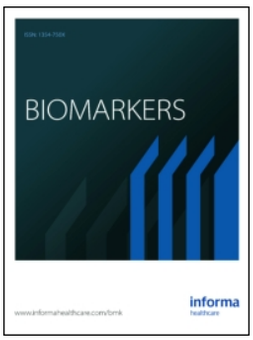

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AT1 receptor blocker Azilsartan Medoxomil normalizes plasma miR-146a and miR-342-3p in a murine heart failure model

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Abstract

Our study measured circulating microRNA (miRNA) levels in the plasma of calsequestrin (CSQ)-tg mouse, a severe heart failure model, and evaluated whether treatment with angiotensin II type 1 receptor blocker, azilsartan medoxomil (AZL-M) influenced their levels using miRNA array analysis. MiR-146a, miR-149, miR-150, and miR-342-3p were reproducibly reduced in the plasma of CSQ-tg mice. Among them, miR-146a and miR-342-3p were significantly restored by AZL-M, which were associated with improvement of survival rate and reduction of congestion. These results suggest that miRNA, especially miR-146a and miR-342-3p, could be used as potential biomarkers for evaluating the efficacy of anti-heart failure drugs.

Introduction

Heart failure is characterized by progressive cardiac remodeling and dysfunction. In the past 20 years, some efficacious therapies including angiotensin II receptor blockers (ARB) (Pfeffer et al., 2003; Cohn & Tognoni, 2001), angiotensin converting enzyme inhibitors (CONSENSUS Trial Study Group, 1987; Pfeffer et al., 1992), beta blockers (CIBIS Investigators and Committees, 1994; Packer et al., 1996), and resynchronization therapy (Bristow et al., 2004; Cleland et al., 2005) have been developed for heart failure. This innovative treatment strategy contributed to the reduction of cardiac death and improvement in the quality of life. Nevertheless, heart failure remains a major cause of morbidity in the world today (Go et al., 2013; Avery et al., 2012). Accurate diagnosis is critical in determining the appropriate medical management; thus, the exploration and establishment of biomarkers are actively in progress. Several useful biomarkers have been identified, such as brain natriuretic peptide (BNP), its biologically inactive split fragment, NT-proBNP, or galectin 3. However, they are not truly sufficient because heart failure is a very complicated disease and is induced by multiple biological factors including oxidative stress, inflammation, fibrosis, neurohumoral activation, and ischemia (Maisel et al., 2008).

MicroRNAs (miRNAs) are endogenous, highly conservative, small non-coding RNAs, which regulate diverse physiological processes including cell differentiation, apoptosis, cellular stress response, and proliferation, by inhibiting protein translation or promoting mRNA degradation (Bartel, 2004). MiRNAs have been reported to play important roles in cardiac function and are

associated with several cardiac diseases, such as hypertrophy and myocardial infarction (Wang & Yang, 2012; Bostjancic et al., 2009). Recent studies have reported that the expression levels of numerous miRNAs were altered during heart failure in both humans and animals (Topkara & Mann, 2011). Genetic deletion or overexpression of miRNAs in mice affects the progression of heart failure (Li et al., 2013; Ucar et al., 2012). Therefore, miRNAs are attractive therapeutic targets, although their exact mechanisms of actions remain unclear. Furthermore, miRNAs have attracted attention as potential biomarkers because circulating miRNAs in blood are resistant to RNase activity, and thus are very stable (Weber et al., 2010). In cardiovascular diseases, research on circulating miRNA profiling was focused mostly on myocardial infarction, because several cardiac-expressed miRNAs are released into the plasma similar to cardiac enzymes (e.g. troponins and creatine kinase MB) (Wang et al., 2010). While several reports have shown circulating miRNA profiling in their studies of heart failure patients, the therapeutic effects of anti-heart failure drugs on circulating miRNA levels were seldom reported. There is also little information on circulating miRNAs in animal models of heart failure. Our study investigates the circulating miRNA levels in the calsequestrin (CSQ)-tg mouse, a severe heart failure model with abnormal Ca^{2+} handling, premature death, and various symptoms of heart failure similar to heart failure patients such as hypertrophy, fibrosis, and pump failure (Jones et al., 1998; Cho et al., 1999), as well as the effects of the potent ARB, azilsartan medoxomil (AZL-M; TAK-491). These findings provide new insights into the diagnosis of heart failure and the use of miRNAs as a

marker to evaluate the efficacy of anti-heart failure drugs.

Materials and methods

Drugs

Azilsartan medoxomil (AZL-M; TAK-491) was synthesized in our laboratories. It was suspended in a 0.5% w/v methylcellulose solution and orally administered at a volume of 10 mL/kg. The vehicle was 0.5% w/v methylcellulose and also orally administered.

Animals

The line of transgenic mice overexpressing canine calsequestrin (CSQ) in the heart was originally established in the Indiana University School of Medicine (Jones et al., 1998), and then bred in Takeda Rabics (Osaka, Japan). The transgenic line was developed on a C57BL/6J background. Male mice were used in this study. They were administered drugs starting at 5 weeks of age. Body weight was measured once a week and survival was monitored once a day. All animal experiments were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals and the guidelines of the Takeda Experimental Animal Care and Use Committee.

