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14. ABSTRACT The purpose of this grant was the synthesis, development, and validation of a PET imaging agent for the non-invasive delineation of hydrogen peroxide (H ₂ O ₂) levels in prostate cancer. We are seeking such an imaging agent because the over-production of hydrogen peroxide in prostate tumors has been linked to with rapid tumor cell proliferation, aggressive tumor growth, and enhanced metastatic ability. We believe that the project has the potential to be paradigm-shifting, as it would be the first PET agent for the <i>in vivo</i> delineation of cellular H ₂ O ₂ production. The central hypothesis of the project is that the selective, H ₂ O ₂ -mediated cleavage of carbon-boron bonds could be used as the centerpiece of a strategy for the PET imaging of H ₂ O ₂ . The majority of the work during this funding period was dedicated to elucidating and executing a synthetic route to an ¹⁸ F-radiolabeled probe containing an H ₂ O ₂ -sensitive carbon-boron bond. Unfortunately, the short radioactive half-life and reactivity with boron of ¹⁸ F ultimately combined with the instability of alkyne-bearing boronates to click chemistry conditions to preclude the synthesis of such an agent. Toward the end of the funding period, we turned to a different positron-emitting radioisotope, ¹²⁴ I, and were subsequently able to synthesize an ¹²⁴ I-labeled, boronate ester-containing probe — ¹²⁴ I-hydroxyphenyl boronate pinacol ester (¹²⁴ I-HPBPE) — that we believe could be a first generation chemoselective imaging agent for the non-invasive <i>in vivo</i> delineation of H ₂ O ₂ levels. Going forward, we plan to further explore the chemical reactivity of ¹²⁴ I-HPBPE, followed by subsequent <i>in vitro</i> and <i>in vivo</i> validation studies. In the end, while the synthesis of the prospective probe required more time and effort than we anticipated, we firmly believe that this research project could lead to the development of a clinically transformative imaging agent.					
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End of Grant Report
Department of Defense CDMRP Prostate Cancer Research Program
Exploration-Hypothesis Development Award
PC110555: “PET Imaging of Oxidative Stress in Prostate Cancer”

Introduction

The original goal of this grant was the development of a strategy to non-invasively delineate intracellular hydrogen peroxide (H_2O_2) levels in prostate tumors using positron emission tomography (PET).¹ It has been well-established that (H_2O_2) is over-produced in many types of prostate cancer, and, more importantly, its over-production has been linked with rapid tumor cell proliferation, aggressive tumor growth, and enhanced metastatic ability.¹⁻⁶ As we wrote in the original proposal, we believe that the “project has the potential to be paradigm-shifting, as it would be the first PET agent for the *in vivo* delineation of cellular H_2O_2 production. The ability to image this novel target would be particularly important in prostate cancer, as the non-invasive determination of intratumoral H_2O_2 levels could be of significant value in the clinic, both as a means of monitoring treatment efficacy and for identifying tumors of aggressive phenotype.”

The central hypothesis of the project is that the selective, H_2O_2 -mediated cleavage of carbon-boron bonds could be used as the centerpiece of a strategy for the PET imaging of H_2O_2 (Figure 1).⁷⁻¹⁰

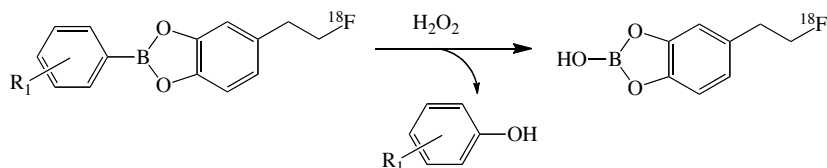


Figure 1. The chemoselective cleavage of carbon-boron bonds by H_2O_2

As we described in the original proposal, “the basic design of the system is straightforward. While whole, the [^{18}F]-fluoroethylarylboronate ([^{18}F]-FEAB) probe will be freely cell permeable, and thus it will pass into and subsequently out of cells with low basal levels of H_2O_2 . In contrast, when the [^{18}F]-FEAB enters a cell with high levels of H_2O_2 , the hydrogen peroxide will oxidatively cleave the boron-carbon bond, producing a [^{18}F]-labeled borinic acid product that, due to its negative charge once deprotonated ($\text{pK}_a < 7$), will become entrapped in the cell (Figure 2).”

