Hydrolysis-resistant ester-based linkers enable ABS probe development for NIR bioluminescent imaging of hypoxia

Anuj K Yadav,¹ Zhenxiang Zhao,¹ Yourong Weng,¹ Sarah H Gardner,² Catharine J Brady,¹ Oliver D Pichardo Peguero,¹ Jefferson Chan¹,²*

¹Department of Chemistry, Beckman Institute for Advanced Science and Technology, and Cancer Center at Illinois, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, United States
²Department of Biochemistry, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, United States

KEYWORDS. Hydrolysis-resistant ester, self-immolative linkers, activity-based sensing, near-infrared bioluminescence imaging, nitroreductase, tumor hypoxia

ABSTRACT: Activity-based sensing (ABS) probes equipped with a NIR bioluminescence (BL) readout are promising chemical tools to study cancer biomarkers owing to their high sensitivity and deep tissue compatibility. However, the standard approach of installing a responsive trigger at the anilne site through a self-immolative linker is not suitable for NIR substrates because they require \( N,N \)-dialkylation at this position to achieve NIR emission. Capping the carboxylate is also unfavorable due to the instability of the resulting ester moiety which would result in high background signals. In this study, we rationally designed a hydrolysis-resistant ester featuring an isopropyl shielding arm. Compared to a benzyl ester analog (proxy for self-immolative linker), the new design is 140.5-fold and 67.8-fold more resistant toward spontaneous and esterase-mediated hydrolysis, respectively. After further \textit{in cellulo} evaluation of stability, this ester moiety was transformed into a general self-immolative linker for ABS probe development via carboxylate masking. We showcased the utility of this technology by developing the first NIR BL probe for hypoxia sensing (BL660-NTR) and applied it in lung cancer cells and in a murine model of non-small cell lung cancer.

INTRODUCTION

The bioavailability and cell permeability of small-molecule drugs, imaging agents, or activity-based sensing (ABS) probes are largely dictated by molecular weight, lipophilicity, hydrogen bonding ability and net charge.¹,² For instance, a positively charged or neutral molecule is more likely to gain entry into a cell compared to an anion owing to Coulombic repulsion caused by the negatively charged membrane. A reliable strategy to improve cell uptake is to temporarily mask the negative functional group. For example, carboxylates can be readily transformed into the corresponding ester moiety, which upon crossing the cell membrane, can be cleaved to unmask the latent charge via intracellular esterase activity.³,⁴ A notable example is the use of this strategy to facilitate uptake of fluorescent indicators for calcium, which are equipped with a tri- or tetra-carboxylate binding motif.⁵,⁶ Under normal circumstances, it would be nearly impossible for a molecule with a net charge of -3 or -4 to enter the cell. However, calcium indicators for cellular imaging are routinely prepared and sold in the acetoxymethyl ester form. Beyond the modification of small molecules, this approach has extended to facilitate the delivery of functional proteins through the conversion of surface-exposed carboxylates to esters. Elegant examples include the delivery of GFP⁷ and human ribonuclease 1.⁸ However, there are limitations that must be considered when carboxylate-masking is employed \textit{in vivo} for the purpose of targeted cargo delivery. First, most esters are prone to spontaneous hydrolysis under acidic, neutral, or basic conditions.⁹,¹⁰ The body features numerous pH gradients (e.g., the pH in the gastrointestinal tract ranges from 5.7 to 7.4), which will result in off-target delivery upon systemic administration. Second, ester-hydrolyzing enzymes are ubiquitously expressed throughout the body in nearly all cell types, leading to indiscriminate cargo release. In general, ester stability is correlated to size and this property has been leveraged to tune the pharmacokinetics of drugs such as androgens. For instance, the \textit{in vivo} half-life of testosterone esters can be

\begin{scheme}
\begin{center}
\includegraphics[width=\textwidth]{scheme1.png}
\end{center}

