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Supplementary Information for

In vivo selective Inhibition of TRPC6 by Novel Antagonist BI 749327 Ameliorates Fibrosis and Dysfunction in Cardiac and Renal Disease

Brian Leei Lin[a], Damian Matera[b], Julia F. Doerner[c], Nan Zheng[c], Donato del Camino[c], Sumita Mishra[a], Hong Bian[b], Svetlana Zeveleva[b], Xiaoguang Zhen[c], Nathaniel T. Blair[c], Jayhong A. Chong[c], David P. Hessler[c], Djahida Bedja[a], Guangshuo Zhu[a], Grace E. Kim[a], Mark J. Ranek[a], Lynn Pantages[b], Mary McFarland[b], Matthew R. Netherton[d], Angela Berry[d], Diane Wong[d], Georg Rast[e], Hu Sheng Qian[b], Steven M. Weldon[b], Jay J. Kuo[b], Achim Sauer[e], Chris Sarko[d], Magdalene M. Moran[c], David A. Kass[a]*, Steven S. Pullen[b]*

Corresponding Authors:
Steven S. Pullen
steven.pullen@boehringer-ingelheim.com

David A. Kass
dkass@jhmi.edu

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Cell Culture and Transfection

TRPC channels: The cDNAs for human versions of TRPC channel subfamily members and mouse TRPC6 were subcloned into pcDNA5/TO. Stable, inducible clonal cell lines were generated in T-REx-HEK293 cells (Invitrogen) constitutively expressing the human muscarinic acetylcholine receptor 1 (M1R). Cells were maintained in DMEM with 4.5 g/L glucose and L-glutamine, supplemented with 10% Fetal Bovine Serum (Sigma Aldrich), 100 U/mL penicillin/100 µg/mL streptomycin, 2 mM glutagro supplement, 5 µg/mL blasticidin, 100 µg/mL hygromycin B, and 1 mg/mL G-418 at 37°C, 10% CO_2. All culture media components were from Corning unless otherwise specified. Plasmids expressing mouse and guinea pig TRPC channels (with the exception of mouse TRPC6) were transiently transfected into T-REx-HEK293 cells stably expressing M1R or parental T-REx-HEK293 cells using Lipofectamine 2000 according to the manufacturer's instructions. A GFP-expression plasmid was co-transfected to identify transfected cells.
TRPM8, TRPA1, and TRPV1: The respective channels were stably expressed in HEK293 cells (TRPA1 and TRM8) or CHO cells (TRPV1) generated by B'SYS GmbH, Switzerland. Cell culture was performed according to the product specifications. Briefly, TRPA1 and TRPM8 cells were cultivated in DMEM with GlutaMAX I and 10% FBS, supplemented with 100 µg/mL hygromycin and 15 µg/mL blasticidin (TRPA1) or 3 µg/mL puromycin (TRPM8). TRPV1 cells were cultivated in F12 (HAM) with GlutaMAX I and 10% FBS, supplemented 500 µg/mL geneticin. Kv11.1 (hERG) and Nav1.5 channels were stably expressed in HEK293 cells and cultivated in DMEM with

GlutaMAX I and 10 % FBS, supplemented with 200 µg/mL geneticin (Nav1.5) or 40 µg/mL hygromycin B (Kv11.1).

Electrophysiology

TRPC channels: cells were voltage-clamped using an EPC10 (HEKA), Axopatch 200B or Axon Multiclamp 700B (Molecular Devices). Currents were acquired at 10 kHz and filtered at 2 or 2.9 kHz. The standard voltage ramp protocol was applied with a 4 - 5 s interval and comprised an 80 ms step at -80 mV, a 320 ms ramp to +80 mV, and a 40 ms voltage step at +80 mV. In some experiments, the ramp was 400 ms flanked by 40 ms voltage steps at -80 and +80 mV. The holding potential in between ramps was -40 mV. Currents were measured as the average of several ms at the voltage steps at -80 and +80 mV. Up to 70% series resistance compensation was used in some recordings. Liquid junction potentials were not corrected. Recordings were performed at room temperature. Electrodes were pulled from borosilicate glass with a resistance of ~1-3 MOhm. The external solution used in TRPC3, TRPC6, and TRPC7 recordings contained in mM: 145 NaCl, 4.5 KCl, 3 $MgCl_2$, 1 EGTA, 10 HEPES, 10 Glucose; pH 7.4 with NaOH. The internal solution contained in mM: 140 Cs-aspartate, 10 EGTA, 10 HEPES, 2.27 $MgCl_2$, 1.91 $CaCl_2$ (~40 nM free Ca^{2+}, MaxChelator); pH 7.2 with CsOH. In case of TRPC5 (see also Just et al. (1)), the external solution contained in mM: 145 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 Glucose; pH 7.4 with NaOH, and the internal solution was in mM: 140 Cs-aspartate, 10 HEDTA, 10 HEPES, 2.27 $MgCl_2$, 1.91 $CaCl_2$ (~1.4 µM free Ca^{2+}, MaxChelator); pH 7.2 with CsOH. Solutions were perfused with a local, gravity fed perfusion system.

For IC_{50} measurements, currents were activated as indicated in Table 1. In some experiments 1-oleoyl-2-acetyl-sn-glycerol (OAG, Avanti Polar Lipids) was applied to elicit TRPC channel currents. Appropriate amounts of OAG in chloroform were dried using a SpeedVac (Thermo Savant) and the resulting residue was dissolved in DMSO to obtain a stock concentration of 10 mM OAG. This stock was then diluted to a final concentration in external solution containing 0.005% Pluronic F-127 (Life Technologies). In the case of TRPC3 channels, constitutive currents were recorded in absence of any agonist. After break-in, variable levels of spontaneous TRPC3 current could be detected. These currents typically ran up over time until reaching a steady-state level which was eventually followed by a slow current decline over time (rundown). BI 749327 was prepared as a 10 mM stock solution in DMSO and diluted in extracellular buffer to $\leq$ 0.1% at the different compound concentrations tested. The compound was perfused when the activated current stabilized or when a steady current rundown could be observed. Following compound addition, the extent of current recovery was assessed by perfusion of (agonist containing) external solution. The amount of leak current was determined by application of the compound at a concentration that produced complete block (control block) at the end of the recording. To estimate percent block at the tested compound concentration, current amplitudes for the current in the presence of the compound, the predicted unblocked current, and the leak current during control block were measured. The predicted unblocked current was derived from linear interpolation of the current amplitudes before and after compound addition. Percent block was then calculated by comparing the current remaining in the presence of the blocker with the predicted unblocked current (after subtracting leak current from both). Cells were

challenged with either a single or multiple compound concentrations. Two to six data points were collected for three concentrations per channel and fitted with a Hill equation where min and max block were set to 0 and 100%, respectively, and HillSlope and IC_{50} were allowed to float.

TRPM8, TRPA1, and TRPV1: Measurements were performed at B'SYS GmbH, Switzerland, according to the respective standard operating procedures. Briefly, cells were voltage-clamped at -40 mV using an EPC10 amplifier (HEKA) and the standard voltage ramp protocol was applied with a 10 s interval. It comprised a step to -80 mV for 80 ms, a 400 ms ramp to +80 mV, followed by a constant segment of +80 mV for 40 ms. Currents of interest were measured at room temperature as the averages of several ms during the constant segments at -80 and +80 mV. For TRPM8 and TRPV1 the following bath solution was used (in mM): 137 NaCl, 4.0 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 10 glucose; pH 7.4 with NaOH. For TRPA1 a calcium-free bath solution was used (in mM): 140 NaCl, 4.0 KCl, 2.0 $MgCl_2$, 10 HEPES, 5.0 EGTA, 10 glucose; pH 7.4 with NaOH. Compounds were always applied on at least three different cells in the following order: vehicle for 30 s, agonist for at least 3 minutes until a steady-state current was reached, agonist and BI 749327 (10 µM) for at least 3 minutes until a steady-state current was reached. Agonists are given in Table 2. The compounds were prepared as 1000x stock solutions in DMSO and subsequently diluted in bath solution in a single step to reach the final concentration. For all channels the same pipette solution was used (in mM): 120 $KMeSO_3$, 6.0 KOH, 20 KCl, 0.8 $MgCl_2$, 0.8 $CaCl_2$, 4.0 MgATP, 1.0 BAPTA, 10 HEPES, pH 7.2 with $MeSO_2OH$. The current of interest readings were leak-

corrected and normalized with respect to steady-state current during agonist application.