Hemodynamic parameter measurement

The systolic blood pressure and heart rate were measured in the conscious condition using the tail-cuff method (BP-98A: Softron, Japan) 2 and 24 h after 4th drug administration in 5-week-old wild type and CSQ-tg mice. Mice at 7 weeks of age were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). A catheter with a pressure sensor diameter of 1.4F (SPR-671: Millar Instruments, USA) was inserted into the left ventricle through the right carotid artery and connected to a polygraph system (NEC San-ei Instruments Ltd. and Nihon Kohden Corporation, Japan). The measurement parameters were mean blood pressure (MBP), heart rate (HR), left ventricular pressure, left ventricular end diastolic pressure (LVEDP), and the rates of intraventricular pressure rise (dP/dt_{max}) and decline (dP/dt_{min}). The data were incorporated into MacLab and analyzed by Chart v.5.3. (AD Instruments, Castle Hill, Australia). Immediately after the measurement, the hearts were rapidly excised, and individual chambers were separated, and weighed.

MiRNA array analysis

Blood was collected from the abdominal vena cava of the wild type and CSQ-tg mice under anesthesia with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). MiRNA was extracted from 200 μl plasma using QIAzol Lysis Reagent and a miRNeasy Micro kit (QIAGEN, Hilden, Germany). MiRNA expression was determined using the TaqMan® Array MicroRNA Card

(Rodent v2.0: Life Technologies, California, USA) containing 585 miRNAs according to the manufacture's protocol. To avoid technical bias, we excluded the miRNAs whose Ct values were above 35 in the more than 3 individuals in each group. Ct value of each miRNA was normalized by subtracting the median Ct value of detectable miRNA within the sample. Relative miRNA expression was calculated by the ΔΔCT method. Hierarchical clustering and heatmap were carried out using MATLAB software (The Mathworks Inc., Natick, USA).

Individual miRNA expression analysis by real-time PCR

For individual miRNA analysis, another set of studies was conducted. Blood was collected from the abdominal vena cava of the wild type and CSQ-tg mice under anesthesia with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). MiRNAs were extracted as described above. Individual miRNAs were then converted into cDNA with the TaqMan MicroRNA Reverse Transcription Kit and the specific real-time (RT) primer (Life Technologies, California, USA), and amplified with the TaqMan PreAmp Master Mix and the specific TaqMan probe. Gene expression was analyzed using the 7900HT Fast Real-Time PCR System (Life Technologies, California, USA) using TaqMan® Fast Advanced Master Mix (Life Technologies, California, USA) and primer-probe sets of TaqMan Gene Expression Assays (Life Technologies, California, USA). Three synthetic *Caenorhabditis elegans* miRNAs (Cel-miR-239, Cel-miR-39, Cel-miR-54) (Hokkaido System Science, Japan) were added to the samples during the assay and their values

were used for data correction (Kroh et al., 2010; Mitchell et al., 2008). The relative miRNA expression was calculated by the ΔΔCT method.

Statistical Analysis

Data are expressed as means ± SD. Survival data were analyzed by using a Kaplan–Meier survival analysis with a log rank method of statistics. Analysis was performed with the Student's *t* test for other experiments.

Results

Mortality

CSQ-tg mice treated with the vehicle (n = 20) died within around 10 weeks as previously reported (Harding et al., 2001). Administration of AZL-M (0.01-1 mg/kg, *p.o.*, n = 20 in each group) from the age of 5 weeks significantly improved survival in a dose-dependent manner (Fig. 1). To determine the contribution of afterload reduction to the improvement of the survival rate by AZL-M, blood pressure was measured under the conscious condition. Administration of AZL-M (0.01-1 mg/kg, *p.o.*) for 3 days significantly reduced the systolic blood pressure only at 2 h, not at 24 h, after administration in the 5-week-old CSQ-tg mice. The systolic blood pressure at 2 and 24 h after administration were 109 ± 8 and 112 ± 11 (wild type mice, n = 9), 100 ± 14 and 102 ± 13 (vehicle, n = 9), 85 ± 11 and 100 ± 8 (0.01 mg/kg, n = 8), 78 ± 8 and 98 ± 13 (0.1 mg/kg, n = 8) and 61 ± 8

and 94 ± 12 mmHg (1 mg/kg, n = 8), respectively. No differences were observed among the CSQ-tg mice groups in terms of heart rate (HR), although the basal HR in the CSQ-tg mice was decreased compared to wild type mice (data not shown).

Hemodynamic parameters

To determine whether AZL-M affects hemodynamic parameters, cardiac catheterization was performed in 7-week-old mice after 2 weeks of drug administration (Fig. 2). Compared with wild type mice, CSQ-tg mice had severe cardiac dysfunction, as shown by the marked reductions in both dP/dt_{max} and dP/dt_{min}, and the elevation of LVEDP, an index of congestion. Oral administration of 1 mg/kg AZL-M significantly reduced LVEDP, but had no effect on both dP/dt_{max} and dP/dt_{min}. MBP and HR were not significantly different among CSQ-tg mice groups. The improvement in the LVEDP was consistent with significant decreases in heart and lung weights in the AZL-M group (Fig. 3).

