Effect of bortezomib on the efficacy of AAV9.SERCA2a treatment to preserve cardiac function in a rat pressure-overload model of heart failure

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Adeno-associated virus (AAV)-based vectors are promising vehicles for therapeutic gene delivery, including for the treatment for heart failure. It has been demonstrated for each of the AAV serotypes 1 through 8 that inhibition of the proteasome results in increased transduction efficiencies. For AAV9, however, the effect of proteasome inhibitors on in vivo transduction has until now not been evaluated. Here we demonstrate, in a well-established rodent heart failure model, that concurrent treatment with the proteasome inhibitor bortezomib does not enhance the efficacy of AAV9.SERCA2a to improve cardiac function as examined by echocardiography and pressure volume analysis. Western blot analysis of SERCA2a protein and reverse transcription-PCR of SERCA2a mRNA demonstrated that bortezomib had no effect on either endogenous rat SERCA2a levels nor on expression levels of human SERCA2a delivered by AAV9.SERCA2a. Similarly, the number of AAV9 genomes in heart samples was unaffected by bortezomib treatment. Interestingly, whereas transduction of HeLa cells and neonatal rat cardiomyocytes by AAV9 was stimulated by bortezomib, transduction of adult rat cardiomyocytes was inhibited. These results indicate an organ/cell-type-specific effect of proteasome inhibition on AAV9 transduction. A future detailed analysis of the underlying molecular mechanisms promises to facilitate the development of improved AAV vectors.

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INTRODUCTION

Adeno-associated virus (AAV)-based vectors are increasingly used as gene transfer vectors in both preclinical disease models as well as clinical trials.1 This is in part due to the lack of pathogenicity of AAVs, their comparatively low immunogenicity and the ability of recombinant AAVs to cause long-term transgene expression even in the absence of genome integration, at least in nondividing cells.1

Excitingly, in Europe, AAV gene therapy has recently been approved for the treatment of lipoprotein lipase deficiency,2 thus bringing AAV gene therapy to the clinic. Other promising results in clinical trials have been obtained in the treatment of ocular diseases, such as inherited retinopathies (reviewed in Colella et al.3) and blood clotting disorders, namely factor IX deficiency.4 Furthermore, we and others have shown that the overexpression of the sarco/endoplasmatic calcium ATPase, SERCA2a, can ameliorate heart failure in both small5,6 and large animal models.7–9 On the basis of these preclinical data, a first-in-man phase I–II clinical trial for patients with advanced heart failure using AAV-based vectors encoding SERCA2a was initiated and recently concluded.10,11 This so-called CUPID trial showed that injection of high-dose AAV1.SERCA2a decreased clinical events in patients with heart failure.

Although these early results from clinical trials show promise, these trials also brought to light challenges that must be overcome to implement broadly AAV gene transfer as a therapeutic modality. Among the difficulties encountered is the need for large vector doses to achieve sufficient transduction efficiencies. The requirement for large vector doses is not only challenging in terms of good manufacturing practice-level AAV vector production but—maybe more importantly—in some trials, large vector doses have been shown to trigger a cellular immune response against the AAV capsids.12,13

It is not surprising then that intense research is taking place to improve transduction efficiencies by isolating new AAV genotypes,14,15 by modifying the AAV capsid by both site-directed16 and directed evolution approaches16,17 as well as by using drugs that enhance AAV transduction (see Nonnenmacher et al.18). These drugs include tyrosine kinase inhibitors,19 topoisomerase inhibitors,20–22 genotoxic agents such as hydroxyurea20,22,23 and proteasome inhibitors.24–28 Proteasome inhibitors are of particular interest as they not only increase transduction by all serotypes studied today24–28 but also recently have been demonstrated to lead to a reduced presentation of AAV capsid-derived peptides on MHC class I molecules,29 thus reducing the risk of an immune response against the transduced cells. A number of studies have shown that proteasome inhibitors can enhance transduction by AAV serotypes 1 through 8.24–28 Most recently, it was also shown that proteasome inhibitors can increase AAV9 transduction in vivo,30 however, the effect of proteasome inhibitors on transduction by AAV9 in vivo has so far not been reported. Hence, we decided to test whether the US Food and Drug Administration-approved proteasome inhibitor bortezomib (also known as Velcade) can increase the efficiency of AAV9.SERCA2a in improving cardiac function in a rat model of pressure overload-induced heart failure.

