# SUPPLEMENTAL MATERIAL

**Supplemental Methods:**

*Rabbit myocyte isolation protocol:*

Cannulated rabbit hearts were rinsed and perfused at 37°C for five minutes with isolation solution containing, in mM: 120 NaCl, 5.4 KCl, 1 $NaH_2PO_4$, 20 $NaHCO_3$, 10 glucose, 1.6 $MgCl_2$, 60 taurine, 20 creatine, 5 L-glutamine, pH 7.2. The solution was then switched to one containing 200 u/ml collagenase type 2 (Worthington) and 0.02 mg/ml protease type 14 (Sigma-Aldrich), for 27-30 minutes. LV tissue was mechanically minced and cells filtered through a 0.2 mm mesh. Cells were briefly spun and supernatant replaced with Tyrode's solution containing BSA (2%). Tyrode's consisted of, in mM: 140 NaCl, 4 KCl, 10 HEPES, 5.5 glucose and 1 $MgCl_2$, pH 7.3. Cells were titrated up to 0.5 mM $Ca^{2+}$ and kept at room temperature.

To measure sarcomere shortening and $Ca^{2+}$ transient changes, cells loaded with the leak-resistant $Ca^{2+}$ indicator Fura \2 LeakRes AM (Abcam) were imaged on an inverted fluorescence microscope (Nikon, TE2000), and studied using the IonOptix imaging system and software (Myocam). Cells were paced at 0.5Hz, and experiments performed at 37°C in Tyrode's containing 1 mM $Ca^{2+}$ and 0.01% DMSO.

*mRNA analysis*

RNA was isolated from frozen tissue by acid guanidinium thiocyanate-phenol-chloroform method using TRIzol Reagent (Thermo Fisher Scientific Inc., Waltham, MA). One μg of RNA was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and a T100 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, California). Real-time PCR was performed with TaqMan Gene Expression Master Mix or Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) using a CFX384 Real-Time System (Bio-Rad Laboratories). TaqMan primers/probes (Thermo Fisher Scientific) were used for PCR analysis

except for rabbit tissue. Relative mRNA expression levels were determined by the ΔΔCt method. Rabbit primer sequences were:

PDE1A (F) 5'-TGGTGGCCCAGTCACAAATA-3' (R) 5'-AATGGTGGTTGAACTGCTTG-3';

PDE1B (F) 5'-CTGTCGGAGATCGAGGTCTTG-3' (R) 5'-GGTGCCCGTGTGCTCATAG-3';

PDE1C(F)5'-CAGATGGAATAAAGCGGCATTC-3' (R)5'GGCAAGGTGAGACGACTTGTAGA-3';

GAPDH(F)5'-TGGTGAAGGTCGGAGTGAAC-3' (R) 5'-ATGTAGTGGAGGTCAATGAATGG-3'.

*Immunoblot protein analysis:*

Frozen tissue was homogenized and sonicated in lysis buffer (Cell Lysis Buffer #9803, Cell Signaling Technology, Danvers, MA). After centrifugation (30 min for tissue), protein concentration was determined from the supernatant by BCA Assay (Thermo Fisher Scientific). Equal amounts of protein were loaded in tris-glycine gels and run at 100V for 75-90 min, transferred to nitrocellulose membranes by semi-dry blotting using a Trans-Blot Turbo Transfer System (Bio-Rad). After blocking at 27°C for 1 hr (Odyssey Blocking Buffer, LI-COR, Inc., Lincoln, NE), membranes were incubated with primary antibodies at 4°C overnight, then with fluorescence-labeled secondary antibodies (LI-COR), washed, and signal detected with the Odyssey Imaging System (LI-COR). The following antibodies were used: PDE1A (sc-50480, Santa Cruz Biotechnology for human, rabbit, mouse, and rat; ab96336, Abcam for dog), PDE1B (ab182565, Abcam), PDE1C (sc-376474, Santa Cruz Biotechnology for human, dog, rabbit, and rat; ab14602, Abcam for mouse), GAPDH (#2118, Cell Signaling Technology for human, mouse, and rat; ab9484, Abcam for rabbit; IMG-3073, Imgenex for dog).

*Plasma Cyclic Nucleotide Assay:*

For plasma samples, a 4x volume of ice cold ethanol was added to each, mixed by inversion, and incubated for 5 min at room temperature. Samples were centrifuged for 10 min at

1500 x g at 4℃. The supernatant was dried using a vacuum centrifuge for 6 hours. Each sample was resuspended in EIA buffer as provided in the cyclic nucleotide ELISA kit (Cayman Chemical cGMP kit #581021, cAMP kit #581001). Samples were measured undiluted and at 1/10 dilution, as per manufacturer's instructions for non-acetylated samples. Myocyte samples were likewise prepared following the same protocol to measure intracellular cAMP. Reaction product was quantitated using a SpectraMax plate spectrophotometer (Molecular Devices, Sunnyvale, CA) reading OD 412 nm.

*Detailed methods for LC/MS-MS analysis of ITI-214 levels*

A single liquid–liquid extraction step with acetonitrile containing 1% formic acid was employed for analysis of ITI-214, followed by a vacuum filtration using Waters Ostro Plates to remove protein and phospholipids. HPLC separation was performed on an Ascentis® Express Phenyl Hexyl column of dimensions 50 mm x 3 mm, 2.7µm with the internal standard of deuterated ITI-214 (d5-ITI-214). The mobile phase consisted of a gradient of 60-100% methanol over 4.5 min in 5mM ammonium bicarbonate with 0.2% $NH_3H_2O$, pumped at a flow rate of 0.8 mL/min. Analysis time was 4.5 min and both the analyte and internal standard eluted around 2.0 min. Multiple reactions monitoring (MRM) mode was used to detect ITI-214. MRM data were collected on a Waters Micromass Quattro Micro API or a Sciex QTrap6500 mass spectrometer. The mass spectrometer was operated in the positive ion detection mode. The precursor to product ions (Q1→Q3) selected for ITI-214 free base and internal standard during quantitative optimization were (m/z) 508.1→321.1 and 513.1→325.1 respectively. Calibration curves were linear from 0.02–500 ng/mL in plasma samples. The lower limit of quantification (LLOQ) for plasma was 0.02 ng/mL.

