Published in final edited form as:

J Cardiovasc Pharmacol. 2013 June ; 61(6): 553–559. doi:10.1097/FJC.0b013e31828e8758.

The therapeutic effect of 2-cyclohexylthio-AMP in heart failure

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Abstract

Aim—to investigate the therapeutic effect of 2-cyclohexylthio-AMP in mice with heart failure (HF).

Methods—2-cyclohexylthio-AMP was dissolved in PBS and infused in mice with ischemic heart failure after permanent left coronary (LAD) ligation and in calsequestrin (CSQ) mice with heart failure. Myocardial function ex vivo was determined in the working heart model. Cardiac function in vivo was assessed by echocardiography.

Results—Injection of 2-cyclohexylthio-AMP induced a dose-dependent increase in +dP/dt, -dP/dt and LVDevP in normal WT mice and in CSQ mice with HF using the ex vivo working heart model. Spontaneous heart rate did not change after injection of 2-cyclohexylthio-AMP. Compared with normal saline treated mice, chronic infusion of 2-cyclohexylthio-AMP in mice with ischemic HF after left coronary artery (LAD) ligation and in CSQ mice resulted in improved +dP/dt, -dP/dt, LVDevP and fractional shortening, restored the β -adrenergic response and decreased heart weight/body weight ratios.

Conclusion—2-Cyclohexylthio-AMP improved the cardiac contractile performance and rescued mice from heart failure. This salutary action may result from reduction of myocardial hypertrophy and the restoration of the β -adrenergic response in both LAD ligation and CSQ mice models of heart failure. That this agent can increase contractile performance without heart rate increase should be desirable in heart failure therapy.

Keywords

heart failure; P2X4 receptor; cardiac contractility; purines; ischemia

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Conflicts of Interest/Disclosures: None

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Introduction

Heart failure (HF) is an emerging epidemic affecting 15 million people in the USA and Europe. HF-related mortality was unchanged between 1995 and 2009, despite a decrease in the incidence of cardiovascular disease. Conventional explanations for this emerging epidemic include an aging population and improved treatment of acute myocardial infarction and HF.¹ Treatment of patients with acutely decompensated HF and signs of low cardiac output remains one of the major challenges of current cardiovascular disease therapy. New agents are needed. Some of the desirable characteristics include lack of tachycardic or bradycardic effect and yet retention of an enhanced myocardial contractile function with less oxygen consumption.²

Recent evidences suggest that P2X receptors (P2XR) may play an important biological role in the heart. P2X4R is an important subunit of the native cardiac myocyte P2XR.³ Mice with cardiac-specific overexpression of the P2X4R (P2X4R Tg mice) showed an enhancement of basal contractile function⁴ and are protected from HF.⁵⁻⁷ Mice with cardiac-specific overexpressing calsequestrin (CSQ mice) develop dilated cardiomyopathy and die prematurely of HF.⁸ The CSQ model is a genetic model of heart failure that responds to drug effective in treating human heart failure.⁹ When CSQ mice also overexpressed P2X4R, they exhibited better cardiac function and survived longer.⁵ Cardiac overexpression of P2X4R was also protective in left anterior descending (LAD) coronary artery ligation model of ischemic HF⁶. These data indicate a salutary role of the cardiac P2X4R in HF.

The P2X4R can be activated by ATP or its potent analogue 2-methylthioadenosine-5'-triphosphate (2-mes-ATP).¹⁰⁻¹² In the heart, extracellular ATP can elicit an increase in contractility without an increase in cardiac myocyte cyclicAMP levels.¹⁰ 2-mes-ATP can induce a membrane current in mouse ventricular myocytes.³ 2-mes-ATP had negligible agonist activity on P2Y receptors in this model and did not stimulate PLC effectively, although in cell models this compound activates a P2Y₁ receptor.¹³⁻¹⁵ 2-mes-ATP increased the +dP/dt and -dP/dt in isolated working heart preparation up to 25% of that caused by the β -adrenergic agonist isoproterenol. The positive inotropic effect of 2-mes-ATP was not accompanied by any increase in heart rate, different from the significant positive chronotropic effect of β -adrenergic agonist. This property may make P2X agonist a desirable agent in the treatment of heart failure since it will not be accompanied by any heart rate-related increase in oxygen consumption.¹¹

