RESEARCH STRATEGY

A. SIGNIFICANCE

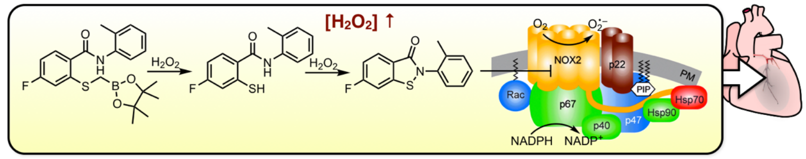


Fig. 1. Approach. A hydrogen peroxide (H_2O_2)-responsive prodrug will be synthesized. In the reperfused myocardium, which has high levels of H_2O_2, the drug will become activated in a two-step sequence, consuming two equivalents of H_2O_2 in the process. The active drug will inhibit Nox2 by perturbing subunit assembly and inhibiting translocation to the plasma membrane (Smith et al., 2012), turning off a major source of ROS production that drives I/R injury. In contrast, the prodrug will remain inactive unaffected tissues. Active Nox2 complex image adapted from Altenhöfer et al., 2015.[80]

Despite tremendous advances in treatment for myocardial infarction over the past few years,[1] mortality has held constant[19] at a rate approaching 10%,[20] and nearly one-fourth of survivors develop heart failure in the ensuing months.[6] Reperfusion, the standard-of-care, paradoxically induces an injury that accounts for up to 50% of infarct size[6] because rapid reintroduction of oxygen to ischemic tissue results in a burst of enzymatic overproduction of harmful reactive oxygen species (ROS).[7,8] The resulting ROS can oxidize proteins, lipids, carbohydrates, and nucleic acids, resulting in irreversible functional losses.[9] Although several enzymes[21] (and the mitochondrial electron transport chain)[22] produce ROS as a by-product, the NADPH oxidase (Nox) family comprises the only enzymes that produce ROS as the primary function,[23,24] implicating Nox proteins as key targets to reduce ROS-mediated tissue damage in ischemia-reperfusion (I/R) injury.

The Nox2 isoform in particular is a key player in I/R injury. At the moment of reperfusion, high Nox2 activity in humans increases the risk that the coronary microvasculature remains obstructed (the "no-reflow" phenomenon)[25,26] by promoting a thromboinflammatory state.[27] Afterwards, Nox2 produces high, sustained levels of ROS for several hours post-reperfusion,[17] leading to further oxidative damage.[28] When Nox2 is knocked out, less vascular oxidative stress occurs in restenosis models,[29] and pharmacologic Nox inhibitors reduce oxidative stress in models of vascular disease.[30] Further, humans with loss-of-function mutations in Nox2 are protected from I/R-induced endothelial dysfunction that contributes to myocardial death.[31] Taken together, these findings suggest that Nox2 is an attractive target for inhibition to ameliorate acute myocardial I/R injury in patients.

Not only does Nox2 drive acute I/R injury, it also drives the chronic injury in the ensuing weeks. Persistent ROS generation from Nox2 in cardiac myocytes causes chronic remodeling that compromises ventricular function and contributes to arrhythmias after MIs.[10–12] Arrhythmias and heart failure in the months following an MI contribute to much of the morbidity and mortality.[32] Current therapeutics for heart failure implicate Nox in the pathophysiology. Angiotensin-converting enzyme (ACE) inhibitors decrease Nox activity in in the vasculature,[33] which may underlie their benefits to heart failure patients.

Clinicians need effective treatments that reduce ROS levels after MI, but no therapies for I/R injury have been successfully translated to the clinic.[15] Although many antioxidant therapies have proven successful in animals, results in humans have been disappointing.[15] Why have these therapies failed? Exogenous antioxidants may reduce ROS levels too slowly imprecisely for clinical benefit. Endogenous antioxidant enzymes (superoxide dismutases, catalase, peroxidases, and peroxiredoxins) have such high activity levels that exogenous antioxidants are unlikely to lower steady-state ROS levels.[14] Further, antioxidant compounds react with ROS too slowly to prevent damage,[15] so even targeted antioxidants[34–36] are not likely to yield clinical benefit on their own. Inhibiting ROS generation in the first place, on the other hand, will likely prove more effective.[37] But since ROS play important homeostatic roles throughout the body,[9] including cell signaling,[38] regulation of cerebral vascular caliber,[39,40] regulation of metabolism,[41] and innate immunity,[37] *an approach that selectively blocks ROS generation in the reperfused myocardium without perturbing cell signaling elsewhere is needed.* We are developing a targeted approach to treating myocardial reperfusion injury by synthesizing H_2O_2-triggered small molecule inhibitors of Nox2, a ROS producer that drives I/R injury both acutely[17,29,5] and in the chronic phase.[10,11]

