Prevention of Heart Failure in Mice by an Antiviral Agent that Inhibits Type 5 Cardiac Adenylyl Cyclase

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Abstract

Despite numerous discoveries from genetically engineered mice, relatively few have been translated to the bedside, mainly because it is difficult to translate from genes to drugs. This investigation examines an antiviral drug, which also has an action to selectively inhibit type 5 adenylyl cyclase (AC5), a pharmaceutical correlate of the AC5 knockout (KO) model, which exhibits longevity and stress resistance. Our objective was to examine the extent to which pretreatment with this drug, adenine 9-β-D-arabinofuranoside (Ara-A), favorably ameliorates the development of heart failure (HF). Ara-A exhibited selective inhibition for AC5 compared to the other major cardiac AC isoform, AC6, i.e., it reduced AC activity significantly in AC5 transgenic (Tg) mice, but not in AC5KO mice and had little effect in either wild type (WT) or AC6Tg mice. Permanent coronary artery occlusion for 3 weeks in C57Bl/6 mice increased mortality and induced HF in survivors, as reflected by reduced cardiac function, while increasing cardiac fibrosis. The AC5 inhibitor, Ara-A significantly improved all these end points, and also ameliorated chronic isoproterenol induced cardiomyopathy. As with the AC5KO mice, Ara-A increased MEK/ERK phosphorylation. A MEK inhibitor abolished the beneficial effects of the AC5 inhibitor in the HF model, indicating the involvement of the downstream MEK-ERK pathway of AC5. Our data suggest that pharmacological AC5 inhibition may serve as a new therapeutic approach for HF.

Key words: Heart failure, AC5 inhibition, Ara-A.
Introduction

Despite gains in the treatment of heart failure (HF) with both angiotensin and β-adrenergic receptor (β-AR) blockers, HF still remains a major cause of death and disability. In addition, some patients do not tolerate β-AR blocking therapy (2). It is conceivable that inhibiting mechanisms distal to the β-AR signaling pathway, identified from genetically engineered mouse models, might be a novel approach. While there have been numerous potential therapeutic approaches discovered from studies in genetically engineered mice in the past two decades, there are relatively few of these discoveries that have been translated to the bedside, mainly because it is difficult to translate the effects of disrupting a gene in a mouse to therapy in patients with HF.

The goal of this investigation was to examine the extent to which a pharmacological inhibitor of type 5 adenylyl cyclase (AC5), Ara-A, could mimic the salutary effects observed in the AC5 knock out (KO) mice model, which protects against cardiac stress (10, 11) and increases longevity (15). The first goal was to determine the extent to which Ara-A selectively inhibits AC5. The next goal was to determine whether pretreatment with the pharmacological AC5 inhibitor ameliorates the development of cardiomyopathy and heart failure following either permanent coronary artery occlusion (CAO) or chronic isoproterenol (ISO) infusion. An additional goal was to determine if the mechanism involved the MEK/ERK pathway, a key protective signaling pathway in the AC5 KO (15). The latter was accomplished by repeating the experiments with permanent CAO in the presence of a specific MEK blocker, U0126.

The AC5 inhibitor (AC5I), Ara-A is a Food and Drug Administration (FDA) approved drug, also known as vidarabine (adenine 9-β-D arabinofuranoside), has been used as an anti-herpes virus drug for many decades and the advantage of identifying Ara-A as a potential drug for heart failure is that the drug as already FDA approved could be rapidly moved to clinical trials.
Methods

Animal Models

Three- to five-month-old male AC5KO (9) (on C57Bl/6 background), and cardiac specific overexpression of AC5 (AC5Tg) (7), or AC6 (AC6Tg) (7) (on FVB background) mice were used in this study. In the CAO model, mini-osmotic pumps delivering Ara-A (15 mg/kg/day), the MEK blocker, U0126 (5 mg/kg/day) (15), or a combination of U0126 with Ara-A, were subcutaneously implanted 1 week before the CAO of the left anterior descending artery. Chronic infusion of ISO (Sigma-Aldrich, Inc., St. Louis, MO) was performed for 7 days at a dose of 60 mg/kg/day with or without Ara-A delivered with the mini-osmotic pumps. The dose of Ara-A was selected on the basis of that previously used for viral encephalopathy (13). Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, revised 2011). This study has been approved by the University of Medicine and Dentistry Institutional Animal Care and Use Committee.