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RESULTS
For our studies, we used a rat pressure overload model in which heart failure (HF) is induced by banding of the ascending aorta (study design: Figure 1a). Echocardiography was employed to assess left ventricular (LV) dimensions and function. Systolic HF was observed between 8 and 12 weeks after aortic banding. Once HF developed, animals were randomized to receive via tail vein injection 1 × 10^{12} genome-containing particles (gcp) of AAV9.SERCA2a alone, 1 × 10^{12} gcp AAV9.SERCA2a + bortezomib or an equivalent amount of empty AAV9 particles. As reported previously, the maximal tolerated dose of bortezomib in rats was 0.1 mg kg⁻¹. At doses of 0.3 or 0.5 mg kg⁻¹ some animals died within 1 week of injection. A dose of 0.1 mg kg⁻¹ of bortezomib, however, was well tolerated in both sham-treated animals and animals with HF. Two months after vector injection, cardiac function was assessed noninvasively by echocardiography and invasively using pressure–volume loop measurements (Figure 1a). At the time of sacrifice, heart and body weight were also measured.

The heart weight to body weight ratio was significantly increased in the HF animals (AAV9.Empty, AAV9.SERCA2a and the AAV9.SERCA2a + bortezomib groups) compared with sham-operated animals (Table 1). LV septal wall thickness as well as LV posterior wall thickness gradually increased in the first few weeks after ascending aortic banding and reached the maximum at about 4 weeks after ascending aortic banding (data not shown) and was preserved up until the onset of HF. At 2 months, the thickness of the septal and posterior wall remained about the same in all vector-injected animals compared with the time of onset of HF and was significantly higher than that in the sham-operated animals (Figure 1b and Table 1).

The echocardiography data shown in Figure 1c and Table 1 also demonstrate that 2 months after treatment, LV end-systolic and LV end-diastolic volumes were significantly lower in the AAV9.SERCA2a, with or without bortezomib treatment, groups compared with AAV9.Empty-treated group (Figure 1c). Also, LV fractional shortening and LV ejection fraction were significantly higher in the above two groups compared with the AAV9.Empty-treated animals (Table 1). Unexpectedly, however, there were no differences in LV diameters and LV volumes or in LV fractional shortening and LV ejection fraction between the AAV9.SERCA2a and the AAV9.SERCA2a + bortezomib-treated groups. Hemodynamic measurements, by P–V loop, were conducted at baseline and during LV unloading by inferior vena cava (IVC) occlusion (Table 2). Figures 2a and b show P–V loop tracings at baseline and during IVC occlusion, respectively. Compared with the AAV9.Empty-treated group, there were significant decreases in LV end-diastolic and end systolic volumes as well as a significant increase in LV ejection fraction in the AAV9.SERCA2a- and AAV9.SERCA2a + bortezomib-treated animals (Figure 2c and Table 2). However, consistent with the echocardiographic data, there were no significant differences in the hemodynamic parameters between the AAV9.SERCA2a and the AAV9.SERCA2a + bortezomib-treated groups. LV contractility, as measured by the end-systolic pressure volume relationship during LV unloading by IVC occlusion, was significantly higher in the AAV9.SERCA2a and AAV9.SERCA2a + bortezomib groups, with a significant shift of V₀ (theoretical volume at which end-systolic pressure is zero) to the left, compared with AAV9.Empty-injected rats. The significant leftward shift of V₀ seen in the AAV9.SERCA2a and in the AAV9.SERCA2a + bortezomib groups is attributed to the reversal of cardiac remodeling with significantly lower LV end-diastolic and LV end-systolic volumes (Figure 2d and Table 2).