**Supplemental Table 1. Hemodynamic data of ITI-214 (oral 10 mg/kg) administration in dogs.**

| | Normal (n=5) | | Heart failure (n=5) | |
|---|---|---|---|---|
| | Baseline | ITI-214 | Baseline | ITI-214 |
| HR [bpm] | 101.3 ± 1.8 | 142.2 ± 4.8 † | 116.9 ± 10.8 | 132.7 ± 10.9 |
| $dP/dt_{max}/IP$ [$s^{-1}$] | 35.4 ± 2.3 | 48.7 ± 1.5 * | 23.1 ± 1.4 ‡ | 28.1 ± 1.8 † |
| PRSW [mmHg] | 84.5 ± 4.4 | 107.9 ± 6.9 † | 38.6 ± 5.7 ‡ | 55.5 ± 4.4 * |
| CO [L/min] | 2.4 ± 0.4 | 3.8 ± 0.6 † | 1.9 ± 0.3 | 2.5 ± 0.3 † |
| LVEDV [mL] | 83.6 ± 9.7 | 77.9 ± 9.3 † | 109.3 ± 11.4 \|\| | 112.2 ± 12.2 |
| LVESV [mL] | 58.2 ± 7.8 | 51.0 ± 6.8 † | 92.7 ± 10.4 \|\| | 93.2 ± 10.8 |
| LVEF [%] | 31.0 ± 2.7 | 34.6 ± 2.2 * | 15.6 ± 2.0 \|\| | 17.4 ± 2.1 |
| SVR [dyne·sec·$cm^{-5}$] | 3830 ± 804 | 2570 ± 795 * | 3445 ± 479 | 2788 ± 369 * |
| Ea [mmHg/mL] | 5.7 ± 1.1 | 5.6 ± 1.3 | 6.1 ± 1.0 | 5.7 ± 1.0 |
| tau [ms] | 25.7 ± 4.5 | 16.7 ± 1.5 | 28.2 ± 2.7 | 23.6 ± 1.4 * |

*p<0.05, †p<0.01 vs baseline of the same group, ‡p<0.05, ||p<0.01 vs normal (baseline) by two-way repeated measures ANOVA. Abbreviation: HR – heart rate, dP/dtmax/IP -- peak rate of pressure rise normalized to instantaneous pressure; PRSW – preload recruitable stroke work, CO – cardiac output, LVEDV – left ventricular end-diastolic volume, LVESV – left ventricular end-systolic volume, LVEF – left ventricular ejection fraction, SVR – systemic vascular resistance, Ea – effective arterial elastance, Tau – relaxation time constant.

**Supplemental Table 2. Hemodynamic effects of ITI-214 with or without β1-adrenergic (esmolol) or A2B-adenosine (MRS1754) receptor blockade in rabbits.**

