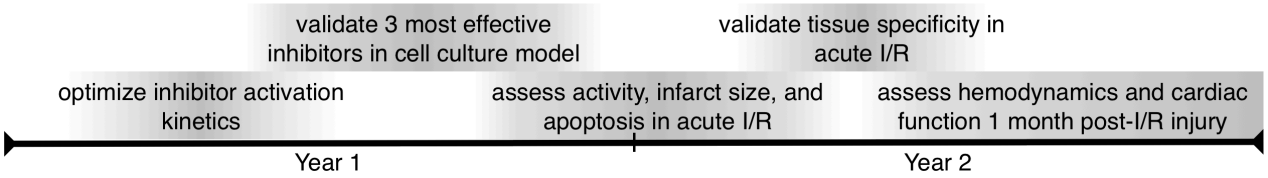
SPECIFIC AIMS. Despite substantial progress in treatment over the past few decades,[1] myocardial infarctions (MIs) kill 120,000 Americans every year.[2] The best available treatment is reperfusion of the heart using percutaneous intervention (PCI).[1,3] Upon reperfusion, however, the return of oxygenated blood stuns the myocardium, induces arrhythmias, and destroys additional myocytes.[4] This injury, known as ischemia-reperfusion (I/R) injury, explains roughly half of infarct size.[5] During reperfusion, a sudden burst of reactive oxygen species (ROS) production causes the injury.[6,7] Acutely, ROS damage biomolecules and induce cell death.[8] Chronically, ROS induce remodeling that leads to heart failure.[9–11] Antioxidants could lessen the damage, but they fail to target the cause of the injury effectively[12,13] and they perturb cell signaling in uninjured tissues.[8,14]

Given the lack of effective therapies for I/R in MI, my long-term goal is to mitigate harmful ROS production selectively in the reperfused myocardium without perturbing redox balance elsewhere. The overall objective of this proposal is to develop a novel anti-ROS approach in which harmful levels of H_2O_2 activate small molecule inhibitors of ROS generation exclusively in infarcted areas. Much of the H_2O_2 in I/R injury (and in subsequent heart failure) comes from NADPH oxidase 2 (Nox2) activity.[9–11,15] Once activated, my proposed agents will locally inhibit Nox2. To achieve selectivity in drug activation, I will leverage my group's expertise in conditional control of cellular processes using "caging groups." These are protecting groups that block a drug's function until removed via a chemical or physical trigger. My proposal builds on my lab's recent development of boronic esters that cage small molecules so they respond to H_2O_2 without producing toxic by-products.[16] I am proposing a drug that becomes an active Nox2 inhibitor after reducing two equivalents of harmful H_2O_2 to harmless water. I hypothesize that a novel boronic ester-based triggering mechanism will allow development of tissue-specific Nox2 inhibitors that selectively target cells accruing pathologic H_2O_2 levels. This will be tested through completion of the following aims within the timeline shown below.

Specific Aim 1: Optimize H_2O_2-responsive Nox2 inhibitors and test them in cells. I will finish preparing a series of boronic ester-caged Nox2 inhibitors and select the most promising lead based on ongoing kinetics experiments and cell culture assays. I will then quantify inhibitor activation, ROS production, and myocyte survival/cardiac enzyme release in a novel cardiac myocyte model with ultrafast changes in oxygen tension.

Specific Aim 2: Test H_2O_2-responsive Nox2 inhibitors in preventing acute and chronic cardiac injury via selective targeting of infarcted tissue in a mouse model of myocardial I/R. I will first measure Nox2 activity, infarct size, and apoptosis in a mouse model of myocardial I/R with and without caged Nox2 inhibitor treatment. Next, I will assess the developed approach's tissue specificity by quantifying cell signaling perturbations in uninjured tissues in animals treated with the caged prodrug or with the active compound. Finally, I will assess cardiac function and histology one month post-I/R injury in treated mice.



SIGNIFICANCE. Although several enzymes[17] (and mitochondria) produce ROS as a by-product, the NADPH oxidase (Nox) family produces ROS as its primary function,[18,19] implicating Nox enzymes as key targets to reduce tissue damage in I/R injury. Of all the Nox isoforms, Nox2 in particular is a key player in all phases of I/R injury, making it a promising therapeutic target.[20] Immediately upon reperfusion, high Nox2 activity induces a thromboinflammatory state[21] that obstructs the coronary microvasculature (the "no-reflow" phenomenon).[23,24] For the next several hours, high, sustained Nox2 activity[15] induces further oxidative damage.[22] Over the ensuing weeks, persistent Nox2 activity causes remodeling that compromises heart function and contributes to arrhythmias after MIs,[9–11] accounting for much of the morbidity and mortality.[23] Nox2 knockout in mice[24] and deficiency in humans[25] protects the vasculature and myocardium from I/R injury—though not without side effects[26–29]—as does pharmacologic inhibition.[20,30–33] Taken together, these findings suggest that inhibiting Nox2 can lessen the acute

damage and chronic sequelae of I/R injury.

