In vivo imaging of hydrogen peroxide production in a murine tumor model with a chemoselective bioluminescent reporter

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Living organisms produce hydrogen peroxide (H$_2$O$_2$) to kill invading pathogens and for cellular signaling, but aberrant generation of this reactive oxygen species is a hallmark of oxidative stress and inflammation in aging, injury, and disease. The effects of H$_2$O$_2$ on the overall health of living animals remain elusive, in part owing to a dearth of methods for studying this transient small molecule in vivo. Here we report the design, synthesis, and in vivo applications of Peroxy Caged Luciferin-1 (PCL-1), a chemoselective bioluminescent probe for the real-time detection of H$_2$O$_2$ within living animals. PCL-1 is a boronic acid-caged firefly luciferin molecule that selectively reacts with H$_2$O$_2$ to release firefly luciferin, which triggers a bioluminescent response in the presence of firefly luciferase. The high sensitivity and selectivity of PCL-1 for H$_2$O$_2$, combined with the favorable properties of bioluminescence for in vivo imaging, afford a unique technology for real-time detection of basal levels of H$_2$O$_2$ generated in healthy, living mice. Moreover, we demonstrate the efficacy of PCL-1 for monitoring physiological fluctuations in H$_2$O$_2$ levels by directly imaging elevations in H$_2$O$_2$ within testosterone-stimulated tumor xenografts in vivo. The ability to chemoselectively monitor H$_2$O$_2$ fluxes in real time in living animals offers opportunities to dissect H$_2$O$_2$'s disparate contributions to health, aging, and disease.


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enzyme activity (33 reaction. Related strategies have been employed for assaying consequently trigger the catalytic bioluminescent luciferin/luciferase mediated cleavage process to generate free luciferin and subse-

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ferase bioluminescent reporter system as a platform for creating minimally invasive, and nontoxic. We chose the firefly luciferin/luciferase bioluminescent reporter system, its physiologically relevant low-micromolar detection efficiency (39), but these strategies have not been used for small-molecule detection. Alkylation of firefly luciferin at the phenolic position prevents signal production even when luciferin’s reactive carboxylic acid moiety remains unaltered (40). Additionally, previous work from our laboratory has shown that the chemoselective deprotection of boronate esters to phenols offers a useful reaction-based method for detecting H2O2 over other ROS (16, 18–21, 41, 42). On the basis of these considerations, we designed the peroxide-sensitive probe PCL-1 (Fig. 1) by attaching an aryl boronic acid to firefly luciferin through a self-immolative benzyl linker (43–45). In the absence of H2O2, PCL-1 is not an active substrate for the luciferase enzyme, but addition of H2O2 triggers cleavage of the boronic acid benzyl ether to release free luciferin, which reacts with firefly luciferase to produce a bioluminescent readout. The synthesis of PCL-1 is depicted in Scheme 1.

**Peroxide Reactivity and Selectivity of PCL-1.** Initial experiments tested the kinetic properties of PCL-1 and the ability of PCL-1 to detect H2O2 in a selective and concentration-dependent manner. First, we performed an in vitro kinetic assay with PCL-1 (5 μM) and H2O2 (1, 5, and 10 mM) to determine the second-order rate constant (k = 3.8 ± 0.34 M−1 s−1) for the reaction. Next, we evaluated the ROS selectivity of the reaction in a bioluminescent assay using purified firefly luciferase. PCL-1 was incubated with a panel of ROS, including H2O2 in the absence and presence of catalase, an H2O2-degrading enzyme, for 5–60 min followed by addition of firefly luciferase. Subsequently, light production was measured over 45 min to determine the relative amount of luciferin released during the ROS incubation period. The relative total photon flux for each condition was calculated by dividing the total photon flux for the experimental condition by the total photon flux for PCL-1 alone to allow a direct comparison between various ROS. Whereas addition of H2O2 showed a ca. sevenfold increase in bioluminescent signal over an hour, there was little to no increase in signal when the boric acid probe was reacted with the other ROS or H2O2 in the presence of catalase (Fig. 2). Additionally, our control compounds, luciferin and valeryl luciferin, which were used for in vivo experiments, showed little to no response to incubation with ROS in vitro (Fig. S1).