\textbf{Scheme 1.} a) Schematic demonstrating the use of standard self-immolative linkers to install ABS triggers will lead to high background. b) Linkers based on hydrolysis-resistant ester ameliorates this concern.
\end{scheme}
increased from the 2-3-day range (propionate ester) to over 12 weeks (undecanate ester) by simply varying the length of the alkyl chain. In the context of ABS, an assortment of ester-based probes exists; however, an important distinction is that the carboxylate is a component of the trigger and not of the reporter. Amongst the ABS probes designed for in vivo applications, those equipped with a bioluminescence (BL) readout are highly coveted. Unlike fluorescence-based probes which require external light excitation to generate a signal, BL results from luciferase-mediated light production and thus, is highly sensitive due to low background. Moreover, the structure of luciferase substrates can be modified to shift the emission wavelength into the NIR as we have done recently, to achieve greater tissue penetration. Regardless of the variant, a viable substrate must contain a free carboxylate group for enzyme recognition and subsequent light generation. Unfortunately, the presence of a negative charge adversely impacts cell permeability. To yield an appreciable BL signal in vivo, luciferin must be administered systemically at exorbitant levels (>150 mg/kg). Recently, we reported the development of BL660-NO (a nitric oxide (NO)-responsive probe) by amidating the carboxylate of BL660 (a NIR BL substrate) with O-phenylene diamine. We found that this modification dramatically improved cell permeability (in vivo dosing decreased to 1 mg/kg), prompting us to explore the installation of other triggers at this position. However, upon surveying the literature, we discovered the available options were limited to hydrolytically stable groups (e.g., amides, aldehydes, etc.), with a few examples of simple esters. We hypothesize the apparent void in this research space is likely due to the general instability of esters in vivo.

In this study, we report the successful development of a new hydrolysis-resistant ester. Moreover, we show that carboxylate-masking using this ester is generalizable through the modification of two sets of molecules, BL660 and methotrexate (a potent anticancer and immunosuppressive drug). Importantly, we converted the optimized ester to a new self-immolative linker, which we used to develop an ester-based probe to sense tumor hypoxia. The utility of the resulting probe (BL660-NTR) was showcased in A549 lung cancer cells, as well as in a murine model of lung cancer via NIR BL imaging.

RESULTS AND DISCUSSION

Design and Synthesis of Substituted Benzyl Esters

Because the primary objective of this work is to develop ABS probes for the BL modality via carboxylate-masking, we chose to investigate the potential of benzyl esters. Triggers based on para-substituted benzyl alcohol are the most ubiquitous self-immolative linkers used in modern probe design. Typically, an electron-deficient analyte-responsive trigger occupies the para-position, and the immolative linker is connected to a reporter through a stable ether, carbamate, or carbonate bond. Upon interaction with the target analyte, the trigger is activated to yield a more electron-rich moiety. Flow of electron density into the aromatic ring facilitates formation of a quinone methide (or aza-quinone methide) intermediate which is coupled to the spontaneous release of the latent reporter. We hypothesized inclusion of additional phenyl or alkyl groups at the benzyl position can impart stability by shielding the ester from water or other nucleophiles. Further, we envisioned installing these onto BL660 would enable monitoring of stability using BL imaging. Our envisioned panel of esters initially consisted of 1 (Ph, H), 2 (Ph, iPr), 3 (PhMe), and later, included optimized ester 4 (Ph, iPr) shown in Figure 1a.

While the synthesis of 1-3 was routine, the esterification of BL660 to afford 4 proved to be challenging owing to the bulky nature of this benzyl alcohol. Conventional reaction conditions to form esters including Fischer, Mitsunobu, Steglich and Yamaguchi esterification failed to give products in appreciable yields beyond trace conversion. Moreover, we observed racemization of the chiral center housing the carboxylate. This is detrimental because stereochemistry at this position is required for luciferase recognition. Similarly, activation to the acyl chloride, followed by addition of the benzyl alcohol gave complex, inseparable mixtures. Our efforts to control the rate of alcohol addition, modularity of the reaction, stoichiometry of the components, and temperature were all unsuccessful. We also found that diazo chemistry was incompatible with our system. Ultimately, we employed cyanuric fluoride at ~20 °C to access the less reactive acyl fluoride intermediate, which upon reaction with the corresponding benzyl alcohol yields the desired esters. Of note, no attempts were made to separate the stereoisomers for 3 and 4. All subsequent analyses were performed on the mixtures and reported as ‘apparent’ results.