Kv11.1 and Nav1.5: Membrane currents were recorded at 31 °C using an EPC-10 patch-clamp amplifier and PatchMaster software (HEKA). The hERG assay has been described previously (2). Briefly, cells were voltage-clamped at a holding potential of -60 mV and currents were elicited using the following a pulse pattern: activation/inactivation: 40 mV for 2000 ms; recovery: -120 mV for 2 ms; ramp to 40 mV within 2 ms; inactivating current: 40 mV for 50 ms, repeated at 15 s intervals. During each inter-pulse interval 4 pulses scaled down by a factor of 0.2 were recorded for a P/n leak subtraction procedure.

Cells were superfused with a bath solution containing (mM): 137 NaCl, 4.0 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 10 Glucose, 10 HEPES; pH 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass tubing and filled with pipette solution containing (in mM): 130 K-aspartate, 5.0 $MgCl_2$, 5.0 EGTA, 4.0 K_2ATP, 10 HEPES; pH 7.2 with KOH. Resistance of the microelectrodes was in the range between 2 and 5 MΩ.

Rs compensation was employed up to a level that safely allowed recording devoid of ringing. The test concentrations of BI 749327 were applied sequentially in an escalating order on each of the three different cells investigated. Final dilutions in extracellular buffer were prepared freshly from 1000x stocks in DMSO by a single 1:1000 dilution step each before starting the experiments. A steady-state level of baseline current was measured for at least 5 sweeps prior to the application of the first compound concentration. For each sweep, peak current amplitudes were measured 3 ms after the ramp to +40 mV. For baseline and each concentration the peak currents of the three

last sweeps before application of the next concentration were averaged. Residual currents were calculated for each cell as the fraction of actual average peak current and average baseline peak current. The IC_{50} was determined by fitting the Hill equation to the residual current data with fixed upper and lower boundaries (100% and 0%) and floating slope and IC_{50} using GraphPad Prism software.

The above procedure was modified as follows for Nav1.5 recordings: cells were voltage-clamped at -110 mV and currents were elicited with 15 ms depolarizing pulses to -20 mV with an inter-pulse interval of 800 ms. A steady-state level of baseline current was measured for at least 20 sweeps prior to the application of the first compound concentration. For each sweep, peak current amplitudes were measured during the -20 mV segment.

CN/NFAT Luciferase Assays in HEK293T Cells

HEK293T cells were cultured in DMEM (10% FBS, 1% penicillin/streptomycin, Thermo Fisher) to 70% confluence, transfected with plasmids expressing a NFAT luciferase reporter (0.3 µg; Promega) and either human WT-TRPC6, one of a group of gain-of-function TRPC6 mutants (P112Q, M132T, R175Q, R895C, R895L), or empty vector pcDNA3.1 (0.3 µg). Renilla-luciferase plasmid (0.001 µg; Promega) was transfected as an internal control. TRPC6-expression plasmids were generated using a PCR-based site-directed mutagenesis kit (QuickChange, Stratagene). After 24 hrs, cells were exposed to BI 749327 at varying doses for an additional 6 hrs, lysate extracted using passive lysis buffer according to manufacturer's instructions (Promega), and luciferase activity measured by Dual-Luciferase Reporter Assay (Promega). For *in vitro* HEK293T

and NRVM (neonatal rat ventricular myocytes) studies, BI 749327 was dissolved in 100% DMSO, and then diluted to final concentrations of 100, 250, and 500 nM (DMSO concentration was 0.01%, and served as vehicle control).

Neonatal Rat Ventricular Myocyte Studies

NRVM were isolated from newborn Sprague-Dawley rat pups, as described (3), plated and cultured in 10% FBS solution in DMEM and maintained at 37ºC, 5% CO_2 for 6 hours. They were then treated with either angiotensin II (100 μM) for 48 hours to stimulate hypertrophic signaling, followed by exposure to either vehicle or BI 749327 (250 nM or 500 nM) for an additional 6 hours in serum-free media. RNA was isolated and analyzed by quantitative polymerase chain reaction.

Plasma Protein Binding

Mouse plasma was spiked with the test compound (1 μM final concentration) and dialyzed in rapid equilibrium dialysis cells (RED-device, Pierce) against phosphate buffered saline pH 7.4 for 5 hours at 37°C. The experiment was performed in triplicate. After sample processing (4), compound concentration in buffer and plasma was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Unbound concentrations in plasma were calculated as:

$$\%\, bound = \frac{plasma\ concentration - buffer\ concentration}{plasma\ concentration} \times 100$$

Pharmacokinetic Studies

Pharmacokinetics of BI 749327 was investigated in male CD-1 mice (Janvier, France) at dose levels of 3, 10, and 30 mg/kg and at 30 mg/kg in B6129F1 mice (Taconic Biosciences, Denmark). BI 749327 was suspended in 0.5% Natrosol® / 0.015% Tween 80 and 10 mL/kg of the final formulation was delivered to 4 mice per dose group via gavage. Serial blood sampling was performed via puncture of the saphenous vein and samples were collected using EDTA coated microtainers (Microvette®, Sarstedt, Germany). Upon centrifugation for 5 minutes, plasma was drawn off and frozen until analysis. Mice for the pharmacokinetic studies were group housed under controlled temperature and light conditions with free access to rodent chow and water ad libitum. The experimental procedures were approved by the local German authorities and were compliant with the German and European Animal Welfare Act.

Plasma levels of BI 749327

Plasma samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) upon protein precipitation. The API 5000 triple quadrupole mass spectrometer was operated in the positive ion mode. The lower limit of quantification was 1 nmol/L. Pharamacokinetic parameters were calculated by means of non-compartmental analysis from the plasma concentration-time curves as previously described (1).

In vivo Pressure-Overload Model

Adult (12+ wks, 28g) C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were used. All mice received daily gavage with methyl-cellulose/tween-80 vehicle starting 1-week prior to transverse aortic constriction (TAC) surgery. This was done to acclimatize the mice to gavage and minimize potential stress effects of the procedure itself when randomizing to active drug. Procedures for TAC have been previously reported (5). Here, we used a 26.5 or 27 gauge needle to size the constriction based on the weight of the mouse. A sham procedure involved similar surgery without aortic constriction. For *in vivo* pressure-overload studies, BI 749327 was stored in powdered form at room temperature until ready to use. BI 749327 was prepared daily in suspension in 0.5% methylcellulose and 0.015% Tween-80 solution, and delivered by oral gavage at a dose of 30 mg/kg/day. A control echocardiogram was obtained before surgery and another 1-week after TAC. All mice with a %FS falling between 20-40% prior to initiating therapy were included for the analysis. Randomization to vehicle or BI 749327 occurred 1-wk after TAC, and continued to week 8, with serial echocardiograms obtained at bi-weekly intervals. In a subset of mice, a tail vein blood draw was performed and samples were put in microvette tubes (Sarstedt) at peak (2 hours post-dosing) and trough (24 hours post-dosing) after 8-weeks chronic dosing. Plasma was separated, and HPLC-MS performed to determine BI 749327 plasma concentration. At the final time point, a subset of mice underwent invasive pressure-volume analysis (n=5-6/group). All protocols were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University.