Existing Nox inhibitors suffer from nonspecificity, off-target effects, and problems in drug delivery.[42] Since Nox enzymes play important homeostatic roles throughout the body, and since ROS from Nox4 may be cardioprotective in I/R injury[43,44], the ideal Nox inhibitor should be isoform-specific for Nox2. Peptidic Nox inhibitors, such as Nox2ds-tat,[45,46] improve isoform specificity, but suffer from poor ADME (absorption, distribution, metabolism, and excretion) characteristics that hamper their clinical use,[47] particularly for a treatment lasting several weeks post-MI. Thus, ideal therapeutics would be small molecules that can be site-specifically activated in injured tissue with isoform specificity that excludes Nox4. **THR101** is a promising starting point. **THR101** inhibits Nox2 with sub-micromolar potency (EC_{50} = 300 nm); its potency is 10-fold lower for Nox1 and >27-fold lower for Nox4,[48] indicating favorable isoform specificity. **THR101** and analogues, however, inhibit off target enzymes (PHOSPHO1[49] and phosphomannose isomerase[50]) with μM potency, so keeping activation inside injured tissues takes on added importance. **THR101** will serve as the starting point for our approach.

The approach proposed here will selectively target tissues with pathologic levels of H_2O_2 (>100 μM),[51–54] as is found in reperfused myocardium[55] in a pattern that correlates well with the infarct.[56] Away from this area, the nanomolar concentrations of H_2O_2 present under physiological conditions[57] will not trigger Nox inhibition. We will achieve specificity by developing a chemical functionality responsive to H_2O_2 at pathologic levels. H_2O_2 will release the Nox2 inhibitor. Additionally, the H_2O_2-sensitive functionality will consume two equivalents of harmful H_2O_2 in the process, and will release only non-toxic by-products. The resulting locally- and temporally-defined Nox inhibition will greatly enhance the therapeutic window of existing inhibitors.

Our proposal builds on several reports[58–61] that use boronates to trigger drug release in the presence of H_2O_2, including a report from our group.[62] Existing chemistries, however, generate toxic quinone methide[63,64] or acrolein[65] by-products, precluding clinical use. Our lab—in collaboration with the Floreancig lab—recently developed a facile method for preparing boron-masked functionalities that react rapidly with H_2O_2 in oxidatively-stressed cells to release alcohols, aldehydes, and ketones without producing toxic by-products.[59]

In this proposal, we will expand the scope of our approach to mask the thiol of **THR101**.[48] SAR studies have demonstrated that **THR101** requires divalent sulfur to be in a benzisothiazolone motif for inhibitory activity;[48] hence, we will cage the sulfur with a boronate ester to prepare a prodrug that will be deprotected and oxidatively cyclized[66] in injured tissues accumulating harmful levels of H_2O_2. Prodrug activation will consume two equivalents of H_2O_2 and the released inhibitor will block Nox2 activity, thereby preventing any further production of tissue-damaging ROS in I/R injury. **The proposed research is significant because it extends cutting-edge chemistry to develop a targeted therapy for I/R injury, arrhythmogenesis, and heart failure after MI**.

B. APPROACH

B.1. Preliminary Results

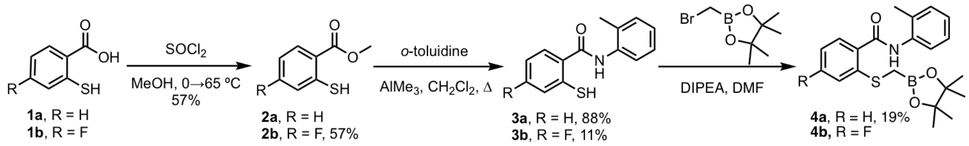


Fig. 2. Preparation of model compound 4a and caged THR101 (4b). Esterification of commercially-available carboxylic acid **1a** with thionyl chloride in the presence of methanol affords methyl ester **2b**. Treatment of commercially-available **2a** and **2b** with *o*-toluidine in the presence of trimethylaluminum generates amides **4a** and **4b**. Finally, treatment of **3b** with $BrCH_2Bpin$ in the presence of DIPEA affords model compound **4a**. A similar sequence is used to prepare caged **THR101** (**4b**).