Figure 1. a) Chemical structures of BL660 esters 1-4. b) Total luminescence count per hour for compounds 1, 2, 3, and 4 (10 µM) incubated in PBS (pH 7.4) at 37 °C for 1, 6, and 12 hrs. (n = 3) c) Total luminescence count per minute for compounds 1, 2, 3, and 4 (10 µM) after treatment with porcine liver esterase (0.1 units/mL) at 37 °C for 20, 40, and 60 mins. (n = 3) d) Total luminescence count per hour for compound 4 (10 µM) at pH 5.0, 7.4, and 9.0 at 37 °C for 2, 3, and 4hrs. (n = 3) e) Normalized total luminescence count for compound 4 (5 µM) exposed to various ester-hydrolyzing enzymes. (n = 3)

Assessment of Hydrolytic Stability and Esterase Reactivity
We began our analysis of stability by incubating BL660 esters 1-3 at 37 °C in PBS (pH 7.4). After 1, 6, and 12 hours, luciferase was added to initiate BL production. The rate of hydrolysis, reported as total luminescence counts per hour, indicates hydrolysis in descending order is: 1 (13.9 ± 1.4 × 10^3), 2 (3.3 ± 0.6 × 10^3), and 3 (1.0 ± 0.1 × 10^3) (Figure 1b and S1). These results show the methyl substituent is over three-fold more effective at blocking hydrolysis than the phenyl group. We speculate hydrolysis of 3 may involve an Sn1 mechanism, thus favoring formation of a double benzylic carbocation. On the contrary, when the panel was treated with porcine liver esterase (0.1 units/mL, 37 °C, pH 7.4) for 20, 40, and 60 minutes, we found that 2 was most resistant toward enzymatic degradation. The enzymatic rates, reported as total luminescence counts per minute, in descending order are: 1 (31.0 ± 6.8 × 10^2), 3 (9.5 ± 1.2 × 10^2), and 2 (0.6 ± 0.1 × 10^2) (Figure 1c and S2). The greater stability imparted by the aromatic ring is not surprising since esterase reactivity can be readily attenuated with bulkier substituents. With this data, we hypothesized the hydrolytic stability of 3 can be retained (or improved) by switching to a larger alkyl group with multiple rotatable bonds (i.e., isopropyl (Pr)), while simultaneously suppressing esterase reactivity. To our delight, we observed significant improvements of both properties with this modification. Relative to the parent benzyl ester, 4 is 140.5-fold more resistant to hydrolysis and exhibits 67.8-fold greater esterase stability (Figure 1b and 1c).

Following this set of experiments, we subjected 4 to acidic (pH 5) and alkaline (pH 9) conditions to account for the various pH gradients in the body. For these experiments we used the Britton–Robinson buffer system. As reported in total luminescence counts per hour, 4 was most stable at pH 7.4 (8.6 ± 1.3 × 10^2), followed by pH 5.0 (25.7 ± 9.6 × 10^2), and finally pH 9.0 (25.8 ± 20.7 × 10^2) (Figure 1d). Lastly, we examined the stability of 4 against a panel of enzymes beyond porcine liver esterase that are known to possess ester cleaving activities. These include lipase, human carboxyesterase-2, monoacylglycerol lipase, fatty acid amide hydrolase, and aldehyde dehydrogenase 1A1. Remarkably, exposure to high levels of these enzymes over a period of one hour resulted in minimal activation (Figure 1e).

**Demonstration of PhPr Ester Stability in Live Cells**

Next, we designed a series of two complementary cellular experiments to determine whether the excellent *in vitro* performance noted above translates within a more complex cellular environment where many esterase isozymes are present. Moreover, we aimed to determine whether the PhPr ester will have a similar effect on other molecules with diverse chemical structures beyond BL660.

First, we prepared BL660-Me, a methyl ester derivative of BL660 (Figure 2a). Next, we cultured 4T1 murine breast cancer cells, stably expressing the luciferase enzyme (4T1-Luc). The cells were then treated with a DMSO vehicle control, BL660-Me or compound 4 (Figure 3a and S3). We postulate that the BL signal will be lower if the optimized ester is indeed more stable in live cells since less of the luciferase substrate will be liberated and made available to generate light. Analysis of the imaging results revealed that the total luminescence count for BL660-Me-treated 4T1 cells (94.1 ± 5.9 × 10^3) were 55-fold and 31-fold more luminescent relative to the vehicle (1.7 ± 0.8 × 10^3) and compound 4 (3.1 ± 1.2 × 10^3) counterparts, respectively (Figure 3b). These results indicate the PhPr ester is stable to the collection of intracellular esterases present in 4T1 cells.

