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TRANSLATIONAL PERSPECTIVES

Translation of Cardiac Myosin Activation With 2-Deoxy-ATP to Treat Heart Failure Via an Experimental Ribonucleotide Reductase-Based Gene Therapy

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SUMMARY

Despite recent advances, chronic heart failure remains a significant and growing unmet medical need, reaching epidemic proportions carrying substantial morbidity, mortality, and costs. A safe and convenient therapeutic agent that produces sustained inotropic effects could ameliorate symptoms and improve functional capacity and quality of life. The authors discovered that small amounts of 2-deoxy-ATP (dATP) activate cardiac myosin leading to enhanced contractility in normal and failing heart muscle. Cardiac myosin activation triggers faster myosin cross-bridge cycling with greater force generation during each contraction. They describe the rationale and results of a translational medicine effort to increase dATP levels using a gene therapy strategy that up-regulates ribonucleotide reductase, the rate-limiting enzyme for dATP synthesis, selectively in cardiomyocytes. In small and large animal models of heart failure, a single dose of this gene therapy has led to sustained inotropic effects with no toxicity or safety concerns identified to date. Further animal studies are being conducted with the goal of testing this agent in patients with heart failure. (J Am Coll Cardiol Basic Trans Science 2016;1:666–79) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Chronic heart failure (HF) is a significant and growing cause of morbidity, mortality, hospitalizations, and medical costs. Current therapies for chronic HF only slow progression or treat complications of the disease but do not rescue cardiac function. In patients with moderate-to-severe HF with reduced ejection fraction, decreased cardiac systolic function is an underlying cause of clinical manifestations, including development and worsening of symptoms, declining functional capacity, episodes of decompensation requiring hospitalization, and premature death. Chronic use of inotropic agents acting

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through the adrenergic system or cyclic AMP has improved symptoms and exercise capacity by increasing ejection fraction, but the benefit of these drugs has been limited by long-term safety and tolerance issues and by the need for parenteral administration (1–3). Importantly, data from patients with cardiac resynchronization and left ventricular (LV) assist devices suggest chronic improvement of cardiac function may prevent or reverse the progression of HF (4,5).

Gene therapy approaches to treat HF have primarily targeted improvement of dysfunctional calcium (Ca^{2+}) cycling that triggers myosin contractions, either directly or through the adrenergic system and cyclic AMP (**Figure 1A**) (6,7). The 3 most advanced programs have completed clinical trials, including gene therapy targeting sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA2a), which regulates Ca^{2+} movement between the cytoplasm and the sarcoplasmic reticulum; stromal cell-derived factor (SDF)-1, which is involved in endogenous myocardial repair; and adenylyl-cyclase type 6 (AC6), which catalyzes cyclic AMP formation (7). Unfortunately, these recently published trials did not meet their stated objectives (8–10).

An alternative and promising method of improving cardiac function in HF patients is to target cardiac myosin directly instead of altering Ca^{2+} regulation within the cell (11). Cardiac myosin activation triggers greater force generation during each contraction. This approach bypasses the adrenergic and cyclic AMP systems, as well as the complex Ca^{2+} regulation mechanisms (**Figures 1A and 1B**). Omecamtiv mecarbil is the only cardiac myosin-activating drug currently in development, and it has advanced to large-scale clinical trials in HF patients with encouraging results (12–17). Omecamtiv is a myocardial ATPase activator that increases LV function independent of Ca^{2+} levels (**Figures 1A to 1C**). However, as a small organic molecule, omecamtiv mecarbil requires delivery by parenteral administration or repeat oral dosing (12). By contrast, a gene therapy approach to cardiac myosin activation would be expected to have a durable effect on cardiac function after a single dose. Here, we review the published work on the development of BB-R12 (AAV6 viral vector with a cardiac-specific promoter cTnT455 to overexpress R1R2 [ribonucleotide reductase, containing R1 (Rrm1) and R2 (Rrm2) subunits] in the heart), a novel gene therapy for HF that targets myocardial contractility directly by increasing production of 2-deoxy-ATP (dATP) in cardiomyocytes.

OVERVIEW

On the basis of observations that dATP is a more efficient contractile substrate for myosin than ATP, we hypothesized that increasing intracellular dATP levels in the myocardium could lead to improved cardiac function. dATP exerts positive inotropic effects on cardiomyocytes as a complementary energy source to ATP via direct myosin interaction. Low-level dATP production occurs normally in mammalian cells via the ribonucleotide reductase (R1R2) enzyme, which catalyzes the removal of a hydroxyl group from the 2 position on the ribose ring of ADP to produce dADP, which is then rapidly converted to dATP via phosphorylation by creatine kinase. Small amounts of dATP (and other deoxynucleoside triphosphates [NTPs]) are normally produced by R1R2 as a substrate for DNA synthesis and repair in replicating cells (**Figure 1C**).

R1R2 is well characterized and consists of 2 subunits: the larger R1 subunit contains the catalytic site and 2 allosteric sites that can bind dATP, whereas the smaller R2 subunit contains the free radical generator. R1R2 is tightly allosterically regulated, with ≤5% of the ATP pool present as dATP (18,19). However, because cardiomyocytes are nonreplicating cells, R1R2 expression is markedly down-regulated and cardiomyocyte dATP levels are <10% of those in other cells (20). dATP concentration can be increased by overexpression of R1R2, the rate-limiting enzyme in its production, using gene therapy.

BB-R12 is a designed multicomponent gene therapy agent consisting of a recombinant serotype-6 adeno-associated viral vector (AAV6) carrying a genome containing a human cardiac troponin T regulatory cassette (hcTnT455) linked to a transgene encoding human sequences of both the large (R1) and small (R2) subunits of R1R2 (separated by a protein cleavage site) to overexpress the enzyme in myocardium. Although BB-R12 is nominally a gene therapy, the therapeutic algorithm employed is novel. Rather than correcting a genetic or proteomic defect, increased dATP levels act as a locally produced inotropic small molecule treatment for the failing heart. In essence, BB-R12 creates a drug production and delivery system within the heart using gene therapy. Local, intracellular production of dATP may also lower the risk of off-target systemic side effects. On the basis of the initial insight that dATP is a more efficient energy source for muscle contraction, the gene therapy

