Prevention of heart failure in mice by an antiviral agent that inhibits type 5 cardiac adenylyl cyclase

Kosaku Iwatsubo,1,3 Claudio Bravo,1 Masami Uechi,2 Erdene Baljinnyam,1 Takashi Nakamura,4 Masanari Umemura,1 L o Lai,1 Shumin Gao,1 Lin Yan,1 Xin Zhao,1 Misun Park,1 Hongyu Qiu,1 Satoshi Okumura,1 Mizuka Iwatsubo,1 Dorothy E. Vatner,1,2 Stephen F. Vatner,1 and Yoshihiro Ishikawa1,2,3

1Department of Cell Biology and Molecular Medicine, Cardiovascular Research Institute, New Jersey Medical School-University of Medicine and Dentistry of New Jersey, Newark, NJ; 2Department of Medicine (Cardiology), New Jersey Medical School-University of Medicine and Dentistry of New Jersey, Newark, NJ; 3Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Yokohama, Japan; and 4Department of Veterinary Medicine, College of Bio-resource Sciences, Nihon University, Fujisawa, Kanagawa, Japan

Submitted 7 March 2012; accepted in final form 11 April 2012


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), 9-β-d-arabinofuranoside (Ara-A), could mimic the salutary effects observed in the AC5 knockout (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 HF following either permanent coronary artery occlusion (CAO) or chronic isoproterenol (ISO) infusion. An additional goal was to determine if the mechanism involved the mitogen/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway in the AC5KO (15). The latter was accomplished by repeating the experiments with permanent CAO in the presence of a specific MEK blocker, U-0126.

The AC5 inhibitor Ara-A is a Food and Drug Administration (FDA)-approved drug, also known as vidarabine (adenine 9-β-D-arabinofuranoside), and has been used as an anti-herpes virus drug for many decades. The advantage of identifying Ara-A as a potential drug for HF is that the drug has already been FDA approved and 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 [AC5 transgenic (TG)] (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−1) (15), the MEK blocker U-0126 (5 mg·kg·day−1) (15), or a combination of U-0126 with Ara-A were subcutaneously implanted 1 wk before the CAO of the left anterior descending artery. Chronic infusion of ISO (Sigma-Aldrich, St. Louis, MO) was performed for 7 days at a dose of 60 mg·kg·day−1 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

Address for reprint requests and other correspondence: S. F. Vatner, Cardiovascular Research Institute, Dept. of Cell Biology and Molecular Medicine, New Jersey Medical School-UMDNJ Newark, NJ 07103 (e-mail: vatnersf@umdnj.edu).
Fig. 1. Selective inhibition of type 5 adenylyl cyclase (AC5) by adenine 9-β-β-arabinofuranoside (Ara-A) (in vitro). Mouse cardiac membrane preparations were used for A and B. Adult mouse cardiac myocytes were used for C and D. A and B: cAMP reduction with Ara-A (10 μM) was measured in cardiac membrane preparations from myocardium of wild-type (WT), AC5 knockout (KO), AC5 transgenic (Tg), and AC6Tg mice with isoproterenol (ISO) (5 μM, A) or with forskolin (50 μM, B). C and 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 A and B and n = 5 for C and D.

Fig. 2. Selective inhibition of AC5 by Ara-A (in vivo). Left ventricle ejection fraction (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 animals/group). D: negative inotropic effect of Ara-A at baseline and at a dose of 0.02 and 0.04 μg·kg⁻¹·min⁻¹ 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-adrenergic receptor (β-AR) blocker metoprolol decreased basal left ventricle (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; and ‡P < 0.05 vs. same ISO dose in WT or vs. same ISO dose in AC5Tg.
Heart failure prevented by AC5 inhibitor

Table 1. Postmyocardial infarction cardiomyopathy model

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 4)</th>
<th>Vehicle (n = 4)</th>
<th>Ara-A (n = 12)</th>
<th>Ara-A + U-0126 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>386 ± 14</td>
<td>517 ± 12‡</td>
<td>520 ± 7</td>
<td>511 ± 13</td>
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<tr>
<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>
</tr>
<tr>
<td>LV end diastolic diameter, mm</td>
<td>3.9 ± 0.2⅃</td>
<td>5.6 ± 0.1⅃</td>
<td>4.8 ± 0.1⅃</td>
<td>5.7 ± 0.2†⅃</td>
</tr>
<tr>
<td>LV end systolic diameter, mm</td>
<td>2.6 ± 0.2⅃</td>
<td>4.7 ± 0.2⅃</td>
<td>3.6 ± 0.1⅃</td>
<td>4.6 ± 0.1⅃</td>
</tr>
<tr>
<td>LV wt/tibial length, mg/mm</td>
<td>5.1 ± 0.4</td>
<td>6.6 ± 0.3§⅃</td>
<td>7.1 ± 0.2⅃</td>
<td>7.0 ± 0.3⅃</td>
</tr>
<tr>
<td>Lung wt/tibial length, mg/mm</td>
<td>7.2 ± 0.3</td>
<td>12.8 ± 0.7§⅃</td>
<td>10.6 ± 0.4§⅃</td>
<td>15.4 ± 0.6§⅃</td>
</tr>
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</table>

Values are means ± SE; n, no. of animals. Ara-A, adenine 9β-t-arabinofuranoside; LV, left ventricular. *P < 0.05, Ara-A different from vehicle (*), Ara-A + U-0126 different from Ara-A (‡), and vehicle different from sham (§).

