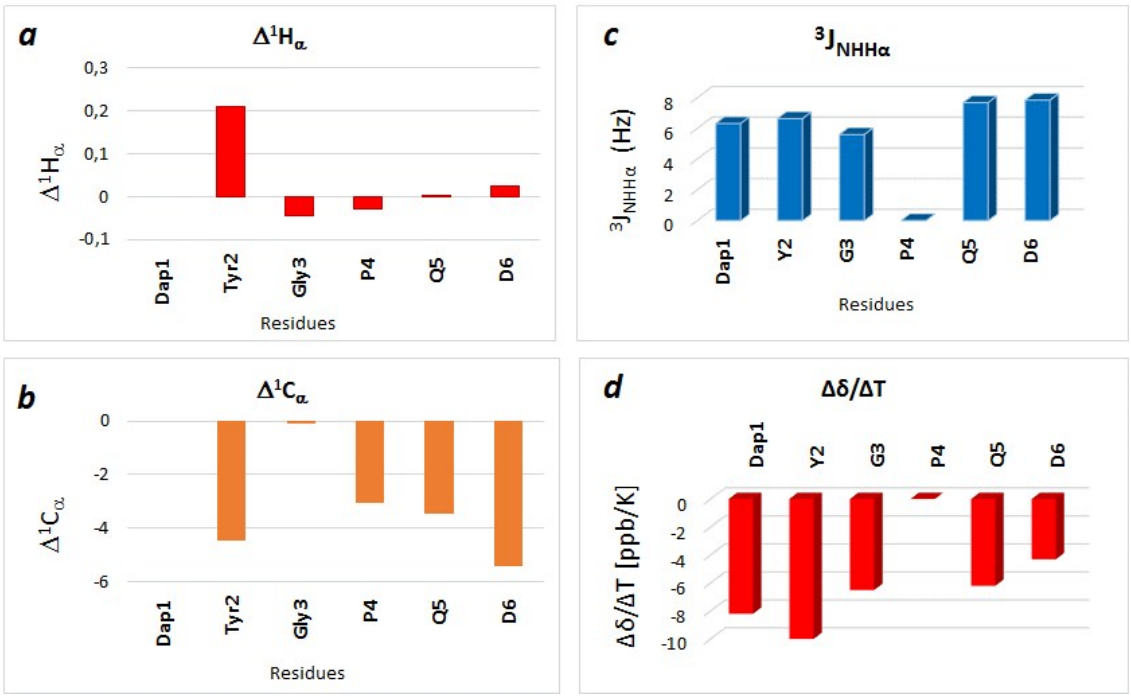


Figure S7. NMR characterization of MiniCTX3. Histograms of chemical shift deviations from random coil values for Hα (**a**) and Cα (**b**). $^3J_{NHH\alpha}$ coupling constants **(c)** and temperature coefficient values (ΔδNH/ΔT) (**d**). Random coil values were extracted from Wishart et al.[6] The chemical shift deviations for the N-terminal residue (Dap1) were not calculated due to the lack of reference parameters.

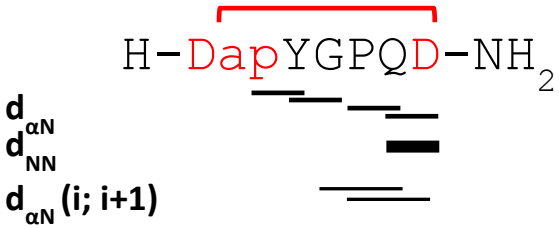


Figure S8. Summary of ROE connectivities observed for MiniCTX3. The thickness of the bar indicates the strength of the ROE (weak, medium or strong).