Next, we modified the two carboxylates in methotrexate (a potent anticancer and immunosuppressive drug) to afford the methyl (MTX-Me) or PhPr (MTX-(PhPr)2) ester variants (Figure 2b). Structure–activity relationship analysis has shown that carboxylate masking decreases target engagement, leading to a significant reduction of cytotoxicity (IC50). We hypothesized if the PhPr esters installed onto methotrexate remain intact, cells treated with MTX-(PhPr)2 will remain viable. To test this, A549 cells were cultured and treated with MTX-Me2 or MTX-(PhPr)2 at various concentrations for 24 hours before they were subjected to MTT analyses. Our results revealed the viability was ~63% and 100% at the highest concentration (10 µM) of MTX-Me2 and MTX-(PhPr)2 tested, respectively (Figure 3c and 3d). This experiment demonstrates that the hydrolysis of the esters on MTX-(PhPr)2 is sufficiently slow that the build up of MTX was inconsequential to the health of the cells.

![Figure 2](image2.png) Figure 2. Chemical structures of a) BL660 and b) MTX and the Me and PhPr ester derivatives.

![Figure 3](image3.png) Figure 3. Representative BL images of 4T1-Luc cells plated in 24-well plates treated with a) DMSO (vehicle); BL660-Me; or Compound 4 for 30 mins at 37 °C. Probe concentration = 10 µM (a) Quantified data from a). n = 4. MTT viability assays of A549 cells treated with c) MTX-Me2 or d) MTX-(PhPr)2 at various concentrations for 24 hours. n = 3. Error bars = SEM. Statistical analysis was performed using a two-tailed t-test (α = 0.05, **** P< 0.0001).
Conversion of PhPr Ester into a Versatile Self-Immolative Linker

While we envision nearly any electron-deficient trigger can be installed at the para (or ortho) position of the PhPr ester to yield an ABS probe for NIR BL imaging, we strategically selected to use the nitro group to develop BL660-NTR. In addition to its common use in probe design for other modalities, this biomarker is a feature of gram positive and negative bacteria and thus, can be leveraged for sensing bacterial infections. Moreover, NTR can also be employed to detect tumor hypoxia owing to overexpression in many types of solid tumors. Once our probe engages its target, the aryl nitro moiety can be reduced to afford the electron-rich hydroxylamine or amino products, which initiates self-immolation to release BL660. In the presence of luciferase, this substrate is then enzymatically converted to a dioxetanone, which decomposes to generate oxy-BL660 in an excited state. Relaxation to the ground state is accompanied by the emission of a photon at 660 nm (Scheme 2).

The synthesis of BL660-NTR began with subjecting 4-nitrobromobenzene to phenyllithium to initiate a lithium-haloegen exchange reaction. The resulting 4-nitrophenyllithium intermediate was used directly without purification to attack 2-methyl propanaldehyde to give benzyl alcohol in 25% yield. Then utilizing the acyl fluoride chemistry we optimized above, BL660-NTR was obtained in 10% yield (Figure 4a).

With BL660-NTR in hand, we performed an in vitro assay to demonstrate that the BL output depends on both NTR and luciferase activity (Figure 4b and S4). When each component is present, we observed a notable increase in the total luminescence count. However, when NTR (column 2) was absent, the signal was significantly attenuated. Likewise, when luciferase (column 3) or BL660-NTR (column 4) was excluded from the reaction, the signal was minimal and comparable to control wells containing only media and a DMSO vehicle. Next, we subjected BL660-NTR to various biologically relevant analytes that may contribute to off-target probe activation in vivo. For instance, transition metals like Cu(II) can facilitate ester hydrolysis via Lewis acid-mediated ester activation. Additionally, various reactive oxygen and nitrogen that can interact with the ester bond were also tested. Lastly, we also examined reactive sulfur species, which are known to reduce aryl nitro groups. Under no circumstance did we observe activation greater than 10% compared to the NTR positive control (Figure 4c). Additional experimental details including NMR and MS characterization and dose dependent NTR studies (Figure S5), can be found in the supporting information document.

