Supplemental data for

Ribonucleotide Reductase-Mediated Increase in dATP Improves Cardiac Performance Via Myosin Activation in a Large Animal Model of Heart Failure

Running title: Gene therapy of BB-R12 improves cardiac function

Shin Kadota MD PhD[1,2]; John Carey BA, SRS[4]; Hans Reinecke PhD[1,2]; James Leggett MD[6]; Sam Teichman MD[6]; Michael A. Laflamme MD PhD[1,2]; Charles E. Murry MD PhD[1,2,3,4]; Michael Regnier PhD[2,3*]; and Gregory G. Mahairas PhD[6*]

[1]Department of Pathology; [2]Center for Cardiovascular Biology, Institute for Stem Cell and Regenerative Medicine; [3]Department of Bioengineering; [4]Department of Medicine/Cardiology, University of Washington, Seattle, WA; [5] North American Science Associates Inc. Northwood, OH, [6]BEAT Biotherapeutics Corp. Seattle WA.

Supplemental Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). All experiments involving animals were conducted in accordance with the Guide for the Use and Care of Laboratory Animals, and all of the animal protocols have been approved by the Institutional Animal Care and Use Committee at North American Science Associates Inc. Northwood, OH (NAMSA).

Swine MI/HF model

Myocardial infarction (MI) was induced in Yucatan mini-pigs (aged 7-11 months, weight 31-50 kgs) by 75 minutes of occlusion with a balloon catheter of the mid-left anterior descending artery. Disposition of the animals is described in **Figure 1.**

Pre-Treatment and Anesthesia Prior to Myocardial Infarct Procedure.

Amiodarone (1000 mg, PO, SID) was administered daily 1-5 days prior to MI induction. Solid food was withheld for approximately 12-24 hours prior to treatment and animals were weighed and given an intramuscular (IM) injection of the antibiotic enrofloxacin at 2-8 mg/kg, 30 minutes prior to surgery. Each animal was subcutaneously (SC) injected with the analgesic buprenorphine at .01-0.1 mg/kg. General anesthesia was induced with an IM injection of Telazol® at 6mg/kg followed by mask administration of isoflurane (0.5-3%), and Propofol (2 mg/kg-10 mg/kg [3-5ml], to effect; ensuring the animal maintain normal breathing during administration. Ophthalmic ointment was applied to both eyes to prevent corneal drying. Intravenous catheter(s) where placed in a peripheral ear vein and amiodarone (150 mg IV over 10 minutes) and lidocaine (1 mg/kg IV) slowly administered during induction to prevent arrhythmia. When a sufficient level of anesthesia is reached, an endotracheal tube was placed and each animal will be attached to an anesthesia machine. Each animal was maintained on isoflurane anesthesia

for the remainder of the preparation and surgical procedure. **Supplemental Table 5** summarizes the agents that were used during induction.

Procedural Preparation and Peri-Procedural Medications

The surgical incision site(s) was cleaned of debris and hair and scrubbed with a germicidal soap, wiped with 70% alcohol, painted with povidone iodine and draped. Surgical incision sites included the left or right femoral/inguinal regions, and the left or right ventral neck area. Animals were placed in a dorsal recumbent position and monitoring leads placed (EKG, pulse oximeter, temperature probe, etc.).

Isoflurane (0.5-3.0%, inhalant) was administered to maintain the anesthetic plane, and LRS, or an equivalent, will be given at 1-10 ml/kg/hr, IV for fluid maintenance. **Supplemental Table 6** outlines additional medications that may be used peri-procedurally to treat arrhythmia, and/or prevent coagulation.

MI Induction

MI induction will be performed by inducing a myocardial infarction of a selected area of the left ventricle, performed by a temporary (at least 75 minutes) 100% occlusion of LAD just past the first diagonal branch and was performed via the arterial access site used in the hemodynamic data collection.