In adult murine ventricular myocytes, the enhanced contractile state induced by 2-mes-ATP was not accompanied by any changes in L-type calcium channel current-voltage relationship.¹² 2-Cyclohexylthio-AMP is a synthetic derivative of AMP¹³⁻¹⁵ and its chemical structure is shown in figure 1. Compared with 2-mes-ATP, 2-cyclohexylthio-AMP is more stable to nucleotidases.¹⁵ The aim of this study is to investigate the therapeutic effect of 2-cyclohexylthio-AMP in two different mouse models of heart failure.

Materials and Methods

Materials

2-Cyclohexylthio-AMP (structure shown in Fig. 1) was synthesized by according to the literature¹³⁻¹⁵ with purity more than 99%. Isoproterenol was purchased from Sigma Company (St. Louis, MO).

Methods

All animal work was reviewed and approved by Institutional Animal Care and Utilization Committee of University of Connecticut Health Center, Farmington, CT.

Systolic heart failure models induced by LAD ligation and calsequestrin overexpression

Left anterior descending artery (LAD) was permanently ligated in mice using the previously established procedure.⁶ After anesthetizing with ketamine (100 mg/kg) and xylazine (10 mg/kg) in WT (BL6) mice 14-16 wk old of either gender, animals were intubated and placed on a rodent ventilator (Hugo Sachs-Harvard Apparatus, Minivent Type 845, Holliston, MA). The respiratory volume was 0.3 ml with a rate of 150 per min. Ligation of LAD was carried out using an 8-0 nylon suture approximately 2 mm below the edge of left atrium. More than three fourths of the animals survived the procedure and recovered that were in turn subjected to drug or vehicle interventions. The calsequestrin (CSQ) mice of severe hypertrophy and dilated cardiomyopathy were generated by cardiac-specific overexpression of CSQ^{5,8,16}. The CSQ mice were kindly provided by Dr. Larry Jones (Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN).

Drug Administration *in vivo*—2-Cyclohexylthio-AMP was dissolved in phosphate-buffered saline (pH 7.4) at 10 μ mol/L, sterilely filtered and infused to mice with HF via a mini-osmotic pump (200 μ L, Alzet, Cupertino, CA) using previously described methods.¹⁷ The drug was infused from mini-osmotic pump at 6 μ l or 0.06 nanomoles per day for 28 days. At various indicated times, echocardiography and isolated working heart preparation were performed to determine the *in vivo* and *ex vivo* heart functions, respectively.

Measurement of intact heart function by an *ex vivo* working heart preparation and determination of infarct size

Various parameters of intact heart function, such as left ventricular developed pressure (LVDevP), rates of pressure increase (+dP/dt) and of pressure decrease (−dP/dt), and total cardiac output were determined using an isolated, *ex vivo* working heart model as described previously.⁴ In brief, after removing lungs and branches of major vessels, aorta was inserted with a 20-gauge catheter. To achieve physiologic flow, pulmonary vein was cannulated with a polyethylene PE-50 catheter connected to a reservoir of Krebs-Henseleit solution (KHS)¹¹ that caused flow into the left atrium, left ventricle and then forward output to the aorta, producing a work-performing heart preparation. Aortic flow represented the amount of perfusing solution obtained from the aortic cannula measured in milliliter per minute while coronary flow was measured via the amount collected from the opening of the pulmonary artery. The sum of aortic flow and coronary flow was equal to cardiac output. A 23-gauge needle connected to a fluid-filled catheter was inserted into the left ventricle and permitted measurement of pressure by a transducer to record left ventricle (LV) pressures and $\pm$ dP/dt. The LVDevP was LV systolic minus LV diastolic pressures. Pressure measurements were obtained by a transducer simulator/calibrator (Kent Scientific; Litchfield, CT) following analysis via computer software (Work-Bench for Windows1, Kent Scientific). A side port of the apparatus delivering perfusion to the pulmonary vein was used to inject 2-cyclohexylthio-AMP or isoproterenol in nM concentration in perfusing solution to test their acute contractile effects. The infarct size was quantitatively determined as the ratio of infarct length to the circumference of endocardium and epicardium according to previously described method.^{6,18,19}

