Perfluoroarene-Based Peptide Macrocycles to Enhance Penetration Across the Blood-Brain Barrier

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Supporting Information

Table of Contents

1. Materials

H-Rink Amide-ChemMatrix resin was obtained from PCAS BioMatrix Inc. (St-Jean-sur-Richelieu, Quebec, Canada). 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium-3-oxid-hexafluorophosphate (HATU), Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Asp(tBu)-OH, Fmoc-L-Glu(tBu)-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Cys(Trt)-OH, Fmoc-L-Gly-OH, Fmoc-L-Ala-OH, Fmoc-L-Val-OH, Fmoc-L-Leu-OH, Fmoc-L-Phe-OH, Fmoc-L-Tyr(tBu)-OH, and Fmoc-L-Trp(Boc)-OH were purchased from Chem-Impex International (Wood Dale, IL). Peptide synthesis-grade N,N-dimethylformamide (DMF), CH_2Cl_2, diethyl ether, *t*-butanol and HPLC-grade acetonitrile were obtained from VWR International (Radnor, PA). Cy5.5-azide and 5-TAMRA azide were purchased from Lumiprobe Corporation (Hallandale Beach, FL). Decafluorobiphenyl was purchased from Oakwood Products, Inc. (Estill, SC). The LDH Assay kit was purchased from Promega (Madison, WI). The primary human astrocytes, astrocyte growth medium and endothelial cell growth medium were obtained from Lonza Bioscience (Walkersville, MD). The human brain microvascular pericytes and the pericyte medium were obtained from ScienCell Research Laboratories (Carlsbad, CA). The hCMEC/D3 human cerebral microvascular endothelial cells were from Cedarlane Labs (Burlington, ON, Canada). The human serum was from Valley Biomedical (Winchester, VA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), water was deionized before use, and reactions were conducted in open-air on the benchtop.

2. Methods for LC-MS Analysis

For all experiments except for the proteolysis assays, LC-MS chromatograms and associated mass spectra were acquired using an Agilent 6520 ESI-Q-TOF mass spectrometer equipped with a C_3 Zorbax column. Mobile phases were: 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following LC-MS method was used for characterization of all TP10 analogues and for the serum stability assay: Zorbax SB C3 column: 2.1 x 150 mm, 5 μm, 5% B from 0 to 2 min, linear ramp from 5% B to 65% B from 2 to 11 min, 65% B from 11 to 12 min and finally 3 min of post-time at 5% B for equilibration, flow rate: 0.8 mL/min. The following LC-MS method was used for characterization of all BIM BH3 analogues: Zorbax SB C3 column: 2.1 x 150 mm, 5 μm, 1% B from 0 to 2 min, linear ramp from 1% B to 61% B from 2 to 11 min, 61% B to 99% B from 11 to 12 min and finally 3 min of post-time at 1% B for equilibration, flow rate: 0.8 mL/min. All data were processed using Agilent MassHunter software package. Y-axis in all chromatograms shown represents total ion current (TIC) unless noted. For the proteolysis assays, LC-MS chromatograms and associated mass spectra were acquired using an Agilent 6550 iFunnel Q-TOF mass spectrometer equipped with a Jupiter C_4 Phenomenex column. The following LC-MS method was used: Phenomenex Jupiter C4: 150 x 1.0 mm ID, 5 μm, linear gradient from 1% B to 91% B over 16 min, flow rate: 0.1 mL/min.

3. General Method for Peptide Preparation

Fast-flow Peptide Synthesis

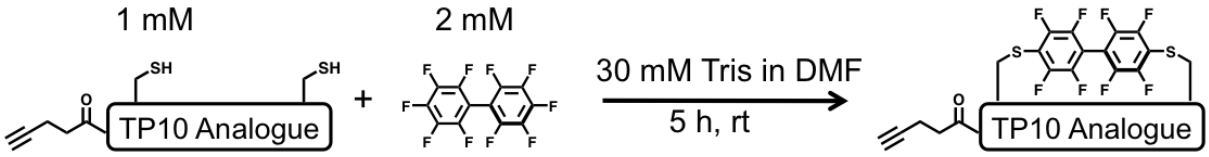
Peptides were synthesized on a 0.1-mmol scale using an automated flow peptide synthesizer.[1] A 200 mg portion of ChemMatrix Rink Amide HYR resin was loaded into a reactor maintained at 90 ºC. All reagents were flowed at 80 mL/min with HPLC pumps through a stainless steel loop maintained at 90 ºC before introduction into the reactor. For each coupling, 10 mL of a solution containing 0.2 M amino acid and 0.17 M HATU in DMF were mixed with 200 μL diisopropylethylamine and delivered to the reactor. Fmoc removal was accomplished using 10.4 mL of 20% (v/v) piperidine. Between each step, DMF (15 mL) was used to wash out the reactor. Special coupling conditions were used for arginine, in which the flow rate was reduced to 40 mL/min and 10 mL of a solution containing 0.2 M Fmoc-L-Arg(Pbf)-OH and 0.17 M PyAOP in DMF were mixed with 200 μL diisopropylethylamine and delivered to the reactor. 4-pentynoic acid was coupled in flow on the automated peptide synthesizer using the same conditions as standard amino acids. After completion of the synthesis, the resins were washed 3 times with DCM and dried under vacuum.

Peptide Cleavage and Deprotection

Each peptide was subjected to simultaneous global side-chain deprotection and cleavage from resin by treatment with 8 mL of 94% (v/v) trifluoroacetic acid (TFA), 2.5% (v/v) 1,2-ethanedithiol (EDT), 2.5% (v/v) water, and 1% (v/v) triisopropylsilane (TIPS) for 6 min at 60 °C. The TFA was evaporated by bubbling N_2 through the mixture until only an oil and the resin remained. Then ~35 mL of cold ether was added to precipitate and wash the peptide (chilled at –80°C). The crude product and resin were pelleted through centrifugation for three minutes at 4,000 rpm and the ether decanted. The ether precipitation and centrifugation was repeated two more times. After the third wash, the pellet was redissolved in 50% water and 50% acetonitrile containing 0.1% TFA, filtered through a fritted syringe to remove the resin and lyophilized. In the re-synthesis of BIM BH3 analogue 4 for Cy5.5-labeled constructs, the crude peptide was redissolved in 70% water, 20% acetonitrile, and 10% DMF, which improved peptide recovery.

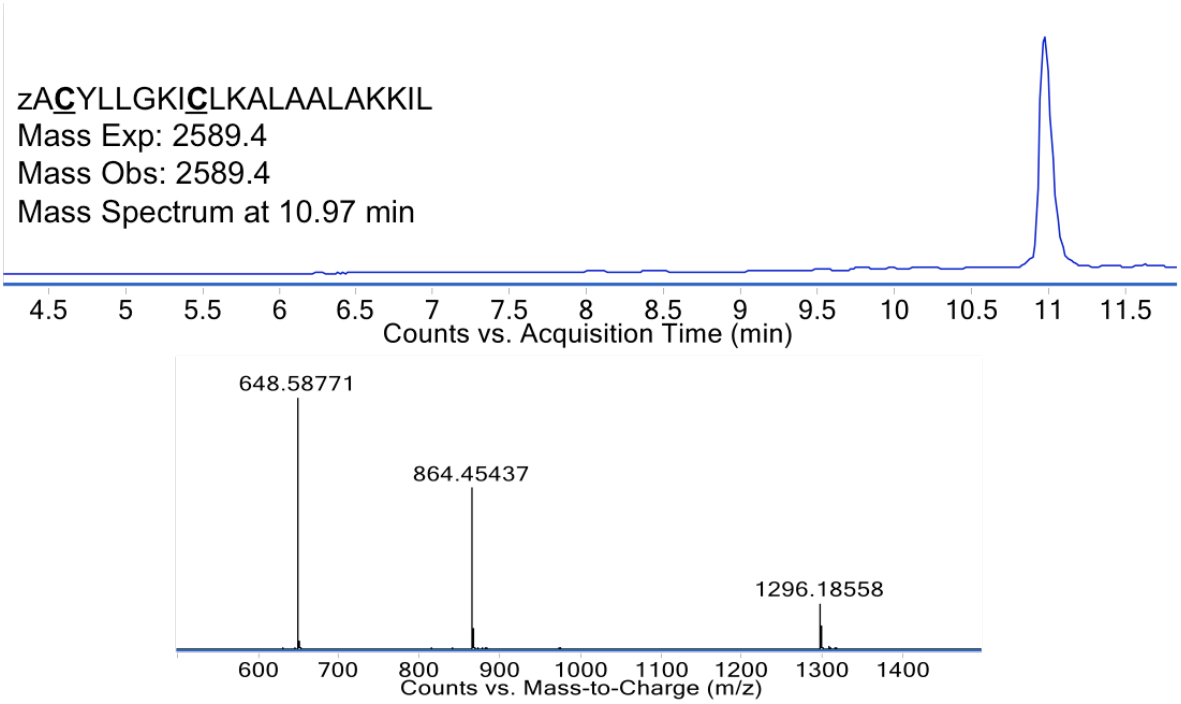
4. Macrocyclization, Alkylation, and Fluorophore Labeling Procedures

Procedure for Macrocyclization of Crude TP10 Peptides



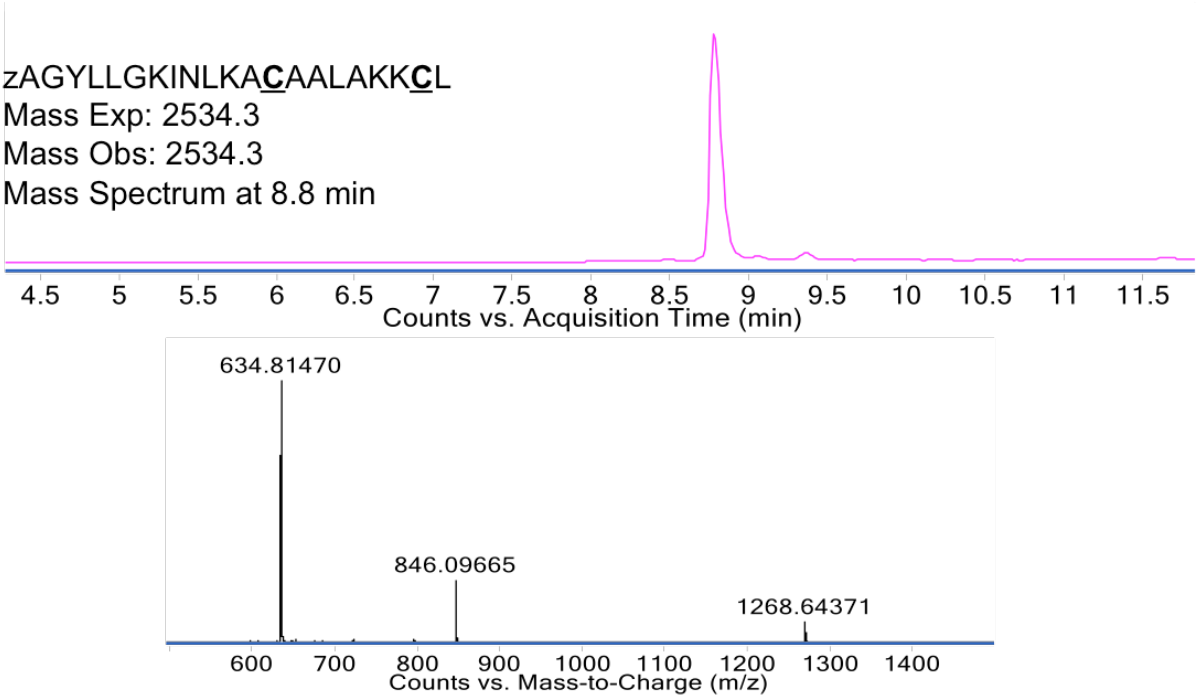
The crude TP10 peptides were dissolved in DMF, and stock solutions of decafluorobiphenyl in DMF and Tris in DMF were added such that the final concentration in the reaction vessel was 1 mM of a given TP10 analogue, 2 mM decafluorobiphenyl, and 30 mM Tris. After 5 h, DMF was removed by rotary evaporation to a final volume of 10 mL and the reaction was quenched by adding 90 mL of 85:15 water:acetonitrile containing 4% TFA. The crude reaction was purified by mass-directed semi-preparative reversed-phase HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 µm). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was run from 20% B to 60% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

M2 Characterization

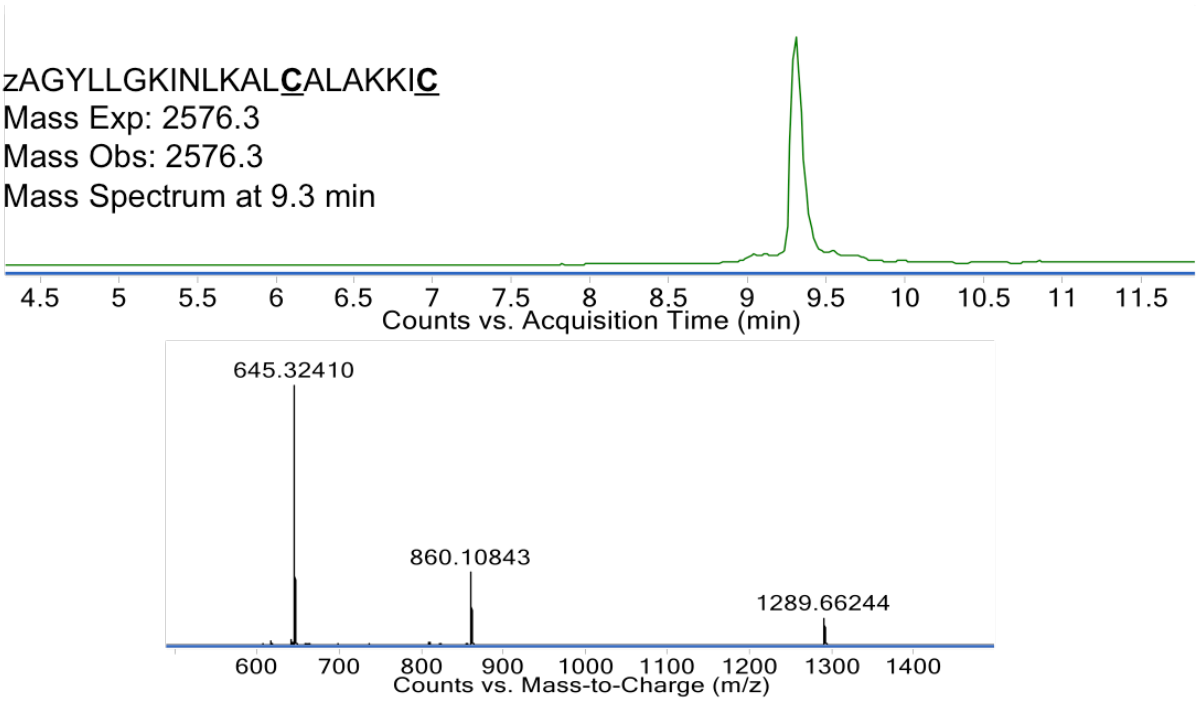


C = site of cyclization z = 4-pentynoic acid

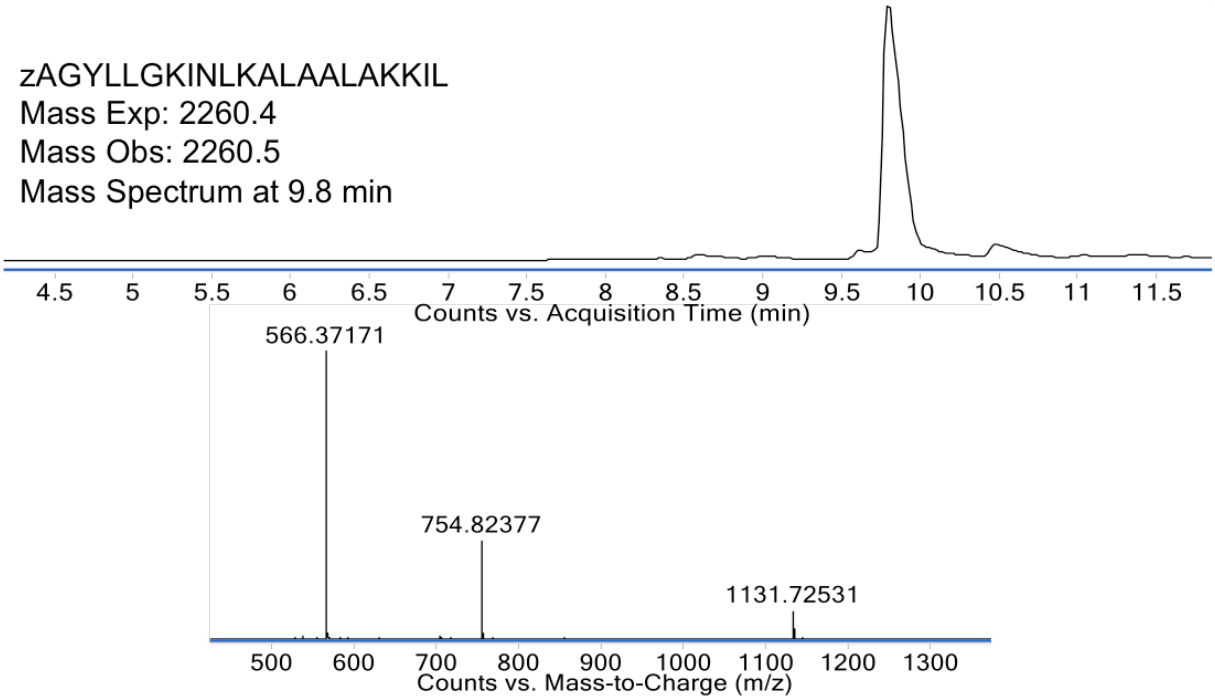
M13 Characterization



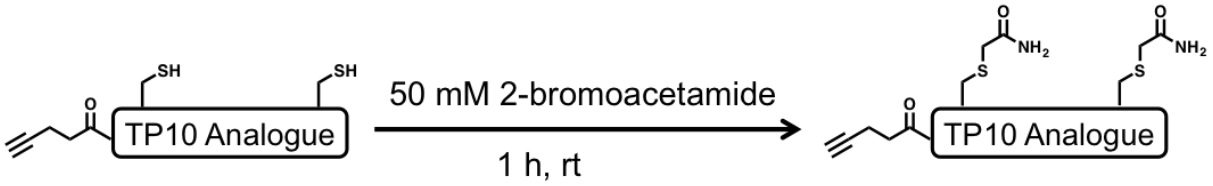
M14 Characterization



Native TP10 Characterization – Note: no macrocyclization reaction. Showing LC-MS of product after purification of the crude peptide by RP-HPLC.