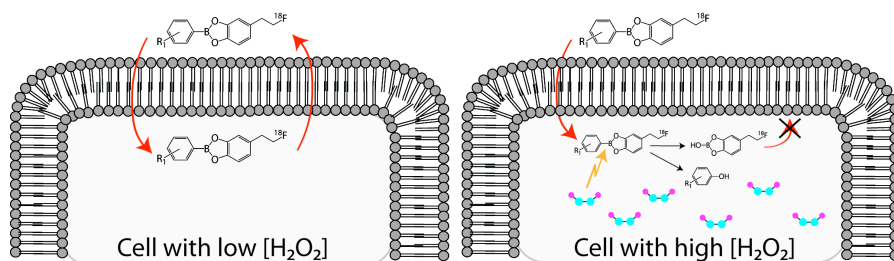


Figure 2. General schematic of the design of the cleavage-based H_2O_2 PET imaging strategy

“By this mechanism, cells with high levels of H_2O_2 will accumulate much higher levels of ^{18}F than cells with lower H_2O_2 levels. Standard organic synthesis and radiochemistry methods will be employed to synthesize the [^{18}F]-FEABs, with the key steps being the boron-carbon bond formation via palladium-catalyzed coupling and the subsequent [^{18}F]-radiolabeling reaction. The substituents on the aryl ring will be strategically varied to optimize the cell permeability of the intact agent (e.g. R_1 = amine, short aliphatic chain, triethylene glycol, monosaccharide, etc.). Once the probes have been synthesized and characterized, $\log P$ values and rates of H_2O_2 -mediated cleavage will be determined for all of the prospective agents. *In vitro* studies will be performed to determine the cellular uptake, efflux, and

retention of the different agents under varying H_2O_2 production conditions. The LNCaP and LNCaP-AR prostate cancer cells lines will be used for all *in vitro* experiments, and intracellular H_2O_2 levels will be modulated in three ways: direct incubation with H_2O_2 ; addition of paraquat (a mitochondrial poison that prompts increased H_2O_2 production); and treatment with testosterone or prostate specific antigen, for these cells are known to increase ROS production when exposed to these agents.⁵ Importantly, independent fluorometric measurements of H_2O_2 concentration will be made in all experiments to verify the amounts of intracellular hydrogen peroxide and allow for the quantitation of trends. Finally, the most promising probes *in vitro* will be evaluated *in vivo* with small animal PET imaging using nude mice implanted with LNCaP and LNCaP-AR xenografts. In both cases, the levels of intratumoral H_2O_2 production will be modulated through exposure to testosterone or prostate specific antigen.'

Body

As may be expected for a hypothesis-based grant like this one, the execution of the project did not quite line up with the plan outlined in the original proposal. However, during the year funded by this grant, we have made massive strides in the synthesis of the H_2O_2 -cleavable imaging agents (Statement of Work Objective #1). This wasn't easy, however, and we have gone through multiple iterations of prospective compounds before finally — and very recently — arriving at a compound that seems to be promising. As we will discuss, in the coming weeks and months, we will use this compound for *in vitro* (Statement of Work Objective #2) and *in vivo* (Statement of Work Objective #3) studies.

The first alteration we had to make was to the nature of the ester on the compound. In the original proposal, we proposed a phenyl-based, catechol-like ester (*Figure 3*). We began synthesizing these compounds; however, we soon learned through a combination of our own experience and an in-depth reading of the literature that unhindered esters like this one are not particularly stable in water or serum.¹¹⁻¹⁴ Therefore, we switched our design to contain a pinacol ester such as those used in the work of Chang, *et al* (*Figure 3*).^{7, 8, 10}

