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for Detection of Extracellular Hydrogen Peroxide**

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Development of an ATP-Independent Bioluminescent Probe for Detection of Extracellular Hydrogen Peroxide

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Abstract

This work reports a new ATP-independent bioluminescent probe (bor-DTZ) for detecting hydrogen peroxide that is compatible with the Nanoluciferase enzyme. The probe is designed with an arylboronate ester protecting group appended to a diphenylterazine core via a self-immolative phenolate linker. Reaction with hydrogen peroxide reveals diphenylterazine, which can then react with Nanoluciferase to produce a detectable bioluminescent signal. Bor-DTZ shows a dose-dependent response to hydrogen peroxide and selectivity over other biologically relevant reactive oxygen species and can be applied to detect either intra- or extracellular species. We further demonstrate the ability of this platform to monitor fluxes in extracellular hydrogen peroxide in a breast cancer cell line in response to the anticancer treatments, cisplatin.

Introduction

Hydrogen peroxide (H₂O₂) is a key molecule in biological redox metabolism and stress.¹ On the one hand, H₂O₂ is a hallmark of oxidative stress² whereby its generation alongside other reactive oxygen species results in direct or indirect oxidation of various biomolecules such as lipids³, DNA⁴, RNA⁵, and proteins⁶. On the other hand, endogenous levels of H₂O₂ have been shown to play important roles in cellular signaling in processes ranging from immune response to cell proliferation.^{7,8,9,10,11} For example, neutrophils have been shown to utilize NADPH oxidase systems to generate millimolar quantities of H₂O₂ as a defense against foreign microbes.¹² H₂O₂ imbalances have been implicated in variety of disease pathologies such as cancer,^{13,14} diabetes,^{15,16} inflammation^{17,18} and cardiovascular diseases^{19,20}. Tools for monitoring H₂O₂ fluxes are vital for understanding the molecular mechanisms at play regarding both the physiological and pathological roles H₂O₂ plays in biological systems.

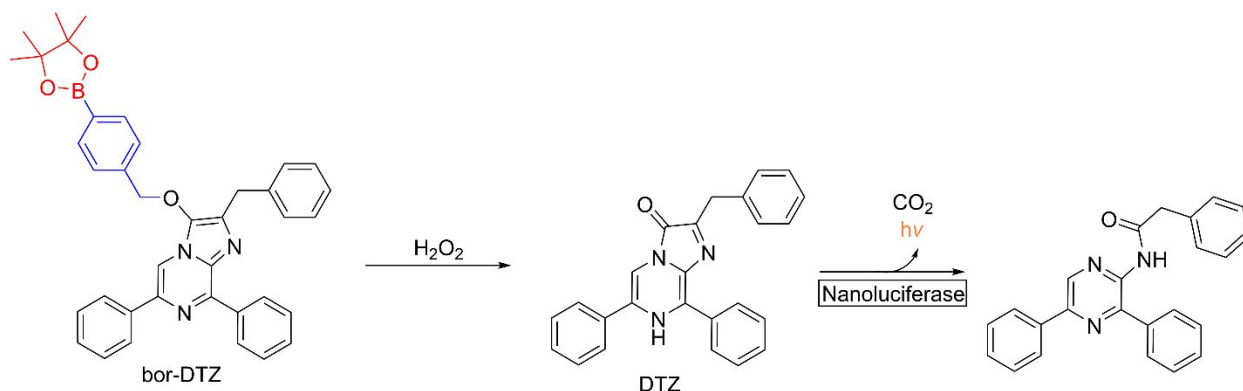
Small molecules^{21,22,23,24,25} employing various reaction triggers such as sulfonic esters²², diketones^{26,27}, and arylboronates²⁸ have been widely employed for fluorescence-based sensing of H₂O₂. In particular, the arylboronate reaction-based triggers have been extensively adopted mainly owing to their high selectivity and sensitivity in addition to their fast reaction kinetics relative to other H₂O₂ reactive moieties.^{29,30,31,32} Although fluorescence-based imaging probes have provided valuable insight into H₂O₂ dynamics, they are primarily applied to monitoring intracellular H₂O₂ dynamics, given that the larger volume and diffuse nature of the extracellular space and extracellular fluids can lead to signal dilution. Aside from fluorescence-based imaging probes researchers have also turned to the development of alternative modalities with improved sensitivity, such as such as electrochemical sensors, but many of these are not amenable to *in vivo* work.^{33,34,35}