Echocardiography

Transthoracic echocardiography was performed in conscious mice (Acuson Sequoia, Siemens, or Vevo 2100 (VisualSonics) as described (6). The operator was blinded to animal treatment.

Pressure-Volume Loop Study

Procedures for performing and analyzing pressure-volume loops for LV mechanical assessment have been previously reported (6). Animals were anesthetized (2% isofluorane), and instrumented with a R-1030 catheter (Millar, Inc), and data recorded at 2kHz (PowerLab, AD Instruments). Signals were analyzed using custom software (WinPVAN, Baltimore).

Tissue Collection and Histology

Heart weight, lung weight, body weight, and tibia length were recorded upon sacrifice of an animal at the terminal point of the experiment. A mid-transverse cross-section of the heart encompassing both the left and right ventricle was dissected using a heart slicer matrix, fixed in 4% paraformaldehyde (overnight) and two transverse sections per animal stained by H&E and Masson's Trichrome, and imaged (Aperio ScanScope CS). The entire section was analyzed for percent fibrosis, and expressed as % total area using two independent programs Aperio ImageScope macro (Leica Biosystems Imaging, Inc.) and ImageJ (NIH)), each done blinded to treatment group. The remaining

myocardium was divided into RV, LV freewall, septum, and atria, frozen in liquid nitrogen, and stored at -80^{o}C.

Quantitative polymerase chain reaction mRNA analysis

RNA was extracted with TRIzol (Ambion), reverse transcribed to cDNA per manufacturer's instructions (Thermo Fisher). Expression was measured using Taqman probes (Applied Biosystems). The following mRNAs were assayed: *Nppa*, *Nppb*, *Myh7, Trpc1, Trpc3, Trpc6, Rcan1*, *Col1a2, Col3a1, Fn1, Mmp2, Tgfb1,* and *Timp2*, normalized to *Gapdh* using the ΔΔCT method (catalog numbers for each primer were Mm01255747_g1, Mm0125577_g1, Mm00600555_m1, Mm00441975_m1, Mm00444690_m1, Mm01176083_m1, Mm01213406_m1, Mm00483888_m1, Mm01254476_m1, Mm01256744_m1, Mm00439498_m1, Mm01178819_m1, Mm01178819_m1, Mm99999915_g1, respectively).

Telemetry instrumented mouse experiments

Male C57BL/6 mice (n=9) were surgically implanted with a radio-telemeter (DSI, St. Paul, MN) to enable measurements of arterial pressure and heart rate (Jackson Laboratories, Bar Harbor, ME). After a two week recovery period, all mice received oral administration of the vehicle for 4 days. Following the 4-day baseline period, all mice received a single daily oral administration of BI 749327 at 30 mg/kg/day for 4 days. Mean arterial pressure (MAP) and heart rate (HR) were determined during the vehicle and BI 749327 treatment at 1 minute intervals for 24 hours. The MAP and HR were calculated as the 24 hour average. The 4-day vehicle treatment for each mouse was

averaged to generate a single baseline MAP and HR. A 1-way ANOVA using Dunnett's post-test utilized to determine a significant effect of daily oral administration of BI 749327 on MAP and HR.

In Vivo Unilateral Ureteral Obstruction Model

Unilateral ureteral obstruction (UUO) surgery was performed under isoflurane-induced anesthesia using aseptic techniques. A midline laparotomy was performed and the left ureter was isolated and ligated at the boundary of the lower renal pole to induce irreversible UUO. Sham animals underwent similar surgical procedures except no ureter ligation was performed. The abdominal incision was closed using sterile sutures and wound clips for the skin closure. Mice recovered in a warm chamber under observation and then returned to single housing in standard plastic caging.

BI 749327 dose response: Approximately 26 and 2 hours prior to UUO surgery, CD-1 mice (Charles River Lab., Kingston, NY) were pre-treated with an oral administration of either vehicle (0.5% methylcellulose / 0.015% Tween 80; Spectrum Chemical, New Brunswick, NJ) or BI 749327 at 3, 10, or 30 mg/kg. Following UUO surgery, vehicle or BI 749327 at the respective doses was orally administered once daily (10 mL/kg) for 5 days.

Prior to the completion of the experiment, BI 749327 treated mice were anesthetized with isoflurane and 100 µL of blood was obtained from the jugular vein for trough plasma concentration measurements. Five days post-UUO surgery, all mice were dosed respectively with vehicle or BI 749327 two hours prior to termination by exsanguination via cardiac puncture under deep anesthesia. The left kidney was removed, blotted, and

a mid-transverse cortical section excised for picosirius red analysis, and the central section was placed into individually labeled histology cassettes and fixed in 10% neutral buffered formalin for 48 hours. The samples were then embedded and sectioned for picosirius red (total collagen) and α-smooth muscle actin (ab124964, Abcam), CD3 (ab16669, Abcam), S100A4 (ab27957, Abcam), F4/80 (14-4801-85, eBioscience), collagen I (ab34710, Abcam), and collagen IV (ab6586, Abcam) staining. Automated multispectral imaging acquisition of positive picosirius red signals and αSMA expression was performed by using a Vectra 3.0 Imaging System (PerkinElmer). Imaging analyses were completed automatically by InForm 2.4 software (PerkinElmer), and the results were expressed as % total positive area per field. Kidney tissue sections (4 μm) were stained with H&E for the general assessment of total number of foci of interstitial lesions. Tubulointerstitial lesions were assessed as the number of foci showing obvious renal damage, which included marked hypercellularity (inflammation or resident myofibroblast increases), fibrosis, tubular hyperplasia, casts, or necrosis. Assessments were performed under blinded conditions at 10 x magnification (Probetex, Inc.). A polar section was collected for BI 749327 concentration determination. Renal cortex from the opposite pole was placed in RNA Later (Qiagen), refrigerated overnight, and stored at -80°C. RNA was extracted with Invitrogen™ TRIzol™ Reagent (ThermoFisher Scientific) and purified with Invitrogen™ PureLink® RNA Mini Kit (ThermoFisher Scientific). Following reverse transcription using Applied Biosystems™ High-Capacity RNA-to-cDNA™ Kit (ThermoFisher Scientific), RNA expression of TRPC1 through TRPC6, Col4a1, Fn1, and Tgfb1 was quantified by qPCR using Applied Biosystems TaqMan

assays (ThermoFisher Scientific). Terminal blood was processed for peak BI 749327 plasma concentration.

Pharmacokinetics and Pharmacodynamics Analysis

The individual αSMA values were plotted as a function of the corresponding individual unbound trough concentrations. For non-linear fitting, the top was fixed to the mean vehicle group value and the bottom to the mean sham group value.

Statistical Analyses

For multiple comparisons, ordinary 1-way ANOVA was used with Tukey pairwise comparison (HEK293T cells), and Holm-Sidak or Kruskal Wallis with Dunn's test (NRVM and TAC models), and Dunnet's (UUO model), as appropriate (GraphPad Prism 7). Time course data based on serial echocardiography was analyzed using an analysis of covariance design, (general linear modeling, SYSTAT 10.2 and Minitab 18). Other measures made at two time points, and/or with a single comparison for drug effect on TAC models was performed using unpaired t-test or Mann-Whitney non-parametric t-test, as appropriate.

