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A Selenium Analogue of Firefly D-Luciferin with Red-Shifted Bioluminescence Emission**

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Bioluminescence imaging (BLI) makes use of light-generating luciferase enzymes, most commonly firefly luciferase (FLuc), *Renilla reniformis* luciferase or *Gaussia* luciferase in combination with their appropriate substrates.^[1] Noninvasive BLI of living subjects has become a routine technique in cancer biology research because it enables monitoring of gene expression, gene delivery, tumor growth, enzyme activity, response to experimental drug therapies, and protein-protein interactions.^[2]

The major limitation in *in vivo* BLI experiments is absorption and scattering of light by tissue, which results in strong attenuation of BL signal emitted below 600 nm.^[3] The yellow-green BL emission from the native FLuc (D-luciferin substrate, $\lambda_{em,max}=553-559$ nm),^[4] which is the most red-shifted native bioluminescent system, decreases substantially with tissue depth. Consequently applications of this system are restricted mainly to small animals at superficial depths. To overcome this limitation, red-shifted variants of native FLuc have been selected using random mutagenesis.^[5] Considerable efforts have also been directed toward the development of analogues of D-luciferin^[6] that produce longer wavelength light, an orthogonal approach to enzyme engineering. A recent report describes the development of a D-luciferin analogue, which emits in the near-infrared region.^[6e] This analogue is an aminoluciferin-Cy5 conjugate and is based on bioluminescence resonance energy transfer (BRET). This type of modification, however, alters the cellular uptake properties of the substrate and likely changes its biodistribution *in vivo*. Here we describe a

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simple modification to D-luciferin, a selenium analogue, which exhibits red-shifted BL emission, and we demonstrate its use for *in vivo* BLI.

We designed a D-luciferin analogue containing a selenium atom substitution for the native sulphur atom at position 1 (**3c**). We hypothesized that this replacement would red shift the emission maximum because of the polar effect of the selenium atom, which has been previously reported in fluorophores.^[7] Furthermore, it is known that several enzymes recognize selenium analogues equally well as the natural sulphur-containing substrates,^[8] making it likely that the derivative would be an efficient luciferase substrate. Finally, on account of the red shift induced by the 6'-amino substituent in **3b**, and the usefulness of the amino group in the preparation of bioluminogenic substrates,^[2d,9] we chose to preserve this functionality in our design.

The synthesis of native luciferin **3a**^[10b-d] and its analogue 6'-aminoluciferin **3b**^[6b] is straightforward and involves a condensation reaction^[10] between 2-cyanobenzothiazole **1a,b** and cysteine **2a**. **3b** is a competent substrate for FLuc and exhibits red-shifted BL with $\lambda_{em,max} = 578$ nm.^[11] The only known D-luciferin analogues tolerated by native FLuc consist of 4'- or 6'-substitutions on the benzothiazole ring^[6a-b] and substitution of the benzothiazole for naphthalene or quinoline,^[12] though aliphatic^[6c] and cyclic alkylaminoluciferin^[6f] substrates can generate light with mutated variants of FLuc. We coupled seleno-D-cysteine **2b** with 2-cyano-6-aminobenzothiazole **1b** at room temperature in 0.5 M Tris-HCl (pH 7.5) buffer with 13% DMSO to afford aminoseleno-D-luciferin **3c**. The product was isolated by HPLC in 78% yield. Because the L-enantiomer of luciferin generates no BL signal but inhibits FLuc,^[13] it is desirable to employ enantiomerically pure seleno-D-cysteine **2b** in the synthesis, which can be prepared from elemental selenium.^[14] In this manner, [⁷⁵Se]**3c** and [⁷⁷Se]**3c** for dual modality BLI/PET and BLI/MRI, respectively, are readily accessible. Finally, we note that other chalcogen-substituted cysteines, such as tellurocysteine **2c**, which is known to react analogously to **2a**, might also be successfully incorporated in the reaction shown in Scheme 1.