ABBREVIATIONS AND ACRONYMS

+dP/dt = maximum rate of pressure rise

−dP/dt = maximum rate of pressure decline

dATP = 2-deoxy-adenosine triphosphate

DCM = dilated cardiomyopathy

hESC-CM = human embryonic stem cell-derived cardiomyocyte

HF = heart failure

LV = left ventricular

LVEDP = left ventricular end-diastolic pressure

LVEF = left ventricular ejection fraction

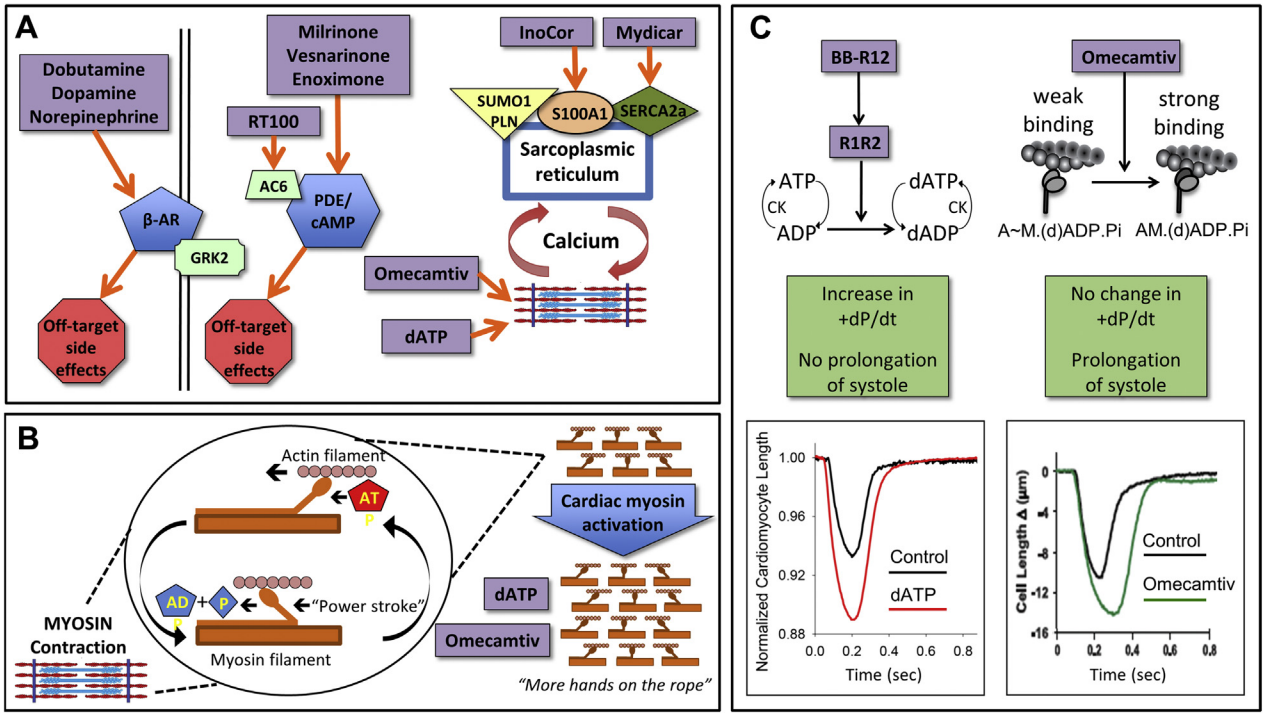
MI = myocardial infarction

NTP = nucleoside triphosphate

vg = vector genome

WT = wild-type

FIGURE 1 Schematic Illustrations of Selected Inotropic Agents and Gene Therapies for Treatment of HF



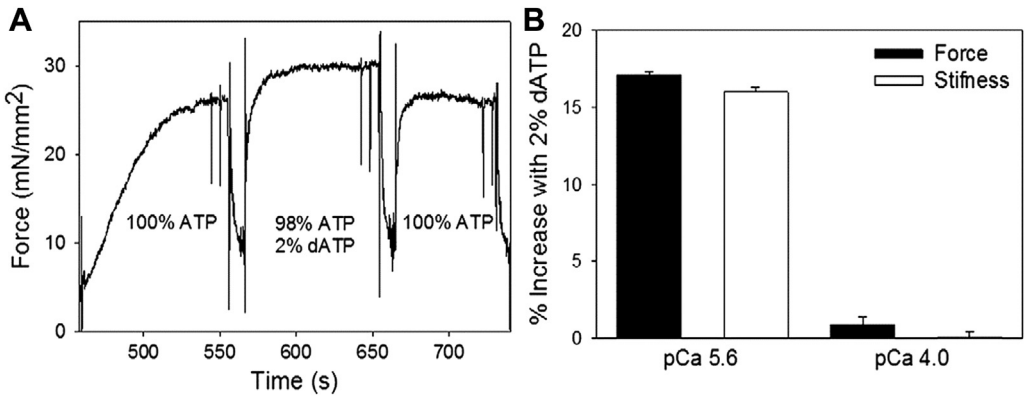
Schematic illustrations depicting targets and mechanisms of action of selected inotropic agents and gene therapies for treatment of heart failure (HF) highlighting cardiac myosin activators BB-R12 and omecamtiv. **(A)** 2-deoxy-ATP (dATP) and omecamtiv are cardiac myosin activators that act directly on the contractile apparatus. Gene therapies that act on calcium cycling and regulation work "upstream" of myosin. Drugs and gene therapies that act on the β-adrenergic system or inhibit phosphodiesterase and cyclic AMP work further upstream of the contractile apparatus and calcium regulation. **(B)** Myosin heads form transient cross-bridges with actin. ATP fuels a conformational change ("power stroke") causing actin to slide past myosin, shortening sarcomere length and causing contraction. Cardiac myosin activators enable more myosin heads (independent force generators) to interact with actin per cardiac cycle (i.e., more active cross-bridges). This has been characterized as "more hands pulling on the rope" and increases the maximum force of contraction. **(C)** Molecular modeling indicates dATP detaches from myosin more rapidly than ATP, enabling faster cross-bridge cycling and more rapid force generation (i.e., increase in maximum rate of pressure rise [+dP/dt]). Replicating cells normally have a small supply of dATP for DNA synthesis and repair. Ribonucleotide reductase (R1R2) converts ATP to dATP under tight allosteric regulation but is down-regulated in nonreplicating cardiomyocytes. BB-R12 provides the gene for R1R2 under control of a cardiac troponin promoter, restricting synthesis to cardiomyocytes. dATP increases the maximum force of contraction, increases +dP/dt and −dP/dt, and does not prolong systole. Omecamtiv activates myosin by increasing the transition rate from weak to strong binding states between myosin and actin, increases the maximum force of contraction without a change in +dP/dt or −dP/dt, and prolongs systole. Omecamtiv figure adapted with permission from Malik et al. (12). AC6 = adenylyl-cyclase type 6; β-AR = beta-adrenergic receptor; BB-R12 = AAV6-cTnT-R1R2; cAMP = cyclic AMP; CK = creatine kinase; GRK2 = G-protein-coupled receptor kinase-2; PDE = phosphodiesterase; PLN = phospholamban; R1R2 = ribonucleotide reductase; SERCA2a = sarcoplasmic/endoplasmic reticulum calcium ATPase; SUMO1 = small ubiquitin-related modifier 1.

approach of up-regulating myocardial R1R2 has progressed from testing in cardiomyocytes and myofibrils, to small animal HF models, and most recently to large animal HF models. The results of these published studies are summarized in the following text. Further testing is underway with the goal of testing this therapy in patients with HF.

dATP IS A MORE POTENT ENERGY SOURCE FOR MYOSIN CONTRACTION. The original pharmacological insight that dATP is a more potent energy source for myosin contraction occurred almost 20 years ago. Studies evaluating ATP and ATP analogs (NTPs) for their effects on skeletal muscle contractility detailed the chemomechanical processes of myosin-actin cross-bridge cycling normally fueled by ATP. These studies showed increased cross-bridge cycling rates with dATP compared with ATP, indicating dATP improved myosin binding with faster detachment from actin, making dATP a more effective contractile substrate. Only dATP increased force generation compared with ATP, whereas all other NTPs and deoxy-NTPs produced weaker contractions (21-23).