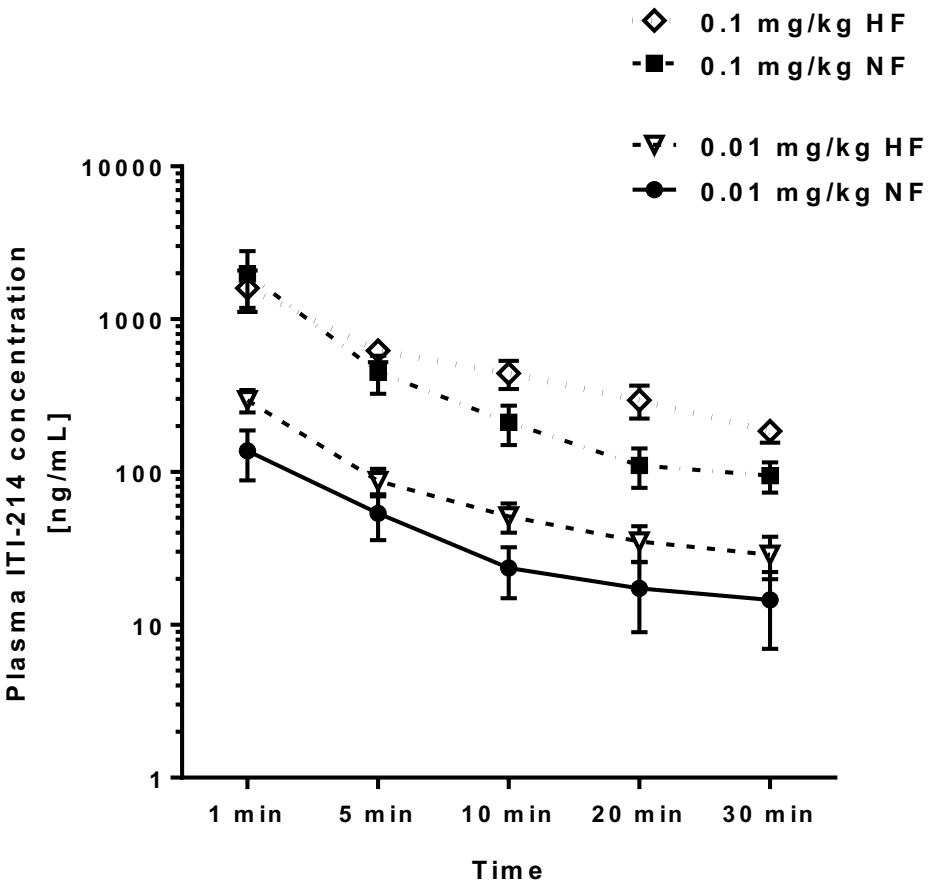
| | Control (n=19) | Esmolol (n=11) | MRS1754 (n=7) |
|---|---|---|---|
| HR [bpm] | | | |
| Baseline | 204.4 ± 11.0 | 207.8 ± 16.5 | 194.6 ± 8.3 |
| Pretreatment | | 200.7 ± 19.6 | 188.3 ± 7.5 |
| ITI-214 | 223.8 ± 14.1 ‡ | 212.4 ± 16.1 | 196.3 ± 7.6 |
| $dP/dt_{max}/IP$ [$s^{-1}$] | | | |
| Baseline | 40.5 ± 1.9 | 43.4 ± 2.3 | 41.0 ± 4.3 |
| Pretreatment | | 44.3 ± 2.8 | 38.4 ± 3.9 |
| ITI-214 | 51.4 ± 2.8 § | 51.0 ± 3.1 ‡ | 42.1 ± 4.1 |
| PRSW [mmHg] | | | |
| Baseline | 26.2 ± 2.4 | 23.8 ± 3.6 | 34.2 ± 6.1 |
| Pretreatment | | 24.6 ± 4.1 | 34.9 ± 4.9 |
| ITI-214 | 39.7 ± 3.0 § | 35.2 ± 2.5 * | 30.9 ± 5.4 |
| CO [L/min] | | | |
| Baseline | 0.11 ± 0.01 | 0.12 ± 0.02 | 0.11 ± 0.02 |
| Pretreatment | | 0.13 ± 0.02 | 0.10 ± 0.02 |
| ITI-214 | 0.14 ± 0.02 † | 0.15 ± 0.02 * | 0.12 ± 0.02 |
| LVEDV [mL] | | | |
| Baseline | 2.3 ± 0.1 | 2.7 ± 0.2 | 2.0 ± 0.1 |
| Pretreatment | | 2.8 ± 0.2 | 2.0 ± 0.1 |
| ITI-214 | 2.4 ± 0.1 * | 2.9 ± 0.2 | 1.9 ± 0.1 |
| LVESV [mL] | | | |
| Baseline | 1.6 ± 0.1 | 2.0 ± 0.2 | 1.4 ± 0.1 |
| Pretreatment | | 1.9 ± 0.2 | 1.4 ± 0.1 |
| ITI-214 | 1.6 ± 0.1 | 2.0 ± 0.2 | 1.3 ± 0.1 |
| LVEF [%] | | | |
| Baseline | 27 ± 4 | 28 ± 5 | 31 ± 4 |
| Pretreatment | | 28 ± 4 | 29 ± 4 |
| ITI-214 | 30 ± 4 * | 29 ± 5 | 30 ± 5 |
| SVR [dyne·sec·$cm^{-5}$] | | | |
| Baseline | 52841 ± 8440 | 48063 ± 13818 | 39992 ± 6441 |
| Pretreatment | | 46113 ± 13996 | 46551 ± 5521 |
| ITI-214 | 46330 ± 8549 * | 44632 ± 14032 | 43548 ± 6238 |
| Ea [mmHg/mL] | | | |
| Baseline | 172 ± 31 | 165 ± 53 | 121 ± 22 |
| Pretreatment | | 158 ± 53 | 135 ± 18 |
| ITI-214 | 175 ± 40 | 161 ± 57 | 132 ± 22 |
| tau [ms] | | | |
| Baseline | 9.6 ± 0.6 | 9.1 ± 0.6 | 11.3 ± 1.9 |
| Pretreatment | | 9.5 ± 0.8 | 14.1 ± 1.8 |
| ITI-214 | 8.8 ± 0.5 * | 8.9 ± 0.9 | 10.4 ± 1.1 |

Data are for baseline and initial ITI-214 exposure (0.1 mg/kg i.v. bolus) , re-baseline 30-40 minutes post dosing, 5 minutes after administration of pre-treatment (esmolol or MRS1754), and then 15 minutes after combining pre-treatment with ITI-214. Different rabbits were used for each of the pretreatment groups, and both contributed to the ITI-214 only control data. p-values for 1-way ANOVA (baseline). *p<0.05, †p<0.01, ‡p<0.001, §p<0.0001 vs baseline by paired t-test (before and after ITI-214). There were no significant changes induced by either pre-treatment alone. Abbreviations are as defined in Supplemental Table S1.

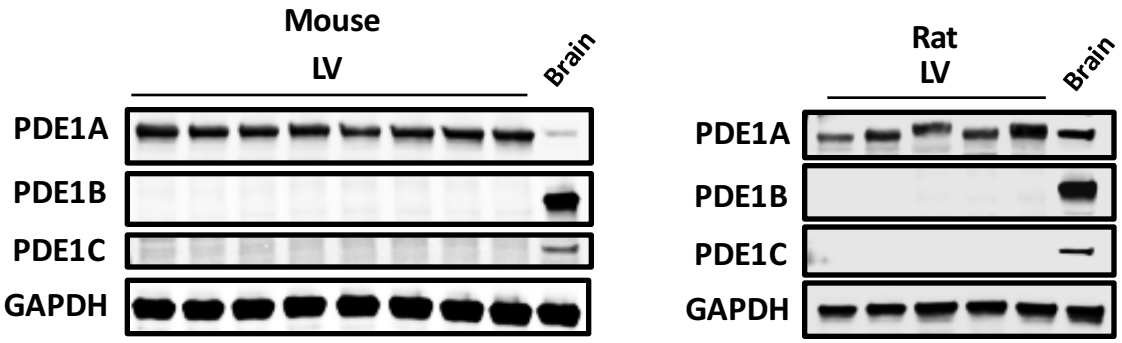
**Supplemental Table 3. Hemodynamic effect of ITI-214 in the anesthetized mouse**