LV end-diastolic pressure volume relationship, obtained during LV unloading by IVC occlusion, revealed no significant differences among the AAV9.Empty and AAV9.SERCA2a groups, with or without bortezomib, indicating that despite the significant improvement in LV contractility, there were no apparent benefits in LV stiffness or LV relaxation among these groups. There was, however, a trend toward improved left ventricular end-diastolic pressure and LV end-diastolic pressure volume relationship and a trend toward decreased interstitial fibrosis (data not shown) in the AAV9.SERCA2a group compared with H + AAV9.Empty group. The lack of increased efficacy in the AAV9.SERCA2a bortezomib group compared with the AAV9.SERCA2a alone group led us to compare the expression levels of SERCA2a in the treatment and control groups. As expected, SERCA2a expression was significantly decreased in the HF animals injected with empty AAV9 particles compared with the sham-operated control group (Figure 3). Similarly, treatment of HF animals with AAV9.SERCA2a, with or without bortezomib, resulted in a significant restoration of SERCA2a expression (Figure 3). Surprisingly, however, there was no difference in SERCA2a protein levels between the AAV9.SERCA2a and the AAV9.SERCA2a + bortezomib groups suggesting that either bortezomib depresses endogenous SERCA2a levels, or that AAV9 transduction is not enhanced in the presence of proteasome inhibitors, at least in rat hearts. To distinguish between these two possibilities we first determined the levels of endogenous, rat SERCA2a mRNA by reverse transcription-PCR. Interestingly, consistent with previous observations and in contrast to SERCA2a protein levels, there was no difference among any of the groups.
including among sham-treated animals and animals with HF injected with AAV9 capsids (Figure 4a). These results suggest that bortezomib does not influence endogenous SERCA2a mRNA levels, and that the observed decrease in SERCA2a protein levels is a result of enhanced SERCA2a degradation.\(^5\) We next examined whether the levels of human SERCA2a mRNA, encoded by AAV9.SERCA2a, were increased in bortezomib-treated animals compared with animals injected with AAV9.SERCA2a alone. Surprisingly, there was a trend toward reduced human SERCA2a expression in the bortezomib-treated animals, although this did not reach statistical significance (Figure 4b). Because the increase in transduction by proteasome inhibitors observed with other serotypes is thought to be, at least in part, a result of the stabilization of AAV virions resulting in increased vector genomes in the transduced tissue, we next analyzed the presence of AAV9.SERCA2a genomes. In agreement with our transcription results, we did not see an increase in AAV9.SERCA2a genomes in rats treated with bortezomib compared with rats that had been injected with AAV9.SERCA2a alone. In contrast, there was a trend toward fewer AAV9.SERCA2a genomes in bortezomib-treated animals, although this did not reach statistical significance (Figure 4c). Together, our results demonstrate that in rat hearts AAV9 transduction is not increased with the proteasome inhibitor bortezomib. 