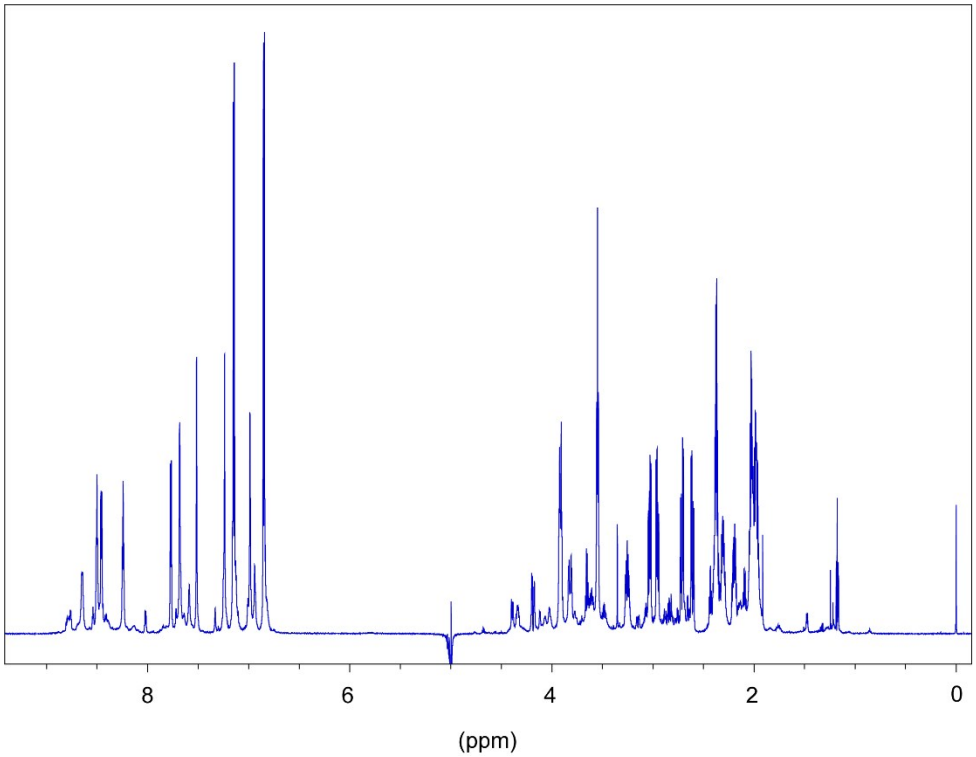


Figure S9. ^{1}H-NMR spectrum of MiniCTX3 peptide in sodium phosphate buffer (pH= 7.0) at 278 K.