| Parameter | ITI-214 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **0.1 mg/kg** (n=7) | | | | | **0.5 mg/kg** (n=6) | | | | |
| | **baseline** | **2 min** | **p value** | **10 min** | **p value** | **baseline** | **2 min** | **p value** | **10 min** | **p value** |
| HR [bpm] | 498.7±15.5 | 504.5±13.8 | 0.27 | 501.2±14.6 | 0.76 | 487.6±22.3 | 504.5±21.7 | ***0.001*** | 507.3±21 | ***0.001*** |
| CO [mL/min] | 13.67±1.4 | 14.6±1.4 | ***0.001*** | 14.32±1.5 | ***0.02*** | 10.46±1.6 | 12.63±1.7 | ***0.0001*** | 12.35±1.7 | ***0.0001*** |
| SVR [dyne·sec·cm-5] | 6.4 ± 0.5 | 5.6 ± 0.4 | ***0.03*** | 6.1±0.5 | 0.54 | 7.6 ± 0.9 | 5.9 ± 0.7 | ***0.0001*** | 6.0 ± 0.7 | ***0.0001*** |
| SBP [mmHg] | 94.7±2.6 | 93.8±2.6 | 0.34 | 94.9±2.8 | 0.93 | 86.3±3.1 | 83.7±2.7 | ***0.0005*** | 84.5±2.9 | 0.07 |
| dP/dtmax [mmHg/s] | 9659±556 | 9789±481 | 0.68 | 9828±456 | 0.53 | 9198±806 | 9818±824 | 0.45 | 9883±925 | 0.31 |
| Ees [mmHg/μL] | 3.5±0.2 | 3.41±0.2 | 0.97 | 3.11±0.2 | 0.61 | 5.4±1.2 | 5.6±1.5 | 0.35 | 4.9±1.1 | ***0.02*** |

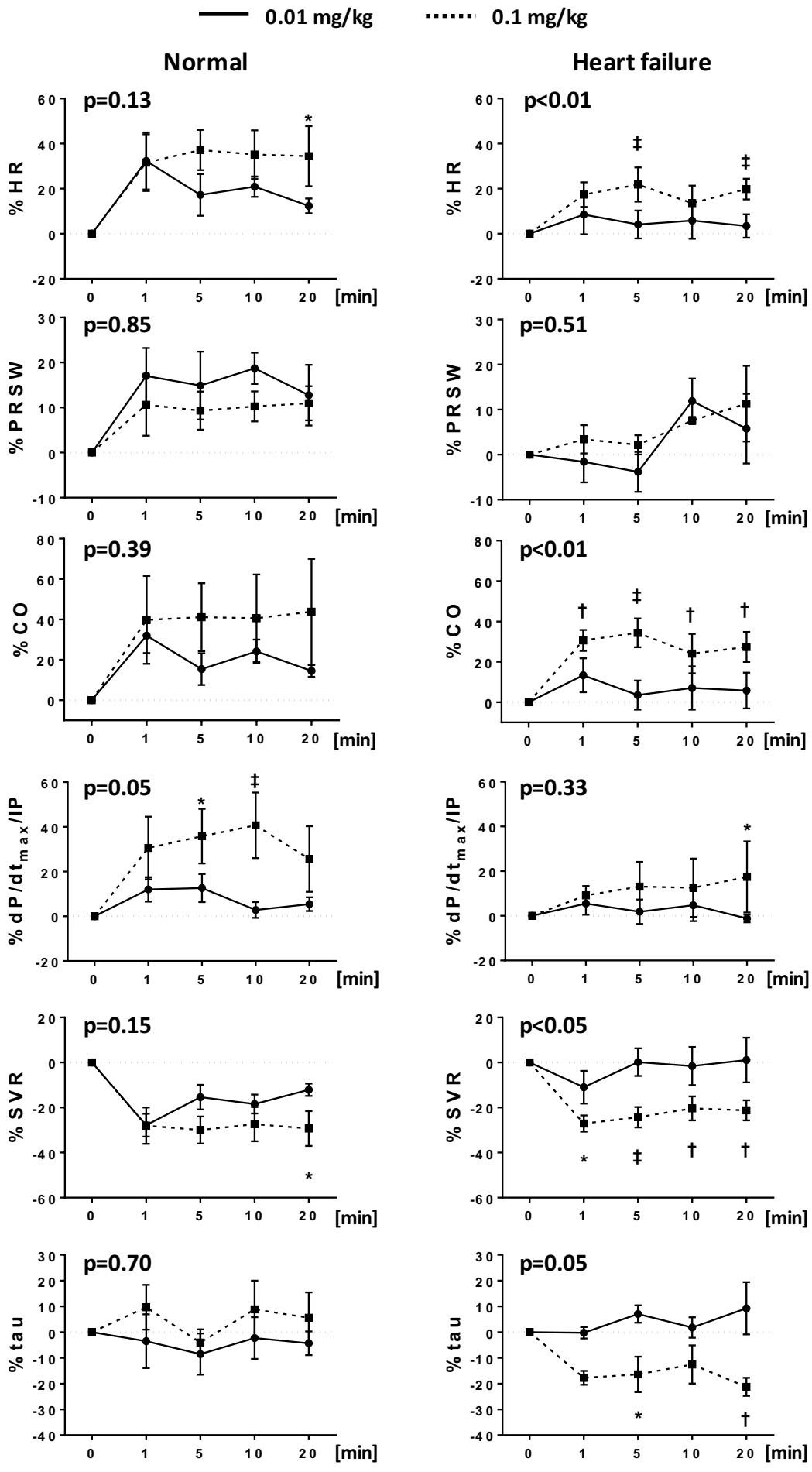
Two-way repeated measures ANOVA with Dunnett's multiple comparison test. Abbreviations: HR – heart rate, CO – cardiac output, SVR – systemic vascular resistance, SBP – systolic blood pressure, dP/dtmax – peak rate of pressure rise, Ees – end-systolic elastance.