Procedure for Alkylation of Crude TP10 Peptides



The crude TP10 peptides were dissolved in a buffer of 6 M guanidine•HCl, 0.2 M sodium phosphate, 50 mM 2-bromoacetamide, and 20 mM *tris*(2-carboxyethyl)phosphine (TCEP)•HCl, pH 7.1 to a final concentration of 1 mM peptide. After one hour, the reaction was quenched by bringing the pH of the solution to 2 with 6 M HCl. The crude reaction was purified by mass-directed semi-preparative reversed-phase HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 µm). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was run from 20% B to 60% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

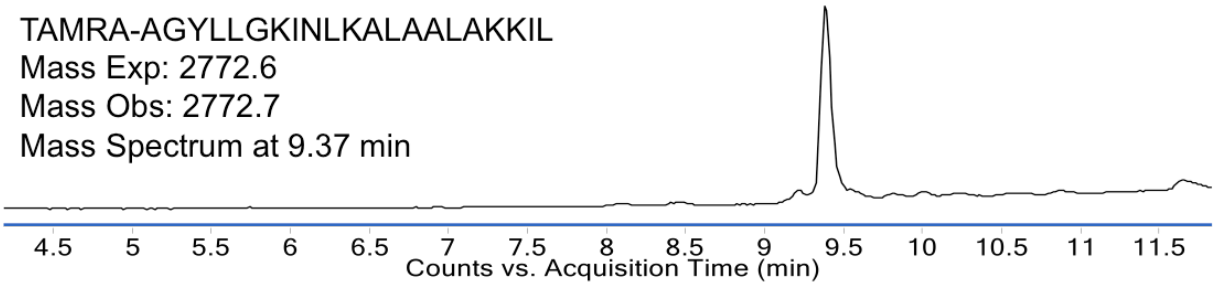
Procedure for Fluorophore Labeling of TP10 Analogues by azide/alkyne Huisgen Cycloaddition

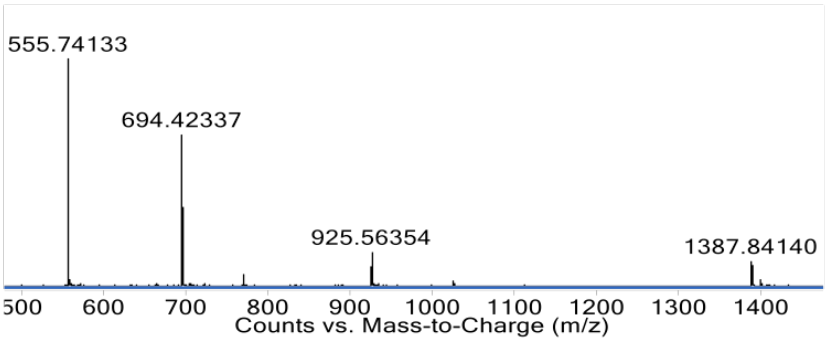
Purified TP10 constructs (0.5 µmol) were each dissolved in 200 µL 50:50 *t*-butanol:water in a 1.7 mL microcentrifuge tube. The following solutions were added to the microcentrifuge tube in order:

10 µL of 50 mM 5-tetramethylrhodamine azide (5-TAMRA azide) in DMSO, 100 µL of 500 mM Tris pH 8 in water, 50 µL of 100 mM copper(II) sulfate in water, 100 µL of 500 mM Tris pH 8, 10 µL of 10 mM Tris(benzyltriazolylmethyl)amine (TBTA) in DMSO, 10 µL of 100 mM TCEP•HCl in water, 100 µL of 1 M ascorbic acid in water and 520 µL 50:50 t-butanol:water. After one hour, the reaction was diluted with 9 mL of 85:15 water:acetonitrile containing 0.1% TFA, filtered and purified by mass-directed semi-preparative RP-HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 µm). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was run from 20% B to 60% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

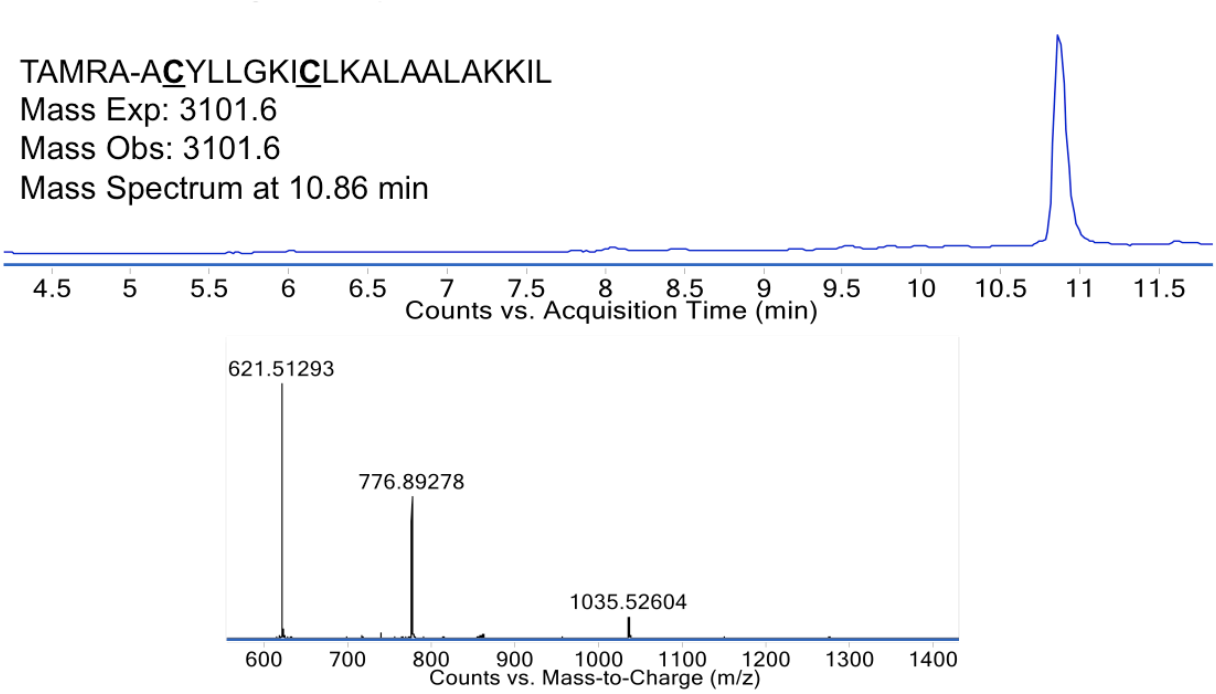
For the Cy5.5 labeled versions of TP10 M13 and Q*13, the same procedure was used, only the concentration of peptide was 1.5 mM and 15 µL of 100 mM Cy5.5 azide in DMSO was added, instead of the 5-TAMRA azide.