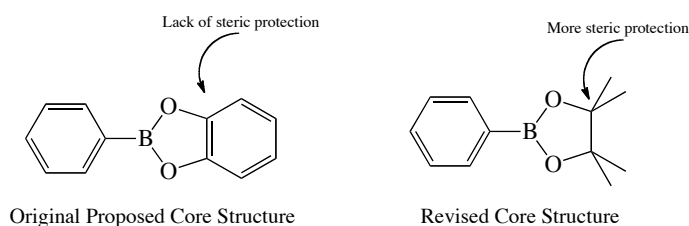


Figure 3. Revision to the core structure of the probe

This change in the core structure of the probe made us think critically about how we planned to incorporate the ^{18}F into the finished probe. There are two essential options: (A) labeling the organic pinacol-based moiety prior to the formation of the boronate pinacol-like ester and (B) labeling the intact ester (*Figure 4*).

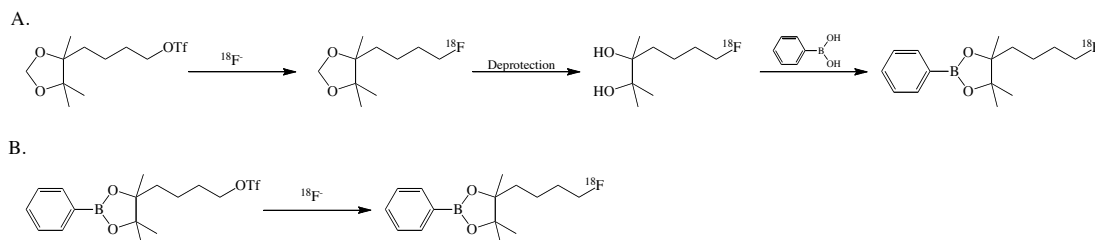


Figure 4. Two possible routes to ^{18}F -labeled probes bearing a boronate pinacol ester: (A) labeling the pinacol-based moiety prior to the creation of the boronate ester; (B) incorporating the radiolabel after the formation of the ester.

Unfortunately, we soon found that neither of these routes would do. We could eliminate Option A rather quickly, because the deprotection and ester formation reactions are far too slow to be compatible with the short radioactive half-life of ^{18}F : ^{18}F 's physical half-life is ~ 2 hr, while each of these reactions (especially the ester formation) requires hours to complete.

The problems with Option B were somewhat more surprising. Recent work in the laboratory of David Perrin at the University of British Columbia has focused on exploiting the incredible strength of the boron-fluorine bond as method of radiofluorination.¹⁵⁻²¹ This, of course, presents a problem for Option B: given the strength of the B-F interaction, any $^{18}\text{F}^-$ we introduce, will almost certainly attach to the boron rather than displace a leaving group (*Figure 5*).

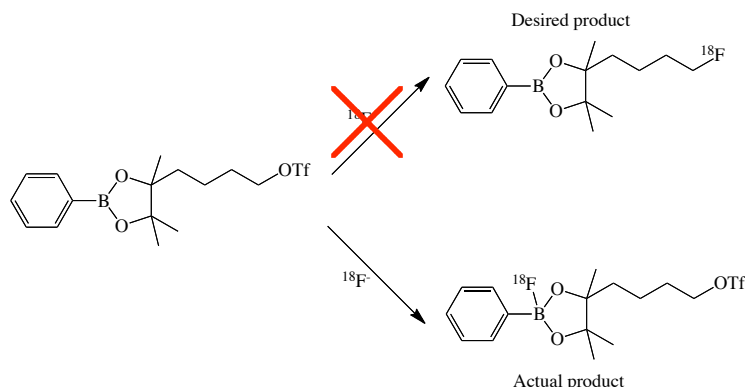


Figure 5. The dangers of trying to incorporate an $^{18}\text{F}^-$ in the presence of a boron

Since the work of Perrin's group strongly suggested that the boron (and not the triflate) will be the target of a radiofluoride nucleophile, we did not want to go through with the time-consuming synthesis of the triflate-bearing pinacol boronate ester.¹⁵⁻²¹ However, in order to confirm the affinity of $^{18}\text{F}^-$ for boron, we did mix together $^{18}\text{F}^-$ with hydroxyphenyl boronate pinacol ester and, as expected, we did see the $^{18}\text{F}^-$ binding to the boron (*Figure 6*). Ultimately, we do believe that displacing a triflate leaving group with $^{18}\text{F}^-$ in the presence of a boron is *possible* with the right combination of electron-donating or electron-withdrawing groups on the phenyl ring attached to the boron. However, we did not believe that pursuing that fundamental radiochemistry problem was worth the time in our execution of this grant.