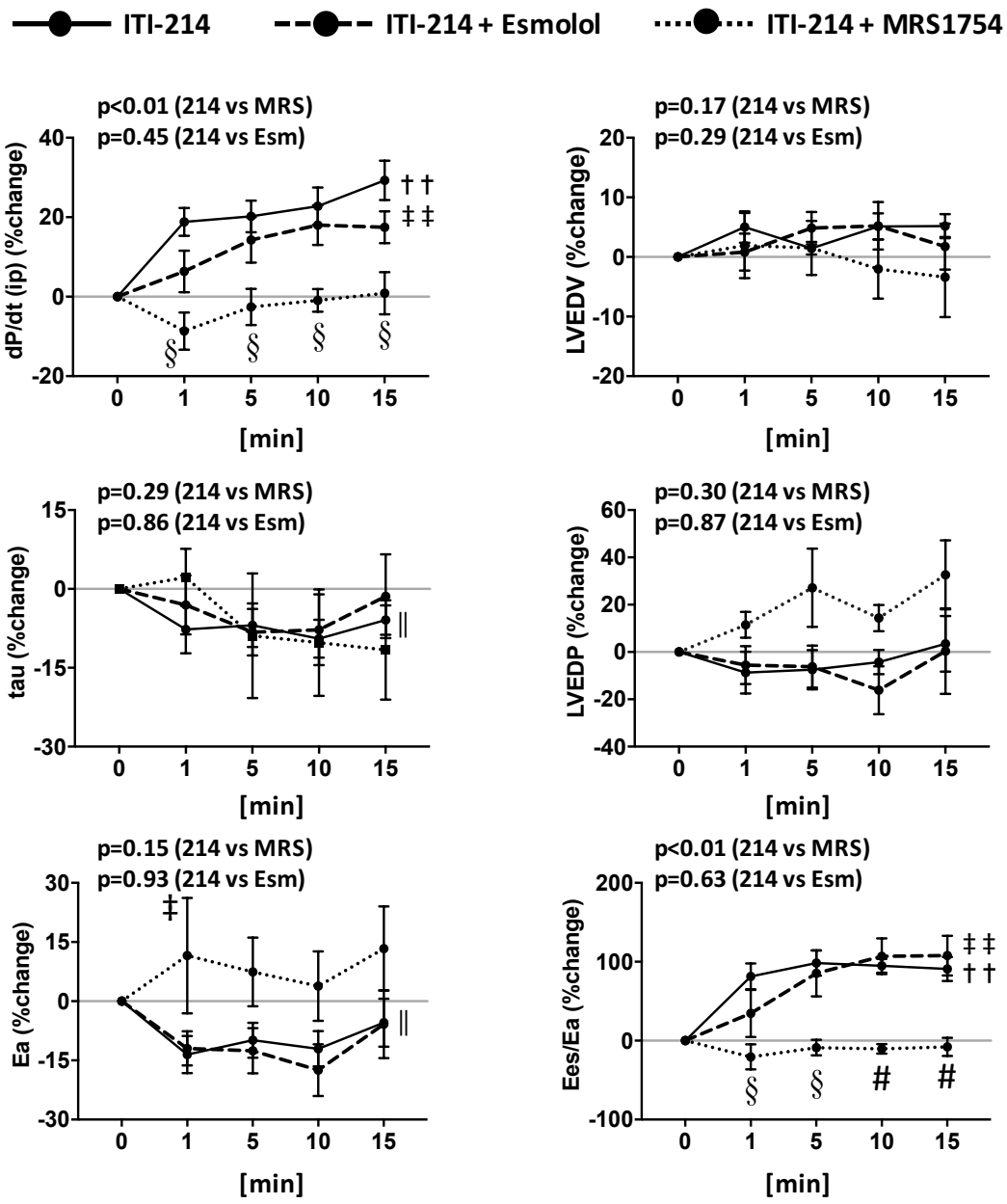

**Supplemental Figure S1. Pharmacokinetic analysis of plasma ITI-214 concentration in intravenous dose titration study in dogs.** A dose-escalation study was performed by increasing drug bolus dose from 0.01 mg/kg to 0.1 mg/kg with a 30-minute interval in normal and HF dogs (n=5-6). NF = non-failing, HF = heart failure.



