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Development of a fluorescent cardiomyocyte specific binding probe

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ABSTRACT

Cardiomyocytes are the major component of the heart. Their dysfunction or damage could lead to serious cardiovascular diseases, which have claimed numerous lives around the world. A molecule able to recognize cardiomyocytes would have significant value in diagnosis and treatment. Recently a novel peptide termed myocyte targeting peptide (MTP), with three residues of a non-natural amino acid biphenylalanine (Bip), showed good affinity to cardiomyocytes. Its selectivity towards cardiac tissues was concluded to be due to the ability of Bip to bind cardiac troponin I. With the aim of optimizing the affinity and the specificity towards cardiac myocytes and to better understand structure–activity relationship, a library of MTP derivatives was designed. Exploiting a fluorescent tag, the selectivity of the MTP analogs to myocardium over skeletal and stomach muscle tissues was assayed by fluorescence imaging. Among the tested sequences, the peptide probe Bip2, H-Lys(FITC)-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Gly-Ser-Gly-Ser-Bip-Bip-NH_2, displayed the best selectivity for cardiomyocytes.

1. Introduction

Cardiovascular diseases are the leading cause of mortality worldwide and more than 7 million patients die per year due to coronary heart disease alone.[1] In light of these facts, it is important to improve the diagnostic techniques available for early detection of cardiac injuries in order to cure in a timely manner the patients affected.

Cardiomyocytes are the main type of cells in the heart and they are responsible for producing contractile force and controlling the regular beating of the heart.[2] Because of their unique biological function, cardiomyocytes are very different from all other types of cells in the body. Cardiac troponin I (cTnI) is a key component of cardiomyocytes and it is involved in heart contraction. In addition, cTnI is an important protein for the detection of ischemia and/or infarction because it is secreted from cardiomyocytes into the blood stream at an early time point after manifestation.[3,4]

Cardiac troponin I is found on the myofilaments as a complex with cardiac troponin T (cTnT) and cardiac troponin C (cTnC). These three components together, respectively the inhibitory unit, the tropomyosin-binding unit and the calcium-binding unit, are responsible for striated myocardium contraction in a calcium dependent fashion.[5,6] cTnI is expressed only in one isoform, which is typical of myocardium and is not expressed in other types of muscles.[7] The significant difference between troponin isoforms (between cardiac and skeletal troponins, but also between cTnI, cTnT and cTnC) makes it possible to develop probes to selectively target one isoform over the others.[5,8]

Recently we have demonstrated the ability of a peptide called myocyte targeting peptide (MTP) to specifically stain cardiac tissues via cardiac troponin I (cTnI) binding (Fig. 1).[9] MTP is composed of three residues of a non-natural amino acid biphenylalanine (Bip) and can potentially be used as a cardiomyocyte-specific labeling agent for myocyte viability study and tissue mapping. Combining MTP with a small collagen binding probe, CAN35, allowed the tissue damage after myocardial infarction to be analyzed in great detail on histological sections.[10]

Despite the promising preliminary results, some issues were encountered working with the MTP probe. Its poor solubility in aqueous media has made the synthesis and application less convenient. Aiming to improve the workability and binding selectivity of MTP, as well as to elaborate the role of non-natural amino acid Bip, a series of MTP derivatives were designed and tested on cardiac muscle tissues. The structure–activity relationship study reveals that both Bip residues and the appended charged motif are required to obtain a strong selective cardiomyocyte recognition. The lead probe Bip2 with improved structure and solubility properties was able to label cardiomyocytes in tissues.

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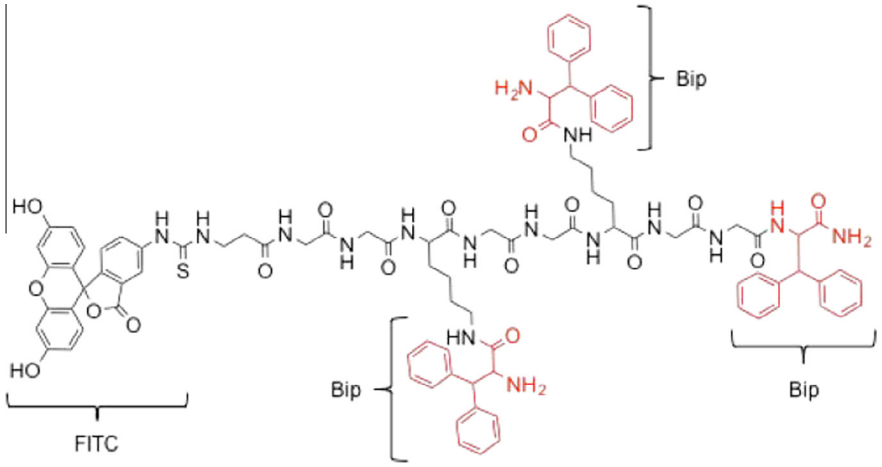


Figure 1. MTP structure (Bip highlighted in red).

2. Material and methods

2.1. General analytical methods for chemical synthesis

NMR spectra were obtained on a Bruker 2014 AvanceHD III 500 MHz spectrometer at 500 MHz for ^{1}H and 126 MHz for ^{13}C spectra. The middle solvent peak for ^{1}H NMR spectra was referenced to 7.26 in deuterated chloroform ($CDCl_3$) and 2.50 in deuterated dimethylsulfoxyde (DMSO-d_6). The middle solvent peak for ^{13}C NMR spectra was referenced to 77.16 in $CDCl_3$ and 39.52 in DMSO-d_6. The coupling constants (J) are in Hz and the chemical shifts (δ) are given in parts per million.

High-performance liquid chromatography (HPLC) was performed on an Agilent Technologies preparative HPLC system. A Grace Vydac 218TP C18 5 μm column was used for analytical HPLC (flow rate 1.0 mL/min), while a GRACE VisionHT High Load C18 5 μm column was used for preparative HPLC (flow rate 8.0 mL/min). The mobile phases used were 0.1% v/v trifluoroacetic acid in water (phase A) and 0.1% v/v trifluoroacetic acid in acetonitrile/water 9:1 (phase B).

Liquid chromatography–mass spectroscopy (LC–MS) analyses were obtained on a Waters Acquity UPLC-H class system operating under electrospray ionization conditions (ESI). The mobile phases used were 0.05% v/v trifluoroacetic acid in water (phase A) and 0.05 v/v% trifluoroacetic acid in acetonitrile (phase B). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses were obtained from the Tufts Medical School, Core facilities, Boston, MA.

Evaporation in vacuo refers to the removal of solvent on a Heidolph rotary evaporator with an integrated vacuum pump. Thin-layer chromatography (TLC) was executed on aluminum backed 60 F254 silica gel. When necessary, dry solvents were obtained from a Pure Process Technology solvent purification system.

2.2. Materials for chemical synthesis

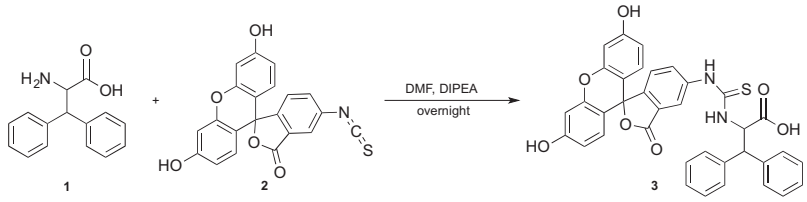
1,2-Ethandithiol (EDT), 3,3,3-triphenylpropionic acid (Trip), acetonitrile (MeCN), anisole, Boc-L-glutamic acid, diisopropylethylamine (DIPEA), dimethylsulfoxide (DMSO), fluorescein isothiocyanate isomer I (FITC), hydrazine, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*,*N*′-dimethylaminopyridine (DMAP), piperidine, thioanisole (TA), oxalyl chloride, triisopropylsilane (TIS), trifluoroacetic acid (TFA) and sodium sulfate anhydrous ($NaSO_4$) were purchased from Sigma Aldrich (St. Louis, MO, USA); dichloromethane (DCM), ethylacetate (EtOAc), hexane, methanol (MeOH), methyl *tert*-butyl ether (MTBE), *N*,*N*′-dimethylformamide (DMF) and sodium chloride were supplied by VWR (Radnor, PA, USA); 2,2-biphenylethylamine, Fmoc-3,3-L-diphenylalanine (Fmoc-Bip) and Fmoc-L-lysine-OH were obtained from Chem Impex International (Wood Dale, IL, USA); 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 3,3-diphenyl-L-alanine (Bip), 6-(Fmoc-amino)hexanoic acid and *N*-hydroxysuccinimide (NHS) were purchased from Alfa Aesar (Ward Hill, MA, USA), Fmoc-*N*-amido-dPEG®4-acid from Quanta Biodesign (Plain City, OH, USA) and acetic acid from Amresco (Solon, OH, USA). All Fmoc protected amino acids for peptide synthesis, (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-methylmorpholine (NMM) were supplied by Protein Technologies (Tuscon, AZ, USA).