Later studies showed dATP substitution for ATP in demembranated rat cardiac trabeculae resulted in increased isometric force at all levels of Ca^{2+} activation. Cardiac muscle "stiffness" (an experimental measurement of cross-bridge binding) and force were

FIGURE 2 Effects on Force Production and Stiffness



Effects on force production and stiffness with [2% dATP/98% ATP] compared with 100% ATP (5 mmol/l total) in rat cardiac muscle. **(A)** Example force trace (pCa 5.6) showing increased force production with transfer from 100% ATP to [2% dATP/98% ATP] and reduction of force after transfer back to 100% ATP. **(B)** % increases in force production and stiffness of cardiac trabeculae with [2% dATP/98% ATP] at submaximal (pCa 5.6) and maximal (pCa 4.0) Ca^{2+} levels. Adapted with permission from Korte et al. (20).

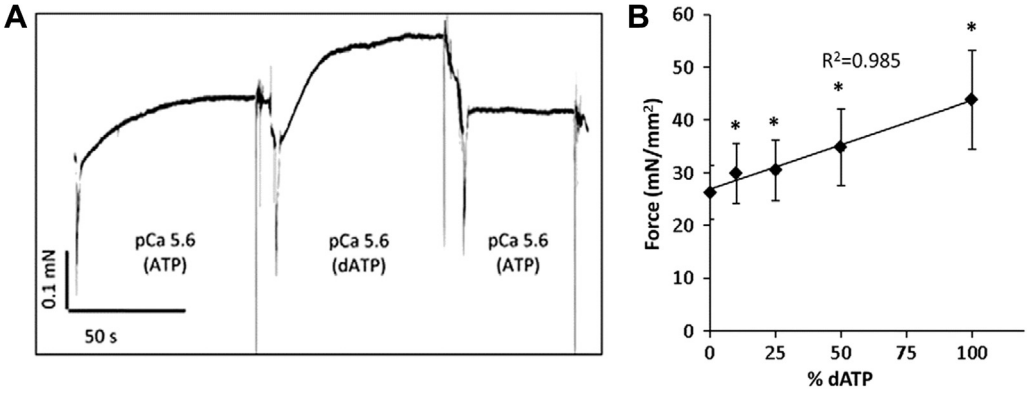
increased similarly with dATP, suggesting that increased isometric force resulted from increased cross-bridge recruitment. By contrast, dATP caused elevated stiffness and force generation in skeletal muscle only at submaximal Ca^{2+} levels, demonstrating a myosin-mediated enhancement of contraction that was more potent in cardiac muscle. dATP substitution for ATP reversibly increased force production, stiffness, cross-bridge cycling, and Ca^{2+} sensitivity of force in cardiac muscle. In both cardiac and skeletal muscle, contractile kinetics were enhanced with dATP; however, increased maximal force production was only seen in cardiac muscle (24,25).

Addition of low levels of dATP to the ATP pool at physiological Ca^{2+} levels resulted in significant increases in force development in rat cardiac muscle. Briefly, demembranated rat cardiac trabeculae were exposed to solutions containing 100% ATP (5 mmol/l) or [2% dATP/98% ATP] (5 mmol/l total) at submaximal (pCa 5.6) and maximal (pCa 4.0) Ca^{2+} concentrations. Force production at pCa 5.6 increased 17% with [2% dATP/98% ATP]. This increase in force was reversible upon transfer back to 100% ATP solution. Stiffness increased in proportion with force with [2% dATP/98% ATP] at pCa 5.6, indicating increased force production with dATP is due to increased cross-bridge binding. At pCa 4.0, these increases in force production and stiffness were not seen (**Figure 2**). This study demonstrated that even small amounts of cellular dATP added to ATP are sufficient to increase contractile force in cardiac tissue by increasing cross-bridge recruitment (20). These data, confirmed by the work of others, suggest that the analogy to a "fuel additive" is more appropriate than "higher-octane fuel" (26–28). These studies were the first indicator of the therapeutic potential of dATP.

Dilated cardiomyopathy (DCM), a prevalent form of HF, is characterized by ventricular dilatation and loss of systolic function. A widely accepted canine model of naturally occurring DCM was used to evaluate whether dATP could improve contractility in DCM cardiac tissues. When myofibrils isolated from DCM canine hearts were treated with dATP, contractility was significantly increased and function was restored to control (nonfailing) cardiac tissue levels, without affecting relaxation (29).

Cardiac tissue from patients with end-stage HF was treated ex vivo with dATP. Adult LV wall tissue was obtained from 16 end-stage HF patients undergoing LV assist device placement or cardiac transplantation. Stiffness and steady-state isometric force of demembranated tissues were measured in the presence of varying ratios of ATP:dATP (5 mmol/l total). Isolated cardiac myofibrils were used to assess the activation and relaxation kinetics with ATP or dATP (2 mmol/l). Contractility of the failing human cardiac muscle was significantly enhanced with dATP without affecting relaxation kinetics. Isometric force at saturating Ca^{2+} (pCa 4.5) increased 11% with dATP and increased 35% at submaximal Ca^{2+} levels in the range where the heart normally operates. Isometric force increased linearly with increasing dATP at pCa 5.6. Myofibrils showed a 13% increase in force production and a 44%

FIGURE 3 Effects of ATP Versus dATP



Effects of ATP versus dATP in ex vivo human cardiac tissue from end-stage HF patients demonstrating dATP increases force. **(A)** Representative isometric force trace of demembranated tissue at pCa 5.6 with ATP or dATP. **(B)** Force measured at pCa 5.6 with 10%, 25%, 50%, and 100% dATP compared with 100% ATP ($n = 4$ at each dATP concentration). Measurements are mean ± SEM. *$p < 0.05$ compared with 100% ATP by paired Student *t* test. Adapted with permission from Moussavi-Harami et al. (30).

increase in activation rate with dATP, with no effect on relaxation rates and no prolongation of systole. In agreement with rat cardiac tissue studies, increased force production, Ca^{2+} sensitivity, cross-bridge cycling, and activation rate at physiological Ca^{2+} levels were seen with low levels of dATP, resulting in increased force production and more robust contractile kinetics (**Figure 3**) (30).

Constitutive dATP up-regulation in vivo was studied in a transgenic mouse model overexpressing R1R2 (TgRR). R1R2 levels are typically higher in replicating than in quiescent cells or in post-mitotic differentiated cells such as cardiomyocytes, because deoxyribonucleotides in their triphosphate form are needed for DNA synthesis. This animal model initially addressed the challenge of providing cardiomyocytes with an adequate supply of dATP in vivo. Both in vivo and ex vivo cardiac assessments were conducted, including cardiac function, energetics, tissue morphology, gene expression, and cardiomyocyte contractility. TgRR mice overexpress both subunits of R1R2 via the chicken β-actin promoter and cytomegalovirus enhancer. Results showed adult TgRR mice have enhanced basal ventricular function in vivo (fractional shortening and ejection fraction) and produce increased contractile force and hemodynamic parameters ex vivo (LV developed pressure, +dP/dt [maximum rate of pressure rise, a parameter of systolic function], −dP/dt [maximum rate of pressure decline, a parameter of early diastolic function]), without exhibiting increased heart rates, cardiac hypertrophy, LV dilation, or adverse cardiac remodeling. Hearts responded to β-adrenergic challenge and performed similarly to normal hearts during high workload for 20 min. High-energy phosphocreatine reserves were mildly reduced at baseline (although levels remained high) but were similar to normal hearts after high workload challenge without affecting cellular ATP levels under normal conditions. No differences in body weight, heart weight, cardiomyocyte size, organization, or fibrosis were seen in hearts from TgRR and wild-type (WT) mice at 3 and 12 months, suggesting no hypertrophy or cardiac remodeling resulted from chronically elevated dATP. This transgenic animal model demonstrated elevated basal cardiac function can be maintained long-term with R1R2 overexpression without noticeable side effects or structural adaptation of the heart (31).