TAMRA-TP10 Characterization

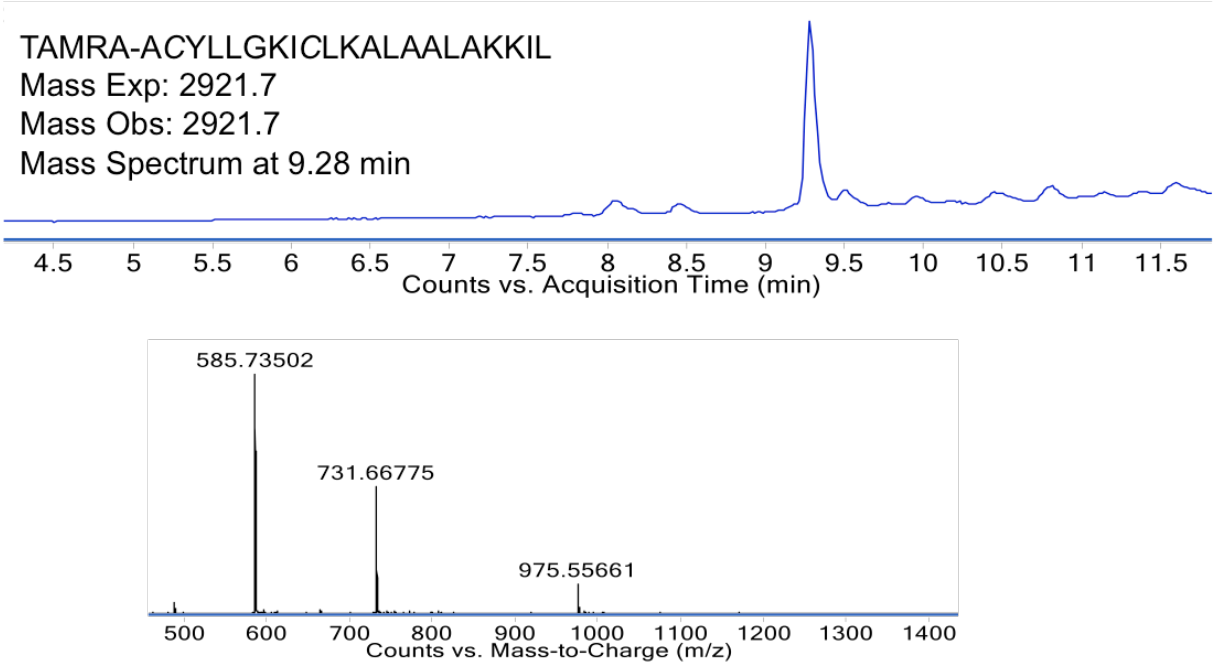




TAMRA-M2 Characterization

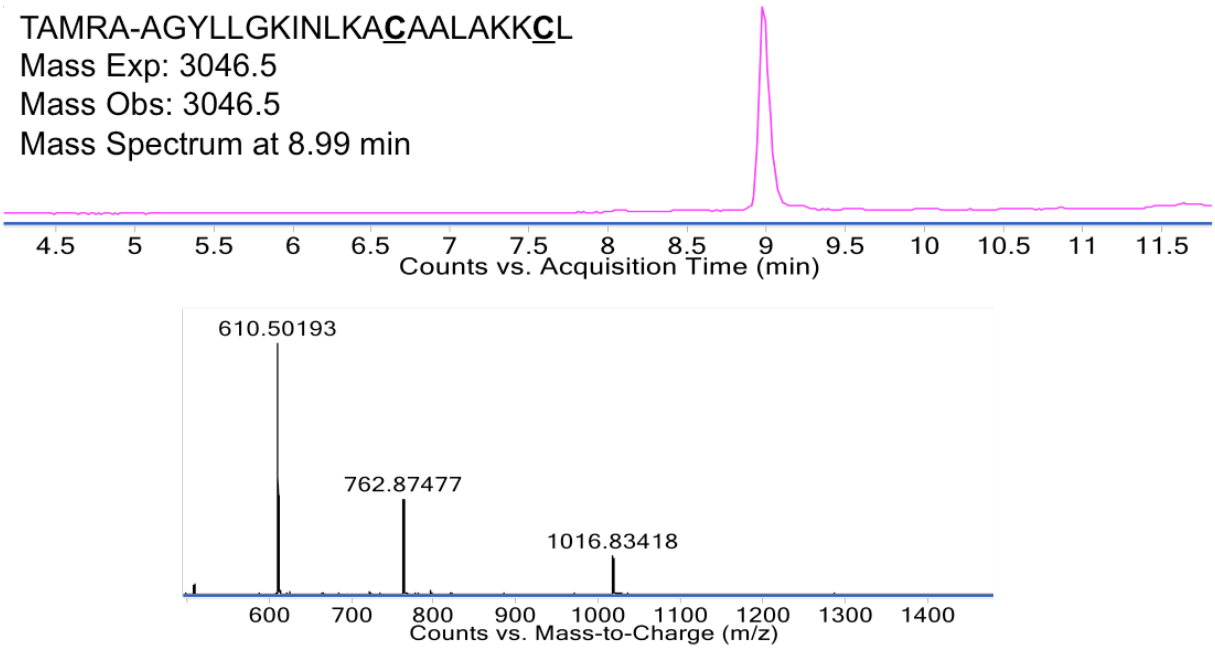


*TAMRA-Q*2 Characterization*



C = site of alkylation

TAMRA-M13 Characterization



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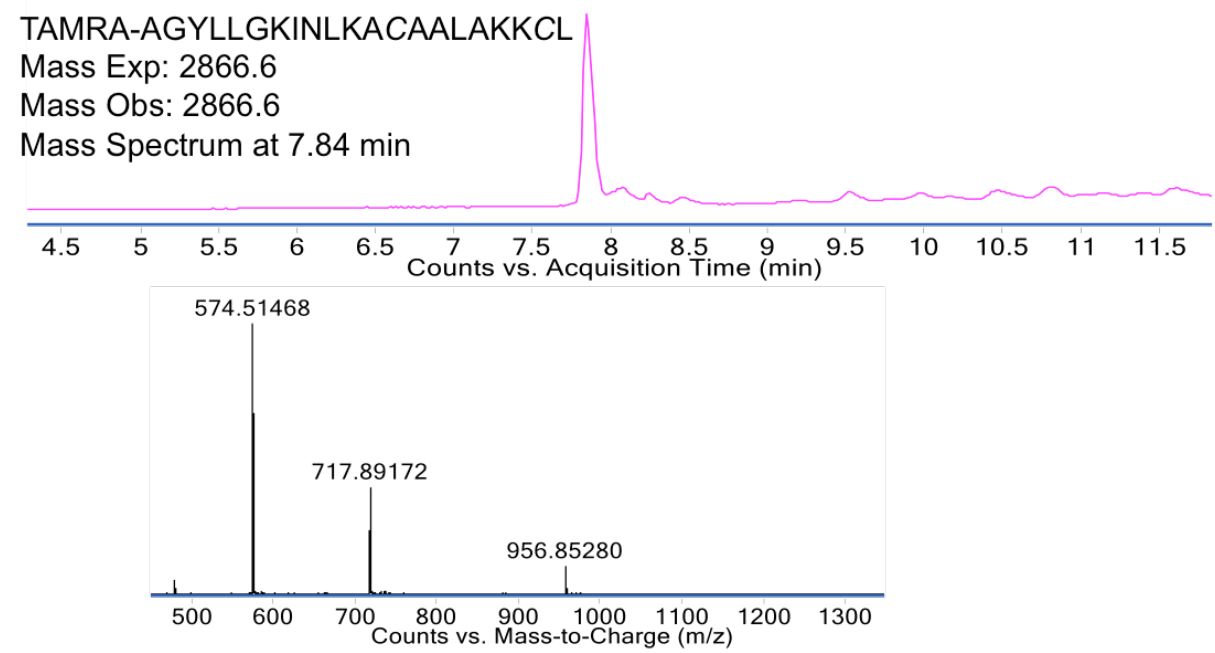
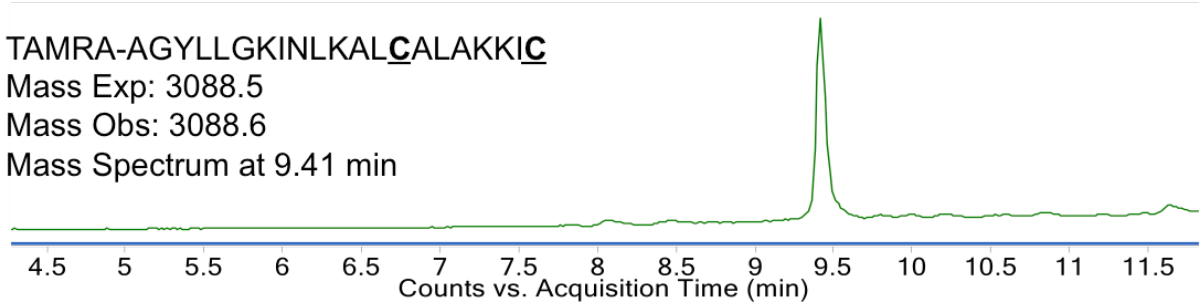
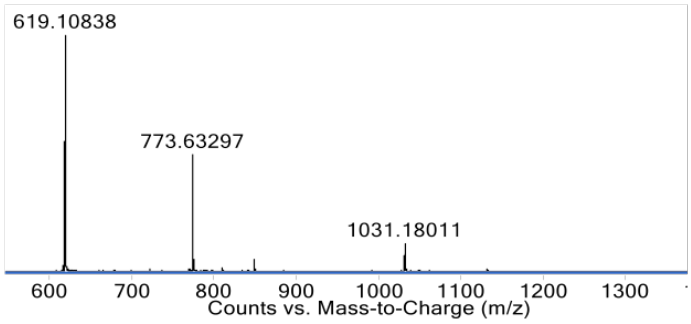
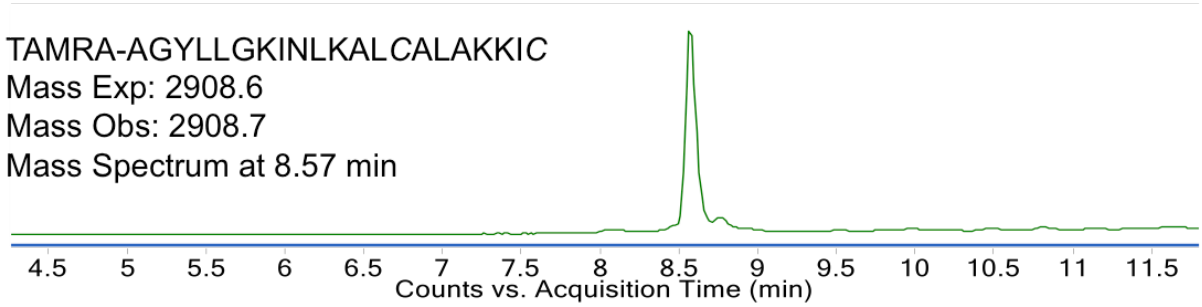


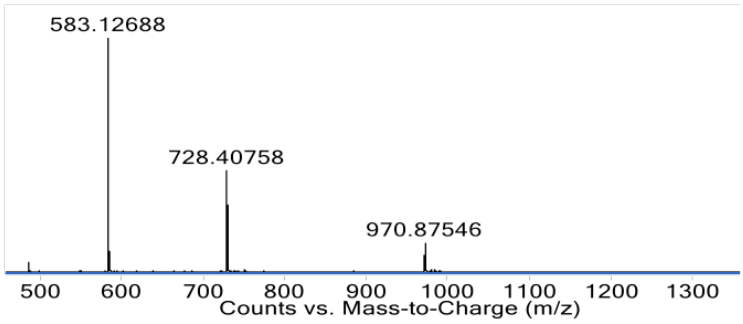
TAMRA-M14 Characterization



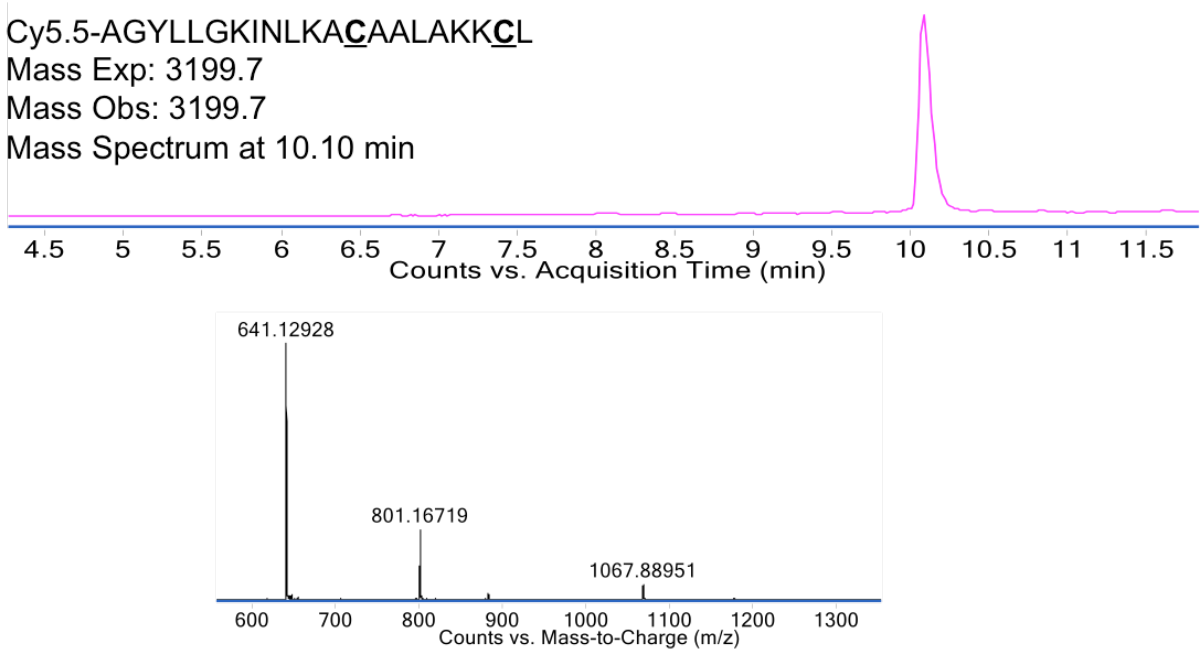


*TAMRA-Q*14 Characterization*

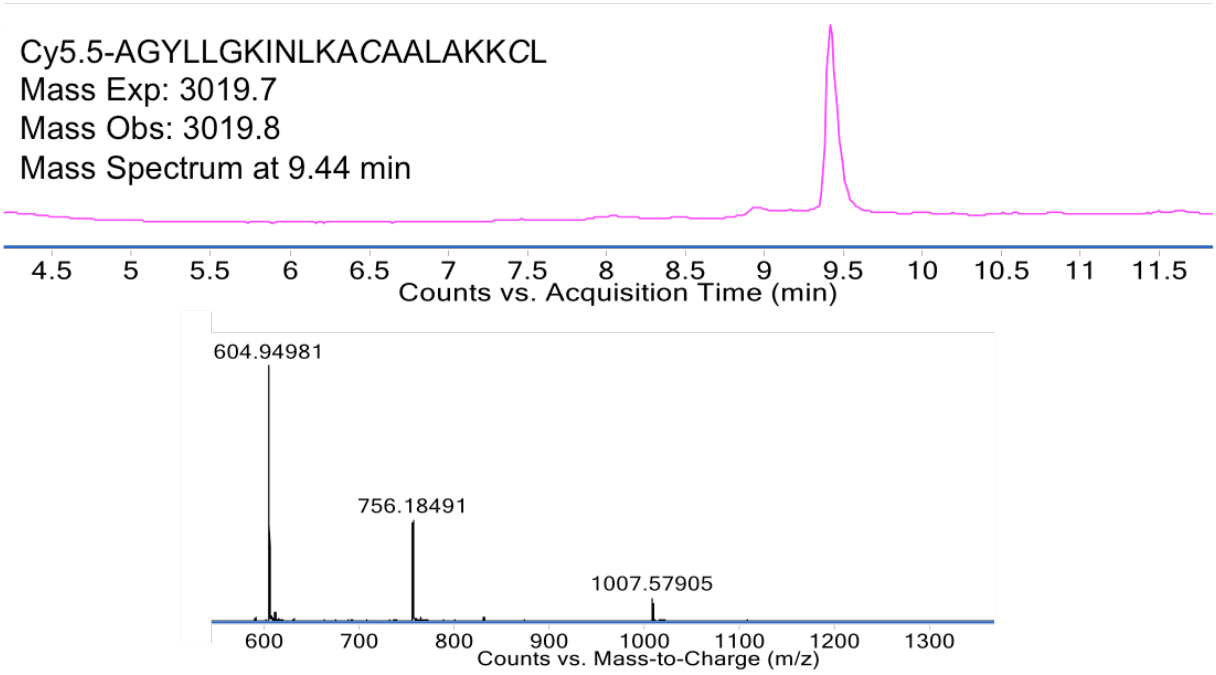




Cy5.5-M13 Characterization



*Cy5.5-Q*13 Characterization*



Purification of Crude BIM BH3 Peptides

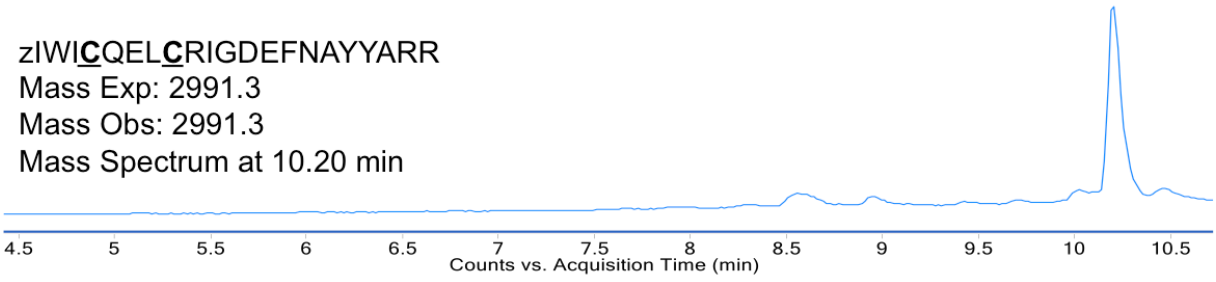
The crude BIM BH3 peptides were redissolved in 70% water and 30% acetonitrile containing 0.1% TFA, filtered through a 0.22 µm nylon filter (the filter was rinsed with 1 mL 50% water and 50% acetonitrile) and purified by mass-directed semi-preparative reversed-phase HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 µm). Solvent A was water with 0.1% TFA additive and Solvent B was

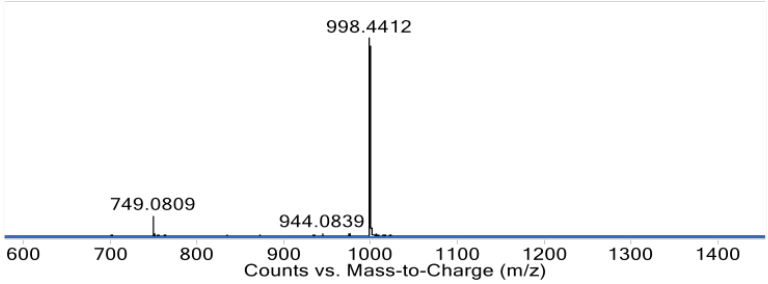
acetonitrile with 0.1% TFA additive. For the native BIM BH3 sequence, a linear gradient that changed at a rate of 0.5%/min was run from 15% B to 55% B. For the two analogues with cysteine substitutions, a linear gradient that changed at a rate of 0.5%/min was run from 30% B to 60% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

Procedure for Macrocyclization of Purified BIM BH3 Peptides

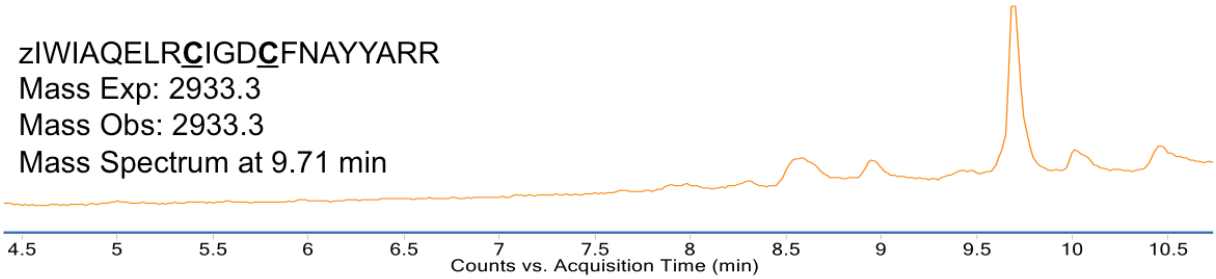
The purified, cysteine-containing BIM BH3 analogues were dissolved in DMF and a stock solution of decafluorobiphenyl in DMF was added such that the final concentration in the reaction vessel was 1 mM of a given BIM BH3 analogue and 2 mM decafluorobiphenyl. Diisopropylethylamine was added such that its final concentration in the reaction mixture was 50 mM. After 2 hours, the reaction mixture was diluted by a factor of two with water containing 0.1% TFA and filtered through a through a 0.22 µm nylon filter. The filter was rinsed with 1 mL 50% water and 50% acetonitrile with no additive. The reaction mixture was then diluted to 30% organic components (DMF and acetonitrile) with water containing 0.1% TFA to prepare the sample for loading onto the HPLC column. The crude reaction was purified by mass-directed semi-preparative reversed-phase HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 µm). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was run from 30% B to 60% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

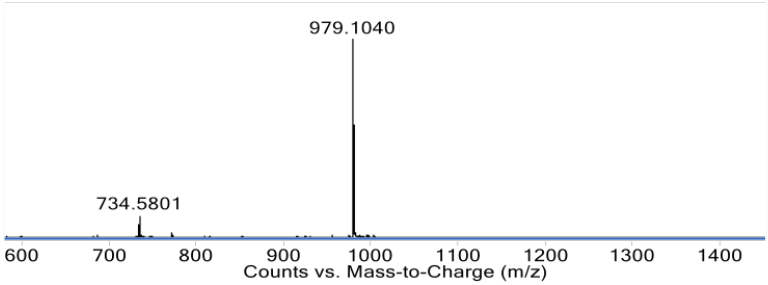
BIM BH3 M4 Characterization





BIM BH3 M9 Characterization





Procedure for Alkylation of Pure BIM BH3 Peptides

Initially, the same conditions were used to alkylate the purified, cysteine-containing BIM BH3 analogues as with the crude TP10 analogues. However, given poor recovery in the original conditions, we tested other conditions for the alkylation of BIM BH3 analogue 4 to ensure we had enough material for *in vivo* studies. BIM BH3 analogue 4 and 2-bromoacetamide were dissolved in 50% buffer containing 0.2 M sodium phosphate and 50 mM *tris*(2-carboxyethyl)phosphine (TCEP)•HCl, pH 8 and 50% DMF to a final concentration of 1 mM peptide and 50 mM 2-bromoacetamide. After one hour, the reaction was quenched by bringing the pH of the solution to 2 with 6 M HCl. The crude reaction was purified by mass-directed semi-preparative reversed-phase HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 µm). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was run from 20% B to 60% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