***In vivo* Echocardiography**—Transthoracic echocardiograms were obtained in isoflurane-anesthetized mice using Vevo 660 High Resolution Imaging System from VisualSonics, Toronto, Canada according to previously described procedure.^{6,17} Echocardiographic measurements were carried out at mid-papillary muscle level and were the averages of three or more cardiac cycles. Fractional shortening (FS) was defined as LV end-diastolic diameter minus LV end-systolic diameter divided by LV end diastolic diameter and served as an indication of *in vivo* cardiac function.

Data Analysis—Data were shown as mean $\pm$ SD. Student's *t* test was used to evaluate the statistically significant differences between two independent groups. Repeated measures ANOVA and posttest comparison were used to analyze differences in the increase of +dP/dt or LVDevP caused by multiple doses of 2-cyclohexylthioAMP; $P < 0.05$ was taken as statistically significant.

Results

2-Cyclohexylthio-AMP enhanced cardiac contractile function when injected in the ex vivo working heart model

The effect of 2-cyclohexylthio-AMP on +dP/dt and LVDevP in normal adult WT mice and CSQ mice with HF are shown in figures 2A and 2B. Injection of 2-cyclohexylthio-AMP induced a dose-dependent increase in +dP/dt and LVDevP in normal WT mice in the ex vivo working heart model. This was an in vitro drug administration to determine the acute hemodynamic effect. The net increased maxima of +dP/dt and LVDevP was 1192.5 ± 285.8 mmHg/sec and 17.6 ± 4.0 mmHg, respectively. In the CSQ mice with HF, 2-cyclohexylthio-AMP also caused an increase in +dP/dt and LVDevP in dose-dependent manner. The net increased maxima of +dP/dt and LVDevP were 1149.3 ± 149.4 mmHg/sec and 17.0 ± 2.7 mmHg, respectively. In either WT or CSQ hearts, the drug was able to also increase the -dP/dt in a dose-dependent manner (Table 1). The spontaneous heart rate did not change after injection of 2-cyclohexylthio-AMP at any of the concentrations in either WT or CSQ hearts. The reason for a lack of any acute effect on heart rate is not known but may be related to the absence of a putative receptor for this agent on the sinoatrial node cells.

Chronic infusion of 2-cyclohexylthio-AMP to mice with ischemic HF results in improved contractile function as determined by ex vivo and in vivo methods

The agent was infused within 2 day after LAD ligation. At 7 days, 1 month and 2 months after drug infusion, the cardiac contractile function was determined by ex vivo and in vivo methods. The results are shown in figure 3. Compared with vehicle-(normal saline, NS) treated mice, 2-cyclohexylthioAMP-treated mice showed higher +dP/dt and cardiac output in the ex vivo working heart preparation. Indicative of a better cardiac performance, mice infused with the drug also showed a greater -dP/dt (NS-infused: -3960 ± -95 mmHg/sec vs. drug-infused -4512 ± -109 mmHg/sec, $P < 0.05$). Consistent with an improved systolic function, the echocardiography-derived fraction shortening (FS) was also higher in 2-cyclohexylthioAMP-treated mice than in NS-treated mice in vivo (Fig. 3). LV wall was better preserved with greater thickness during both systole and diastole in drug- vs. NS-infused mice with ischemic heart failure (Table 2). As further evidence for a therapeutic effect of 2-cyclohexylthioAMP, the heart weight/body weight ratio in drug- (ratio= 5.22 ± 0.41 mg/g) is lower than that in NS- (ratio= 6.08 ± 0.31 mg/g) treated mice with ischemic HF. The therapeutic effect of the drug was associated with a restoration of beta-adrenergic responsiveness (Fig. 4) as determined by isoproterenol-stimulated increase in +dP/dt. When isoproterenol was injected into the heart through pulmonary vein at 20 nmol/L in working heart model, HF mice previously infused with 2-cyclohexylthio-AMP showed a greater increase in +dP/dt than HF mice that were infused with NS. The beneficial effect of 2-cyclohexylthio-AMP in mice with ischemic HF was not due to a decrease in infarct size since drug- (infarct size: 37.32 ± 7.61 %) and vehicle- (infarct size: 38.80 ± 6.15 %) infused mice showed similar infarct size ($P > 0.05$). It is of interest that the therapeutic effect of 2-cyclohexylthio-AMP was maintained even at 1 month after the cessation of its infusion. The spontaneous heart rate of vehicle-infused mice was similar to drug-treated mice in the ex vivo preparation (not shown).