dATP MECHANISM OF ACTION

The potential underlying chemomechanical mechanisms for improved contractility seen with dATP were studied using molecular modeling and confirmed with motility assays. A well-characterized myosin structure in the pre-powerstroke state was used as a starting structure for molecular dynamics simulations (32), and atom-level differences in myosin structure and dynamics with dADP binding were evaluated. Simulations showed that when dADP.Pi (the nucleotide form present for actin binding) is in the nucleotide binding pocket of myosin, the PHE129 that normally binds to O2′ at the 2 position of the ribose

ring (missing in dATP) binds elsewhere on dADP and precipitates other changes in intramolecular contacts within the nucleotide binding pocket compared with ADP.Pi. This results in a change in binding pocket structure and nucleotide position within the pocket (**Figure 4A**). This local change causes myosin to undergo global conformational changes toward a conformation seen in actin binding states. dADP binding stabilizes a myosin conformation that is more energetically favorable for actin binding (closed cleft conformation), resulting in more exposed polar residues on the actin binding surface of myosin, thus increasing the probability of electrostatic interactions between actin and myosin (**Figure 4B**). Molecular dynamics simulation results were supported by motility assays, which indicated that dATP enhances weak binding electrostatic interactions between actin and myosin (S.G. Nowakowski, M. Regnier, V. Daggett, unpublished data, October 2016). These studies suggest that the missing hydroxyl group in dATP modifies myosin head structure in a manner resulting in an increased rate and affinity of actin binding and explain the previously observed increase in cross-bridge cycling (**Figure 4C**) (33). Due to increased electrostatic interactions, more myosin heads (independent force generators) interact more quickly with actin during each cardiac cycle, leading to a faster, stronger contraction. This has been measured as an increase in maximum force, increase in +dP/dt, increase in −dP/dt, and no prolongation of systole (**Figure 1C**).

Recently reported data from an independent group showed dATP increased myosin-actin sliding velocities by 40%, and increased ATPase activity, ADP release rates, and actin-binding affinities (34).

Omecamtiv mecarbil also increases Ca^{2+} sensitivity and force of contraction, but by contrast, through an increase in the probability of transition from weak to strong (force-producing) actin-myosin binding states (12,35). No increase in the kinetics of cross-bridge cycling has been reported, but it has been suggested that more myosin heads are recruited and have a prolonged interaction with actin. This has been measured as no change in +dP/dt or −dP/dt, and a prolongation of systole (**Figure 1C**).

TRANSLATIONAL MEDICINE CHALLENGE

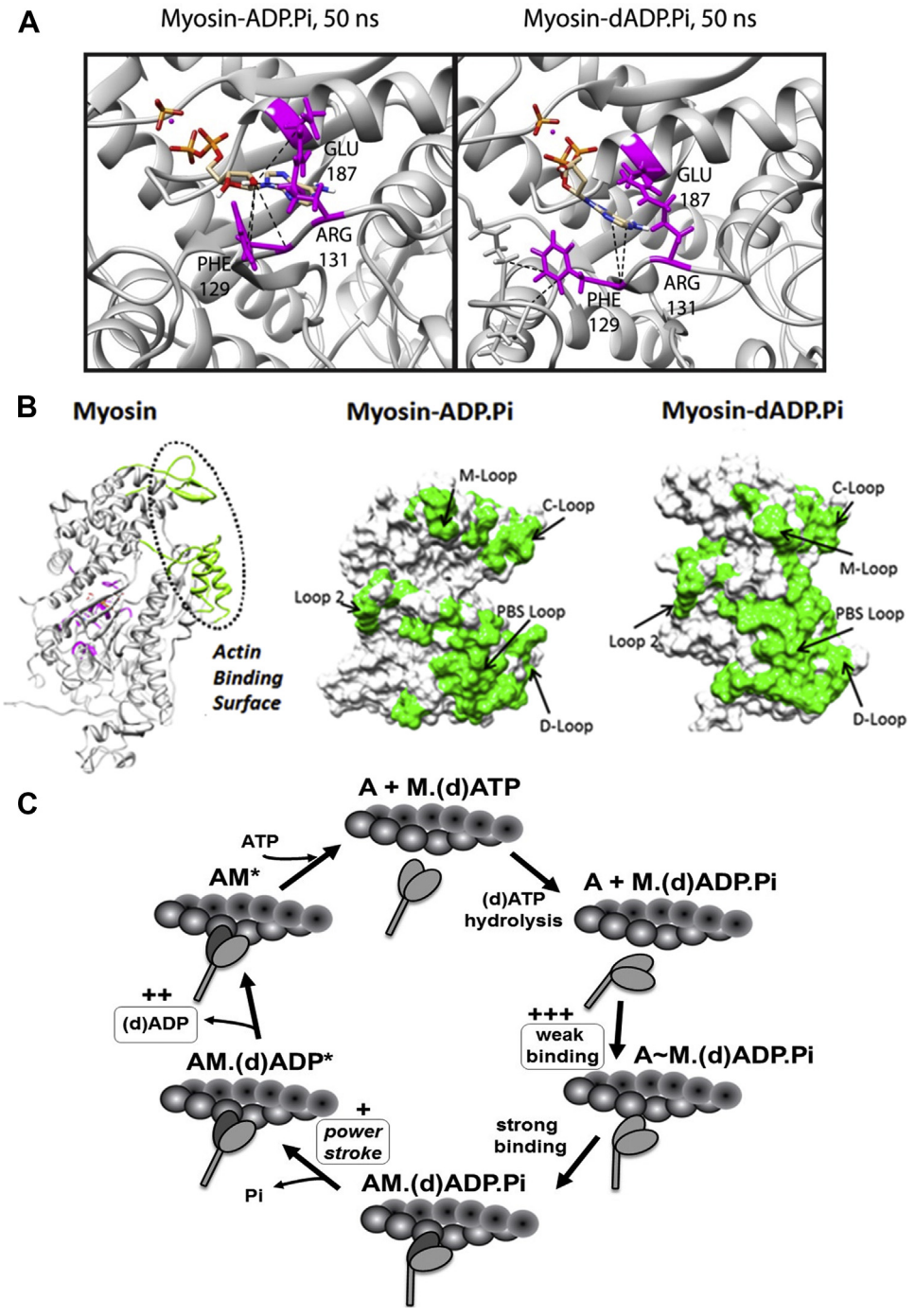
DEVELOPING A GENE-DELIVERY SYSTEM TO INCREASE dATP SPECIFICALLY IN CARDIAC TISSUE. Chronic administration of dATP directly to the heart is not a feasible strategy to achieve long-term therapeutic cardiac effects. The transgenic mouse model demonstrated sustained systemic overexpression of R1R2 increases basal cardiac function. To translate these findings into a viable therapy, a method of increasing intracellular dATP specifically in cardiomyocytes was needed, and viral vector systems were designed to overexpress R1R2.

