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Cardiac Myosin Activation with Gene Therapy Produces Sustained Inotropic Effects and May Treat Heart Failure with Reduced Ejection Fraction

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Abstract

Chronic inotropic therapy is effective for the treatment of heart failure with reduced ejection fraction, but has been limited by adverse long-term safety profiles, development of tolerance, and the need for chronic parenteral administration. A safe and convenient therapeutic agent that produces sustained inotropic effects could improve symptoms, functional capacity, and quality of life. Small amounts of 2-deoxy-adenosine triphosphate (dATP) activate cardiac myosin leading to enhanced contractility in normal and failing heart muscle. Cardiac myosin activation triggers faster myosin crossbridge cycling with greater force generation during each contraction. This paper describes the rationale and results of a translational medicine effort to increase dATP levels using a gene therapy strategy to deliver and upregulate ribonucleotide reductase (R1R2), the enzyme responsible 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 a benign safety profile. Further animal studies are appropriate with the goal of testing this agent in patients with heart failure.

Keywords

Cardiac myosin activation; dATP; Gene therapy; Heart failure; Inotropic therapy; Ribonucleotide reductase; Translational medicine

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1 Introduction

Chronic heart failure (HF) remains a significant, growing cause of morbidity, hospitalizations, medical costs, and mortality and the condition has been characterized repeatedly as an epidemic (Redfield 2002; Lüscher 2014; Yancy et al. 2013). In patients with moderate-to-severe HF with reduced ejection fraction, decreased cardiac systolic function and compensatory mechanisms are the underlying causes of the clinical manifestations of HF, including the development and worsening of symptoms, declining functional capacity, episodes of decompensation requiring hospitalization, progressive dysfunction, and premature death. Chronic use of inotropic agents acting through the adrenergic system or cyclic AMP has improved symptoms and exercise capacity through an increase in ejection fraction, but the benefit of these drugs has been limited by problematic long-term safety and tolerance, and by the need for parenteral administration (Cohn et al. 1998; Lowes et al. 2000; Francis et al. 2014). Data from the use of cardiac resynchronization and left ventricular (LV) assist devices suggest that chronic improvement of cardiac function may prevent or reverse the progression of HF (Solomon et al. 2010; Ambardekar and Buttrick 2011).

Recently, gene therapy has been tested for the treatment of HF offer the promise of durable results after a single exposure through sustained expression of a therapeutic gene. These therapies primarily target improvement of dysfunctional calcium cycling that triggers myosin contractions, either directly or through the adrenergic system and cyclic AMP (Tilemann et al. 2012; Pleger et al. 2013). The three most advanced programs have completed clinical trials, including gene therapy targeting sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a) which regulates calcium movement between the cytoplasm and the sarcoplasmic reticulum, stromal cell-derived factor 1 (SDF-1) which is involved in endogenous myocardial repair, and adenylyl-cyclase type 6 (AC6) which catalyzes cyclic AMP formation (Pleger et al. 2013).

A new and promising method of improving cardiac function in HF patients is to target cardiac myosin directly instead of altering calcium regulation within the cardiomyocyte (Teerlink 2009). Cardiac myosin activation triggers faster myosin crossbridge cycling and greater force generation during each contraction. This approach bypasses the adrenergic and cyclic AMP systems, as well as the complex calcium regulation mechanisms. Omecamtiv mecarbil is the only cardiac myosin activating drug currently in development and encouraging clinical results have been reported preliminarily (Malik et al. 2011; Cleland et al. 2011; Greenberg et al. 2015; Teerlink et al. 2016). Omecamtiv is a myocardial ATPase activator that increases LV function independent of calcium levels by prolonging systole without an increase in +dP/dt. However, as a small organic molecule, omecamtiv mecarbil requires chronic repeat administration via injections or oral dosing.

A gene therapy strategy to cardiac myosin activation could represent the inter-section between the promise of a single-exposure therapy leading to a durable improvement in cardiac function. Here we review the published work on the development of BB-R12, a novel gene therapy for HF that targets cardiac myosin contraction directly by increasing production of 2-deoxy-adenosine triphosphate (dATP) in cardiomyocytes.