AC assay

AC activity was measured by a modification of the method of Salomon et al. (12), as we previously described (12). When the AC assays were performed using crude membranes from AC6Tg mice heart, manganese instead of magnesium was used in the assay buffer to obtain maximum enzymatic catalytic activity because AC6 is stimulated more by manganese than by magnesium (16).

Adult cardiac myocytes.

Adult cardiac myocytes were isolated from Langendorff-perfused mouse hearts as previously described (14). Enzyme solution containing 1 mg/ml collagenase (type II; Worthington), 0.1 mg/ml protease (type XIV; Sigma) and 10 μM blebbistatin (Toronto Research Chemicals) was perfused in a heart for 15-20 min followed by washing. The heart was removed from the
perfusion apparatus and swirled in a culture dish. Ca\(^{2+}\) was gradually added to the dish until the concentration reached 1 mM. The cells were filtered with a cell strainer and cultured in DMEM/F12 medium with 5% horse serum until used for the cAMP accumulation assay.

\[^{3}\text{H}\]Adenine labeling and cAMP accumulation assay

cAMP accumulation assays in adult mouse cardiac myocytes were performed as done previously (8). Briefly, cells were incubated with \[^{3}\text{H}\]adenine (3 µCi/ml) for 3 hours, and cells were washed and pretreated with 20 mM HEPES-balanced serum-free minimum essential medium containing 0.5 mM 3-Isobutyl-1-methylxanthine. After preincubation with Ara-A for 10 min, reactions were started by the addition of 50 µM of the nonspecific AC agonist forskolin or 5 µM of the \(\beta_1/\beta_2\)-AR agonist ISO. Ten minutes after the addition of forskolin or ISO, reactions were terminated by the addition of 12% (w/v) trichloroacetic acid containing 0.25 mM ATP and 0.25 mM cAMP. The \[^{3}\text{H}\]ATP and \[^{3}\text{H}\]cAMP were separated with single acidic alumina columns. The cAMP production was calculated as \[^{3}\text{H}\]cAMP/(\[^{3}\text{H}\]cAMP + \[^{3}\text{H}\]ATP) x 10^4.

Isoproterenol challenge

Mice were anesthetized with 2.5% tribromoethanol (0.015 ml/g of body weight) injected intraperitoneally, and echocardiography was performed. For acute injection of ISO, a PE-10 catheter was inserted into the right jugular vein, and an ISO solution was injected at the rate of 1 µl/s. For ISO challenge experiments, Ara-A, metoprolol or their vehicle, were administered by mini-osmotic pumps from 7 days before the experiment, in a dose 15 mg/kg/day and 5 mg/kg/day, respectively.

Histological analyses

Heart specimens were fixed with formalin, embedded in paraffin, and sectioned at 6-µm thickness. Interstitial fibrosis was evaluated by picric acid sirius red (PASR) staining and
ImagePro-Plus software analysis, as previously described (4, 10).

Western blotting

Western blotting in tissue lysate from the viable region of the LV was conducted with commercially available antibodies against the phosphorylated form and total MEK or ERK (Cell Signaling). Western blotting was performed as previously described (8).

Data and statistical analysis

All data are reported as mean±SEM. Statistical comparisons were calculated using a Student’s t-test and ANOVA analysis with Newman-Keuls post hoc comparison test. The groups passed the normality test and had similar variation. In addition, the Mann-Whitney test confirmed the results from the t-test for the critical data points, e.g., responses of LVEF. Survival curves were compared using log-rank test, and Kaplan-Meier survival analysis. P values of <0.05 were considered significant.
Results

AC5 inhibition decreases cAMP production in the heart.

The hearts of AC5Tg mice showed a tenfold increase in cardiac membrane AC activity using forskolin, indicating that AC5 represented most of the AC activity in the AC5Tg heart, in contrast to the wild type (WT) or AC6Tg heart, where AC5 expression represented a relatively minor fraction of total AC. AC5KO mice showed, as expected, the null AC5 expression (9). When cardiac membrane preparations were used, Ara-A reduced cAMP production much more in AC5Tg than in WT, and not in AC5KO (Figure 1A and 1B). When cultured adult cardiac myocytes were used, Ara-A also demonstrated more effective inhibition in AC5Tg than in WT (Figure 1C and D). We found that the inhibitory effect was similar in WT and AC6Tg, but significantly less than observed in AC5Tg. These data suggest that Ara-A inhibits AC5, more than AC6, in the heart.