To fill this gap, bioluminescence has become an increasingly favorable candidate for molecular imaging platforms.³⁶ Bioluminescence results in the emission of photons via the enzymatic oxidation of a small molecule luciferin by its cognate luciferase enzyme. Imaging with this technique offers the benefits of high signal-to-noise ratio as well as the ability to endow cell and tissue specificity through the genetic encoding of the luciferase.³⁷ The phenomenon has been adapted for sensing molecular analytes of interest

by chemical modification of the luciferin, termed “caging”, which precludes interaction with its luciferase pair.^{38,39} Upon chemo-selective reaction with the analyte the native luciferin is restored and subsequent oxidation by its luciferase results in the production of detectable photons. This strategy has been successfully employed for the detection of intracellular H_2O_2 using a firefly luciferin caged with an arylboronic acid.^{40,41} While capable of detecting intracellular H_2O_2 and in the tissues of living mice, an inherent property of the firefly luciferin/luciferase system is its dependence on ATP, therefore limiting the probes’ applicability to intracellular applications and regimes where ATP levels are not significantly perturbed. Development of probes for the extracellular space are of particular interest due to the transient nature of signaling biomolecules like H_2O_2 . The ability to monitor extracellular H_2O_2 would provide valuable insight into both the physiological roles that this oxidative metabolite plays in extracellular signaling as well as a means to monitor oxidative stress in disease progression. Unlike the firefly luciferases, marine luciferases and their derivatives do not require ATP, making these platforms adaptable to extracellular applications.^{42,43}

To this end, we report the design, synthesis, and evaluation of a new bioluminescent based probe for detecting H_2O_2 in the extracellular space using a marine luciferin/luciferase pair. We report a boronate ester caged diphenylterazine (bor-DTZ), a small molecule luciferin with an H_2O_2 reactive boronate ester and self-immolative linker attached to the carbonyl of the parent diphenylterazine (DTZ). Upon H_2O_2 induced oxidative hydrolysis and subsequent self-immolation, DTZ is generated which can then interact with the engineered marine luciferase, Nanoluciferase (Nluc)⁴⁴, to produce a detectable bioluminescent signal. We demonstrate the ability of bor-DTZ to selectively detect H_2O_2 over other biologically relevant ROS and show that it can detect exogenous and endogenous H_2O_2 in live cells. We further show the probes utility in monitoring changes to extracellular H_2O_2 in response to a clinically relevant cancer treatment in a human breast cancer cell model.

Scheme 1. Design of bor-DTZ for H_2O_2 -responsive Bioluminescence^a



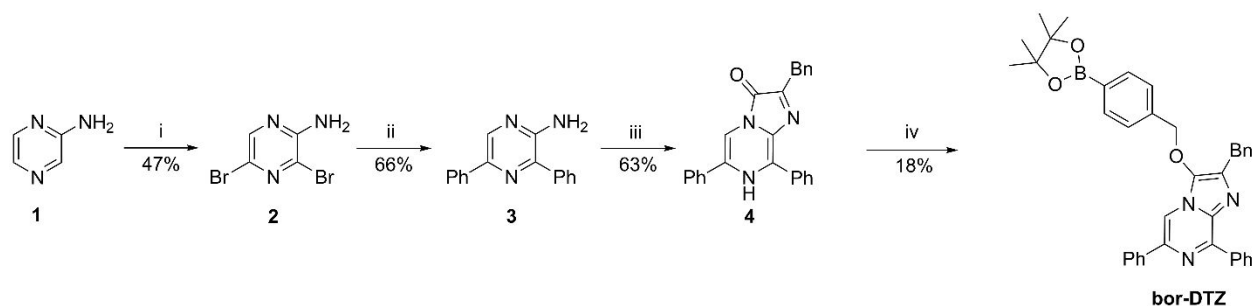
^a Red: H_2O_2 -responsive cage, blue: self-immolative linker, black: diphenylterazine (DTZ) core.