Chronic infusion of 2-cyclohexylthio-AMP in CSQ mice with heart failure caused better cardiac contractile performance

At one month after 2-cyclohexylthio-AMP infusion, the cardiac contractile function was determined. The results are shown in figure 5. Infusion of the drug caused an enhanced cardiac function as determined by an improved $+dP/dt$, $LVDevP$ and cardiac output by the ex vivo working heart model. There was also an improvement in $-dP/dt$ after drug infusion (NS-infused: -3843 ± -163 mmHg/sec vs drug-infused -4351 ± -113 mmHg/sec, $P < 0.05$). Indicative of an enhanced systolic function, drug infusion also caused an increased FS by in vivo echocardiography (Fig. 5). LV dimensions at systole and diastole were significantly smaller in drug- vs. vehicle-infused animals (Table 2). LV wall thickening was also greater at systole in drug-treated mice, providing another indication of the salutary effect of 2-cyclohexylthio-AMP. The highly hypertrophic CSQ heart also showed a lower heart weight/body weight ratio in drug-(ratio: 9.56 ± 0.98 mg/g) vs. NS-(ratio: 11.42 ± 1.48 mg/g) infused animals. The spontaneous heart rate of NS-infused CSQ mice (320 ± 47 bpm) was similar to that in 2-cyclohexylthio-AMP infused (301 ± 28 bpm) CSQ mice.

Discussion

The actions of extracellular nucleotides in cell signaling are mediated by two classes of cell surface purinergic receptors. P2X receptors are ligand-gated ion channels activated by extracellular ATP, and P2Y receptors are G protein-coupled receptors activated by both adenine and uracil nucleotides.^{20,21, 22} In the heart, a number of P2 receptors are expressed.²³ Recent findings suggested that cardiac P2X receptors represent a novel and potentially important therapeutic target for the treatment of heart failure.^{12,17} Extracellular ATP or 2-mes-ATP could stimulate an ionic current in mouse, rat, and guinea pig cardiac ventricular myocytes.^{3, 24, 25} This P2X current was up-regulated in cardiac ventricular myocytes of the CSQ hearts.²⁶ The P2X4 receptor is an important subunit of the endogenous cardiac myocyte P2X receptor. In previous studies, cardiac performance and survival were enhanced in P2X4 transgenic mice subjected to ischemic HF due to LAD ligation⁶. The beneficial effect of cardiac-specific overexpression P2X4R can be mimicked by chronic infusion of a nucleotidase-resistant analog of AMP, MRS2339¹⁷. MRS2339 is an (N)-methanocarba monophosphate derivative of 2-chloro-AMP that contains a rigid bicyclic ring system (bicyclo[3.1.0]hexane) in place of ribose.¹⁵ MRS2339 has an agonist-like activity at the endogenous cardiac P2XR.²⁶ Chronic MRS2339 infusion in post-infarct and CSQ mice with HF resulted in higher $+dP/dt$, $LVDevP$, and cardiac output in the ex vivo working heart model. Heart function in vivo, as determined by echocardiography-derived FS, was also improved in MRS2339-infused mice. MRS2339-infused mice also exhibited improved survival in both CSQ and ischemic HF models.