2.3. Synthetic procedure

2.3.1. General procedure for peptide synthesis

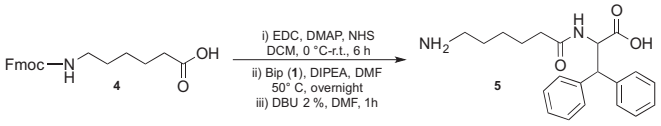
Solid phase peptide synthesis (SPPS) was performed on an automatic synthesizer (PS3, Protein Technologies, Tuscon, AZ, USA) using standard Fmoc chemistry. Rink amide-MBHA LL resin (Novabiochem, Billerica, MA, USA) with a loading capacity of 0.38 mmol/g was employed to afford peptide amides. Fmoc-amino acids (4 equiv) were coupled on the resin (0.1 mmol) using HBTU (4 equiv) and the base NMM. Fmoc deprotection was achieved by exposure to 20% piperidine in DMF. FITC (6 equiv) was dissolved in DMSO anhydrous (4 mL) and it was added, together with 1 mL of DIPEA, to the resin-bound peptide. The reaction was agitated in darkness overnight to achieve the fluorescent labeling on the N-terminal beta alanine (β-Ala). If the labeling was executed on the side chain of a lysine instead, the (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) protecting group was removed before FITC grafting with hydrazine 2% in DMF (10 mL) for 30 min. In the latter case an extra glycine was added at the N-terminus, which was removed spontaneously through Edman degradation during the subsequent TFA cleavage. The final FITC labeled peptide sequence was then cleaved from the resin with cleaving cocktail A (95% TFA, 2.5% TIS, 2.5% H_2O, 5 mL for 4 h) or cleaving cocktail B (90% TFA, 5% TA, 3% EDT, 2% anisole, 5 mL for 3 h) and precipitated from MTBE. The precipitate was centrifuged, washed further with MTBE and dried in vacuo. The crude, fully deprotected FITC labeled peptides were purified by HPLC (220 nm and 460 nm). The major peak was analyzed by MALDI-TOF in the case of peptides over 1700 Da and by LC–MS for all the other compounds.

2.3.2. Synthesis of 2-(3-(3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthen]-5-yl)thioureido)-3,3-diphenylpropanoic acid (BipAA, 3)



3,3-Diphenyl-L-alanine (**1**, 50.0 mg, 0.21 mmol) was dissolved in DMF anhydrous (8 mL) and FITC (**2**, 160.0 mg, 0.41 mmol) was added together with DIPEA (358 μL, 2.07 mmol). The solution was left stirring in darkness overnight. The solvent was removed in vacuo, the crude product was redissolved in EtOAc and acetic acid 5% was mixed. The aqueous layer was extracted with EtOAc (3 × 20 mL), the organic layers were combined, washed with brine, dried over $NaSO_4$ and concentrated in vacuo. The crude product was purified via prep HPLC from 30% to 100% of phase B over 45 min to achieve **3** as a yellow solid (102 mg, 77% yield). Retention time analytical HPLC from 30% to 100% of phase B over 30 min: 14.183 min. NMR, δ (500 MHz, DMSO), 10.10 (2H, s, OH FITC), 8.26 (1H, s, CH FITC), 8.07 (1H, d, J = 8.4 Hz, NHCO Bip), 7.54 (1H, d, J = 8.3 Hz, CH FITC), 7.39–7.18 (10H, m, Ph Bip), 7.09 (1H, d, J = 8.3 Hz, CH FITC), 6.66 (2H, s, 2CH FITC), 6.59–6.53 (4H, m, CH FITC), 5.78 (1H, t, J = 9.2 Hz, CHNH Bip), 4.54 (1H, d, J = 9.9 Hz, $CHPh_2$ Bip). ^{13}C NMR δ (126 MHz, DMSO) 180.51 (CS FITC), 172.08, 168.33, 159.45, 151.84 and 147.27 (FITC), 140.91, 140.81, 140.68, 129.15, 129.04, 128.46, 128.36, 128.33, 128.22, 126.77 and 126.70 (Ph Bip and FITC), 126.50, 124.05, 116.22, 112.52, 109.61 and 102.16 (FITC), 59.75 (*C*HNH Bip), 52.78 ($CHPh_2$ Bip). ESI-MS m/z, $M+H^+$ = 631.1, calculated for $C_{36}H_{26}N_2O_7S$ = 630.2.

2.3.3. Synthesis of 2-(6-aminohexanamido)-3,3-diphenylpropanoic acid (5)



6-(Fmoc-amino)hexanoic acid (**4**, 50 mg, 0.14 mmol), EDC (35 mg, 0.18 mmol) and DMAP (2 mg, 0.015 mmol) were dissolved in DCM anhydrous (4 mL). The temperature was brought to 0 °C and NHS (21 mg, 0.18 mmol) was added. The solution was allowed to reach room temperature and it was left stirring for 6 h. Water was subsequently mixed to the solution. The aqueous layer was extracted with DCM (4 × 10 mL), the organic layers were combined, washed with brine, dried over $NaSO_4$ and concentrated in vacuo to afford the product as a white powder. The crude product was dissolved in DMF anhydrous (10 mL) with 3,3-diphenyl-L-alanine (**1**, 44 mg, 0.18 mmol) and DIPEA (73 μL, 0.42 mmol) was added to the solution. The temperature was brought to 50 °C and the reaction was left under stirring overnight. The white precipitate formed was filtered off, the solvent was removed in vacuo, the crude product was redissolved in DCM and water was mixed. The aqueous layer was extracted with DCM (4 × 20 mL), the organic layers were combined, washed with brine, dried over $NaSO_4$ and the solvent was removed in vacuo. The collected white powder was combined and dissolved in DBU 2% in DMF (5 mL). After 1 h the solvent was removed in vacuo, the crude product was redissolved in water and DCM was added. The aqueous layer was extracted with DCM (3 × 10 mL), neutralized and lyophilized. The crude product was purified by prep HPLC from 0% to 100% of phase B over 45 min to achieve the pure product **5** as a white powder (20 mg, 40% yield over three steps). Retention time analytical HPLC from 0% to 100% of phase B over 30 min: 13.861 min. R_f = 0.91 in EtOAc/MeOH 8:2. NMR, δ (500 MHz, DMSO), 12.46 (1H, br s, COOH), 8.25 (1H, d, J = 9 Hz, NHCO Bip), 7.76 (2H, br s, NH_2), 7.32–7.14 (10H, m, Ph Bip), 5.19 (1H, dd, J = 9 Hz and 11.7 Hz CHNH Bip), 4.30 (1H, d, J = 11.7 Hz, $CHPh_2$ Bip), 2.73–2.62 (2H, m, CH_2NH), 1.98–1.87 (2H, m, CH_2CO), 1.43–1.34 (2H, m, CH_2CH_2NH), 1.28–1.18 (2H, m, CH_2CH_2CO), 1.03–0.94 (2H, m, $CH_2CH_2CH_2CO$). ^{13}C NMR δ (126 MHz, DMSO) 172.16 (COOH), 171.39 (CONH), 141.09, 141.06, 128.20, 127.97, 126.47, 126.17, (Ph Bip), 54.66 (CHNH Bip), 52.90 ($CHPh_2$ Bip), 38.42 (CH_2NH), 34.58 (CH_2CO), 26.53 (CH_2CH_2NH), 24.89 ($CH_2CH_2CH_2CO$), 24.42 (CH_2CH_2CO). ESI-MS m/z, $M+H^+$ = 355.3, calculated for $C_{21}H_{26}N_2O_3$ = 354.2.

2.3.4. Synthesis of 2-(6-(3-(3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthen]-5-yl)thioureido)hexanamido)-3,3-diphenylpropanoic acid (BipHA, 6)

2-(6-Aminohexanamido)-3,3-diphenylpropanoic acid (**5**, 19 mg, 0.054 mmol) was dissolved in DMF anhydrous (5 mL) and FITC (44 mg, 0.113 mmol) was added together with DIPEA (97 μL, 0.56 mmol). The solution was left stirring in darkness overnight. The solvent was removed in vacuo, the crude product was redissolved in EtOAc and acetic acid 5% was mixed. The aqueous layer was extracted with EtOAc (3 × 10 mL), the organic layers were combined, washed with brine, dried over $NaSO_4$ and the solvent was removed in vacuo. The crude product was purified via prep HPLC from 20% to 100% of phase B over 45 min to achieve **6** as a yellow solid (24 mg, 60% yield). Retention time analytical HPLC from 20% to 100% of phase B over 30 min: 16.217 min. NMR, δ (500 MHz, DMSO), 10.11 (1H, br s, OH FITC), 9.84 (1H, br s, OH FITC), 8.22 (1H, s, CH FITC), 8.21 (1H, d, J = 9.1, NHCO Bip), 8.00 (1H, br s, NH FITC), 7.71 (1H, br s, CH FITC), 7.32–7.15 (11H, m, 10 CH Ph Bip and 1 CH FITC), 6.67 (2H, d, J = 2.3 Hz, 2CH FITC), 6.61–6.55 (4H, m, 4 CH FITC), 5.19 (1H, dd, J = 9.0 Hz and 11.6 Hz, *C*HNH Bip), 4.29 (1H, d, J = 11.7 Hz, *C*HPh_2 Bip), 3.38 (2H,

br s, CH_2NH), 1.99–1.89 (2H, m, CH_2CO), 1.48–1.38 (2H, m, C$H_2$$CH_2$-NH), 1.31–1.25 (2H, m, C$H_2$$CH_2$CO), 1.05–0.98 (2H, m, C$H_2$$CH_2$$CH_2$-CO). ^{13}C NMR δ (126 MHz, DMSO) 180.44 (CS FITC), 172.37, 171.65, 168.51, 159.46, 151.84 and 146.95 (FITC), 141.37, 141.28 and 141.17, 129.01, 128.35, 128.19, 128.11, 126.60 and 126.37 (Ph Bip and FITC), 124.10, 116.69, 114.28, 112.55, 109.70 and 102.19 (FITC), 54.80 (CHNH Bip), 53.03 ($CHPh_2$ Bip), 43.70 (CH_2NH), 34.93 (CH_2CO), 28.03 ($CH_2$$CH_2$NH), 25.69 ($CH_2$$CH_2$$CH_2$CO), 24.90 ($CH_2$$CH_2$CO). ESI-MS m/z, M+H$^+$ = 744.3, calculated for $C_{42}H_{37}N_3O_8S$ = 743.2.

2.3.5. Synthesis of sBip2

Fmoc-Bip-OH, Fmoc-Bip-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-β-Ala-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the N-terminal beta alanine. The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail A, purified with prep HPLC from 10% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 10% to 100% of phase B over 30 min: 17.257 min. ESI-MS m/z, M+H$^+$ = 1155.3, calculated for $C_{62}H_{58}N_8O_{13}S$ = 1154.4.