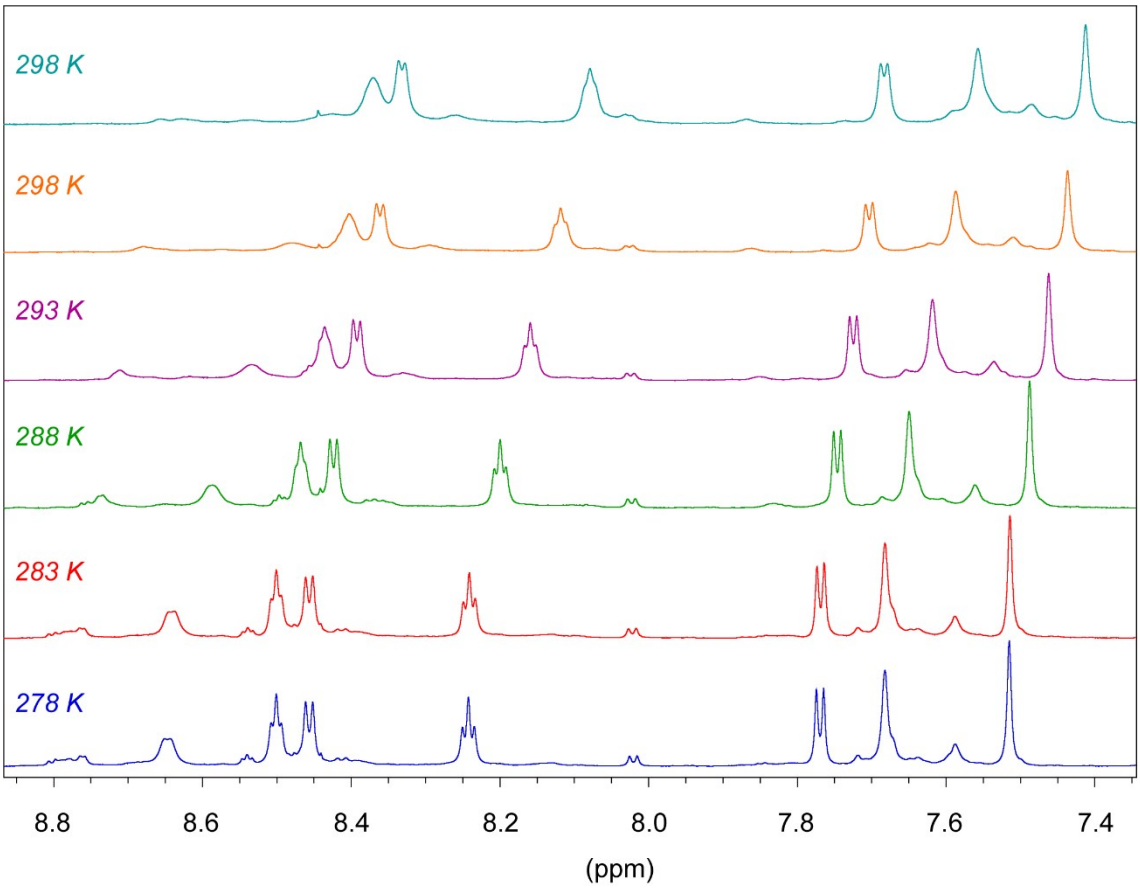


Figure S10. Comparison of the amide regions of the 1D ^{1}H NMR spectra of MiniCTX3 acquired between 278 K and 298 K with 5 K increments.