Scheme 2. Schematic showing NTR-mediated activation of BL660-NTR under hypoxic conditions to afford BL660. Subsequent luciferase-catalyzed conversion to the dioxetanone intermediate is followed by spontaneous decomposition to the oxy-BL660 product to generate NIR BL.

Figure 4. a) Synthetic route to access BL660-NTR. b) In vitro assay demonstrating that the probe, NTR, and luciferase must all be present to generate a signal: BL660-NTR (5 μM), NTR (0.1 units), and luciferase (0.0125 mg/mL final concentration). c) Response to biologically relevant analytes that may cause interference. d) MTT assay at various concentrations of BL660-NTR (0, 5, 10, 20 μM). Green and orange represents 1- and 3-hour incubation, respectively. e) Representative BL images of A549 cell lysates obtained after cells were cultured in a 1% or 20% oxygen atmosphere for 2 days prior to incubation with BL660-NTR for 60 mins. f) Quantified data from e). n = 4. Error bars = SEM. Statistical analysis was performed using a two-tailed t-test (α = 0.05, **** P < 0.001).

Figure 4. a) Synthetic route to access BL660-NTR. b) In vitro assay demonstrating that the probe, NTR, and luciferase must all be present to generate a signal: BL660-NTR (5 μM), NTR (0.1 units), and luciferase (0.0125 mg/mL final concentration). c) Response to biologically relevant analytes that may cause interference. d) MTT assay at various concentrations of BL660-NTR (0, 5, 10, 20 μM). Green and orange represents 1- and 3-hour incubation, respectively. e) Representative BL images of A549 cell lysates obtained after cells were cultured in a 1% or 20% oxygen atmosphere for 2 days prior to incubation with BL660-NTR for 60 mins. f) Quantified data from e). n = 4. Error bars = SEM. Statistical analysis was performed using a two-tailed t-test (α = 0.05, **** P < 0.001).
In vivo Application of BL660-NTR to Detect Tumor Hypoxia

Finally, we employed BL660-NTR in vivo to image tumor hypoxia (via NTR activity). Hypoxia is defined as a condition where the demand for oxygen from rapidly dividing cancer cells, outweigh the available supply. This condition emerges as the distance between the cancer cells and nutrient-rich blood vessels become greater as a function of tumor growth. It is estimated that ~50% of solid tumors are hypoxic, it is impossible to know whether a given lesion is oxygen-deficient without a reliable readout. This knowledge becomes critical when using luciferase-expressing cells to study cancer biology because hypoxia can significantly influence tumor properties (e.g., drug resistance, tumorigenesis, etc.). Of note, although several NTR probes for BL have been developed previously, the first example capable of deep-tissue detection of hypoxia via NIR BL imaging.

NU/J mice (male, ~5-weeks old) were inoculated with A549-Luc2 lung cancer cells (5 × 10⁶) in both flanks. Testing of BL660-NTR commenced after tumors had grown to a volume of ~300 mm³ (~4 weeks). First, we performed imaging using BL660-NTR, which was administered systemically via retroorbital injection (Figure 5a and S7). ROIs were drawn around each tumor and the luminescent count was summed for each animal. By the first scan (5 min), strong BL signals were apparent in all tumors. The BL intensity appears to decrease slightly after 15 mins and levels off until the end of the experiment (75 mins) (Figure 5c). Next, to distinguish between NTR activation and off-target ester hydrolysis, we employed compound 4 as a control reagent, which only differs in that the nitro trigger is absent (Figure 1a). After approximately 20 hours, the same animals were treated with compound 4 and imaged (Figure 5b and S8). This delay was to ensure the probe had fully cleared from the first experiment. Despite dosing at the same concentration, the BL signal in the tumors at all time points were barely discernable from background (Figure 5c). The total luminescent count after 15 mins for compound 4 was only 4.0 ± 0.7 × 10⁴, whereas the corresponding readout was 16.6 ± 2.5 × 10⁴ for BL660-NTR. This represents a 5.2-fold difference, indicating successful detection of tumor hypoxia (Figure 5d).