Under fluoroscopy an appropriately sized guide catheter (5-6 Fr JR, MPA1, AL1-LBT or HS, Cordis, Bridgewater, NJ) was advanced to the left main coronary artery with the use of a 0.035" guidewire. A coronary angiogram was performed to visualize the major branches of the left coronary circuit and its tributaries. The target site for occlusion will then be identified and recorded. A balloon catheter (2.5-4.0 × 15 mm, Cordis EMPIRA) was then advanced to the occlusion site, with the aid of a 0.014" guidewire (Cho ICE Floppy, Boston Scientific, Marlborough, MA). The balloon was inflated for 75 minutes. Upon inflation, an angiogram was performed to ensure 100% occlusion of the target site.

After the ischemic period is complete, the balloon was slowly deflated over up to three minutes (if tolerated) and the balloon and guide catheters removed from the left main coronary artery. Upon

confirming the animal is stable, a final angiogram was performed. All catheters and introducers were then removed, and the access site closed using standard technique.

Management of Critical Reactions to Model Creation

Critical reaction to the model induction procedure was anticipated and treated if necessary by the veterinary staff. Expected adverse events include electrocardiographic changes resulting in asystole and ventricular fibrillation. These instances were treated with immediate defibrillation and administration of the medications listed in **Supplemental Table 6**.

Infarct Recovery Phase

Once the animal is observed to be stable (extubated and sternal), it was transferred to its individual pen. Post-operative analgesia (buprenorphine at 0.01-0.02 mg/kg) and antiarrhythmics (Amiodarone 1000 mg, PO, SID) were administered at the discretion of the test site veterinary staff. The animals were injected IM with the antibiotic enrofloxacin at 2-8 mg/kg for the first 2 post-operative days.

Vector design and Production

AAV6 ^{cTnT455} Rm1 Rm2 (BB-R12) contains a 4,431 base pair (bp) transgene cassette consisting of the 585 bp upstream promoter region of cTnT containing a duplication of the E1 enhancer element fused to 2379 bp Rm1 of human RNR codon optimized from GENBANK NM_001033.3 followed sequentially by a 63 bp porcine 2A sequence fused to the 1170 bp Rm2 of RNR codon optimized from GENBANK NM_001034 followed by a 163 bp synthetic poly A sequence. The entire transgene cassette is 4,431 bp in size and is cloned between the ITR sequences of AAV2. BB-R12 was produced in insect cells.[1, 2] High titer BB-R12 stocks (1.5 X 10^{13} Viral genome (VG)/ml) was formulated in PBS containing 5 mM Ca and 5 mM $MgCl_2$ and further supplemented with 35mM $MgCl_2$.

Test & Control Article Preparation

Two weeks after induction of MI, survived animals received intracoronary infusions of BB-R12. Antegrade coronary infusion of BB-R12 was performed based on the previous reports.[3, 4] To administer

the test or control article, femoral access was via percutaneous puncture or surgical cutdown for the placement of a 6/7-Fr sheath. After sheath insertion, heparin (100-200 units/kg) was administered IV to maintain an activated coagulation time (ACT) of 250-300 seconds. A 5/6-Fr guiding catheter was advanced to the left coronary artery and after angiogram, two 0.014-inch guide wires advanced, one into the left anterior descending (LAD) artery and one into the left circumflex (LCX) artery to fix the position of the catheter. To prepare the control article or BB-R12 test material, arterial blood (~10 ml) was drawn into a 20 ml syringe from the femoral sheath or catheter and diluted with 10 ml of 0.9% sodium chloride solution (saline) and the appropriate amount of PBS or BB-R12 added and mixed into the syringe using a needle. For flushing the residual test or control article from the catheter lumen, arterial blood (~10 ml) was drawn into a second 20 ml syringe from the femoral sheath or catheter and diluted with 10 ml of 0.9% sodium chloride solution (saline).