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. (10), 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. Ca²⁺⁺ was gradually added to the dish until the concentration reached 1 mM. The cells were filtered with a cell strainer and cultured in DMEM/F-12 medium with 5% horse serum until used for the cAMP accumulation assay.

[3H]adenine labeling and cAMP accumulation assay. cAMP accumulation assays in adult mouse cardiac myocytes were performed as previously done (8). Briefly, cells were incubated with [3H]adenine (3 μCi/ml) for 3 h, 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 β₁/β₂-AR agonist ISO. Ten minutes after the addition of forskolin or ISO, reactions were terminated by the addition of 12% (wt/vol) trichloroacetic acid containing 0.25 mM ATP and 0.25 mM CAMP. The [3H]ATP and [3H]cAMP were separated with single acidic alumina columns. The cAMP production was calculated as [3H]cAMP/[([3H]cAMP + [3H]ATP)] × 10⁴.

ISO challenge. Mice were anesthetized with 2.5% tribromoethanol (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. Ca²⁺⁺ was gradually added to the dish until the concentration reached 1 mM. The cells were filtered with a cell strainer and cultured in DMEM/F-12 medium with 5% horse serum until used for the cAMP accumulation assay.

RESULTS

AC5 inhibition decreases cAMP production in the heart. The hearts of AC5Tg mice showed a 10-fold 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 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 LV ejection fraction (LVEF). Survival curves were compared using the log-rank test and Kaplan-Meier survival analysis. *P values of <0.05 were considered significant.
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 (Fig. 1, A and B). When cultured adult cardiac myocytes were used, Ara-A also demonstrated more effective inhibition in AC5Tg than in WT (Fig. 1, C 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 LVEF only slightly (Fig. 2A) in WT mice. In contrast, metoprolol depressed LV function significantly and essentially eliminated the inotropic effects of ISO challenge (Fig. 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 (Fig. 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 (Fig. 2C), similarly to the WT group response, further indicating the selectivity of Ara-A for AC5 (Fig. 2, A–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 postmyocardial infarction HF. Next, we examined the extent to which Ara-A ameliorated postmyocardial infarction (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%), whereas the LV end-diastolic diameter 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) compared 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 (Fig. 3B and Table 1). Ara-A also significantly improved survival rate compared with vehicle \( (P < 0.05, \text{log-rank test}) \) (Fig. 3A), and reduced, \( P < 0.05, \) intestinal fibrosis (Fig. 4, A and B). At autopsy, the cause of death in the mice that died was either due to cardiac rupture or HF.

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 (Fig. 5A) and in the post-MI cardiomyopathy model (Fig. 5B). U-0126, a MEK inhibitor, inhibited basal and Ara-A-induced ERK phosphorylation, suggesting that U-0126 indeed inhibits ERK signaling in the heart in vivo, and Ara-A activates ERK via MEK phosphorylation. U-0126 inhibited activity of MEK, but not phosphorylation itself, which is consistent with a previous report by Favata et al. (4). We found that U-0126 abolished the salutary effects of Ara-A in terms of survival (Fig. 3A), preservation of LV function (Fig. 3B), and histological evidence of fibrosis (Fig. 4, A and B).

Fig. 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 picric acid Sirius red (PASR) staining from animals treated with vehicle or Ara-A; \( n = 16 \) animals/group. B: fibrosis was increased post-MI both adjacent and remote from the infarcted area and was partially protected by Ara-A. U-0126 blocked this protection with Ara-A; vehicle group, \( n = 4 \); Ara-A group, \( n = 10 \); and Ara-A + U-0126, \( n = 6 \) animals. t-Test: \( ^* P < 0.05 \) vs. vehicle; \( ^\dagger P < 0.05 \) vs. Ara-A within the respective zone (either adjacent or remote).
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) (Fig. 6A). Ara-A showed preserved contractile function as measured by LVEF (Fig. 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 in vitro and in vivo. AC5Tg showed enhanced cAMP production compared with 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 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 nonselective 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 \( \beta \)-AR blocker, rather than specifically on AC5, since AC5 is involved in \( \beta \)-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 in vitro and 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 β-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 used in the treatment of HF through mechanisms that have never been considered previously, i.e., inhibition of AC5.

Because 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 of 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 U-0126.

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 HF, either by chronic ISO or chronic MI, 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 MI model in the mouse, since there is so little salvaged myocardium adjacent to the infarct. Accordingly, we examined a rat model of chronic MI 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 (Fig. 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.

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ACKNOWLEDGMENTS

This study was presented at the American Heart Association Melvin Marcus Young Investigator competition, November 2010.

GRANTS

This study is supported in part by grants from the American Heart Association (SDG 0835596D), the Foundation of the University of Medicine and Dentistry of New Jersey, the Melanoma Research Foundation, the Japanese Ministry of Health Labor and Welfare, Grant-in-Aid for Scientific Research on Innovative Areas (22136009), and National Institutes of Health Grants GM-067773, HL-102472, AG-027211, HL-033107, HL-059139, HL-069752, HL-067773, HL-095888, HL-069020, HL-062863, AG-023137, AG-014121, and AG-023567.

DISCLOSURES

No conflicts of interest are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES