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A Cardiac Tissue-Specific Binding Agent of Troponin I

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Material and methods

Materials

All reagents used were of analytical or HPLC grade. Dicholormethane (DCM), N-Methylpyrolidone (NMP) and methanol (MeOH) were purchased from Fisher (Fair Lawn, NJ, USA). *N*,*N*-dimethylformamide (DMF), diethylether, acetonitrile (MeCN), diisoproylethylamine (DIPEA), piperidine, actin protein and collagen type I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triisopropylsilane (TIS) and triethylamine (TEA) and Kaiser test kit were purchased from Sigma-Aldrich (Milwaukee, WI, USA). HOBt and HBTU were purchased from Applied Biosystems (Foster City, CA, USA). Fmoc protected amino acids were purchase from AnaSpec (Fremont, CA, USA). Fmoc-L-3,3-biphenylalanine was purchased from Synthetech (Albany, OR, USA). Fmoc-Rink amide MBHA-resin was purchase from Novabiochem (La Jolla, CA, USA). Collagen Type I, and III were purchased from Rockland (Gilbertsville, PA, USA). Non-fat dry milk was purchased from Santa Cruz (Santa Cruz, CA, USA). Novex sharp pre-stained protein standards and X cell surelock were purchased from Life Technologies (Grand Island, NY, USA). Human cTnI was purchased from Life Diagnostics (West Chester, PA, USA). Primary antibodies for cTnI and collagen I, human cardiac troponin T, and actinin protein were purchased from Abcam (Cambridge, MA, USA). Tris-HEPES-SDS precast polyacrylamide mini gels, lane marker reducing sample buffer, and CB imperial protein stain were purchased from Thermo Scientific (Rockford, IL, USA). Citrate buffer, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody, 3,3-diaminobenzidine tetrahydrochloride (DAB), and DAPI medium

were purchased from Vector Laboratories (Burlingame, CA, USA). Mayer's hematoxylin and 3% hydrogen peroxide were purchased from Dako (Carpinteria, CA, USA).

Bip Peptides Synthesis and Characterization

All fluorescent cTnI staining peptides were synthesized by solid-phase peptide synthesis (SPPS) using the standard Fmoc strategy on an automatic synthesizer (ABI-433A, Applied Biosystems). Rink amide MBHA resin, 250 μmol, with a substitution level of 0.7 μmol/mg was used as the support for peptide amide synthesis. Four-fold molar excess, relative to the resin loading, of each Fmoc protected amino acids, L-3,3-biphenylalanine, glycine, glycine, lysine, glycine, glycine, lysine, glycine, glycine, and beta-alanine were coupled sequentially to the solid support using the HBTU/HOBT coupling strategy.

After completion of the peptide chain elongation, fluorescein 5-isothiocyanate (FITC), was coupled to the last *N*-terminal amino acid, beta alanine (βAla). Three equivalents of FITC (750 μmol, 292 mg), relative to the resin loading, were dissolved in 4 mL NMP followed by the addition of 1 mL DIPEA. This solution vortexed thoroughly for 2-3 min then added to Rink amide MBHA resin-bound peptide in a manual SPPS reaction vessel and agitated gently overnight under N_2 at RT in the dark. The reagents were drained and washed five times with NMP. The completion of FITC coupling was confirmed by a Kaiser Test. Selective removal of the Mtt protecting groups from the side chain of the Lys residues in Bip1-Bip3 peptides was achieved using 10 ml of a solution of 1%TFA, 5% TIS, and 94% DCM, 10 times for 3 min each. Subsequent coupling of

Fmoc-L-3,3-biphenylalanine (six equivalent relative to the resin loading) to the free ε-amino group of the Lys residues was carried out using HBTU/HOBT.

Thereafter, the dry peptidyl resin was mixed with the deprotection cleavage cocktail (96% TFA, 3% H_2O, 1.0% TIS, 10-20 mL cocktail/g resin) in a manual SPPS reaction vessel at RT in the dark with gentle agitation under N_2 for three hours. The cleavage cocktail containing peptides were filtered and reduced in a volume to ~1 mL. Addition of cold diethyl ether provided peptide amides precipitates of Bip1-Bip3.

Fluorescent peptides, Bip1-Bip3, were dissolved in ~6 mL MeCN:H_2O (50:50 v/v) and purified by reversed phase high performance liquid chromatography (RP-HPLC) using Nova-Pak® HR C18 Waters preparative column, 6 μm, 60 Å, 19 mm ID × 300 mm L, (Waters, Milford, MA, USA) on a Varian-ProStar 210 Chromatography system (Varian, Palo Alto, CA, USA). Targeting peptides, Bip1-Bip3*,* were purified by eluting the column at 8 ml/min with a linear gradient from 0 % B to 50 % B in 60 min. Detection was carried out at 220 nm and 280 nm using a Varian-ProStar L-345 UV-Vis detector (Varian, Palo Alto, CA, USA). The purity of targeting peptides was determined by analytical RP-HPLC using a C-18 Vydac column, 5 μm, 4.6 mm ID × 150 mm L (GRACE, Deerfield, IL, USA) on a Varian 920-LC liquid chromatography system coupled to a UV-Vis/fluorescence diodarray detector, and equipped with Galaxie Chromatography Data system™ (version 1.9, Varian, Palo Alto, CA, USA). The identity of the purified peptides was confirmed by ESI-MS using a Thermo Finnigan LCQ Fleet mass spectrometer, and the raw data were analyzed using Xcalibur software (ThermoFisher Scientific, West Palm Beach, FL, USA). The purity of the purified targeting peptides fractions were confirmed by LC-MS on a C-18 Vydac column, 5 μm,