Results And Discussion

Synthesis and reactivity of bor-DTZ. The recently engineered Nanoluciferase (Nluc) is a marine based luciferase derived from a deep sea shrimp which has gained attention due to its small size, high thermal stability, and highly intense luminescence in the presence of its engineered substrate, furimazine, relative to firefly luciferase.⁴⁴ We recently demonstrated that modifying imidazopyrazinone substrates at the imidazolyl carbonyl with a reactive group responsive to copper(II) could yield caged luciferins compatible with Nluc, with the synthetic imidazopyrazinone, diphenylterazine (DTZ), yielding optimal response among a series of caged imidazopyrazinone derivatives.⁴⁵ Thus, to design a bioluminescence agent for

the Nluc system that could respond to hydrogen peroxide, we paired the well-studied arylboronate ester cage to diphenylterazine (DTZ) with a self-immolating phenolate linker (scheme 2). The arylboronate ester cage is expected to undergo selective oxidative hydroxylation by H_2O_2 , which in turn triggers an electron cascade to reveal the parent DTZ for recognition by Nluc. We synthesized the native DTZ according to previously published methods^{45,46} and then performed a nucleophilic substitution with 4-bromomethylphenylboronic acid pinacol ester under basic conditions to afford bor-DTZ in 18% yield. We attributed the low yield due to a side product where the pinacol ester adds to the alkene at the C2 position as well as DTZ being inherently prone to oxidation. However, it is important to note that for subsequent biological assays bor-DTZ can be used in concentrations as low as 1 μM such that low milligram quantities of bor-DTZ can be used for hundreds of assays depending on sample size.

Scheme 2. Synthesis of bor-DTZ.



Reagents and conditions: (i) NBS, CHCl_3 , 3 h; (ii) Phenylboronic acid, $\text{Pd}(\text{PPh}_3)_4$, K_2CO_3 , 1,4-dioxane, H_2O , 80 $^\circ\text{C}$, 12 h; (iii) 1,1-diethoxy-3-phenylpropan-2-one, EtOH, H_2O , cat. HCl, 80 $^\circ\text{C}$, 12 h; (iv) 4-Bromomethylphenylboronic acid pinacol ester, Cs_2CO_3 , KI, MeCN, 12 h.

To evaluate the reactivity of bor-DTZ, its luminescence output was measured upon addition of analytes of interest in the presence of purified recombinant Nanoluciferase (rNluc) in aqueous buffer. Selectivity was assessed by comparing reactivity with H_2O_2 to a panel of reactive oxygen species as well as glutathione, a biologically relevant reducing agent. The normalized total photon flux was determined by calculating the area under the curve of the kinetic reading over 30 minutes and normalizing values from all ROS tested to that of bor-DTZ alone (Figure 1a). A representative kinetic curve for bor-DTZ in the presence of 100 μM H_2O_2 can be seen in the supplementary information (Figure S1). We observed that bor-DTZ exhibits exceptional selectivity towards H_2O_2 relative to the other ROS tested. We confirmed that the turn-on response was due to H_2O_2 by observing a loss in signal upon addition of catalase, an enzyme that rapidly degrades H_2O_2 . The DTZ/Nluc system has been previously reported to have a λ_{max} of 500 nm.⁴⁶ The bor-DTZ/Nluc system similarly yielded a λ_{max} emission of 500 nm in the presence of H_2O_2 (Table S1), further confirming that the parent DTZ is released upon reaction with the analyte. Moreover, bor-DTZ demonstrated a dose-dependent response to the H_2O_2 from 50-250 μM (Figure 1b), which is within a biologically-relevant range in the context of the tumor microenvironment.^{47,48,49} A 10-fold change in molar luminescence is observed from the lower limit of detection (10 μM H_2O_2) to the point of signal saturation (250 μM) (Table S1).

