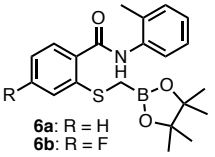
RESUBMISSION MODIFICATIONS

This AHA Predoctoral Fellowship application was previously reviewed in 2018 and received significant enthusiasm, though reviewers also pointed out several weaknesses. I would like to thank the reviewers for their careful evaluation and insightful critiques. These valuable comments helped me to strengthen the proposal. I have made several revisions to the proposal to address all reviewer comments.

1. *Reviewer 2 asked about the persistence of inhibitor activation after Nox2 inhibition, and the extent of drug activation*: continued drug activation will occur at the infarct as long as pathologic H_2O_2 levels arise from continued Nox2 expression, infiltrating leukocytes, and XO activity.[1] The approach I have developed is a "smart" system that consumes harmful H_2O_2 to release a functional Nox2 inhibitor *when* and *where* Nox2 is hyperactive. Thus, this approach shuts off the major driver of I/R injury without perturbing physiologic Nox2 activity in uninjured tissues. Dosing was calculated to maintain a reserve of caged inhibitor that will respond to evolving, pathologic Nox2 activity in the injured area.

2. *Reviewer 3 asked how dosing for* in vivo *experiments will be determined*: Dosing will be initially based on published potency data for the active form of the lead compound[2] and PK/PD data for analogue **2**.[3] For treatment of acute heart attacks, the compound will be dosed at 71 mg kg^{-1} for high level Nox2 inhibition (90%). For the long-term experiment in the chronic setting, literature values for 50% inhibition with 35% bioavailability indicate 36 mg kg^{-1} day^{-1} as a starting point. We will adjust the dose using in-house PK data generated from the University of Pittsburgh Cancer Institute (UPCI) Cancer Pharmacokinetics and Pharmacodynamics Facility (Dr. Deiters holds an appointment in the UPCI).

3. *Reviewers 1 and 3 expressed concerns that the caged Nox2 inhibitors were not yet prepared and that the completion of Aim 2 depends on the development of these inhibitors in Aim 1.* I have now completed the synthesis of Nox2 inhibitor pro-drugs **6a** and **6b**. Furthermore, reaction yields of these syntheses have increased substantially since the initial submission, due to my increasing expertise in handling easily-oxidized compounds (I work with phosphines in other projects, which are easily oxidized, like the thiol intermediates in this proposal). Having lead compound **6b** and analogue **6a** in-hand shows that the reaction conditions are robust, so no synthetic difficulties are expected in preparing other analogues in the proposed panel. Further, successful deprotection and cyclization of both compounds to the corresponding Nox2 inhibitors under physiologically-relevant conditions has been confirmed by NMR spectroscopy and mass spectrometry, validating the feasibility of the overall strategy.



4. *Reviewer 2 noted that my initial submission ignored other sources of ROS, including mitochondrial respiration, xanthine oxidase (XO), and nitric oxide synthase (NOS) uncoupling.* While mitochondria release significant ROS in the first few minutes of ischemia,[4] Nox2's ROS production begins immediately[5] and creates high, sustained levels of ROS for several hours after reperfusion.[4] Cellular mitochondrial ROS production may be overestimated in the literature due to reliance on data from isolated mitochondria,[6] and it is difficult to target this source without compromising cell health.[7] Careful experimentation has revealed XO to be an important ROS source in reperfusion injury,[8] and its high H_2O_2 generation in ischemic conditions[1] is expected to activate my proposed inhibitors selectively at sites of injury. These inhibitors, however, should target Nox2 rather than XO. Results from human trials using XO inhibitors to block ROS formation have been inconsistent,[7,9] and XO's production of cardioprotective nitric oxide at injury sites further weakens the rationale for targeted XO inhibition,[1] which is unlikely to improve clinical outcomes.[9] Lastly, superoxide from NOS uncoupling in I/R injury presents another challenge. To address this, Nox2 inhibition is expected to prevent the "no-reflow" phenomenon[5] in the coronary microvasculature and thus minimize the hypoxia that contributes to uncoupling.[1] Thus, Nox2 inhibition may reduce NOS's contribution to overall ROS production. Co-administering caged Nox2 inhibitors with tetrahydrobiopterin to reduce NOS uncoupling further[10] is a promising strategy for future work. Notably, the proposed approach is expected not only to inhibit local Nox2-mediated ROS production at injury sites, but also to inhibit Nox2-mediated ROS production from infiltrating leukocytes (another key source of ROS in I/R injury).[7] Taken together, these observations

suggest that Nox2 is the most practical and important target for targeted inhibition in MI, and that other ROS sources are likely to enhance localized Nox2 inhibitor activation.