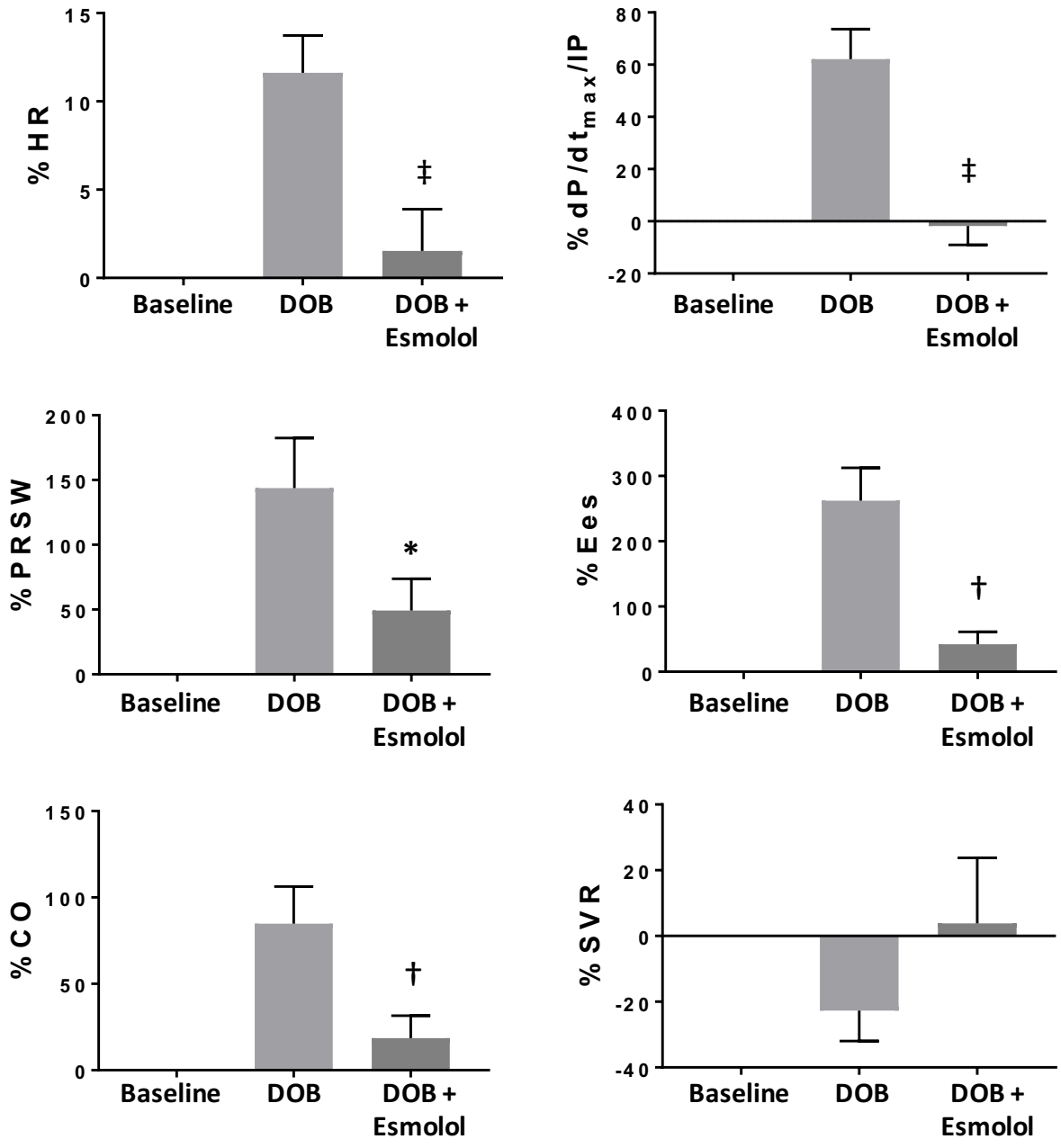

**Supplemental Figure S2. PDE1 isoform expression in heart tissue from rodents.** PDE1 isoform protein expression in normal rat and mouse left ventricle (n=8 for mouse, n=5 for rat). Equal amount of brain lysate is provided as a positive control. LV = left ventricle.


0.01 mg/kg
0.1 mg/kg
Normal
Heart failure
p=0.13
p<0.01
% HR
p=0.85
p=0.51
% PRSW
p=0.39
p<0.01
% CO
p=0.05
p=0.33
% dP/dtmax/IP
p=0.15
p<0.05
% SVR
p=0.70
p=0.05
% tau
0 1 5 10 20 [min]