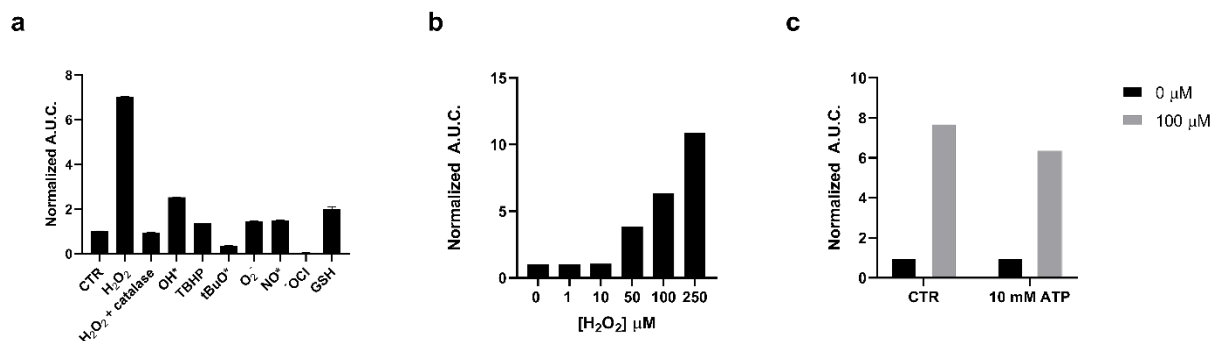


Figure 1. Reactivity of bor-DTZ (1 μM) reported as calculated area under the curve of luminescence output over 20 minutes in the presence of analytes and rNluc (0.4 μg/mL), normalized to bor-DTZ in the absence of analytes; all solutions in DPBS, pH 7.4, 37°C, emission range: 300-850 nm. (a) Reactivity of bor-DTZ in the presence of various biologically relevant ROS (100 μM) and glutathione (GSH, 10 mM). Error bars denote n=3, SEM. (b) Light output of bor-DTZ in the presence of various concentrations of H₂O₂ (0-250 μM) and rNluc (0.4 μg/mL). (c) Responsiveness of bor-DTZ to H₂O₂ (100 μM) in the presence of ATP (10 mM). Data points are normalized to the control without H₂O₂ added.

We next assessed whether the signal produced by bor-DTZ was affected by ATP. Previously reported bioluminescence probes for H₂O₂ rely on the firefly luciferase enzyme, which utilizes ATP as a cofactor. The need for ATP limits the probe to mainly intracellular applications where ATP is abundant; additionally, various cellular processes can result in significant changes to ATP levels, making this cofactor a confounding variable for firefly luciferase-based sensors.

Detection of intracellular H₂O₂ by bor-DTZ.

Having established the selectivity and sensitivity of bor-DTZ towards H₂O₂ in buffer we next evaluated the H₂O₂ responsiveness in cells. We utilized a breast cancer cell line, MDA-MB-231, engineered to express Nluc intracellularly. To stimulate an increase in intracellular H₂O₂ we treated cells with 500 μM paraquat for 24 hours, which generates intracellular ROS through disruption of mitochondrial respiration.⁵⁰ We monitored light output in the presence of both bor-DTZ, and analogous experiments were performed using the parent DTZ. The data is presented as the ratio of the area under the curve of the 1 hour luminescence of bor-DTZ:DTZ in order to normalize for any changes in Nluc expression or cell proliferation due to paraquat treatment (Figure 2a). Representative kinetic curves for the luminescence of both bor-DTZ and DTZ are presented in the supplementary information (Figure S2). Indeed, bor-DTZ detected a significant ($p < 0.005$) increase in signal of paraquat-treated cells relative to untreated cells showing the biological utility of this probe in cell-based models. As this particular cell-line is expressing a form of Nluc without a secretion signaling domain the observed signal indicates that bor-DTZ is able to diffuse through the cell membrane. Additionally, the cells are washed with PBS prior to imaging in order to remove any extracellular hydrogen peroxide or Nluc that may be present. We also performed an experiment to assess the toxicity of bor-DTZ towards the cells using an MTS assay, and found that when Nluc MDA-MB-231 cells are incubated with bor-DTZ (10 μM) for 8 hours there is no significant effect on cell viability (Figure 2b), suggesting minimal to no toxicity.