Ara-A attenuates contractile response to β-AR stimulation in AC5Tg, but little in WT.

ISO was administered in mice. Ara-A did not reduce baseline LV function and reduced ISO-increased LV ejection fraction (LVEF) only slightly (Figure 2A) in WT mice. In contrast, metoprolol depressed LV function significantly and essentially eliminated the inotropic effects of ISO challenge (Figure 2A). In AC5Tg mice, the acute ISO challenge increased LVEF even more in the vehicle group, but this increased inotropic effect was not observed in the Ara-A group (Figure 2B). Thus, the ability of Ara-A to block the inotropic effects of ISO is obvious only when AC5 is overexpressed. The response to ISO challenge in the AC6Tg group was similar to vehicle (Figure 2C), similarly to the WT group response, further indicating the selectivity of Ara-A for AC5 (Figure 2A-D). Therefore, in contrast to metoprolol, Ara-A did not act as a β-AR blocker, i.e., did not depress cardiac function and did not eliminate the inotropic effects of ISO.
**Ara-A attenuates the progression of post-myocardial infarction (MI) HF.**

Next, we examined the extent to which Ara-A ameliorated post-MI cardiomyopathy. The post-MI cardiomyopathy model was induced by permanent ligation of the left anterior descending coronary artery, which results in an infarct size of 30-40% of the LV (Data not shown). In this model the LVEF was reduced significantly, p<0.05, 42.2% compared with sham 71.6%, while the LV end-diastolic diameter (LVEDD) was increased, p<0.05, from 3.9 in the sham to 5.6 mm in the vehicle treated post-MI cardiomyopathy group. Lung weight / tibial length, which is an indicator of HF, was increased, p<0.05, in the post-MI cardiomyopathy group (12.8) in comparison with the sham group (7.2) (Table 1). Ara-A improved LVEF by 38% and reduced LV diastolic end-diastolic diameter by 14% compared with the vehicle group (Figure 3B and Table 1). Ara-A also significantly improved survival rate compared to vehicle (p<0.05, log-rank test) (Figure 3A), and reduced, p<0.05, intestinal fibrosis (Figure 4A and 4B). At autopsy, the cause of death in the mice that died was either due to cardiac rupture or heart failure.

**MEK-ERK pathway mediates the salutary effects of Ara-A.**

Administration of Ara-A increased the phosphorylation of MEK, ERK1 and ERK2 in WT mouse hearts (Figure 5A) and in the post-MI cardiomyopathy model (Figure 5B). U0126, a MEK inhibitor, inhibited basal and Ara-A-induced ERK phosphorylation, suggesting that U0126 indeed inhibits ERK signaling in the heart in vivo, and Ara-A activates ERK via MEK phosphorylation. U0126 inhibited activity of MEK, but not phosphorylation itself, which is consistent with a previous report by Favata et al (6). We found that U0126 abolished the salutary effects of Ara-A in terms of survival (Figure 3A), preservation of LV function (Figure 3B) and histological evidence of fibrosis (Figure 4A and 4B).

**Ara-A preserves cardiac function with chronic catecholamine stress.**

We next examined whether the AC5 inhibitor, Ara-A, attenuates cardiac dysfunction induced by
excessive catecholamine stress with chronic ISO infusion. Survival rate during chronic ISO
infusion was higher in the Ara-A group than in the vehicle group (p<0.05, log-rank test) (Figure
6A). Ara-A showed preserved contractile function as measured by LVEF (Figure 6B),
suggesting that Ara-A protects against ISO-induced cardiac dysfunction. These data indicate
that Ara-A retards the progression of ISO-induced cardiomyopathy.
Discussion

There are many discoveries in genetically altered mice that cannot be applied clinically, since there is no pharmacological counterpart that can be given to animals or patients with cardiovascular disease. The major finding of this investigation is that a drug, which has been commercially available for decades, but only as an anti-viral agent, has potent and selective AC5 inhibitory properties and that this drug ameliorates the progression of cardiomyopathy in animals induced with either chronic ISO or MI. With both interventions, Ara-A demonstrated increased survival, preserved contractile dysfunction, and reduced cardiac interstitial fibrosis.