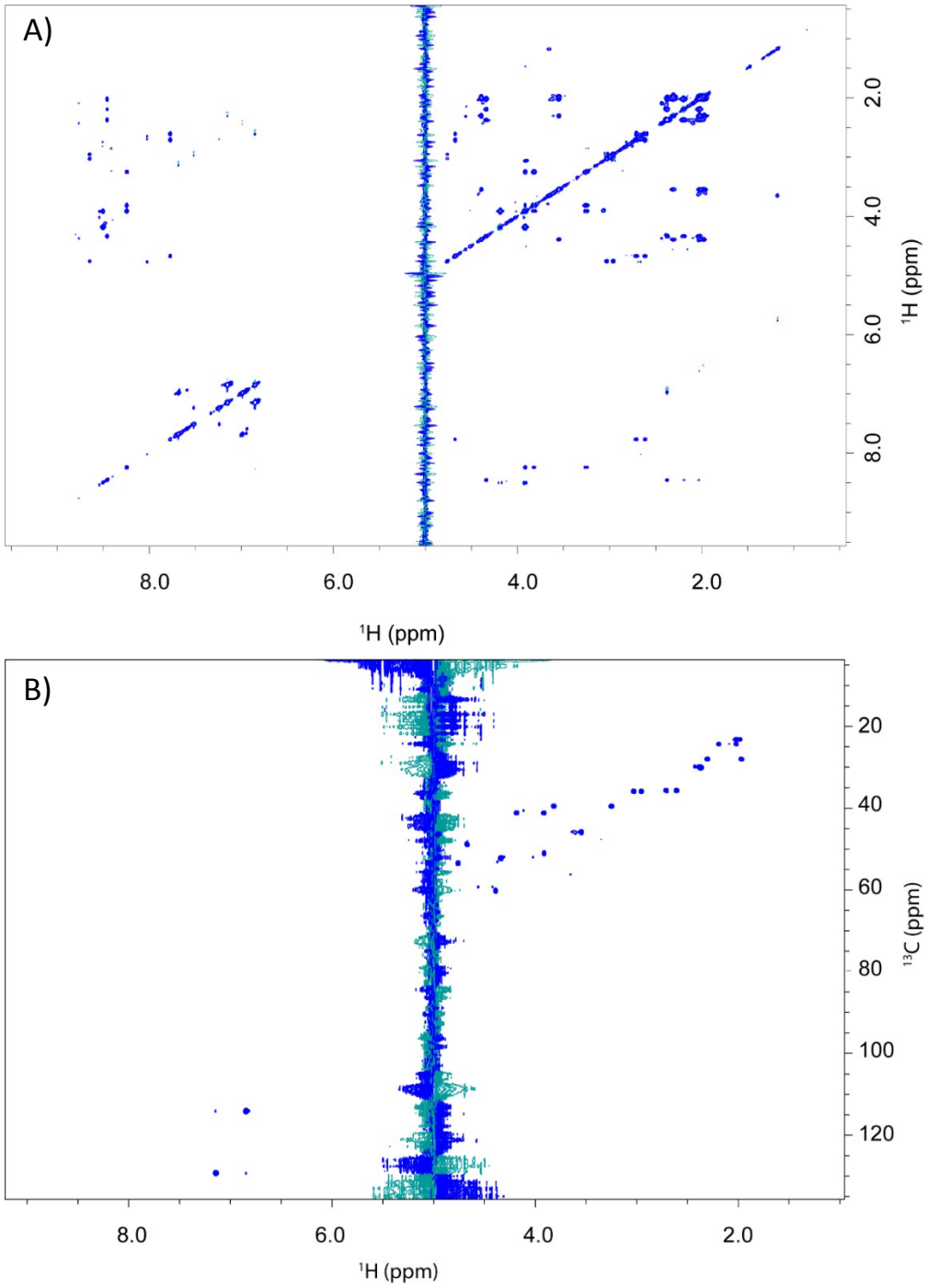


Figure S11. A) ^{1}H ^{1}H 2D TOCSY NMR spectrum and B) ^{1}H ^{13}C HSQC NMR spectra of MiniCTX3.