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4.6 mm ID x 150 mm L, (GRACE, Deerfield, IL, USA) using Accela HPLC coupled with a PDA detector and autosampler (ThermoFisher Scientific, West Palm Beach, FL, USA). Fractions with the same purity were collected together and lyophilized to yield yellowish powders with >98% purity. ESI-MS show the molecular ions (*m/z*) of each targeting peptide (Table S1). The conjugates were stored at 4°C in the dark.

SDS-PAGE Analysis and Staining Imaging

Protein electrophoresis analyses were performed using SDS-PAGE mini gels (4%-20%). For subsequent staining with coomassie blue, 20-μl aliquots of premixed loading buffer (5X) and 2 μg of each protein were loaded per sample well. For fluorescent staining with FITC-peptides, 20 μl aliquots of premixed loading buffer (5X), fluorescent peptide (1 μM) and 2 μg of each protein were loaded per sample well. The protein gels in 1X running buffer of 100mM Tris, 100mM HEPES, 0.1%(~3mM) SDS were subjected to 100V (76 mA) for 60 min in a gel running apparatus. After electrophoresis, the gel was fixed in 500 mL of solution with 50% ethanol, 10% acetic acid, and 40% H_2O for 30 min at RT with gentle agitation on a rotating platform. The fixing solution was removed by aspiration, and the gels were washed with 500mL of H_2O. The gels were then stained with 400 mL Coomassie blue at RT for 3-4 h with gentle agitation. Staining was stopped when the gel develop a uniform blue color. The Coomassie stain was removed by aspiration after staining, and the gel was de-stained with ~250 mL of 5% (v/v) methanol in water with 7.5% (v/v) acetic acid with gentle agitation. The destaining solution was replaced several times over 1-2 hours until the blue protein bands began to appear with amber background staining. After the gel

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equilibrated in 500 mL of the storage solution of 5% (v/v) acetic acid for at least 1 h, it was then transferred to a transparent sealable bag for bright field imaging.

Fluorescent stained gels were fixed, washed extensively with water, and transferred to transparent sealable bag for imaging. The fluorescence intensities were monitored using the optical imaging system IVIS-200. Images were acquired using FITC fluorescence filters (410-440 nm background bandpass filter, 445-490 nm excitation bandpass filter, and 515-575 nm emission bandpass filter). Images were collected and corrected for flat field and cosmic fluorescence. The radiance scale of the color bar represents the fluorescence emission normalized to the illumination intensity.

Spectroscopic Studies

Absorption spectra of 100 μL of MTP probe, cTnI and cTnT proteins (10 μM each) as well as mixtures of cTnI and cTnT proteins with MTP probe (10 μM, each) were measured in triplicate in 96 well black walls, clear-bottom plates (Corning, NY, USA). The changes in absorption were monitored using absorption spectrophotometer (SpectraMax.M2^{e}, Molecular Devices, Sunnyvale, CA, USA) in the UV/Vis region between 350 nm and 550 nm at 21 ^{o}C.

Immunohistochemical Staining

All animal studies were performed in compliance with the approved animal protocols and guidelines of Institutional Animal Care and Use Committee at The Methodist Hospital Research Institute. Collected mouse heart and skeletal muscles

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were fixed in 10% natural buffered formalin for 18 h, embedded in paraffin wax, sliced into 4-μm sections, and mounted on positively charged slides. Unstained tissue sections were deparaffinized with xylene for 13 min and rehydrated with ethanol and water for 12 min. Heat mediated antigen retrieval was performed with citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) using a steamer at 98°C for 20 min and cooled down to RT for another 20 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. Protein was blocked with 5% normal horse serum in PBS for 5 min. Tissue sections were incubated with primary antibodies against troponin I and collagen I in blocking solution at a 1/100 and 1/200 dilutions, respectively, for 30 min at RT followed by HRP conjugated goat anti-rabbit IgG secondary antibody for 15 min at RT. Slides were developed with DAB substrate for 5 min and counterstained with Mayer's hematoxylin for 30 seconds. Slides were then washed and dehydrated with ethanol, cleared with xylene, and mounted.

Histofluorescence Staining

Deparaffinized tissue sections were blocked with 5% BSA for 30 min followed by incubation with 10 μM solution of Bip3 fluorescent probe (~500 μl/section) for 1 h in the dark using a humidified chamber at RT. Tissue sections rinsed extensively with PBS three times, 5 min each, counter stained with DAPI (DNA Satin), coverslipped with mounting medium, and stored in the dark. The FITC fluorescent signal of the tissue sections was collected using fluorescent microscopy.