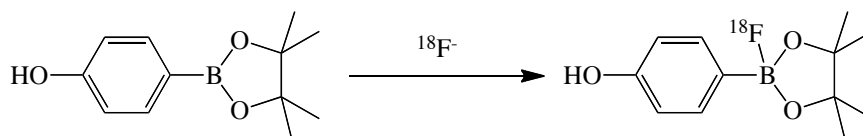


Figure 6. The reaction between $^{18}\text{F}^-$ and a 4-hydroxyphenyl boronate pinacol ester

Not to be deterred, our next plan was to incorporate the ^{18}F into the finished probe using copper-catalyzed click chemistry.²²⁻²⁶ This method, shown below in *Figures 7 and 8*, allows for the rapid incorporation of the ^{18}F into the compound without having to label with ^{18}F in the presence of the boron. A number of literature reports describing the synthesis of ^{18}F -labeled azides can be found in the literature, and standardized techniques for the copper-catalyzed click reaction between ^{18}F -labeled azides and alkynes have been reported as well.^{22, 27-30}

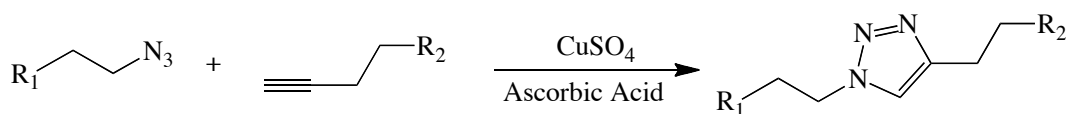


Figure 7. The Cu-catalyzed [3+2] Huisgen cycloaddition between an azide and an alkyne

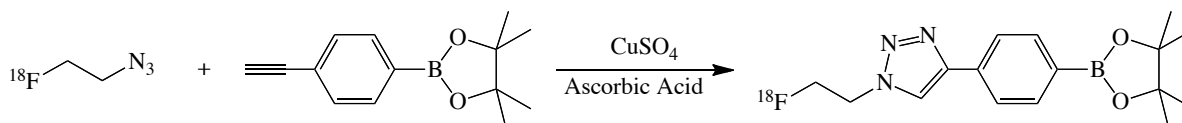


Figure 8. Proposed structure of a H₂O₂-reactive PET probe based on Cu-catalyzed click chemistry

Prior to performing any radiochemistry with this system, we wanted to see if the pinacol boronate ester was stable under click chemistry conditions. To this end, we had 4-ethynylbenzyl boronate pinacol ester specially made for our laboratory by Frontier Scientific, and we purchased azidoaniline from Sigma-Aldrich as a model compound for the reaction (*Figure 9*).

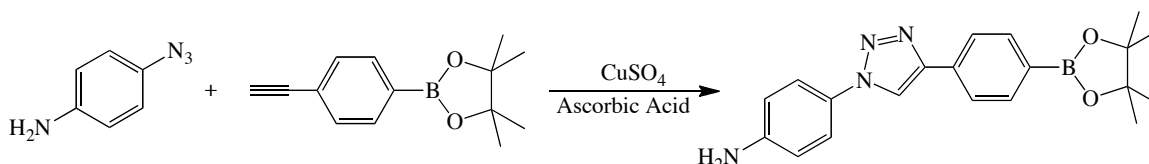


Figure 9. Model click reaction between azidoaniline and an alkyne-containing pinacol boronate ester.

In subsequent HPLC-based stability studies, we learned that 4-ethynylbenzyl boronate pinacol ester was completely stable in the presence of Cu(II)SO₄ or ascorbic acid. When mixed together, however, Cu(II)SO₄ and ascorbic acid create Cu(I), a species necessary for the click reaction. Unfortunately, our stability experiments suggest that 4-ethynylbenzyl boronate pinacol ester is unstable in the presence of Cu(I) (*Figure 10*). Future experiments are currently underway on this system, because we firmly believe this click chemistry approach represents the best the best way to create an ¹⁸F-based, boron-containing PET probe.