Circulating miRNA profiling by miRNA array

MiRNA expression profiles of plasma samples were determined using a TaqMan-based miRNA array. Hierarchical clustering of microarray data with the detected 386 probes revealed that the profiles of circulating miRNAs were remarkably different between wild type and CSQ-tg mice (Fig. 4). On the other hand, profiles of circulating miRNAs in the CSQ-tg mice treated with AZL-M were

similar to that in wild type mice rather than the vehicle-treated CSQ-tg mice. To prioritize these miRNAs, the following criteria were applied: Either <0.5- or >2- fold changes with p values less than 0.05 between vehicle treated CSQ-tg and wild type mice. MiRNAs that fulfilled these criteria were listed in Table 1, showing that 9 miRNAs were up-regulated and 27 miRNAs were down-regulated. AZL-M restored the levels of several of these miRNAs (miR-149, miR-150, let-7d*, miR-342-3p, miR-146a, miR-28*, miR-138*, and miR-702) back towards normal. MiR-137, miR-197, miR-208, miR-544, and miR-804 also showed expression patterns suitable for use as biomarkers, which are almost inexistent in the wild type mice, but remarkably increased in the CSQ-tg mice although they were not consistent with the above criteria (data not shown).

Individual miRNA expression analysis

To confirm the array results, some intriguing miRNAs in the plasma (the top 5 miRNAs that were either up-regulated or down-regulated in CSQ-tg mice, and the 8 miRNAs that were significantly restored by AZL-M, total: 16 miRNAs) were individually measured using real-time PCR in another set of studies. As shown in Fig. 5, miR-146a, miR-149, miR-150 and miR-342-3p were significantly down-regulated in the plasma of CSQ-tg mice, and miR-146a and miR-342-3p were significantly restored by treatment with AZL-M. MiR-146a and miR-342-3p showed correlation with atrial weight, an index of congestion, in CSQ-tg mice treated with vehicle and AZL-M (r=0.75 and 0.59, respectively).

Discussion

Heart failure is a heterogeneous disease with various causes, symptoms, magnitudes of severity, and numerous complications. To manage this complex disease, biomarkers are valuable in understanding the disease condition and choosing the appropriate therapeutic approach. Our study conducted a comprehensive analysis of the plasma miRNA levels in CSQ-tg mice. We found remarkable changes in the miRNA profiles of this model by array analyses. Among these miRNAs, we confirmed that four specific ones, miR-146a, miR-149, miR-150, and miR-342-3p, were significantly down-regulated in the plasma of CSQ-tg mice using individual TaqMan analysis. While there is limited understanding of the physiologic roles of miRNAs, their function and expression are rapidly being elucidated in the current research. The expressions of these four miRNAs are not heart-specific, but some of them could be related to cardiovascular disease. For example, miR-146a has been reported to regulate toll-like receptor 4 (TLR4) signaling, which is associated with immune responses through the modulation of downstream proteins, such as interleukin-1-receptor-associated kinase 1 (IRAK1) and tumor-necrosis-factor-receptor-associated factor 6 (TRAF6) (Takahashi et al., 2010). In the plasma of the patient with coronary artery disease, the miR-146a level was significantly higher, followed by the elevation of IRAK1, TRAF6, and TLR4 mRNA. Furthermore, treatment with telmisartan and enalapril decreased this elevation, in contrast to the results of our study

(Takahashi et al., 2010). Another study showed that miR-146a protected the heart against myocardial ischemia/reperfusion injury in mice (Wang et al., 2013). Although the role of miR-342-3p in the body's cardiovascular system remains unclear, its plasma level was significantly reduced in heart failure patients compared with healthy volunteers (Ellis et al., 2013), which is consistent with our results. MiR-149 expression was reduced in the hearts of myocardial infarction-induced heart failure mice (van Rooij et al., 2008), but the functional study of their hearts has not been performed yet. MiR-150 is known for playing an important role in the heart and in cardiovascular diseases. Its level in the heart was decreased in hypertrophic mice (thoracic aortic banding mice and constitutive active calcineurin transgenic mice) and its overexpression caused a reduction in the cell size of primary neonatal rat cardiomyocytes (van Rooij et al., 2006). Thus, miR-150 could contribute to the inhibition of hypertrophy. In addition, a low circulating level of miR-150 was associated with left ventricular remodeling in patients with ST elevation myocardial infarction (Devaux et al., 2013). However, it is unknown whether these miRNAs are directly associated with progression of heart failure. Future studies will determine the mechanism by which these miRNAs are associated with disease state.

Tijsen *et al.* (2010) using miRNA array analysis, have identified miR-423-5p as a circulating miRNA specific to heart failure that can be highly elevated. Another group confirmed their results and found an additional three miRNAs, miR-320a, -22, and -92b, in the serum of patients with systolic heart failure (Goren et al., 2012). Fukushima *et al.* (2011) found that circulating miR-126

was down-regulated in patients with heart failure, and Corsten *et al.* (2010) found that miR-499 was up-regulated in patients with acute heart failure. However, these miRNAs did not change in our study. This discrepancy might be associated with the different stages of disease progression, different analysis method, and species difference.