First, it was important to demonstrate that Ara-A impairs AC5 activity selectively, both \textit{in vitro} and \textit{in vivo}. AC5Tg showed enhanced cAMP production compared to WT, and the effects of Ara-A were greater in AC5Tg than that either in WT or in AC6Tg, which represents the other major AC isoform in the heart, indicating a high selectivity for AC5. This was supported by the data showing that Ara-A does not inhibit cAMP production in AC5KO and inhibits cAMP almost identically in AC6Tg and WT. We also examined this inhibitor \textit{in vivo} and demonstrated in parallel experiments that Ara-A reduced ISO stimulated LVEF more in AC5Tg than WT, and reduced the ISO response minimally in AC6Tg, similar to that in WT. If Ara-A was a non-selective AC inhibitor, then it should have shown enhanced AC inhibition with overexpressed AC6 as it did with overexpressed AC5. However, although we demonstrated relatively specific selectivity for AC5, and the MEK/ERK pathway, this does not mean that the inhibitor may also have other actions as well.

One could argue that Ara-A exerts its beneficial effect in ameliorating the extent of HF, simply acting as another β-AR blocker, rather than specifically on AC5, since AC5 is involved in β-AR signal transduction. We do not subscribe to this view for several reasons. First, Ara-A reduced cAMP production relatively modestly in response to ISO in WT mice \textit{in vitro} and \textit{in vivo},
particularly compared with the effects of metoprolol, which essentially abolished the inotropic response to ISO. Ara-A inhibited the inotropic response to ISO significantly only in the presence of elevated AC5, as in the AC5Tg, which was still less of a negative inotropic effect than metoprolol. Furthermore, the AC5 KO mouse does not show a decreased heart rate and only minimally reduced LV function (7), which is not consistent with the actions of a beta blocker. In support of this, heart rate was not lower in animals with chronic MI treated with Ara-A (Table 1).

Ara-A was previously found to inhibit AC5 activity by the computer-based drug screening system and its inhibition was confirmed in in vitro AC assays (8). The major finding of the current investigation was to demonstrate that pretreatment with this drug ameliorated the development of HF through the MEK-ERK pathway. Thus, an anti-herpes viral drug could be utilized in the treatment of HF through mechanisms that have never been considered previously, i.e. inhibition of AC5. Since the animals that died after the intervention most likely suffered more severe LV dysfunction, and more animals died without treatment, then it could be argued that the salutary effects Ara-A with both chronic ISO and post-MI may be underestimated since the mortality was also reduced.

It is important to point out that the cellular mechanism mediating the beneficial effects of Ara-A does not involve simple β-AR blockade, but rather involves MEK-ERK signaling. The link between reduced AC5 as in the AC5KO mouse and the increase in Raf-1-MEK-ERK signaling was elucidated in a prior study from our laboratory demonstrating that in the AC5KO mouse this pathway was involved in enhanced longevity in this mouse model (15). The current investigation demonstrates that the MEK-ERK pathway is also involved in the protection afforded in the heart by Ara-A during the development of HF and cardiac remodeling induced by MI, as evidenced by the increase in MEK-ERK signaling with Ara-A and the blockade of the salutary effects of the AC5 inhibition with the MEK inhibitor U0126.
It is well recognized that acutely administered ISO improves LV function, whereas chronic ISO induces LV dysfunction and eventually HF along with increased mortality (1, 14). Ara-A also preserved cardiac function and reduced mortality with chronic ISO in the current study. Thus, it is tempting to speculate that pharmacological inhibition of AC5 could enhance survival in HF patients and preserve their cardiac function.

An underlying assumption of the current study is that the induction of heart failure, either by chronic ISO or chronic myocardial infarction, induces upregulation of AC5 in the heart. Indeed this was found in a prior study in the chronic ISO model (9). This was more difficult to demonstrate in the chronic myocardial infarction model in the mouse, since there is so little salvaged myocardium adjacent to the infarct. Accordingly, we examined a rat model of chronic myocardial infarction and analyzed AC5 protein content, using a specific AC5 antibody (5), in myocardial samples, both adjacent and remote to the infarct. The upregulation of AC5 in the remote zone was only modest, but was more striking in the adjacent tissue (Figure 7).