2 Overview

Original observations showed that dATP is a more efficient contractile substrate for myosin than ATP, and 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 a direct interaction with myosin. dATP increases $+dP/dt$ (with a corresponding increase in $-dP/dt$) and does not prolong systole. Cellular production of low levels of dATP occurs normally in mammalian cells by action of ribonucleotide reductase (R1R2), the enzyme that 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 deoxy-NTPs) are normally produced by R1R2 as a substrate for DNA synthesis and repair in replicating cells.

R1R2 has been extensively characterized and consists of two subunits: the larger R1 subunit contains the catalytic site and two allosteric sites that can bind dATP, while the smaller R2 subunit contains the free radical generator. R1R2 is tightly allosterically regulated, with no more than approximately 5% of the ATP pool present as dATP (Caras and Martin 1988; Ahmad and Dealwis 2013). However, since cardiomyocytes are non-replicating cells, the expression of R1R2 is normally markedly downregulated and dATP levels in cardiomyocytes are less than 10% of that in other cells (Korte et al. 2011). dATP concentration can be increased by the overexpression of R1R2, the rate-limiting enzyme in its production, using gene therapy.

BB-R12 is a designed, multi-component gene therapy consisting of a recombinant serotype-6 adeno-associated viral vector (AAV6) that carries a genome containing a human cardiac troponin-specific promoter (cTnT455) linked to a transgene that codes for the human sequences of both the large (R1) and small (R2) subunits of R1R2 to overexpress the enzyme selectively in myocardium. BB-R12 increases dATP levels in cardiomyocytes for the treatment of chronic HF with reduced ejection fraction. Though BB-R12 is nominally a gene therapy, the therapeutic algorithm employed is novel. Rather than correct a genetic or proteomic defect, increased levels of dATP act as a locally produced inotropic small molecule drug 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 cardiac or systemic side effects. Based on the initial insight that dATP is a more efficient energy source for muscle contraction, the gene therapy approach of upregulating myocardial R1R2 has progressed from testing in cardiomyocytes and myofibrils to small animal HF models, and most recently to pilot large animal HF models. The results of these published studies are summarized below. Further testing is underway with the goal of testing this therapy in patients with HF.

3 dATP Is a More Potent Source of Energy for Myosin Contraction

The original pharmacologic insight that 2-dATP is a more potent source of energy for myosin contraction occurred almost 20 years ago. Studies initiated by Dr. Michael Regnier evaluating ATP and ATP analogs (NTPs) for their effects on skeletal muscle contractility

provided a detailed analysis of the chemo-mechanical processes of myosin–actin crossbridge cycling that is normally fueled by ATP. These studies showed increased crossbridge cycling rates with dATP compared to ATP, indicating dATP improved myosin binding with faster detachment of the myosin head from actin, thus making dATP a more effective contractile substrate than ATP (Regnier et al. 1998a; Regnier et al. 1998b; Regnier and Homsher 1998). Only dATP increased force generation compared to ATP, while all other NTPs and dNTPs produced weaker contractions (Fig. 1). Furthermore, dATP increased force generation through an increase in $+dP/dt$ (with a corresponding increase in $-dP/dt$) and no prolongation of systole (Fig. 1).

Later studies investigating how the chemo-mechanical transduction pathway differs in cardiac muscle showed dATP substitution for ATP in demembranated cardiac trabeculae isolated from adult rats resulted in increased isometric force at all levels of calcium activation. Muscle “stiffness” (an experimental measurement of crossbridge binding) and force were increased similarly with dATP in cardiac muscle, suggesting increased isometric force resulted from increased strong crossbridge binding. In contrast, dATP caused elevated stiffness and force generation in skeletal muscle only at submaximal levels of calcium, demonstrating a myosin-mediated enhancement of contraction that was more potent in cardiac muscle. The substitution of dATP for ATP reversibly increased force production, stiffness, crossbridge cycling, and calcium 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 (Regnier et al. 2000; Regnier et al. 2004).