It is important to determine whether circulating miRNAs can be associated with the therapeutic effect of drugs. In our research, AZL-M significantly restored the changes in miR-146a and miR-342-3p levels. This result suggests that these circulating miRNAs could be used as biomarkers in reflecting the effects of anti-heart failure drugs. Interestingly, our preliminary miRNA array analysis showed that all four miRNA (miR-146a, miR-149, miR-150 and miR-342-3p) decreased in the plasma of CSQ-tg mice were also reduced in the heart, in addition, AZL-M restored all of them (data not shown). It is likely that the cardiac levels of miR-146a and miR-342-3p reflected the plasma levels in the heart failure model, but we have no clear explanation why plasma levels of miR-149 and miR-150 did not change with AZL-M. In contrast to our observations, other inhibitors of the renin angiotensin aldosterone system (RAAS) decreased the circulating miR-146a level in the patient with coronary artery disease as mentioned above. The value of miR-146a and miR-342-3p as circulating biomarker candidates and mechanisms of their restoration by AZL-M should be confirmed in future research. For further validation of these miRNAs, we need to evaluate their sources, targets, and functions, in addition to their associations with parameters of cardiac function. Furthermore, additional researches in other

heart failure models such as myocardial infarction-induced heart failure model, transverse aortic constriction model, isoproterenol-induced heart failure model or heart failure patients are also required. Comparison of other drugs, such as beta blocker, angiotensin-converting inhibitor, and mineralcorticoid receptor antagonist, with AZL-M in CSQ-tg mice would also provide us useful information. Accumulation of results from these studies will lead the development of reliable biomarkers for heart failure in the future.

ARBs reportedly have beneficial effects on heart failure in clinical (Pfeffer et al., 2003; Cohn & Tognoni, 2001), and preclinical studies (Matsumoto et al., 2004; Pourdjabbar et al., 2005). Currently, three ARBs, valsartan, losartan and candesartan cilexetil, are clinically available for the treatment of heart failure. AZL-M improved mortality, congestion, and remodeling in CSQ-tg mice. We found that candesartan cilexetil showed anti-heart failure effects similar to AZL-M in this model (data not shown). Therefore, AZL-M would also be expected to provide beneficial effects on heart failure in the clinical setting. Several mechanisms of actions have been reported so far for anti-heart failure effects by ARBs. Afterload reduction due to lowering blood pressure is a primary contributor to the improvement of heart failure. In the present study, AZL-M lowered blood pressure only at 2 hours after 3-day-administration, and the effects did not last 24 hours; thus, other mechanisms such as inhibition of angiotensin II-induced hypertrophy, fibrosis, inflammation, and production of reactive oxygen species in the heart might contribute to its therapeutic effects. Its short-lasting effect on blood pressure in the CSQ-tg mice might have been due to the different

pharmacokinetics between humans and mice. In our preliminary study, we observed that AZL-M concentration rose sharply 0.5 hours after administration and disappeared after 24 hours in both the normal and CSQ-tg mice (data not shown), in contrast to long-lasting pharmacokinetics in humans (Angeli et al., 2013). Also, 3-day-administration in CSQ-tg mice might be too short to achieve steady anti-hypertensive effect. It was reported that losartan, another ARB, improved not only mortality but also cardiac function including dP/dt_{max} and dP/dt_{min} in CSQ-tg mice at 2 and 5 months of age (Gunther et al., 2010). The discrepancy in our study might be attributed to the difference in backgrounds of the CSQ-tg mice. CSQ-tg mice showed strain-specific variation. The C57BL/6J background used in our study is known to have the most severe symptoms of heart failure (Suzuki et al., 2002; Wheeler et al., 2005), and thus it might be difficult for drugs to improve the extensive reduction of dP/dt_{max} and dP/dt_{min}. Moreover, the losartan treatment data was obtained in isolated hearts, in comparison to our systemic measurement *in vivo*.

Conclusions

In summary, circulating miRNA profiles in the CSQ-tg mice were remarkably different from those in wild type mice. AZL-M restored the changes in miR-146a and miR-342-3p, in addition to its other anti-heart failure effects, suggesting that these miRNAs could be potential biomarkers for the therapeutic effects of anti-heart failure drugs, especially RAAS inhibitors.

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Declaration of interest

The authors declare there are no conflicts of interest.

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Table 1

Significantly up-regulated (fold change > 2, $p < 0.05$) or down-regulated (fold change < 0.5, $p < 0.05$) circulating miRNAs in CSQ-tg (CSQ) compared to wild type mice (WT). (#) showed the miRNAs subjected to the individual expression analysis. Analysis was performed with the Student's *t* test.

Up-regulated miRNAs in vehicle-treated CSQ-tg mice compared to WT mice

	CSQ (Vehicle) vs. WT			CSQ (AZL-M) vs. CSQ (Vehicle)		
	fold	SD	t-test	fold	SD	t-test
mmu-miR-133a (#)	10.10	6.28	0.004	16.63	23.66	0.47
mmu-miR-133b (#)	10.79	6.77	0.004	17.55	28.06	0.53
mmu-miR-136 (#)	2.87	1.32	0.005	3.71	6.06	0.71
mmu-miR-411 (#)	3.87	2.55	0.02	2.28	1.53	0.15
mmu-miR-130a (#)	3.54	2.31	0.02	8.50	12.11	0.29
mmu-miR-1	5.95	4.67	0.02	6.06	7.82	0.97
mmu-miR-127	2.15	1.21	0.04	2.06	2.17	0.92
mmu-miR-29c	3.32	2.69	0.04	5.14	7.15	0.52
mmu-miR-376c	2.11	1.30	0.048	4.03	6.49	0.44