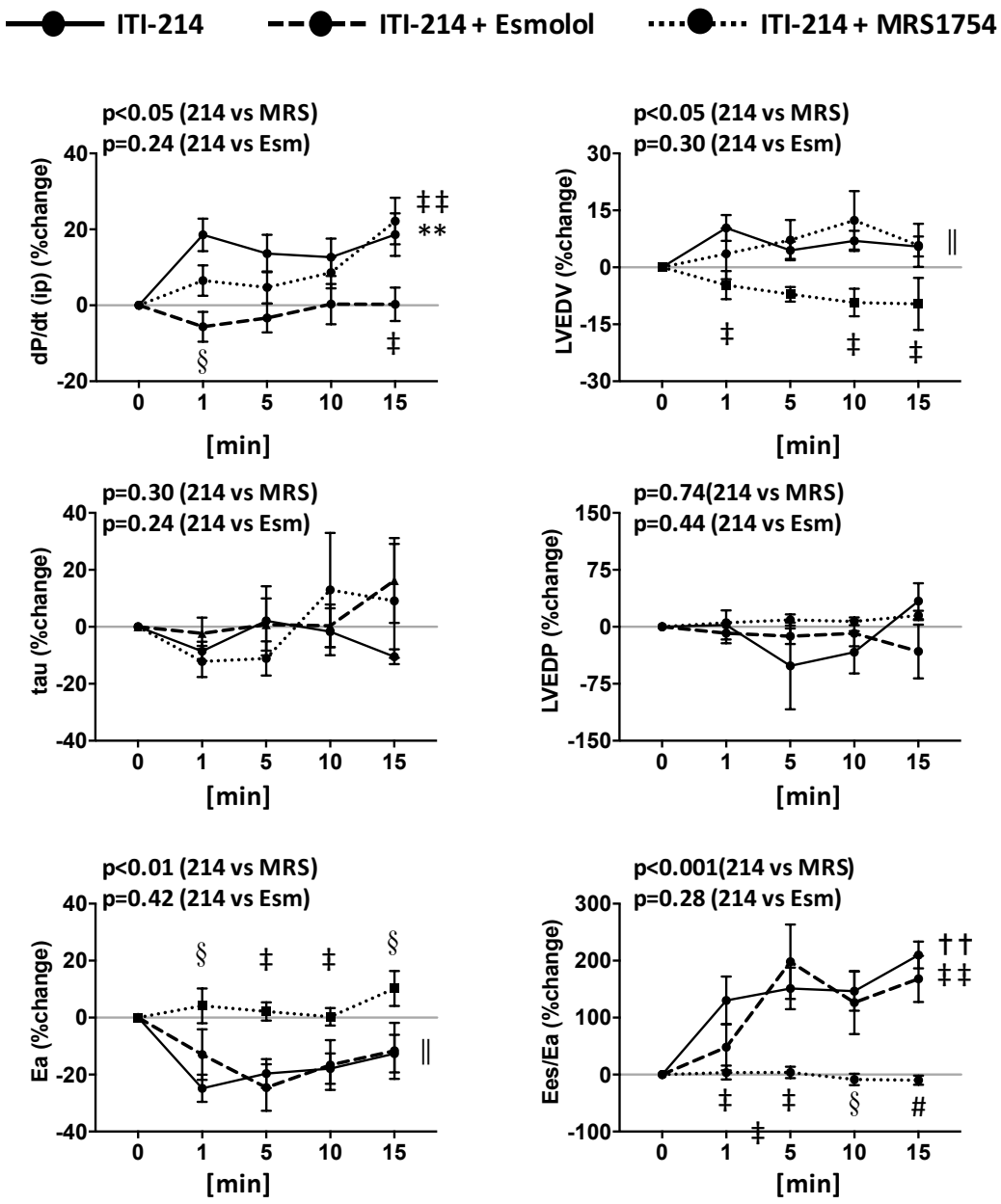
**Supplemental Figure S3. Hemodynamic effect of acute PDE1 inhibition by intravenous administration of ITI-214 in dogs with non-failing and failing hearts.** Summary time course plots of the response to ITI-214 are shown. The dogs were initially given a bolus dose of 0.01 mg/kg dose, and again 0.1 mg/kg 30 minutes after the first. *$p<0.05$, †$p<0.01$, ‡$p<0.001$ for 0.1 mg/kg vs 0.01 mg/kg at each time point (n=6). P values for each graph are for interaction of time and dose by two-way repeated measures ANOVA.



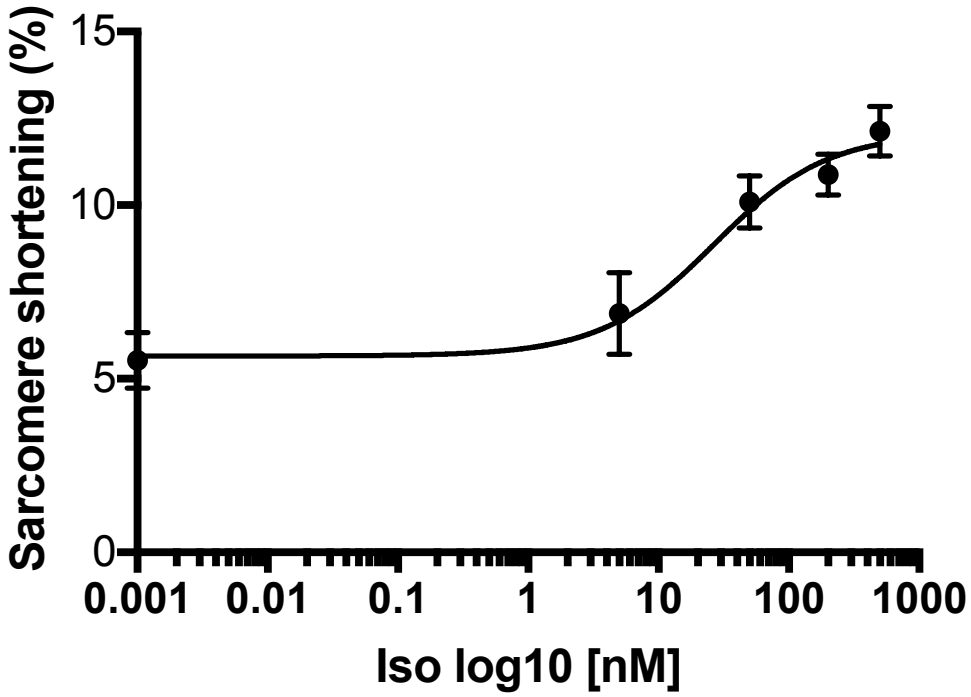

**Supplemental Figure S4. Influence of β-adrenergic or adenosine $A_{2B}$ receptor blockade on ITI-214 response.** Summary time course plots of the response to ITI-214 in rabbits are shown (n=19 vehicle+ITI-214 0.1 mg/kg i.v.; n=11 for esmolol (β-blocker)+ITI-214; and n=7 for MRS1754 ($A_{2B}$R blocker+ITI-214). ‡p<0.05, §p<0.01, #p<0.0001 for ITI-214 vs ITI-214+MRS1754 (two-way ANOVA with Sidak's post-hoc test), ||p<0.05, ††p<0.001 (one-way ANOVA for ITI-214), ‡‡p<0.05 (one-way ANOVA for ITI-214+Esmolol). P values above each graph are for interaction of time and drug intervention from 2-way repeated measures ANOVA. 214 = ITI-214, MRS = MRS1754, Esm = esmolol.