In the present study, we investigated a structurally different AMP analog in 2-cyclohexylthio-AMP and tested its therapeutic effect in the same ischemic and CSQ models of heart failure. This AMP analog has an agonist-like effect in the isolated working heart model, capable of increasing $LVDevP$, $+dP/dt$, $-dP/dt$ and cardiac output in a dose-dependent manner. This agonist-like effect was observed in both WT mice and in mice with CSQ heart failure. The agent had no effect on the spontaneous heart rate, suggesting a selective effect on the contractile state without an associated chronotropic effect. A selective contractile effect may be advantageous in heart failure because it would not be accompanied by a heart rate-related increase in oxygen demand. It is of interest that acute injection of 2-cyclohexylthio-AMP can also increase $-dP/dt$ in not only WT but also CSQ hearts, implying a potential relaxation effect in mice with heart failure. Mice with ischemic or CSQ heart failure also exhibited a greater $-dP/dt$ in response to infusion of the drug. These results raised the possibility of an improved diastolic function in drug-treated animals. In both the ischemic and CSQ HF models, chronic 2-cyclohexylthio-AMP infusion conferred a

therapeutic effect. This salutary effect was manifested by an improved cardiac function as assessed via either ex vivo working heart preparation or in vivo echocardiography in both HF models. As another indication of improved cardiac function in HF, drug-treated animals showed a greater beta-adrenergic responsiveness than vehicle-infused animals with ischemic heart failure. Compared with vehicle-infused mice, 2-cyclohexylthio-AMP infusion decreased the heart weight/body weight ratio, implying that 2-cyclohexylthio-AMP reduced myocardial hypertrophy in both models of HF. That the infarct sizes were similar in drug- vs. vehicle-infused mice indicates that the beneficial effect of 2-cyclohexylthio-AMP was not due to a smaller infarct. Finally, the therapeutic efficacy was maintained well after drug infusion has stopped in that one month after infusion has ceased, cardiac function remained improved in mice with ischemic HF. There is currently no bio-analytical method to measure 2-cyclohexylthio-AMP or its metabolite. Therefore, its pharmacokinetics and pharmacodynamics in vivo are not known. The target of this agent is presumably a purinergic receptor although the identity, selectivity and affinity of the putative receptor for the compound are not known. Overall, the therapeutic effect of this agent mimicked that of MRS2339 and the salutary effect of cardiac transgenic overexpression of P2X4 receptors.

Recently, the X-ray crystallographic structure and site-specific mutagenesis of zebrafish P2X4R have elucidated the ATP binding pocket, which resides at each pair of subunit interfaces in the trimeric channel.^{27,28} Four positively charged lysine and arginine residues line the binding pocket for ATP, whose phosphate groups mediate ligand specificity. There are also hydrophobic interactions between the adenine base and leucine and isoleucine. The hydrophobicity of these residues is conserved, and is suggested to be involved in the recognition of the adenine base.^{27,28} A phosphate group and adenine base appear to be common pharmacophore features of an agonist at this form of the P2X4R, which may contain other P2X subunits. Since 2-cyclohexylthio-AMP contains both a phosphate group and an adenine base, it is possible that 2-cyclohexylthio-AMP can activate the cardiac P2X receptor and that such activation contributes to therapeutic efficacy in HF. It is known that extracellular ATP can stimulate an increase in cardiac contractility in human atrial myocardium.²⁹ It is possible that the modest increase in cardiac contractile performance induced by 2-cyclohexylthio-AMP contributes to its therapeutic effect in heart failure. The pharmacokinetic characteristics and exact mechanism of action of 2-cyclohexylthio-AMP need further investigation.

Conclusions

2-Cyclohexylthio-AMP improved the cardiac contractile performance and rescued mice from heart failure. The beneficial effect of this agent may be mediated by activation of the endogenous cardiac P2X receptor in mice with heart failure. The therapeutic action may result from reduction of myocardial hypertrophy and restoration of the β -adrenergic response in both LAD ligation and CSQ models of heart failure.

Acknowledgments

None

Source of funding: This work was supported in part by RO1-HL48225 to Bruce T. Liang. This research was supported in part by the Intramural Research Program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases.