Down-regulated miRNAs in vehicle-treated CSQ-tg mice compared to WT mice

	CSQ (Vehicle) vs. WT			CSQ (AZL-M) vs. CSQ (Vehicle)		
	fold	SD	t-test	fold	SD	t-test
mmu-miR-297a* (#)	0.31	0.13	0.000005	2.80	3.14	0.06
mmu-miR-132 (#)	0.38	0.18	0.00001	4.72	5.22	0.051
mmu-miR-149 (#)	0.22	0.08	0.00005	0.56	0.37	0.03
mmu-miR-150 (#)	0.33	0.14	0.0001	1.82	1.55	0.03
mmu-miR-592 (#)	0.32	0.26	0.0001	0.83	0.69	0.08
mmu-miR-151-3p	0.37	0.14	0.0001	3.06	3.27	0.053
mmu-miR-674*	0.48	0.22	0.0001	1.38	1.28	0.09
mmu-let-7d* (#)	0.44	0.15	0.0002	1.65	1.33	0.04
mmu-miR-574-3p	0.30	0.18	0.0004	2.05	2.51	0.09
mmu-miR-455*	0.31	0.21	0.001	0.52	0.46	0.25
mmu-miR-455	0.12	0.08	0.001	0.25	0.18	0.10
mmu-miR-322	0.29	0.21	0.001	0.41	0.26	0.33
mmu-miR-200b*	0.35	0.35	0.002	1.27	1.69	0.20
mmu-miR-181a-1*	0.45	0.19	0.003	1.57	1.76	0.12
mmu-miR-134	0.33	0.28	0.004	1.23	1.29	0.09
mmu-miR-342-3p (#)	0.37	0.24	0.004	2.11	2.06	0.048
mmu-miR-218-1*	0.35	0.21	0.005	0.98	1.27	0.21
mmu-miR-146a (#)	0.48	0.23	0.005	2.76	2.68	0.047
mmu-miR-503*	0.40	0.23	0.007	1.57	1.94	0.13
mmu-miR-28* (#)	0.50	0.33	0.009	1.97	1.57	0.03
mmu-miR-138* (#)	0.28	0.13	0.010	1.70	1.51	0.03
mmu-miR-214	0.25	0.31	0.013	1.70	2.99	0.25
mmu-miR-667	0.40	0.45	0.02	1.92	2.67	0.15
mmu-miR-702 (#)	0.22	0.08	0.02	0.84	0.55	0.015
mmu-miR-877*	0.47	0.31	0.02	1.71	1.73	0.08
mmu-miR-129-3p	0.40	0.22	0.02	1.70	1.58	0.054
mmu-miR-801	0.43	0.37	0.046	0.96	0.84	0.13

Figure legends

Figure 1

Survival curve in wild type (WT) and CSQ-tg mice (CSQ) treated with vehicle or AZL-M. The drugs were orally administered from the age of 5 weeks. Kaplan–Meier survival analysis with a log rank method of statistics was used to calculate the significant improvement in survival rate.

Figure 2

Hemodynamic parameters in wild type (WT) and CSQ-tg mice (CSQ) orally treated with vehicle or AZL-M (1 mg/kg) at 7 weeks of age. (A) LVEDP, (B) dP/dt_{max}, (C) dP/dt_{min}, (D) MBP, (E) HR. Values represent the mean ± SD, $^{**}P < 0.01$ vs. WT + Vehicle, $^{\#}P < 0.05$ vs. CSQ + Vehicle by Student's t-test.

Figure 3

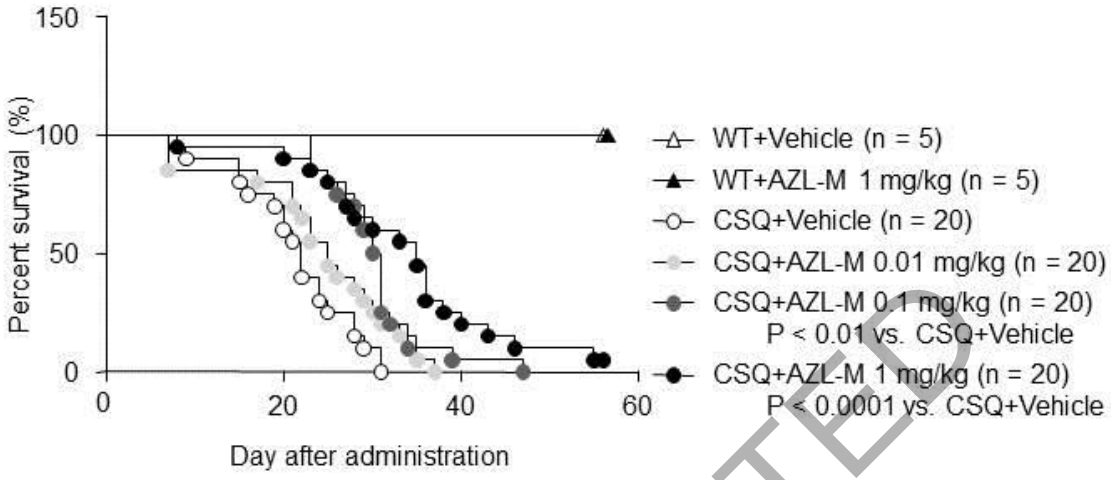
Heart and lung weight in wild type (WT) and CSQ-tg mice (CSQ) orally treated with vehicle or AZL-M (1 mg/kg) at 7 weeks of age. (A) LV/BW (left ventricular weight/body weight), (B) RV/BW (right ventricular weight/body weight), (C) AW (atria weight/body weight), (D) LW/BW (lung weight/body weight). Values represent the mean ± SD, $^{**}P < 0.01$ vs. WT + Vehicle, $^{\#\#}P < 0.01$ vs. CSQ + Vehicle by Student's t-test.