CONCLUSION

The growing diversity of ABS probes for BL imaging has enabled the study of various important analytes using luciferase-expressing cells and animal models. Most of these examples are prepared by capping the phenolic alcohol of luciferin (or related molecules) with an analyte-responsive trigger, which upon removal can interact with luciferase to yield BL. While luciferin and probes derived from this parent molecule boast excellent BL properties (e.g., strong signal output), the emission wavelength is still in the visible window (λ_max ~ 560 nm), limiting the attainable imaging depth. A transition to the NIR region (>650 nm) can improve tissue penetration since there are less optical absorbers (e.g., amino acids and nucleic acids) that can intercept, and scatter the emitted light. However, red-shifting luciferase substrates often entails exchanging the alcohol group with a dialkylamino substituent, which prevents installation of triggers at this position. This was in fact the case with NIR emitting BL660, which features a N,N-diethylamino group. Thus, our only option was to cap the carboxylate with a trigger; however, as discussed previously, the variety of suitable triggers are severely limited.

In this work, we overcame this limitation by rationally developing a hydrolysis-resistant ester that is also remarkably stable against enzymatic degradation. By installing a trigger at the para- or ortho-positions of this ester, we can leverage
self-immolative chemistry to afford NIR ABS probes via carbonate-masking and subsequent unmasking. Specifically, inclusion of a nitro group transformed the phenylacetate ester into an ABS linker for sensing hypoxia in vivo via NTR activity. Our probe, BL660-NTR, is the first NIR BL probe for this target, and only the second NIR emitting ABS probe ever developed. Beyond sensing hypoxia, our new self-immolative linker can be readily adapted to develop probes for other analytes.

In addition to modifying BL660, we have also successfully masked methotrexate, which is notorious for severe side-effects when administered to patients owing to non-specific destruction of healthy cells. Moreover, carbonates are found in many other medications, including antibacterial agents (ceftazidime, ciprofloxacin, and sulbactam). As such, the pursuit of prodrugs via carbonate masking using our technology is an exciting avenue.

**EXPERIMENTAL DETAILS**

**Synthesis of BL660-NTR**

2-Methyl-1-(4-nitrophenyl)propan-1-ol (6). A solution of phenyllithium in dibutyl ether (1.9 M, 1.69 mL, 3.21 mmol, 1 equiv.) was slowly added to a cooled (-78 °C) solution of 4-nitro iodobenzene (0.8 g, 3.21 mmol, 1 equiv.) in anhydrous THF (16 mL) under N₂. The reaction was stirred for 45 min at the same temperature to afford 5 in situ, then a solution of isobutyraldehyde (0.32 mL, 3.50 mmol, 1 equiv.) in anhydrous THF (3 mL) was added dropwise. The reaction was stirred for an additional one hour at -78 °C before warming to room temp. The reaction mixture was then quenched with saturated aqueous NH₄Cl, transferred to a separatory funnel, and extracted with diethyl ether (3 × 15 mL). The combined organic layers were dried over Na₂SO₄, filtered, concentrated, and purified using silicon gel column chromatography (eluents: 3:1 v/v EtOAc:Hexanes) to afford 6 in 20% yield. (0.126 g, 0.64 mmol). ¹H NMR (500 MHz, CDCl₃) δ 8.20 (d, J = 8.9 Hz, 2H), 7.49 (d, J = 8.7 Hz, 2H), 4.56 (dd, J = 6.1, 3.2 Hz, 1H), 2.01 – 1.94 (m, 2H), 0.95 (d, J = 6.7 Hz, 3H), 0.87 (d, J = 6.9 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 175.00, 150.15, 135.56, 123.53, 123.41, 127.41, 147.33, 140.07, 133.33, 140.07, 147.20, 170.41. HRMS [M+H⁺] calculated mass for C₂₃H₁₆N₂O₃S = 508.2270, found = 508.2262.

**MTT Assay using MTX-Me₂ and MTX-Pr₂**

48-well plates were seeded with 30,000 A549 cells per well (500 µL of 60,000 cells/mL) and incubated at 37 °C with 5% CO₂ for 24 hours. Media was removed and fresh serum-free RPMI 1640 containing 0.001, 0.01, 0.1, 1, 10 µL of MTX-Me₂ or MTX-(Pr₂) were added (0.1% DMSO final v/v). The media was removed after 24 hours incubation and replaced with 500 µL of a 20:1 mixture of serum-free RPMI 1640 and (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL stock in PBS). The cells were incubated for 2 hours under the same condition and then the medium was removed and replaced with DMSO (500 µL/well). The absorbance of each well was recorded at 555 nm on a microplate reader. Viability was calculated by the absorbance relative to the vehicle control.