B.1.1. Synthesis and testing of model compound. H_2O_2-responsive **THR101** analogue **4a** was prepared from commercially-available starting materials (**Fig. 2**). Next, model compound **4a** was treated with low millimolar concentrations of H_2O_2, and cyclization kinetics were determined using nuclear magnetic resonance spectroscopy (NMR, **Fig. 3**).[59,67] NMR showed complete consumption of starting material to form cyclized product **5** in under 20 minutes, presumably through a hemithioacetal intermediate. This assay is still under development, and the yield of **5** has yet to be determined. **5** is the predominant product, however, and as the intermediate hemithioacetal was not observed, it is not so stable as to preclude prodrug activation. These preliminary results suggest that the proposed inhibitors are likely to become activated rapidly in the presence of pathophysiologically-relevant levels of H_2O_2.

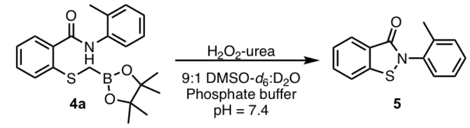


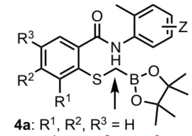
Fig. 3. Determination of activation kinetics of a model compound. Boronate ester **1** was treated with H_2O_2, and the activation of the compound was monitored by ^{1}H-NMR. Full activation took <20 min.

B.1.2. Synthesis of the caged analogue of published Nox2 inhibitor THR101. With the model compound in-hand, further work has been directed to the preparation of caged **THR101** (**4b**) (**Fig. 2**), which is nearly complete. During the first reaction in this sequence, the thiol **2b** and its corresponding disulfide were formed in a 42:58 ratio, suggesting that fluorination *meta* to the thiol substantially increases the propensity of these compounds to oxidize. Consequently, it is likely that **4b** and analogues are likely to oxidatively cyclize even faster than **4a**, making them more useful treating the acute reperfusion injury induced by percutaneous intervention.[68]

B.2. Specific Aim 1: Development and cell-based testing of H_2O_2-responsive Nox2 inhibitors.

B.2.1. Sub-Aim 1A: Synthesize a series of boronate-caged benzoisothiazolone Nox2 inhibitors. *Rationale*: To treat I/R injury in the acute setting, we need a Nox2 inhibitor that rapidly responds to H_2O_2. The initial lead compound **4b** may not have optimized solubility and cyclization kinetics. The first step of activation—oxidation of the boronate ester to the hemithioacetal—proceeds to completion in under 5 minutes for a variety of substrates,[67] consuming one equivalent of H_2O_2 in the process. This step was similarly rapid for model compound **4a** (see **C.1** and **Fig. 3**). By contrast, the second step, oxidative cyclization to the active Nox inhibitor, takes 90 minutes rapidly at room temperature in the literature.[66] Although this is likely to proceed nearly four times as fast at body temperature, this may be too slow for treating acute I/R injury. Since rapid activation kinetics are essential for use in the acute setting, we will explore several analogues likely to cyclize even faster while maintaining inhibitory potency and isoform selectivity for Nox2.

Experimental Design: We hypothesize that substitution near to the thiol—positions highly tolerant of substitution without diminishing potency[48]—could enhance cyclization kinetics through 3 potential mechanisms. As our synthesis (**Fig. 2**) is highly modular, different commercially-available or easily-prepared[69,70] analogues of **1a** may be used to vary the substitution here to test the three hypotheses. First, improved transition state alignment of the thiol with an H_2O_2 oxygen atom can enhance the rate by several orders of magnitude.[71] Thus, positioning a hydrogen bond donor nearby to stabilize H_2O_2, as in proposed compounds **6** and **7**, is likely to improve cyclization kinetics (**Fig. 4**). Second, theoretical calculations show that thiol solvation plays a key role in the bimolecular rate-determining step of the oxidation.[72,73] Consequently, adding hydrophilic substituents to promote solvation (as in compounds **6** and **7**) is likely to increase the rate of cyclization. Third, modulating the electronics of the thiol could improve reactivity. Lowering the pK_a via the inductive effect, as in compounds **4b**, **9**, and **10**, will promote ionization to the more reactive thiolate,[73] which promotes sulfenic acid formation[74,16] and therefore cyclization. On the other hand, addition of electron donors to the pi system, as in **6**, **7**, **8**, and **11**, is will increase the nucleophility of the thiol, which could speed up its reaction with H_2O_2. A control compound with a methylene group replacing the amide nitrogen will be prepared to establish the role of cyclization.