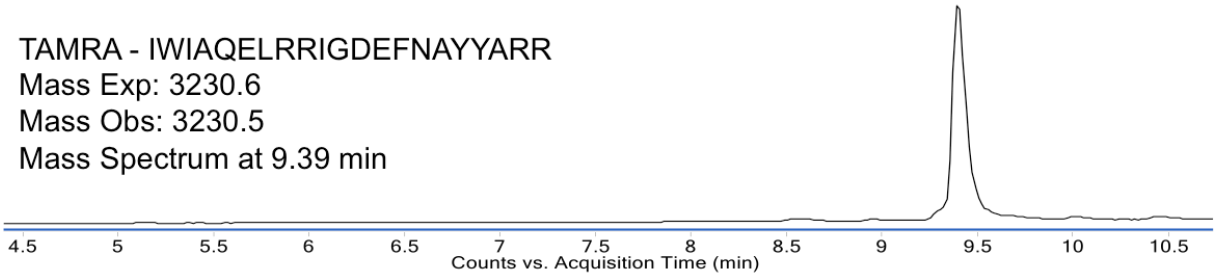
Procedure for Fluorophore Labeling of BIM BH3 Analogues by azide/alkyne Huisgen Cycloaddition

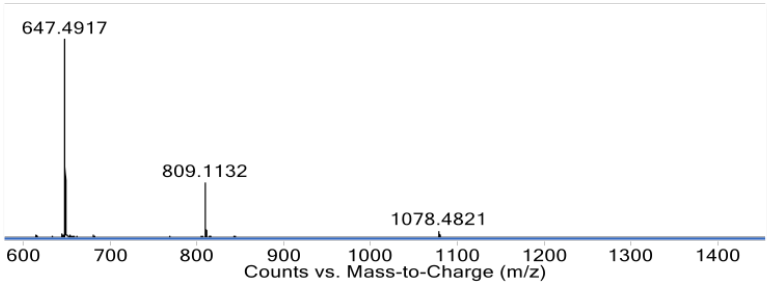
Purified BIM BH3 analogues were labeled with 5-TAMRA azide using copper-catalyzed "click" chemistry. (see "Procedure for Fluorophore Labeling of TP10 Analogues by azide/alkyne Huisgen Cycloaddition"). For BIM BH3 wt, BIM BH3 Q*4 and BIM BH3 Q*9: After one hour, the reaction was diluted with 9 mL of water containing 0.1% TFA, filtered through a 0.22 µm nylon filter (rinsed with 1 mL of 50:50 water:acetonitrile) and purified by mass-directed semi-preparative RP-HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 µm). Solvent A was water with 0.1% TFA

additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was run from 25% B to 55% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS. For BIM BH3 M4 and BIM BH3 M9: After 1 h, the reaction was diluted with 1 mL of water containing 0.1% TFA, filtered through a 0.22 μm nylon filter (rinsed with 1 mL of 50:50 water:acetonitrile) and purified by mass-directed semi-preparative RP-HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 μm). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was run from 35% B to 65% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

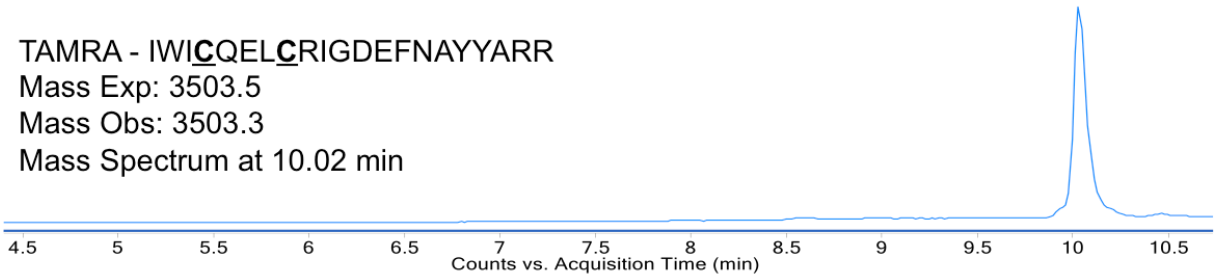
For the Cy5.5 labeled versions of BIM BH3 M4 and Q*4, the same procedure was used only Cy5.5 azide in DMSO was added instead of 5-TAMRA azide. Also, a different linear gradient was used for purification, which changed at a rate of 0.5%/min and ran from 40% B to 70% B.

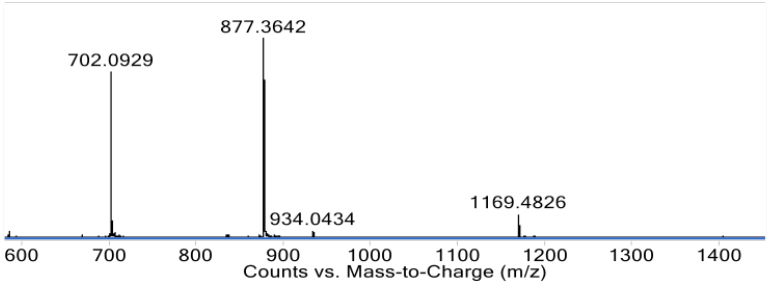
TAMRA – BIM BH3 Characterization



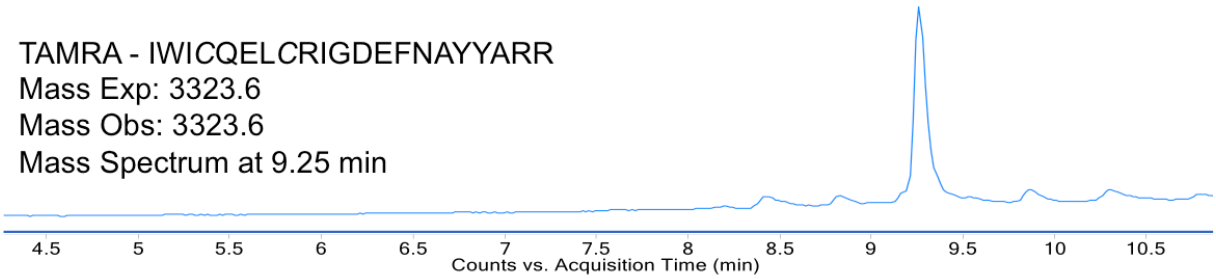


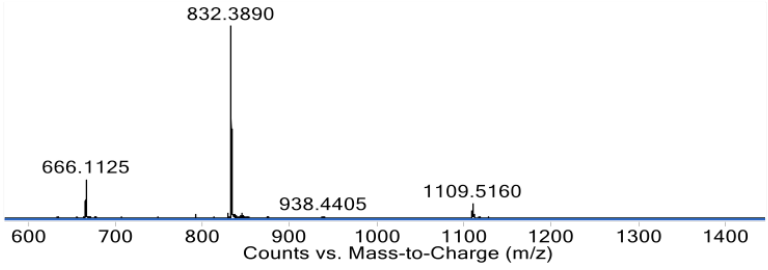
TAMRA – BIM BH3 M4 Characterization



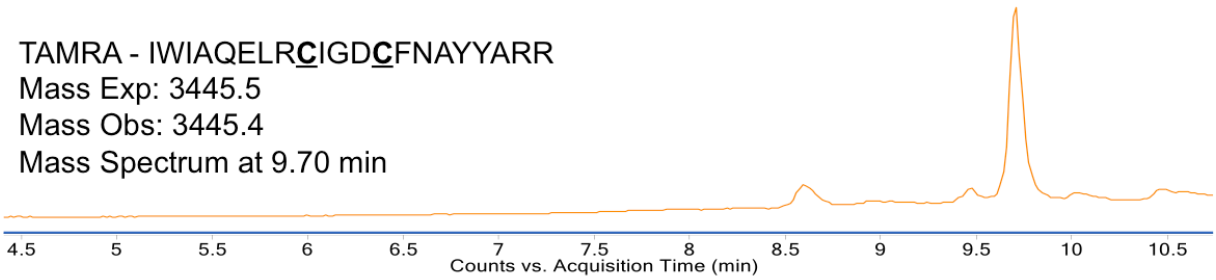


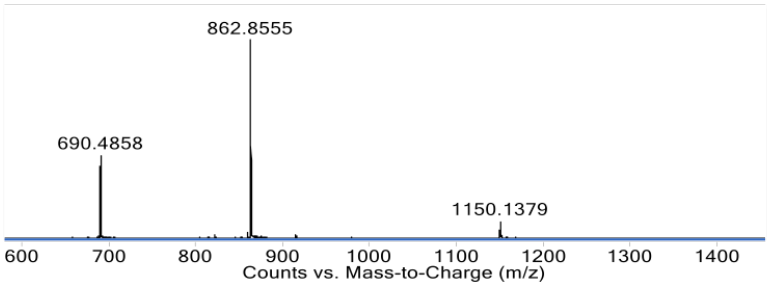
*TAMRA – BIM BH3 Q*4 Characterization*



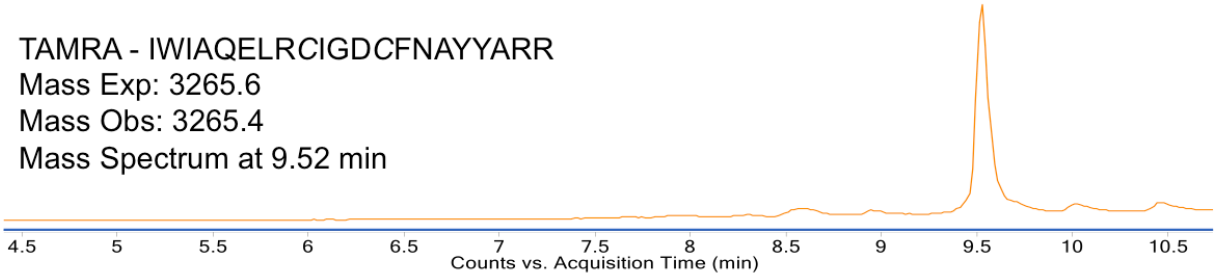


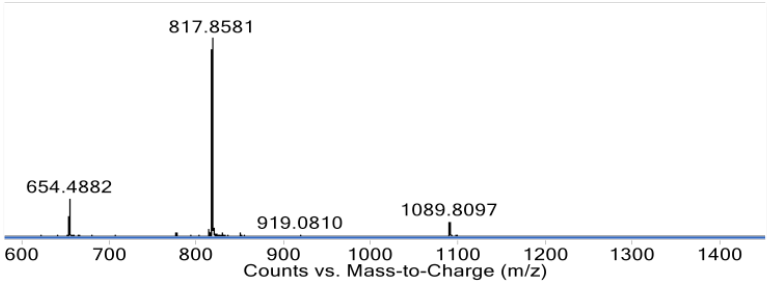
TAMRA – BIM BH3 M9 Characterization



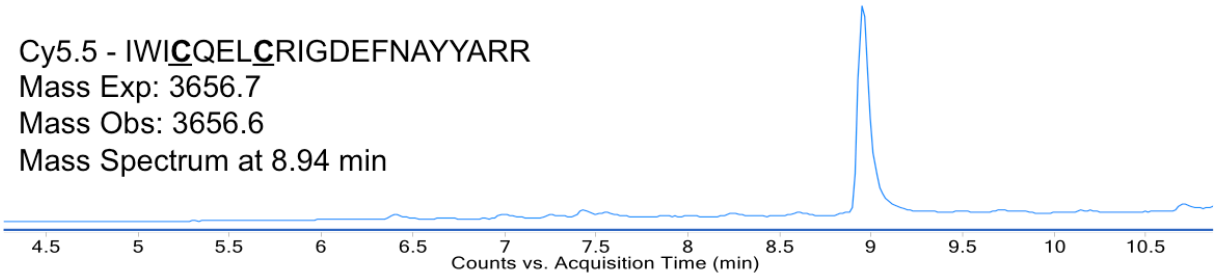


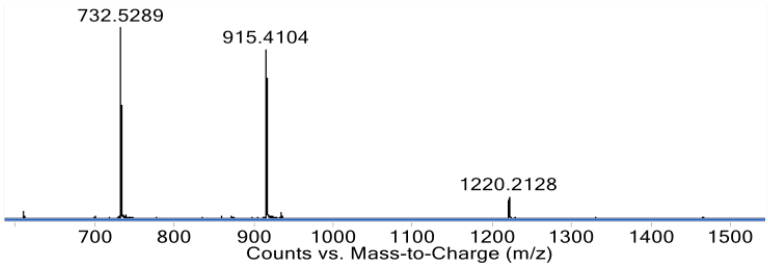
*TAMRA – BIM BH3 Q*9 Characterization*



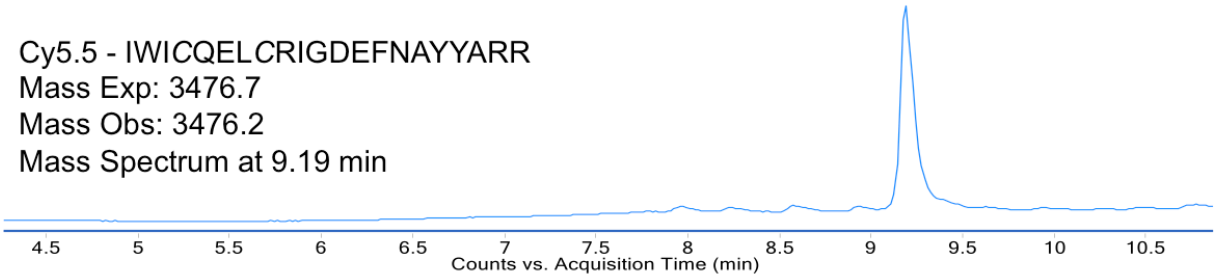


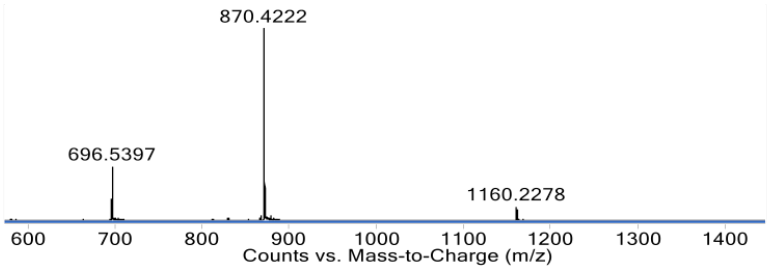
Cy5.5 – BIM BH3 M4 Characterization





*Cy5.5 – BIM BH3 Q*4 Characterization*





Preparation of Hydrocarbon Cyclized BIM BH3 Peptide

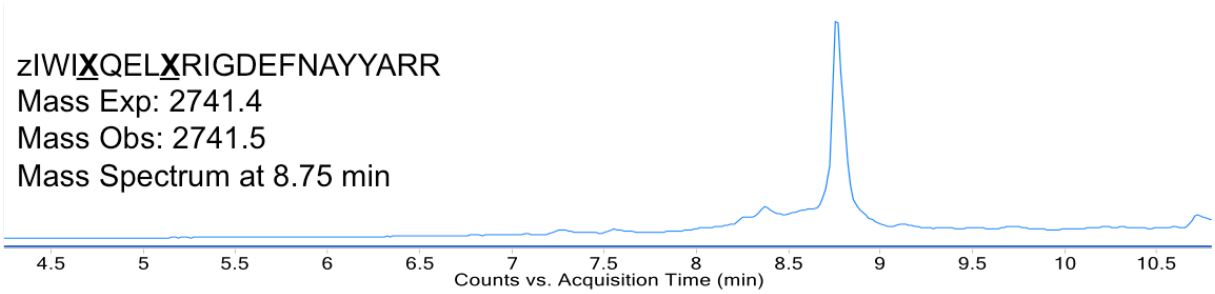
The peptide was synthesized using our standard fast-flow solid phase synthesis conditions with a 50 mg portion of ChemMatrix Rink Amide HYR resin. For each Fmoc-(*S*)-2-(4-pentenyl)Ala-OH residue and the residue immediately following, modified coupling conditions were utilized. For these residues, amino acid and HATU (150 μmol) were dissolved together in 2 mL NMP. Next, 120 μL of diisopropylethylamine was added to the vial and shaken to mix. The activated amino acid

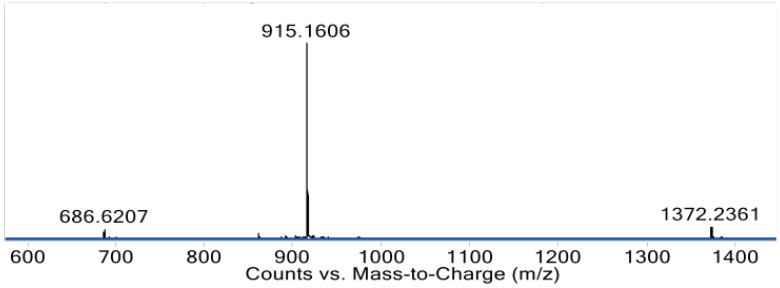
solution was flowed over the resin bed at a flow rate of 0.2 mL/min at a temperature of 90 ºC. For the N-terminal isoleucine residue, the Fmoc protecting group was not removed prior to olefin metathesis.