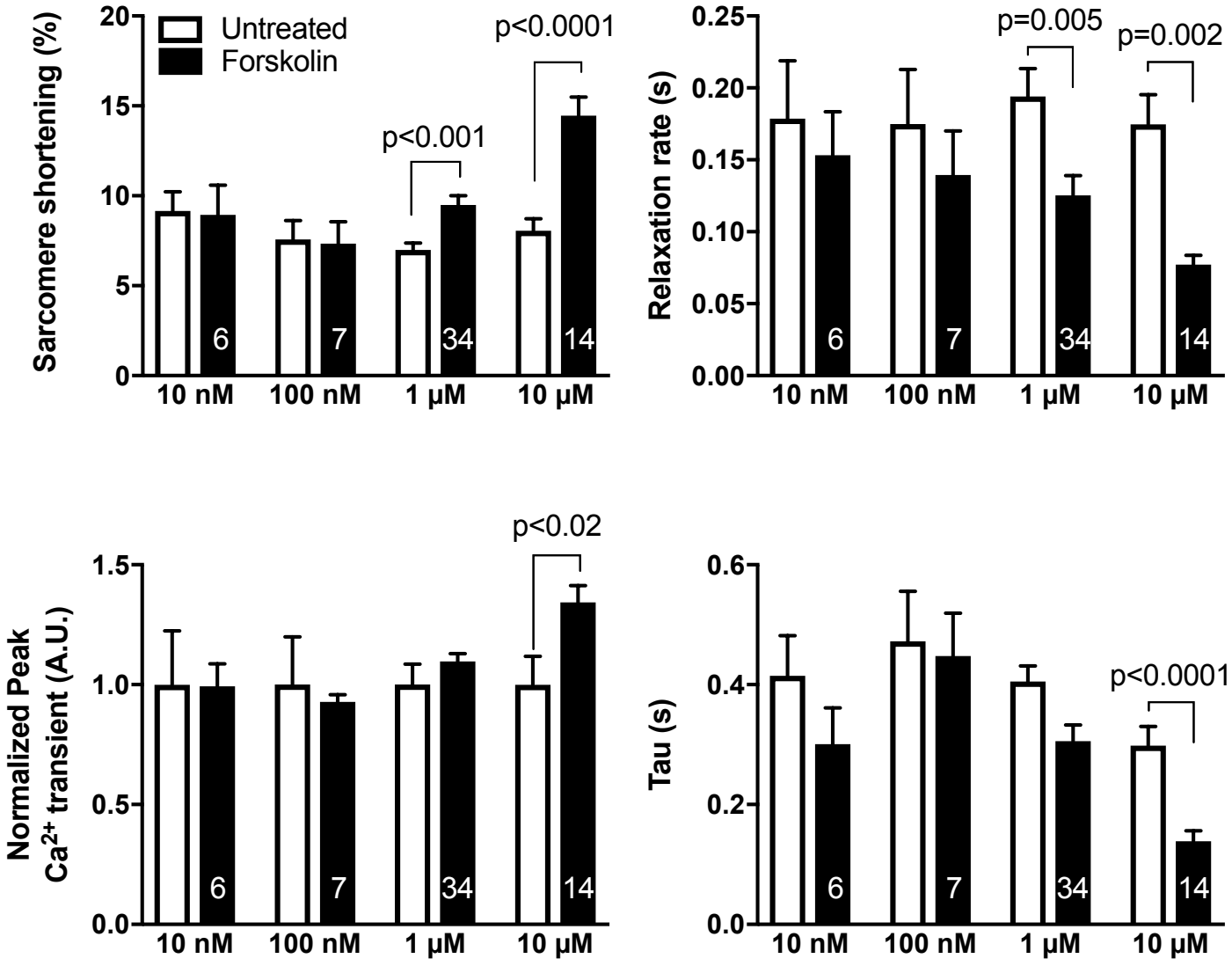

**Supplemental Figure S5. Esmolol blunts dobutamine-induced hemodynamic effects.** Rabbits were infused with dobutamine at 10 μg/kg/min for 10-15 min prior to treatment with esmolol (0.5 mg/kg bolus injection followed by 0.05 mg/kg/min infusion). Data are presented as % change relative to baseline. *$p<0.05$, †$p<0.01$, ‡$p<0.001$ vs DOB by paired t-test (n=7). DOB = dobutamine.




**Supplemental Figure 6. Influence of β-adrenergic or adenosine $A_{2B}$ receptor blockade on ITI-214 response at fixed heart rate.** Summary data for three conditions with constant atrial pacing in rabbits. (n=13 vehicle+ITI-214 0.1 mg/kg i.v.; n=8 for esmolol (β-blocker)+ITI-214; and n=6 for MRS1754 ($A_{2B}$R blocker)+ITI-214). ‡p<0.05, §p<0.01, #p<0.0001 for ITI-214 vs ITI-214+MRS1754 (two-way ANOVA with post hoc Sidak's test), ‖p<0.05, **p<0.01, ††p<0.001 (one-way ANOVA for ITI-214), ‡‡p<0.05 (one-way ANOVA for ITI-214+Esmolol). (n=13 vehicle+ITI-214 0.1 mg/kg i.v.; n=8 for esmolol (β-blocker)+ITI-214; and n=6 for MRS1754 ($A_{2B}$R blocker)+ITI-214). P values above each graph are for interaction of time and drug intervention from 2-way repeated measures ANOVA. (214 = ITI-214, MRS = MRS1754, Esm = esmolol).




**Supplemental Figure S7. Dose-response curve for functional response to isoproterenol (iso).** Change in cell sarcomere shortening in response to increasing dose of isoproterenol was fitted with a nonlinear fit (n=5-12 from two rabbits) to estimate the $EC_{50}$. This dose was then used as the priming dose to which either ITI-214 or Cilostamide was added.




**Supplemental Figure S8. Dose-dependent effect of forskolin on rabbit LV myocyte sarcomere shortening and calcium transients**. Grouped average changes in (top) sarcomere shortening and (bottom) $Ca^{2+}$ transients and their respective kinetics (P values from multiple T-tests, n=indicated, from 8 rabbits).