SI Figure Legends

Figure S1. BI 749327 effects on angiotensin-induced TRPC3/6/7-mediated NFAT signaling and baseline hypertrophic gene expression. (A) Angiotensin II induced NFAT signaling in TRPC3/6/7-expressing HEK cells by 7.3-fold, 2.3-fold, and 2.9-fold respectively over pcDNA-expressing control (control data not shown). Data for each TRPC channel transfection are shown normalized to results without BI 749327 co-treatment, and there were significant declines in NFAT activation with TRPC6 and TRPC7 transfection (displayed p-values for Mann Whitney U test). The reduction in NFAT activity by BI 749327 in cells receiving TRPC6 transfection was greater than that with TRPC3 (*p=0.005) and borderline over TRPC7 (†p=0.06) (Kruskal-Wallis test, with Dunn's multiple comparisons). (B) In unstimulated neonatal rat myocytes, BI 749327 exposure at 500 nM did not alter the mRNA expression for *Rcan1, Nppa, Nppb,* or *Myh7* (unpaired t-test).

Fig. S1

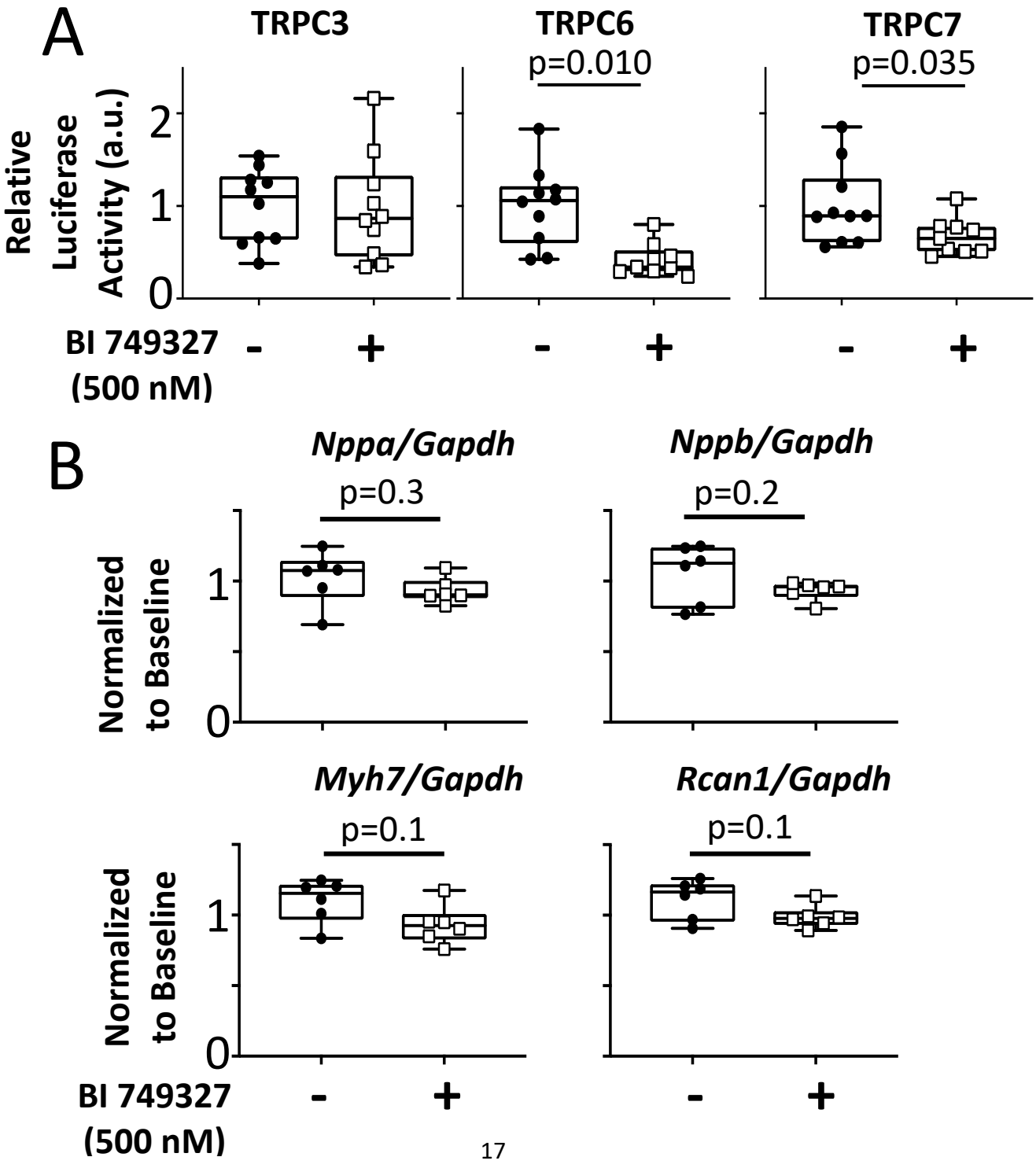
A
TRPC3
TRPC6
TRPC7
p=0.010
p=0.035
Relative Luciferase Activity (a.u.)
0
1
2
BI 749327 (500 nM)
-
+
B
Nppa/Gapdh
Nppb/Gapdh
Myh7/Gapdh
Rcan1/Gapdh
p=0.3
p=0.2
p=0.1
p=0.1
Normalized to Baseline
0
1

Figure S2. Comparison of measured and simulated mouse PK. (A) Oral pharmacokinetics of BI 749327 was investigated in male CD-1 mice after oral administration of 3 mg/kg (closed triangles), 10 mg/kg (closed squares), and 30 mg/kg (closed circles) BI 749327 and in the TRPC6 wild-type (B6129F1) mice after oral administration of 30 mg/kg BI 749327 (open circles). (n=4/group, serial blood sampling). (B) Pharmacokinetics of BI 749327 in male CD-1mice after oral administration of 3 mg/kg, 10 mg/kg, or 30 mg/kg BI 749327 and in the TRPC6 wild-type (B6129F1) mice after oral administration of 30 mg/kg BI 749327. For single dose PK (black symbols), (n=4/group). Data are mean ± SEM. The multiple dose simulations (lines) were derived from compartmental modeling of the single dose pharmacokinetic data. The measured exposures in the UUO studies are depicted as blue symbols (n=7-10).