2.3.6. Synthesis of sPheBip

Fmoc-Bip-OH, Fmoc-Phe-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-β-Ala-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the N-terminal beta alanine. The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail A and purified with prep HPLC from 10% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 10% to 100% of phase B over 30 min: 16.543 min. ESI-MS m/z, M+H$^+$ = 1079.7, calculated for $C_{56}H_{54}N_8O_{13}S$ = 1078.4.

2.3.7. Synthesis of sGlyBip

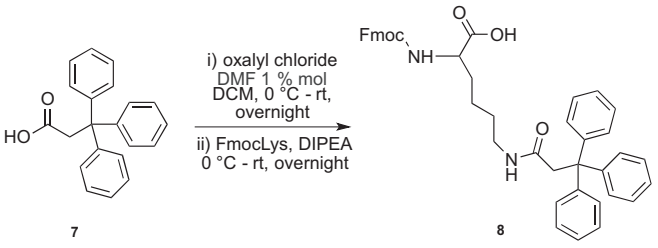
Fmoc-Bip-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-β-Ala-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the N-terminal beta alanine. The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail A and purified with prep HPLC from 10% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 10% to 100% of phase B over 30 min: 14.323 min. ESI-MS m/z, M+H$^+$ = 989.7, calculated for $C_{49}H_{48}N_8O_{13}S$ = 988.3.

2.3.8. Synthesis of sPhePhe

Fmoc-Phe-OH, Fmoc-Phe-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-β-Ala-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the N-terminal beta alanine. The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail A and purified with prep HPLC from 10% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 10% to 100% of phase B over 30 min: 14.670 min. ESI-MS m/z, M+H$^+$ = 1003.3, calculated for $C_{50}H_{50}N_8O_{13}S$ = 1002.3.

2.3.9. Synthesis of N^2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-N^6-(3,3,3-triphenylpropanoyl)lysine (Fmoc-Lys(Trip)-OH, 8)

3,3,3-Triphenylpropionic acid (**7**, 150 mg, 0.50 mmol) was dissolved in DCM anhydrous (10 mL) and oxalyl chloride (1.50 mmol, 275 μL) was added at 0 °C. The solution was allowed to reach room temperature and stirred overnight. The solvent was removed in vacuo to afford a yellow solid. The crude product was dissolved in DCM anhydrous (15 mL) and Fmoc-Lys-OH (0.22 g, 0.6 mmol) and DIPEA (1.04 mmol, 180 μL) were added at 0 °C. The reaction was stirred overnight at room temperature. The solvent was removed in vacuo, the crude product was redissolved in DCM and water was mixed. The aqueous layer was extracted with DCM (4 × 20 mL), the organic layers were combined, washed with acetic acid 5% and brine, dried over $NaSO_4$ and the solvent was removed in vacuo. The crude product obtained was dissolved in the minimum quantity of EtOAc and precipitated with hexane. The product was reprecipitated in EtOAc/hexane to give product **8** as a white solid (83 mg, 25% yield). R_f = 0.44 in EtOAc/MeOH 8:2; NMR, δ (500 MHz, $CDCl_3$), 7.79 (2H, d, J = 7.5, Fmoc), 7.62 (2H, t, J = 6.6, Fmoc), 7.42 (2H, t, J = 7.5, Fmoc), 7.34–7.24 (17H, m, Ph and Fmoc), 5.55 (1H, d, J = 8.1, NH Fmoc), 5.23 (1H, br s, N*H*CH_2CPh_3), 4.49–4.39 (2H, m, CH_2 Fmoc), 4.32–4.30 (1H, m, CH Lys), 4.23 (1H, t, J = 6.7 Hz, CH Fmoc), 3.67 (2H, s, CH_2CPh_3), 3.00–2.91 (2H, m, CH_2NH Lys), 1.80–1.73 and 1.64–1.57 (2H, m, CHCH_2 Lys), 1.15–0.98 (4H, m, $2CH_2$ Lys). ^{13}C NMR δ (126 MHz, $CDCl_3$), 175.85 (COOH), 172.85 (CONH), 156.49 (OCONH), 145.77, 143.83, 143.66, 141.41, 129.20, 129.16, 129.12, 128.48, 128.45, 128.41, 127.89, 127.22, 126.89, 125.15, 125.11 and 120.13 (Ph Trip and Fmoc), 67.29 (CH_2 Fmoc), 56.06, 53.59 (CH Lys), 48.12 (CH_2-CPh_3), 47.18 (CH Fmoc), 39.47 (CH_2NH Lys), 31.71 ($CHCH_2$ Lys), 28.15 and 22.25 ($2CH_2$ Lys). ESI-MS m/z, M+H$^+$ = 653.4, calculated for $C_{42}H_{40}N_2O_5$ = 652.3.

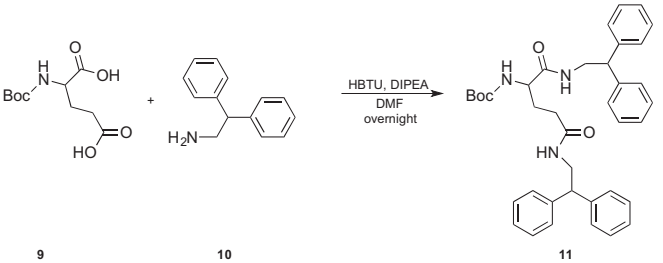


7 8

2.3.10. Synthesis of sTrip

Fmoc-Lys(Trip)-OH (**8**), Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-β-Ala-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the N-terminal beta alanine. The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail A and purified with prep HPLC from 10% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 0% to 100% of phase B over 30 min: 19.263 min. ESI-MS m/z, M+H$^+$ = 1121.6, calculated for $C_{59}H_{60}N_8O_{13}S$ = 1120.4.

2.3.11. Synthesis of *tert*-butyl (1,5-bis((2,2-diphenylethyl) amino)-1,5-dioxopentan-2-yl)carbamate (11)

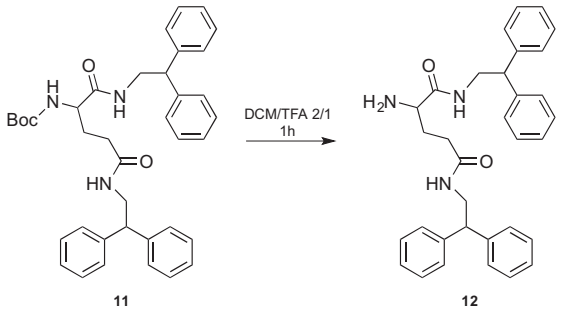


9 10 11

Boc-Glu-OH (**9**, 100 mg, 0.40 mmol) was dissolved in DMF anhydrous (10 mL) with HBTU (0.32 g, 0.85 mmol). 2,2-

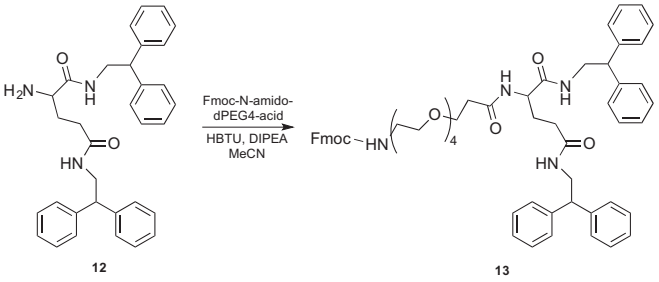
Biphenylethylamine (**10**, 0.17 g, 0.85 mmol) was added followed by DIPEA (207 μL, 1.20 mmol). The reaction was left stirring overnight. The solvent was removed in vacuo, the crude product was redissolved in EtOAc and acetic acid 5% was mixed. The aqueous layer was extracted with EtOAc (4 × 20 mL), the organic layers were combined, washed with brine, dried over $NaSO_4$ and the solvent was removed in vacuo. The crude product was redissolved in the minimum quantity of EtOAc and precipitated with hexane. The product was then reprecipitated using DCM/hexane to achieve **11** as a white solid (182 mg, 75% yield). R_f = 0.83 in EtOAc 100%; ^{1}H NMR, δ (500 MHz, $CDCl_3$) 7.31–7.16 (20H, m, Ph), 6.21 (1H, br s, NHCO), 5.89 (1H, br s, NHCO), 5.45 (1H, br s, NH Boc), 4.20–4.13 (2H, 2t, J = 8.05 Hz and J = 8.05 Hz, $2CHPh_2$), 3.99–3.87 (2H, m, CH_2-$CHPh_2$), 3.77–3.69 (3H, m, CH_2CHPh_2 and CH Glu), 2.06–1.89 and 1.88–1.80 (2H, m, CH_2CO Glu), 1.77–1.68 (2H, m, $CHCH_2$ Glu), 1.40 (9H, s, $C(CH_3)$). ^{13}C NMR δ (126 MHz, $CDCl_3$) 172.50 (CONH), 171.38 (CONH), 155.78 (OCONH), 141.95, 141.90, 141.84, 141.80, 128.78, 128.72, 128.15, 128.09 and 126.89 (Ph), 79.95 ($C(CH_3)_3$), 53.53 (CH Glu), 50.65 ($CHPh_2$), 50.52 ($CHPh_2$), 43.88 (CH_2CHPh_2), 43.76 (CH_2CHPh_2), 32.25 (CH_2CO Glu), 29.49 ($CHCH_2$ Glu), 28.35 ($C(CH_3)$). ESI-MS m/z, M+H$^+$ = 606.5, calculated for $C_{38}H_{43}N_3O_4$ = 605.3.

