Cardiac-Targeting Peptide

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Despite significant strides in prevention, diagnosis, and treatment, cardiovascular diseases remain the number one cause of mortality in the United States, with rates climbing at an alarming rate in the developing world. Targeted delivery of therapeutics to the heart has been a lofty goal to achieve with strategies ranging from direct intra-cardiac or intra-pericardial delivery, intra-coronary infusion, to adenoviral, lentiviral, and adeno-associated viral vectors which have preference, if not complete cardio-selectivity, for cardiac tissue. Cell-penetrating peptides (CPP) are 5–30-amino-acid-long peptides that are able to breach cell membrane barriers while carrying cargoes up to several times their size, in an intact functional form. Identified nearly three decades ago, the first of these CPPs came from the HIV coat protein transactivator of transcription. Although a highly efficient CPP, its clinical utility is limited by its robust ability to cross any cell membrane barrier, including crossing the blood–brain barrier and transducing neuronal tissue non-specifically.

cell-penetrating peptides protein transduction domains cardiac-targeting peptide

1. Identification of Cardiac-Targeting Peptide

Researchers utilized a combinatorial in vitro and in vivo phage screening of a large, commercially available M13 phage display library to identify CTP, a cardiomyocyte-specific cell-penetrating peptide [1]. The heart is a very vascular organ with only a small minority of the intravascularly injected phage being internalized, with the blood pool contaminating what is recovered from the cardiomyocytes themselves. To enrich for cardiac-targeting peptides, researchers attempted to decrease non-specific phage by pre-screening in a rat cardiomyoblast, H9C2, cell line. Cells were plated in a six-well plate and allowed to reach 70% confluency before being incubated with the phage library for 6 hrs. At the end of the incubation period, cells were washed extensively, trypsinized, collected and lysed using freeze–thaw cycles. Phage recovered from the lysed cell was expanded, injected into a mouse and allowed to circulate for 24 h. The rationale for prolonged circulation time was to allow for non-specific phage from the blood pool to clear out [2], allowing for only internalized phage to be recovered from heart tissue. In order to maximize viability of the internalized phage, mice were treated 24 h before the injections and on the day of the injection with Chloroquine to prevent acidification of lysosomes and resulting breakdown of internalized phage. Recovered phage from the heart was expanded and reinjected for subsequent cycles. After the 4th cycle, 10 plaques were picked and sent for sequencing, with 6 out of 10 bearing the identical sequence of APWHLSSQYSRT, a peptide that researchers later named CTP or cardiac-targeting peptide. In vitro incubation of a number of different cell lines with fluorescently tagged CTP revealed a preferential uptake of the peptide by H9C2 cells [1]. Injecting mice with fluorescently tagged CTP showed robust uptake by heart in 30 min. Later biodistribution

studies revealed peak uptake at 15 min (earliest time point tested) with almost complete disappearance of fluorescence by 6 h [3].

Phage display had been utilized prior to the above experiments in an in vitro display methodology to identify another, longer cardiomyocyte-homing peptide. McGuire and colleagues used an in vitro phage display approach to identify a 20-mer amino acid (WLSEAGPVVTVRALRGTGSW) which had partial sequence homology to tenascin-X, an extracellular matrix protein [4]. This peptide had a 180-fold preference for cardiomyocytes in vitro, the cell line used for phage display. They were able to show preferential isolation of this phage from heart tissue as compared to a random peptide-bearing phage [4]. More recently, an independent group were able to engineer cardiosphere-derived cells to produce exosomes labeled with Lamp2b and WLSEAGPVVTVRALRGTGSW, which showed anti-apoptotic effects in vitro in primary mouse neonatal cardiomyocytes, and increased cardiac retention in vivo compared with non-labeled exosomes [5]. However, it is worth noting that all in vivo injections into mice were intramyocardial, and not systemic or intravenous, bringing into question the strength of the targeting as well as the clinical applicability of this approach for use in humans. This is an important point as intra-myocardial delivery is clinically feasible in a very limited number of patients such as those undergoing coronary artery bypass grafting or another open-heart procedure, precluding repeat therapies. In another application, L-arginine-loaded gold nanoparticles modified with WLSEAGPVVTVRALRGTGSW exhibited increased cellular uptake by cardiomyocytes with resulting improved photoacoustic imaging in vitro and in vivo [6]. These nanoparticles were able to increase nitric oxide production, and decrease apoptosis, fibrosis and infarct size in a rat model of ischemia–reperfusion [6]. Hopefully, larger animal studies will be forthcoming and show similar efficacy, as this was a very recent publication.