Fig. S2

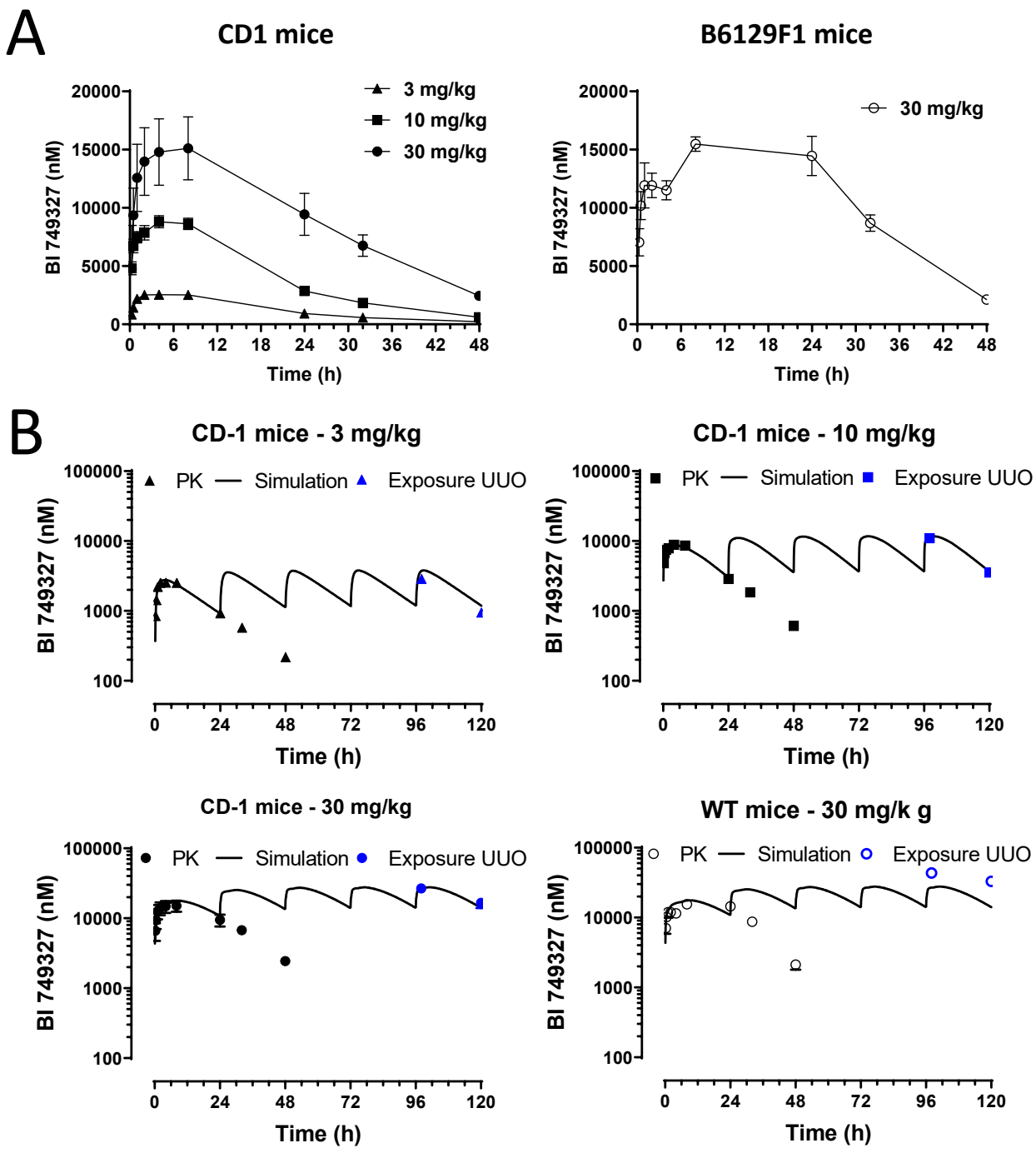
A
CD1 mice
B6129F1 mice
3 mg/kg
10 mg/kg
30 mg/kg
BI 749327 (nM)
Time (h)
B
CD-1 mice - 3 mg/kg
CD-1 mice - 10 mg/kg
CD-1 mice - 30 mg/kg
WT mice - 30 mg/k g
PK
Simulation
Exposure UUO

Figure S3. Exposure of BI 749327 in sham and TAC mice. Mice undergoing sham or TAC surgery enrolled in the pressure-overload heart disease study were dosed with 30 mg/kg BI 749327 via oral gavage daily up to 8-weeks post-surgery, after which plasma was collected from tail vein at peak (2 hours post-gavage) and trough (24 hours post-gavage) timepoints. Data are for unbound (free plasma) concentration. The trough level of ~180 nM supports full coverage against TRPC6 within a selective range for the 24 hour period.

Fig. S3

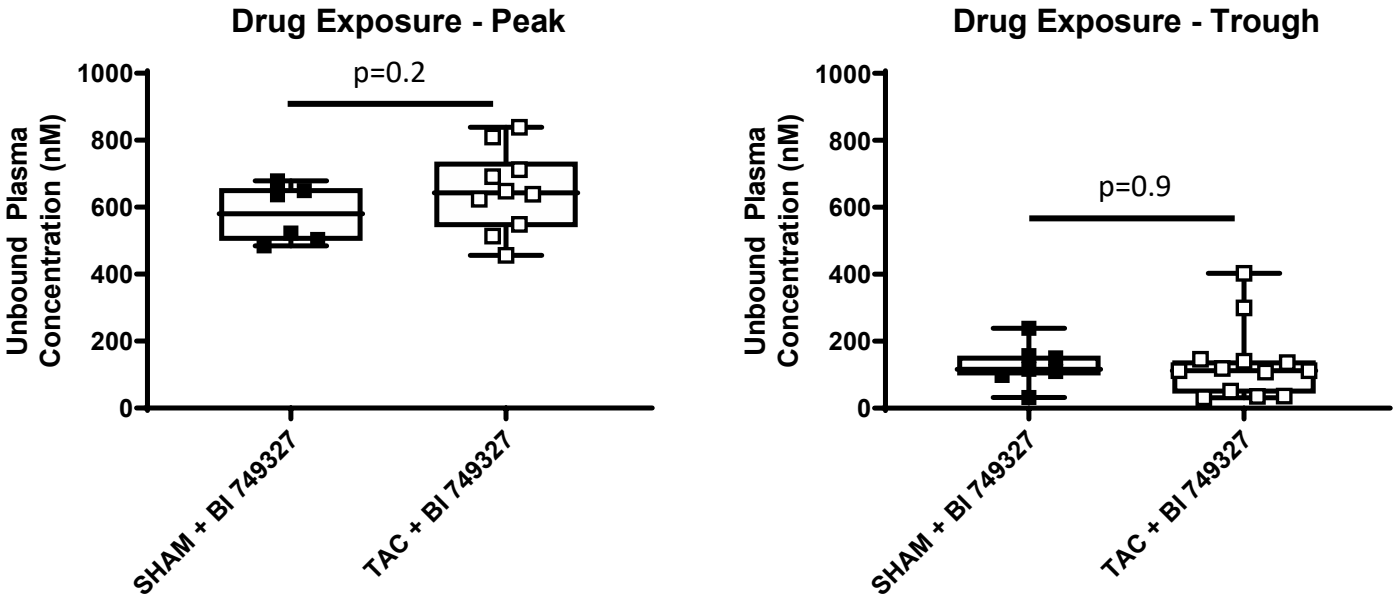
Drug Exposure - Peak
p=0.2
Unbound Plasma Concentration (nM)
1000
800
600
400
200
0
SHAM + BI 749327
TAC + BI 749327
Drug Exposure - Trough
p=0.9
Unbound Plasma Concentration (nM)
1000
800
600
400
200
0
SHAM + BI 749327
TAC + BI 749327

Figure S4. BI 749327 has no effect on MAP or HR. Normal telemetry instrumented mice were monitored for (A) MAP and (B) HR after administering vehicle or 30 mg/kg BI 749327 once daily for four days.