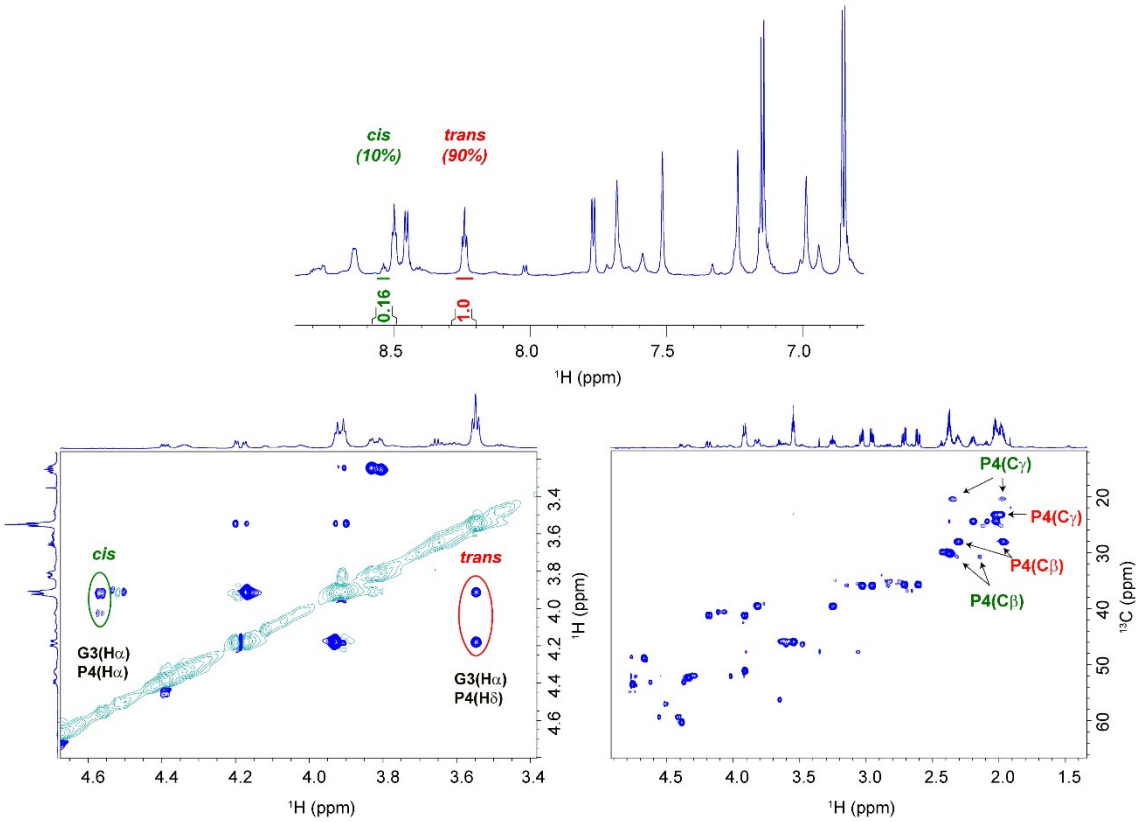


Figure S12. Quantification of the cis and trans isomers of MiniCTX3 peptide in 25 mM Na_2HPO_4 buffer pH=7.0 at 278 K. a) Expanded regions of the ^{1}H NMR spectrum showing the presence of different spin systems corresponding to the trans (~ 90%) and cis (~ 10%) conformations observed for this peptide. b) Selected region of ^{1}H ^{1}H 2D ROESY spectrum. Two sets of peaks are clearly observed, one set showing Gly3(H*α*)-Pro4(Hδ) ROE whereas the other Gly3(H*α*)-Pro4(Hα) ROE, diagnostic of trans and cis conformation respectively. c) Similar doubling is shown in ^{1}H-^{13}C HSQC from which ^{13}C chemical shifts diagnostics of cis and trans are obtained.