Despite promising results in animals, no therapies to reduce ROS in I/R injury (e.g., antioxidants) have been successfully translated to the clinic.[13] But why? First, *endogenous* antioxidant enzymes have such high activity that *exogenous* antioxidants are unlikely to lower steady-state ROS levels.[12] Second, antioxidant compounds react with ROS too slowly to prevent damage,[13] so even targeted antioxidants are unlikely to work on their own. Inhibiting ROS generation in the first place, by contrast, holds more promise.[34] But since ROS play important homeostatic roles throughout the body,[8,35] including cell signaling,[36] regulation of cerebral vascular caliber[37,38] and of metabolism,[39] circadian rhythm,[40] protein folding,[41,42] innate immunity,[34] and even cardioprotection in the case of Nox4,[43,44] the ideal approach will selectively block ROS generation in the reperfused myocardium without perturbing cell signaling elsewhere.

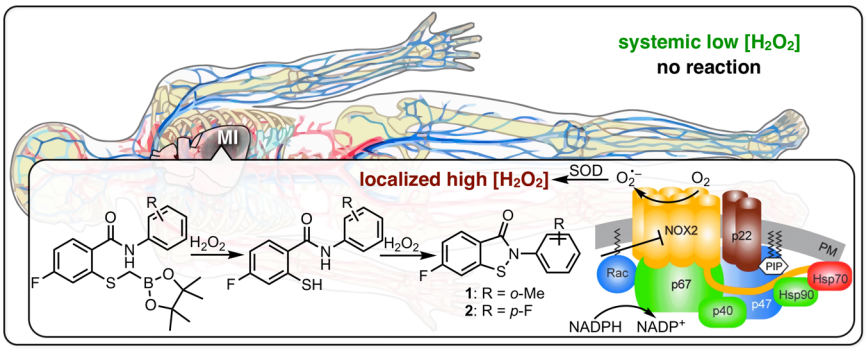


Fig. 1. Approach. High levels of H_2O_2 in the reperfused myocardium will activate the Nox2 inhibitor in a two-step sequence, consuming two equivalents of H_2O_2 in the process. This will turn off a major source of ROS that drives I/R injury. Nox2 complex adapted from Altenhöfer et al., 2015.[129]

To this end, I have selected **6a** based on known Nox2 inhibitor **THR101** (**1**)[45] as the starting point to generate my pilot data. **1** inhibits Nox2 with high potency (EC_{50} = 300 nM); its potency is 10-fold weaker for Nox1 and weaker still (>27-fold) for Nox4,[45] indicating favorable isoform specificity. Although some of the superoxide that Nox2 produces can react with nitric oxide to form peroxynitrite instead of dismutating to H_2O_2,[12] pathologic levels of H_2O_2 ($\geqslant$100 μM)[46–49] quickly build up in the reperfused myocardium[50] in a pattern that correlates tightly with the infarct.[51] In the reperfused myocardium, H_2O_2 reaches up to 51 ± 15 μM after thirty minutes of ischemia and increases to 100 ± 7 μM two minutes after reperfusion.[50] Outside the infarct, the nanomolar concentrations of H_2O_2 under physiological conditions[52–54] will not trigger compound activation[16] at concentrations sufficient for Nox inhibition.[45]

Our proposal builds on several reports of H_2O_2-triggered drug release using boronic esters,[55–58] including our group's.[59] Existing chemistries, however, generate toxic quinone methide[60,61] or acrolein[62] by-products, precluding clinical use. Our lab—collaborating with Paul Floreancig's—recently published a method for releasing several functional groups in response to cellular H_2O_2 without producing toxic by-products.[16] The 100 μM local H_2O_2 concentration found in reperfused infarcts[50] rapidly activates small molecules caged using our approach.[16,56] For this proposal, I expanded the scope to mask the thiol of a precursor to **1**. In injured tissues with damaging levels of H_2O_2, the boronic ester will be deprotected and the released thiol will oxidatively cyclize[63] to afford a benzoisothiazolone moiety that is essential for Nox2 inhibitory activity (**Fig. 1**).[45] Drug activation will consume two equivalents of H_2O_2, and the released inhibitor will block Nox2 to stop further production of tissue-damaging ROS. The proposed research is significant and supports the AHA's mission because it extends cutting-edge chemistry to develop a targeted therapy for I/R injury, arrhythmogenesis, and heart failure after MI.