Figure 4

Clustering analysis of miRNA levels in the plasma of wild type (WT) and CSQ-tg mice (CSQ) treated orally with vehicle or AZL-M (1 mg/kg) at 7 weeks of age.

Figure 5

MiRNA expression in the left ventricle of wild type (WT) and CSQ-tg mice (CSQ) treated orally with vehicle or AZL-M (1 mg/kg) at 7 weeks of age. (A) miR-146a, (B) miR-342-3p, (C) miR-150, (D) miR-149. Values represent the mean ± SD, $^{**}P < 0.01$ vs. WT + Vehicle, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. CSQ + Vehicle by Student's t-test.



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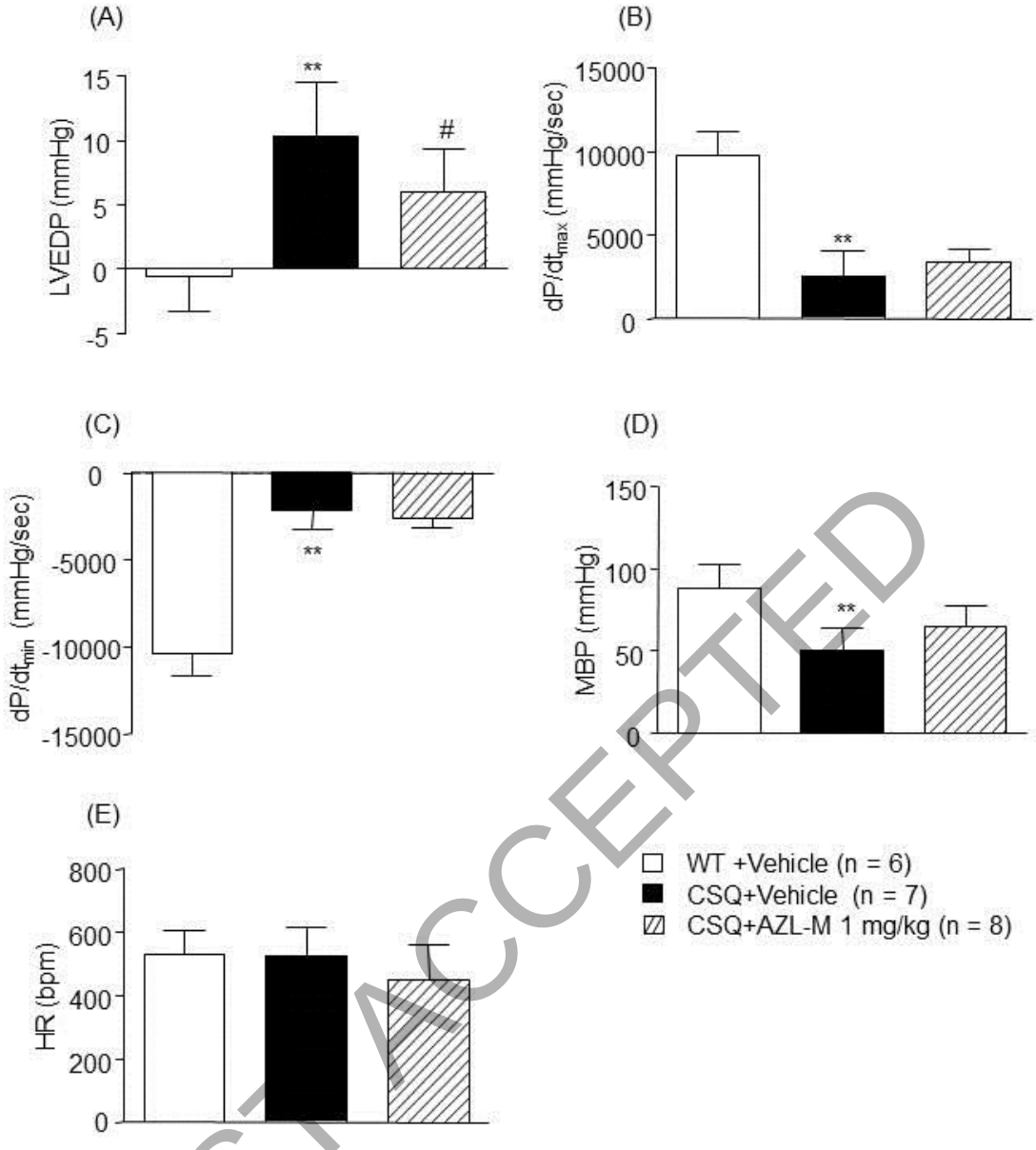