The olefin metathesis was carried out using a modified protocol from Kim *et al.*[2] Briefly, the peptidyl resin was swelled with 2 mL of DCE. The resin was treated with 1 mL of a 6 mM solution of Grubbs' first-generation catalyst in DCE under constant nitrogen bubbling at room temperature. After 2 hours, the reaction progress was assessed by removing a small portion of resin, subjecting the resin to cleavage, and analyzing the peptide by LC-MS. Given incomplete metathesis, the reaction was then allowed to proceed overnight. The resin was washed 3 times with DCE, then 3 times with DMF, and the Fmoc group was removed by treatment with 20% piperidine in DMF (2 x 4 minutes). Then, 4-pentynoic acid was coupled at room temperature (1 mmol in 2.5 mL of 0.4 M HATU with 500 μL diisopropylethylamine). The peptide was cleaved using a cocktail of 95% (v/v) TFA, 2.5% (v/v) water, and 2.5% (v/v) TIPS for 6 min at 60 °C, triturated three times with ether, resuspended in 50% water and 50% acetonitrile, and lyophilized.

The crude, lyophilized hydrocarbon cyclized BIM BH3 peptide was redissolved in 70% water and 30% acetonitrile containing 0.1% TFA, filtered through a 0.22 µm nylon filter and purified by mass-directed semi-preparative reversed-phase HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 μm). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was conducted from 25% B to 55% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

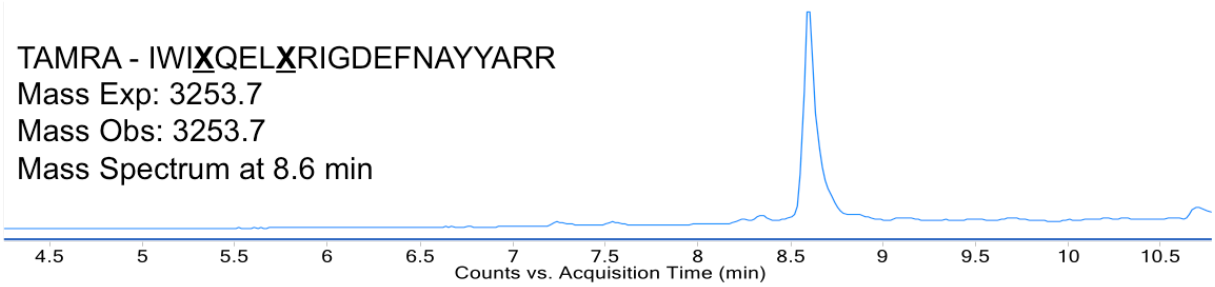
BIM BH3 HC4 Characterization

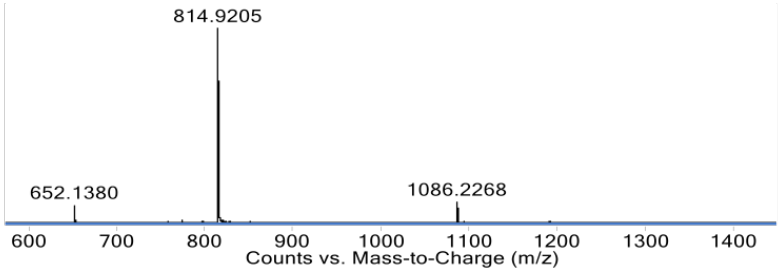




Finally, 5-TAMRA was conjugated the purified hydrocarbon cyclized BIM BH3 peptide using copper-catalyzed "click" chemistry. (see "Procedure for Fluorophore Labeling of TP10 Analogues by azide/alkyne Huisgen Cycloaddition"). Again, the reaction was purified by mass-directed semi-preparative RP-HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 μm). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was run from 25% B to 55% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

TAMRA - BIM BH3 HC4 Characterization





5. Flow Cytometry Assay

HeLa Flow Cytometry Experiment

HeLa cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) Pen Strep at 37 °C and 5% CO_2. Twelve hours before treatment, HeLa cells were plated at a density of 60,000 cells per well in a 24-well plate. 1 mM stocks of each of the peptides were prepared in DMSO. Concentration of the stocks was quantified by absorbance using a UV/Vis spectrophotometer and a 5-TAMRA extinction coefficient of ε=54,124 $M^{-1}cm^{-1}$ at λ=552 nm (determined experimentally by creating a standard curve of 5-TAMRA azide dissolved in DMSO/PBS). Then, each peptide was diluted to a final concentration of 2.5 μM in serum-free DMEM. To treat the cells, the overnight growth media was aspirated from each well and 200 μL of a given 2.5 μM peptide stock was applied. Cells were incubated for 2 hours at 37 °C and 5% CO_2 with the peptide treatment, and then the treatment media was aspirated. Trypsin-EDTA 0.25 % (100 μL) was added to the cells and incubated for 15 min at 37 °C and 5% CO_2. To quench the trypsin, 400 μL of phenol-red free DMEM supplemented with 10% (v/v) fetal bovine serum was added to each well. The dissociated cells in media were transferred to microcentrifuge tubes and spun at 500 rcf for 3 min. The supernatant was removed, the pellets were washed with 1 mL of phosphate-buffered saline (PBS), and the tube was spun again. The supernatant was again removed and the pellets were resuspended in 500 μL PBS with 2% FBS (v/v) along with 1 μL of 1 mg/mL propidium iodide in water. Flow cytometry analysis was carried out on a BD LSRII flow cytometer. Gates were applied to the data to ensure that only data from healthy, living cells were taken into account. Cells that were highly positive for propidium iodide or had forward/side scatter readings that were sufficiently different from the main cell population were excluded. Each histogram contains at least 5,000 gated events.

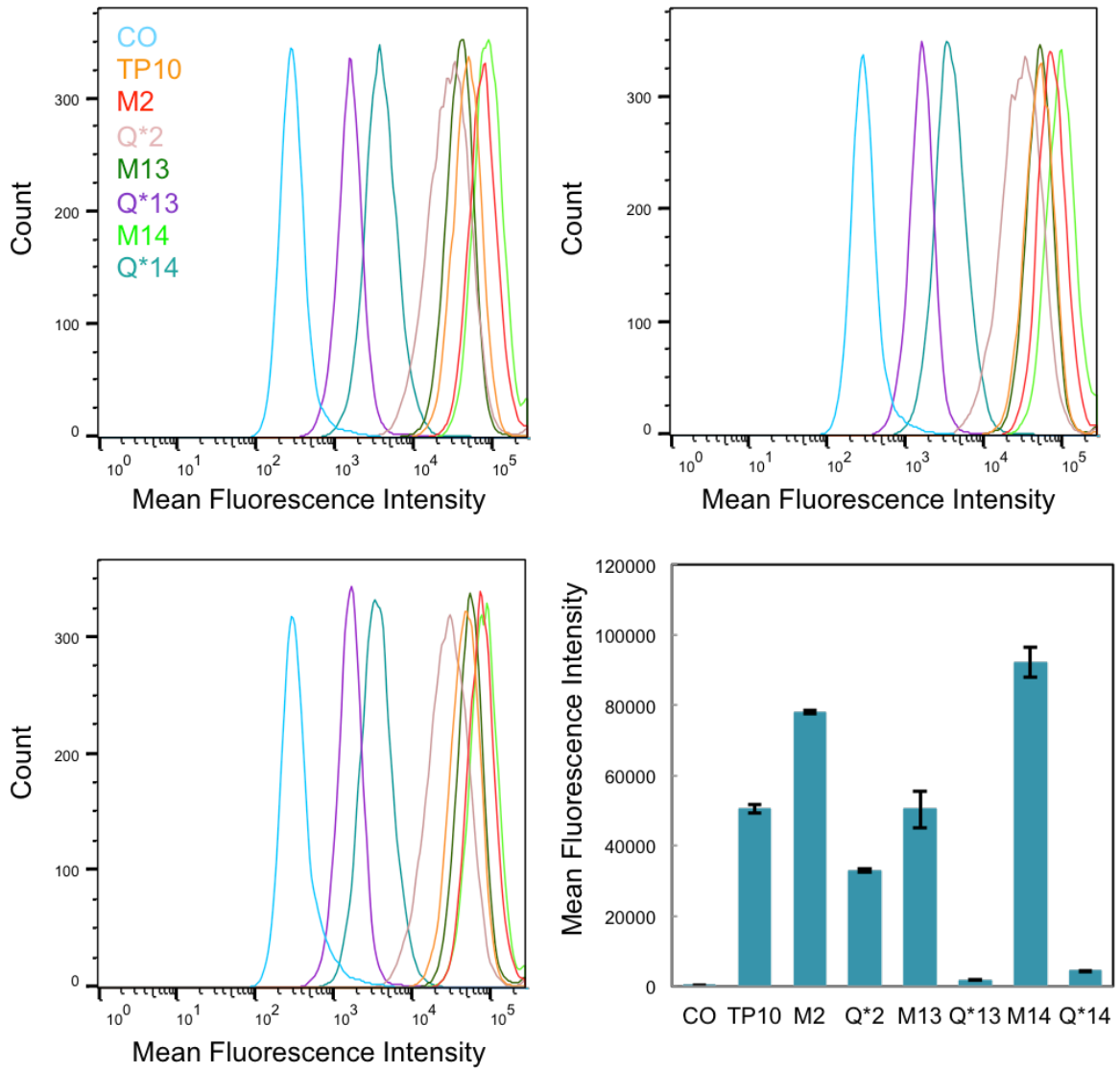


Figure S1. Flow cytometry data (n = 3) obtained from the experiments with HeLa cells. CO is a cell only control.

hCMEC/D3 Cell Flow Cytometry

hCMEC/D3 cells were maintained in EGM-2 media with all included growth factors, cytokines, and supplements at 37 °C and 5% CO_2. Twelve hours before treatment, hCMEC/D3 cells were plated at a density of 60,000 cells per well in a 24-well plate. The same 1 mM DMSO stocks of the peptides from the HeLa cell experiment were used. Each peptide was diluted to a final concentration of 2.5 µM in BBB-media (EGM-2 with 2% human serum without FBS or VEGF). To treat the cells, the overnight growth media was aspirated from each well and 200 µL of a given 2.5 µM peptide stock was applied. Cells were incubated for 2 hours at 37 °C and 5% CO_2 with the peptide

treatment, and then the treatment media was aspirated. Trypsin-EDTA 0.25 % (200 µL) was added to the cells and incubated for 15 min at 37 °C and 5% CO_2. To quench the trypsin, 800 µL of BBB media was added to each well. The dissociated cells in media were transferred to microcentrifuge tubes and spun at 500 rcf for 3 minutes. The supernatant was removed, the pellets were washed with 1 mL of PBS, and the tube was spun again. The supernatant was again removed and the pellets were resuspended in 500 µL PBS with 2% FBS (v/v) along with 1 µL of 1 mg/mL propidium iodide in water. Flow cytometry analysis was carried out on a BD LSRII flow cytometer. Gates were applied to the data to ensure that only data from healthy, living cells were taken into account. Cells that were highly positive for propidium iodide or had forward/side scatter readings that were sufficiently different from the main cell population were excluded. Each histogram contains at least 5,000 gated events.

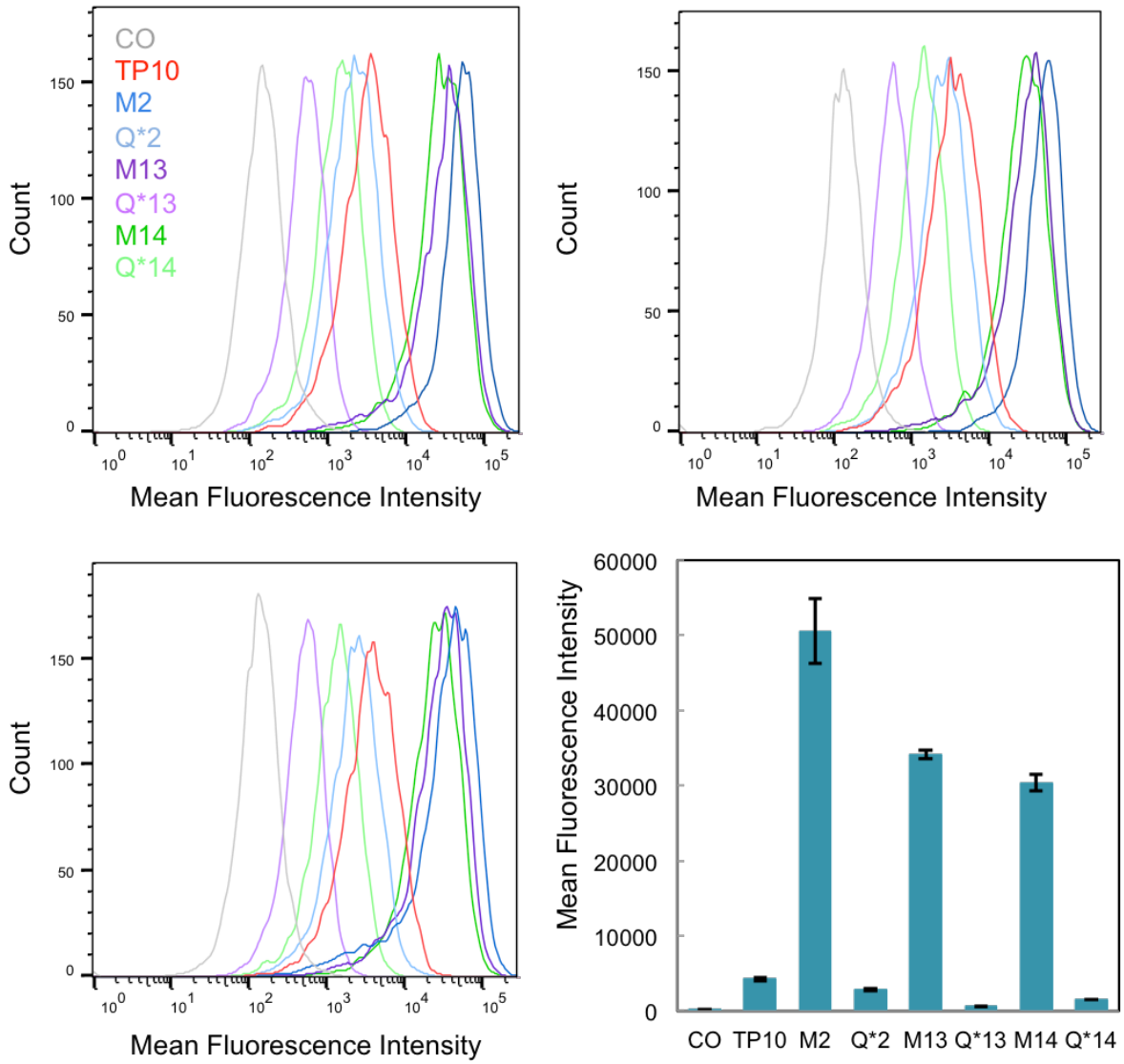


Figure S2. Flow cytometry data (n=3) obtained from the experiments with hCMEC/D3 cells. CO is a cell only control.

6. Proteolysis assay

Proteolysis Assay for Macrocyclic TP10 Analogues

For each peptide, 10 µL of PBS, 0.1 µL of Proteinase K (0.005 mg/mL stock solution in PBS), and 1 µL of peptide (1 mM DMSO stock solution) were combined in a PCR tube. The resulting reaction mixture was capped and incubated at 37 °C for 2 hours. At each time point, 0.5 µL of the crude reaction was transferred to an LC-MS vial and quenched by addition of 39 µL of 50:50 water:acetonitrile. A 0.8 µL portion of the quenched reaction was injected onto an Agilent 6550 iFunnel Q-TOF MS. Time points were taken at t = 0 min, 30 min, 60 min, and 120 min. An extracted ion current (EIC) for the +4 charge state m/z was analyzed using the MassHunter software. The EIC peak was integrated and percent peptide intact was determined by $(EICt_1/EICt_0) * 100$ in which $EICt_1$ is the peak integration at a given time point and $EICt_0$ is the peak integration at time t = 0 (results in main text).