Test & Control Article Delivery

The tubing and catheter was primed with 4 ml of diluted blood from the flushing syringe. Intravenous nitroglycerin (1µg/kg/min) was initiated through the ear vein. Control or test article was administered through the guide catheter which was placed at the proximal left main tract and the test or control article solution (12-15 ml) injected at a rate of 1 mL/min through the guide over 12-15 minutes using an infusion pump into the left coronary artery. This was followed by injection of the flush solution (5 ml for 5 minutes). A wire and a catheter was then be fixed to right coronary artery the same way as left coronary artery. The remaining test or control article solution (5-8 ml) was injected into the right coronary artery for 5-8 minutes and followed by injection of the flush solution (5 mL over 5 minutes). After completing the administration of the test or control article, the guide catheter and introducer will be removed. Before removal of the sheath, ACT was allowed to return to baseline.

Pathology

Animal wellness was monitored through daily observations, adverse events and body weight. Clinical pathology samples were analyzed for clinical chemistry panel (electrolytes, hepatic, renal,

metabolic), CBC and coagulation panel. At necropsy, all macroscopic alterations in the examined tissues and organs were recorded and selected organs weighed. Fresh specimens for the biodistribution analysis were collected from the heart, liver, kidney, spleen, lungs, brain, skeletal muscle and testes. Microscopic analyses of the following tissues were performed: Adrenals, aorta, brain, heart, kidneys, liver, lungs and bronchi, lymph nodes, ovaries, spleen, testes and thymus.

The heart ventricles were trimmed by serially slicing the ventricles in a plane parallel to the atrioventricular groove. The ventricular slice that incorporated the central area of the infarct was further subdivided to provide sections of infarct border, central infarct and uninfarcted left ventricular free wall, interventricular septum and right ventricular free wall for microscopic evaluation. In addition, sections of the left and right coronary groove were collected. Heart ventricular tissues were routinely processed, embedded in paraffin, and sectioned at approximately 5 µm. Heart ventricular tissues were stained with H&E and Masson's Trichrome for evaluation.

Echocardiography

Transthoracic echocardiography (Acuson Cypress, Siemens Medical Solutions, Malvern, PA) was performed on anesthetized animals prior to beginning the surgical procedure, and at each of the specified follow-up timepoints. Images were obtained of multiple heart cycles in orthogonal long axis views, as well as short axis views of the left ventricle. End systolic and end diastolic images were captured for measurement of left ventricular volumes and quantification of ejection fractions. Echocardiography data was blinded for analysis.

Hemodynamics

Hemodynamic data collection was performed prior to infarct creation and during scheduled follow-up procedures to obtain information on cardiac performance. Arterial and venous pressure monitoring catheters were introduced via the left or right femoral vein and artery, or the left or right

carotid artery and right jugular vein. These access sites were exposed through surgical cut-down and instrumented with a 6-9F vascular introducer (Avanti+, Cordis). Under fluoroscopy, a Swan-Ganz CCO catheter (Edwards LifeSciences, Irvine, CA) was advanced to various locations of the right heart for data collection, including pulmonary capillary wedge pressure (PCWP), pulmonary artery pressure (PAP), central venous pressure (CVP), and cardiac output (CO). A Millar Mikro-Tip catheter (Millar Inc., Houston TX) was advanced through the arterial circuit via an appropriately sized guide catheter (Wiseguide, Boston Scientific) for data collection of AoP and LVP. For each of the pressure endpoints, a minimum of 5 heart cycles was recorded with PowerLab data acquisition platform (ADInstruments, Colorado Springs, CO).

PCR

Frozen mini-pig tissue plugs of liver, lung, right ventricle, left atrium, and 3 samples from the left ventricle were obtained. Tissue samples were homogenized in Qiagen Buffer ATL containing proteinase K using the Omni Bead Ruptor homogenizer with 2.8 mm ceramic beads. The homogenized sample was incubated at 56°C for 10 minutes. DNA was extracted from the homogenized tissue using the Qiagen QIAamp 96 DNA QIAcube HT Kit. Blood samples were incubated in Buffer ATL containing proteinase K at 56°C for 10 minutes. DNA was extracted from lysed blood samples using the Qiagen 96 QIAamp QIAcube HT Kit.