An experiment in rat cardiac muscle demonstrated that addition of low levels of dATP to the ATP pool at physiological calcium levels resulted in significant increases in force development. Briefly, demembranated rat cardiac trabeculae were exposed to solutions containing 5 mM NTP (either 100% ATP or [2% dATP/98% ATP]) at submaximal (pCa 5.6) and maximal (pCa 4.0) calcium concentrations. Force production at pCa 5.6 was increased by 17% with a solution of [2% dATP/98% ATP] compared to 100% ATP solution. Furthermore, the resultant increase in force was reversible upon transfer back to 100% ATP solution. Stiffness was increased with [2% dATP/98% ATP] compared to 100% ATP at pCa 5.6, indicating that increased force production with dATP is due to increased crossbridge binding. At pCa 4.0, these increases in force production and stiffness were not seen (Fig. 1). This study demonstrated that even small amounts of cellular dATP added to ATP are sufficient to significantly increase contractile force in cardiac tissue by increasing the number of strong crossbridges (Korte et al. 2011). These data, confirmed by the work of others, suggest that the analogy to a “fuel additive” is more appropriate than “higher-octane fuel” (Schoffstall et al. 2006; Schoffstall and Chase 2008; Baker 2011).

Dilated cardiomyopathy (DCM), a prevalent form of HF, is characterized by ventricular dilatation, as well as a 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 the control (non-failing) cardiac tissue levels, without affecting relaxation (Cheng et al. 2016).

In order to evaluate the potential for a human therapy, cardiac tissue obtained from patients with end-stage HF was treated ex vivo with dATP to determine whether the same effects would be seen. Adult LV wall tissue was obtained from 16 end-stage HF patients undergoing LV assist device placement or cardiac transplantation. Demembranated tissues were mounted on a custom biomechanical assay platform where stiffness and steady-state isometric force were measured at varying calcium concentrations (pCa) in the presence of varying ratios of ATP:dATP (5 mM total). Cardiac myofibrils isolated from the tissue samples were used to assess the activation and relaxation kinetics with ATP or dATP (2 mM). Contractility of the failing human cardiac muscle was significantly enhanced in the presence of dATP without affecting relaxation kinetics. Isometric force at saturating calcium levels (pCa 4.5) was increased by 11% with dATP compared to ATP, and increased by 35% at submaximal calcium levels in the range where the heart normally operates. Isometric force was also shown to increase linearly ($R^2 = 0.985$) with increasing dATP content at pCa 5.6. Myofibrils showed a 13% increase in force production and a 44% increase in activation rate with dATP compared to ATP, with no effect on relaxation rates and no prolongation of systole. In agreement with previous studies in rat cardiac tissues, increased force production, calcium sensitivity, crossbridge cycling, and rate of activation at physiological calcium levels were seen with low levels of dATP, resulting in increased force production and more robust contractile kinetics (Fig. 2) (Moussavi-Harami et al. 2015).

The studies of rat, canine, and human tissues ex vivo characterized the increased magnitude and rate of force production with dATP in cardiac muscle. However, the structural basis for improved myosin binding and detachment with dATP in muscle was not yet understood. In order to investigate the underlying chemo-mechanical mechanisms for improved contractility seen with dATP, atom-level differences in pre-powerstroke myosin structure and dynamics with dADP binding were evaluated with molecular dynamics (MD) simulations. Effects of dADP on myosin affinity for actin were measured by an in vitro motility (IVM) assay to validate MD simulation results. A well-characterized myosin structure in the pre-powerstroke state was used as a starting structure for MD simulations. Simulation results showed that binding of dADP in the myosin cleft alters the intramolecular contacts within the nucleotide binding pocket (starting with Phe129) compared to ADP, resulting in a change in both the structure of the binding pocket and the position of the nucleotide within the pocket. This initiates a cascade of altered contacts in the protein structure, causing myosin to undergo global conformational changes towards a conformation seen in strong 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. MD simulation results were supported by IVM analysis, which indicated that dATP enhances weak binding electrostatic interactions between actin and myosin. These studies suggest that the missing hydroxyl group in dATP modifies the chemo-mechanical interaction of the nucleotide with the binding site on myosin, inducing global conformational changes in the myosin head structure. These changes result in an increased rate and affinity of actin binding. The increased frequency and strength of crossbridge cycling ultimately increase force production in cardiac muscle (Nowakowski et al., 2014, unpublished data) (Nowakowski et al. 2013a).

To evaluate the effects of constitutive upregulation of dATP in vivo, a transgenic mouse model that overexpresses R1R2 (TgRR) was studied. R1R2 levels are typically higher in replicating than in quiescent cells or in post-mitotic differentiated cells such as cardiomyocytes, since deoxynucleotides are needed for DNA synthesis and repair. The development of this animal model therefore initially addressed the challenge of providing heart cells in an intact animal with an adequate supply of dATP. 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 the R1 and R2 subunits of R1R2 and wild-type (WT) control transgenic mice were bred similarly to TgRR mice. Results showed that 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, positive and negative maximum LV pressure waves), without exhibiting increased heart rate, cardiac hypertrophy, LV dilation, or adverse cardiac remodeling. Hearts were able to respond to high workload β -adrenergic challenge (dobutamine infusion) and performed similarly to normal hearts during the high workload for 20 min. High-energy phosphocreatine reserves were mildly reduced at baseline (though the 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 WT mice at 3 and 12 months, suggesting no hypertrophy or cardiac remodeling resulted from chronically elevated dATP. This transgenic animal model demonstrated that elevated cardiac function can be maintained over the long-term with R1R2 overexpression without noticeable side effects or structural adaptation of the heart, indicating that long-term upregulation of dATP may be a viable therapeutic mechanism (Nowakowski et al. 2013b).

4 Translational Medicine Challenge: Developing a Gene-Delivery System to Increase dATP Specifically in Cardiac Tissue

The body of work characterizing the effects of dATP on cardiac muscle contractility demonstrates how this ATP analog increases the magnitude and rate of force development. However, chronic administration of dATP directly to the heart is not a viable strategy to achieve long-term therapeutic cardiac effects. The transgenic mouse model demonstrated sustained systemic overexpression of R1R2 increases basal cardiac function. In order to translate these findings into a viable therapy for cardiac diseases, 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 overexpression of R1R2 to increase cellular dATP concentration used a delivery system consisting of two separate recombinant adenoviral (AV) vectors that expressed the R1 or R2 subunits separately with a GFP reporter under the cytomegalovirus (CMV) promoter. These vectors were administered simultaneously in equal doses (this treatment is referred to as AV-R1 +AV-R2, Table 1). When cultured rat cardiomyocytes (both adult and neonatal) were transduced with AV-R1 +AV-R2, the treatment resulted in increased intracellular dATP content and an increased

magnitude of contraction and rate of contraction and relaxation, without affecting calcium transient properties. These results suggest that the increased contractility seen with R1R2 overexpression is due to increased myofilament responsiveness to calcium. 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 (defined as fractional shortening/maximal Fura-2 fluorescence) was increased in cells treated with AV-R1 +AV-R2 compared to controls at all stimulation frequencies (Fig. 3a). Measurement of cellular R1, R2, and dATP levels indicated a significant increase in R1 and R2 protein content, and an approximate tenfold increase in dATP over that seen in control cells, equating to approximately 1% of the total adenine nucleotide pool (Fig. 3b–d). This study provided the first proof of principle that dATP levels could be increased within cardiomyocytes, and that small elevations of dATP levels enhanced contractility (Korte et al. 2011).

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 (Lundy et al. 2014). To evaluate the therapeutic potential of this delivery system, AV-R1 +AV-R2 was used to transduce cardiomyocytes isolated from infarcted adult rat hearts. Contractility of the treated infarcted cardiomyocytes was significantly improved compared to untreated infarct cells with the magnitude and rate of contraction restored to levels comparable to healthy (uninfarcted) cardiomyocytes (Feest et al. 2014).