The initial in vitro evaluation of viral-mediated R1R2 overexpression to increase cellular dATP concentration used a delivery system consisting of 2 separate recombinant adenoviral (AV) vectors expressing rat R1 or R2 subunits with a GFP reporter under the cytomegalovirus promoter. Vectors were administered simultaneously in equal doses (treatment referred to as AV-R1 + AV-R2 [single adenoviral vector expressing R1 subunit + single adenoviral vector expressing R2 subunit]) (**Table 1**). When rat cardiomyocytes were transduced with AV-R1 + AV-R2, intracellular dATP content, magnitude, and rate of contraction and relaxation all increased, without affecting Ca^{2+} transient properties. These results suggest that the increased contractility seen with R1R2 overexpression is due to increased myofilament responsiveness to Ca^{2+}. Cells treated with AV-R1 + AV-R2 maintained the relative increase in relaxation kinetics at all stimulation frequencies, indicating no impairment of relaxation. Contractile response was increased in treated cells compared with controls at all stimulation frequencies (**Figure 5A**). Cellular R1, R2, and dATP levels indicated a significant increase in R1 and R2 protein content, and a 10-fold increase in dATP over control cells, equating to approximately 1% of the total adenine nucleotide pool (**Figures 5B and 5D**). This study provided the first proof of principle that dATP levels could be increased in cardiomyocytes and that small elevations of dATP levels enhanced contractility (20).

When the AV-R1 + AV-R2 system was used analogously to transduce human cells (human embryonic stem cell-derived cardiomyocytes [hESC-CMs]), contractility was again significantly increased (36). To evaluate the therapeutic potential of this delivery system, cardiomyocytes isolated from infarcted adult rat hearts were transduced. Contractility of the treated infarcted cardiomyocytes was significantly improved compared with untreated infarct cells, with the magnitude and rate of contraction restored to levels comparable to healthy (uninfarcted) cardiomyocytes (37).

The transfer of small molecules such as ATP and dATP between cells is facilitated by gap junctions. It was hypothesized that dATP could diffuse through gap junctions between physically coupled cardiomyocytes to enhance contractility of neighboring cells that were not overexpressing R1R2. To test this, the transfer of fluorescein-labeled dATP via gap junctions between

FIGURE 4 dATP Mechanism of Action



(A) Molecular simulation results show the loss of O2′ on dADP disrupts contacts in the nucleotide binding pocket on myosin. Representative structures showing conformational changes within the nucleotide binding pocket at 50 ns with ADP and dADP. Phe129 **(magenta)** and the primary contacts it makes are shown **(dotted black lines)**. **(B)** Representative acting binding surface structures on myosin (**circled area** on ribbon structure) with ADP and dADP simulations at 50 ns, showing conformational changes in myosin resulting in increased exposure of polar residues **(green regions)** in the actin binding surface with dADP binding. Modeling figures adapted with permission from S.G. Nowakowski, M. Regnier, V. Daggett, unpublished data, October 2016. **(C)** Schematic illustrating the chemomechanical cycle of muscle contraction. Transitions between actin (A) and myosin (M) binding states are labeled. Transition states in **boxes** are where dATP alters the cycle, with the magnitude of the dATP effect indicated by the number of **plus symbols**. Adapted with permission from Regnier et al. (22).

AV-R1 + AV-R2 transduced and nontransduced (WT) cardiomyocytes (rat and human) and the resulting effects on contractility were measured. Rapid transfer of dATP between coupled cells (Figure 6A) was demonstrated, and this effect was blocked with a gap junction inhibitor. R1R2 overexpressing hESC-CMs had significantly larger contraction magnitudes than WT cells (Figures 6B and 6C). WT hESC-CMs coupled to AV-R1 + AV-R2–treated hESC-CMs also had increased contraction magnitudes and velocity, but showed no significant differences in time to peak contraction or time to 90% relaxation (Figure 6D). When R1R2-overexpressing hESC-CMs were transplanted into adult rat hearts in vivo, they delivered dATP to the host myocardium and significantly increased global cardiac performance within 5 days (Figure 6C) (36).

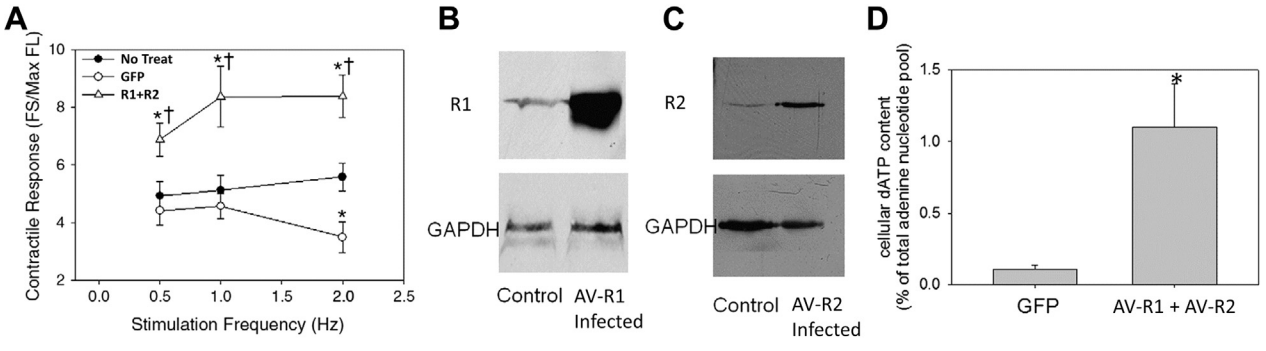
To reduce immune responses to the AV delivery system and achieve long-term expression of R1R2 in myocardium, AAV6 systems were developed that targeted cardiac tissue (Table 1). The next translational step was to develop a dual vector system consisting of 2 separate recombinant AAV6 vectors expressing rat sequences of R1 or R2 transcribed by the cardiac-specific regulatory cassette cTnT455 (containing highly modified enhancer and promoter regions of the human cardiac troponin gene *TNNT2*), to be delivered simultaneously at equal doses (referred to as Rat AAV6-R1 + AAV6-R2 [single adeno-associated virus serotype 6 (AAV6) vector expressing R1 subunit + single AAV6 vector expressing R2 subunit]). When delivered systemically via intravenous infusion to healthy mice, the resulting R1R2 overexpression increased global cardiac systolic function at all doses (1.5×10^{13}, 4.5×10^{13}, or 1.35×10^{14} total vector genomes (vg)/kg), and effects persisted for at least 13 weeks post-injection. A larger effect in the high-dose group was seen at 1 to 3 weeks and persisted, suggesting an early dose-response, but by 6 weeks, the effect in lower-dose groups reached the level seen in the high-dose group. These results indicate improvements in heart function seen with higher doses of vector-mediated R1R2 overexpression can be achieved over time with lower doses of vector (38). This is consistent with the hypothesis that transduction of fewer cardiomyocytes in the lower-dose groups required more time to establish a concentration gradient for dATP to diffuse from these cells to distal non-transduced cells.