In summary, AC5 inhibition with Ara-A could be a new approach to the treatment of HF. In addition to its favorable action on halting the progression to HF due to cardiomyopathy following either permanent CAO or chronic ISO, it exerts little cardiac depression, potentially making the drug more tolerable for patients with compromised cardiac function. Importantly, since the drug studied is already FDA approved, the time from bench to bedside may be accelerated.
Acknowledgements

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Author Contributions

Kousaku Iwatsubo, Dorothy E. Vatner, Stephen F. Vatner and Yoshihiro Ishikawa directed the study, created experimental designs, analyzed data, and wrote the paper. Claudio Bravo conducted experiments, analyzed data, and revised the manuscript. Shumin Gao handled all echocardiography. Lin Yan and Lo Lai performed the biochemistry work. Hongyu Qiu, Erdene Baljinnyam, Xin Zhao, Takashi Nakamura, and Masami Uechi conducted the experiments. Misun Park was responsible for all histology work. Satoshi Okumura’s responsibilities involved writing and editing the manuscript. Mizuka Iwatsubo performed data analysis and also wrote the manuscript. And Masanari Umemura conducted experiments.
References


Figure Legends

Figure 1. Selective inhibition of AC5 by Ara-A (in vitro)
Mouse cardiac membrane preparations were used for panels A and B. Adult mouse cardiac myocytes were used for panels C and D. (A, B) cAMP reduction with Ara-A (10 µM) was measured in cardiac membrane preparations from myocardium of WT, AC5KO, AC5Tg and AC6Tg mice with ISO 5µM (A) or with forskolin 50 µM (B). (C, D) cAMP reduction with Ara-A (10 µM) was measured in adult cardiac myocytes from myocardium of WT, AC5KO, AC5Tg and AC6Tg mice with ISO (C) or with forskolin (D). cAMP reduction by Ara-A was greater in AC5Tg than in WT, was similar between WT and AC6Tg, and was absent in the AC5KO. These data indicate that Ara-A selectively suppresses AC5 enzymatic activity. T-test *p<0.01 vs. vehicle. n=4 experiments for panels A and B and n=5 for panels C and D.

Figure 2. Selective inhibition of AC5 by Ara-A (in vivo)
LVEF was measured in response to ISO challenges in WT (A), AC5Tg (B), and AC6Tg (C) in the presence of either vehicle, Ara-A or metoprolol (n=6 per group). (D) Shows the negative inotropic effect of Ara-A at baseline and at a dose of 0.02 and 0.04 µg/kg/min of ISO. Ara-A decreased ISO-induced increases in LVEF more in AC5Tg mice than WT or AC6Tg mice (A-D). These data show that Ara-A selectively suppresses AC5 in vivo. In contrast to Ara-A, the β-AR blocker, metoprolol, decreased basal LV function and also completely blocked the positive inotropic response to ISO (A). T-test *p<0.05 Ara-A vs. vehicle, †p<0.05 metoprolol vs. Ara-A or vs. vehicle; †p<0.05 vs. same ISO dose in WT or vs. same ISO dose in AC6Tg.

Figure 3. Ara-A protects against post-MI cardiomyopathy.
WT C57Bl/6 mice after permanent CAO (A) Follow up for 21 days. Kaplan-Meier graph of post-MI animals treated with Ara-A, Ara-A+U0126 or Vehicle. Ara-A enhanced the survival post-MI in WT C57Bl/6 mice. Log-Rank Test *p<0.05 vs. Ara-A. n=16 animals per group (B) in survivors
from experiment in panel A, LVEF was improved by Ara-A (Ara-A n=12, Ara-A +U0126 n=5 and vehicle n=4). U0126 blocked the protective effects of Ara-A in terms of mortality and LVEF (A and B). T-test *p<0.05 vs. Ara-A; †p<0.05 vs. vehicle, Ara-A or Ara-A+U0126.

