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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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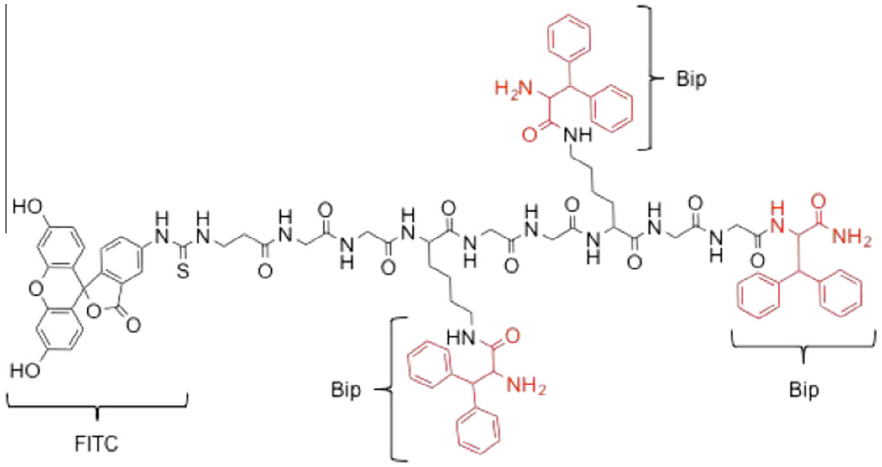

Figure 1. MTP structure (Bip highlighted in red).

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.