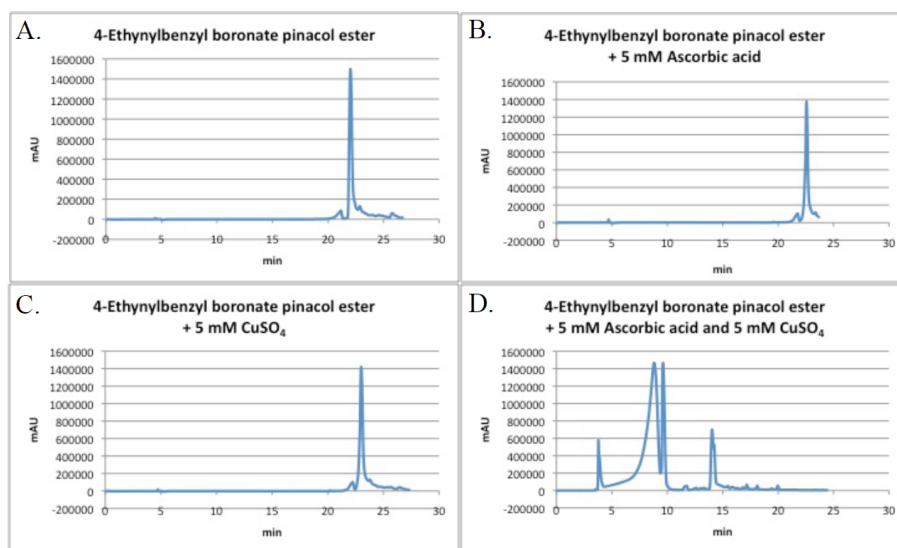


Figure 10. Illustration of the decomposition of 4-ethynylbenzyl boronate pinacol ester in the presence of Cu(I). While the compound is stable alone (A), in the presence of 5 mM ascorbic acid (B), and in the presence of 5 mM CuSO₄ (C), the compound falls apart to a number of other species in the presence of a combination of 5 mM ascorbic acid and 5 mM CuSO₄ (a combination that readily forms Cu(I)).

At this point, we felt to some degree like we were out of ^{18}F -related options. The radionuclide's short half-life and high affinity for boron abrogated our first two synthetic routes, and the instability of 4-ethynylbenzyl boronate pinacol ester in the presence of Cu(I) seems to preclude the use of Cu-catalyzed click chemistry. Luckily, ^{18}F is not the only radiohalogen available. As an alternative, we have decided to turn to ^{124}I , a positron-emitting radiohalogen with a longer half-life (~4 days) and without the affinity of $^{18}\text{F}^-$ for boron. Radiolabeling proteins with isotopes of radioiodine most often centers upon radiolabeling tyrosines, and we have recently used this chemistry to create a prospective first generation H_2O_2 -sensitive PET imaging agent. We have labeled 4-hydroxyphenyl boronate pinacol ester with ^{124}I using a Chloramine T based procedure to produce ^{124}I -hydroxyphenyl boronate pinacol ester (^{124}I -HPBPE) in high yield and purity (see *Figures 11 and 12*).

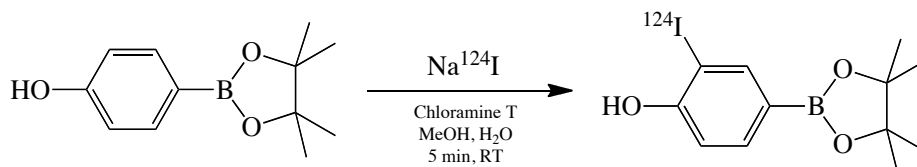


Figure 11. The synthesis of ^{124}I -hydroxyphenyl boronate pinacol ester (^{124}I -HPBPE)

