SUPPLEMENTAL MATERIAL

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Supplemental Methods

Pressure-Volume Loop Haemodynamics and Tissue Collection

Pressure-volume loop analysis was performed in a subset of mice 21 days after TAC as previously described,[15] immediately prior to sacrifice. Pressure-volume loop data was collected and analyzed using IOX version 2.1.10 software (EMKA Instruments). At sacrifice, venous blood from the inferior vena cava was collected into a heparinized syringe and immediately centrifuged at 16,100 x G for 5 minutes at room temperature. Plasma was separated and snap frozen in liquid nitrogen. Heart, lungs and kidneys were removed, weighed, and either snap frozen in liquid nitrogen or placed in 10% formalin for histological analysis.

Measurement of cGMP

The final concentration of cGMP was determined in both plasma and LV tissue. LV tissue was immediately snap frozen in liquid nitrogen following sacrifice. Approximately 30mg of tissue was then crushed into a fine powder over dry ice using a pre-chilled cryo tissue grinder. On ice, 6% trichloroacetic acid was added to samples (10µL/mg of tissue) followed by vortexing for 10 seconds. Proteins were precipitated out by centrifuging at 2000 x G for 15 minutes at 4°C and the supernatant was transferred to a 15 mL falcon tube on ice. Trichloroacetic acid in the supernatant was extracted by addition of approximately five volumes of diethyl ether. Samples were mixed by gentle rocking, then allowed to equilibrate into two layers. The top layer was discarded, and the sample washed a further three times by repeat additions of diethyl ether. After the final wash, the bottom layer was transferred to a microcentrifuge tube, snap frozen in liquid nitrogen and later lyophilized. Samples were resuspended in assay buffer and cGMP concentration determined in duplicate using the cGMP Enzyme Immunoassay Biotrak (EIA) system (RPN226, GE Healthcare, Amersham, UK), according to protocol #2. For plasma, frozen samples were thawed on ice, gently mixed by inversion, then cGMP concentration determined in duplicate as for LV tissue.

Histology

Mid-papillary sections of the left ventricle were fixed in 10% formalin for 24 hours and embedded in paraffin. Sections were cut to a 5µm thickness, placed onto glass slides and stained with picrosirius red for analysis of cardiac fibrosis. Slides were imaged using a light microscope (Olympus BX40) using SPOT Imaging software (18.2 Color Mosaic; SPOT v5.3, MI, USA) in a blinded fashion at 20x magnification. Cardiac fibrosis was determined based on red-orange stain intensity, analyzed using Fiji-ImageJ.[35]

Gene Expression

Gene expression of left ventricular apex tissue was performed as previously described,[15] using the following TaqMan™ probes (ThermoFisher Scientific, USA): *Nppa* (Assay ID: Mm01255747_g1), *Nppb* (Assay ID: Mm01255770_g1), *Col1a1* (Assay ID: Mm00801666_g1), *Ctgf* (Assay ID: Mm01192933_g1), *Fn1* (Assay ID: Mm01256744_m1), *Myh7* (Assay ID: Mm00600555_m1), and *Gapdh* (Assay ID: Mm99999915_g1) for normalization.

Protein Phosphorylation and Expression

Protein expression of calcineurin (1:500 dilution, BD Biosciences, #610260), and phosphorylation of cardiac myosin binding protein-C at Ser^{273} (1:1000 dilution, antibody as described by Thoonen et al),[18] was determined in basal left ventricular tissue by western blot and normalized to GAPDH (EMD Millipore, MAB374). Tissue was crushed, protein extracted in protein lysis buffer using 30 µg protein, and protein concentration was then determined using BCA assay as previously described.[36] Lysates were run on gels alongside a protein standard ladder (Bio-Rad Precision Plus Dual Color Standards no. 161-0374) and transferred to nitrocellulose membranes as previously described.[36] Membranes were blocked in 7.5% milk or BSA and incubated overnight at 4°C with primary antibody, washed, then incubated with HRP-conjugated secondary antibody (GE Healthcare, NA931V and NA934V) at room temperature

for 1 hour, before incubation with ECL substrate (Li-Cor, no. 926-95000), as previously described.[36]