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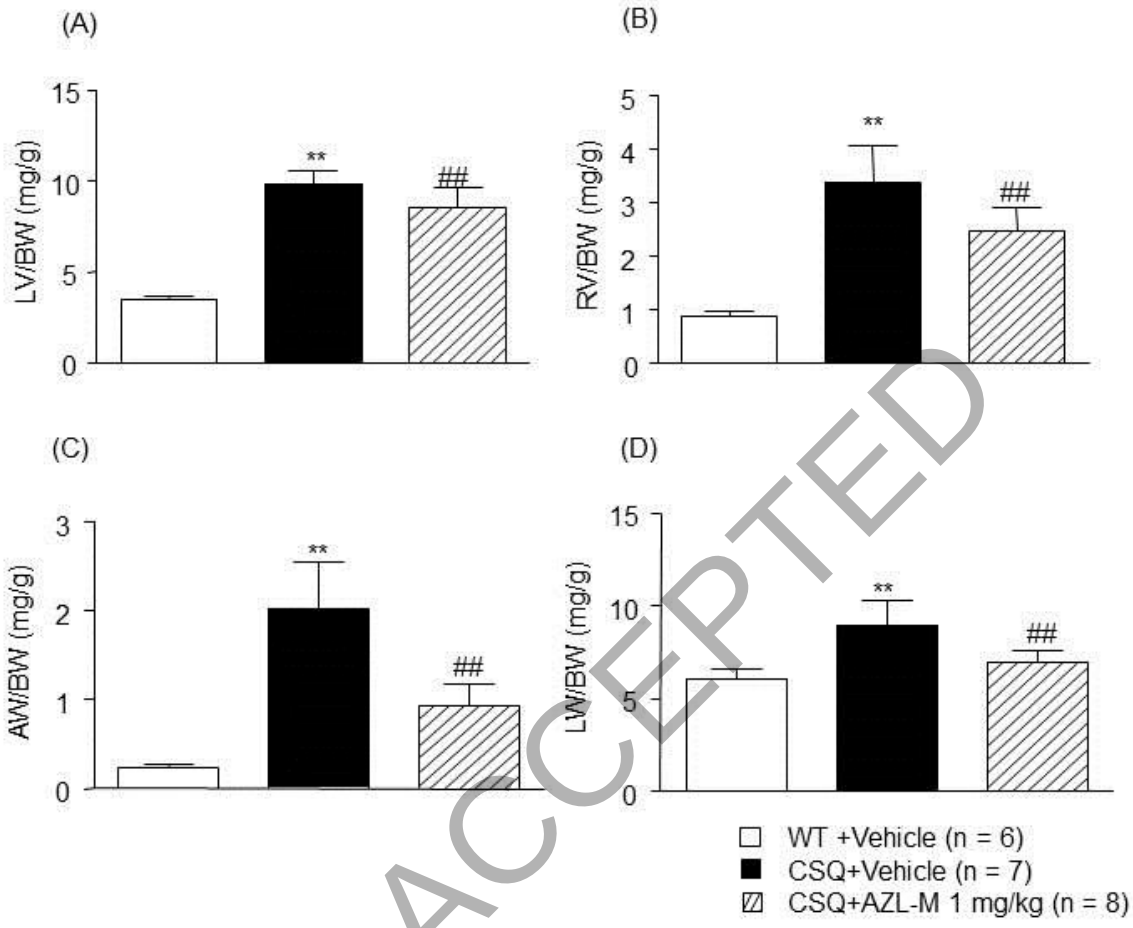

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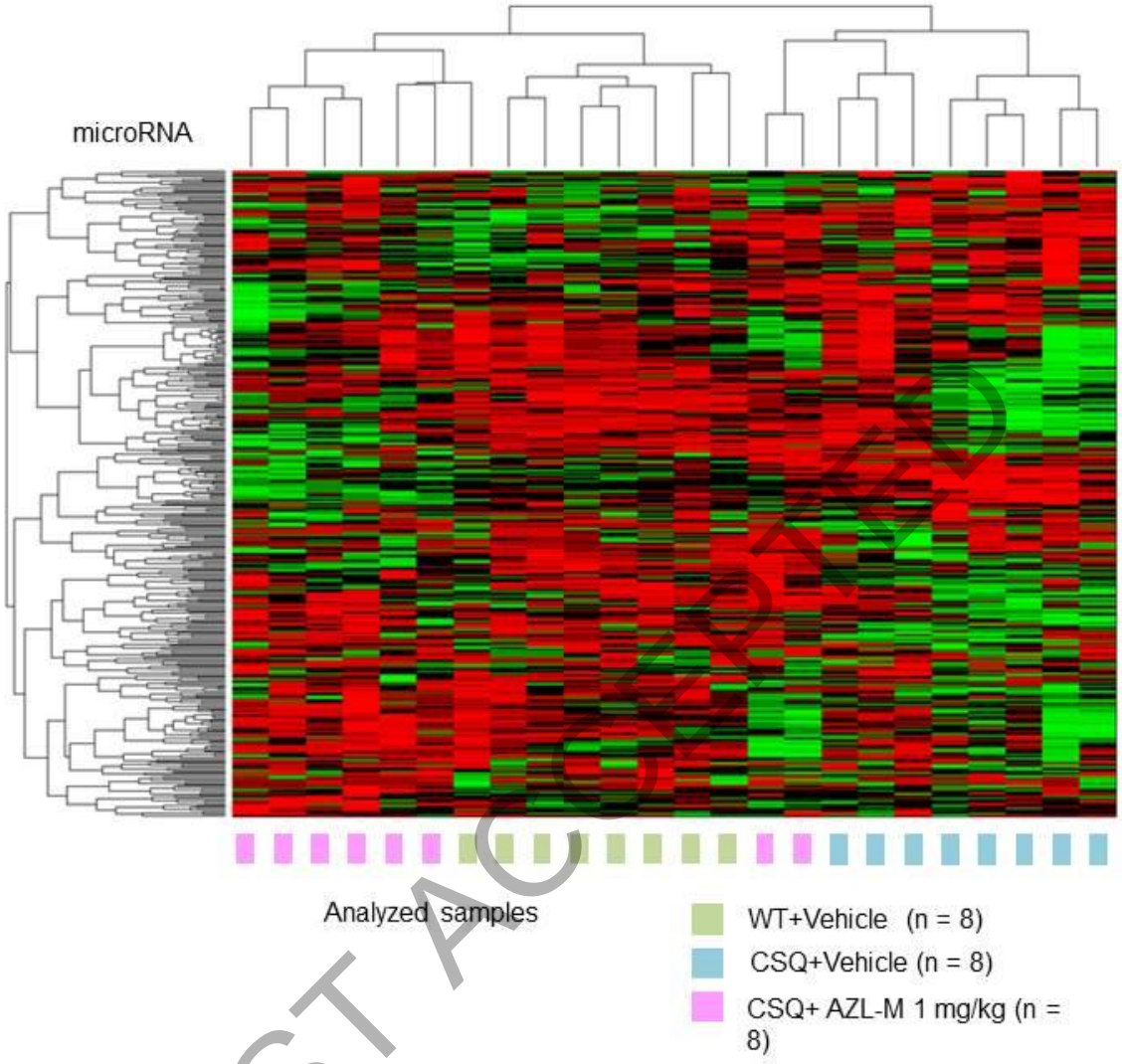