The absorbance spectra of amino-D-luciferin **3b** and its selenium analogue, aminoseleno-D-luciferin **3c**, are nearly identical with both containing a local maximum at 350 nm in 50 mM Tris-HCl (pH 7.5) buffer, and the molar absorptivities were determined to be 15,100 M⁻¹cm⁻¹ and 15,500 M⁻¹cm⁻¹, respectively. The value for **3b** is in good agreement with a previous measurement in 95% ethanol (15,500 M⁻¹ cm⁻¹).^[6a]

In the presence of ATP, Mg²⁺, and oxygen, **3c** is a competent substrate for purified native FLuc, emitting red BL that is readily distinguished from the yellow-green BL of D-luciferin **3a** and the orange BL of amino-D-luciferin **3b** (Figure 1a). The BL emission maxima for **3a**, **3b**, and **3c** were 559 nm, 588 nm, and 600 nm, respectively (Figure 1b). The red-shifted BL of **3c** also persists at 37 °C (data not shown). The percentage of integrated BL emission above 600 nm—an important figure of merit for transmission through tissue in *in vivo* BLI—for **3c** is 55%, which is higher than the values for **3a** and **3b**, 23% and 41%, respectively.

To determine the apparent Michaelis constant K_m of **3c**, which is defined as the substrate concentration at half of the maximum reaction velocity, the initial rate of luminescence was measured as a function of substrate concentration and plotted in Figure 2a. The reaction of FLuc with **3c** exhibits typical Michaelis-Menten kinetics, as evidenced by the linearity of the Lineweaver-Burk plot (Figure 2b). Nonlinear regression analysis of the Michaelis-Menten plot (Figure 2a) generated a K_m of 0.70 ± 0.04 μ M for **3c**, which is not statistically different by *t*-test from the value reported for **3b**, 0.62 ± 0.05 μ M (see Supporting Information).^[11] Thus, substitution of the sulphur atom for selenium does not appreciably perturb the affinity of the substrate for FLuc.

To compare the emission rates of **3b** and **3c** under biologically relevant conditions, both substrates were incubated with a human breast adenocarcinoma cell line (MDA-MB-468) that stably expressed FLuc. The time course of BL emission in cell culture for each substrate, shown in Figure 3, qualitatively resembles the *in vitro* time course obtained using purified FLuc (see Supporting Information). Following an initial period characterized by flash kinetics, **3c** exhibited a lower and more stable basal emission rate relative to **3b**; after 10 min, the signal from **3b** decayed in a quasi-linear manner with an emission rate decrease of approximately 2.5×10^4 photons $\text{s}^{-1} \text{min}^{-1}$ ($R^2 = 0.994$) while **3c** decayed considerably slower. Extrapolation of each line to $y = 0$ followed by integration of the area under each curve indicates that **3c** emitted approximately 74% the number of photons of **3b** (see Supporting Information), suggesting a lower quantum yield for **3c** if equal numbers of molecules reacted. Indeed, more efficient non-radiative relaxation would be expected for **3c** on account of the well known heavy-atom effect.^[15] A reduced quantum yield is not sufficient to explain the differences in the kinetic profiles of **3b** and **3c**, however. Recent work by Ribiero *et al.*^[16a] indicates that dehydroluciferyl adenylate (L-AMP), a non-BL byproduct of the reaction,^[17] behaves as a potent competitive inhibitor of FLuc ($K_i = 3.8$ nM),^[16,18] and is responsible for much of the early decrease in BL. The faster initial decay of **3c** and its lower basal level could be explained by the dehydroamino-selenoluciferyl-AMP byproduct having a lower K_i than the corresponding sulphur analogue.

The *in vivo* performance of **3c** was also compared to **3b** in nude mice bearing subcutaneous tumor xenografts of an MDA-MB-468 cell line constitutively expressing FLuc (FLuc+). Each mouse was sequentially injected with either substrate, with a 5 h delay observed between substrates to allow for enzyme recovery and substrate clearance. Following tail vein injection of either substrate into mice ($n = 4$), BL emission was imaged at several time points (Figure 4a). To account for differences in tumor sizes among the mice, BL at each time point and for each substrate was normalized to the maximum BL signal. This always occurred with substrate **3b** at the first time point ($t = 1$ min). The normalized BL values for each substrate were averaged at each time point and plotted with their standard deviations (Figure 4b).