2.3.12. Synthesis of 2-amino-N^1,N^5-bis(2,2-diphenylethyl) pentanediamide (12)



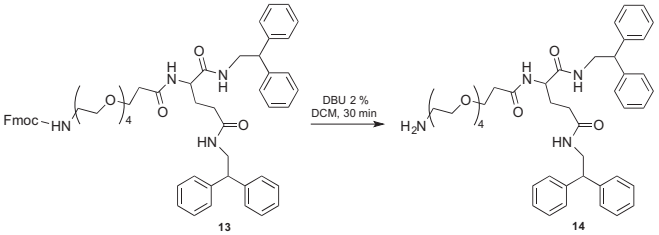
11 (150 mg, 0.25 mmol) was dissolved in DCM/TFA 2:1 (9 mL) and stirred for 1 h. The solvent was evaporated under compressed air, redissolved in DCM and water was mixed. The aqueous layer was extracted with DCM (4 × 20 mL), the organic layers were combined, washed with brine, dried over $NaSO_4$ and the solvent was removed in vacuo. The crude product was redissolved in the minimum quantity of DCM and precipitated with hexane. The product was reprecipitated with DCM/hexane to give product **12** as a white solid (106 mg, 84% yield). ^{1}H NMR, δ (500 MHz, $CDCl_3$) 8.21 (1H, br s, NHCO), 7.20–6.85 (20H, m, Ph), 6.05 (1H, br s, NHCO), 4.17 (1H, t, J = 7.8 Hz, $CHPh_2$), 4.05 (1H, t, J = 8.1 Hz, $CHPh_2$), 4.01–3.97 (1H, m, CH_2CHPh_2), 3.74–3.69 (2H, m, CH Glu and CH_2CHPh_2), 3.59–3.53, (1H, m, CH_2CHPh_2), 3.42–3.38 (1H, m, CH_2CHPh_2), 1.85–1.81 (1H, m, CH_2CO Glu), 1.68–1.65 (1H, m, CH_2CO Glu), 1.61–1.57 (2H, m, $CHCH_2$ Glu). ^{13}C NMR δ (126 MHz, $CDCl_3$) 172.67 (CONH), 168.24 (CONH), 142.04, 141.92, 141.88, 128.85, 128.82, 128.71, 128.63, 128.41, 128.25, 128.14, 128.10, 126.97, 126.77, 126.67 (Ph), 52.58 (CH Glu), 50.38 ($2CHPh_2$), 44.05 (CH_2CHPh_2), 43.93 (CH_2CHPh_2), 31.04 ($CHCH_2$ Glu), 27.02 (CH_2CO Glu). ESI-MS m/z, M +H$^+$ = 506.5, calculated for $C_{33}H_{35}N_3O_2$ = 505.3.

2.3.13. Synthesis of (9*H*-fluoren-9-yl)methyl (17-((2,2-diphenylethyl)carbamoyl)-15,20-dioxo-23,23-diphenyl-3,6,9,12-tetraoxa-16,21-diazatricosyl)carbamate (13)



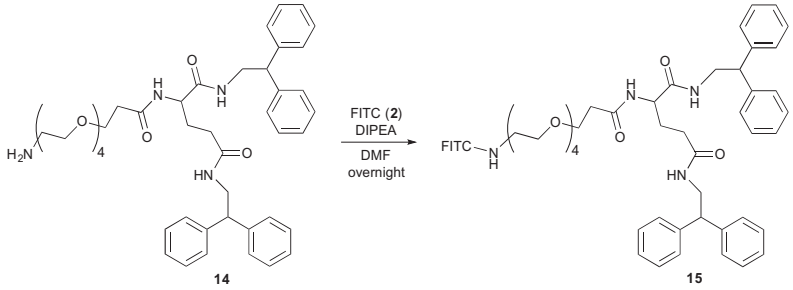
12 (200 mg, 0.40 mmol) was added to a solution of Fmoc-*N*-amido-dPEG®4-acid (220 mg, 0.44 mmol) and HBTU (156 mg, 0.41 mmol) in MeCN anhydrous (10 mL). DIPEA (208 μL, 1.2 mmol) was added and the solution was left stirring overnight. The solvent was removed in vacuo, the crude product was redissolved in EtOAc and water was mixed. The aqueous layer was extracted with EtOAc (3 × 20 mL), the organic layers were combined, washed with acetic acid 5% and brine, dried over $NaSO_4$ and the solvent was removed in vacuo. The crude product was purified by silica gel column chromatography (EtOAc/MeOH from 100:0 to 80:20) to give the pure product **13** as a yellow solid (0.2 g, 51% yield). R_f = 0.68 in EtOAc 100%; ^{1}H NMR, δ (500 MHz, $CDCl_3$), 7.75 (2H, d, J = 7.4 Hz, Fmoc), 7.60 (2H, d, J = 7.1 Hz, Fmoc), 7.38 (2H, t, J = 7.5 Hz, Fmoc), 7.30 (2H, t, J = 7.3 Hz, Fmoc), 7.29–7.14 (21H, m, Ph and CHN*H*CO), 6.39 (1H, br s, N*H*CH_2CHPh_2), 6.26 (1H, br s, N*H*CH_2CHPh_2), 5.67 (1H, br s, NHCO Fmoc), 4.36 (2H, d, J = 6.7 Hz, CH_2 Fmoc), 4.19–4-12 (3H, m, CH Fmoc and $2CHPh_2$), 4.05–3.98 (1H, m, CH Glu), 3.95–3.81 (2H, m, CH_2CHPh_2), 3.78–3.67 (2H, m, CH_2CHPh_2), 3.62–3.52 (16H, m, PEG), 3.36 (2H, br s, NH_2CH_2 PEG), 2.36 (2H, br s, $COCH_2$ PEG), 2.01–1.95 (1H, m, CH_2CO Glu), 1.91–1.86 (1H, m, CH_2CO Glu), 1.79–1.68 (2H, m, $CHCH_2$ Glu). ^{13}C NMR δ (126 MHz, $CDCl_3$) 172.81 (CONH), 171.88 (CONH), 171.25 (CONH), 156.83 (OCONH), 144.17, 142.20, 142.12, 142.03, 141.93, 141.46, 128.83, 128.78, 128.30, 128.26, 128.21, 127.82, 127.21, 126.92,126.85, 125.29 and 120.11 (Ph and Fmoc), 70.60, 70.56, 70.45, 70.35 and 67.26 (PEG), 66.76 ($FmocCH_2$), 52.56 (CH Glu), 50.69 ($CHPh_2$), 50.66 ($CHPh_2$), 47.40 (CH Fmoc), 43.97 ($2CH_2$-$CHPh_2$), 41.03 (NH_2CH_2 PEG), 36.95 ($COCH_2$ PEG), 32.47 (CH_2CO Glu), 28.99 ($CHCH_2$ Glu). ESI-MS m/z, M+H$^+$ = 975.7, calculated for $C_{59}H_{66}N_4O_9$ = 974.5.

2.3.14. Synthesis of 2-(1-amino-3,6,9,12-tetraoxapentadecan-15-amido)-N^1,N^5-bis(2,2-diphenylethyl)pentanediamide (14)



13 (170 mg, 0.174 mmol) was dissolved in DCM and 2% v/v of DBU was added. The reaction was stirred for 30 min and then water was mixed. The aqueous layer was extracted with DCM (3 × 20 mL), the organic layers were combined, washed with acetic acid 5% and the solvent was removed in vacuo. The product was purified by prep HPLC from 50% to 100% of phase B over 45 min, to achieve product **14** as a clear solid (94 mg, 72% yield). Retention time analytical HPLC from 30% to 100% of phase B over 30 min: 19.295. ^{1}H NMR, δ (500 MHz, $CDCl_3$), 8.12 (2H, br s, NH_2), 7.94 (1H, d, J = 6.8 Hz, CHN*H*CO), 7.34–7.14 (21H, m, Ph and N*H*CH_2-$CHPh_2$), 6.24 (1H, t, J = 5.5 Hz, N*H*CH_2CHPh_2), 4.26 (1H, t, J = 8.1 Hz, $CHPh_2$), 4.22–4.14 (2H, m, $CHPh_2$ and CH Glu), 4.00–3.95 (1H, m, C*H*$_2CHPh_2$), 3.81 (2H, t, J = 6.5 Hz, C*H*$_2CHPh_2$), 3.76–3.73 (17H, m, C*H*$_2CHPh_2$ and 16 PEG), 3.12 (2H, br s, NH_2C*H*$_2$ PEG), 2.51–2.38 (2H, m, $COCH_2$ PEG), 1.93–1.83 (2H, m, CH_2CO Glu), 1.78–1.72 (2H, m, CHC*H*$_2$ Glu). ^{13}C NMR δ (126 MHz, $CDCl_3$) 173.23, 172.25 and 171.68 (3 CONH), 142.16, 128.82, 128.76, 128.73, 128.29, 128.25, 128.21, 126.90 and 126.83 (Ph), 70.39, 70.28, 70.11, 69.97, 69.83 and 67.14 (PEG), 53.33 (CH Glu), 50.57 (2$CHPh_2$), 44.06 (C*H*$_2CHPh_2$), 43.95 (C*H*$_2CHPh_2$), 39.92 (NH_2CH_2 PEG), 35.78 ($COCH_2$ PEG), 32.38 (CH_2CO Glu), 28.57 (CH*C*H_2 Glu). ESI-MS m/z, M+H$^+$ = 753.7, calculated for $C_{44}H_{56}N_4O_7$ = 752.4.