Fig. S4

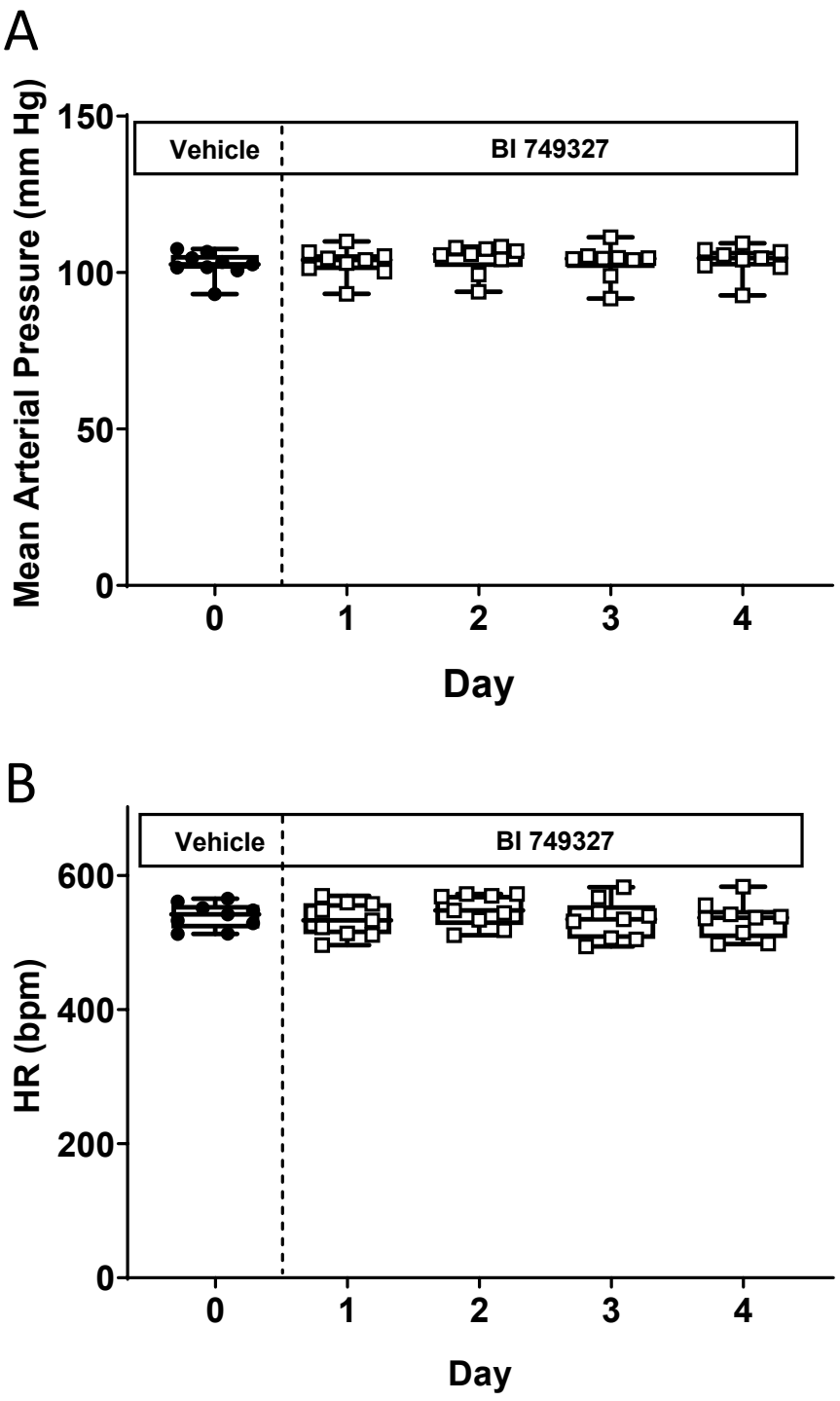
A
Mean Arterial Pressure (mm Hg)
150
100
50
0
Vehicle
BI 749327
0
1
2
3
4
Day
B
HR (bpm)
600
400
200
0
Vehicle
BI 749327
0
1
2
3
4
Day

Figure S5. Morphometric parameters from pressure-overload study.

Echocardiographic measures of (A) left ventricular cross sectional dimension at end-diastole (LVID;d) and (B) end-systole (LVID;s) (C) left ventricular posterior wall thickness measured in diastole (LVPW;d) (D) heart rate and (E) direct measurements of body weight. (ANCOVA, general linear modeling); (F) lung weight/body weight ratio ($p=0.65$ between TAC-VEH and TAC-BI 749327, 1-way ANOVA, Tukey's multiple comparisons).

Fig. S5

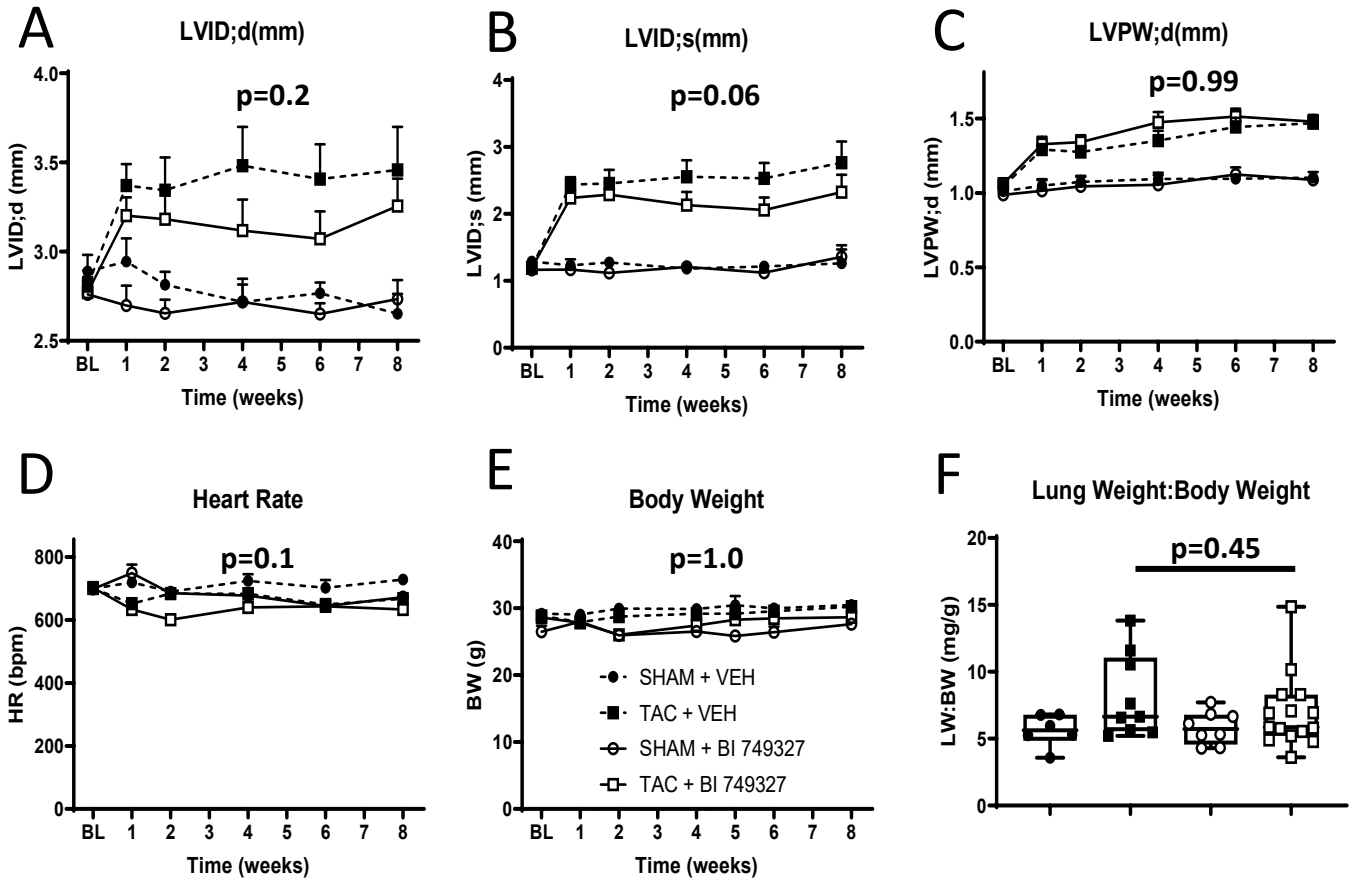
A
LVID;d(mm)
p=0.2
LVID;d (mm)
Time (weeks)
B
LVID;s(mm)
p=0.06
LVID;s (mm)
Time (weeks)
C
LVPW;d(mm)
p=0.99
LVPW;d (mm)
Time (weeks)
D
Heart Rate
p=0.1
HR (bpm)
Time (weeks)
E
Body Weight
p=1.0
BW (g)
SHAM + VEH
TAC + VEH
SHAM + BI 749327
TAC + BI 749327
Time (weeks)
F
Lung Weight:Body Weight
p=0.45
LW:BW (mg/g)

Figure S6. BI 749327 has no effect on TRPC1 or TRPC3 *in vivo*. Expression of related TRPC channel genes (A) *Trpc1* and (B) *Trpc3* in the LV were unaltered by BI 749327 (Mann-Whitney test between drug treatment in TAC group. No differences were significant in sham group. n=6/sham group, n=12 TAC-VEH group, n=15 TAC-BI 749327 group).