2. Cardiac-Targeting Peptide for Diagnostic Purposes

Coronary artery disease is the most common form of cardiovascular disorder. Atherosclerotic plaque buildup leads to progressive narrowing of coronary artery lumen. This is a chronic process, with symptoms developing only after a significant portion of the lumen (>50–70%) has been compromised. Symptoms, once they develop, range from chest pain, shortness of breath on exertion, to fatigue and decreased exercise tolerance, and are responsible for the most frequent reason for presentation to the emergency rooms in the US. These symptoms are investigated in patients by putting them through cardiac stress testing while continuously monitoring the heart with electrocardiograms (ECGs). The heart is stressed with exercise, the most physiological form of stressor, or chemicals in patients who cannot exercise. Commonly, ECG alone has low sensitivity and specificity for detection of occlusive coronary artery disease, and hence is combined with an imaging modality, in the form of nuclear cardiac imaging or echocardiography using ultrasound. Myocardial perfusion imaging is most commonly performed through using cardiac tracers like Thallium, or, more recently, Technetium 99^{m}. Uptake of this tracer by the liver and gut loops interferes with heart imaging, lowering the sensitivity of the examination. Targeting the radioisotopes to the heart would, in theory, improve cardiac uptake and decrease radio-isotope dose. CTP was tagged with Technetium 99^{m} via a chelator, HYNIC, and showed almost exclusive uptake by the heart with predominantly renal excretion, when compared with Technetium Sestamibi, the formulation of Technetium 99^{m} in wide-spread clinical use [3].

Cardiac PET imaging is a more recent addition to the clinician armamentarium of diagnostics. Compared to SPECT imaging, PET has a higher sensitivity (80.3% versus 68.7%) and similar specificity (63.8% versus 61.7%) and therefore overall superior accuracy in predicting occlusive coronary artery disease with significantly lower radiation exposure (by ~50%) than SPECT. Yet, it remains grossly under-utilized with data from 2019, indicating that of all nuclear cardiac stress tests, PET studies represented only 10% of them. The underlying reason for this is lack of widespread availability of cardiac PET tracers. The only FDA-approved cardiac PET tracers in clinical use are ^{15}O water, ^{13}N ammonia, and ^{82}Rubidium [7][8]. The availability of these tracers is limited by the need for an on-site (^{15}O water and ^{13}N ammonia) or nearby (^{13}N ammonia) cyclotron, or commitment to costly generators (^{82}Rb). Due to the short half-lives ranging from 76 s for ^{82}Rb, to 2.1 min for ^{15}O water and 10 min for ^{13}N ammonia, their use in conjunction with treadmill exercise stress testing is either not possible (^{82}Rb and ^{15}O water) or not practical (^{13}N ammonia). Furthermore, the long positron range of ^{82}Rb makes image resolution suboptimal and its low extraction limits its spatial resolution. Of all stress modalities (exercise, dobutamine, dipyridamole, adenosine, and regadenoson), exercise is the most physiological stressor, and provides important additive prognostic information not available with other stressors.

With this background in mind, CTP could provide a solution. A recent work showed that labeling of CTP with Gallium-68, another PET radioisotope, can be successfully performed using 1,4,7-Triazacyclononane-1,4,7-triacetic acid (NOTA) as a chelator, with preliminary animal studies showing promise. This conjugation was optimized to yield 98% radiolabeling efficiency at room temperature in ten minutes, and with >97% radiochemical purity, without the need of a heating block. The CTP-NOTA-Gallium 68 conjugate was taken up by heart tissue in as little as 5 min with isotope leaving the heart in ~30 min. At later time points, the gall bladder lit up on PET imaging, with a significant portion being secreted by the kidneys (presented in abstract form at Society of Nuclear Medicine, Chicago, 2023).

