

Online Supplement

c-Src Kinase Inhibition Reduces Arrhythmia Inducibility and Connexin43 Dysregulation after Myocardial Infarction

Cody A. Rutledge, BS^{1,2}, Fu Siong Ng, MD, PhD³, Matthew S. Sulkin, MS³, Ian D. Greener, PhD², Artem M. Sergeyenko, BA², Hong Liu, MD, PhD², Joanna Gemel, PhD⁴, Eric C. Beyer, MD, PhD⁴, Ali A. Sovari, MD², Igor R. Efimov, PhD³, Samuel C. Dudley, MD, PhD²

¹Department of Physiology, University of Illinois at Chicago, Chicago, IL, USA

²Section of Cardiology, University of Illinois at Chicago, Chicago, IL, USA

³Department of Biomedical Engineering, Washington University, St. Louis, MO, USA

⁴Department of Pediatrics, University of Chicago, Chicago, IL, USA

Supplemental Methods:

Coronary Artery Ligation: Twelve-week old male C57 Black mice (Charles River, Wilmington, MA) were anesthetized using vaporized isoflurane then intubated and ventilated by positive pressure respirator (Harvard Apparatus, Holliston, MA). A left thoracotomy was performed just lateral to the sternum to expose the heart. The pericardium was removed, and an 8-0 monofilament suture was looped around the proximal left anterior descending coronary artery. The suture was either knotted to induce infarction or removed in the sham surgery. The intercostals muscles, pectoralis muscle, and skin were sutured and the animal allowed to recover

under heat lamp. The mice were treated with bupornephrine twice daily and monitored post-operatively for five days. The animal experiments were conducted according to the National Institutes of Health Guide for Care and Use of Experimental Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee.

PP1, PP3, and AZD0530 Treatments: Two weeks following surgery, MI mice were randomized into treatment groups with either the Src inhibitor PP1 {1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolol[3,4-d]-pyrimidin-4-amine} or the inactive analogue PP3 {1-phenyl-1H-pyrazolol[3,4-d]-pyrimidin-4-amine} (Enzo Life Sciences, Farmingdale, NY). Mice were injected intraperitoneally three times a week for two weeks at 1.5 mg/kg. Additionally, a separate group was treated with either saline or the Src family tyrosine kinase inhibitor AZD0530 (Selleck Chem, Houston, TX) at 25 mg/kg/day by gastric gavage. 24 sham, 42 PP3-, 49 PP1-, 12 saline-, and 12 AZD0530-treated mice were analyzed in this study.

Echocardiography: Echocardiography was performed as previously described¹. Briefly, mice were anesthetized two and four weeks following surgery using isoflurane applied by a nose cone. Transthoracic echocardiography was performed using the Vevo 770 high-resolution in vivo imaging system (Visual Sonics, Toronto, Canada). Heart rate was maintained around 500 bpm by adjusting isoflurane concentration. M-mode and B-mode images were taken for at least 5 s along the parasternal long axis and left ventricle short axis. Percent fractional shortening (%FS) was calculated as: $100 \times (LVEDd) - (LVESd) / (LVEDd)$.

Percent LV ejection fraction (EF) was calculated as:

$100 \times [(7/2.4 + LVEDd) \times LVEDd^3] - [(7/2.4 + LVESd) \times LVESd^3] / [(7/2.4 + LVEDd) \times LVEDd^3]$. MI

animals were only included in the study of EF was less than 45%.