In contrast to *in vitro* studies, the time courses of *in vivo* BL for **3b** and **3c** are comparable; with the exception of the 5 min time point in Figure 4b, there is no statistically significant difference between the substrates ($P > 0.05$). The discrepancy between *in vitro* and *in vivo* experiments cannot be fully explained by the theoretically higher penetration of the red-shifted BL of **3c** through tissue (~34%) because this effect is more than offset by its slower emission rate (see Figure 3). The differentially improved performance of **3c** in *in vivo* imaging may be attributed to some organism-level process, such as temperature regulation, perfusion, biodistribution, or elimination.^[19] We note that such a process need only induce a small change in the steady-state concentration of a potent inhibitor species (e.g., L-AMP) relative to *in vitro* conditions to induce a large change in the BL emission kinetics.

In conclusion, we have described the first selenium-containing luciferin **3c** and demonstrated that it is a competent substrate for native FLuc, exhibiting a red-shifted BL maximum relative to its sulphur-containing analogue **3b**. Though the substitution does not decrease the substrate's affinity for FLuc, it results in less light output *in vitro*, due in part to a lower quantum yield. For *in vivo* BLI applications, the substitution is benign; it has no net effect on the emission kinetics, likely due to a trade-off between greater tissue penetration of the BL emission and lower quantum yield.

Our demonstration of the feasibility of chalcogen substitution without a detrimental effect upon *in vivo* BLI enables the prospect of luciferin probes for multimodal imaging. One implementation for BLI/MRI relies upon the ability of FLuc to catalyze the conversion of

luciferin to oxyluciferin, for which the enol tautomer is the predominant ground-state species.^[20] Thus, the chalcogen at position 1, which is singly allylic in the substrate, becomes doubly allylic in the product. As both ⁷⁷Se and ¹²⁵Te are stable spin-½ nuclei with narrow lines and a wide range of chemical shifts, this chemical shift change is, in principle, detectable by MRI; we suspect that hyperpolarization might be required for the low sensitivity ⁷⁷Se nucleus.²¹ If successful, this method could be generally applied to BL/MR imaging of any luciferase-expressing mouse tumor model.

Experimental Section

Synthesis of **3c**

Seleno-D-cystine (5.7 mg, 0.017 mmol), synthesized according to ref. 14, was reduced to **2b** by incubation in a Tris-HCl (0.5 M, pH 7.5, 2.1 mL) solution of tris(2-carboxyethyl)phosphine hydrochloride (14.7 mg, 0.0513 mmol) for 5 min. The aqueous solution of **2b** (0.034 mmol, 2 eq) was added to a solution of **1b** (3.0 mg, 0.017 mmol, 1 eq) in DMSO (300 µL), mixed thoroughly, and allowed to react for 1.5 h at room temperature under an atmosphere of nitrogen. Aliquots of the reaction mixture were separated by reverse-phase HPLC using an Acclaim 120 C18 column with gradient elution (0% to 85% acetonitrile with water balance over 30 minutes; both elution solvents contained 0.1% trifluoroacetic acid; 1 mL/min flow-rate). Upon elution, fractions of **3c** were combined and subjected to rotary evaporation, yielding **3c** as a light-yellow solid (78%).

Bioluminescence photos

The photos in Figure 1a were taken in the dark with a Nikon D70 digital camera equipped with an AF-S Nikkor 18–70 mm DX lens. BL solutions were prepared as described for the BL spectra (*vide infra*), but on a larger scale and with 10× the concentration of FLuc. To collect the maximum amount of light, the largest available aperture size was employed (f3.5). Integration times were 10 s for **3a** and 30 s for **3b** and **3c**.