2.3.15. Synthesis of 2-(1-((3′,6′-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9′-xanthen]-5-yl)amino)-1-thioxo-5,8,11,14-tetraoxa-2-azaheptadecan-17-amido)-*N*^{1},*N*^{5}-bis(2,2-diphenylethyl)pentanediamide (PEG4Bip2, 15)



14 (94 mg, 0.12 mmol) was dissolved in DMF (5 mL) and FITC was added (0.140 g, 0.36 mmol), followed by DIPEA (1 mL). The reaction was left stirring in darkness overnight. The solvent was removed in vacuo, the crude product was redissolved in DCM and brine was mixed. The aqueous layer was extracted with DCM (5 × 20 mL), the organic layers were combined, dried over $NaSO_4$ and the solvent was removed in vacuo. The crude product was purified by prep HPLC from 30% to 100% of phase B over 45 min to achieve product **15** as a yellow solid (75 mg, 55% yield). Retention time analytical HPLC from 30% to 100% of phase B over 30 min: 17.923 min. ^{1}H NMR, δ (500 MHz, DMSO), 10.14 (1H, br s, OH FITC), 10.05 (1H, br. s, OH FITC), 8.30 (1H, br s, FITC), 8.11 (1H, br s, N*H*CH_2CHPh_2), 7.90 (1H, d, J = 8.1 Hz, CHN*H*CO), 7.85 (1H, t, J = 5.6 Hz, N*H*CH_2CHPh_2), 7.76–7.71 (2H, m, FITC and NH PEG), 7.31–7.15 (22H, m, Ph, NH FITC and FITC), 6.70 (2H, d, J = 2.3 Hz, FITC), 6.63 (2H, d, J = 8.7 Hz, FITC), 6.59–6.57 (2H, dd, J = 2.3 and 8.7 Hz, FITC), 4.19–4.15 (2H, dd, J = 7.7 and 15.1 Hz, 2$CHPh_2$), 4.13–4.08 (1H, m, CH Glu), 3.78–3.46 (22H, m, 2C*H*$_2$-$CHPh_2$ and PEG), 2.40–2.27 (2H, m, $COCH_2$ PEG), 1.84–1.81 (2H, m, CH_2CO Glu), 1.66–1.61 (1H, m, CHC*H*$_2$ Glu), 1.51–1.49 (1H, m, CHC*H*$_2$ Glu). ^{13}C NMR δ (126 MHz, DMSO) 180.54 (CS FITC), 171.56, 171.20, 169.88, 168.52, 159.50, 151.89 and 147.10 (FITC), 142.93, 142.71, 141.34, 129.04, 128.39, 128.36, 127.85 and 126.29 (Ph and FITC), 124.06, 116.35, 112.59, 109.72 and 102.24 (FITC), 69.75, 69.65, 69.45, 68.43 and 66.73 (PEG), 51.99 (CH Glu), 50.07 ($CHPh_2$), 49.96 ($CHPh_2$), 43.70 (CH_2CHPh_2), 43.11 (CH_2-$CHPh_2$), 35.79 ($COCH_2$ PEG), 31.53 (CH_2CO Glu), 28.22 (CHCH_2 Glu). ESI-MS m/z, M+H$^+$ = 1142.9, calculated for $C_{65}H_{67}N_5O_{12}S$ = 1141.5.

2.3.16. Synthesis of MTP

Fmoc-Bip-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Lys(ivDde)-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Lys(ivDde)-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, and Fmoc-β-Ala-OH were coupled sequentially to the solid support, Fmoc-Bip-OH was then coupled to the free ε-amino group of the Lys residues and FITC was grafted to the peptide on the N-terminal beta alanine as described in previous work.[9] The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail A and purified with prep HPLC from 0% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 0% to 100% of phase B over 30 min: 18.143 min. MALDI-TOF m/z, M+H$^+$ = 1745.5, calculated for $C_{93}H_{100}N_{16}O_{17}S$ = 1744.7.

2.3.17. Synthesis of Bip1

Fmoc-Bip-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(ivDde)-OH, Fmoc-Gly-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the ε-amine of lysine. The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail B and purified with prep HPLC from 0% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 0% to 100% of phase B over 30 min: 13.823 min. MALDI-TOF m/z, M+H$^+$ = 2139.2, calculated for $C_{94}H_{139}N_{37}O_{20}S$ = 2138.1.

2.3.18. Synthesis of Bip2

Fmoc-Bip-OH, Fmoc-Bip-OH, Fmoc-Ser(Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(ivDde)-OH, Fmoc-Gly-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the ε-amine of lysine. The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail B and purified with prep HPLC from 0% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 0% to 100% of phase B over 30 min: 16.063 min. MALDI-TOF m/z, M+H$^+$ = 2362.2, calculated for $C_{109}H_{152}N_{38}O_{21}S$ = 2361.2.

2.3.19. Synthesis of RRsBip2

Fmoc-Bip-OH, Fmoc-Bip-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(ivDde)-OH, Fmoc-Gly-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the ε-amine of lysine. The

fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail A and purified with prep HPLC from 0% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 0% to 100% of phase B over 30 min: 17.183 min. ESI-MS *m/z*, M+H$^+$ = 1581.9, calculated for $C_{79}H_{92}N_{18}O_{16}S$ = 1580.7.

2.3.20. Synthesis of RGRGBip2

Fmoc-Bip-OH, Fmoc-Bip-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Arg (Pbf)-OH, Fmoc-Gly-OH, Fmoc-Lys(ivDde)-OH, Fmoc-Gly-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the ε-amine of lysine. The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail A and purified with prep HPLC from 0% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 0% to 100% of phase B over 30 min: 16.437 min. MALDI-TOF *m/z*, M +H$^+$ = 1966.4, calculated for $C_{93}H_{116}N_{26}O_{21}S$ = 1964.9.

2.3.21. Synthesis of R7GSGS

Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(ivDde)-OH, Fmoc-Gly-OH were coupled sequentially to the solid support as described in the general procedure. FITC was grafted to the peptide on the ε-amine of lysine. The fully deprotected FITC labeled peptide was cleaved from the resin with cleaving cocktail B and purified with prep HPLC from 0% to 100% of phase B over 45 min to achieve the product as a yellow powder. Retention time analytical HPLC from 0% to 100% of phase B over 30 min: 12.363 min. MALDI-TOF *m/z*, M +H$^+$ = 1916.8, calculated for $C_{79}H_{126}N_{36}O_{19}S$ = 1915.0.

2.4. General analytical methods for histology

Ultraviolet absorbance measurements were performed on an Agilent Technologies Cary 60 UV–Vis. All the tissue samples were sectioned using Thermo Scientific Microm HM550 Cryostat and were analyzed with an EVOS®FL Life technologies fluorescence microscope.

2.4.1. Materials for histology

BSA was purchased from Sigma Aldrich (St. Louis, MO, USA), DAPI containing medium from Vector Laboratories Inc. (Burlingame, CA, USA) and PBS pH 7.4 from Corning (Tewksbury, MA, USA).

2.5. Histology analysis

2.5.1. Fluorescent probe preparation

The solutions were prepared freshly before each experiment by dissolving a small portion of each peptide in 100 μL of MeOH. The concentration was determined, after dilution in PBS, by UV absorbance according to the predetermined extinction coefficient of FITC (ε = 67,000 M^{-1} cm^{-1} at 493 nm) in PBS. Final working solutions (10 μM) were obtained diluting the appropriate volume of stock solution in PBS (final MeOH concentration <1.5%).

2.5.2. Tissues preparation and fluorescence stain

All animal tissues were collected in compliance with the approved animal protocols and guidelines of the Institutional Animal Care and Use Committee at Weill Cornell Medical College. Mouse left ventricle, skeletal muscles and stomach were harvested, then embedded in OCT and frozen at −80 °C. Samples were sectioned at 10 μm and stored at −80 °C. The frozen sections were fixed with 50% MeOH and 50% acetone for 5 min, blocked with 5% BSA for 30 min and then incubated with the fluorescent probes solutions (10 μM, 300 μL/slide) for 1 h at room temperature. The slides were washed with PBS (×3) and subsequently mounted with DAPI containing medium. FITC and DAPI fluorescent signals were analyzed using GFP (Ex: 470/22 nm; Em: 510/42 nm) and DAPI (Ex: 357/44 nm; Em: 447/60 nm) fluorescence filters with a fluorescence microscope.

2.5.3. Immunofluorescence stain of cTnI

Tissues were prepared as described above. The frozen sections were blocked with 1% BSA for 1 h after fixation and then treated with anti-cardiac troponin I antibody (Abcam Inc., Cambridge, MA, USA, 5 μg/mL, 300 μL/slide, buffer: PBS, sodium azide 0.02%, BSA 1%) for 1 h. After washing with PBS (×3), the frozen sections were incubated with goat anti-rabbit IgG fc (Alexa Fluor®647) secondary antibody (Abcam Inc., Cambrige, MA, USA, 4 μg/mL, 300 μL/slide, buffer: PBS, glycerol 30%, BSA 1%) for 1 h. The slides were washed with PBS and then coverslipped with DAPI containing medium. Alexa Fluor®647, and DAPI fluorescent signals were analyzed using cy5 (Ex: 628/40 nm; Em: 692/40 nm) and DAPI (Ex: 357/44 nm; Em: 447/60 nm) fluorescence filters with a fluorescence microscope.

2.5.4. Fluorescence intensity calculation

Numerical values for fluorescence intensities were obtained using the software ImageJ 1.48, analyzing the mean pixel intensity of selected regions of interest (ROI). The 25 brightest ROI were selected in each muscle tissue and the 25 brightest ROI were selected in the relative background area. The average was calculated and the background signal was subtracted from the tissue signal. The measurement was repeated 5 times by two researchers independently. The relative fluorescence intensity between cardiac and skeletal muscle tissues was calculated for MTP, Bip1 and Bip2 as ratio of the cardiac tissues fluorescence intensity to the skeletal tissues fluorescence intensity:

$$\frac{Cf - Cb}{SKf - SKb}$$

where *Cf* is the cardiac muscle tissue fluorescence intensity, *Cb* is the cardiac tissue background, *SKf* is the skeletal tissue fluorescence intensity and *SKb* is the skeletal tissue background.

In the same way the relative fluorescence intensity between cardiac and stomach muscle tissues was calculated:

$$\frac{Cf - Cb}{STf - STb}$$

where *Cf* is the cardiac muscle tissue fluorescence intensity, *Cb* is the cardiac tissue background, *STf* is the stomach muscle tissue fluorescence intensity and *STb* is the stomach tissue background.