Abbreviations

AMP adenosine 5'-monophosphate

ATP	adenosine 5'-triphosphate
CSQ	calsequestrin
FS	fractional shortening
HF	heart failure
KHS	Krebs-Henseleit solution
LAD	left anterior descending coronary artery
LV	left ventricular
LVDevP	left ventricular developed pressure
2-mes-ATP	2-methylthioadenosine 5'-triphosphate
MRS2339	(1'S,2'R,3'S,4'R,5'S)-4-(6-amino-2-chloro-9H-purin-9-yl)-1-[phosphoryloxy-methyl]bicyclo[3.1.0]hexane-2,3-diol
NS	normal saline
PBS	phosphate buffered saline
SR	sarcoplasmic reticulum
WT	wild type

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Figure 1. The chemical structures of 2-cyclohexylthio-AMP (A) and 2-mes-ATP (B)

Figure 2. An agonist-like effect of 2-cyclohexylthio-AMP

The effects of 2-cyclohexylthio-AMP on various working heart function parameters were determined in WT (A) and CSQ (B) mice. The agent caused an increase in +dP/dt and LVDevP without any change in heart rate in either WT (n=8) or CSQ (n=8) mice. In both WT and CSQ hearts, the increase in +dP/dt caused by 10 nM of the drug was less than that induced by 100 nM; the increase by 100 nM was in turn less than that caused by 1000 nM (Repeated Measures ANOVA and post-test comparison, $P<0.05$). Similarly, the increase in LVDevP was sequentially greater as the drug concentrations increased from 10 to 1000 nM except that in the WT hearts, the LVDevP was not statistically different between 10 and 100 nM of the drug (Repeated Measures ANOVA and post-test comparison, $P<0.05$).

Figure 3. Chronic infusion of 2-cyclohexylthio-AMP improved contractile performance in WT mice with LAD ligation

Myocardial function ex vivo was determined in the working heart preparation. Cardiac function in vivo was assessed by echocardiography. Separate series of mice with ischemic heart failure were infused with either drug or vehicle for 7 days, 1 month or 2 months after LAD ligation. The sample size and P values were indicated.

Figure 4. Chronic infusion of 2-cyclohexylthio-AMP restored β -adrenergic responsiveness in mice with ischemic HF using the working heart preparation

Isoproterenol was injected in the KHS buffer at the concentration 20 nmol/L to perfuse the heart via the coronary artery circulation. The effect of isoproterenol was expressed as the percentage of increase in +dP/dt. The sample size and P values were indicated.

Figure 5. Chronic infusion of 2-cyclohexylthio-AMP improved contractile performance in CSQ mice with HF

Cardiac function was determined by the ex vivo working heart preparation and by in vivo echocardiography. The sample size and P values were indicated.

Table 1
2-cyclohexylthioAMP causes increases in $-dP/dt$ of both WT and CSQ hearts

Increase in $-dP/dt$ (mm Hg)	10 nM	100 nM	1000 nM
WT (n=8)	303 $\pm$ 89	488 $\pm$ 148	604 $\pm$ 101
CSQ (n=8)	141 $\pm$ 73	270 $\pm$ 84	637 $\pm$ 181

Effects of 2-cyclohexylthio-AMP on $-dP/dt$ were determined in WT and CSQ mice. In both WT hearts, the increase in $-dP/dt$ caused by 10 nM was less than that induced by either 100 nM or 1000 nM while there was no difference in the increase by 100 nM vs. 1000 nM. In CSQ hearts, the increase caused by 10 nM and 100 nM of the compound was less than that induced by 1000 nM while there was no difference in the increase by 10 nM vs. 100 nM. Repeated Measures ANOVA and post-test comparison were carried out.

Table 2
Effects of chronic 2-cyclohexylthio-AMP infusion on cardiac dimensions and wall thickness

CSQ	LVID@D	LVPW@D	IVS@S	LVID@S	LVPW@S
Vehicle	4.1±0.11	0.38±0.007	0.46±0.007	3.54±0.14	0.46±0.007
Drug	*3.67±0.12	0.39±0.004	*0.49±0.005	*2.89±0.13	*0.49±0.005
Ischemic HF					
Vehicle	3.99±0.19	0.400±0.004	0.48±0.005	3.25±0.18	0.482±0.004
Drug	3.92±0.07	*0.416±0.004	*0.51±0.003	2.98±0.077	*0.508±0.002

Drug administered was 2-cyclohexylthio-AMP, which was infused via SC route for 4 weeks as described in Methods. Ischemic heart failure was produced by ligation of LAD that was then followed by drug infusion within 24 hr.

* P<0.05 in drug vs. vehicle (NS) comparison.