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 the contractility of neighboring cells that were not themselves overexpressing R1R2. To test this hypothesis, the transfer of fluorescein-labeled dATP via gap junctions between AV-R1 + AV-R2 transduced and non-transduced (WT) cardiac cells (both rat and human) and the resulting effects on contractility were measured. Rapid transfer of dATP between coupled cells (Fig. 4a, b) was demonstrated, and this effect was blocked when a gap junction inhibitor was introduced. R1R2-overexpressing human cardiomyocytes (hESC-CMs) had significantly larger contraction magnitudes than WT cells (Fig. 4c, d). WT hESC-CMs coupled to AV-R1 + AV-R2 treated hESC-CMs also had increased contraction magnitudes and contraction velocity, but showed no significant differences in time to peak contraction or time to 90% relaxation (Fig. 4d). When these R1R2-overexpressing human cardiomyocytes were transplanted into adult rat hearts in vivo, they delivered dATP to the host heart muscle and significantly increased global cardiac performance within 5 days (Fig. 4e) (Lundy et al. 2014).

In order to reduce immune responses to the delivery system relative to AV and achieve long-term expression of R1R2 in the myocardium, adeno-associated virus vector (AAV6) systems were developed that targeted cardiac tissue (Table 1). The next translational therapy was to develop a dual vector system consisting of two separate recombinant AAV6 vectors that express rat sequences of R1 or R2 under the cardiac-specific promoter cTnT455 (a regulatory cassette composed of enhancer and promoter portions of the human cardiac troponin gene (TNNT2)), which would be delivered simultaneously at equal doses

(treatment referred to as Rat AAV6-R1 +AAV6-R2). 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 the effects persisted for at least 13 weeks post-injection. A larger effect in the high dose group was seen at 1–3 weeks and persisted, suggesting an early dose-response, but by 6 weeks the effect in the lower dose groups had increased to reach the level seen in the high dose group. These results indicate that the improvements in heart function seen with higher doses of vector-mediated R1R2 overexpression can be achieved with lower doses of the vector over time (Kolwicz et al. 2016). This pattern is consistent with the hypothesis that transduction of fewer cardiomyocytes in the lower dose groups required more time to establish a sufficient concentration gradient for dATP to diffuse from these cells to distal non-transduced cells.

Further translational research led to the development of a single vector system using an AAV6 vector containing a transgene cassette expressing optimized human sequences of both R1 and R2 subunits separated by a protein cleavage site under a single cardiac-specific promoter (cTnT455) (Table 1). This vector system, called BB-R12 (AAV6-R1.2 in this publication, Fig. 5a), was designed to restrict the increase of intracellular R1R2 to cardiac muscle cells. To evaluate this single vector delivery system and determine if the increased cardiac function seen with the dual vector systems used previously 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 and compared to other vector systems as described in Table 1. While significant viral genome uptake was seen in liver and ventricular tissues of treated mice compared to controls (with negligible uptake in the gastrocnemius tissue, Fig. 5b), increased levels of R1 and R2 proteins were only found in ventricular tissue (Fig. 5c), confirming the specificity of the cTnT455 promoter. BB-R12 significantly increased basal cardiac function by ~20–30%. Responsiveness to high workload challenge (dobutamine infusion) indicated that β -adrenergic signaling remained intact with AAV6 treatment, and responses were similar to those seen in transgenic mice (Kolwicz et al. 2016).

Finally, to determine whether the BB-R12 system was capable of improving in vivo cardiac function in a disease model, BB-R12 (2.5×10^{13} vg/kg) was injected directly into the adjacent non-infarcted myocardium of adult rats 5 days after myocardial infarction (MI) induced by permanent ligation 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 compared to sham controls (uninfarcted). However, by 4 and 8 weeks, cardiac function had recovered in BB-R12 treated rats to levels comparable to the sham control hearts (Kolwicz et al. 2016).

5 Large Animal Model of HF

After characterization in vitro, ex vivo, and in vivo in rodent systems and models, 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 (Dixon and Spinale 2009; Ishikawa et al. 2012).

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 that all groups showed the effects of the 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 post-MI (day 0). Doses were 1×10^{13} (high), 5×10^{12} (medium), or 1×10^{12} (low) doses of vector genomes. The cardiovascular responses over the 2 months post-treatment were compared to sham controls (MI + saline). As expected, during the 2-month observation, the sham group showed progression of HF with continued deterioration in LV ejection fraction (LVEF), increased LV end diastolic pressure (LVEDP), and decreased maximum rate of pressure rise ($+dP/dt$), a parameter of systolic function.