Primers and probes specific for the transgene, *rm1*, inserted in the AAV vector were designed, qualified, and shown to be fit for purpose. qPCR was performed using the Qiagen QuantiFast Pathogen PCR plus IC kit and the AAV specific primers and probes. The QuantiFast Pathogen PCR plus IC kit contained internal amplification control (IAC) primers, probes, and control IAC DNA that was added to each PCR reaction. The IAC assay was used to monitor each PCR reaction for inhibition. The IAC DNA was added to the reaction at a low level to prevent competition with the Rm1 assay, but at a level to monitor for inhibitors in the extracted DNA samples. Samples that had lower levels of AAV amplification (30 Ct or higher), and a Ct difference between the IAC amplification of the negative control and the

sample of 2 Cts or more were considered inhibitory and diluted for reanalysis. The use of the IAC mitigates false negative reporting due to inhibitors in the extracted sample.

PCR analysis was conducted in an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City CA) with software SDS 2.2.2, using the following PCR run conditions: activation at 95°C for 5 minutes, 45 cycles of denaturing at 95°C for 15 seconds and annealing and elongation at 60°C for 30 seconds. Fluorescence was monitored during the annealing and elongation portion of the 45 cycles. The DNA samples were analyzed in triplicate. The copy number for each DNA sample was calculated from the average cycle threshold (Ct) values for each sample using the linear regression analysis of the standard curve for each PCR plate. The DNA concentration was measured for each sample and the quantity of AAV was reported as copy number per microgram of DNA. The assay has a limit of detection of 10 copies. The lower limit of quantification for the assay was 40 copies per µg of DNA.

A 6-point standard curve was prepared fresh on the day of analysis. These standards were run in duplicate on each PCR plate. The copy number of the standards ranged from 9.95×10^6 per reaction to 99.5 copies per reaction.

Blood Sampling and testing

Whole blood and serum (5 ml ea.) was collected at baseline, treatment, and days 14, 28 and 56 post treatment and subjected to a full hematology, coagulation and clinical chemistry panel (Antec GLP, Morrisville, NC).

Statistical analysis

All values are reported as mean ± standard error of the mean (SEM). Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd, Japan) was used for all statistical analyses. Student's t test was used to test for differences between two groups if normal distribution was assumed. Normal distribution was examined with frequency histograms. In case of nonparametric distribution, Mann-

Whitney U test was used. Analysis of variance (ANOVA) was used to test for differences among at least three groups. A P value of <0.05 was considered statistically significant.

Supplemental References

1. Smith RH, Levy JR, Kotin RM. A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells. Molecular therapy : the journal of the American Society of Gene Therapy 2009;**17**(11):1888-96.
2. Li L, Dimitriadis EK, Yang Y, Li J, Yuan Z, Qiao C, Beley C, Smith RH, Garcia L, Kotin RM. Production and characterization of novel recombinant adeno-associated virus replicative-form genomes: a eukaryotic source of DNA for gene transfer. PloS one 2013;**8**(8):e69879.
3. Ishikawa K, Aguero J, Naim C, Fish K, Hajjar RJ. Percutaneous approaches for efficient cardiac gene delivery. Journal of cardiovascular translational research 2013;**6**(4):649-59.
4. Ishikawa K, Fish KM, Tilemann L, Rapti K, Aguero J, Santos-Gallego CG, Lee A, Karakikes I, Xie C, Akar FG, Shimada YJ, Gwathmey JK, Asokan A, McPhee S, Samulski J, Samulski RJ, Sigg DC, Weber T, Kranias EG, Hajjar RJ. Cardiac I-1c Overexpression With Reengineered AAV Improves Cardiac Function in Swine Ischemic Heart Failure. Molecular therapy : the journal of the American Society of Gene Therapy 2014;**22**(12):2038-45.