2.6. Statistical analysis

Statistical analyses were performed using one-way and two-way Anova with the software Prism 6 (GraphPad Software, Inc.). Values of *P* less than 0.05 were considered statistically significant.

3. Results and discussion

Previous studies of MTP showed its preferential binding to cardiac muscle over striated skeletal muscle.[9] In this work the tissue selectivity of MTP and of its derivatives was assayed using cardiac, skeletal as well as stomach muscle tissues, which are representative of the three different kinds of muscle tissue present in the

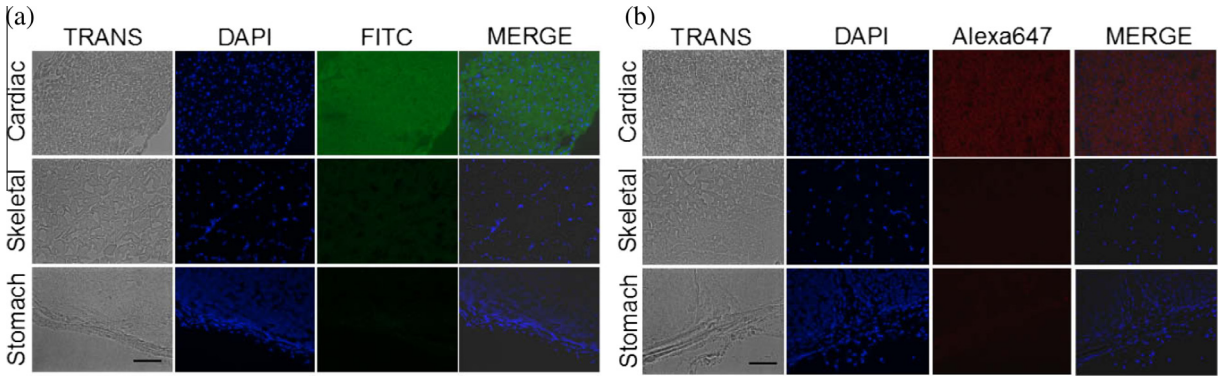


Figure 2. Comparison between MTP probe and troponin I specific antibody. Cardiac, skeletal and stomach muscle tissues stained with (a) MTP solution (10 μM), exposure time 12 ms, and (b) anti-cardiac troponin I antibody (1 μg/mL) followed by goat anti-rabbit IgG fc (Alexa Fluor®647) secondary antibody (2 μg/mL), exposure time 100 ms. Original magnification 200×, scale bar 100 μm.

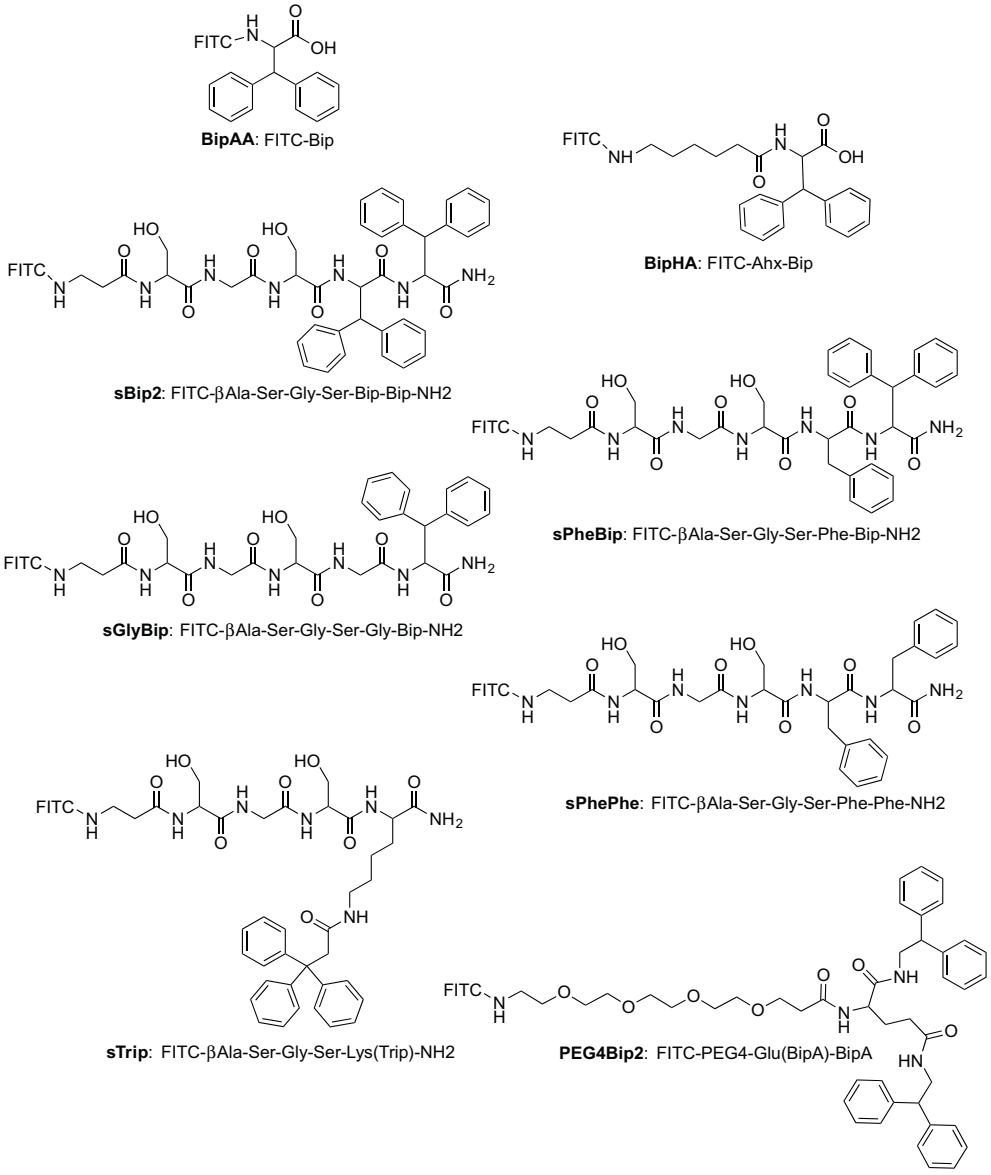


Figure 3. Structures of BipAA, BipHA and of the hexapeptide MTP derivatives.

body. The tissues were collected from mice and prepared following the standard histological fixation protocol. The tissue sections were then incubated with the probe for an hour, washed with PBS, and then examined and quantitated by a fluorescence microscope (Fig. 2).

The distribution of the signal localization and intensity obtained after staining of the three different tissues with the fluorescein (FITC)-labeled MTP (Fig. 2a) was similar to the one obtained from anti-cTnI antibody (Fig. 2b). Due to the lack of cTnI expression in skeletal and stomach muscles, no signal was seen in these control tissues.

The capability of MTP to bind cTnI was previously demonstrated with the isolated cTnI protein.[9] It was also observed that MTP with three Bip residues bound better than the ones with two or one Bip residues. The removal of the two binding groups from the Lys side chain of the MTP structure decreased its staining ability, but nonetheless the signal was still present. With the aim of investigating whether the Bip amino acid alone could stain muscle tissue, FITC was directly grafted to Bip (BipAA, Fig. 3). To minimize the steric hindrance between the bulky FITC and the Bip binding group, an analog incorporating a C_6 spacer (6-amino hexanoic acid, Ahx) was also synthesized, conjugating FITC to Bip via Ahx (BipHA, Fig. 3). However, both compounds failed to stain cardiomyocytes (data not shown), demonstrating that Bip alone does not provide a strong enough binding to cTnI and therefore new peptidic compounds, having a more similar structure to MTP, were designed.

A series of six hexapeptides was prepared using an automated synthesizer on a solid support, via Fmoc chemistry, to investigate the importance of the unusual Bip amino acid and the number of phenyl groups in the peptide structure (Fig. 3).

The presumably hydrophobic cTnI binding residues were placed consecutively at the C-terminus, and a solubility enhancing domain, β-Ala-Ser-Gly-Ser, was added to the N-terminus to separate the fluorescent reporter from the binding domain. The number of phenyl groups was adjusted by varying the hydrophobic residues. It was brought down from four to three, substituting one Bip with a phenylalanine in sPheBip and from four to two, using a glycine instead of the second Bip in sGlyBip. In the case of sPhePhe, two phenylalanine residues replace the two Bip. The last two compounds in the group are sTrip, where a triphenyl moiety (Trip) was connected via amide bond to the side chain of a lysine, and PEG4Bip2, a synthetic analog prepared in solution over 5 steps (Scheme S1 in the supplementary data), where the two Bip amino acids were replaced by two 2,2-diphenylethylamine (BipA) and where the peptide chain was exchanged for a polyethylene glycol (PEG) of comparable length. The Bip amines were linked to the PEG through a glutamic acid residue via peptide bonds.

Histologic analysis was performed on this hexapeptide series, using 2, 5 and 10 μM solutions of the compounds in PBS. Only the best results obtained with 10 μM concentration are here reported. In the first four compounds, where the number of phenyl groups was decreased in the order sBip2, sPheBip, sGlyBip and sPhePhe, the fluorescence signal in cardiomyocytes was also reduced: halved in sPheBip compared to sBip2 and brought to the minimum in sGlyBip and sPhePhe (Fig. 4a).