Fig. S6

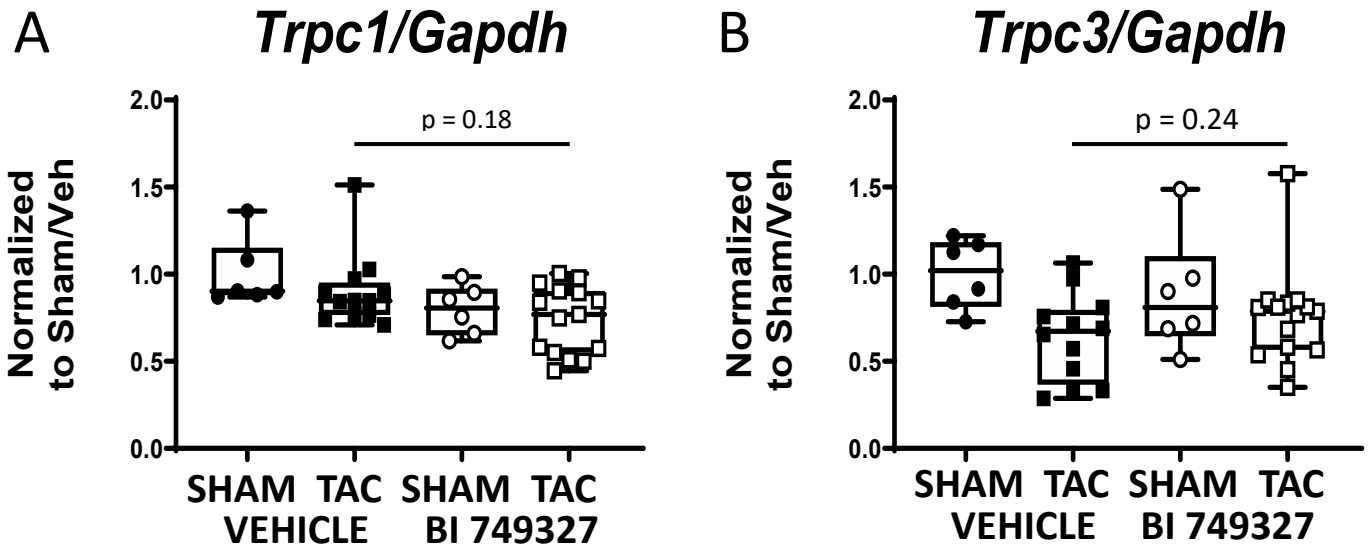
A
Trpc1/Gapdh
Normalized to Sham/Veh
2.0
1.5
1.0
0.5
0.0
p = 0.18
SHAM
TAC
SHAM
TAC
VEHICLE
BI 749327
B
Trpc3/Gapdh
Normalized to Sham/Veh
2.0
1.5
1.0
0.5
0.0
p = 0.24
SHAM
TAC
SHAM
TAC
VEHICLE
BI 749327

Figure S7. BI 749327 treatment inhibits renal injury of mice after UUO. (A-B) mRNA for extracellular matrix genes *Fn1* and *Col4a1* were upregulated in vehicle treated UUO mice and reduced by 30 mg/kg BI 749327 treatment. (C-F) S100A4, collagen I, collagen IV, and F4/80 immunohistochemical staining of kidney cortex is increased in mice after UUO and is diminished by treatment with 30 mg/kg BI 749327 (*$p<0.05$ vs vehicle, 2-tail unpaired t-test).

Fig. S7

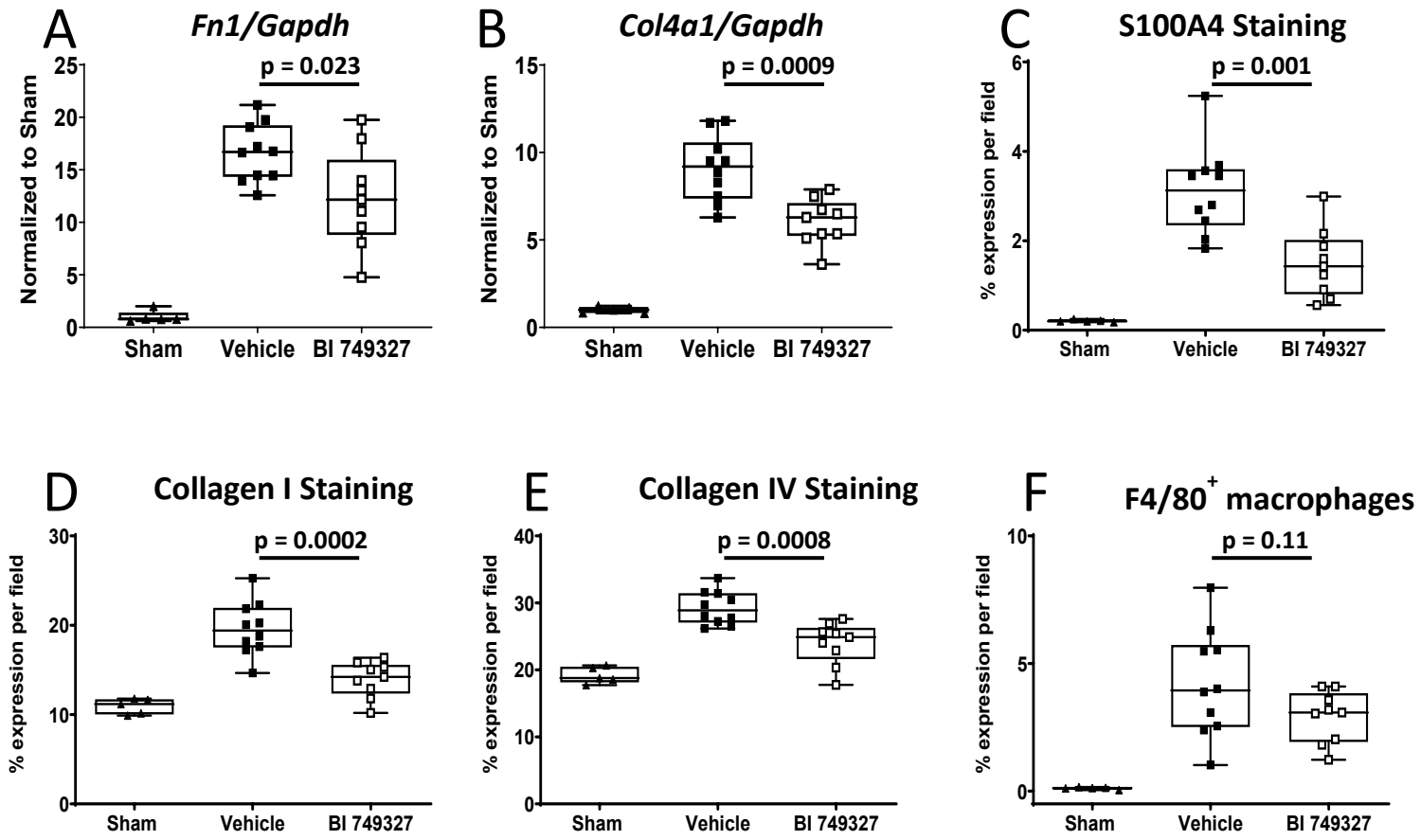
A
Fn1/Gapdh
p = 0.023
Normalized to Sham
25
20
15
10
5
0
Sham
Vehicle
BI 749327
B
Col4a1/Gapdh
p = 0.0009
Normalized to Sham
15
10
5
0
Sham
Vehicle
BI 749327
C
S100A4 Staining
p = 0.001
% expression per field
6
4
2
0
Sham
Vehicle
BI 749327
D
Collagen I Staining
p = 0.0002
% expression per field
30
20
10
0
Sham
Vehicle
BI 749327
E
Collagen IV Staining
p = 0.0008
% expression per field
40
30
20
10
0
Sham
Vehicle
BI 749327
F
F4/80+ macrophages
p = 0.11
% expression per field
10
5
0
Sham
Vehicle
BI 749327

Figure S8. Pharmaco-kinetics and dynamics analysis of BI 749327 effects on α-smooth muscle actin as marker for activated fibroblasts in UUO renal fibrosis model. Histochemistry derived αSMA staining level is plotted versus corresponding unbound trough plasma BI 749327 concentration ($C_{plasma,unbound}$) from the same animal. Black circles: data from the inhibitor treated animals from the dose-dependence study in CD-1 mice; open circles: data from untreated mice (vehicle and sham); straight line: non-linear fit; dotted line: 95% confidence interval.