4a: R^1, R^2, R^3 = H
4b: R^1 = H, R^2 = F, R^3 = H
6: R^1 = NH_2, R^2 = H, R^3 = H
7: R^1 = OH, R^2 = H, R^3 = H
8: R^1 = OCH_3, R^2 = H, R^3 = H
9: R^1 = F, R^2 = H, R^3 = H
10: R^1 = CF_3, R^2 = H, R^3 = H
11: R^1 = H, R^2 = H, R^3 = OCH_3
Key: Electron-poor; electron-rich; improved solvation.
: Vary substitutents to increase water solubility and promote hemithioacetal breakdown.
Z: Vary to improve cyclization kinetics (alternative approach).

Fig. 4. Proposed analogues of caged THR101.

Anticipated Results, Possible Pitfalls, and Alternative Approach: We anticipate that several compounds in this series will have enhanced cyclization kinetics over model compound **4a** and parent compound **4b**, though it is difficult to speculate about which of the three competing theoretical hypotheses will be empirically correct. In the unlikely possibility that none of the proposed modifications enhances the kinetics, modifications to promote nucleophilic attack of the amide nitrogen on the sulfur atom by altering the attached ring can be made without diminishing inhibitory potency.[48] A pitfall so far has been the low yield obtained for coupling ester **2b** to *o*-toluidine to afford amide **3b**. Alternative conditions have been validated for this transformation in the literature.[75,76]

B.2.2. Sub-Aim 1B: Determine the kinetics of decaging and activation of the caged inhibitors. *Rationale:* To select the best compound for biological testing, we will quantify the kinetics and yield of the activation of the caged Nox2 inhibitors synthesized in Sub-Aim 1A. The resulting data will illustrate the relationship between the electronics of the thiol and the rate of oxidative cyclization, providing information about the hotly-debated[71,77] and biologically-important[16] mechanism by which H_2O_2 oxidizes thiols.

Experimental Design: Kinetics will be determined using NMR as in **B.1.1**.[59,67,78] ^1H NMR spectra will be acquired every 32 scans (every 3'24"). Compounds **4b**, **6-11**, and the negative control compound will be dissolved in 9:1 DMSO-d_6:D_2O with pH 7.4 phosphate buffer. After acquiring a baseline, repeated measurements will begin after adding H_2O_2. Final concentrations will be 20 mM for the compounds being tested and 100 μM for H_2O_2, as is found in the reperfused myocardium.[55] We have observed significant downfield shifts in the aryl hydrogens upon cyclization, making it easy to measure Nox inhibitor activation. Activation will be confirmed using ^{11}B NMR.[67]

Anticipated Results, Possible Pitfalls, and Alternative Approach: We expect that some of the proposed inhibitors **6-11** will cyclize faster in with greater yields than parent compound **4b**, though it is difficult to predict which hypothetical mechanism of rate enhancement will prevail (see **B.2.2**). The pitfall of limited aqueous solubility became evident during preliminary NMR experiments (see **B.1.1**), which necessitated 90% DMSO to dissolve the model compound **4a**. To get around this limitation, we envision two approaches. First, heating the sample and spectroscope bore to 37 ºC is likely to improve solubility (and is also more physiologically relevant than room temperature). Second, we predict that adding a polar ammonium or sulfonate group on a short alkyl chain at the α-boryl methylene position will enhance water solubility (**Fig. 4**, arrow). This modification is likely to increase the rate of hemiacetal collapse (and, consequently, the rate of Nox2 inhibitor activation), improving the compound for use in the acute setting.