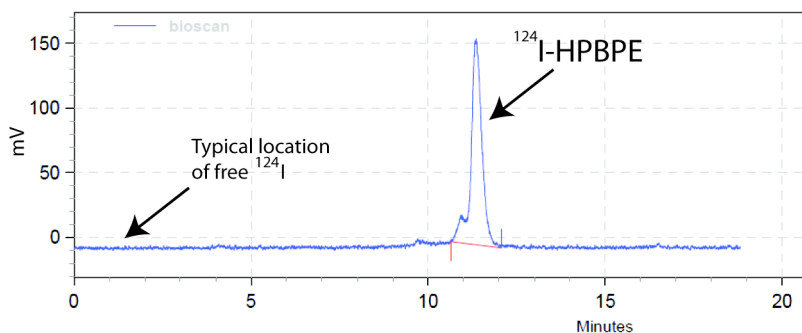


Figure 12. A radio-HPLC trace of ^{124}I -hydroxyphenyl boronate pinacol ester (^{124}I -HPBPE)

Further, while the data is very preliminary at the time of writing this report, initial studies seem to indicate that the ^{124}I -HPBPE is, indeed, specifically cleaved by H_2O_2 . However, much more validation is needed prior to asserting this with full confidence. Once the chemistry has been further elucidated, we plan to take ^{124}I -HPBPE into both *in vitro* (Statement of Work Objective #2) and *in vivo* (Statement of Work Objective #3) experiments as soon as possible.

In the end, we admittedly and regrettably were unable to move past the synthesis phase of the experimentation during the funding period (Statement of Work Objective #1). However, we made incredible strides toward the synthesis of an H_2O_2 -sensitive PET imaging agent during the funding period, and we are thus incredibly grateful to the Department of Defense and the CDMRP for the funds. They were essential to getting the project off the ground and moving it from an exciting idea towards a reality.

Key Research Accomplishments

- The synthesis of an ^{124}I -labeled radiopharmaceutical bearing a boronate pinacol ester that may be developed into an chemoselective PET imaging agent for the non-invasive *in vivo* delineation of H_2O_2 levels.

Reportable Outcomes

- A manuscript on the synthesis and characterization of the ^{124}I -labeled hydroxyphenyl boronate pinacol ester is planned, but more data must be collected first.
- The general theory behind this work was used to apply for a Department of Defense Breast Cancer Research Program Idea Award. However, the proposal, entitled “PET Imaging of Highly Reactive Oxygen Species in Breast Cancer” was not funded.
- The progress achieved during this funding period on the development of ROS-sensitive PET imaging agents was used to support a successful application for an intramural Seed Grant (entitled “PET Imaging of Reactive Oxygen Species”) from the Memorial Sloan-Kettering Cancer Center Imaging and Radiation Sciences Program.
- The PI (Brian Zeglis) has used the overall mission of this grant (*i.e.*, the development of reactive oxygen species-sensitive PET imaging agents) as a centerpiece of the research plan that he has used to apply for faculty positions at universities across the country, including the University of Chicago, Ohio State University, Penn State University, Dartmouth College, Brown University, Columbia University, and New York University.

Conclusion

The majority of the work during this funding period was dedicated to elucidating and executing a synthetic route to an ^{18}F -radiolabeled probe containing an H_2O_2 -sensitive carbon-boron bond. Unfortunately, the short radioactive half-life and reactivity with boron of ^{18}F ultimately combined with the instability of alkyne-bearing boronates to click chemistry conditions to preclude the synthesis of such an agent. Toward the end of the funding period, we turned to a different positron-emitting radioisotope, ^{124}I , and were subsequently able to synthesize an ^{124}I -labeled, boronate ester-containing probe — ^{124}I -hydroxyphenyl boronate pinacol ester (^{124}I -HPBPE) — that we believe could be a first generation chemoselective imaging agent for the non-invasive *in vivo* delineation of H_2O_2 levels. Going forward, we plan to further explore the chemical reactivity of ^{124}I -HPBPE, followed by subsequent *in vitro* and *in vivo* validation studies. In the end, while the synthesis of the prospective probe required more time and effort than we anticipated, we firmly believe that this research project could lead to the development of a clinically transformative imaging agent.

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Appendices

Because all figures have been embedded in the text, there are no appendices for this report.