Supplemental Tables

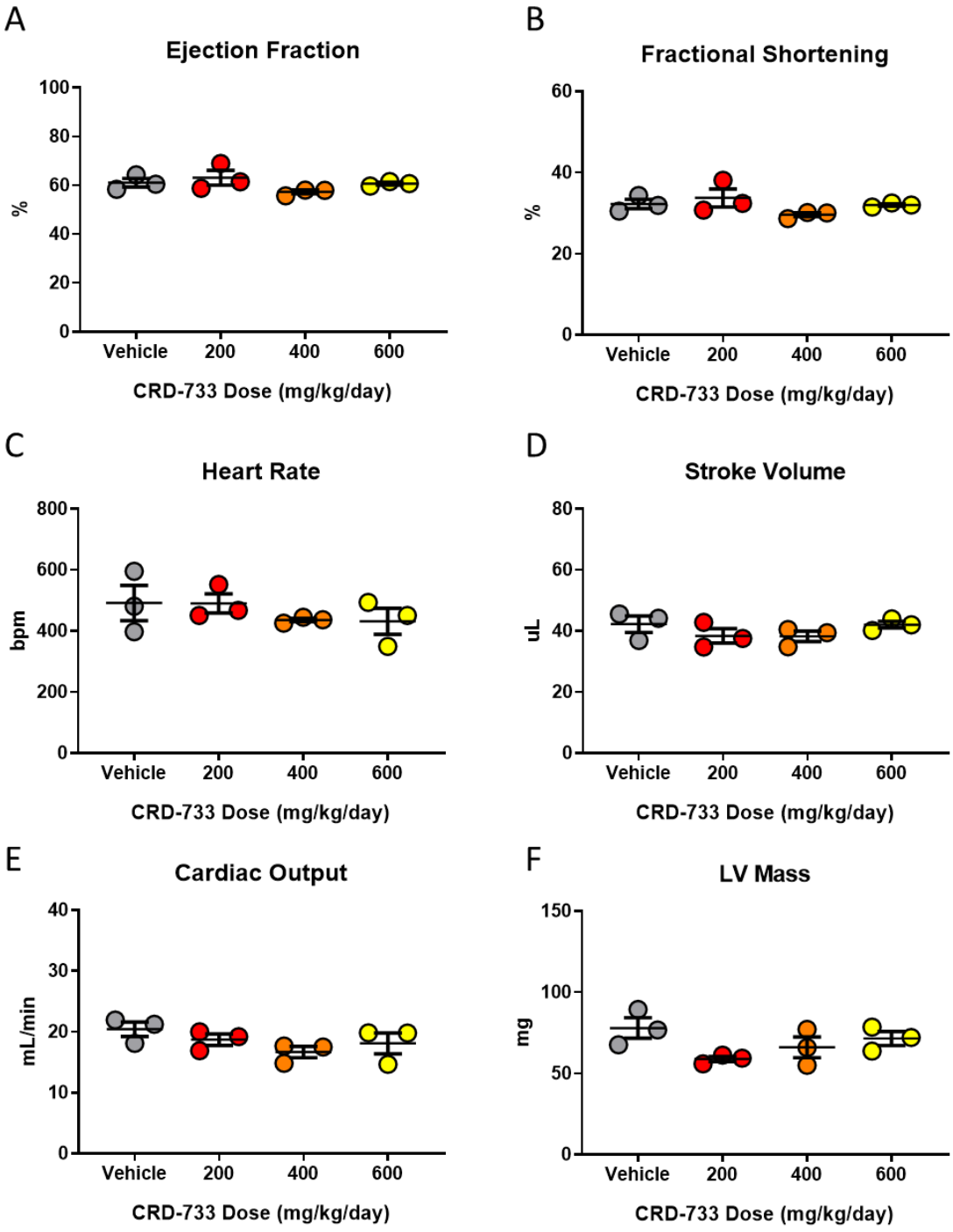
Supplemental Table S1. Hemodynamic indices.