There are two obvious reasons for these results: (1) in contrast to other AAV serotypes, inhibition of proteasome activity does not increase AAV9 transduction or (2) the lack of an increase in transduction by AAV9 is specific to rat cardiomyocytes. To distinguish between these two possibilities, we first analyzed the effect of bortezomib on AAV9 transduction in HeLa cells. Consistent with recently reported results,\(^3\) bortezomib treatment resulted in an \(~10\)-fold and \(20\)-fold increase in transduction by AAV9 and AAV2, respectively (Figure 5a). These results demonstrate that similar to other serotypes, proteasome inhibition can increase AAV9 transduction, at least in HeLa cells, thus ruling out that the lack of increased transduction is AAV9 specific. Rather, these results suggest that the lack of an increase in transduction is specific to rat cardiomyocytes. To test this hypothesis, we analyzed the effect of bortezomib on AAV2, AAV6 and AAV9 transduction in neonatal rat cardiomyocytes and adult rat cardiomyocytes. Interestingly, bortezomib enhanced transduction of neonatal rat cardiomyocytes by AAV2, AAV6 and AAV9 by \(~10\)-, \(~2\)- and \(~15\)-fold, respectively (Figure 5b). Strikingly—and in line with the in vivo data—bortezomib did not enhance AAV9 transduction in adult rat cardiomyocytes (Figure 5c). In contrast, bortezomib treatment resulted in \(~10\)-fold reduction in AAV9 transduction and decreased transduction by AAV2 and AAV6 by \(~10\)- and \(~15\)-fold, respectively. The reason for this decrease in transduction is unknown, but these results demonstrate that the effect of bortezomib on AAV9 transduction is not species-specific but rather cell type-specific.

### Table 1. Echocardiography data of HF animals treated with AAV9.Empty vs AAV9.SERCA2a with or without bortezomib

| Group                  | Onset of heart failure | Two months post injection |
|------------------------|------------------------|---------------------------|
|                        | Sham (n = 3)           | HF + AAV9.Empty (n = 5)   |
|                        |                        | HF + AAV9.SERCA2a (n = 5) |
|                        |                        | HF + bortezomib + AAV9.SERCA2a (n = 3) |
| HW/BW (mg g\(^{-1}\))  |                        |                          |
| BW (g)                 | 439 ± 47.77            | 562.71 ± 87.44           |
| ISVd (cm)              | 0.21 ± 0.01            | 0.27 ± 0.03              |
| LVPWd (cm)             | 0.22 ± 0.01            | 0.29 ± 0.02              |
| LVId (cm)              | 0.68 ± 0.04            | 0.87 ± 0.06              |
| LVdS (cm)              | 0.27 ± 0.02            | 0.5 ± 0.06               |
| LVFS (%)               | 59.41 ± 2.35           | 42.32 ± 4.77             |
| LVEDV (mL)             | 455.55 ± 38.37         | 1002.37 ± 168.51         |
| LVEF (%)               | 81.45 ± 12.79          | 408.92 ± 124.34          |

### Table 2. Hemodynamic data of HF animals treated with AAV9.Empty vs AAV9.SERCA2a with or without bortezomib

| Hemodynamic data        | Maximum pressure (mm Hg) | End diastolic pressure (mm Hg) | End systolic pressure (mm Hg) | EF (%) | ESPVR mm Hg \(\mu\) L\(^{-1}\) | V\(_0\) (mL) | EDPRV mm Hg \(\mu\) L\(^{-1}\) |
|-------------------------|--------------------------|--------------------------------|-------------------------------|--------|-----------------------------|-------------|-----------------------------|
| Sham (n = 3)            | 133 ± 26.53\(^a\)        | 7.4 ± 2.09\(^a\)              | 119.2 ± 36.69\(^a\)          | 60.93 ± 12.52 | 0.15 ± 0.04                 | 449.80 ± 149.7\(^a\) | 0.018 ± 0.01\(^a\) |
| HF + AAV9.Empty (n = 5) | 245.67 ± 15.26           | 20.73 ± 5.19                  | 165.2 ± 11.46                | 32.67 ± 9.19\(^bc\) | 0.22 ± 0.10                 | 265.41 ± 157.5\(^b\) | 0.04 ± 0.02\(^b\) |
| HF + AAV9.SERCA2a (n = 5) | 251 ± 15.03              | 18.27 ± 7.03                  | 177.93 ± 13.45               | 57.47 ± 7.34 | 0.41 ± 0.12\(^c\)          | 152.34 ± 68.68         | 0.03 ± 0.01\(^c\) |
| HF + Bortezomib + AAV9.SERCA2a (n = 3) | 261.38 ± 19.86 | 20.66 ± 1.15                  | 178.11 ± 20.1               | 62.55 ± 121 | 0.33 ± 0.05\(^d\)          | 216.89 ± 85.99         | 0.037 ± 0.006\(^d\) |