PRELIMINARY STUDIES. I already completed the synthesis of the analog **6a** and expect to have **6b** in hand before the start of this fellowship, since I am only one step away from completing its synthesis (**Fig. 2**). I treated **6a** with H_2O_2 at low mM concentration and determined activation kinetics using NMR spectroscopy (**Fig. 3**).[16,56] Gratifyingly, complete conversion of **6a** to the cyclized product **7** was observed in <20 min. These preliminary results show that the proposed inhibitors activate rapidly

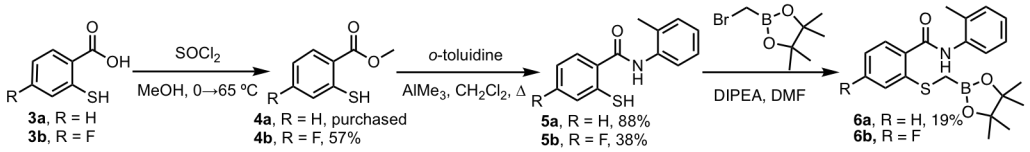


Fig. 2. Preparation of caged Nox2 inhibitor 6a and caged THR101 (6b). 6a was prepared in three steps as shown, and I am only one step away from completing the synthesis of **6b.**

in the presence of pathophysiologically-relevant levels of H_2O_2. We also prepared a latent fluorophore that senses H_2O_2 from cellular Nox2 using H_2O_2-induced boronic ester oxidation in a mechanism identical to the proposed inhibitors. We observed significant fluorophore activation[16] in HeLa cells stimulated with phorbol 12-myristate 13-acetate, a potent activator of Nox2,[64] (**Fig. 4**) giving us a probe to monitor Nox2-induced drug activation.

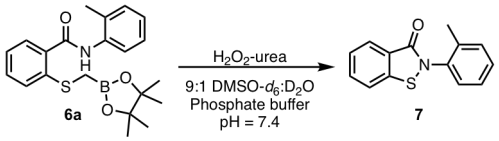


Fig. 3. Determination of activation kinetics of Nox2 inhibitor 6a. H_2O_2 treatment fully activated **6a** in <20 min (measured by ^{1}H-NMR).

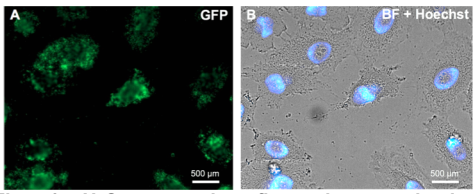


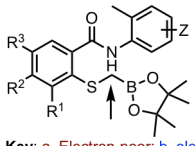
Fig. 4. H_2O_2-responsive fluorophore activation. HeLa cells pretreated with phorbol 12-myristate 13-acetate (PMA) (1 μM) for 60 min were incubated with boronic ester-caged coumarin (10 μM) for 60 min and Hoechst 33258 (2 μM) for 15 min, and were imaged (Zeiss Axio Observer Z1, 20x lens) using filters for GFP (ex, 470 nm; em, 525 nm) and Hoechst (ex, 377 nm; em, 464 nm). (**A**) GFP channel demonstrating fluorophore response to Nox2-mediated H_2O_2 production after treatment with PMA. (**B**) Merged brightfield and Hoechst channels indicating positions of cells and nuclei.

APPROACH.

Specific Aim 1: Develop H_2O_2-responsive Nox2 inhibitors and test them in cells. *Treating acute I/R injury requires a Nox2 inhibitor that rapidly responds to H_2O_2.* I will therefore optimize activation kinetics starting with the initial lead compound **6a**. Based on computational[65–67] and experimental evidence,[14,68] I will prepare and test several analogues designed to cyclize faster while maintaining inhibitory potency and isoform selectivity for Nox2 (**Fig. 5**), and I will test them in a cell culture model.