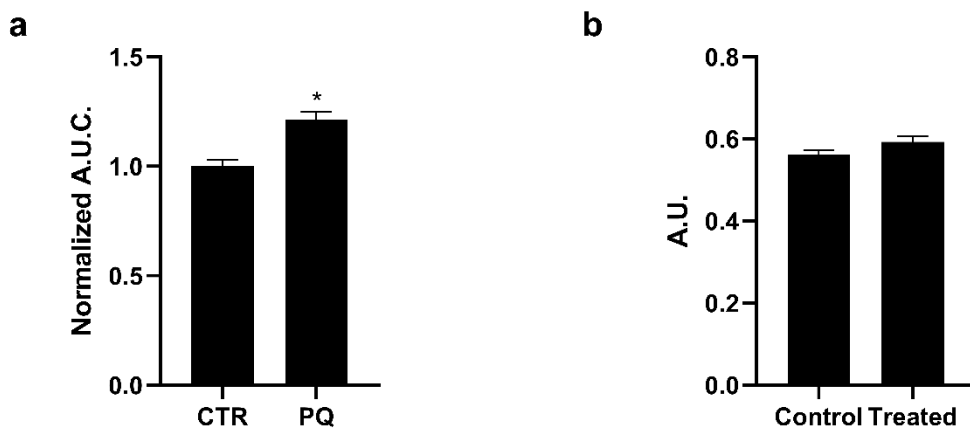


Figure 2. (a) Calculated ratio of the area under the curve over 1 hour of bor-DTZ:DTZ (10 μ M for both) luminescence from MDA-MB-231 cells expressing Nluc in the absence (control, CTR) or presence of 500 μ M paraquat (PQ) for 24 hours. Error bars denote $n=3$, SEM. Statistical significance was assessed by calculating p -values using unpaired t-test, * $p < 0.005$. Measurements performed in DPBS, pH 7.4, 37°C, emission range: 300-850 nm. (b) Average absorbance at 490 nm of Nluc MDA-MB-231 cells after an 8-hour incubation with bor-DTZ (10 μ M) followed by the addition of MTS for one hour. Error bars denote $n=6$.

Detection of extracellular H_2O_2 by bor-DTZ.

To determine the ability of bor-DTZ to detect changes in extracellular H_2O_2 , we applied bor-DTZ in the media of MDA-MB-231 cells engineered to stably express and secrete Nanoluciferase (secNluc MDA-MB-23). Cells were plated and cultured in Optimem media for 24 hours, then media was removed and placed in a new well plate, H_2O_2 was spiked in and the samples were treated with bor-DTZ, then analyzed for light output over a 20-minute period. As the cells secrete Nluc, no addition of rNluc was required for these experiments. Furthermore, media was removed from cells and the assay was done in the absence of cells to prevent any signal occurring from intracellular activation. We observed slight increases in light output in the 0-10 μ M range followed by large increases from 50-250 μ M (Figure 3a). This range is in line with physiologically-relevant levels of tumor microenvironments wherein H_2O_2 concentrations can reach as high as 50-100 μ M.^{47,48,49}

To determine if bor-DTZ could detect endogenous levels of H_2O_2 in the media, the bioluminescent response of the media was measured in the absence or presence of catalase without addition of exogenous H_2O_2 (Figure 2b). We observe a drastic decrease in light output in cell media that had been spiked with catalase suggesting bor-DTZ could detect basal levels of H_2O_2 in the media. Notably, catalase has little to no effect on the native DTZ/Nluc system (Figure S3).