Proteolysis Assay for Linear TP10 Analogues

For each peptide, 10 µL of PBS, 0.1 µL of Proteinase K (0.05 mg/mL stock solution in PBS), and 1 µL of peptide (1 mM DMSO stock solution) were combined in a PCR tube. The resulting reaction mixture was capped and incubated at room temperature for 2 hours. At each time point, 0.5 µL of the crude reaction was transferred to a LC-MS vial and quenched by addition of 39 µL of 50:50 water:acetonitrile. A 0.8 µL portion of the quenched reaction was injected onto an Agilent 6550 iFunnel Q-TOF MS. Time points were taken at t = 0 min, 20 min, 40 min, 60 min, and 120 min. An extracted ion current (EIC) for the +4 charge state m/z was analyzed using the MassHunter software. The EIC peak was integrated and percent peptide intact was determined by $(EICt_1/EICt_0) * 100$ in which $EICt_1$ is the peak integration at a given time point and $EICt_0$ is the peak integration at time t = 0.

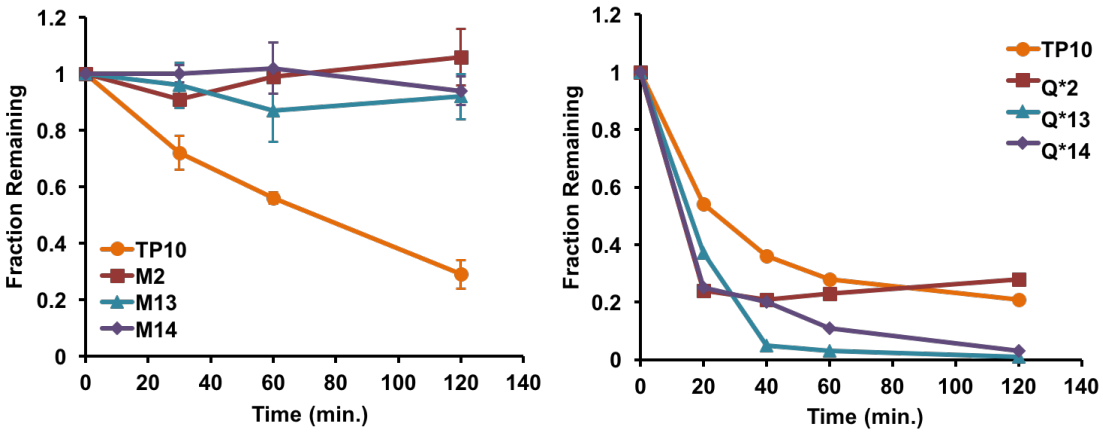


Figure S3. Proteolysis assay data obtained from incubation with Proteinase K. Over two hours, the macrocyclic variants are stable to Proteinase K, while native TP10 is degraded (n=3). All alkylated controls degraded to the same or greater extent than the native TP10 peptide.

7. LDH Assay

HeLa cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) Pen Strep at 37 °C and 5% CO_2. Twelve hours before treatment, HeLa cells were plated at a density of 10,000 cells per well in a 96-well plate. Stocks (1 mM) of each of the peptides were prepared in DMSO. Concentration of the stocks was confirmed by absorbance using a UV/Vis spectrophotometer and a 5-TAMRA extinction coefficient of ε=54,124 $M^{-1}cm^{-1}$ at λ=552 nm. Each peptide was diluted to a final concentration of 10 μM in serum-free DMEM. To treat the cells, the overnight growth media was aspirated from each well and a serial dilution was performed, such that the total volume in each well was 100 μL and concentrations of 5 μM, 2.5 μM, and 1 μM were evaluated. Additional wells included a DMSO control, to ensure the quantities of DMSO used to solubilize the peptide would not cause membrane leakage and two sets of cell only wells - one to serve as the negative control and the other to be frozen and thawed after incubation to serve as a positive control. Cells were incubated for 2 hours at 37 °C and 5% CO_2 and then the supernatant treatment media was transferred to another clear-bottom 96-well plate for the assay. The assay was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the included technical bulletin. After completion of the assay, the final solution in each well was diluted 1:1 with PBS such that the measured absorbance was in the linear range of the instrument. The absorbance was measured on a BioTek Epoch Microplate Spectrophotometer. Additional controls at this step included DMEM only and 5 μM TAMRA-azide, to confirm that there was no background absorbance at 490 nm.

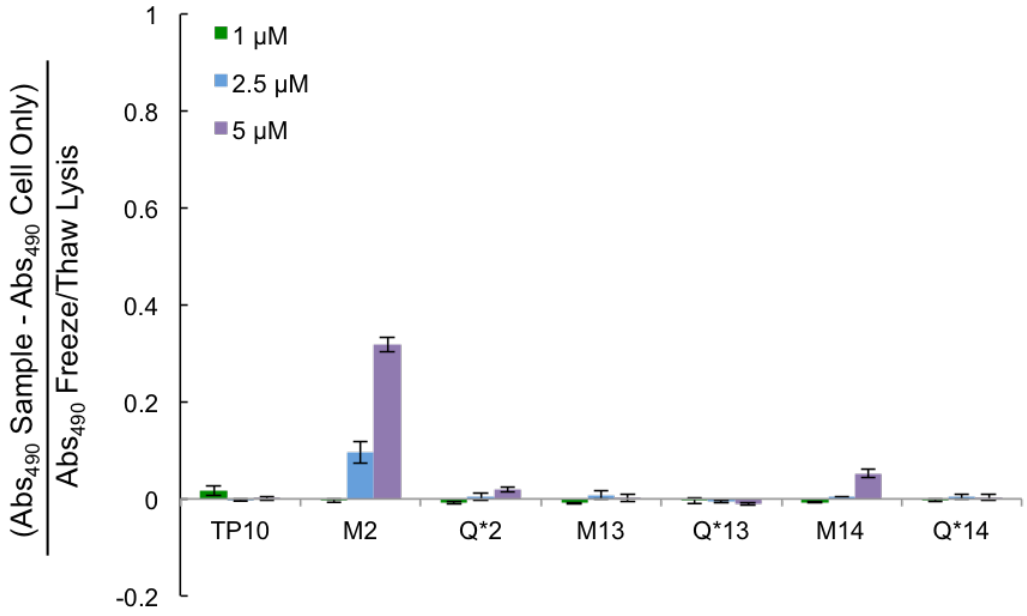


Figure S4. LDH assay data (in triplicate) obtained from the experiments with HeLa cells. With increasing concentrations, M2 did display some LDH leakage compared to a cell only control. With M13 and M14, however, little if any LDH leakage was observed.

8. Spheroid Assay

Primary human astrocytes were cultured in AGM media with all included growth factors, cytokines, and supplements at 37 °C and 5% CO_2. Astrocytes used in spheres were between passage number two and five. Human brain microvascular pericytes (HBVP) were cultured in pericyte medium containing 2% FBS, pericyte growth supplement and Pen Strep. HBVPs used in spheres were between passage number two and ten. hCMEC/D3 endothelial cells were maintained in EGM-2 with all included growth factors, cytokines, and supplements at 37 °C and 5% CO_2. hCMEC/D3 endothelial cells were between passage 27 and 32. All cells were grown in T75 tissue culture flasks. For co-culture conditions for spheroid formation and functional assays, spheroids were maintained in BBB-media (EGM-2 with 2% human serum without FBS or VEGF) at 37 °C and 5% CO_2.

Sterile 1% agarose (w/v) was transferred into a tissue culture hood and 50 μL of the solution was dispensed into each well of a 96-well plate and allowed to cool and solidify. Primary human astrocytes, HBPVs, and hCMEC/D3 cells were treated with trypsin-EDTA 0.25% and incubated for 15 minutes at 37°C and 5% CO2. The trypsin in each flask was quenched with BBB-media. Each cell type was counted with a hemocytometer and 1000 cells of each cell type were seeded onto the 96-well plate containing the solidified agarose in a 1:1:1 ratio to a final volume of 100 μL. Cells were incubated in a humidified incubator at 37 °C and 5% CO_2 for 48 hours, in order to allow the cells to assemble into multicellular BBB spheroids.

Microcentrifuge tubes containing a 5 μM solution of each peptide analogue in BBB-media were prepared. Five to ten multicellular BBB spheroids were added to each tube. Spheroids were incubated for 3 hours at 37 °C on a rotator. The spheres were then removed and washed three times with PBS, fixed in 3.7% formaldehyde, and transferred into Nunc Lab-Tek II thin-glass 8-well chambered coverglass (Thermo Scientific). The spheroids were imaged using a confocal microscope under a 20x objective. Z-slices captured through each spheroid (up to 100 μm depth) were analyzed using the ZEN imaging software (blue edition; 2012 version). In order to quantify the mean fluorescence intensity, the image at a depth of 88 μm in the z-plane was used. The mean fluorescence of a 100 μm circle centered on the center of the sphere in the XY-plane was quantified using ImageJ (Figure S6).

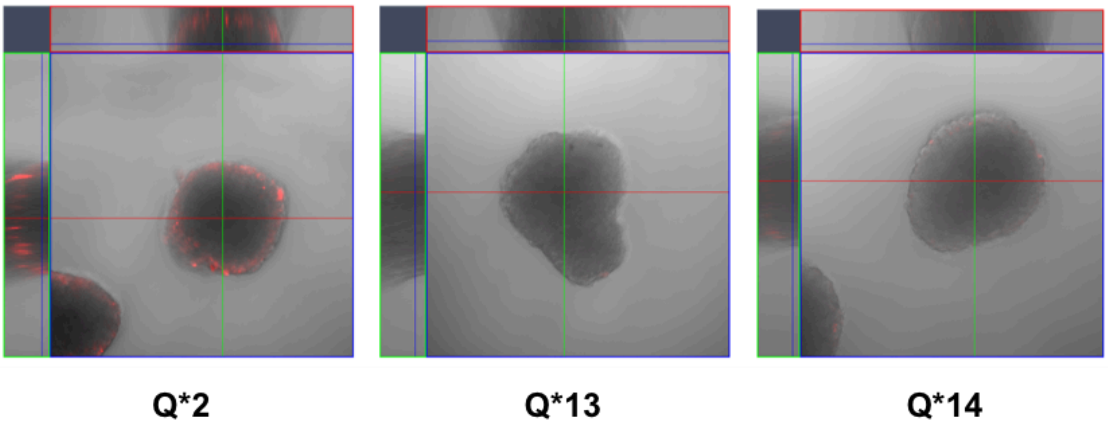


Figure S5. The confocal microscopy images display a representative spheroid after incubation in a solution of BBB media with 5 μM of each alkylated TP10 control (Q*2, Q*13, Q*14) showing an overlay of both fluorescence (5-TAMRA) and brightfield. The images are at a z-depth of 88 μm into the core of the sphere. These images are the controls from the experiment presented in Figure 4 in the main text.

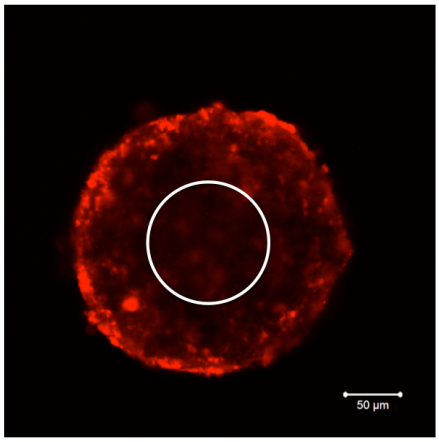


Figure S6. Example of fluorescence quantification for a sphere treated with 5 μM BIM BH M4. For each sphere, a z-stack is collected. The image corresponding to a z-depth of 85-90 μm into the sphere is extracted for quantification. The mean fluorescence intensity of the circle shown above is quantified using ImageJ. This is to ensure that the results are reflective of fluorescent material in the core of the sphere, rather than on the outside in the endothelial cells.

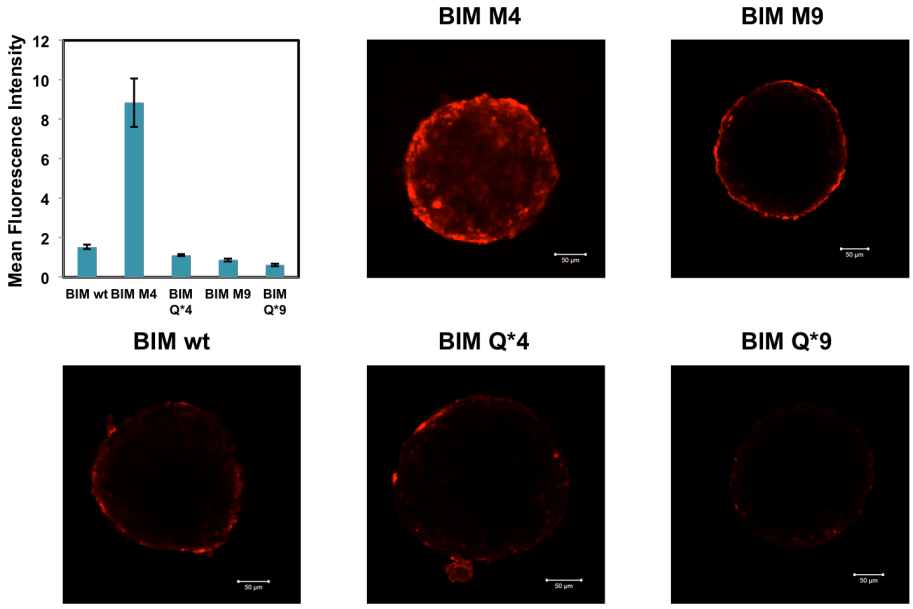


Figure S7. The confocal microscopy images display a representative spheroid after incubation in a solution of BBB media with 5 μM of each BIM BH3 analogue. The images show fluorescence (5-TAMRA) and are at a z-depth of 85 μm into the core of the sphere. The plot (also Figure 6B in main text) shows quantification of the mean fluorescence intensity in the core of the sphere for all BIM BH3 analogues (n=6).

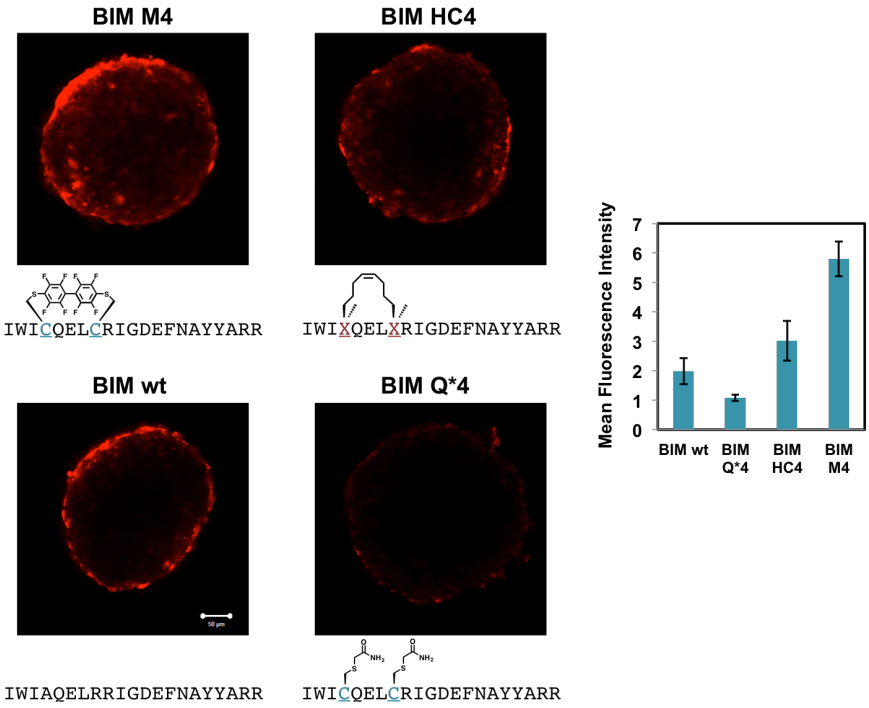


Figure S8. Comparison of perfluoroarene-cyclized BIM BH3 to hydrocarbon-cyclized BIM BH3. The confocal microscopy images display a representative spheroid after incubation in a solution of BBB media with 5 μM of each BIM BH3 analogue. The images show fluorescence (5-TAMRA) and are at a z-depth of 85 μm into the core of the sphere. The plot shows quantification of the mean fluorescence intensity in the core of the sphere for all BIM BH3 analogues (n=8).