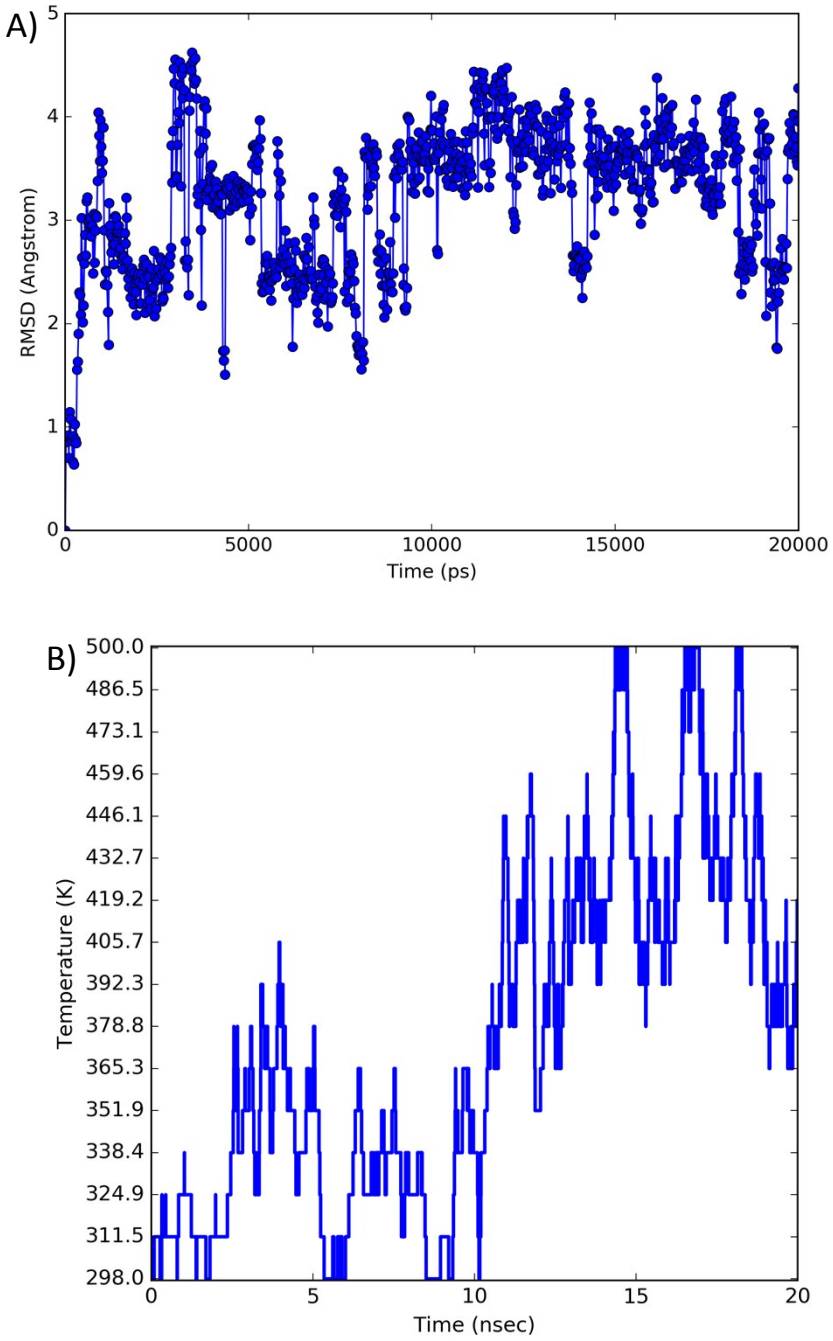


Figure S13. A) RMSD of MiniCTX3 backbone for the 298 K trajectory during the REMD simulation. B) Temperature evolution along the REMD simulation of the system starting at 298 K.

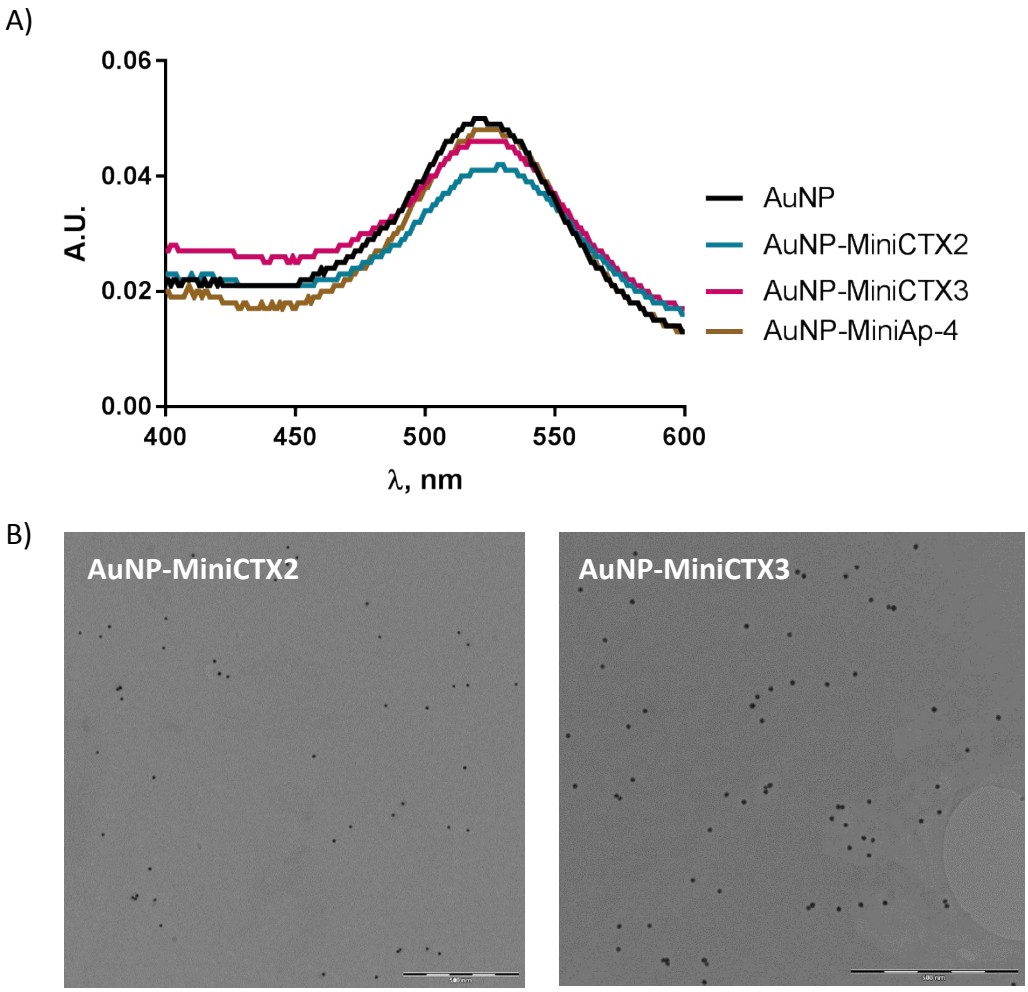


Figure S14. Synthesis and conjugated AuNPs. A) Absorption spectra. B) TEM micrographies. Scale bar: 500 nm.