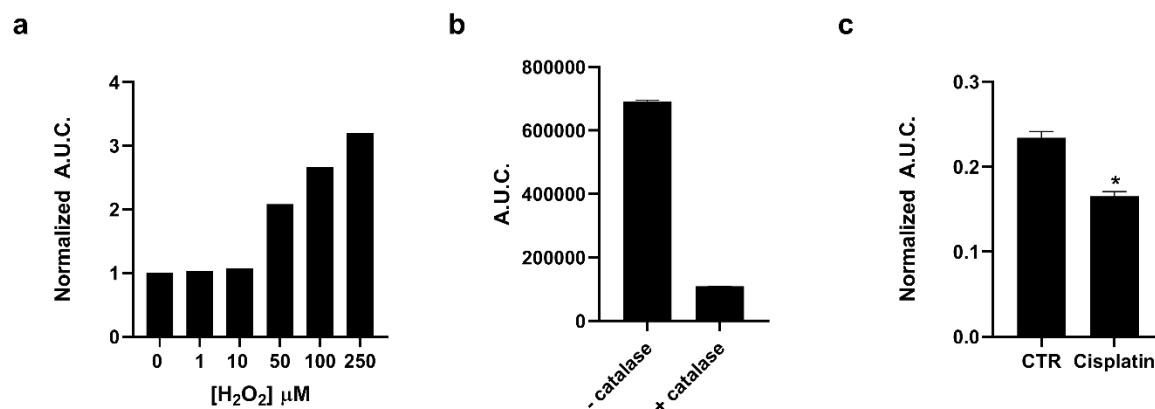


Figure 3. (a) Dose-dependent light output as monitored by calculated area under the curve for luminescence over 20 minutes of bor-DTZ (1 μM) in the presence of various concentrations of H_2O_2 (0-250 μM) in cell media removed from secNluc MDA-MB-231 cells. Data points are normalized to the control without H_2O_2 added. (b) Calculated area under the curve of luminescence over 20 minutes of cell media from secNluc MDA-MB-231 cells in the presence or absence of catalase (1×10^4 U/L) with 1 μM bor-DTZ. Error bars denotes SEM, $n=3$. (c) Calculated ratio of the area under the curve over 20 minutes of bor-DTZ:DTZ luminescence from secNluc MDA-MB-231 cells treated with cisplatin (30 μM). Error bars denote SEM, $n=3$. Statistical significance was assessed by calculating p -values using unpaired t-test, * $p < 0.05$. Measurements performed in Optimum, pH 7.4, 37°C, emission range: 300-850 nm.

As oxidative stress and ROS production is a hallmark of breast cancer,⁵¹ we applied bor-DTZ to assess how extracellular H_2O_2 levels are perturbed in the presence of cisplatin, a well-studied anti-cancer agent.⁵² The secNluc MDA-MB-231 cells were plated and treated with cisplatin for 16 hours before removing the media from the cells and monitoring luminescence in the presence of bor-DTZ or the parent DTZ for 20 minutes. The ratio of the area under the curve for bor-DTZ:DTZ was calculated in order to account for any effects that the treatments had on secNluc expression or cell proliferation (Figure 3c). We observe that cisplatin treatment induces a decrease in light output relative to the control. Interestingly, our previous report on a copper(II) responsive DTZ showed that cisplatin also decreases extracellular levels of copper(II). These findings provide a basis and tool for further investigations into the interplay between trafficking of H_2O_2 and copper in both physiological and pathological states. The above study also demonstrates the high-throughput and accessible nature that the bor-DTZ probe offers through use of a 96-well plate and luminometer or plate reader. This lays the foundation for a unique new tool for future high-throughput studies investigating the effect of external stimuli on H_2O_2 dynamics.

Experimental Methods

General Methods. Reactions using moisture- or air-sensitive reagents were carried out in dried glassware under an inert N_2 atmosphere. DTZ (**4**) was synthesized according to previously published methods.^{45,46} Dry solvents were all purchased from Sigma-Aldrich and used immediately. All commercially purchased chemicals were used as received without further purification. 2-aminopyrazine was purchased from Oakwood Products. All other chemicals were purchased from Sigma-Aldrich unless otherwise noted. Silica Gel 60 F254 (precoated sheets, 200 μm thickness, MilliporeSigma) were used for analytical thin layer chromatography. Silica gel sorbent (230-400 mesh, grade 60, ThermoFisher) or aluminum oxide (neutral, Brockmann I, 50-200 μm , grade 60, Sigma-Aldrich) were used for column chromatography. ^1H and ^{13}C NMR spectra were collected at room temperature in DMSO- d_6 (Sigma-Aldrich) on a 600 MHz Varian NMR spectrometer. All chemical shifts are reported as δ parts per million relative to the residual solvent peak at 2.50 (DMSO- d_6) for ^1H and 39.52 (DMSO- d_6) for ^{13}C . Multiplicities are reported as s

(singlet), d (doublet), t (triplet), q (quartet), p (pentet), h (hexet), m (multiplet), dt (doublet of triplets), or br (broad). Heated electrospray ionization mass spectral analyses were performed using an ThermoQ Exactive HF (High field Orbitrap) at the UC Davis Campus Mass Spectrometry Facilities.