Abbreviations: AAV, adeno-associated virus; BW, body weight; HF, heart failure; HW, heart weight; ISVd, interventricular septum at diastole; LV, Left ventricle; LVEDV, LV end-diastolic volume; LVFS, LV fractional shortening; LVId, LV end-diastolic diameter; LVdS, LV end-systolic diameter; LVPWd, LV wall thickness of the posterior wall. There was no significant difference between HF + AAV9.SERCA2a vs HF + AAV9.SERCA2a + bortezomib. \(^{a}\)P < 0.05 sham vs all HF groups (AAV9. Empty, AAV9.SERCA2a ± bortezomib). \(^{b}\)P < 0.05 HF + AAV9. Empty vs HF + AAV9.SERCA2a ± bortezomib.

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Both in small and large animal models,\textsuperscript{6,8,33} treatment of HF by overexpressing SERCA2a with AAV-based vectors has been shown to be successful at improving cardiac dysfunction. Furthermore, a recently completed phase I–II clinical trial for the treatment of advanced HF with AAV\textsuperscript{1} SERCA2a yielded encouraging results.\textsuperscript{10,11} Despite—or perhaps because of—these promising results, further improvements in cardiac delivery with AAV-based vectors remains an essential focus of investigation. For both safety and efficacy reasons, high-level expression of SERCA2a with the smallest possible vector dose remains an important goal.

In 2001, Danos and colleagues demonstrated that AAV\textsuperscript{2} transduction can be greatly enhanced by treating cells with the proteasome inhibitor MG132.\textsuperscript{24–28} To date, there is no clear understanding of the exact mechanism by which proteasome inhibitors enhance AAV transduction. It was initially proposed that proteasome inhibitors increased AAV-mediated transduction simply by protecting viral capsids against cytoplasmic degradation, but subsequent investigations suggested that additional mechanisms were involved, including enhanced AAV trafficking to the nucleus\textsuperscript{34} and mobilization in and out of the nucleolus.\textsuperscript{35}

Since the original observation by Danos et al.\textsuperscript{26} with AAV2, an enhancement of transduction by proteasome inhibitors has been demonstrated for all the serotypes 1 through 8.\textsuperscript{8,24–28} Most recently, it has also been reported that bortezomib can increase AAV9 transduction \textit{in vitro}.\textsuperscript{125} But, to our knowledge, the effect of proteasome inhibitors on AAV9 transduction \textit{in vivo} has so far not been evaluated.\textsuperscript{26} Here, we demonstrate that transduction of rat
hearts by AAV9 is not enhanced at the maximum tolerated dose of bortezomib. The easiest explanation for this lack of enhancement of transduction would be that the bortezomib dose was too low. We deem this unlikely, however, because a slightly higher (and presumably toxic) dose of 0.2 mg kg\textsuperscript{-1} resulted in a rapid, profound (>80%) and prolonged (>72 h) inhibition of rat proteasome activity \textit{in vivo} (Figure 1 in Adams et al.\textsuperscript{32}). Furthermore, bortezomib significantly inhibits transduction of adult rat cardiomyocytes (Figure 5c), demonstrating that bortezomib can enter adult rat cardiomyocytes and presumably inhibit proteasome activity.

Figure 4. Bortezomib treatment does not affect endogenous SERCA2a expression and fails to increase transduction by AAV9-SERCA2a. (a) Rat SERCA2a and GAPDH expression were measured by RT-PCR with specific primers against rat SERCA2a and GAPDH. (b) Human SERCA2a expression was measured with specific primers against human SERCA2a and normalized to rat GAPDH expression. (c) AAV9 vector genomes were normalized to diploid genomes. The measurements were done at 2 months post vector injection.