Bioluminescence spectra

To a solution containing 40 µL of pH 7.4 PBS buffer (Dulbecco's, 1×), 20 µL of ATP buffer (Stratagene; 0.5 mM ATP, 0.5 mM CoA, 10 mM Mg(II) sulfate, pH 7.8), and 1 µL of 1.5 mM luciferin substrate **3** in pH 7.4 PBS buffer was added 5.0 µL of FLuc solution (130 µg mL⁻¹ in pH 7.4 PBS buffer containing 1% BSA) with mixing, and incubated in a small-volume cuvette (Starna, 16.40F-Q-10/Z15) for 2 min to allow the BL signal to stabilize. Spectra were collected using a wavelength-calibrated FluoroMax-3 fluorimeter (Horiba Jobin Yvon) with a blocked excitation path (0.5 s integration time; 1 nm increments; 10 nm emission slit).

Determination of K_m

Two-fold serial dilutions of **3c** were performed using 1× PBS (pH 7.4) to give eight 50 µL solutions ranging in concentration from 32.3 µM to 0.252 µM, each in a 1.5 ml microfuge tube. To each tube was added 100 µL of Luciferase Assay Buffer II (Promega) supplemented with 3.31 mg/ml ATP (6 mM). Immediately before measuring the luminescence of a sample, 50 µL of a freshly prepared 1.1 µg/ml QuantiLum Recombinant Luciferase (Promega) solution in 1× PBS was added to the tube. The solution was briefly vortexed, and the resulting luminescence emission was measured over 10s using a Turner Biosystems 20/20n luminometer. K_m was determined by nonlinear regression analysis of the Michaelis-Menten plot using the Enzyme Kinetics Wizard in the SigmaPlot 12.0 software package.

Cell culture kinetics

To a 60 μL suspension of $\sim 6 \times 10^4$ MDA-MB-468 FLuc+ cells in pH 7.4 PBS buffer was added 20 μL of 100 μM substrate **3b** or **3c** in pH 7.4 PBS buffer with gentle mixing. The time course of BL was measured using an IVIS200 with 10 s integration at each time point (no emission filter).

In vivo kinetics

All animal handling was performed in accordance with Stanford University's Animal Research Committee guidelines. Nude, athymic mice were subcutaneously implanted with 10^7 MDA-MB-468 FLuc+ cells at a single site. Once tumors grew to ~ 5 mm in diameter (~ 10 days), the mice were injected with 100 μL of 2.5 mM substrate **3b** or **3c** via the tail vein. Mice were anesthetized with isoflurane, and the time course of BL emission from the tumor was measured using IVIS200 with 30 s integration at each time point (no emission filter). Each substrate was tested in four different mice, and a 5 h delay between same-mouse experiments was employed to allow for clearance and FLuc recovery. In the absence of substrate, mice exhibited no BL. No toxic effects were observed with **3c**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) Bioluminescence photographic images of **3a** (left), **3b** (middle), and **3c** (right). b) *In vitro* bioluminescence spectra of **3a**, **3b**, and **3c** at 23 °C.

Figure 2.

a) Michaelis-Menten plot for **3c** using light output from luminescence as a readout of the reaction rate. b) The same data represented in a Lineweaver-Burk plot.

Figure 3.

a) Time course of baseline-corrected BL from equimolar solutions of **3b** and **3c** in cell suspensions of MDA-MB-468 expressing FLuc. Lines represent the least squares best fits for the quasi-linear basal signals from **3b** and **3c** after 10 min. b) False-color BL intensity images superimposed on the corresponding white light images of the wells at various times.

Figure 4.

a) Representative BLI of a nude mouse implanted with FLuc+ MDA-MB-468 xenografts after i.v. injection of either **3b** or **3c**. b) Time course of tumor-localized BL after i.v. injection of either **3b** or **3c** into mice implanted with FLuc+ MDA-MB-468 xenografts. Each data point represents the average baseline-corrected BL signal from four mice. To account for variations in tumor size, BL signals for each tumor were normalized to the maximum signal obtained from that tumor before averaging; the maximum signal was always obtained with substrate **3c** at $t = 5$ min. y -Error bars show the standard deviation of BL.

Scheme 1.

Structure and synthesis of D-luciferin (**3a**), amino-D-luciferin (**3b**) and designed aminoseleno-D-luciferin (**3c**).