INVASIVE LV HEMODYNAMICS AND PRESSURE-VOLUME LOOPS				
	Sham Vehicle (n=5)	**TAC Vehicle (n=6)**	**TAC CRD-733 (n=8)**	**P-Value**
Cardiac Output (mL/min)	13.49±2.69	8.56±1.97	9.54±1.06	0.19
Stroke Volume (µL)	25.40±4.64	17.50±3.38	19.13±2.35	0.28
Stroke Work (µL*mmHg)	1971±383.8	1891±477.9	1777±240.6	0.93
Heart Rate (bpm)	526.2±22.55	489.2±44.88	498±13.37	0.68
dP/dt Max vs. EDV	138.9±19.32	74.76±31.78	78.73±27.90	0.28
Emax	11.94±2.63	10.0±4.14	16.33±4.98	0.57
ESPVR	2.70±0.54	4.039±1.367	7.25±2.44	0.26
EDPVR	0.19±0.02	0.85±0.14	1.02±0.43	0.24
Effective Arterial Elastance (mmHg/µL)	4.80±1.32	11.17±2.36	8.00±1.12	0.06
Preload-Recruitable Stroke Work	48.75±4.15	39.52±7.97	68.57±15.03	0.22
ECHOCARDIOGRAPHY				
	Sham Vehicle (n=10)	**TAC Vehicle (n=17)**	**TAC CRD-733 (n=10)**	**P-Value**
Isovolumic Contraction Time (ms)	11.36±0.75	11.08±0.92	11.25±0.95	0.98
Isovolumic Relaxation Time (ms)	15.03±0.73	14.91±0.63	15.28±0.73	0.93
Mitral Valve Deceleration Time (ms)	28.97±1.53	20.46±1.04***	22.97±2.04*	<0.001
Mitral Valve Ejection Time (ms)	50.78±1.30	59.17±1.21**	60.61±2.37**	<0.001
LV Volume during Diastole (µL)	69.21±3.597	74.40±4.439	55.82±3.76†	0.0148
LV Volume during Systole (µL)	29.35±3.304	36.88±4.378	23.37±1.919	0.0572

Supplemental Table S1. Hemodynamic indices derived from pressure-volume loops and echocardiography following 21 days of TAC or sham control and 14 days of PDE9 inhibition with CRD-733 or vehicle treatment. EDV: end diastolic volume, ESPVR: end systolic pressure-volume relationship, and EDPVR: end diastolic pressure-volume relationship. Units are given in parenthesis where applicable. Data is expressed as mean ± SEM. Right hand column represents overall P-value for 1-way ANOVA. *P<0.05, **P<0.01, and ***P<0.001 vs. Sham Vehicle by 1-way ANOVA with Tukey's post-test. †P<0.05 vs. TAC vehicle by 1-way ANOVA with Tukey's post-test.

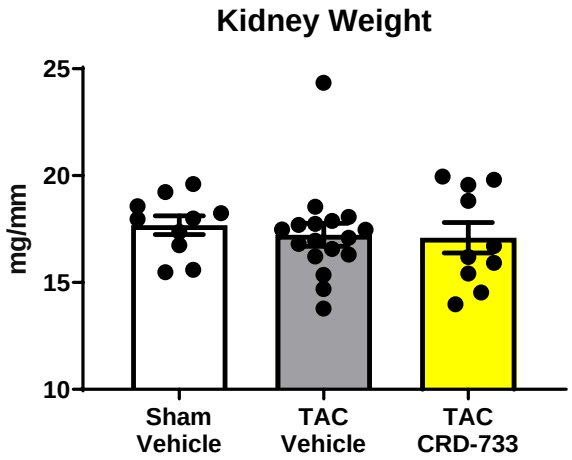
Supplemental Figures and Figure Legends

Supplemental Figure S1.



Supplemental Figure S1: Echocardiography-derived indices for cardiac function in naïve mice treated with increasing doses of CRD-733 for 14 days. CRD-733 does not affect indices of cardiac function, including ejection fraction **(A)**, fractional shortening **(B)**, heart rate **(C)**, stroke volume **(D)**, and cardiac output **(E)**. Additionally, CRD-733 treatment did not affect LV mass **(F)**. Data analyzed by 1-way ANOVA with Tukey's post-test; $P>0.05$ for all indices; n=3.

Supplemental Figure S2.



Supplemental Figure S2. Kidney weights after 21 days TAC and 14 days drug treatment. Kidney weights did not significantly differ between groups. Kidney weights were normalized to tibia length and data was analyzed by 1-way ANOVA with Tukey's post-test. Data presented as mean ± SEM; n=10-17.

Supplemental References

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18. Thoonen R, Giovanni S, Govindan S, Lee DI, Wang GR, Calamaras TD, Takimoto E, Kass DA, Sadayappan S, Blanton RM. Molecular screen identifies cardiac myosin-binding protein-c as a protein kinase g-ialpha substrate. Circ Heart Fail. 2015;8:1115-1122.

35. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. Fiji: An open-source platform for biological-image analysis. Nature methods. 2012;9:676-682.

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