B.2.3. Sub-Aim 1C: Quantify inhibitor activation, ROS production, and cell survival/cardiac enzyme release upon treatment with caged inhibitors. *Rationale*: These cell culture experiments are crucial next steps in determining the spatial control and therapeutic potential of our approach. I/R injury may be modeled by subjecting cells to hypoxia-reoxygenation. Hypoxia-reoxygenation increases Nox2's ROS-generating activity in cultured myocytes,[43] making this a good model for I/R injury. Further, Nox2 knockdown has been shown to increase cell survival during I/R; by contrast, knockdown of both Nox2 and Nox4 *decreases* survival,[43,44] underscoring the need for a targeted Nox inhibitor that is selective for Nox2 and activated only in tissues exposed to pathologic levels of H_2O_2.[47,79,80]

Cultured cardiac myocytes[81,82] and endothelial cells (ECs)[83] are useful, well-validated models for studying pathophysiology. ECs, which are key players in reperfusion injury, express high levels of Nox2[84] that generates a great deal of diffusible H_2O_2 in reperfusion injury.[55] As both ECs and myocytes are involved in the Nox2-driven component of cardiac reperfusion injury, a co-culture of ECs and cardiac myocytes[85] will provide a sophisticated and relevant cell culture model for testing our approach (**Fig. 5A**).

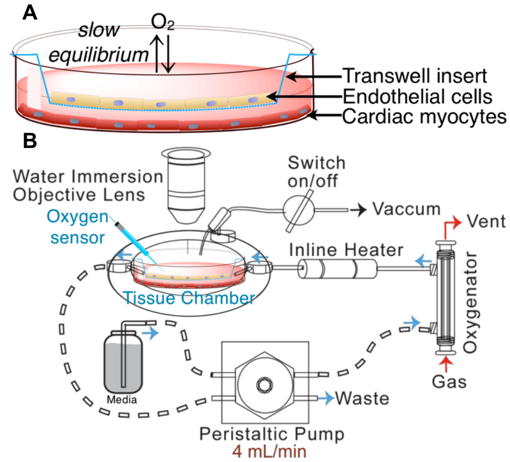


Fig. 5. Proposed hypoxia-reoxygenation model. Primary cardiac myocytes will be isolated from Sprague Dawley rats.[86] Extrapolating from growth curves, 2 rats will be needed to obtain the 1 to 2 x 10^8 cells required for each experiment.[81] Coronary artery ECs will be obtained from American Type Culture Collection (ATCC PCS-100-020). Published hypoxia-reoxygenation co-culture models[85] (**A**) change tissue oxygen tension by changing the gas composition in the incubator, resulting in gradual equilibration between the gas, culture medium, and cells that slowly changes oxygen tension, in contrast to rapid changes in real-life I/R. Our proposed model (**B**) takes the Weber group's recent system for modeling I/R injury in stroke[89] and applies it to myocardial I/R. This system can change tissue oxygen tension from >700 mmHg to <20 mmHg in under 30 seconds, recapitulating real-life I/R with unprecedented accuracy[89]. This system pumps media through a microfluidic oxygenator. The media is warmed and gently superfused over cultured tissue, and its oxygen level is constantly monitored. Vacuum aspiration can rapidly remove media to allow for rapid changes in oxygen tension. Mindful that shear stress from short-term fluid flow can activate endothelial Nox2,[114] we will minimize flow rates over the endothelial cell layer during steady-state perfusion to reduce this source of Nox2 activation that is extraneous to the I/R injury model. Image adapted from Yin et al. 2015.

Typical tissue culture hypoxia-reoxygenation models do not recapitulate the rapid changes in tissue oxygen tension that occur in myocardial I/R. The ischemic injury results from cause sudden obstructions to coronary blood flow due to vasospasms, hemorrhage into plaques, and/or luminal thrombi,[87,88] resulting in acute myocardial oxygen deprivation.[68] Reperfusion injury results when PCI rapidly restores oxygen by physically recanalizing the stenotic vessel. Unlike real-life I/R injury, existing hypoxia-reoxygenation models change oxygen levels slowly and gradually (**Fig. 5A**). We propose taking the ischemic stroke I/R injury apparatus from our collaboration with Dr. Weber and applying it to myocardial I/R (**Fig. 5B**). This setup changes tissue oxygen levels nearly instantaneously.[89] *We hypothesize that our proposed inhibitors will activate specifically in cultured cells exposed to hypoxia-reperfusion, where they will downregulate ROS production, improve cell survival, and decrease cardiac enzyme release*