Tissue Collection and Western Blots: Four weeks following surgery, mice were euthanized, and hearts were excised. Left ventricular tissue was dissected under microscope into scar, scar border, and distal ventricle regions. After excision, the atria were removed, and the ventricles were placed into a pre-chilled heart tissue matrix (Zivic Instruments, Pittsburgh, PA) for dissection into 1-mm transverse sections. Sections were then individually examined under dissection microscope and separated into scar, scar border, and distal ventricle areas. Scar tissue was identified as blanched, necrotic tissue at the anterior of the heart, consistent with a left anterior descending coronary artery occlusion. The scar border was defined as up to 2 mm of viable tissue adjacent to the edge of the infarcted tissue. Remaining viable tissue was considered distal ventricle. Tissue sections were kept on ice throughout the dissection process. Tissues were prepared for Western blots as previously described². Briefly, tissue samples were homogenized in lysis buffer and protease/phosphatase inhibitor cocktail (Pierce, Rockford, IL) and normalized for protein concentration. Samples were separated on gradient SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA then incubated with antibodies against c-Src, phosphorylated c-Src (Tyr 416), and Cx43 (Cell Signaling, Danvers, MA) and normalized against glyceraldehydes-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotech, Santa Cruz, CA). Membranes were incubated then in secondary anti-rabbit or anti-mouse antibodies and developed using Western Lightning chemiluminescence kits (PerkinElmer, Waltham, MA) and ChemiDoc XRS imaging system (BioRad, Hercules, CA). Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunohistochemistry: Frozen tissues were sectioned and stained using primary antibodies directed against Cx43 (Santa Cruz Biotechnology), n-cadherin (Life Technologies, Grand Island, NY), and nitrotyrosine (Santa Cruz Biotechnology). Additional slides were stained with

Masson's Trichrome (Thermo Scientific, Rockford, IL) for determining scar size as a percentage of total circumference at three cross sections of the ventricle. Briefly, slides were fixed in formaldehyde, blocked, and then stained overnight. Anti-rabbit and anti-mouse fluorescent antibodies were applied (Invitrogen, Eugene, OR). Slides were analyzed using Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY), and 40x magnification images were taken of distal ventricle and scar border. Images were analyzed for fluorescence intensity using Zeiss Zen software or for correlation coefficient using ImageJ and the JACOP colocalization plugin³.

Histology and imaging services were provided by the Research Resources Center - Research Histology Core at the University of Illinois at Chicago. To examine any alterations in Cx43 distribution (including lateralization) multiple images (≥ 6) were acquired from Cx43 and N-cadherin double-labeled slides prepared from each mouse and examined using a Leica SP2 AOBs laser scanning confocal microscope in the Integrated Microscopy Facility at the University of Chicago. The extent of colocalization of Cx43 and N-cadherin were determined using the JACoP plugin for ImageJ software.

PCR: Total RNA was isolated using RNeasy Minikit (Qiagen, Valencia, CA) from frozen distal ventricle tissue samples taken from sham and MI mice. Equal quantities of total RNA were used to generate cDNA using the High Capacity cDNA synthesis kit (Applied Biosystems, Carlsbad, CA), and quantitative PCR was performed using Fast SYBR green chemistry (Applied Biosystems, Carlsbad, CA) on an ABI 7500 platform. Primers were designed against mouse Cx43 (Cx43_F TACTTCAATGGCTGCTCCTCA and Cx43_R GTAATTCGCCAGTTTTGCTC) and Hypoxanthine phosphoribosyltransferase (HPRT) (HPRT_F AGGCCAGACTTTGTTGGATTT and HPRT_R GGCTTTGTATTTGGCTTTTCC) using Primer3 plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus>) and synthesized

by MWG (Huntsville, AL). HPRT acted as the housekeeping gene by which to normalize Cx43 and sodium channel cDNAs. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification between groups.

Optical Mapping: Optical mapping studies were performed in 17 mice (7 PP1, 6 PP3 and 4 sham). Mice were anaesthetized with a mixture of ketamine and xylazine and injected with heparin to prevent thrombus formation. Hearts were rapidly excised through a midsternal incision, washed in oxygenated (95% O₂-5% CO₂) modified Tyrode solution [(in mM) 128.2 NaCl, 4.7 KCl, 1.19 NaH₂PO₄, 1.05 MgCl₂, 1.3 CaCl₂, 20.0 NaHCO₃, and 11.1 glucose (pH 7.35 ± 0.05)], and then retrogradely perfused via an aortic cannula at a constant pressure of 60 mmHg using a peristaltic pump (Peri-Star, WPI, Sarasota, FL). The heart was placed then in custom-made optical mapping chamber, attached to a pacing electrode and oriented so that the optical mapping field contained infarcted tissue, infarct border zone and non-infarcted myocardium (Figure 3). After 10 min of stabilization, hearts were stained with the voltage-sensitive dye RH-237, (Invitrogen, Carlsbad, CA) and perfused with the excitation-contraction uncoupler blebbistatin (10µM, Tocris Bioscience) to prevent motion artifacts. The heart was excited with a light source at an excitation wavelength of 530 nm, and optical signals were recorded using a MiCAM Ultima-L CMOS camera (SciMedia) with high spatial (100 × 100 pixels) and temporal (1,000 frames/s) resolution (emission filter >650nm). Optical action potential were recorded during sinus rhythm and during ventricular pacing at a number of S1-S1 cycle lengths. Optical mapping data were processed and analyzed offline as previously described⁴.