9. Serum Stability Assay

PBS (95 µL), mouse serum (5 µL), and peptide solution (0.5 µL of a 10 mM DMSO stock solutions of Cy5.5-TP10 M13 and Cy5.5-TP10 Q*13 quantified by mass) were combined in a 0.7 mL microcentrifuge tube. The resulting mixture was capped and incubated at 37 °C for 24 hours. At each time point, 5 µL of the solution was transferred to a different 0.7 mL microcentrifuge tube, flash frozen in liquid N_2, and lyophilized. Upon drying, the samples were redissolved in 30 µL of 50:50 water:acetonitrile containing 0.1% TFA, transferred to an LC-MS vial, and 1 µL was injected onto the Agilent 6520. Time points were taken at t = 0 h, 1.5 h, 3 h, 6 h, 12 h, and 24 h. An extracted ion current (EIC) for the +5 charge state m/z was analyzed using the MassHunter software. The EIC peak was integrated and the fraction of peptide intact was determined by $EICt_1/EICt_0$ in which $EICt_1$ is the peak integration at a given time point and $EICt_0$ is the peak integration at time t = 0.

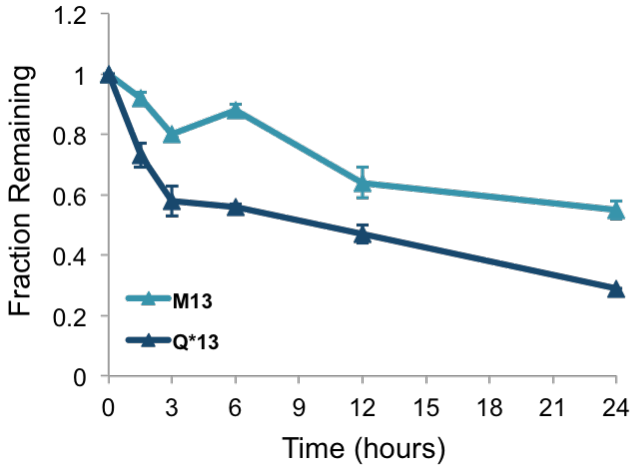


Figure S9. Serum stability data (in triplicate) obtained from incubating M13 and Q*13 with 5% mouse serum. While degradation of both peptides was seen over time, there was a greater fraction remaining of M13 at time t = 24 h.

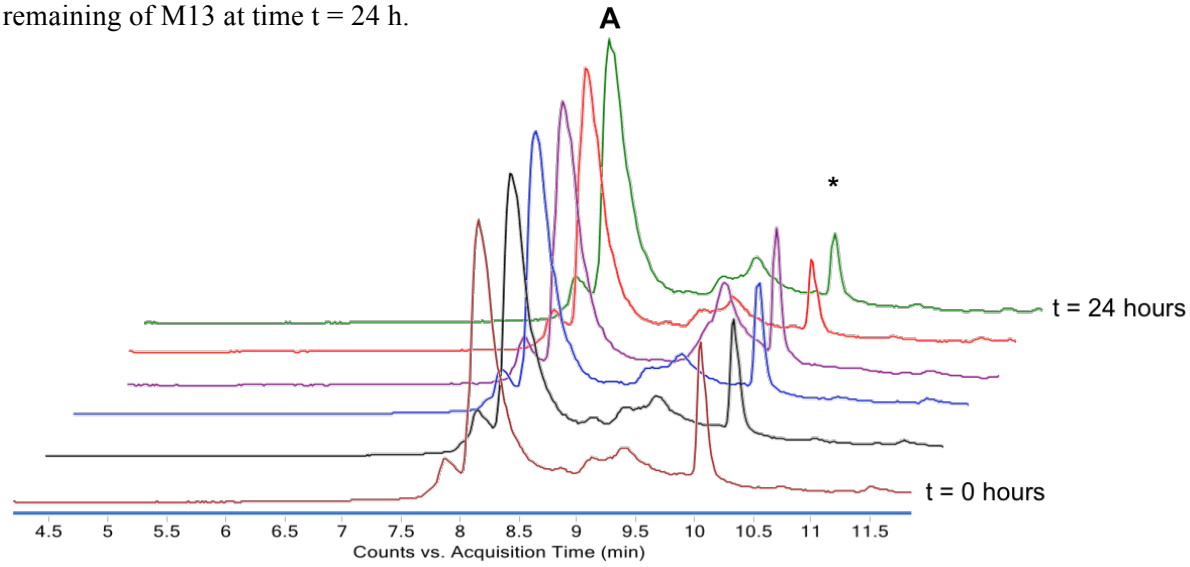


Figure S10. Total ion current (TIC) chromatograms of one representative trial of the serum stability assay with peptide M13. A = albumin * = M13

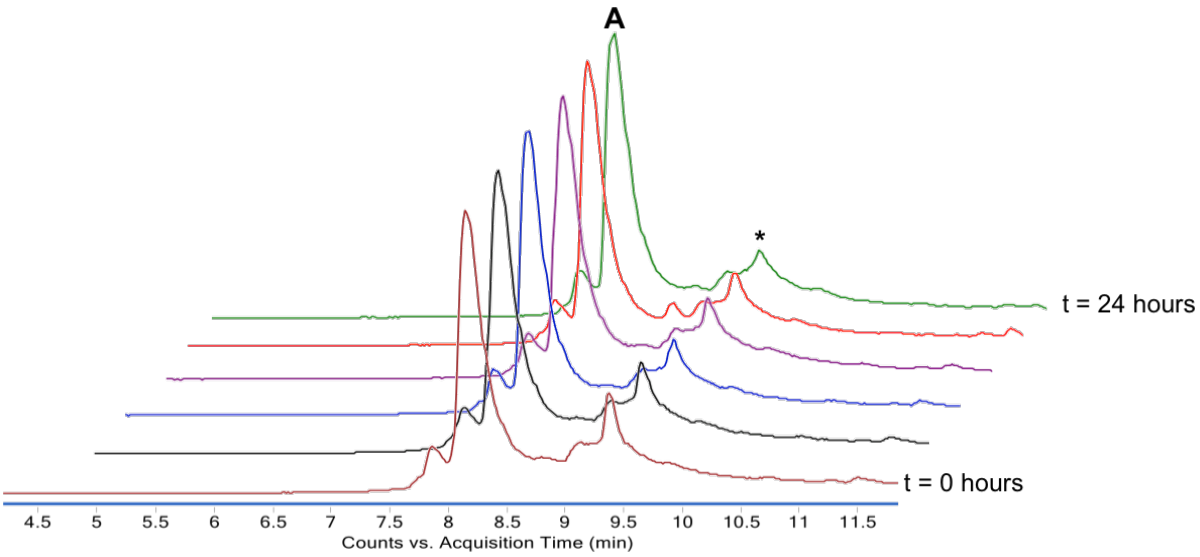


Figure S11. Total ion current (TIC) chromatograms of one representative trial of the serum stability assay with peptide M13. A = albumin * = Q*13

10. *In Vivo* Imaging System Analysis

The lyophilized Cy5.5-TP10 M13, Cy5.5-TP10 Q*13, Cy5.5-BIM M4, and Cy5.5-BIM Q*4 powders were dissolved to a final concentration of 2 mM in DMSO by mass. Each peptide was then diluted to a concentration of 100 µM in a solution of 50:50 polyethylene glycol (PEG)-300:0.9% sodium chloride (v/v) irrigation solution. A 100 µL dose of each peptide solution was administered intravenously via the tail vein into healthy 8-week old female nude mice. After 4 hours, 100 µL of 50 mg/mL tetramethylrhodamine isothiocyanate-labeled dextran (155 kDa) in 0.9% sodium chloride was injected via the tail vein. Mice were sacrificed 30 min later by cervical dislocation. The brain, heart, lungs, kidneys, spleen, and liver were excised from each mouse, frozen on dry ice and imaged using an *In Vivo* Imaging System (Perkin Elmer) at an excitation of 640 nm. Using Living Image software, regions of interest were drawn around each organ and the total radiant efficiency of Cy5.5 from each organ was quantified.

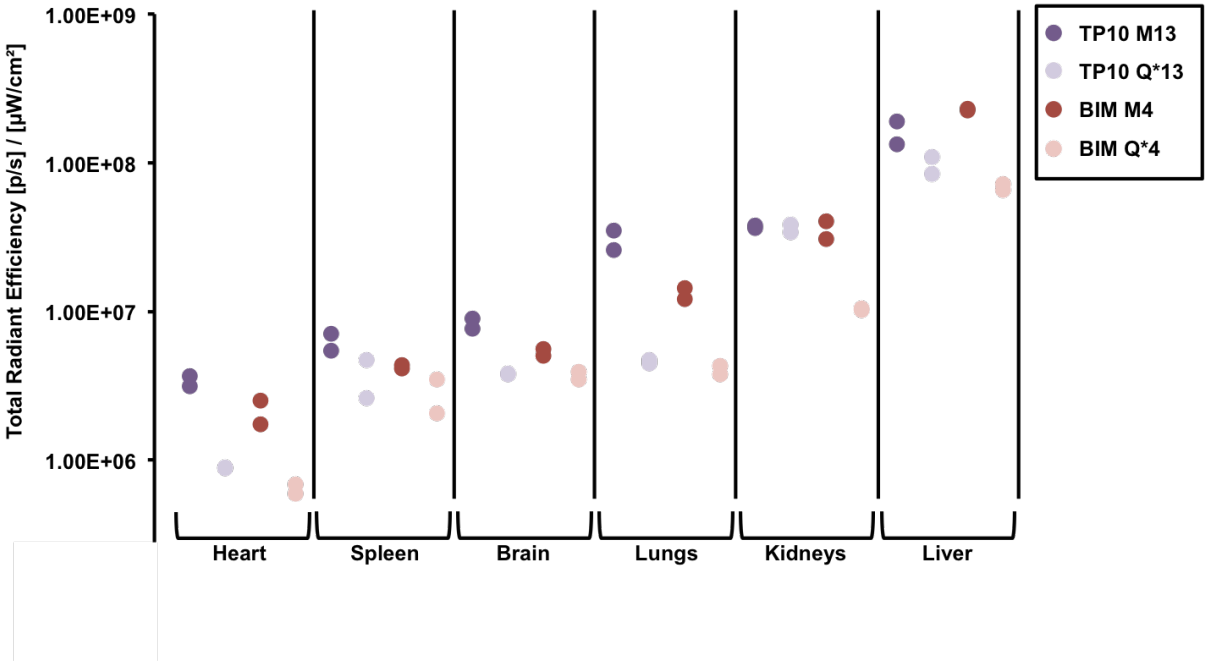


Figure S12. Biodistribution. Total Cy5.5 radiant efficiency of organs from mice treated with Cy5.5-labeled versions of TP10 M13, TP10 Q*13, BIM M4, and BIM Q*4. Two mice were in each treatment group and the data points from each mouse organ are plotted individually. The fluorescent material primarily ends up in the liver and kidneys for both the macrocyclic analogues and the alkyl analogues. The macrocycles have increased accumulation in the brain, heart, and lungs compared to their respective alkyl controls.

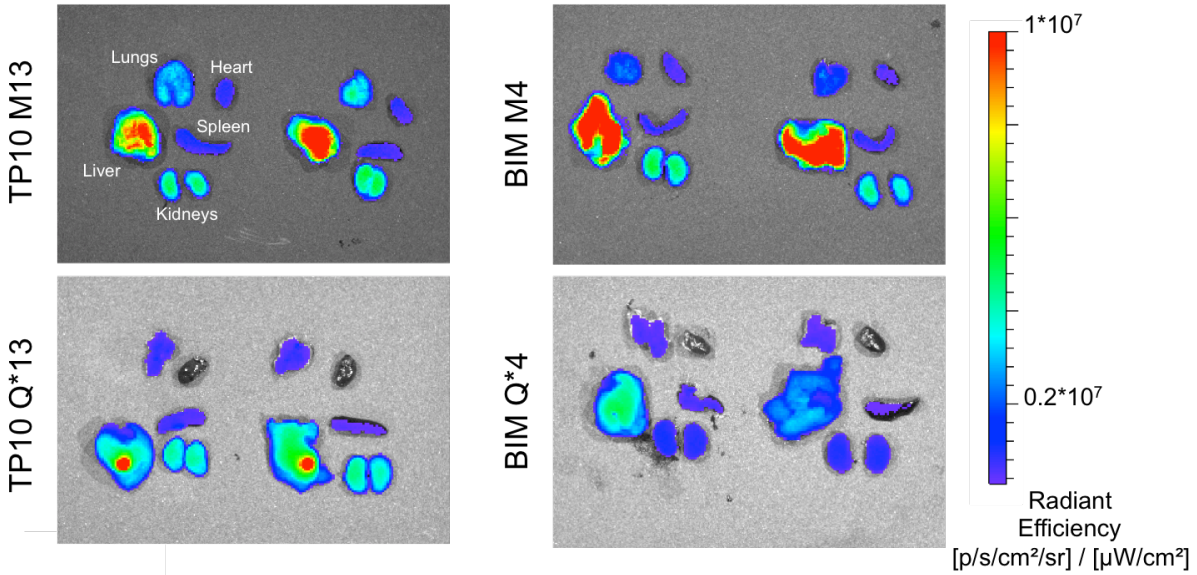


Figure S13. Cy5.5 radiant efficiency of each set of organs from mice treated with Cy5.5-labeled versions of TP10 M13, TP10 Q*13, BIM M4, and BIM Q*4. For imaging, the excitation wavelength was at 640 nm and the emission was monitored through the Cy5.5 filter on the instrument.

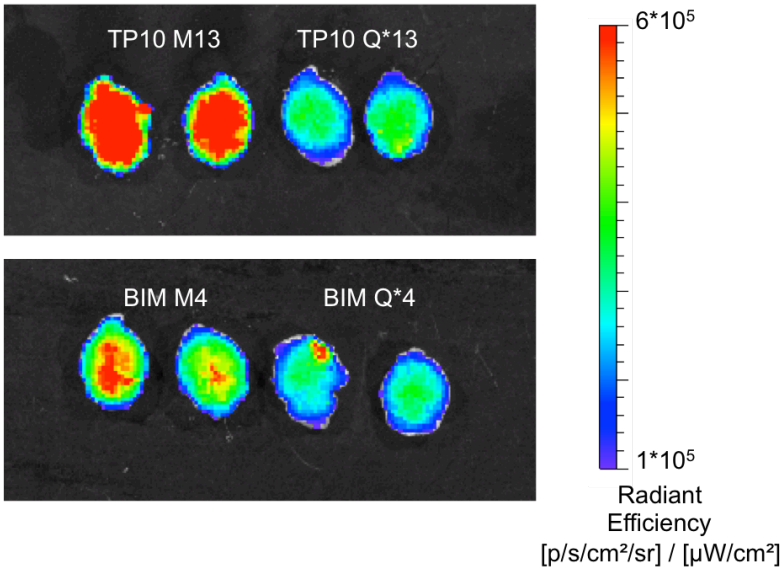


Figure S14. Cy5.5 radiant efficiency of each set of brains from mice treated with Cy5.5-labeled versions of TP10 M13, TP10 Q*13, BIM M4, and BIM Q*4. For imaging, the excitation wavelength was at 640 nm and the emission was monitored through the Cy5.5 filter on the instrument. One brain from each group is presented in the main text Figures 4 and 5. The bright spot on the left brain in the BIM Q*4 group is due to a small amount of spleen that was frozen to the brain. The organs were all frozen next to each other and removal risked damage to the brain by removing the small piece of spleen.

IVIS Study of TP10 Constructs 24 hours after treatment with lower concentration

The lyophilized Cy5.5-TP10 M13 and Cy5.5-TP10 Q*13 powders were dissolved to a final concentration of 2 mM in DMSO by mass. Each peptide was then diluted to a concentration of 10 µM in a solution of 50:50 polyethylene glycol (PEG)-300:0.9% sodium chloride irrigation solution. A 100 µL dose of each 10 µM peptide solution was administered intravenously via the tail vein into healthy 8-week old female nude mice. The mice were sacrificed 24 hours after treatment (by CO_2 asphyxiation). Brains were excised and immediately imaged using an *In Vivo* Imaging System (Perkin Elmer).