Experimental Design: Three experiments will be run using our EC-cardiac myocyte co-culture system: we will quantify prodrug activation, ROS production, and cell survival/cardiac enzyme release upon treatment with caged inhibitors. Our group recently published a fluorophore that becomes activated in cells stimulated to produce ROS via Nox2 (**Fig. 6**).[67] The fluorophore is activated by H_2O_2-induced boronate oxidation in a mechanism identical to proposed Nox2 inhibitors. First, we will quantify inhibitor activation in our rapid hypoxia-reoxygenation model by using confocal microscopy and quantifying signal from the fluorescent probe. Second, we will measure the extent to which **6** (or the best inhibitor identified in Sub-Aim 1B) lowers ROS production in hypoxia-reoxygenation. Superoxide levels will be measured using the SOD-inhibitible cytochrome c assay[45,46,90] and H_2O_2 levels with Amplex Red.[90,91] Third, we will evaluate our inhibitors' abilities to protect cardiac myocytes from injury. We will quantify survival using CellTiter Blue. As an additional clinically-relevant marker of myocyte injury,[92,93] we will measure the amount of creatine kinase-MB and lactate dehydrogenase[94] that dying cells release. We will test positive control compound **5** to gauge the importance of the boronate cage.

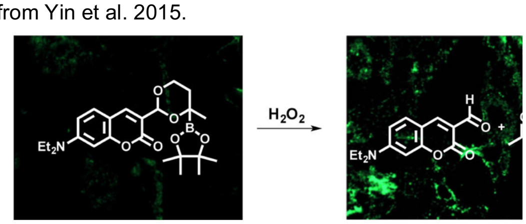


Fig. 6. H_2O_2-responsive fluorophore activation. This fluorophore responds to H_2O_2 generated by HeLa cells treated with phorbol 12-myristate 13-acetate (PMA), a well-known activator of Nox2. Image adapted from Hanna et al. 2016.

Anticipated Results, Potential Pitfalls, and Alternative Approach: We anticipate rapid Nox2 inhibitor activation, decreased ROS production, improved survival, and decreased cardiac enzyme leakage in cells treated with the proposed inhibitors and subjected to hypoxia-reoxygenation. As an alternative approach, in the unlikely situation that our cell culture model does not induce inhibitor activation, a well-validated line of COS-22 cells stably transfected with Nox2 and all subunits required for activity is available from Dr. Pagano.[46] These cells may be used in place of ECs, as they produce high levels of diffusible[23] H_2O_2 in response to hypoxia-reoxygenation. They also are expected to have a lesser response to shear stress from fluid flow than ECs. As another pitfall, if the laser in confocal microscopy is found to induce excessive cellular damage, one of the two spinning disk confocal microscopes at the Center for Biologic Imaging will be used instead.

B.3. Specific Aim 2: Evaluate caged Nox2 inhibitors in preventing acute injury and chronic dysfunction via targeting injured tissue in a rat model of myocardial I/R injury.

B.3.1. Sub-Aim 2A: Measure Nox2 activity, infarct size, and apoptosis in the ischemic area in a rat model of myocardial I/R. *Rationale*: First, we will test our inhibitors in the acute setting. Since Nox2 has been shown to drive substantial ROS production in the hours after I/R,[17,29] and since Nox2 knockout has been shown to decrease infarct size in I/R injury,[31,43] we hypothesize that our targeted approach to Nox2 inhibition will reduce infarct size in a rat model of I/R injury.

Experimental Design: We will induce I/R injury by ligating and later reopening the left anterior descending (LAD) coronary artery. Ligating the LAD produces the largest infarcts in animal models,[95] is easiest to reproduce,[96] and is the most common site of occlusion in humans.[97] We will carry through the best compound from Sub-Aim 1B and use it for this experiment as well. Based on pharmacokinetic data for the related compound **Jm-77c**[50] and potency data for **THR101**,[48] our lead compound will be dosed at 71.3 mg/kg to achieve a high level of Nox2 inhibition (90%). The compound will be administered in a way that mimics our approach's application to PCI in humans: we will inject the compound (or saline) into the tail vein immediately before reperfusion.