3. Cardiac-Targeting Peptide for Therapeutic Purposes

The first therapeutic application of CTP was studied by Avula and colleagues in order to perform cell-selective arrhythmia ablation [9]. They engineered an 8-arm pegylated nanoparticle with an average of 2.6 CTP molecules attached to the pegylated arms along with a photosensitizer chlorin e6. Injecting these nanoparticles into rats followed by laser illumination of the heart induced localized myocyte-specific ablation restoring normal rhythm with 85% efficiency. Of note, there was no damage to “innocent, bystander” cells like endothelial cells or myofibroblasts. They were also able to demonstrate this cardiomyocyte-specific ablation in sheep heart ex vivo. This led to the formation of complete heart block at the ablated region leading to restoration of normal sinus rhythm [9].

Extracellular vesicles, or exosomes, are heterogeneous, membrane bound vesicles, ranging in size from nanometers to micrometers, and originate as extrusions from the endosome or plasma membrane. They are being actively explored by a number of researchers as delivery vehicles for cardiac and other applications. In a series of experiments, researchers from South Korea were able to engineer exosomes expressing CTP along with LampB, and showed ~15% increased uptake in vitro and in vivo compared to non-targeted (LampB but no CTP bearing) exosomes [10]. In another demonstration of this targeted delivery, exosomes labeled only with CTP were loaded

with curcumin and miR-144-3p, and showed not only in vivo delivery to the murine heart, but also cardioprotection in a mouse infarct model [11]. In a separate application, Kim and colleagues engineered extracellular vesicles to express high levels of CTP [12]. These vesicles were loaded with anti-RAGE (receptor for advanced glycation end products) siRNA. RAGE has been shown to contribute to inflammation in a number of cardiac pathologies that lead to myocarditis. Such labeled vesicles were injected intravenously into a rat model of myocarditis induced by immunization with cardiac myosin. On day 7 after induction of myocarditis, rats were treated with a single injection of non-targeted vesicles loaded with anti-RAGE siRNA or targeted vesicles labeled with CTP. Targeted vesicles significantly decrease RAGE, IL-6, and TNF-α levels in heart tissue. This decrease in multiple inflammatory markers was accompanied by a significant increase in left ventricular ejection fraction, and improvements in left ventricular end-systolic and end-diastolic dimensions [12]. Treatment also led to a significant decrease in protein levels of RAGE (as expected), but also levels of Il-6, TNF-α, COX2 and proportion of phosphorylated p65, an activated component of the NF-κappa-B pathway [13].

As stated earlier, heart failure with preserved ejection fraction is growing in incidence as well as a disease particularly recalcitrant to treatments by drugs and interventions that have been shown to be beneficial in heart failure with reduced ejection fraction. This is likely due to significant differences in underlying pathophysiology. One of the hallmarks of heart failure with preserved ejection fraction is a thick hypertrophied left ventricle with increased myocyte stiffness. To target this particular pathology at the cardiomyocyte level, Gallicano and colleagues worked to deliver microRNA106a to the cardiomyocyte. MicroRNAs are small non-coding, ~22-nucleatide-long RNAs that silence gene expression via a post-translation modification of mRNAs. They target the 3′UTR of an mRNA through a 7–8 bases long seed sequence that marks the complex for degradation in the RISC or RNA-induced silencing complex. One of the targets of miRNA106a is calcium calmodulin kinase IIδ that is upregulated in heart failure and responsible for the calcium mishandling intrinsic to these hypertrophied cardiomyocytes. In order to deliver miRNA106a specifically to hypertrophied cardiomyocytes, it was conjugated to the N-terminus of CTP via a disulfide linker. This linker was chosen due to its covalent nature and high likelihood of remaining stable in serum, as well as being broken down in the reducing intracellular environment, leading to release of cargo miRNA106a from its vector CTP, allowing it to bind to calcium calmodulin kinase Iiδ mRNA marking it for degradation in the RISC complex. Incubation of human left ventricular myocytes with fluorescently labeled CTP-miRNA106a conjugate led to robust uptake by the cells [14]. This conjugate was selectively taken up by human cardiomyocyte cell line and not by HEK293 cells [14]. Additionally, this conjugate was able to reverse angiotensin–phenylephrine-induced hypertrophy of myocytes by decreasing expression of both HDAC4 and calcium calmodulin kinase IIδ, both targets of miRNA106a [13]. Studies on the effect of this conjugate in reversing cardiac hypertrophy and ameliorating heart failure in an in vivo mouse model are ongoing.

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