No selectivity for cardiomyocytes was observed with sPheBip, sGlyBip and sPhePhe. In the case of sBip2 instead, the fluorescence signal in cardiomyocytes was more than two times higher than the signal in skeletal and stomach muscles (p <0.0001). This trend shows, consistently with previous findings, that the number of phenyl groups plays a role in the selective binding of heart muscle. Probably the binding of the peptides to cTnI happens between the hydrophobic Bip moieties and the protein hydrophobic pocket. This behavior would be consistent with reports on the cardiac troponin complex, where cTnI showed binding to cTnC through hydrophobic interactions, which keep the two units together during the contraction mechanism.[11,12] Other studies also showed that small calcium-sensitizing molecules incorporating one or more aromatic rings, for instance Levosimendan which helps the heart contraction in case of malfunctioning, bind at the hydrophobic interface between cTnC and cTnI.[12] In the case of sTrip and PEG4-Bip2, no selectivity was achieved since the fluorescence signal was higher in skeletal muscles rather than in cardiac muscles as desired (Fig. 4a). These non-selective sequences were then discarded. sBip2 was the best staining probe in the first series (Fig. 4b). It is interesting to observe the dramatic difference in tissue selectivity between sBip2 and PEG4Bip2. Although they have similar hydrophobic domains, the hydrophilic spacer domains altered the tissue selectivity, hinting that the solubility enhancing region also contributed to the recognition.

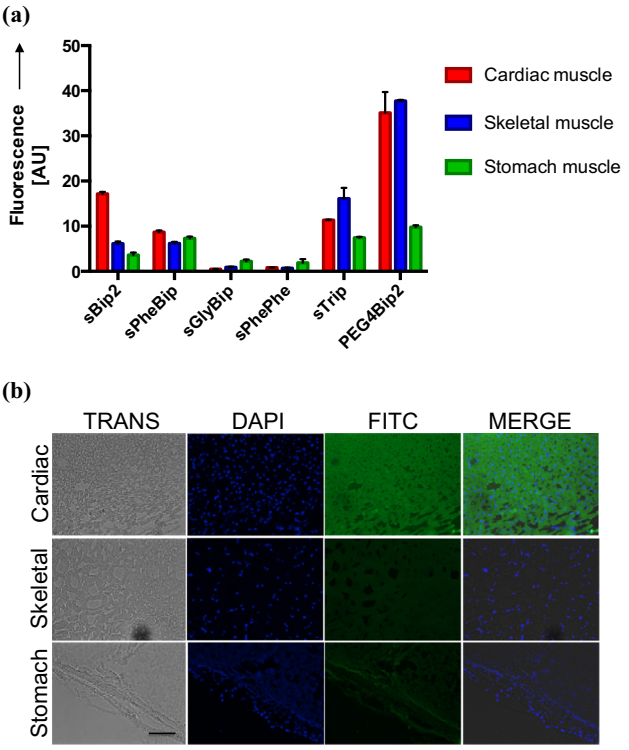
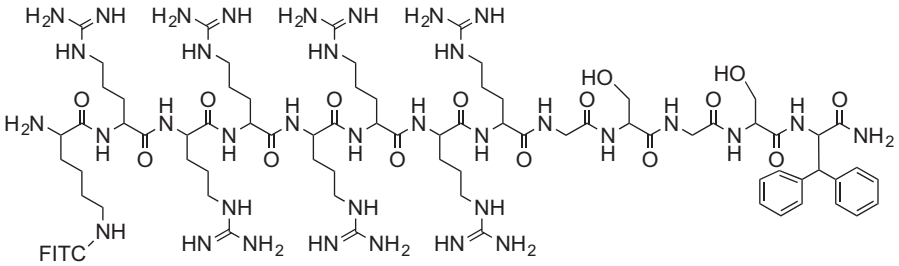


Figure 4. Tissue selectivity in the hexapeptide series. (a) Graph showing the background corrected fluorescence intensities and (b) fluorescence microscopy images of the best probe, sBip2. Staining was performed with 10 μM solutions in PBS, for 1 h. Microscope exposure time 50 ms (exposure time reduced to 20 ms for PEG4Bip2 to avoid signal saturation) original magnification 200×, scale bar 100 μm.

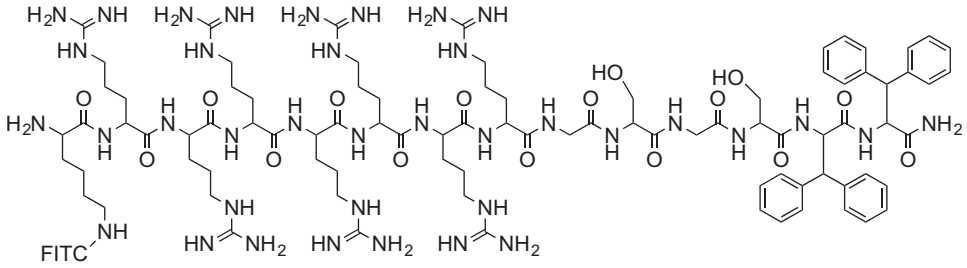
Despite sBip2 showed good cardiomyocyte staining properties and selectivity over the other types of muscle tissues, yet its solubility in aqueous media was not as good as hoped. To overcome this solubility issue, a hydrophilic hepta-arginine chain was added to the sBip2 leading to the Bip2. Other Bip2 analogs with various numbers of arginine residues were synthesized. In RGRGBip2 four of the seven arginines were replaced by glycines and in RRsBip2 the number of arginines was reduced to two (Fig. 5).

In addition, an analog containing only one Bip residue, Bip1, was also prepared. R7GSGS, which has the same amino acids sequence of Bip1 and Bip2, but lacks the Bip terminal residues, was synthesized to confirm the importance of the Bip amino acid and to investigate the effect of the poly-arginine chain.

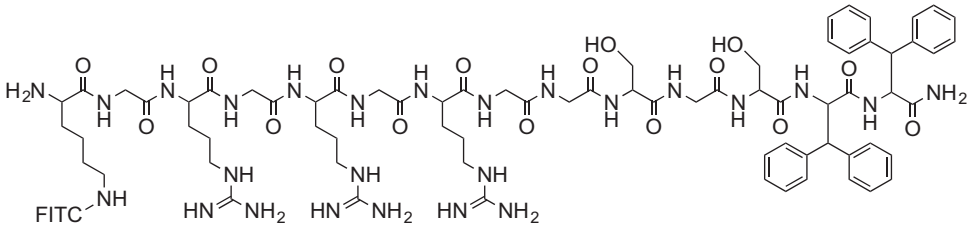
Histologic analyses of this second series were performed staining the tissues with 10 μM solutions of the compounds. The original MTP probe was included as a reference. As shown in Figure 6a, highest fluorescence intensity in cardiomyocytes was obtained with Bip1 and Bip2, which performed remarkably better than



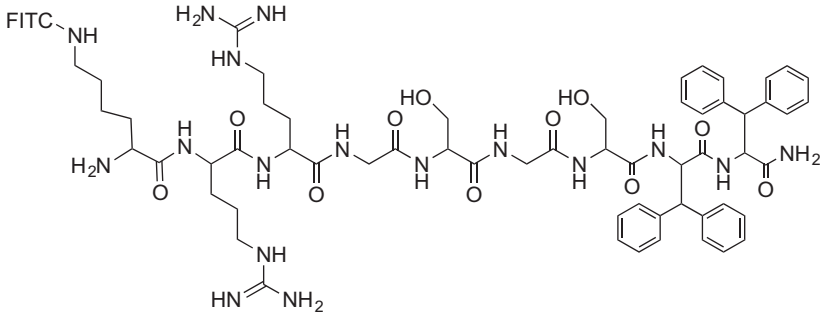
Bip1: Lys(FITC)-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Gly-Ser-Gly-Ser-Bip-NH_2



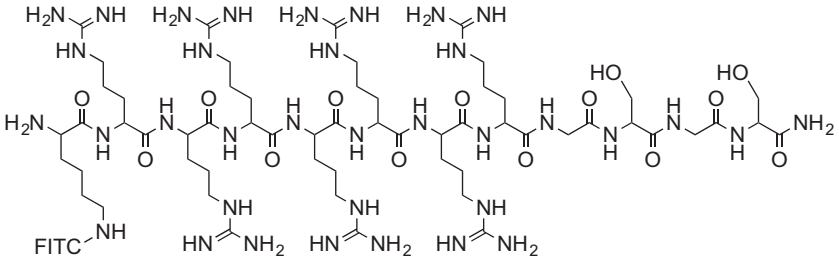
Bip2: Lys(FITC)-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Gly-Ser-Gly-Ser-Bip-Bip-NH_2



RGRGBip2: Lys(FITC)-Gly-Arg-Gly-Arg-Gly-Arg-Gly-Gly-Ser-Gly-Ser-Bip-Bip-NH_2



RRsBip2: Lys(FITC)-Arg-Arg-Gly-Ser-Gly-Ser-Bip-Bip-NH_2



R7GS GS : Lys(FITC)-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Gly-Ser-Gly-Ser-NH_2

Figure 5. Structures of probes in the second series of MTP derivatives.