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S1. List of calculated and observed molecular masses of cTnI targeting peptides.

Targeting peptide	Calculated Mol. Wt. (Da)	Observed $[M+H]^{1+}/1$	Observed $[M+2H]^{2+}/2$	Observed $[M+3H]^{3+}/3$
Bip1	1298.52	1299.42	650.45	433.72
Bip2	1522.68	1523.46	762.11	508.39
Bip3 (MTP)	1745.82	1746.70	874.27	580.95

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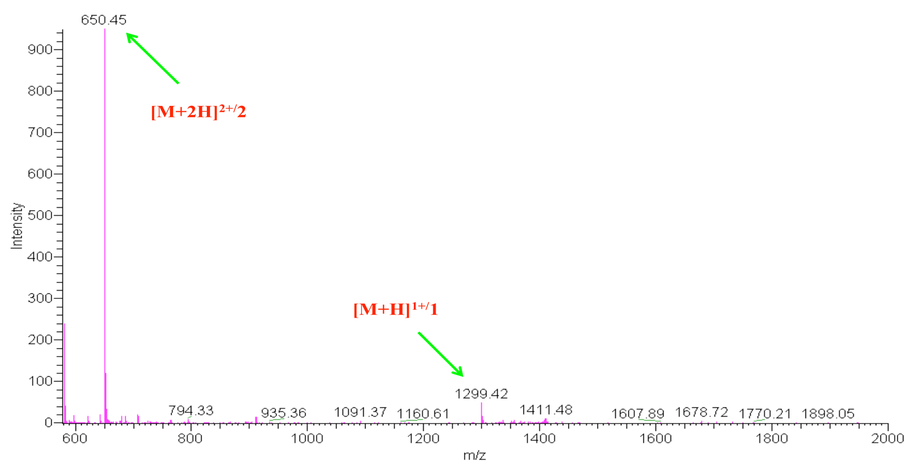
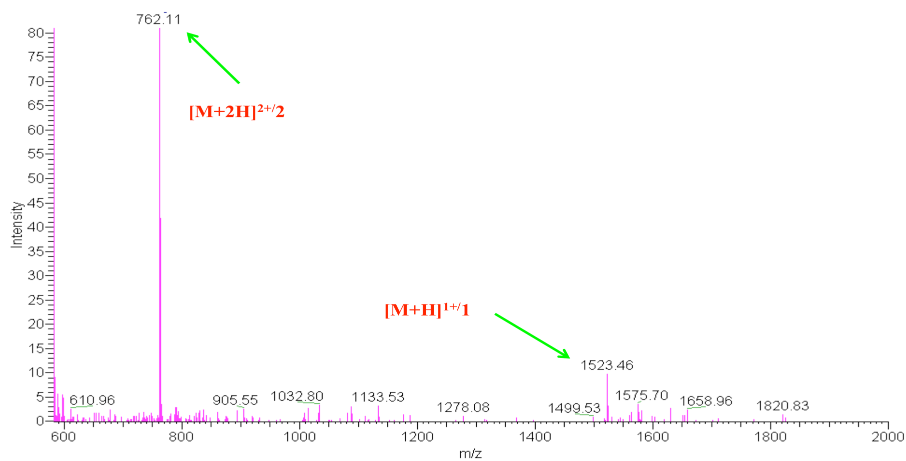
S2. Mass spectra characteristics and HPLC trace of the developed biphenylalanine-rich **Bip1- Bip3** peptides.

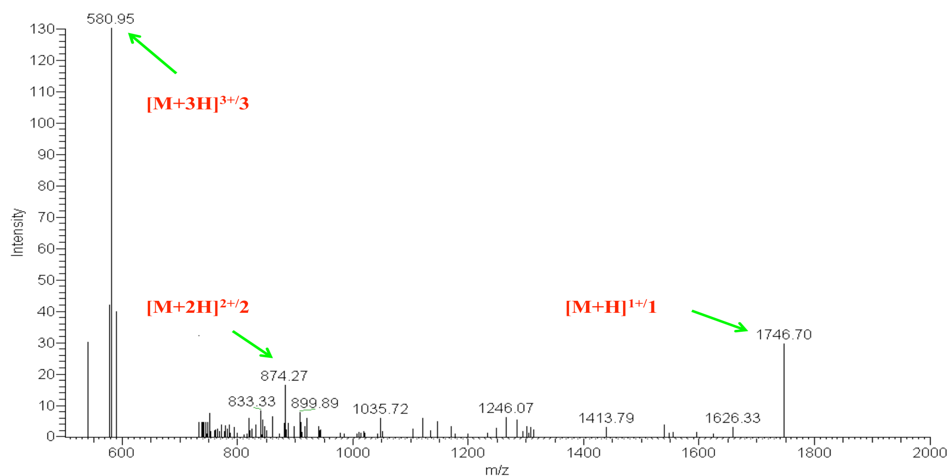
Bip1-Mass Spec



Bip2-Mass Spec



Bip3-Mass Spec



HPLC Trace