TABLE 1 Description of Viral Vector Systems

Sequence Species	Viral Vector	Gene(s) Expressed	Vector System Design	Treatment Name
Rat	AV	R1 + GFP	2 separate vectors	AV-R1 + AV-R2
Rat	AV	R2 + GFP		
Rat	AAV6	R1	2 separate vectors	Rat AAV6-R1 + AAV6-R2
Rat	AAV6	R2		
Human	AAV6	R1	2 separate vectors	Human AAV6-R1 + AAV6-R2
Human	AAV6	R2		
Human	AAV6	R1-P2A-R2*	Single vector	Human AAV6-R1.2 (BB-R12)

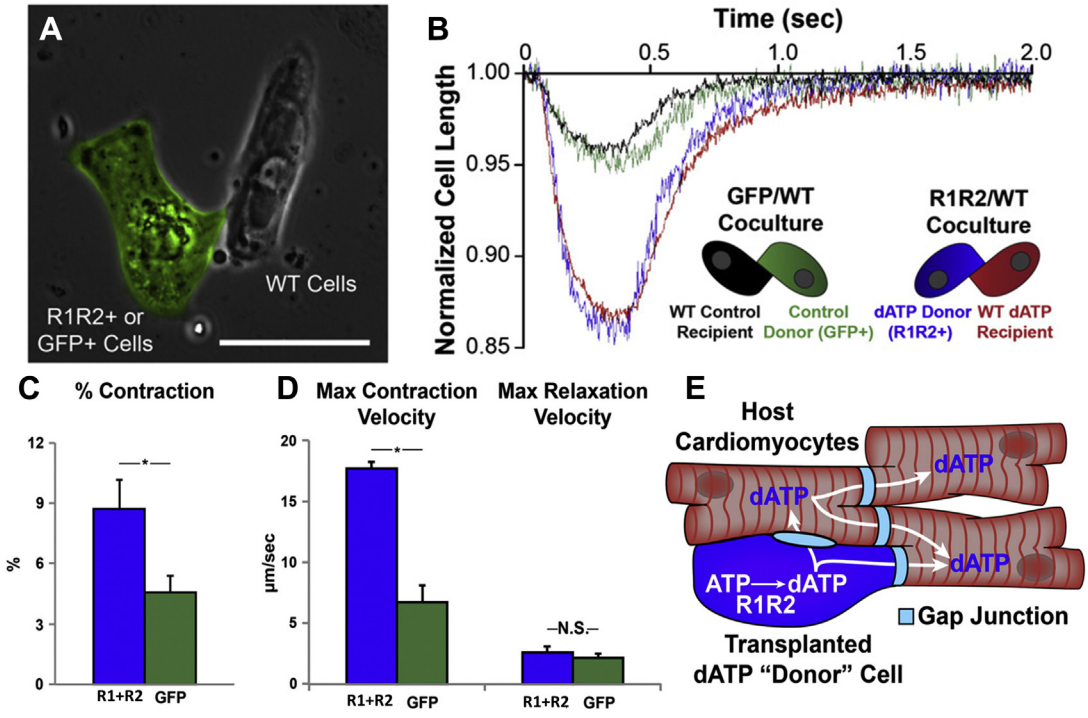
*P2A is a 20-amino acid peptide linker that enables cotranslation of 2 proteins from a single strand of mRNA and subsequent cleavage of the 2 proteins.

FIGURE 5 Effects of AV-R1 + AV-R2 Treatment on ARCs



(A) Contractile response of adult rat cardiomyocytes (ARCs) at different stimulation frequencies. AV-R1 + AV-R2–treated cells **(triangles)** showed significantly greater response to Ca^{2+} at all frequencies. AV-GFP–treated cells **(open circles)**; nontransduced cells **(solid circles)**. *$p < 0.05$ compared with nontransduced; †$p < 0.05$ compared with AV-GFP–treated. **(B to D)** R1R2 protein expression in ARCs after AV-R1 + AV-R2 treatment. Increased R1 **(B)** and R2 **(C)** protein expression in AV-R1 + AV-R2–treated neonatal rat ventricular myocytes (NRVMs). **(D)** Increased intracellular dATP in AV-R1 + AV-R2–treated NRVMs. *$p < 0.05$ compared with AV-GFP–treated NRVMs. GFP = green fluorescent protein. Adapted with permission from Korte et al. (20).

FIGURE 6 R1R2 Overexpression Increases hESC-CM Contractility and Enhances Contractility of Coupled Cardiomyocytes

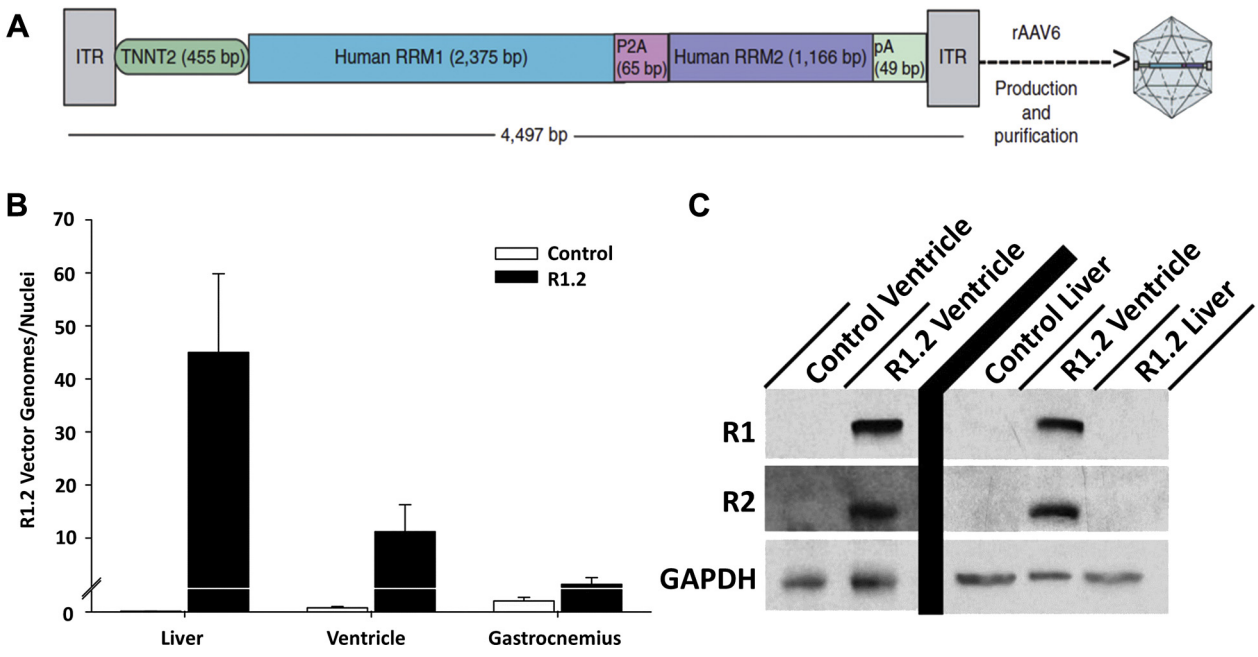


(A) Example of coupled green fluorescent protein (GFP)-expressing cell and WT cell. Contractile measurements were made individually on each cell in a doublet, as well as on uncoupled WT cells in culture. **(B)** Representative traces showing increased contractility of AV-R1 + AV-R2–treated cells and coupled WT cells compared with control cells. AV-R1 + AV-R2–treated hESC-CMs showed **(C)** significantly increased magnitude of contraction compared with AV-GFP–treated cells and **(D)** significantly increased maximum contraction velocity without affecting relaxation velocity. *$p < 0.05$; N.S. = not significant. **(E)** Schematic showing dATP generation in transplanted R1R2-overexpressing cells (dATP "Donor" cells) and the gap junction-mediated transfer of dATP through coupled host cardiomyocytes. hESC-CM = human embryonic stem cell-derived cardiomyocyte; WT = wild-type. Adapted with permission from Lundy et al. (36).