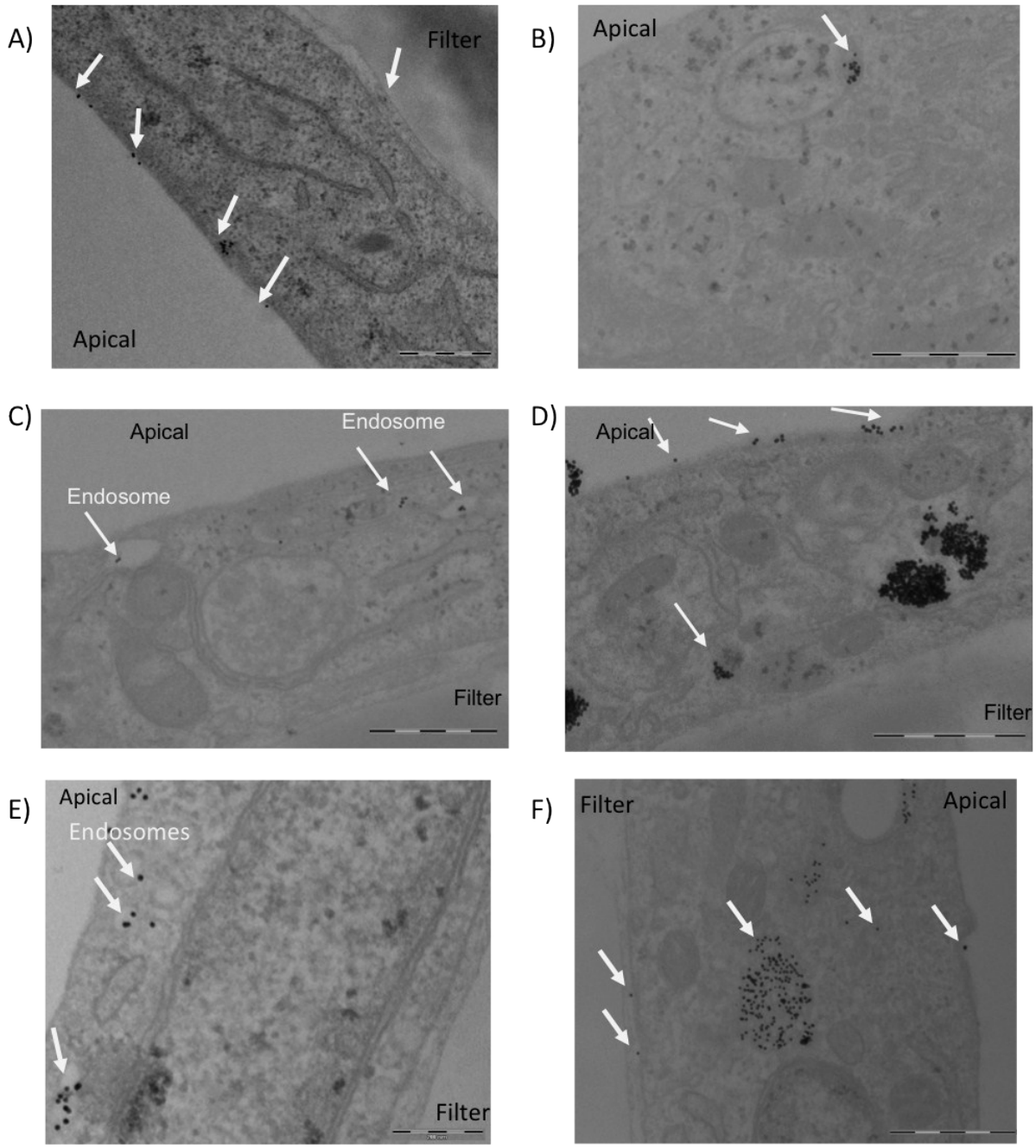
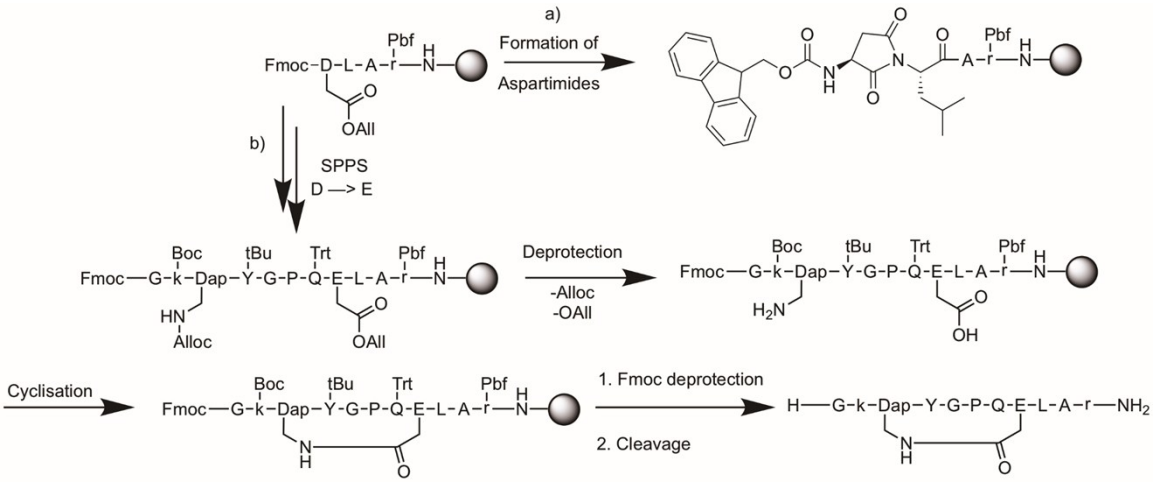
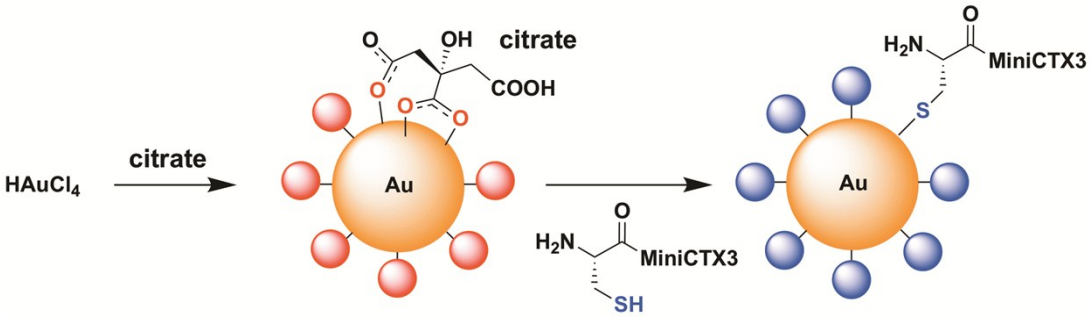


Figure S15. Transcytosis of 12 nm AuNP in the human BBB cellular model. The endothelial cells in the transwells after the 2h assay are shown. Arrows indicate nanoparticles. A) AuNP, B) AuNP-MiniCTX2, C) - E) AuNP-MiniCTX3 and F) AuNP-MiniAp-4. Panel C) shows the presence of AuNP-MiniCTX2 inside the cells located in endosomes. Panel D) shows that AuNP-MiniCTX2 are aggregated inside the cells or accumulated in the surface of the endothelial cells. Scale bar: 500 nm, except when indicated.



Scheme S1. Synthesis of MiniCTX2. Pathway a) shows the formation of aspartimides due to the lability of the OAll group protecting the Asp. In pathway b), by changing Asp by Glu, the secondary reaction is not present and the desired product is obtained by the formation of the lactam bridge.



Scheme S2. Scheme of the conjugation of the synthesis of gold nanoparticles and conjugation to the peptides with an extra Cys.

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