Myocardial I/R will be modeled using a well-validated surgical technique,[43,96,98] which merely adds a reperfusion step to the ligation procedures routinely carried out in the Wang lab.[99–102] Briefly, a left thoracotomy will be made at the level of the fourth intercostal space, and the pericardium will be gently pulled apart. Under a dissecting microscope, polypropylene suture will be passed under the LAD 2 mm distal to the tip of the left auricle. A loose double knot will be tied, through which a short length of PE-10 tubing will be placed, and the loop will be secured with a slipknot. Occlusion will be verified by confirming pallor in the LAD's territory. Ischemia will be maintained for 60 minutes. Afterwards, the knot will be untied and the tubing removed. The suture will be left in place for determination of infarct size and area-at-risk (AAR) by triphenyltetrazolium chloride (TTC) and Alcian blue staining.[103] After 24 hours of reperfusion, the animals will be reanesthetized and the chest reopened. Analysis of the infarct will be completed after intracardiac injection of potassium chloride and excision of the heart.

We will measure infarct size and area-at-risk (AAR) using triphenyltetrazolium chloride (TTC) and Alcian blue staining. In the ischemic area, the extent of apoptosis will be determined using a Western blot for cleaved Caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).[43] Lastly, efficacy of the inhibitor in reducing ROS generation in the AAR will be determined by measuring H_2O_2 levels in homogenized tissue using Amplex Red.[43] These readouts will be compared for rats injected with the compound versus with saline. We performed a power analysis to determine the minimal number of rats necessary to detect an effect. Assuming an effect size and variance for infarct size/AAR consistent with Nox2 knockout,[43] finding a significant difference with $\alpha = 0.05$ powered at 80% necessitates a sample size of 11 animals per treatment group.

Anticipated Results, Possible Pitfalls, and Alternative Approach: We anticipate significant reductions in infarct size/AAR, apoptosis, and H_2O_2 production in the infarcted area comparing animals treated with the caged inhibitor to vehicle-treated animals. In the event that the reperfusion period is suboptimal, timing can be adjusted to 90 minutes of ischemia with >1 hour of reperfusion, which is well-validated for this method.[98]

B.3.2. Sub-Aim 2B: Assess specificity of caged Nox2 inhibitor activation for the injured area. *Rationale*: Due to the important homeostatic roles that ROS from Nox2 play throughout the body[9] and the potential off-target effects of **THR101** analogues,[49,50] it is essential that our approach inhibits Nox2 in tissues accruing pathologic levels of H_2O_2. To determine our approach's selectivity for such tissues, we will compare inhibitor activation and markers of excessive Nox2 inhibition between the infarcted myocardium and unaffected areas of the heart and other organs.

Experimental Design: First, to determine the spatial distribution of inhibitor activation, we will administer our published H_2O_2-responsive fluorophore (**Fig. 6**)[67] to rats subjected to myocardial I/R. The organs will be removed, treated with catalase to prevent further fluorophore activation, subjected to 3 freeze-thaw cycles with liquid nitrogen, lysed, and homogenized.[101] For the heart, the infarct and the uninjured areas will be carefully dissected and treated separately. Fluorophore activation in the organ homogenate will be measured using a fluorimeter. Fluorescence will be compared between animals treated with the caged and uncaged (active) fluorophore, and between animals subjected to I/R injury and sham-operated controls. We used power analysis to calculate the minimum number of animals needed ($\alpha = 0.05$, $1-\beta = 0.8$) based on the compound's response to H_2O_2 generated by PMA-stimulated cells.[67] In each of the 4 treatment groups, 2 animals are necessary.

Second, we will quantify markers of off-target Nox2 inhibition in animals treated with our lead compound and subjected to I/R injury. In previous studies, Nox2 knockout was found to perturb cell signaling by increasing Erk2 and Stat3 phosphorylation in the myocardium.[31,104] This illustrates an important role of Nox2 in regulating key cell signaling pathways.[105] Erk2 and Stat3 phosphorylation will be probed using Western blot, comparing the injured and uninjured portions of the myocardium. Additionally, pStat3 will be measured in the lungs, adipose tissue, and thymus *ex vivo*, where it is highly expressed,[106] and pErk2 will be measured in the liver, where it is highly expressed.[107] Erk3 and Stat2 phosphorylation will be compared in animals treated with the caged compound, the corresponding active compound, or saline, across I/R and sham-operated treatment groups. Once again, we performed a power analysis to determine the necessary sample size. Using literature values for Erk2 phosphorylation in a Nox2 knockout mouse,[31] 5 animals will be needed for each of the 6 treatment groups.