In 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 to sham (Figs. 6 and 7a). This is consistent with the hypothesis that fewer transduced cells in the medium dose group required more time for dATP to diffuse from these cells to non-transduced cells and produce a response. Hemodynamic data show that LVEDP and $+dP/dt$ were significantly improved in the high dose groups by 2 months post-treatment, with similar results in $-dP/dt$, a parameter of early diastolic function (Fig. 7b–d). There was 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 findings in any organ nor any histopathologic differences in the infarct area. The results of this pilot large animal cardiac pharmacological study of the effects 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 (Kadota et al. 2015).

6 Limitations and Future Directions

Data from the studies described above are consistent with the interpretation that elevated dATP levels increase cardiac contractility due to a direct interaction with myosin. dATP increases myosin–actin crossbridge cycling rates causing increased force generation. dATP levels can be increased with a single dose of gene therapy that selectively upregulates R1R2 in transduced cardiomyocytes. dATP can diffuse through gap junctions from a small number of transduced cells into a larger number of neighboring, non-transduced cells amplifying the effect.

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

Additional studies are warranted to further our understanding of important translational aspects of this technology. These include replication of 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 the effects on oxygen consumption (both at the sarcomere and, more importantly, the whole-heart level), and further assess the acute and longer-term cardiac and non-cardiac safety profile, in particular the arrhythmic potential.

From a practical perspective, gene therapy may have a valid target and fail due to insufficient transduction efficiency, i.e., too few cells are transduced to produce the desired effects. BB-R12 contains an AAV6 viral vector, which has greater affinity for the heart compared to most other myogenic AAV serotypes (Palomeque et al. 2007; Arnett et al. 2013; Gao et al. 2011). The effect of dATP appears potent, since small increases in dATP can increase cardiac contractility, and the diffusion of dATP from transduced (“factory”) cells through gap junctions to neighboring and distal non-transduced cells amplifies the effect. Even though the potency and amplification effects may make BB-R12 less critically dependent upon transduction efficiency, optimization of dose and delivery techniques will increase the likelihood of observing a beneficial therapeutic response. Antegrade intracoronary infusions produced a favorable effect in pigs, and systemic injections and direct myocardial injections have also been evaluated preliminarily. 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.

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 may include alternative AAV vectors, cell-based therapies, or direct myocardial injections.

7 Conclusion

The observation that dATP activates cardiac myosin in healthy and failing cardiomyocytes has been translated into BB-R12, a gene therapy agent capable of upregulating R1R2 selectively in the heart leading to the local synthesis of dATP. Testing to date suggests that a durable increase in cardiac performance can be produced safely after a single exposure. The approach represents the intersection of the promise of both cardiac myosin activation and gene therapy. The extensive in vitro, ex vivo, and in vivo research into experimental HF described here support 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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Non-Standard Abbreviations and Acronyms

AAV6-R1	Single AAV6 vector expressing R1 subunit
AAV6-R1.2	Single AAV6 vector expressing R1 & R2 subunits, linked by P2a
AAV6-R2	Single AAV6 vector expressing R2 subunit
AV-R1	Single AV vector expressing R1 subunit
AV-R2	Single AV vector expressing R2 subunit
BB-R12	AAV6 viral vector with a cardiac-specific promoter cTnT455 to overexpress R1R2 in the heart
dATP	2-Deoxy-adenosine triphosphate
HF	Heart failure
R1	Rrm1 subunit of ribonucleotide reductase
R1R2	Ribonucleotide reductase
R2	Rrm2 subunit of ribonucleotide reductase
TgRR	Transgenic mouse model overexpressing R1R2

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Fig. 1.