MTP (respectively 5 and 6.5 times higher signal), while sBip2, RRsBip2 and RGRGBip2 gave lower fluorescence signal (half of the MTP intensity or less). This difference could be explained for the first two derivatives with the shorter length of the peptidic chain, but the explanation for RGRGBip2, where the length was conserved, must be the reduction in the number of the positively charged arginine residues. Looking at the selectivity, all Bip containing compounds preferentially stained cardiomyocytes over skeletal and stomach muscles to different extents, but the most effective one was Bip2 (Fig. 6b). Despite its high affinity, its

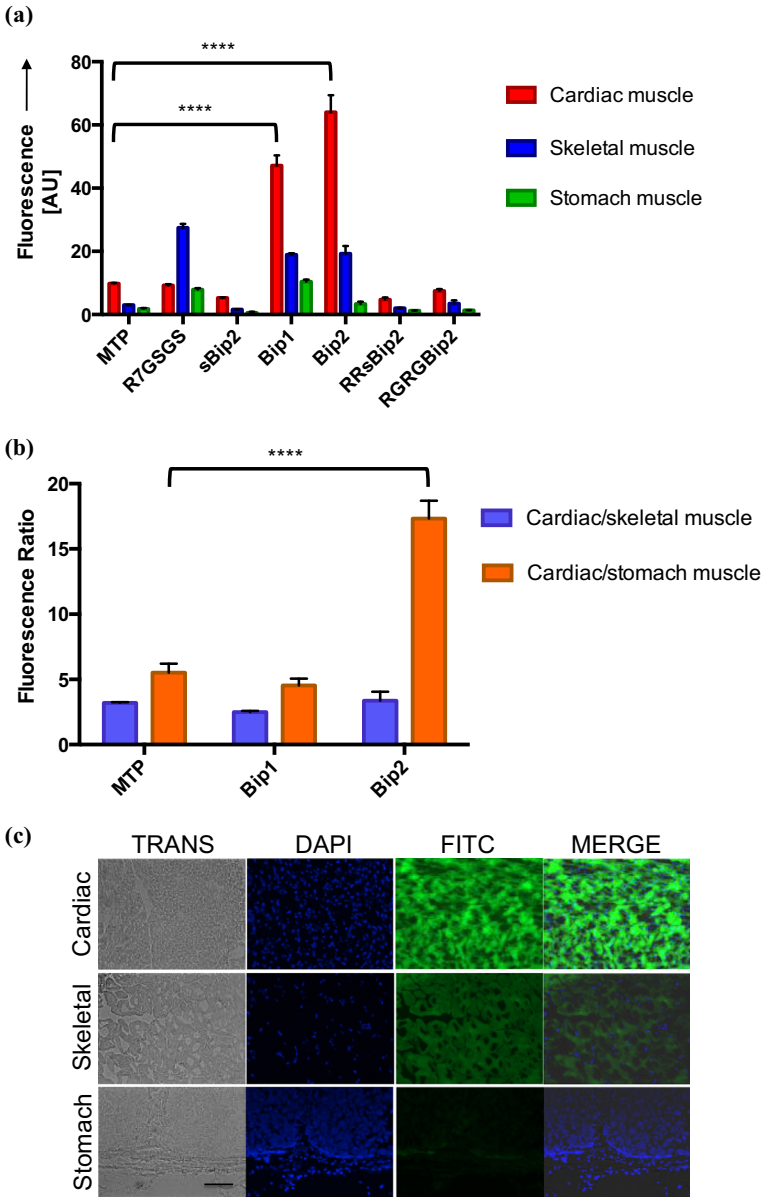


Figure 6. Tissue selectivity in the second series of peptides. (a) Graph showing the background corrected fluorescence intensity; **** means p <0.0001; (b) selectivity of the best candidate probes reported as ratio between cardiac and skeletal or stomach muscles and (c) fluorescence microscopy images of the best probe, Bip2. Staining was performed with 10 μM solutions in PBS, for 1 h. Fluorescence microscope exposure time 12 ms, original magnification 200×, scale bar 100 μm.

selectivity for cardiac over skeletal tissues was comparable to the one of MTP; instead, its selectivity towards stomach tissue was significantly improved (over three times, p <0.0001).

Interestingly R7GSGS showed in cardiac muscle a comparable signal to MTP despite the absence of the Bip moiety, however the signal intensity was 5 and 6.5 times lower than the signal obtained with Bip1 and Bip2 respectively (Figs. 6a and S2 in Supplementary information). The highest fluorescence signal registered for this R7GSGS was in skeletal muscle, with a similar value to the ones registered for Bip1 and Bip2. It is also noteworthy that the fluorescence was present in all three kinds of tissue, including the nuclei of the cells. This behavior could be attributed to the poly-arginine cell-penetrating motif that is known to be unselective unless a targeting vector is present,[13] and, as already demonstrated in the literature for fixed cells, could give false results due to fixation, like apparent nuclear localization.[14] This result underlines the importance of the biphenylalanine residues in selectively targeting cardiomyocytes.

Of all compounds tested, Bip2 showed the best selectivity and the highest fluorescence signal in cardiac muscle (p <0.0001). These results suggest that, on top of the phenyl groups, which are important for the hydrophobic interaction, the peptide chain and its charge (Table 1) also play a critical role in the cTnI binding. The charged domain may interact with a neighboring hydrophilic region and further stabilize the peptide-protein interaction.

Table 1
Charge and number of phenyl groups in the peptides of the second series

Peptide	Charge	# phenyl
MTP	+2	6
sBip2	0	4
RRsBip2	+3	4
RGRGBip2	+4	4
R7GSGS	+8	0
Bip1	+8	2
Bip2	+8	4

Additionally, the cell-penetrating motif may enhance the compounds' internalization and therefore increase the contact with the available cTnI.

Though the exact binding mechanism remains to be explored, the addition of a positively charged poly-arginine sequence improved tissues staining, selectivity towards cardiomyocytes, and solubility in aqueous media. Furthermore, compared to MTP, the new probe is also easier to synthesize and purify.

In this study, fluorescein was used as the reporter. It emits bright fluorescence in the visible window, but the molecule itself is not photostable. It is not suitable for repetitive imaging. As fluorescein was attached to Bip2 through a reactive amino group, Bip2 probes with different fluorescent reporters can be prepared conveniently. If the probe were tested in vivo, near infrared fluorophores, which have better tissue propagation properties, should be used.

The newly identified cardiomyocyte-specific probe Bip2 could find application as a probe for heart injury diagnosis, not only in the study of myocyte viability and in tissue mapping, but also in blood assays. Following heart damage, troponins are released from the necrotic cardiac cells into the blood, thus the concentration of troponin in the blood is clinically used to diagnose early cardiac injuries.[15,16] At present, all the assays in the clinics are immunoassays, which are selective, but also costly and subject to laborious manipulation and false positive results due to the presence of autoantibodies.[17] For these reasons it would be interesting to study peptidic probes, which are easier to operate and more cost effective. Some work has already been done on this field, using screening of phage-displayed combinatorial libraries[18,19] or other strategies involving structure-based design of analogs of cTnI's natural ligands.[20] Recently, anti-cTnI antibody has been used to deliver the modified liposomes loaded with the anti-arrhythmia drug AMO-1 to ischemic myocardium.[21] Bip2 could also be applied similarly to transport drugs for heart disease medications. However, the capability of Bip2 for in vivo cardiomyocyte-specific binding remains to be investigated.

4. Conclusions

In conclusion, a library of MTP derivatives was developed and its structure–activity relationship was studied attesting the importance of the biphenyl moiety for the cardiomyocyte recognition, but also the importance of the positively charged peptidic chain. The MTP probe was exceeded by the new cardiomyocyte-specific probe Bip2, composed of a Bip dimer located on the C-terminus and a poly-arginine motif on the other side. The new staining agent demonstrated enhanced cardiomyocyte staining ability, higher cardiac muscle tissue selectivity and total solubility in aqueous media. It is demonstrated that this new MTP is very useful in histological tissue analysis. Potentially it could be applied to isolate primary cardiac cells, to detect circulating cTnI for early cardiac injury diagnosis, and to transport therapeutic agents to the heart.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.02.042.

References and notes

1. World Health Organization, http://www.who.int/mediacentre/factsheets/fs317/en/, (accessed 11-11-2015, 2015).
2. Woodcock, E. A.; Matkovich, S. J. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 1746.
3. Kemp, M.; Donovan, J.; Higham, H.; Hooper, J. *Br. J. Anaesth.* **2004**, *93*, 63.
4. Korff, S.; Katus, H. A.; Giannitsis, E. *Heart* **2006**, *92*, 987.
5. Babuin, L.; Jaffe, A. S. *Can. Med. Assoc. J.* **2005**, *173*, 1191.
6. Gomes, A. V.; Potter, J. D.; Szczesna-Cordary, D. *IUBMB Life* **2002**, *54*, 323.
7. Ricchiuti, V.; Voss, E. M.; Ney, A.; Odland, M.; Anderson, P. A. W.; Apple, F. S. *Clin. Chem.* **1998**, *44*, 1919.
8. Ricchiuti, V.; Zhang, J.; Apple, F. S. *Clin. Chem.* **1997**, *43*, 990.
9. Abd-Elgaliel, W. R.; Tung, C.-H. *Mol. Biosyst.* **2012**, *8*, 2629.
10. Chen, J.; Lee, S. K.; Abd-Elgaliel, W. R.; Liang, L.; Galende, E.-Y.; Hajjar, R. J.; Tung, C.-H. *PLoS ONE* **2011**, *6*, e19097.
11. Sorsa, T.; Pollesello, P.; Solaro, R. J. *Mol. Cell. Biochem.* **2004**, *266*, 87.
12. Lindert, S.; Li, M. X.; Sykes, B. D.; McCammon, J. A. *Chem. Biol. Drug Des.* **2015**, *85*, 99.
13. Zhang, Q.; Gao, H.; He, Q. *Mol. Pharm.* **2015**, *12*, 3105.
14. Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B. *J. Biol. Chem.* **2003**, *278*, 585.
15. Amundson, B. E.; Apple, F. S. *Clin. Chem. Lab. Med.* **2015**, *53*, 665.
16. De Lemos, J. A. *J. Am. Med. Assoc.* **2013**, *309*, 2262.
17. Korley, F. K.; Jaffe, A. S. *J. Am. Coll. Cardiol.* **2013**, *61*, 1753.
18. Kim, I. S.; So, I-S.; Son, H-N.; Cha, K. PCT/KR2012/010705 [WO/2013/085367], 2013.
19. Park, J. P.; Cropek, D. M.; Banta, S. *Biotechnol. Bioeng.* **2010**, *105*, 678.
20. Chandra, D.; Sankalia, N.; Arcibal, I.; Banta, S.; Cropek, D.; Karande, P. *Biopolymers* **2014**, *102*, 97.
21. Liu, M.; Li, M.; Sun, S.; Li, B.; Du, D.; Sun, J.; Cao, F.; Li, H.; Jia, F.; Wang, T.; Chang, N.; Yu, H.; Wang, Q.; Peng, H. *Biomaterials* **2014**, *35*, 3697.