After confirming the selectivity of PCL-1 for H2O2 over other biologically relevant ROS, we examined the responsiveness of PCL-1 to alterations in peroxide levels. Incubation of PCL-1 with various concentrations of H2O2 for 60 min was followed by addition of firefly luciferase and detection of the released luciferin through measurement of the bioluminescent signal. This assay established the linear dependence (R2 = 0.9927) of luciferin release from PCL-1 on the concentration of H2O2 over a 2-order-of-magnitude range, from 5 to 250 μM (Fig. 2 and Fig. S2). Taken together, the high selectivity of the PCL-1 reporter system, its physiologically relevant low-micromolar detection limit, and its dose-dependent response are necessary features of a

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**Results and Discussion**

**Design and Synthesis of PCL-1.** Desirable properties for an effective H2O2 reporter in living animals include selectivity for H2O2 over other biologically relevant ROS, a good signal-to-noise contrast ratio, high-efficiency signal production, and deep tissue signal penetration. In addition, practical molecular imaging probes for use in whole organisms should be readily transported in vivo, minimally invasive, and nontoxic. We chose the firefly luciferin/luciferase bioluminescent reporter system as a platform for creating in vivo H2O2 imaging agents because it meets all of the aforementioned chemical and biological criteria. In particular, the firefly luciferin substrate is a nontoxic small molecule that easily enters the blood stream, produces tissue-penetrable signal in all organs of transgenic mice, and is metabolized within hours (30–32).

We envisioned a bioluminescent H2O2 reporter in which an appropriately caged firefly luciferin that is unreactive toward the luciferase enzyme could be unmasked by a selective H2O2-mediated cleavage process to generate free luciferin and subsequently trigger the catalytic bioluminescent luciferin/luciferase reaction. Related strategies have been employed for assaying enzyme activity (33–38) and cell-penetrating peptide transport

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**Fig. 1.** Design strategy for H2O2-mediated release of firefly luciferin from PCL-1.

**Scheme 1.** Synthesis of PCL-1.
practical probe that can detect changes in H₂O₂ levels in living systems in the presence of oxidative or reductive stimuli.

**PCL-1 Visualizes Changes in H₂O₂ Levels in Living Cells by Bioluminescent Imaging.** We next determined whether the performance of PCL-1 in aqueous solution translated to cell culture. Initial experiments using live-cell assays with exogenous H₂O₂ addition indicated that the presence of cells did not interfere with production of the H₂O₂-dependent bioluminescent signal and demonstrated a low, biologically relevant detection limit (2.5 μM) as well as a linear response to H₂O₂ in cellulo (Fig. S3) (2, 46).

In subsequent experiments, to determine whether PCL-1 could detect endogenously produced H₂O₂ in living cells, we incubated LNCaP-luc cells with 500 μM paraquat for 24 h because previous work established that paraquat triggers elevations in intracellular H₂O₂ through disruption of the mitochondrial electron transport chain (47). Following paraquat stimulation, the LNCaP-luc cells were loaded with PCL-1 and the bioluminescent signal was measured. Paraquat-treated cells showed significantly (p < 0.005) higher levels of PCL-1 derived luminescence compared to unstimulated control cells (Fig. 3).

Because luciferin is a cell-permeable small molecule, three possible modes of action for PCL-1 are (i) reaction of this probe with H₂O₂ in the extracellular medium followed by cellular uptake of the free luciferin product, (ii) reaction of PCL-1 with intracellular H₂O₂ to generate luciferin within cells, or (iii) both of the above. To probe whether PCL-1 was reacting with H₂O₂ in the intra- and/or extracellular space, we added catalase to paraquat-stimulated cells as a cell-impermeable scavenger for extracellular H₂O₂ (48). We observed that the addition of catalase with paraquat causes a decrease in bioluminescent signal compared to addition of paraquat alone, but the signal did not decrease to the level of background signal of cells treated only with catalase in the absence of paraquat (Fig. 3). These data suggest that PCL-1 is capable of interacting with and detecting both extracellular and intracellular H₂O₂ pools. Moreover, because treatment with H₂O₂-scavenging catalase reduced the level of PCL-1 bioluminescence in cells compared to untreated specimens (Fig. 3), the data also suggest that PCL-1 is sensitive enough to detect basal levels of H₂O₂ that are endogenously produced without addition of compounds that stimulate H₂O₂ production, highlighting the potential utility of PCL-1 for in vivo studies.