(a) Relative contraction velocity with different NTP and dNTP substrates (ATP = 1). (b) The effect of 2-deoxy-adenosine triphosphate (dATP) on cardiomyocyte contraction compared to control. (a) and (b) adapted from Regnier et al. (1998a). (b) Effects on force production and stiffness with [2% dATP/98% ATP] compared to 100% ATP (5 mM total) in rat cardiac muscle. 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 solution. (c) % increases in force production and stiffness of cardiac trabeculae with [2% dATP/98% ATP] at submaximal (pCa 5.6) and maximal (pCa 4.0) calcium levels. (c) and (B) adapted from Korte et al. (2011)

Fig. 2.

Effects of ATP vs. dATP in ex vivo human cardiac tissue from end-stage HF patients demonstrating dATP increases force. (a) Representative isometric force trace of demembrated tissue at pCa 5.6 with ATP or dATP. (b) Force measured at maximal (pCa 4.5) and submaximal calcium concentrations (pCa 5.6) with ATP or dATP. Measurements are mean±SEM. * $P < 0.05$ by paired student's t -test. Adapted from Moussavi-Harami et al. (2015)

Fig. 3.

Effects of AV-R1 + AV-R2 treatment on adult rat cardiomyocytes (ARCs). **(a)** Contractile response of ARCs at different stimulation frequencies. AV-R1 + AV-R2 treated cells (*open triangles*) showed significantly greater response to calcium at all frequencies. AV-GFP treated cells (*open circles*); non-transduced cells (*closed circles*). * $P < 0.05$ compared to non-transduced; † $P < 0.05$ compared to AV-GFP treated. **(b–d)** R1R2 protein expression in ARCs after AV-R1 + AV-R2 treatment. Increased **(b)** R1 and **(c)** R2 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 to AV-GFP treated NRVMs. Adapted from Korte et al. (2011)

Fig. 4.

R1R2 overexpression increases human embryonic stem cell-derived cardiomyocyte (hESC-CM) contractility and enhances contractility of coupled cardiomyocytes. (a) Example of coupled 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 to control cells with no prolongation of systole. AV-R1 + AV-R2 treated hESC-CMs showed (c) significantly increased magnitude of contraction compared to AV-GFP treated cells, and (d) significantly increased maximum contraction velocity without affecting relaxation velocity. * $P < 0.05$; NS 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. Adapted from Lundy et al. (2014)

Fig. 5. 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. qPCR results quantifying the BB-R12 vector genomes/nuclei of liver, heart (ventricle), and gastrocnemius tissues. Bars represent the mean $\pm$ 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 heart (ventricle) and liver tissue, probing for R1, R2, and a loading control (GAPDH). Adapted from Kolwicz et al. (2016)

Fig. 6. BB-R12 effect on LV ejection fraction (LVEF) in swine model of MI/HF. LVEF at each time point in the 2-month study of swine MI/HF for sham (MI + saline) vs. BB-R12 therapy at low, medium, and high doses ($n = 4-5$ per group). * $P < 0.05$, ** $P < 0.01$ compared to sham, mixed effects regression model; data represented as mean $\pm$ SEM. Adapted from Kadota et al. (2015)

Fig. 7. BB-R12 effect on hemodynamic parameters in swine model of MI/HF. Day 0 (pre-treatment) to Day 56 changes in (a) LVEF, (b) LV end diastolic pressure (LVEDP), (c) +dp/dt, and (d) -dp/dt. Data are mean change ± SEM. * $P < 0.05$. Adapted from Kadota et al. (2015)

Table 1

Description of viral vector systems

Sequence species	Viral vector	Gene(s) expressed	Vector system design	Treatment name
Rat	AV	R1 + GFP	Two separate vectors	AV-R1 + AV-R2
Rat	AV	R2 + GFP		
Rat	AAV6	R1	Two separate vectors	Rat AAV6-R1 + AAV6-R2
Rat	AAV6	R2		
Human	AAV6	R1	Two separate vectors	Human AAV6-R1 + AAV6-R2
Human	AAV6	R2		
Human	AAV6	R1-P2A-R2 ^a	Single vector	Human AAV6-R1.2 (BB-R12)

^aP2A is a 20 amino acid peptide linker that enables co-translation of two proteins from a single strand of mRNA and subsequent cleavage of the two proteins