An alternative reason for the lack of enhanced transduction with bortezomib is that the effect of proteasome inhibitors on transduction is serotype specific, possibly because the AAV9 capsid might be inherently more resistant to proteasomal degradation. Serotype-specific effects of proteasome inhibitors on AAV transduction have been reported in vascular endothelial cells where AAV2 transduction is greatly enhanced by LnLL and MG132, but transduction by AAV7 or AAV8 are unaffected.\textsuperscript{25} However, our data do not support the conclusion that proteasome inhibitors act in a serotype-specific manner as bortezomib treatment had a similar effect on transduction by AAV2 and AAV6 but decreases transduction in adult rat cardiomyocytes. Nor is it apparent why bortezomib stimulates transduction of neonatal rat cardiomyocytes but decreases transduction in adult rat cardiomyocytes. (a) HeLa cells were pretreated for 30 min with dimethyl sulfoxide (DMSO) or bortezomib (1.25 μM) and infected with AAV2-luciferase or AAV9-luciferase (1e4 gcpl/cell). After 24 h, cells were lysed and firefly luciferase activity was measured. (b) Neonatal rat cardiomyocytes were infected with AAV2-luciferase, AAV6-luciferase or AAV9-luciferase (1e4 gcpl/cell) in the presence of DMSO or bortezomib (1 μM). Luciferase activity was measured 24 h post infection. (c) Adult rat cardiomyocytes were infected with AAV2-luciferase, AAV6-luciferase or AAV9-luciferase (1e4 gcpl/cell) in the presence of DMSO or bortezomib (1 μM). Luciferase activity was measured 48 h post infection. *P<0.05.
cardiomyocytes by these AAV serotypes. It is clear, however, that serotype-specific or species-specific effects of bortezomib cannot explain the results. Rather the differences appear to be cell type specific. A dissection of the biological basis for the lack of enhancement of AAV9 transduction of rat myocardium by bortezomib is beyond the scope of this manuscript. But the differential effects of bortezomib on AAV9 transduction of adult and neonatal rat cardiomyocytes provides us and others with an in vitro system to start dissecting the multifaceted mechanisms of proteasome inhibition on AAV transduction in various cell types and tissues. Thus, while it might be disappointing that proteasome inhibition does not result in increased heart transduction with AAV9 vectors, a dissection of the complex processes at work may reveal novel and important mechanistic insights that will facilitate the development of future AAV vectors with higher infectivities and/or better defined tissue tropism.

**MATERIAL AND METHODS**

Study design and induction of HF by aortic banding

All procedures involving the handling of animals were approved by the Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai and in adherence with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The aortic banding model was used to generate pressure overload-induced hypertrophy and HF. Sprague-Dawley rats weighing 180–200 g underwent ascending aortic banding, as previously described in detail. For the HF experiment, animals that developed systolic HF were randomized to receive an injection of AAV9.SERCA2a or AAV9.SERCA2a+a-bortezomib (Velcade, Millenium, Cambridge, MA, USA) (n = 3) for 2 months. Age-matched sham-operated animals were used as control (n = 3). The viral dose used was 1 × 10^12 gcp and the bortezomib dose used was 0.1 mg/kg. The virus and the bortezomib were injected simultaneously via the tail vein.

**Echocardiography**

Transthoracic echocardiography was performed using a Vivid 7 echocardiography apparatus with a 14 MHz probe (i13L probe, General Electric, New York, NY, USA). Animals were sedated with ketamine 80–100 mg/kg injected intraperitoneally. Long and short axes parasternal views and short axis parasternal two-dimensional views, at the mid-papillary level, of the LV were obtained to calculate the LV end-diastolic and end-systolic volumes as well as the ejection fraction of the LV. Volumes were calculated by using the formula of the area length method (V = 0.65 × A × L, where A is the area of the LV cavity in cm^2, obtained from the mid-papillary short parasternal image in diastole and in systole, and L is the length of the LV cavity in cm, measured from the long parasternal axis image as the distance from the endocardial LV apex to the mitral–aortic junction in diastole and in systole). M-mode images were obtained by two-dimensional guidance from the parasternal short axis view for the measurements of LV wall thickness of the interventricular septum (IVSd, cm) and the posterior wall (LVPw, cm), LV end-diastolic diameter (LVIDd, cm) and LV end-systolic diameter (LVIDs, cm) as well as to calculate the LV fractional shortening (LVFS, %).