**Molecular Imaging of H₂O₂ Fluxes in Living FVB-luc⁺ Mice.** We next sought to apply PCL-1 to molecular imaging of H₂O₂ in living animals. Initial studies utilized FVB-luc⁺ mice (30) that ubiquitously express firefly luciferase along with exogenous peroxide addition. Several concentrations of H₂O₂ were injected into the i.p. cavity of unshaven FVB-luc⁺ mice with subsequent i.p. injection of PCL-1, and the bioluminescent signal produced by these living animals was imaged in real-time using a CCD camera. Differences in bioluminescent signal from mice injected with different amounts of H₂O₂ can be detected within the first few minutes following injection, and monitoring of the integrated total photon flux for each mouse reveals a dose-dependent increase in signal as a function of the H₂O₂ concentration (Fig. 4). Although H₂O₂ clearly elevates the signal detected from PCL-1 in vivo, it has no impact on the signal produced by luciferin.
Interestingly, mice treated with only PCL-1 with no added peroxide also show modest but measurable bioluminescence throughout their bodies, suggesting that PCL-1 may be detecting basal levels of \(\text{H}_2\text{O}_2\) produced in these living animals. To determine whether this emission signal was due in part to the detection of endogenous \(\text{H}_2\text{O}_2\), we injected PCL-1 into unshaven FVB-luc\(^+\) mice in the presence and absence of NAC, a commonly used antioxidant (49). We were pleased to observe that the NAC-treated animals exhibit a significantly reduced bioluminescent signal compared to vehicle control animals (Fig. 4). Control experiments in FVB-luc\(^+\) mice with luciferin and NAC (Fig. S4) indicated that NAC has no effect on the bioluminescent production of photons in vivo, establishing that PCL-1 is sensitive enough to visualize basal levels of \(\text{H}_2\text{O}_2\) in healthy, living animals without external stimulation of peroxide production.

Detection of Endogenous \(\text{H}_2\text{O}_2\) in a Prostate Tumor Model. After establishing that the \(\text{H}_2\text{O}_2\)-mediated boronic acid deprotection of PCL-1 provides a selective and sensitive platform for real-time imaging of \(\text{H}_2\text{O}_2\) in aqueous solution, in living cells, and in living mice, we sought to apply this bioluminescent reporter to studies of \(\text{H}_2\text{O}_2\) physiology at an in vivo level. In the present study, we targeted the roles that \(\text{H}_2\text{O}_2\) plays in the development and progression of cancer because recent reports suggest that tumor cells produce an elevated level of \(\text{H}_2\text{O}_2\) compared to noncancerous cells and that this ROS increase is correlated with cancer cell growth and malignancy (50). Initial experiments focused on prostate cancer using androgen-sensitive prostate cancer cells (LNCaP). In dissociated cell culture, LNCaPs respond to testosterone by increasing their proliferation rate (51, 52) and elevating their ROS production (53), suggesting a link between oxidative stress and development of this disease. However, the probes previously used to detect oxidant production in LNCaPs do not selectively detect any particular ROS (53), and the environment that tumor cells experience in vivo is greatly different from dissociated cell culture conditions with variances in oxygen level, nutrient supply, and acidity (54). Thus, the application of tools to probe specific ROS molecules, like \(\text{H}_2\text{O}_2\), in the context of a living animal is critical to elucidating the relationships between redox biology and cancer.