**Detection of NTR Activity in A549 Lung Cancer Cells**

A549 cells were seeded in T75 culture flasks and incubated in 1% or 20% oxygen atmosphere for 48 hours. A solution of BL660-NTR in DMSO (2 mM, 50 µL) was quickly added into each flask such that the final concentration of the probe is 10 µM and 0.5% DMSO. The cells were incubated with the probe for 60 minutes (under the respective culture conditions), washed with fresh PBS, and detached from the culture flasks. The cells were then transferred to a 15 mL centrifuge tube and pelleted at 1000 rpm for 5 min at room temp. The cells (4.25 × 10⁶ cells/mL) were then resuspended in PBS along with 10% protease inhibitor solution (1 protease inhibitor mini tablet per 10 mL PBS, Pierce, Thermo Fisher Scientific) and sonicated on ice for 2.5 minutes (pulse 01, 01, 40%). The cell debris was removed via centrifugation at 4 °C. The cell lysates were further diluted 2.5-fold by PBS and 475 µL were transferred into 24-well plates (n = 4 for each condition), treated with luciferase (25 µL, 0.25 mg/mL), and imaged immediately using the IVIS imaging system. Bioluminescence light was collected in open mode (no filters were applied). ROIs were drawn around each well. The signal intensity was quantified using the Living Image Analysis Software.

**BL Imaging of Hypoxia in a Lung Cancer Model**

NU/J mice bearing A549-Luc2 tumors were anesthetized using isoflurane (1-3% for maintenance; up to 5% for induction) in oxygen from a precision vaporizer. After testing to ensure animals are fully under anesthesia, an initial background scan is recorded. BL660-NTR or compound 4 formulated in 3:7 v/v DMSO:PBS (see SI for details) was then administered via retroorbital injection. Images were captured using the IVIS imaging system at 5,
15, 30, 45, 60, and 75 minutes after injection. Bioluminescence light was collected in open mode (no filters were applied). ROIs were drawn around each tumor and summed for each animal. The signal intensity was quantified using the Living Image Analysis Software.

Supporting Information.
Further experimental details, including synthetic procedures, spectral data, and supplemental in vitro and in vivo procedures and data are supplied in the Supporting Information document. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
* Jefferson Chan
Email = jeffchan@illinois.edu

Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources
This work was supported by the National Institutes of Health (R35GM133581) and in part by a grant awarded to JC from the Chemistry Discovery Fund established by Ving & May Lee.

ACKNOWLEDGMENT

CJB acknowledges the Chemistry-Biology Interface Training Grant (T32-GM136629) and previous support from the Robert C. and Carolyn J. Springborn Graduate Fellowship. SHG thanks the Cancer Center at Illinois for a Graduate Student Cancer Scholarship. JC thanks the Helen Corley Petit Scholar Program and the Camille and Henry Dreyfus Foundation. Major funding for the 500 MHz Bruker CryoProbe was provided by the Roy J. Carver Charitable Trust (Muscatine, Iowa; Grant No. 15-4521) to the School of Chemical Sciences NMR Lab. The Q-ToF Ultima mass spectrometer was purchased in part with a grant from the National Science Foundation, Division of Biological Infrastructure (DBI-0100085). We also acknowledge Dr. Iwona Dobrucka and the Molecular Imaging Laboratory at the Beckman Institute for use of the IVIS imaging system.

ABBREVIATIONS

BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; GFP, green fluorescent protein; IC_{50}, half maximal inhibitory concentration; MS, mass spectrometry; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIR, near-infrared; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; ROI, region of interest; rpm, rotations per minute; SEM, standard error of the mean; S_A, unimolecular nucleophilic substitution.

REFERENCES


(35) Hoy, J.; Pinto da Silva, L.; Esteves da Silva, J. C. G.


8565. https://doi.org/10.1021/ja101766r.