**Figure 4. Ara-A protects against cardiac fibrosis post MI**

WT C57Bl/6 mice after permanent CAO. (A) Representative images of fibrosis adjacent to infarcted myocardium with PASR staining from animals treated with vehicle or Ara-A. n=16 animals per group. (B) Fibrosis was increased post-MI both adjacent and remote from the infarcted area and was partially protected by Ara-A. U0126 blocked this protection with Ara-A. Vehicle group n=4, Ara-A group n=10 and Ara-A+U0126 n=6 animals. T-test *p<0.05 vs. vehicle; †p<0.05 vs. Ara-A within the respective zone (either adjacent or remote).

**Figure 5. MEK/ERK signaling is activated by Ara-A.**

(A) Western blot analyses showed that Ara-A increased phosphorylation of MEK and ERK in the mice hearts with chronic infusion of Ara-A for 6 days. Control mice did not undergo any pump implantation. (B) Animals treated with vehicle, Ara-A and Ara-A+U0126 post MI and sham were evaluated for MEK/ERK activation by western blot. Ara-A increased ERK phosphorylation, which was inhibited by U0126. U0126 selectively inhibited ERK phosphorylation but not phosphorylation of MEK. The numbers at the base of the bars refer to different groups. n=4 animals per group. T-test *p<0.05 vs. vehicle or vs. control; †p<0.05 vs. Ara-A.

**Figure 6. AC5 inhibition attenuates chronic ISO-induced HF.**

WT C57Bl/6 mice for 7 days of ISO infusion. (A) Ara-A increased the survival rate during the period of chronic ISO infusion. n=32 for ISO and n=18 for ISO+Ara-A group. (B) Ara-A prevented LVEF dysfunction induced by chronic ISO. ISO n=28 and ISO+Ara-A n=16. Vehicle n=4, Ara-A n=6 and Ara-A+ISO n=6. T-test *p<0.05 vs. vehicle or vs. Ara-A+ISO.
Figure 7. AC5 Protein levels are increased adjacent to infarct.

Western blotting demonstrated that AC5 protein levels, assessed with a specific AC5 antibody (5), were significantly upregulated adjacent to the infarct in the chronic CAO model and less so in the remote zone. In this experiment we used a rat model, because there was not enough tissue adjacent to the infarct in the mouse chronic MI model. * p<0.05 from sham; † p<0.05 from remote.
Figure 1.

A. Cardiomyocyte Membranes

B. Cardiomyocyte Membranes

C. Isolated Cardiomyocytes

D. Isolated Cardiomyocytes

WT, AC5KO, AC5Tg, AC6Tg

Isoproterenol

Forskolin

Vehicle • Ara-A

cAMP production (pmol/mg/min)
cAMP production (cAMP/Total x 10^-4)

* indicates significant difference.
Figure 2.

A. WT

B. AC5Tg

C. AC6Tg

D. $\Delta$LVEF (Ara-A minus Vehicle)

- Vehicle
- Ara-A
- Metoprolol
Figure 3.

A

![Graph showing survival rate over days after CAO for different treatments: Ara-A, Ara-A+U0126, and Vehicle.](image)

B

![Bar chart showing % LVEF for different treatments: Sham, Vehicle, Ara-A, and Ara-A+U0126.](image)
Figure 4.
Figure 5.

A. Drug infusion only

B. Drug infusion plus MI

Legend:
- Control
- Vehicle
- Ara-A
- Ara-A+U0126
- U0126

**Ratio of Phosphorylated form/total protein**
Figure 6.
Figure 7.
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<th></th>
<th>Sham (n=4)</th>
<th>Vehicle (n=4)</th>
<th>Ara-A (n=12)</th>
<th>Ara-A + U0126 (n=6)</th>
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<tr>
<td>Heart Rate (Beats per minute)</td>
<td>386 ± 14</td>
<td>517 ± 12 ‡</td>
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<td>LV Ejection Fraction (%)</td>
<td>71.6 ± 1.6</td>
<td>42.2 ± 4.6 ‡</td>
<td>56.9 ± 1.8 *</td>
<td>47.9 ± 1.3 †</td>
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<td>LV End Diastolic Diameter (mm)</td>
<td>3.9 ± 0.2</td>
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<td>5.7 ± 0.2 †</td>
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<td>LV End Systolic Diameter (mm)</td>
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* p<0.05: Ara-A different from vehicle
† p<0.05: Ara-A+U0126 different from Ara-A
‡ p<0.05: Vehicle different from Sham