To study the production of \(\text{H}_2\text{O}_2\) and stimulatory effects of testosterone in prostate cancer in living animals, we developed an i.p. LNCaP-luc xenograft model in immunodeficient SCID hairless outbred (SHO) mice (Fig. S5). To determine the baseline levels of \(\text{H}_2\text{O}_2\) generation in the tumors as well as alterations in \(\text{H}_2\text{O}_2\) fluxes over 24 h, mice were injected with PCL-1 on day 1 and with PCL-1 and sesame oil, a vehicle used for all experiments, on day 2. No change in basal bioluminescent signal from the mice over the time course of the experiments was found (Fig. S6). We then moved on to test the effects of testosterone (in the form of testosterone propionate) on \(\text{H}_2\text{O}_2\) production within the prostate cancer tumors. For these experiments, mice were injected with PCL-1 on day 1 and the baseline signal was measured. On day 2, the mice were injected with either testosterone propionate or empty vehicle, followed 1.5 h later by PCL-1. Mice treated with testosterone propionate showed a ca. 41% increase in total photon flux compared to vehicle control mice (Fig. 5). These data suggest that LNCaP-luc tumors produce elevated levels of \(\text{H}_2\text{O}_2\) in vivo upon testosterone stimulation.

To ensure that the observed signal enhancement from testosterone stimulation of the LNCaP tumors was due to an increase in \(\text{H}_2\text{O}_2\) production and not a result of nonspecific cellular and metabolic changes, we utilized a non-ROS responsive control compound, valeryl luciferin (Scheme S1) (55), in experiments identical to those outlined above for PCL-1. This esterase-cleavable luciferin was chosen as the control compound instead of firefly luciferin because the peak for signal production by luciferin in LNCaP-luc cells, as opposed to many other luciferase transfected cells, is reached prior to the first imaging time point (<1 min after injection). In contrast, because valeryl luciferin requires cleavage of the valeryl ester prior to light production, the signal peak is shifted to later time points and can be detected within the time frame of the imaging experiments to ensure consistent quantitation of the bioluminescent signal. We observed no change in the bioluminescent signal from valeryl luciferin from day 1 to day 2 when mice were injected with vehicle alone or vehicle plus testosterone on the second day (Fig. S7). These results clearly indicate that testosterone does not alter the expression of firefly luciferase in the LNCaP-luc xenografts nor change the interactions between the luciferin derivatives and these tumors, which further validates that PCL-1 is imaging changes in tumor production of \(\text{H}_2\text{O}_2\) upon testosterone stimulation.

In a final set of control experiments to confirm that PCL-1 was detecting a testosterone-triggered increase in tumor \(\text{H}_2\text{O}_2\) production, we utilized NAC as a general chemical scavenger for \(\text{H}_2\text{O}_2\). We performed these experiments by injecting mice on day 2 with testosterone propionate, followed 1.5 h later by serial administration of NAC and PCL-1. As shown in Fig. 5, NAC treatment causes a reduction in bioluminescent signal in testosterone-stimulated animals back to baseline levels, with light production comparable to vehicle control tumors. The collective data establish that androgen-sensitive prostate tumors respond to the proliferation signal of testosterone in vivo by elevating their production of \(\text{H}_2\text{O}_2\).

Concluding Remarks
Hydrogen peroxide contributes to a diverse array of physiological and pathological events in living organisms, but there is an insufficient understanding of how local fluxes of this potent small-molecule oxidant can be beneficial or damaging to the entire an-
inal in stages of health, aging, injury, and disease. An emerging set of chemical tools for selective imaging of $\text{H}_2\text{O}_2$ at the cellular level continues to help elucidate the functions of this ROS in simplified model systems, but analogous approaches to probe such questions in vivo remain limited. In this study, we have presented a unique chemoselective reporter for real-time imaging of $\text{H}_2\text{O}_2$ produced in living animals. PCL-1 is a caged luciferin derivative that is activated upon selective cleavage of its $\text{H}_2\text{O}_2$-sensitive boronic acid group to generate free luciferin, which can then undergo reaction with luciferase to generate light through a bioluminescent reaction. We have shown that this reporter system is able to selectively detect $\text{H}_2\text{O}_2$ in a concentration-dependent manner in living cells and in living animals, demonstrating that the $\text{H}_2\text{O}_2$-mediated cleavage of boronic acids is a bioorthogonal reaction for $\text{H}_2\text{O}_2$ sensing that can reliably operate in vivo. In addition, live-mouse imaging experiments with the antioxidant NAC establish that PCL-1 is sensitive enough to visualize basal luc cell line. PCL-1 imaging reveals that these tumors trigger animal model of cancer through use of an androgen-sensitive species.

Materials and Methods

Materials and procedures for the synthesis of compounds, bioluminescent assays, and animal experiments are described in SI Text.

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