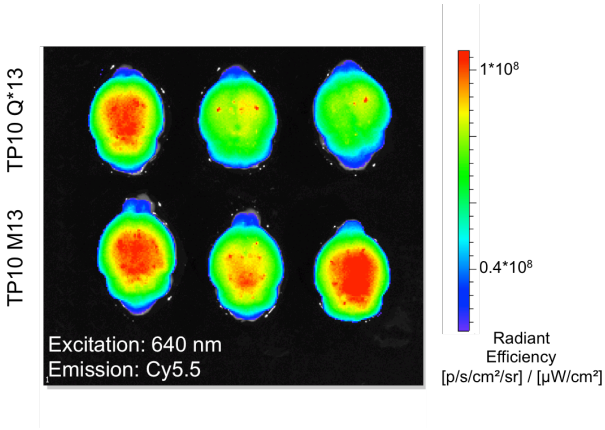


Figure S15. Three brains treated with Cy5.5-TP10 Q*13 (top) and three brains treated with Cy5.5-TP10 M13 (bottom) after excitation at 640 nm to image Cy5.5.

11. Confocal Microscopy of *Ex Vivo* Brain Slices

After imaging the frozen brains with the IVIS instrument, cryosections (30 µm) of the brains were obtained using a Microm HM 550 cryostat. Brain slices were fixed in 3.7% formaldehyde for 10 min, stained with Hoechst dye (1:1000 in PBS), mounted with a coverslip in VectaShield Antifade mounting media and imaged under a confocal microscope (63x oil-immersion objective). Tile scans (2x2) and z-slices were merged to generate a 2D maximum intensity projection using ZEN imaging software (black edition; 2012 version).

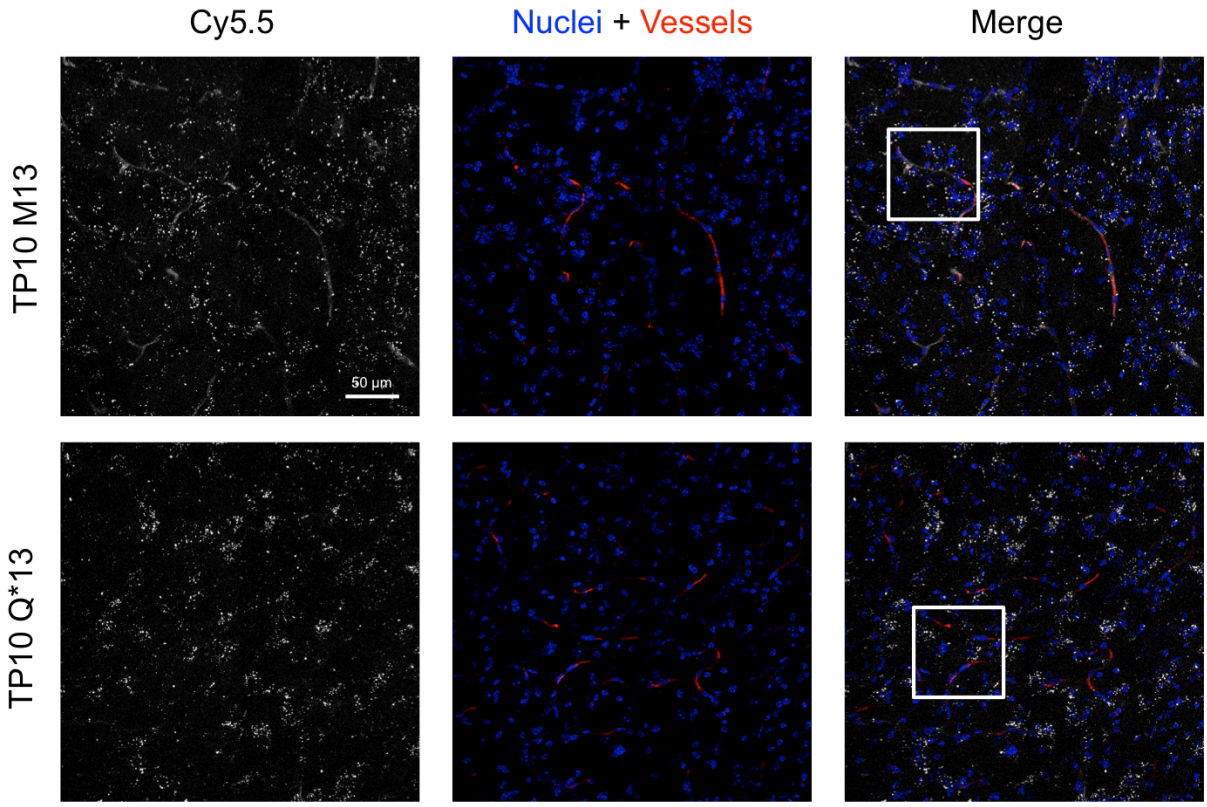


Figure S16. Images of brain slices after treatment with Cy5.5-TP10 M13 (top) and with Cy5.5-TP10 Q*13 (bottom) (nuclei – Hoechst – blue, dextran – TRITC – red, peptide – Cy5.5 – white). The left panel is only the Cy5.5 channel, the middle panel is a merge showing Hoescht for identification of nuclei and tetramethylrhodamine-labeled dextran in the vessels, and the right panel is a merge of all three channels. The square selections in each image are enlarged and shown in the main text in Figure 5B.

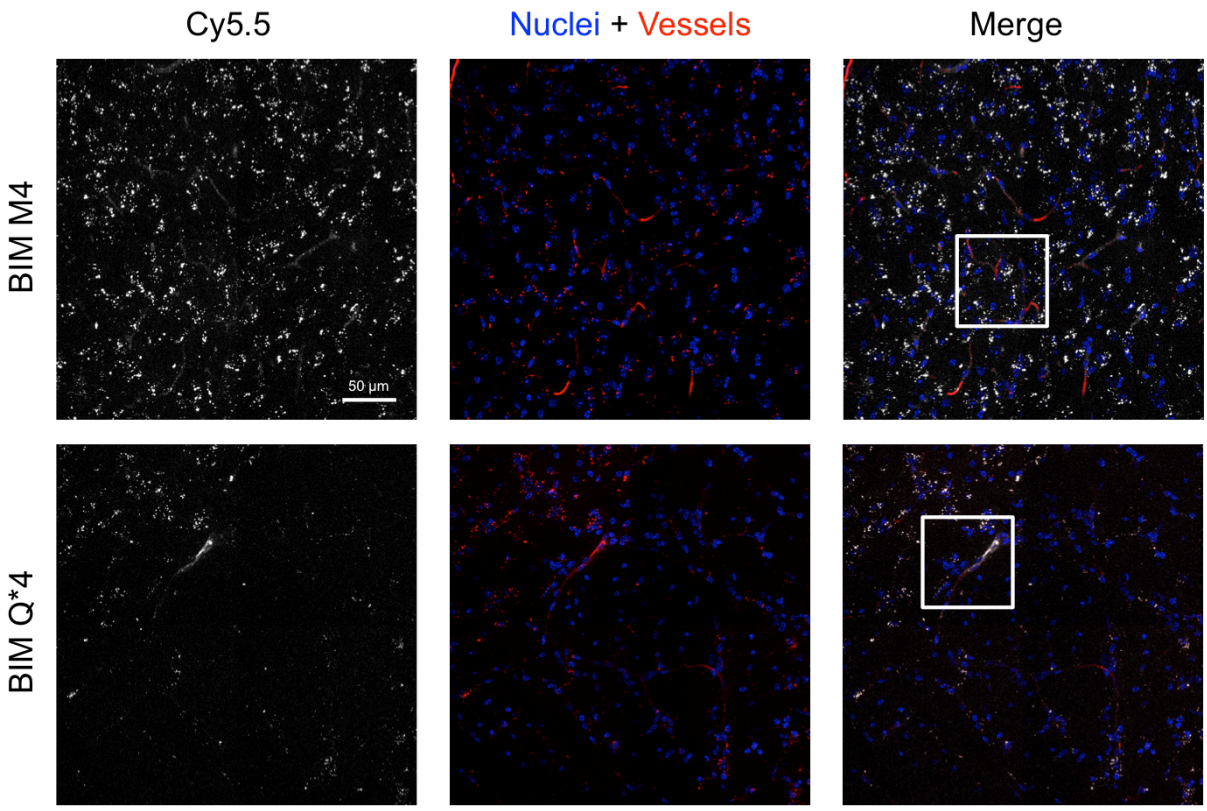


Figure S17. Images of brain slices after treatment with Cy5.5-BIM M4 (top) and with Cy5.5-BIM Q*4 (bottom). The left panel is only the Cy5.5 channel, the middle panel is a merge showing Hoescht for identification of nuclei and tetramethylrhodamine-labeled dextran in the vessels, and the right panel is a merge of all three channels. The square box is the area that has been enlarged and displayed in the main text in Figure 6D.

Imaging of TP10 Constructs after 1 hour at lower concentration

The 2 mM DMSO stocks of each Cy5.5-labeled TP10 analogue were diluted to a final concentration of 50 µM in a solution of 50:50 polyethylene glycol (PEG)-300:0.9% sodium chloride irrigation solution. A 100 µL dose of each 50 µM peptide solution was administered intravenously via the tail vein into healthy 8-week old female nude mice. After 45 min, 100 µL of tetramethylrhodamine isothiocyanate-labeled dextran (155 kDa; 50 mg/mL) was injected via the tail vein. Mice were sacrificed 15 min later by CO_2 asphyxiation and cervical dislocation. Brains were excised and flash frozen in a dry ice/ethanol bath. Cryo-sections (30 µm) of the brains were obtained using a Microm HM 550 cryostat. Brain slices were fixed in 3.7% formaldehyde for 10 min, stained with Hoechst dye (1:1000 in PBS), mounted with a coverslip in VectaShield Antifade mounting media and imaged under a confocal microscope (63x oil-immersion objective). Tile scans (2x2) and z-slices were merged to generate a 2D maximum intensity projection using ZEN imaging software (black edition; 2012 version).

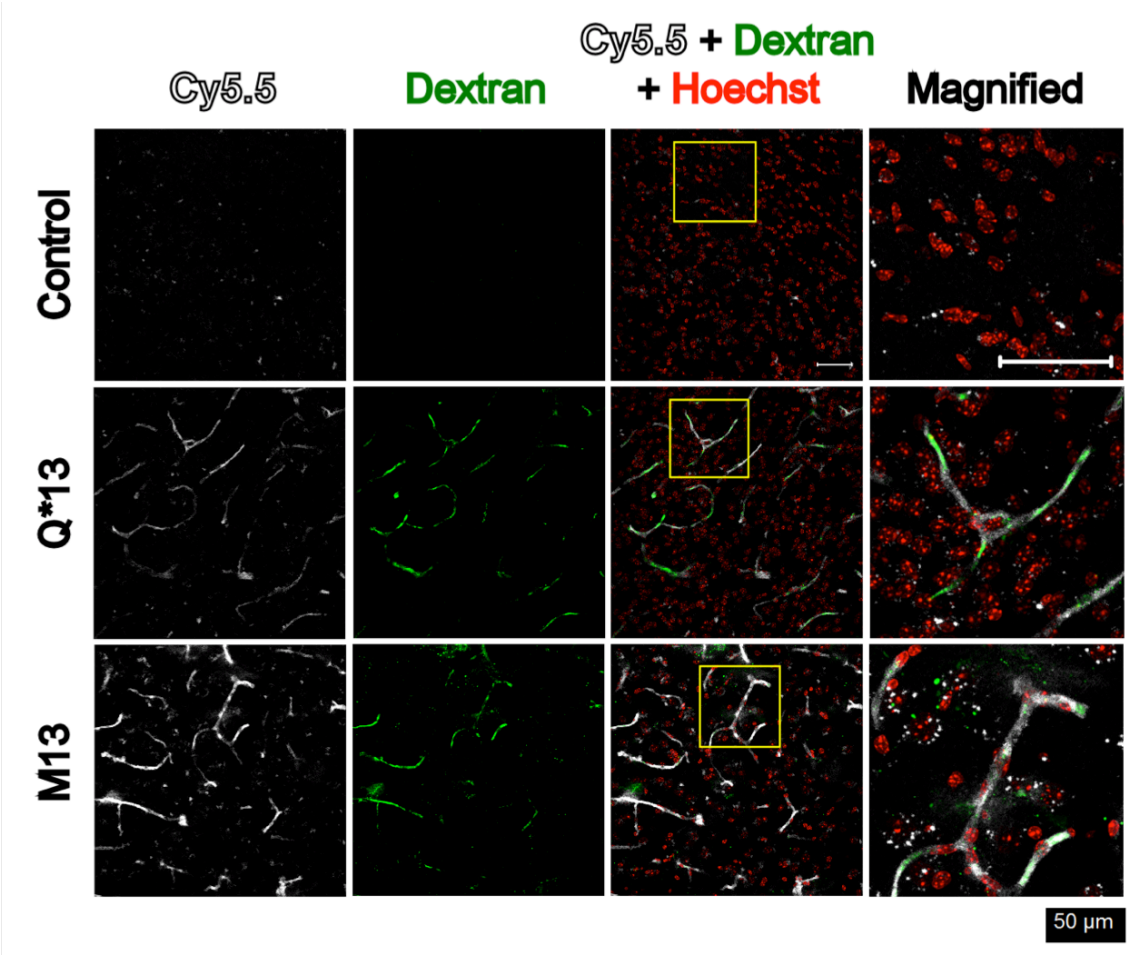


Figure S18. Confocal imaging of frozen sections of mouse brains after treatment for one hour with half the amount of each Cy5.5-labeled TP10 analogue (nuclei – Hoechst – red, dextran – TRITC – green, peptide – Cy5.5 – white). The control mice were not injected with any compound.

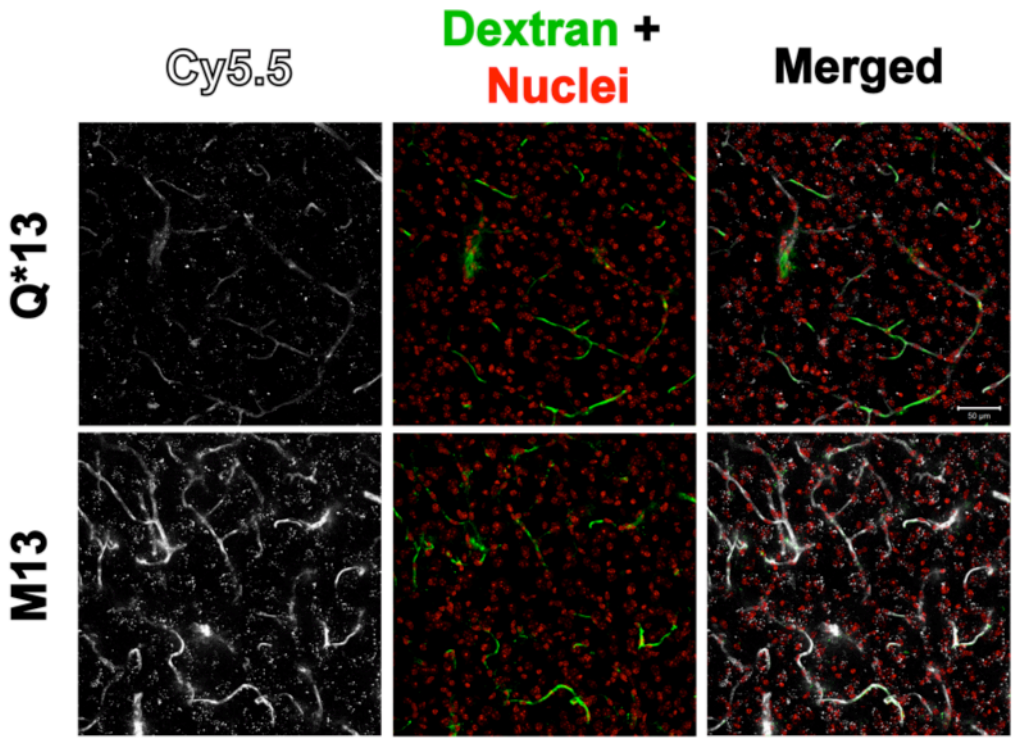


Figure S19. An additional set of confocal images of frozen sections of mouse brains after treatment for one hour with half the amount of each Cy5.5-labeled TP10 analogue (nuclei – Hoechst – red, dextran – TRITC – green, peptide – Cy5.5 – white).

12. References

(1) Mijalis, A. J.; Thomas III, D. A.; Simon, M. D.; Adamo, A.; Beaumont, R.; Jensen, K. F.; Pentelute, B. L. *Nat. Chem. Biol.* **2017**, *13* (5), 464–466.
(2) Kim, Y.-W.; Grossmann, T. N.; Verdine, G. L. *Nat. Protoc.* **2011**, *6* (6), 761–771.