Further translational development led to a single AAV6 vector system containing a transgene cassette expressing optimized human sequences of both R1 and R2 subunits separated by a protein cleavage site (P2A internal ribosome entry site peptide) under a single cardiac-specific promoter (hcTnT455) (Table 1). This vector system, called BB-R12 (AAV6-R1.2 [single AAV6 vector expressing R1 & R2 subunits, linked by P2a] in this publication) (Figure 7A), was designed to restrict the intracellular R1R2 increase to cardiomyocytes. To evaluate this single vector system and determine whether the increased cardiac function seen with the dual vector systems could be replicated, BB-R12 was delivered systemically via tail vein injection (7×10^{13} vg/kg) to healthy adult mice, and cardiac function was monitored for 4 weeks compared with other vector systems (described in Table 1). Although significant viral genome uptake was seen in liver and ventricular tissues of treated mice (with negligible uptake in gastrocnemius tissue) (Figure 7B), increased levels of R1 and R2 proteins were only found in ventricular tissue (Figure 7C), confirming the cTnT455 promoter specificity. BB-R12 significantly increased basal cardiac function by 20% to 30%. β-Adrenergic signaling remained intact with BB-R12 treatment, and responses to high workload challenge were similar to those seen in transgenic mice (38).

Finally, to determine whether BB-R12 was capable of improving in vivo cardiac function in a disease model, BB-R12 (2.5×10^{13} vg/kg) was injected directly into adjacent noninfarcted myocardium of adult rats 5 days after myocardial infarction (MI) induced by permanent ligature of the left anterior descending coronary artery. Effects on cardiac function were monitored with echocardiography for 8 weeks post-MI. At 2 weeks post-treatment, fractional shortening remained significantly depressed in the MI group

FIGURE 7 BB-R12 Vector in Healthy Mouse Tissues

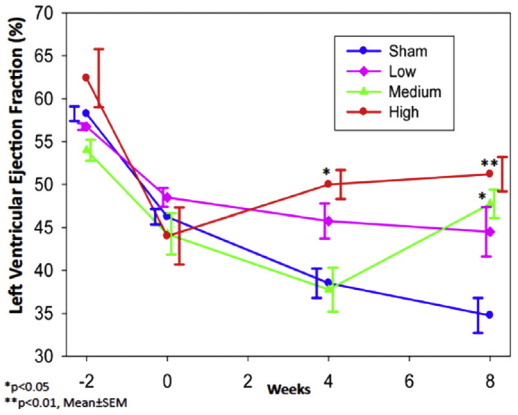


(A) Schematic diagram detailing the BB-R12 (AAV6-R1.2) vector construct containing a cardiac-specific promoter (cTnT455) and human sequences of the R1 (RRM1) and R2 (RRM2) subunits separated by a self-cleaving P2A peptide linker. **(B)** BB-R12 vector genome biodistribution in healthy mice tissues. Quantitative polymerase chain reaction results quantifying BB-R12 vector genomes/nuclei of liver, heart (ventricle), and gastrocnemius tissues. **Bars** represent mean ± SD. **(C)** R1R2 is selectively overexpressed in cardiac tissue of healthy mice treated with BB-R12. Western blot analysis of control and BB-R12–treated mouse ventricle and liver tissue, probing for R1, R2, and a loading control (GAPDH). Adapted with permission from Kolwicz et al. (38).

compared to sham controls (uninfarcted). However, by 4 and 8 weeks, cardiac function had recovered in BB-R12 treated rats to levels comparable to sham controls (38).

LARGE ANIMAL HF MODEL. The definitive translational evaluation of BB-R12 was to determine whether viral-mediated overexpression of R1R2 could increase dATP levels and improve cardiac function in a standard large animal (swine) MI/HF model (39,40). Yucatan minipigs were screened for AAV6 neutralizing antibodies. Seronegative animals had MI induced by balloon occlusion of the left anterior descending coronary artery. At 2 weeks post-MI, echocardiographic and hemodynamic data confirmed all groups showed the effects of induced MI and ensuing HF. Animals were not immunosuppressed and were administered BB-R12 via free-flowing, antegrade intracoronary infusion after onset of HF 2 weeks following MI (day 0). Doses were 1×10^{13} (high), 5×10^{12} (medium), or 1×10^{12} (low) vg. Cardiovascular responses over 2 months post-treatment were compared with sham controls (MI + saline). As expected, the sham group showed progression of HF with continued deterioration in LV ejection fraction (LVEF), increased LV end-diastolic pressure (LVEDP), and decreased +dP/dt. By contrast, animals treated with the high dose of BB-R12 had improved LVEF by 1 month post-treatment, with further improvement after 2 months. Interestingly, the medium-dose group showed no response at 1 month but had significantly increased LVEF by the 2-month time point compared with sham (**Figures 8 and 9A**). This is consistent with the hypothesis that fewer transduced cells in the medium-dose group required more time for dATP to diffuse to nontransduced cells and produce a response. Hemodynamic data show LVEDP and +dP/dt were significantly improved in the high-dose group by 2 months post-treatment, with similar results in −dP/dt (**Figures 9B and 9D**). No effect on LV end-diastolic diameter and no differences in blood pressure or heart rate were seen. No humoral or cellular immune responses to BB-R12 occurred, and no adverse effects on blood counts, chemistries, or liver enzymes were seen at any time point. At the end of the study, there were no gross or microscopic pathological

FIGURE 8 BB-R12 Effect on LVEF in Swine Model of MI/HF



Left ventricular ejection fraction (LVEF) at each time point in the 2-month study of swine myocardial infarction (MI)/heart failure (HF) for sham (MI + saline) versus BB-R12 therapy at low, medium, and high doses (n = 4 to 5 per group). *p < 0.05, **p < 0.01 compared with sham, mixed effects regression model; data are represented as mean ± SEM. Adapted with permission from Kadota et al. (41).

findings in any organ. The results of this pilot cardiac pharmacological study of BB-R12 in an MI/HF swine model extended the findings from rodent pharmacology studies, with safe and sustained increases in global cardiac function observed following a single administration and in a dose-responsive manner (41).

BB-R12 AS INOTROPIC THERAPY

Inotropic agents have been shown to be effective for both short- and long-term treatment of HF, but their utility has been limited primarily by safety concerns. These inotropic agents work upstream of calcium signaling and myosin contractions via stimulation of the adrenergic system (dopamine, dobutamine, norepinephrine) or by inhibiting phosphodiesterase and cAMP production (milrinone, vesnarinone, enoximone) (**Figure 1A**) (3). Briefly, these safety problems are tachyarrhythmias and myocardial ischemia/hypotension due to the primary mechanisms of action (i.e., "on-mechanism" but off-target) (42). The promise of new therapies targeting calcium-cycling or activating myosin is that these work downstream from the signaling mechanisms that can cause the side effects. This more targeted mechanism may yield a more acceptable benefit-risk profile.