Fig. S8

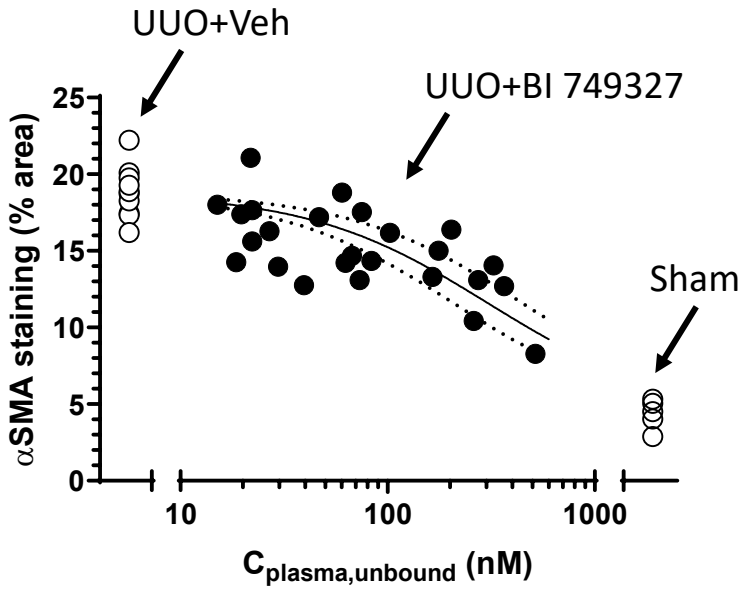
UUO+Veh
UUO+BI 749327
Sham
αSMA staining (% area)
25
20
15
10
5
0
10
100
1000
C plasma,unbound (nM)

Figure S9. BI 749327 has no effect on TRPC6 expression in the UUO model.

Relative TRPC6 mRNA expression for sham, vehicle, and BI 749327 at 3, 10, and 30 mg/kg/day are 1.00±0.05, 5.41±1.08 (p=0.004, 2-tail unpaired t-test Welsh's correction), 5.55±1.05, 5.09±0.68, 5.59±1.01, (n=3, 9, 10, 9, 7) respectively.

Fig. S9

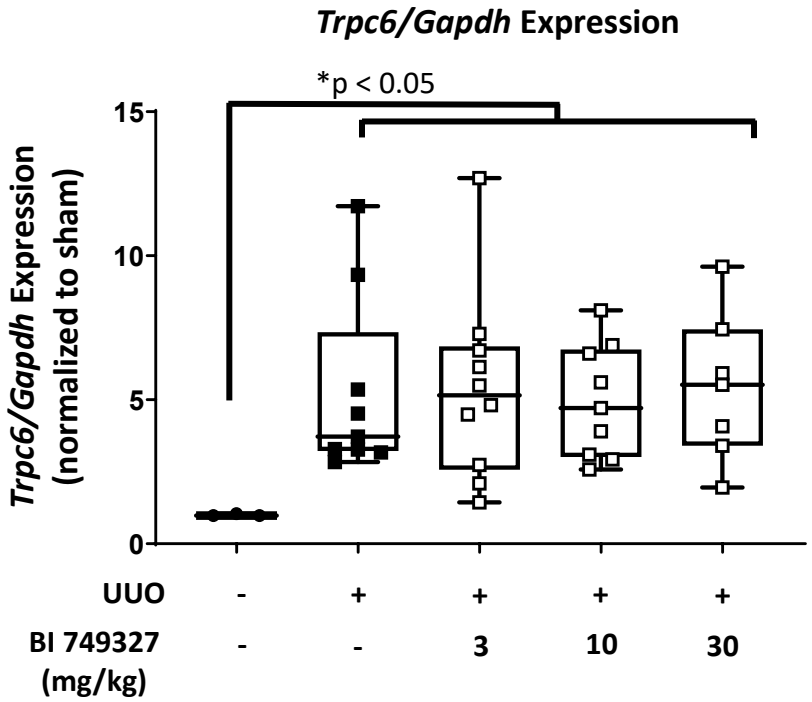
Trpc6/Gapdh Expression
*p < 0.05
Trpc6/Gapdh Expression (normalized to sham)
15
10
5
0
UUO - + + + +
BI 749327 (mg/kg) - - 3 10 30

Table S1: Potency of block (IC_{50}) for BI 749327 on recombinantly expressed TRPC channel subfamily members from different species.

Channel	Current Activation	IC_{50} ± SE [nM]
mTRPC6	1 µM OAG	13 ± 1.4
hTRPC6	1 µM OAG	19 ± 0.2
gpTRPC6	1 µM OAG	15 ± 1.6
mTRPC3	No agonist	1100 ± 330
hTRPC3	No agonist	940 ± 8.2
mTRPC7	1 µM OAG	550 ± 11
hTRPC7	1 µM OAG	580 ± 47
mTRPC5	80 µM La^{3+}	>10000
hTRPC5	80 µM La^{3+}	>10000

m, mouse; h, human; gp, guinea pig

Table S2: Selectivity against human non-TRPC subfamily members.

Channel	Current Activation	IC_{50} ± SE [nM]
hTRPA1	1 μM supercinnamaldehyde	>10000
hTRPM8	30 μM menthol	>10000
hTRPV1	300 nM capsaicin	>10000
hNav1.5	No agonist	>10000
hKv11.1(hERG)	No agonist	3000 ± 98

h, human

Table S3: Mouse PK parameters.

Strain	Dose [mg/kg]	C_{max} [nM]	T_{max} [h]	MRT_{tot} [h]	$T_{1/2}$ [h]
CD1	3.0	2730 ± 139	5.5 ± 1.5	18.0 ± 0.5	11.3 ± 0.25
	10	9340 ± 223	5.0 ± 1.0	17.0 ± 0.7	10.6 ± 0.56
	30	16100 ± 2760	5.5 ± 1.5	24.3 ± 3.9	13.5 ± 2.6
B6129F1/Tac	30	16200 ± 768	10.3 ± 4.9	21.1 ± 0.6	8.5 ± 0.26

N=4

Supplemental References

1. Just S*, et al.* (2018) Treatment with HC-070, a potent inhibitor of TRPC4 and TRPC5, leads to anxiolytic and antidepressant effects in mice. *PLoS One* 13(1):e0191225.
2. Rast G & Guth BD (2014) Solubility assessment and on-line exposure confirmation in a patch-clamp assay for hERG (human ether-a-go-go-related gene) potassium channel inhibition. *J Pharmacol Toxicol Methods* 70(2):182-187.
3. Takimoto E*, et al.* (2005) Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med* 11(2):214-222.
4. Braun C*, et al.* (2017) Quantification of Transporter and Receptor Proteins in Dog Brain Capillaries and Choroid Plexus: Relevance for the Distribution in Brain and CSF of Selected BCRP and P-gp Substrates. *Mol Pharm* 14(10):3436-3447.
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6. Chung HS*, et al.* (2017) Transient receptor potential channel 6 regulates abnormal cardiac S-nitrosylation in Duchenne muscular dystrophy. *Proc Natl Acad Sci U S A* 114(50):E10763-E10771.