Experimental Design: I hypothesize that substitution near the thiol—which does not reduce potency[45]—will enhance cyclization kinetics through three potential mechanisms: improved transition state alignment, improved thiol solvation, or altered electronics of the thiol. As our synthesis (**Fig. 2**) is highly modular, different commercially-available or easily-prepared[69,70] analogues of **3a** will be used to vary the substitution here to test these hypotheses. To determine the relative importance of oxidation vs. cyclization, I will synthesize a negative control by replacing the amide nitrogen with a methylene group. Inhibitory potency will be verified by cell-free and whole-cell Nox2 activity assays[45,71] using COS-22 cells stably transfected with Nox2 and all subunits required for activity (COS-Nox2 cells).[72] I have worked with these cells extensively and performed both assays during my lab rotation with Dr. Pagano. After preparing the compounds (**Fig. 5**), I will determine activation kinetics by ^{1}H-NMR.[16,56,73] Spectra of compounds **6b**, **8**-**13**, and the negative control (20 mM) will be recorded in deuterated 9:1 DMSO:PBS after treating with 100 μM H_2O_2, as found in the reperfused myocardium.[50]

I will test the three most effective analogues in cell culture to determine the therapeutic potential of our approach. I/R injury will be modeled by subjecting rat cardiac myocytes,[74,75] which increase Nox2 activity in hypoxia-reoxygenation,[43] to this insult. To induce hypoxia/reoxygenation, we will use a system that produces rapid changes in oxygen tension to mimic real-life I/R injury.[76–78] Existing models change oxygen levels slowly and gradually by diffusion (**Fig. 6A**). In contrast, our apparatus, which our collaborator Dr. Weber first used to mimic the I/R injury of ischemic stroke, will achieve nearly instantaneous (<30 sec) changes in oxygen tension (**Fig. 6B**).[79]



6a (R^1, R^2, R^3 = H)
6b (R^1 = H, R^2 = F, R^3 = H)[a]
8 (R^1 = NH_2, R^2 = H, R^3 = H)[b,c]
9 (R^1 = OH, R^2 = H, R^3 = H)[b,c]
10 (R^1 = OCH_3, R^2 = H, R^3 = H)[b]
11 (R^1 = F, R^2 = H, R^3 = H)[a]
12 (R^1 = CF_3, R^2 = H, R^3 = H)[a]
13 (R^1 = H, R^2 = H, R^3 = OCH_3)[b]

Key: a, Electron-poor; b, electron-rich; c, improved solvation.
↑: Vary substitutents to increase water solubility and promote hemithioacetal breakdown.
Z: Vary to improve cyclization kinetics (alternative approach).

Fig. 5. Proposed analogues of caged THR101. Hypotheses: Cyclization kinetics may be improved by (1) improving transition state alignment of the thiol with an H_2O_2 oxygen atom by placing a hydrogen bond donor nearby[65] (compounds **8** and **9**), (2) improving thiol solvation in the bimolecular rate-determining step of the oxidation[66,67] (compounds **8** and **9**), and/or (3) modulating the electronics of the thiol. Lowering thiol pK_a via induction (compounds **6b**, **11**, and **12**) will promote ionization to the more reactive thiolate,[67] promoting sulfenic acid formation[14,68] and therefore cyclization. On the other hand, adding electron donors (compounds **8**, **9**, **10**, and **13**) will increase the thiol's nucleophility, which could accelerate the reaction.

We will use our system to measure three parameters: prodrug activation, ROS production, and cell survival/enzyme release. First, using our latent fluorophore that responds to H_2O_2 with a mechanism identical to that of the Nox2 inhibitors (**Fig. 4**),[16] I will quantify inhibitor activation in our hypoxia-reoxygenation model via fluorescence microscopy. Second, I will measure the effect of the fastest activated inhibitors on ROS levels in hypoxia-reoxygenation using cytochrome c[72,80,81] and Amplex Red assays.[80,82] Third, I will quantify survival using CellTiter Blue. As clinically-relevant markers of myocyte injury,[83,84] I will measure creatine kinase-MB and lactate dehydrogenase[85] release.

Anticipated Results, Possible Pitfalls, and Alternative Ap-

proaches: I anticipate that several proposed compounds will cyclize faster than **6a** and **6b**. If not, modifications to promote cyclization by altering the *N*-aryl system can be made without diminishing inhibitory potency.[45] For NMR and cell culture experiments, limited solubility may be remedied by using the corresponding boronic acid instead of the ester. If proposed pinacol boronic esters are unstable under biological conditions, more stable,[86–88] easily-synthesized[89,90] 6-membered ring systems will be prepared. As an alternative approach, a boronamide can be used in place of the boronic ester, which not only increases stability but allows for even greater spatial selectivity: boronamides hydrolyze under the acidic conditions[86] present in ischemic heart tissue,[91,92] thereby creating an "AND gate" requiring both the high H_2O_2 levels and low pH found in the ischemic myocardium for drug activation.