LIMITATIONS AND FUTURE DIRECTIONS

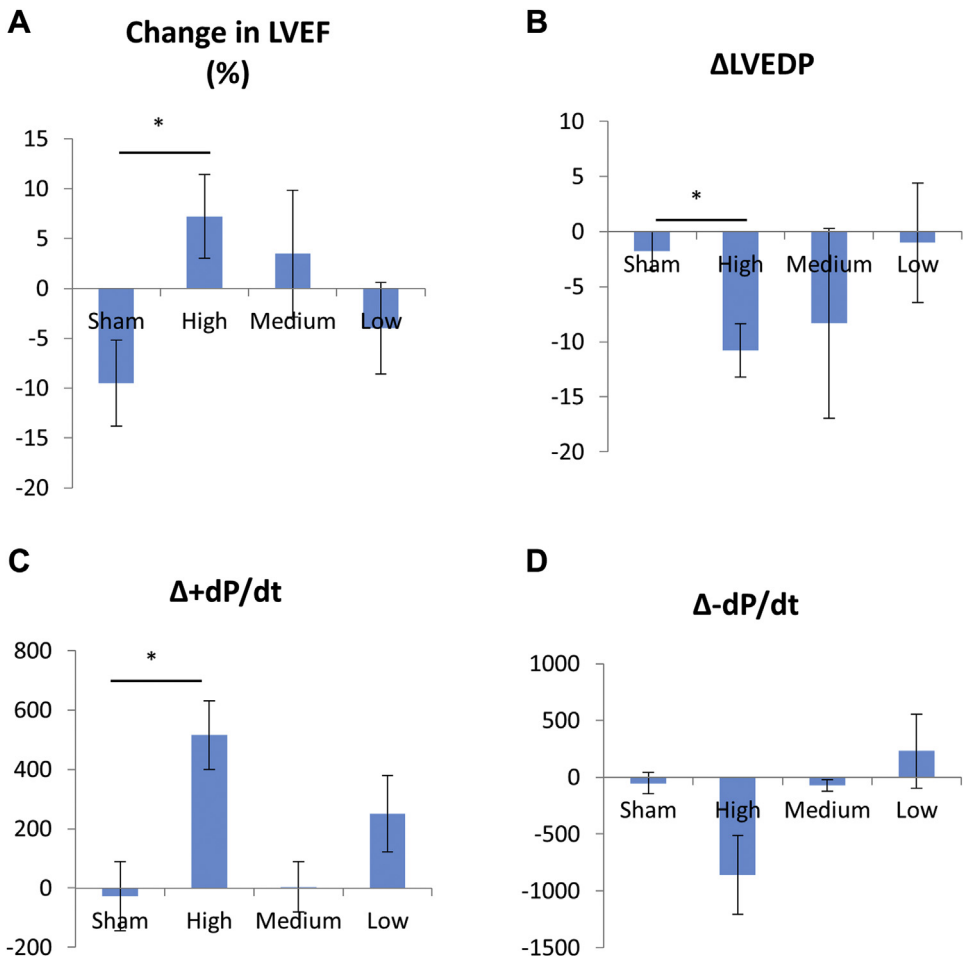
Data from the studies described in the preceding text are consistent with the interpretation that elevated dATP levels increase cardiac contractility due to direct interaction with myosin. dATP increases myosin-actin cross-bridge recruitment and cycling rates, causing increased and more rapid force generation. dATP levels can be increased with a single dose of gene therapy that selectively up-regulates R1R2 in transduced cardiomyocytes. dATP can diffuse through gap junctions from a small number of transduced cells into a larger number of neighboring, nontransduced cells, amplifying the effect.

However, dATP may exert additional effects via enhancing functions of other excitation-contraction coupling components that bind ATP, or enhancing metabolic or mitochondrial energy pathways. Because R1R2 also converts other NTPs into deoxy-NTPs, elevated levels of 1 or more of these could affect cardiac contractility. A more complete understanding of the mechanisms underlying this approach is desirable but not essential to further evaluating its therapeutic potential.

Additional studies are warranted to further our understanding of important translational aspects of this technology. These include replicating the pilot swine study findings in a larger study, direct measurements of dATP and R1R2 activity in treated animals, and confirmation of the response in a second large animal experimental model of HF. Further work will assess the durability of the observed response, evaluate effects on oxygen consumption (at the sarcomere and, more importantly, the whole-heart level). Importantly, in light of experience with other inotropic agents, further work must assess short- and longer-term cardiac and noncardiac safety profiles, in particular the arrhythmic potential.

From a practical perspective, gene therapy may have a valid target and fail due to insufficient transduction efficiency, that is, too few cells are transduced to produce the desired effects. The authors of the recently published report of the CUPID-2 (A Study of Genetically Targeted Enzyme Replacement Therapy for Advanced Heart Failure) trial of SERCA2a gene therapy speculate this may have contributed to their results (8). BB-R12 contains an AAV6 viral vector, which has greater affinity for the heart compared with most other myogenic AAV serotypes (43–45). The effect of dATP appears potent because small increases in dATP can increase cardiac contractility, and diffusion of dATP from transduced (factory) cells through gap junctions to neighboring and distal

FIGURE 9 BB-R12 Effect on Hemodynamic Parameters in Swine Model of MI/HF



Day 0 (pre-treatment) to Day 56 changes in **(A)** LVEF, **(B)** LVEDP, **(C)** +dP/dt, and **(D)** −dP/dt. Data are mean change ± SEM. *$p < 0.05$. Adapted with permission from Kadota et al. (41). LVEDP = left ventricular end-diastolic pressure; other abbreviations as in Figures 1 and 8.

nontransduced cells amplifies the effect. Even though the potency and amplification effects may make BB-R12 less critically dependent on transduction efficiency, optimization of dose and delivery technique will increase the likelihood of a beneficial therapeutic response. Antegrade intracoronary infusions produced a favorable effect in pigs and translate readily to the clinical setting. Systemic injections and direct myocardial injections have also been evaluated. Before human testing, further animal studies should evaluate antegrade intracoronary infusions with balloon occlusions, as well as retrograde coronary sinus infusions that may increase transduction efficiency (6).

Lastly, because adeno-associated viruses are endemic, there is antiviral immunity in the human population. The animals in the swine MI/HF study were from a closed herd and were all seronegative. Circulating neutralizing antibodies to AAV6 are present in a large proportion of the human population and the clinical utility of this approach would be limited. However, if the therapeutic utility can be demonstrated in an initial, carefully selected population, then alternative approaches to delivery can be considered. These include alternate AAV vectors, cell-based therapies, or direct myocardial injections.

CONCLUSIONS

The observation that dATP activates cardiac myosin in healthy and failing cardiomyocytes has been

translated into BB-R12, a gene therapy agent capable of up-regulating R1R2 selectively in the heart, leading to localized synthesis of dATP. Testing to date suggests that a durable increase in cardiac performance can be produced safely after a single exposure. The extensive in vitro, ex vivo, and in vivo research into experimental HF described here supports further animal studies and may eventually lead to clinical testing of BB-R12 as a therapy for the many patients with HF due to systolic dysfunction.

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