Telemetry: Sham, PP1-treated, and PP3-treated mice were implanted with ETA-F10 telemetry monitors (Data Sciences International, St. Paul, Minnesota). While anesthetized for coronary artery ligation, a skin incision was made in the lower right abdomen, and a transmitter was

implanted subcutaneously. Two electrocardiographic leads were tunneled under the skin and sutured near the right shoulder and left abdomen to create a lead II configuration. Signals were recorded continuously for four weeks. Signals were evaluated for 10 minutes at 6:00, 12:00, 18:00, and 24:00 for presence of arrhythmia. Heart rate calculations and cardiac rhythm analysis was performed using LabChart 7 Pro (ADInstruments, Colorado Springs, CO).

Arrhythmia Inducibility: Mice were anesthetized using vaporized isoflurane then intubated and ventilated by positive pressure respirator (Harvard Apparatus). The heart was exposed by piercing the abdominal skin and muscle and cutting upwards through the diaphragm and ribs on either side of the chest, then pulling back the chest wall. A bipolar 0.25mm silver pacing electrode (Warner Instruments, Hamden, CT) was placed on the base of the right ventricle. A STG1008 constant-current stimulator (Multi-channel Systems, Reutlingen, Germany) was used along with MCStimulus software to burst pace the heart. Fifteen second burst pacing protocols of 4 V, 1 ms square waves began at 80 ms intervals, then were decreased by 5 ms iterations until arrhythmia was induced or the heart could only be captured at a 2:1 interval. Arrhythmias were classified according to waveform morphology and number of unpaced arrhythmic beats. Non-sustained ventricular tachycardia (NSVT) was defined as 3-5 extra beats. Polymorphic ventricular tachycardia (PMVT) was defined as 5+ extra beats with variations in morphology between beats. Sustained monomorphic ventricular tachycardia (SMVT) was defined as continuous tachycardia lasting 5 or more seconds with repetitive, single peaked waveforms. ECG patterns were recorded and analyzed using LabChart 7 Pro (ADInstruments).

Statistical Analysis: Data are expressed as the mean \pm standard error. Comparisons between groups were evaluated using one-way ANOVA tests with Tukey post-hoc tests of significance in all figures except Supplemental Figure 2, which employed a Kruskal-Wallis test to account for

heterogeneity of variance.. Fisher exact tests were used to evaluate inducibility data. $p \leq 0.05$ was considered significant. Statistical analysis was completed using Graphpad Prism 6 software (San Diego, CA).

References

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- (3) Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* 2006;224:213-32.
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Supplemental Figure 1. c-Src inhibition with AZD0530 improves Cx43 levels after MI.

Graphs show the quantification of p-Src and Cx43 band intensities in immunoblots (of homogenates from distal ventricle or scar border regions) after correction based on GAPDH levels and normalization to sham expression levels.

Supplemental Figure 2. Sodium channel and Cx43 mRNA levels. mRNAs for sodium channel α -subunit (Nav1.5) were determined by real time RT-PCR and are shown in graphs after normalization to levels in sham operated animals. A) Sodium channel expression in the distal ventricle was unchanged between treatment groups B) mRNA levels of Cx43 were unchanged following MI with PP3 treatment, but there was a trend for an increase after PP1 treatment.