**Invasive P–V loop measurements of the LV**

At the study endpoint, LV P–V loop measurements were obtained as previously described. Briefly, rats were anesthetized with inhaled 5% isoﬂurane for induction, and subsequently intubated and mechanically ventilated in an identical fashion to the aortic banding model and as described previously. Isoflurane was lowered to 2–3% (volume/volume) for surgical incision. The chest was opened through a median sternotomy. A 1.9F rat P–V catheter (Sciensc, London, ON, Canada) was inserted into the LV apex through an apical puncture performed with a 25G needle. Hemodynamic recordings were performed after 5 min of stable heart rate and anesthesia was maintained at 0.75–1.0% isoflurane to keep the animals sedated and maintain a stable heart rate of around 350 beats min^-1. Hemodynamics was recorded subsequently through a Sciensec P–V Control Unit (FY8978). The intrathoracic IVC was transiently occluded to decrease venous return during the recording to obtain load-independent P–V relationships. Linear fits were obtained for end-systolic and end-diastolic pressure–volume relationships. At the end of the experiment, 50 μl of 30% NaCl was slowly injected into the external jugular vein for ventricular parallel conductance (Gp) measurement as previously described. Blood resistivity was measured using a special probe (Sciensc). Volume measurements were initially obtained as blood conductance and were calibrated using the Baan equation, and pressure sensors were calibrated according to the manufacturer’s instructions.

**AAV vector production, purification and characterization**

AAV9.SERCA2a contains a SERCA2a expression cassette flanked by two AAV2 inverted terminal repeats and is pseudotyped with an AAV9 capsid. The SERCA2a expression cassette consists of a CMV promoter followed by the human SERCA2a coding sequence and a SV40 polyadenylation signal. AAV9.SERCA2a was produced in 293 cells with the two-plasmid method and polyethyleneimine. The virus was purified by double-iodixanol purification followed by dialysis by lactated Ringer’s solution. Vector purity, genome containing particle and viral particle titers were determined as described. For in vitro experiments, rat cardiomyocytes were cultured encoding firefly luciferase under the control of a CAG promoter was packaged into AAV2, AAV6 or AAV9 capsids and purified on single-iodixanol gradients to yield AAV2-Luc, AAV6-Luc or AAV9-Luc virus.

**Western blotting**

Protein lysates were obtained from LV tissue that was homogenized in RIPA lysis and extraction buffer which contained protease and phosphatase inhibitors (Pierce, Rockford, IL, USA). Twenty micrograms of total protein extracts were mixed with Laemmli sample buffer containing 5% β-mercaptoethanol (Bio-Rad, Hercules, CA, USA). Samples were heated at 95 ºC for 5 min and then loaded onto 12% SDS-PAGE gels. After electrophoresis, proteins were transferred onto PVDF Membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% fat-free milk in Tris-buffered saline (TBS) for 1 h at room temperature and incubated with the primary antibodies diluted in blocking solution overnight at 4 ºC. The following primary antibodies were used: GAPDH (Sigma, St. Louis, MO, USA; 1:10,000 dilution) and SERCA2a (21st Century Biochemicals, Marlboro, MA, USA; 1:30,000 dilution). The second day, after three washing steps with TBS 0.05% Tween-20, the membrane was incubated with secondary fluorescent anti-mouse and anti-rabbit antibodies (LI-COR Biosciences, Lincoln, NE, USA; 1:10,000 dilution) for 45 min then was washed three times with TBS 0.05% Tween-20. The membrane was then scanned with an Odyssey imaging system (LI-COR Biosciences) and bands were analyzed with Odyssey software, version 3.0 (LI-COR Biosciences). The values obtained were normalized to GAPDH to correct for variation in protein loading.