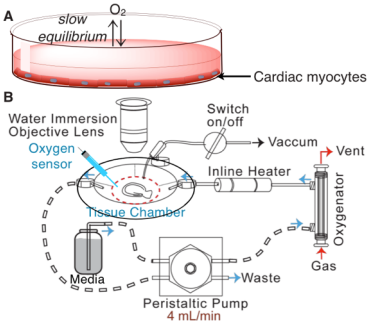


Fig. 6. Cell culture hypoxia-reoxygenation model. Primary cardiac myocytes will be isolated from Sprague Dawley rats.[130] Extrapolating from growth curves, 2 rats will be needed to obtain the 1 to 2 x 10^8 cells required for each experiment.[74] Existing hypoxia-reoxygenation co-culture models[131] (**A**) slowly change tissue oxygen tension by changing the gas composition in the incubator; the gas, media, and cells slowly equilibrate. Our model (**B**) takes the Weber group's system[79] and applies it to myocardial I/R. This system can change tissue oxygen tension from >700 mmHg to <20 mmHg in under 30 seconds. Image adapted from Yin et al. 2015.[79]

In the cell culture model, I anticipate rapid Nox2 inhibitor activation, decreased ROS production, improved survival, and decreased cardiac enzyme leakage in myocytes treated with the proposed inhibitors and subjected to hypoxia-reoxygenation. In the unlikely situation that the model fails to activate the inhibitors, COS-Nox2 cells[72] will be added via transwell inserts. These cells produce high levels of diffusible[18] H_2O_2 under hypoxia-reoxygenation conditions. In the unlikely scenario that the proposed Nox2 inhibitors—which are based on validated scaffolds[45,93]—fail to prevent cellular injury, I will prepare a new series of inhibitors based on **2** or other validated Nox1/2 inhibitors with μM potency.[71]

Specific Aim 2: I hypothesize that our approach to Nox2 inhibition will reduce infarct size without perturbing cell signaling in healthy tissues. I also hypothesize that our approach will to prevent hemodynamic compromise in the chronic setting of I/R injury (in which Nox2 drives much of the morbidity and mortality).[23]

Experimental Design: We will induce I/R injury by ligating (60 min) and reopening the left anterior descending (LAD) coronary artery in 10-12 week-old C57BL/6 mice. This well-validated surgical technique[43,94,95] merely adds a reperfusion step to the ligations routinely performed in the Dutta lab.[96–103] To control for the effects of surgery, we will perform sham operations comprising all steps except tightening the ligature.[94,104] The lead compound will be injected IV (tail vein) before reperfusion and will be dosed for high Nox2 inhibition (90%). For chronic treatment, the lead compound will be dosed for 50% Nox2 inhibition.

I will use the best compound identified in the cell culture experiments for the animal experiments. I will use a 2 $\times$ 2 design, randomizing mice into I/R injury vs. sham surgery and caged compound vs. vehicle treatment groups. To test efficacy in the acute setting, after 24 hours, infarct size and area-at-risk will be determined by triphenyltetrazolium chloride[100] and Alcian blue staining.[43] I will quantify apoptosis by TUNEL and will measure ROS generation in the AAR by the Amplex Red assay.[43] Power analysis based on infarct size/AAR observed in Nox2 knockout mice[43] and compensating for surgical variability from our institutional experience[105] shows that 15 mice are needed for each treatment group.

To localize inhibitor activation in the acute setting, I will trace activation using *in vivo* bioluminescence imaging and the aforementioned I/R injury protocol. I will use commercially available mice globally expressing firefly luciferase (Jackson, No. 025854), and to probe for areas of inhibitor activation, I will apply our caging chemistry[16] to luciferin to generate an H_2O_2-responsive luciferase substrate. Similar H_2O_2-responsive luciferase substrates are well-validated in mice.[106,107] I will use a 2 $\times$ 2 design (sham operation vs. I/R sugery and vehicle vs. probe). Power analysis based on the similar published probes' response to H_2O_2 in mice[106] and once again compensating for variability in surgical outcomes[105] shows that in each of the 4 treatment groups, 6 mice will be needed. Cellular uptake, tissue-selective activation, and pharmacokinetic parameters of the inhibitors will be verified by LC-MS.[108]