5. *Reviewer 2 noted that both male and female mice should be included to generate robust, generalizable results.* I apologize that my justification for the use of male mice was in the vertebrate animals section and not mentioned in the main text of the proposal. Male mice have a significantly stronger I/R phenotype than females.[11,12] The power analysis calculations in this proposal were repeated using a published analysis of sex differences in the I/R phenotype to estimate the decrease in effect size from including female mice.[11] The total number of mice required for this study to be adequately powered increased substantially (to 282 animals), and repeated power analysis accounting for the greatly increased variance from including both sexes will call for an even higher sample size. Performing this many surgeries is impractical in the context of this 2-year fellowship proposal. As a long-term goal, we will complete future experiments in female mice using a lengthier 2-hour coronary artery ligation. These experiments may comprise part of a follow-up proposal (e.g., AHA Postdoctoral Fellowship or NIH K Award).

6. *Reviewer 1 noted that (few) rats were chosen for cell culture work, but mice were chosen for in vivo experiments*: Rats were initially chosen for three reasons: (1) literature precedent for using these cells for hypoxia-reoxygenation experiments, (2) Dr. Weber's experience with rat models, and (3) the wide availability of kits to facilitate rat myocyte isolation. Given the movement of the field toward murine myocytes[13] and the need for concordance between the proposed experiments pointed out by Reviewer 2, we will use 10-12 week-old C57BL/6J mice instead of rats as the source for the primary cell culture experiments, which have been validated as a hypoxia-reoxygenation model.[14] The number of mice required for Aim 1 has been updated to reflect the requirements of the model.

7. *Reviewers 1, 2, and 3 discussed productivity*: I have now submitted a first-author manuscript on complementary work with exogenous small-molecule triggers to control protein function, which is under review at *ChemBioChem*. As an undergraduate, my research centered on developing a new treatment for GM3 Synthase Deficiency; this work was carried out in collaboration with the Clinic for Special Children, which has an interest in retaining intellectual property rights. Dr. Deiters and I have laid out a plan for promptly publishing the results of my proposed research (see Sponsor's Training Plan).

8. *Reviewer 3 asked for clarification of the rationale for treatment in the chronic setting*: Nox2 drives the chronic sequelae of I/R injury responsible for much of the morbidity and mortality of myocardial infarctions,[15–17] making it an attractive target for long-term treatment. Effective targeting to the infarcted area is even more important in the chronic setting, given Nox2's essential homeostatic roles as discussed in the Research Plan.

9. *Reviewer 3 asked about the time point for evaluating off-target effects*: The compound will be re-dosed every 8 h (approx. 5 half-lives[3]); samples for qRT-PCR will be collected after 32 hours to allow sufficient time for transcript up-/downregulation of Wnk2, which has the longest transcript $t_{½}$ at 12 h.[18]

10. *Reviewers 2 and 3 expressed concerns that Dr. Deiters has limited time for mentoring given the large size of our research group. Further, Reviewer 3 mentioned our lack of expertise with the animal models.* The Deiters group's size ensures that significant combined knowledge of chemical and biological methods is available. Our group has extensive experience developing new chemical tools and applying them in animal models, which has led to 9 publications. The group has comprised up to 20 trainees in the past without affecting productivity or time to graduation, and a recent analysis found that lab size during graduate training correlates positively with success in continuing in academia.[19] The results of Dr. Deiters' mentorship are evident in the success his trainees enjoy during and after graduate school (see Sponsor's Past and Current Trainees). Dr. Deiters was recently a finalist for our joint MD-PhD program's highest mentorship award (the William E. Brown Outstanding MSTP Mentor Award) out of all of the faculty at CMU and Pitt. He and I have a standing one-on-one meeting every Monday for 30-45 minutes. Additionally, Dr. Dutta has offered to devote 5% of his effort to help me design, conduct, and analyze the mouse experiments (see letter of support), and has made arrangements for a postdoctoral fellow and two full-time animal surgeons to train and assist me with this model.