PROGRESS REPORT: 2/28/2017

"Novel Cell-Free Peptide Therapeutics for Cardiac Repair and Regeneration"; Award #: RMM 11215 TR001

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Summary of Problem To Be Investigated: Myocardial infarction (MI) is a major consequence of coronary heart disease despite current treatment strategies, progression from MI to heart failure (HF) occurs in up to 1/3 of MI patients and markedly increases risk for hospitalization and death. Thus the prevention of post-MI HF remains an unmet clinical challenge, despite advances in cell-based therapies. The objective of this highly translational application is to advance an innovative **cell-free peptide therapeutic strategy** that has been engineered for optimizing the heart's intrinsic reparative and regenerative systems following experimental MI in the presence of permanent myocardial injury. Our long-term goal is to prevent and/or reverse post-MI HF.

We will advance in our proposed research a highly translational plan investigating a cell-free peptide therapeutic strategy for post-MI HF with **NPA7**, which is our most innovative, and paradigm shifting **first-in-class multivalent therapeutic peptide.** Here we engineered a single peptide through the structural modification of two endogenous cardioprotective peptides, which function through two separate receptor and second messenger pathways which are present in all cell types in the human heart. **NPA7**'s architecture is constructed around the key amino acid sequence of B-type natriuretic peptide (BNP), which we reported possesses reverse remodeling properties in two proof of concept trials related to acute MI and chronic HF. BNP activates the guanylyl cyclase A receptor (GC-A) and elicits potent anti-apoptotic, anti-hypertrophic, aldosterone inhibiting and vascular regenerating properties through generation of cGMP. In engineering **NPA7,** we fused BNP_{10-32} to $angiotensin_{1-7}$ (ANG_{1-7}) to recruit cardioprotective actions of ANG_{1-7} via the Mas receptor (MasR) and the second messenger cAMP. Importantly, ANG_{1-7}/MasR also inhibits apoptosis but has additional potent anti-inflammatory actions and stimulates progenitor cells *in vitro* and *in vivo.*

Our **hypothesis** is that the **cell-free therapeutic peptide NPA7** promotes optimal healing and recovery by facilitating reparative and regenerative processes in the heart. Specifically, NPA7 will reduce apoptosis and inflammation, increase angiogenesis and cardiomyocyte proliferation and inhibit pathological fibrosis. Studies are proposed in human myocardial cells (cardiomyocytes, endothelial cells and cardiac fibroblasts) *in vitro* and in a rodent model of MI *in vivo*.

Our **Specific Aims** are:

Aim 1: Establish *in vitro* the protective properties of **NPA7** in suppressing apoptosis in human CMs, promoting angiogenesis in human ECs and reducing proliferation of human CFs.
Aim 2: Establish *in vivo* in a model of MI-induced permanent myocardial injury the protective actions of **NPA7** in enhancing myocardial function with suppression of apoptosis, increasing angiogenesis, and inhibiting fibrosis.

Summary of Progress Year 1: Below we summarize major progress in Year One of funding. Studies have validated activation of dual pGC-A/MasR activation by NPA7 and activation of respective second messengers; presence of both pGC-A and MasR in human cardiomyocytes and suppression of cardiomyocyte apoptosis by NPA7 *in vitro.*

NPA7 binds to pGC-A and MasR in GCA++ and MasR++ HEK293cells

We used HEK293 cells overexpressing either human pGC-A or human MasR to assess *in vitro* binding of NPA7 with pGC-A receptors and MasR in GCA++ and MAS++ HEK293 cells, respectively. These studies demonstrated that NPA7 successfully binds to pGC-A and MasR in GCA++ and MAS++ cells. Furthermore, we demonstrated that addition of MasR-i (MasR inhibitor) was a dose-independent competitive inhibitor for binding of NPA7 to the MasR underscoring the binding of NPA7 to MasR.

NPA7 activates second messengers cGMP (pGC-A) and cAMP (MasR) production in HEK293 cells overexpressing pGC-A and MasR in vitro

We demonstrated the action of ANG1-7, BNP and NPA7 on generation of the second messengers of the pGC-A receptor and MasR, cGMP and cAMP, respectively also in HEK293 cells. In GCA++ cells, treatment with ANG1-7 did not induce cGMP production, while treatment with BNP and NPA7 resulted in significant and comparable cGMP activation. Further, when compared with ANG1-7 and BNP treatment, only treatment with 10^{-7} M NPA7 significantly increased production of the second messenger cAMP in MAS++ cells.

NPA7s receptor targets, pGC-A and MasR are present in human cardiac fibroblasts

We performed immunofluorescent staining to determine presence of NPA7s receptor targets in heart. We observed that pGC-A receptors and Mas receptors are ubiquitously expressed in human cardiac fibroblasts, providing further rationale for NPA7 treatment *in vivo*.

NPA7 increases NO production in human endothelial cells in vitro

Importantly, ANG1-7 via the MasR activates the PI3-K/AKT/NOS3 pathway, stimulating cGMP production in an NO-dependent manner. Therefore, treatment with NPA7 would yield higher NO activity than single-BNP treatment in cells expressing NOS. Accordingly, we studied Nitrite/Nitrate ratio as a read-out of NO production. Treatment with ANG1-7 and NPA7 significantly increased NO production in HAECs. Treatment with BNP on the other hand, had no effect on NO generation *in vitro.* Importantly, addition of the MasR-i A779 to NPA7-treatment significantly reduced NO production. This action may contribute to NPA7 induced angiogenesis.

NPA7 suppresses Staurosporine induced apoptosis in human cardiomyocytes

Human cardiomyocytes (HCM) were isolated and cultured. We assessed real time apoptosis employing an imaging system (Incucyte) over a 32-hour period. Apoptosis was induced at time 0 hour with Staurosporine (100 mg/ml) with peak apoptosis at 15 hr which decreased 25%

from peak at 32 hours. As controls, HCMs were treated with NPA7 at two doses (10^{-8} and 10^{-6}M) alone without Staurosporine and imaged real time for 32 hours. NPA7 had no effect and was devoid of intrinsic apoptotic actions. In Staurosporine treated cells, NPA7 was potently anti-apoptotic in a dose dependent manner reducing apoptosis throughout the 32-hour period by 50% at low dose and by 75% at high dose. Thus, a major goal of Aim 1 was successful and demonstrated that NPA7 has potent anti-apoptotic actions *in vitro* in HCM.

Presentations and Publications: The data described above will be presented at the Annual Meeting of the American College of Cardiology meeting in Washington DC in March 2017 and specifically in the Young Investigator Competition. A manuscript is being prepared for the *Journal of the American College of Cardiology.*

Grants: In 2016, an NIH grant was awarded (HL36634) to support laboratory-based studies on NPA7 to develop it as a peptide for the treatment of heart failure. Support is from 2016 to 2020.

Patents: No new patents.

Budget Update: All funds ($125,000) for Year One have been spent.

Reporting to all Minnesotans: While more people are surviving a heart attack, the heart muscle remains injured and can lead to congestive heart failure with retention of salt and water, shortness of breath and weakness. Our drug being developed at Mayo Clinic could protect the heart and even heal the heart after heart attack thus reducing the risk for heart failure.