Synthesis of bor-DTZ. Diphenylterazine, **4**, (14 mg, 0.037 mmol, 1 equiv.), 4-bromomethylphenylboronic acid pinacol ester (11 mg, 0.037 mmol, 1 equiv.), Cs₂CO₃ (5 mg, 0.015 mmol, 0.4 equiv.), and KI (7 mg, 0.041 mmol, 1.1 equiv.) were added to a flame-dried round-bottom flask that was then purged with N₂. The reagents were then dissolved in anhydrous acetonitrile (1 mL) and the reaction was allowed to stir overnight. The reaction mixture was subsequently quenched in 10 mL of water and extracted in DCM (3 x 5 mL). The combined DCM extract was then washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure. The crude product was then purified by reverse-phase HPLC (isocratic, 80/20 MeCN:H₂O over 2 hours on a T3 Atlantis column (Waters)) and dried under reduced pressure to obtain **bor-DTZ** (4 mg, 18% yield) as a yellow-brown solid. ¹H NMR (600 MHz, DMSO-d₆) δ 8.83-8.85 (d, 2H), 8.44 (s, 1H), 8.09-8.11 (d, 2H), 7.68-7.70 (d, 2H), 7.50-7.60 (m, 5H), 7.46-7.47 (d, 2H), 7.41-7.43 (t, 1H), 7.27 (m, 4H), 7.18 (m, 1H), 5.25 (s, 2H), 4.07 (s, 2H), 1.27 (s, 12H). ¹³C NMR (151 MHz, DMSO-d₆) δ 146.08, 139.64, 139.59, 137.25, 137.11, 136.84, 136.19, 135.09, 134.15, 131.55, 130.77, 129.75, 129.17, 129.13, 129.03, 128.88, 128.86, 128.82, 128.74, 126.62, 126.48, 110.99, 84.18, 77.02, 32.95, 25.11. High-resolution mass spectrometry (HRMS) (m/z): [M+H]⁺ calculated for : C₃₈H₃₇BN₃O₃, 594.2927; found, 594.2915.

In Vitro Luminescence Assays. Milli-Q water (18.2 MΩ) was used to prepare all aqueous solutions. Reactive oxygen species solutions were prepared to 10 mM in water. Hydrogen peroxide (H₂O₂), *tert*-butyl hydroperoxide (TBHP), and hypochlorite (OCl⁻) stock solutions were prepared from 30%, 70%, and 2-3% aqueous solutions, respectively. Hydroxyl radical (*OH) and *tert*-butoxy radical (*OtBu) were generated in situ by reaction with excess Fe(II). Nitric oxide (NO*) was generated using PROLI NONOate (Cayman Chemical). Superoxide (O₂⁻) was delivered from a stock solution of potassium superoxide (KO₂) in DMSO. A 100 μM solution of bor-DTZ was prepared by dissolving bor-DTZ in pure ethanol. A 0.4 μg/mL solution of rNluc was prepared by adding 1 μL of 0.4 mg/mL stock solution (Promega, Nano-Glo Assay Kit) into 999 μL of DPBS at pH 7.4. 100 μL of rNluc solution was added to the wells of a white, opaque, flat-bottom 96-well plate followed by 1 μL of ROS 10 mM stock solution or 1 μL of a 1 M GSH stock solution. Finally, bor-DTZ (1 μL) was added to all the wells using a multi-channel pipette and mixed well. The bioluminescent signal was immediately measured using a Molecular Devices SpectraMax i3x plate reader at 37 °C for 1 hour. For dose-dependence studies 100x stock solutions of H₂O₂ were prepared in Millipore water from a 30% (w/w/) aqueous solution and 1 μL of these stock solutions were added to 100 μL of rNluc solution in wells of a 96-well white plate. Bor-DTZ (1 μL of a 100 μM solution) was added via a multichannel pipette and the luminescence was immediately measured. For ATP studies a 10 mM solution of ATP was prepared in DPBS pH 7.4 by adding 100 μL of a 100 mM stock (ThermoScientific) into 900 μL of DPBS. To this was added 1 μL of 0.4 mg/mL rNluc (Promega) to get a final volume of 0.4 μg/mL rNluc. The control rNluc solution was prepared to 0.4 μg/mL as described previously and 100 μL aliquots of control and ATP containing solutions were plated in 96-well white plate. Bor-DTZ (1 μL of a 100 μM solution) was added to the wells and the luminescence was measured immediately. When reporting area under the curve, these values were calculated using GraphPad Prism software.

Cell Culture. MDA-MB-231 cells stably expressing secreted or intracellular Nanoluciferase (secNluc MDA-MB-231 or Nluc MDA-MB-231 respectively) were a kind gift from Drs. Gary and Kathy Luker (University of Michigan). Cells were maintained in Dulbecco's modified medium (DMEM, 4.5 g/L

glucose) supplemented with 10% fetal bovine serum (FBS) (Gibco) 1x penicillin-streptomycin (Corning), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco) at 37 °C and 5% CO₂.