Next, I will quantify markers of off-target Nox2 inhibition in treated mice. Overuse of antioxi-

dants or underproduction of ROS have a characteristic transcriptomic signature:[109] (1) increased *Ankrd1*, a transcription factor upregulated in heart failure,[110] (2) decreased *Klf15*, a cardioprotective transcription regulator,[111] and (3) decreased *Wnk2*, a tumor-suppressing Ser/Thr kinase.[112] I will quantify all 3 transcripts in the myocardium by qRT-PCR;[109] *Wnk2* will also be quantified in the brain and pancreas given its high expression in those tissues.[113,114] Power analysis using published qRT-PCR data for all three genes[109] with compensation for variability in the surgical outcome[105] indicates that detecting *Klf15*—which has the smallest effect size and greatest variance—requires 11 animals per treatment group. I will use a 3 × 2 design (I/R or sham-operated × vehicle, caged compound, or active compound).

For a more comprehensive picture of selective Nox2 inhibition, I will quantify Erk2, Stat3, and Akt phosphorylation by Western blot. Myocardial Erk2 and Stat3 phosphorylation—which increase in Nox2 knockouts[115,116]—will be compared in injured and uninjured areas by Western blot. Additionally, I will measure pStat3 in the lungs, adipose tissue, and thymus, where it is highly expressed,[117] and pErk2 in the liver.[118] As a functionally significant readout, I will measure hippocampal Akt phosphorylation. Nox2 knockdown (and pharmacologic inhibition) reduces Akt phosphorylation in adult hippocampal stem/progenitor cells, resulting in impaired neurogenesis;[119] this may underlie the cognitive impairments found in Nox2-deficient mice[120] and adult humans.[26,119] I will quantify transcription and protein expression in organs from mice used for the initial animal experiment so that no additional mice will need to be sacrificed in the caged compound-I/R and vehicle-I/R treatment groups. Thus, I will need only an additional 44 mice to ensure that all 6 treatment groups have n = 11.

For the experiment in the chronic setting, I will randomize I/R-injured mice to four groups: vehicle, acute treatment (injection just before reperfusion), chronic treatment (as an additive to drinking water), and acute plus chronic treatment; vehicle/sham-operated mice will serve as a negative control. Hemodynamics will be evaluated by transthoracic echocardiography[121] at five time points (baseline, then 3, 7, 14, and 28 days) to approximate ejection fraction[122] and estimate wall thickness and volume.[105] After the final reading, I will record PV loops[123] and will assess cardiac fibrosis and inflammation histologically (Masson trichrome and hematoxylin-eosin (H&E) stains, respectively). Angiogenesis will be evaluated based on both H&E staining[124] and *in situ* hybridization using a VEGF-A RNA probe.[125]. Power analysis based on left ventricular (LV) dP/dt_{max} in Nox2 knockout mice[11] indicates that 6 animals per treatment group are needed. To compensate for attrition,[105,126] however, I will have 9 animals in each of the 4 groups.

Anticipated Results, Possible Pitfalls, and Alternative Approaches: In treated animals, I anticipate significantly decreased infarct size/AAR, apoptosis, and H_2O_2 production in the infarcted area. Further, I anticipate reduced off-target effects using our caging approach, and I anticipate long-term protection of cardiac function. If the surgeries produce too weak of an effect, the period of ischemia can be increased to 90 minutes.[95] For the bioluminescence experiments, if tissue penetration is insufficient, chemiluminescence will be red-shifted by using an aminoluciferin scaffold[107] and replacing the thiazole sulfur with selenium,[127] or by conjugation to Cy5 for BRET imaging.[107,128] I anticipate that hemodynamic parameters from PV catheterization will be ranked as follows: vehicle < acute treatment only < chronic treatment only < acute and chronic treatment. If mice refuse oral agents in the chronic treatment, the compounds will be administered using osmotic pumps, which I used during my first lab rotation. In the unlikely scenario that surgical difficulties arise in placing the PV conductance catheter via the carotid artery, the catheter will be placed by apical stab with an open chest approach.[123]

STATISTICAL ANALYSIS. Power calculations were performed in consultation with the University of Pittsburgh Statistical Consulting Center. The distribution of outcome readouts will be analyzed using histograms, q-q plots, and the Shapiro-Wilk test (if required). Data that appear fairly symmetrically distributed will be summarized as mean ± SEM and analyzed by ANOVA with Tukey's post-hoc test. If the underlying data distribution is highly skewed, data will be presented as median (range) and analyzed using the Kruskal-Wallis test with Dunn's test for pairwise comparisons. I will perform all analyses in GraphPad Prism.