**Quantitation of vector DNA and RNA in tissue samples**

Frozen heart samples from rats injected with AAV9.SERCA2a or AAV9 empty capsids were ground in liquid nitrogen and stored at −80 ºC until extraction. Total DNA and RNA were purified from ~30 mg of tissue using Oneysec blood and tissue kit and Rnasey mini kit from Qiagen (German-town, MD, USA). mRNA extraction included on-column purification followed by dialysis against lactated Ringer's solution. Vector DNA was quantified using primers specific for human SERCA2a. Primers specific for the intron in the rat SERCA2a gene (forward 5'-TGTGCTGCTTGTGTCTTTGG-3' and reverse 5'-AGCATACGTCGCTGGAAGCCA-3') were used to standardize the number of rat genomes present in each sample. AAV-encoded human SERCA2a RNA was quantified after reverse transcription from cDNA samples with the same primer pair used for viral DNA. Endogenous rat SERCA2a mRNA was quantified after reverse transcription with primers specific for rat SERCA2a that showed no cross-reaction with the human SERCA2a (forward 5'-ATTGAGCATTCCATCTT-3' and reverse 5'-ATCTGATGTTACCTTCACG-3'). Rat GAPDH mRNA was quantified after reverse transcription with the primers rGAPDH forward 5'-ACAAAGTTGGACTGCTGAGA-3' and rGAPDH reverse 5'-AGCTCCTGACATTCCG-3' and was used to normalize the RNA content of all samples.
In vitro transduction assays
HeLa cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin/streptomycin, rat neonatal cardiomyocytes were isolated with the Neonatal Cardiomyocyte Isolation System from Worthington (Lakewood, NJ, USA) according to the manufacturer’s instructions and plated on laminin-coated plates in medium (F10, 10% horse serum, 5% bovine serum albumin, 100 IU/ml-1 penicillin, 100 µg/ml-1 streptomycin). Rat adult cardiomyocytes were isolated by standard procedures45 and plated on laminin-coated plates in Dulbecco’s modified Eagle’s medium, 132 µg/ml-1 bovine serum albumin, 20 µM glutamine, 100 IU/ml-1 penicillin, 100 µg/ml-1 streptomycin, 1% insulin-transferrin-selenium-ethanolamine (Life Technologies, Grand Island, NY, USA); cat. no. 51500-056), 200 µM bortezomib (1.25 µM for HeLa cells) or with 0.01% dimethyl sulfoxide and infected with AAV1-Luc, AAV2-Luc, AAV6-Luc or AA9-Luc at an multiplicity of infection of 1e4 gcp/cell. Transduction assays were performed 16 h post-isolation for neonatal cardiomyocytes and 3 h post isolation for adult cardiomyocytes. Cells were pre-treated for 30 min with 1 µM bortezomib (1.25 µM for HeLa cells) or with 0.01% dimethyl sulfoxide and infected with AAV1-Luc, AAV2-Luc, AAV6-Luc or AA9-Luc at an multiplicity of infection of 1e4 gcp/cell. Lysates were obtained after 24 h for HeLa cells or 48 h for both neonatal and adult cardiomyocytes, and luciferase activity was measured using luciferase assay reagent (Promega, Madison, WI, USA) and a Synergy 2 plate reader (BioTek, Winooski, VT, USA).

Statistical analysis
Results are shown as mean ± s.d. Statistical significance was determined using the Student–Newman–Keuls test. A P-value of <0.05 was considered statistically significant.

CONFLICT OF INTEREST
Dr Hajir is the scientific co-founder of Celladon, which plans to commercialize AAV1:SERCA2a for the treatment of HF. The remaining authors declare no conflict of interest.

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