Detecting H₂O₂ intracellularly in Nluc MDA-MB-231 Cells. Cells were plated at 10,000 cells per well in a 96-well white, opaque, flat bottom plate. Twenty-four hours later media was replaced with fresh media and paraquat was added from a freshly prepared stock solution to a final concentration of 500 μM. Cells were then incubated for 24 hours and then the media was removed and washed with warm DPBS before adding 100 μL of fresh DPBS to wells. Bor-DTZ (1 μL of a 1 mM solution) was added to wells and luminescence was measured immediately over one hour.

MTS Assay of Bor-DTZ treated Nluc MDA-MB-231 Cells. Cells were plated at 10,000 cells per well in a 96-well black, clear bottom plate. Twenty-four hours later the media was replaced with fresh media (containing no FBS) and Bor-DTZ (stock solution in ethanol) was added to wells at a final concentration of 10 μM and the plate was incubated for 8 hours in a 37°C incubator and 5% CO₂. Following incubation MTS (CellTiter 96, Promega) was added to wells according to the manufacturers protocol. The plate was incubated for one hour at 37 °C before measuring the absorbance at 490 nm on a Molecular Devices SpectraMax i3x.

Detecting H₂O₂ in Media of secNluc MDA-MB-231 Cells. Cells were plated at 10,000 cells per well in a 96-well black clear bottom plate. Twenty-four hours later the media was removed from the wells and added to a fresh 96-well white, opaque, flat bottom plate. Catalase (Sigma Aldrich) was added to wells in triplicate to a final concentration of 1 x 10⁴ U/L from a stock solution. Following this bor-DTZ (1 μL of a 100 μM solution) was added to wells and luminescence was measured immediately. For dose-response experiments 100X solutions of H₂O₂ were prepared in water and 1 μL of this was added to secNluc MDA-MB-231 cells 24 hours after plating as previously described. Bor-DTZ (1 μL of a 100 μM solution) was added to wells and luminescence was measured immediately.

Monitoring secNluc MDA-MB-231 response to cisplatin. Cells were plated at 10,000 cells per well in a 96-well, white, opaque flat-bottom plate. Eight hours later, the media was removed, and the cells were washed with pre-warmed (37 °C) DPBS and 100 μL of OptiMem (Gibco) was added to each well. A 3 mM solution of cis-platin was prepared in water. 1 μL of this was added to the wells for a final concentration of 30 μM cisplatin. At the 16-hour time point solutions of 100 μM bor-DTZ and 100 μM DTZ (in ethanol) were prepared and 1 μL was added per well per treatment (n=3). Luminescence was immediately recorded as previously described. The area under the curve was calculated using GraphPad Prism software and the ratio of the A.U.C. from bor-DTZ to DTZ was calculated.

Conclusions

We have demonstrated the design, synthesis, and evaluation of a new bioluminescent probe, bor-DTZ, for monitoring H₂O₂ in vitro in an ATP-independent manner. Bor-DTZ shows a high level of selectivity towards H₂O₂ over other biologically relevant reactive oxygen species and shows sensitivity down to the low-mid μM range. The probe can detect H₂O₂ in either intracellular or extracellular environments based on where the Nluc enzyme is expressed. We also demonstrate that bor-DTZ can be applied to a 96 well-plate for cell-based assays for high-throughput analysis of H₂O₂ dynamics in a cancer cell line.

Bioluminescence-based sensing of H₂O₂ offers the advantages of in vivo compatibility as well as high sensitivity due to near-zero background and signal output that does not require an excitation light source. Among the caged bioluminescence agents that have been reported, bor-DTZ is one of the first diphenylterazine-based imaging probes to be paired with the bright, thermostable Nluc. The ATP-independent nature of Nluc allows bor-DTZ to be used in both intracellular and extracellular applications broadening the scope of currently available tools for H₂O₂. Paired with Nluc and emerging analogs for

deep-tissue imaging, bor-DTZ has broad potential for investigating oxidative biology in both cell-based and live-animal studies.

Conflict of Interest Statement

The authors report no conflicts of interest.

Author Contributions

J.J.O. and M.C.H. designed all experiments. J.J.O. performed all experiments and data collection. J.J.O. and M.C.H. analyzed the data. J.J.O. took the lead in writing the manuscript, and J.J.O. and M.C.H. wrote and edited the manuscript. M.C.H. conceived the study and was the major supervisor on overall direction of the study.

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