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Clustering analysis of miRNA levels in the plasma of wild type (WT) and CSQ-tg mice (CSQ) treated orally with vehicle or AZL-M (1 mg/kg) at 7 weeks of age.

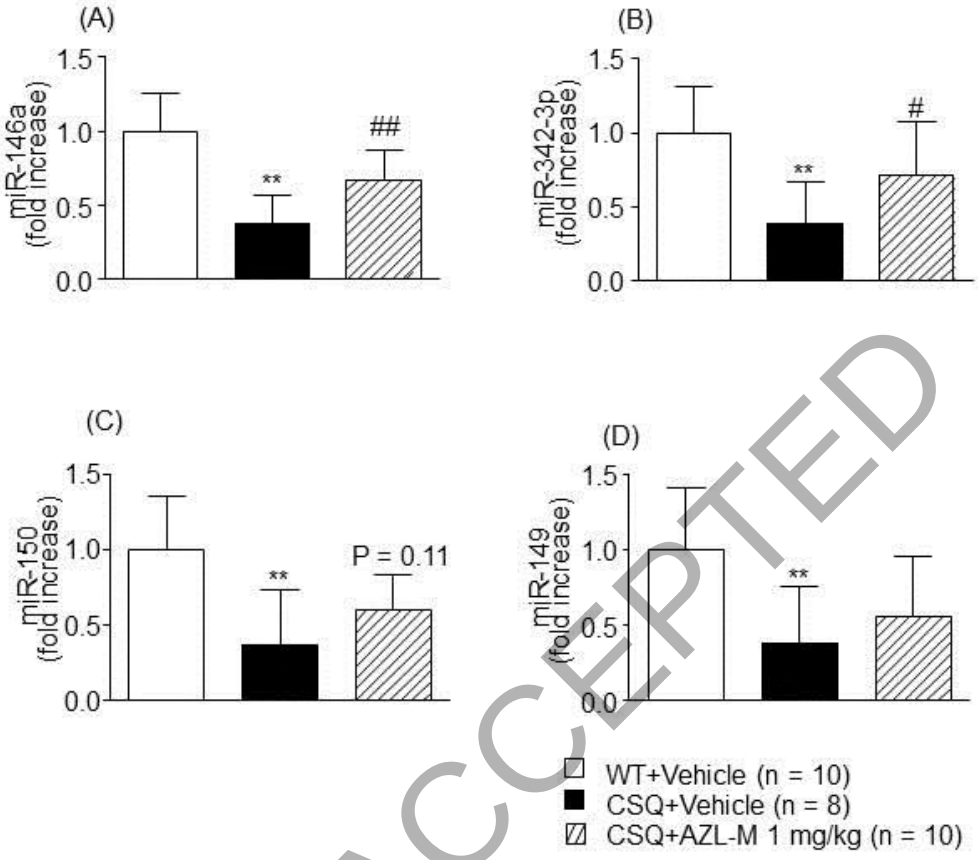

Figure 5

MiRNA expression in the left ventricle of wild type (WT) and CSQ-tg mice (CSQ) treated orally with vehicle or AZL-M (1 mg/kg) at 7 weeks of age. (A) miR-146a, (B) miR-342-3p, (C) miR-150, (D) miR-149. Values represent the mean ± SD, $^{**}P < 0.01$ vs. WT + Vehicle, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. CSQ + Vehicle by Student's t-test.