Anticipated Results, Possible Pitfalls, and Alternative Approach: We anticipate spatially-controlled inhibitor activation specifically in the area of infarction. Additionally, we anticipate that the active compound will significantly increase off-target Erk2 and Stat3 phosphorylation compared to the caged prodrug and to the saline control. Although our caged fluorophore has proven to be quite sensitive to ROS generated in cell culture,[67] two pitfalls may arise for its use in live animals. First, the fluorophore is not particularly bright (brightness ≈ 10^4 M^{-1} cm^{-1}),[108] so the resulting signal may fall below the limit of quantification. Second, the activated fluorophore is small and lipophilic, so it may diffuse between tissue compartments in animals, compromising our ability to detect tissue-specific activation. As an alternative approach, caged fluorescein (**Fig. 7**) could be used instead as a facile application of our phenol-caging chemistry.[67] Fluorescein is significantly brighter,[108] can be detected with commercially-available antibodies, and will stay in cells after decaging,[109] solving these potential problems.

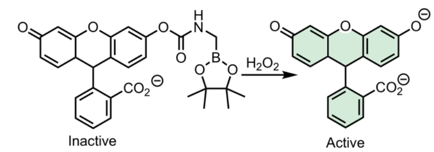


Fig. 7. Alternative H_2O_2 fluorophore strategy. Here fluorescein is caged with a self-immolative spacer.[93] Oxidation will release the phenol, which is an efficient fluorophore (brightness ≈ 10^5 M^{-1} cm^{-1}).[108] Anti-fluorescein antibodies are available commercially for sensitive quantification by Western blot or ELISA. The decaged fluorophore is a large and highly charged molecule which is likely to remain trapped in cells.[109]

B.3.3 Sub-Aim 2C: Assess cardiac function 1 month post-I/R injury in treated rats. *Rationale*: Nox2 drives the chronic sequelae of I/R injury[10–12] responsible for much of the morbidity and mortality.[32] Thus, our approach's potential to preserve cardiac function in the weeks following I/R injury is of significant public health importance. We hypothesize that caged Nox2 inhibitors will lessen the extent of cardiac dysfunction secondary to I/R injury.

Experimental Design: Because our proposed inhibitors are likely to be bioavailable orally,[50] which is preferable for a long-term treatment, we will treat mice post-I/R with caged Nox2 inhibitors by mouth (PO). We will divide animals into 5 groups: saline, treated acutely (tail vein injection at the time of reperfusion), treated chronically (PO after reperfusion), treated acutely and chronically, and aline/sham-operated (stopping after opening the pericardium). For 50% inhibition with a bioavailability of 35%,[49] our lead compound will be dosed at 36 mg/kg.

Transthoracic echocardiography provides a reproducible, noninvasive method for measuring clinically relevant hemodynamic parameters in the rat.[110] To this end, echocardiographic readings will be taken for all rats in the experiment at five time-points (pre-I/R baseline, 3 days, 7 days, 14 days, and 28 days). These readings will be used to calculate fractional area change (FAC),[102] which closely approximates ejection fraction, an important parameter for monitoring the progression of heart failure.[111] After the final echocardiographic reading, a pressure-volume (PV) catheter will be placed. Catheterization with a pressure-volume conductance catheter is a well-established method widely considered to be the gold standard for measuring cardiac function in live animals.[112] Once again, power analysis ($\alpha = 0.05$, $1-\beta = 0.8$) was carried out to determine the number of minimum animals required. Based on literature values for left ventricular (LV) dP/dt_{max} (normalized to end-diastolic volume, EDV) in Nox2 knockout mice,[12] 9 animals per treatment group are needed. To compensate for attrition, however, based on our past experience,[102] we will have 13 animals in each of the 5 treatment groups.

Anticipated Results, Potential Pitfalls, and Alternative Approach: We anticipate that FAC values and hemodynamic parameters from PV catheterization will be ranked as follows: saline < acute treatment only < chronic treatment only < acute and chronic treatment ≈ untreated/sham-operated. In the rare scenario that technical issues preclude the recording of PV loops, we will obtain hemodynamics measurements using the Langendorff preparation instead. The Langendorff (isolated perfused) heart is well-validated model with over a century of use,[113] and the VMI uses this technique extensively. We will measure measure left ventricle end diastolic pressure (LVEDP) and ventricular contractility (dP/dt_{max})[43] while obtaining electrocardiographic recordings directly from the epicardium so that mechanical and electrical cardiac function may be coordinated.[113]