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Evaluation of cyclic luciferin as a substrate for luminescence measurements in *in vitro* and *in vivo* applications

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ABSTRACT

Bioluminescence imaging (BLI) is a powerful tool for cell tracking, monitoring of gene delivery and expression in small laboratory animals. An alternative luciferase (Luc) substrate cyclic luciferin (Cycluc) was recently advanced for BLI applications as providing a stronger, more stable signal at significantly lower doses than the classical substrate D-luciferin (D-Luc) increasing sensitivity of Luc detection 10 to 100 times. We evaluated benefits of using Cycluc in *in vivo* studies in mice injected with murine adenocarcinoma 4T1 cells expressing Luc, and in single-cell organisms, the oocytes of *Xenopus laevis*. No significant increase in the efficacy of detection of the luminescent signal was recorded in either of the systems. Kinetic studies demonstrated that Km for Cycluc was 10000 higher, whereas Vmax was 100 lower than that of D-Luc. Cycluc efficiently bound to the active center of luciferase, but its turnover was extremely low, leading to actual inhibition of bioluminescence. This compromises Cycluc as a substrate for measurement of the activity of the wild-type luciferases, still widely used as reporters for *in vivo* monitoring microorganisms and tumor cells. It may find better applications with the development of *in vivo* imaging based on the genetically engineered mutant luciferases with different substrate requirements.

1. Introduction

Bioluminescence imaging is a powerful tool for both *in vitro* and *in vivo* monitoring of cell growth and migration [1,2]. Luciferases which generate a bioluminescent signal through oxidation of a luciferin substrate, are the most common reporter enzymes employed for high throughput screening assays. The two most common are the luciferases from the firefly *Photinus pyralis* and the sea pansy *Renilla reniformis*. In the case of firefly luciferase (Luc), bioluminescent light is produced by the chemical reaction of Luc enzyme with its substrate, D-luciferin (D-Luc) or its analogues [3,4]. Luc uses D-Luc substrate and ATP to form a luciferyl-adenylate intermediate. This intermediate undergoes nucleophilic attack by molecular oxygen with subsequent displacement of AMP, leading to formation of an unstable dioxetanone. The latter spontaneously degrades to oxyluciferin, and CO_2 with the emission of a photon [5].

Generation of signal requires no excitation, translating into a very low background signal, and high signal to background ratio inherent to bioluminescence assays making them specifically attractive for *in vivo* bioluminescent imaging (BLI). The most common *in vivo* BLI method entails genetic or (bio)chemical labelling of biological objects with Luc introduced into an animal model, with subsequent intraperitoneal administration of D-luc followed by capture of the light emission with a CCD camera [6].

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Photon emission by Luc using D-Luc as a substrate peaks at 560 nm [7]. The wavelength of the emitted light greatly impacts the signal to noise ratio as the light is subject to absorption and scattering while passing through tissue. The near-infrared window of 650–900 nm is optimal for measurements due to a lower tissue absorption capacity [8]. Several modifications to luciferin were proposed which would allow to shift the wave-length of emitted light into the near-infrared spectrum, and also to increase the quantum yield [4,9,10]). One such recently proposed is a synthetic Luc substrate 2-(6,7-Dihydro-5H-thiazolo[4,5-f]indol-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (Cycluc) [11]. Enzymatic digestion of Cycluc leads to emission at 599 nm, i.e. a notable shift to the infrared spectrum [10]. Cycluc was promoted as allowing to use a lower dose while providing stronger and more stable signal [11]. However, earlier when used as a Luc substrate in *in vitro* tests, it was shown to generate a lower light output than the classical D-Luc substrate [10].

The focus of this study was to evaluate the putative improvement offered by Cycluc in monitoring Luc expressing cells *in vivo*. As we could not confirm such improvement, we measured the kinetics of the enzymatic reaction involving Cycluc and found high affinity of Cycluc towards luciferase and at the same time, its low turnover, which turns it into an inhibitor of photon flux, and limits its applicability for *in vivo* measurements of the activity of the wild-type luciferases.

2. Methods

2.1. Kinetic parameters for firefly luciferin (D-Luc) and Cycluc

In vitro studies employed luciferase enzyme purified from *P. pyralis* (Sigma-Aldrich). To determine K_m values for D-Luc and Cycluc, bioluminescence activity assays were performed at the saturating concentration of ATP (1,2 mM) and concentrations of D-Luc (D-luciferin, Promega) or Cycluc (Aobious, Gloucester, MA) between 0.1 μM and 1.4 mM. The activity was defined as the maximum intensity of emitted light and expressed in relative light units (RLU). The measurements were performed on an FB12 luminometer (Zylux) at 20 °C. In typical experiment mimicking measurement at the physiological conditions *in vivo*, 350 μL of D-Luc or Cycluc solution in PBS buffer containing 10 mM MgSO4, and 2 mM EDTA were added to a cuvette and supplemented with 5 μL of luciferase solution (0.01 mg/mL). The cuvette was placed into a sample compartment of the luminometer and the reaction was initiated by injecting 150 μL of 4 mM ATP in the same buffer solution. A neutral filter (1.25%) was placed in the cuvette compartment of the luminometer to keep bioluminescence intensity values within the dynamic range of the luminometer. The values of Km(D-Luc) and Km(Cycluc) were calculated using Lineweaver-Burk plot. Consequently, the final reaction mixture comprised 1.2 mM ATP, 10 mM $MgSO_4$, ~0.1 μg/mL luciferase (~1.6 nM luciferase) and varying concentrations of D-Luc or Cycluc substrates. Measurements of flash and glow intensity were performed as described by Thermofisher (https://assets.thermofisher.com/TFS-Assets/LCD/Application-Notes/D10338~.pdf).

2.2. BLI of luciferase expression in X. laevis oocytes

Oocytes of the African frog *Xenopus laevis* (Nasco, Fort Atkinson, WI) were surgically obtained as previously described [28]. Handling of frogs and experiments has been approved by the Central Commission for Animal Research (Centrala Försökdjursnämnden; #N21/12).

Adult female frog ovarian tissue was homogenized, rinsed in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM Na2HPO4, 5 mM HEPES [pH 7.8]) and digested using 8 U of Liberase Blendzyme 3 (Roche) in OR2 medium for 2 h at 19 °C upon gentle rotation. Stage VI oocytes were incubated overnight at 19 °C in OR2 medium containing gentamicin (10 μg/mL Sigma-Aldrich). Plasmid DNA encoding luciferase (pvax-luc; kind gift of AK Roos, Karolinska Universitet, Sweden) in the amount of 10 ng was subsequently injected into nuclei. Oocytes expressing luciferase were maintained in OR2 medium for 24 h prior to immersion in varying concentrations of D-luciferin (Promega) or Cycluc (Aobious, Gloucester, MA). Serial dilutions of each of the substrates were added to 8 to 10 oocytes placed in the sterile 96-well OptiPlate™ plates (Perkin Elmer) adapted for bioluminescence measurement. Oocyte bioluminescence was measured using the IVIS Spectrum (Perkin-Elmer) 10 min after addition of substrate, and subsequently every 10 min for a total of 3 h.

2.3. Implantation of luciferase expressing mammary adenocarcinoma cells into mice

Eight-week old female BALB/c mice were purchased from Charles River Laboratories (Germany). Animals were housed 6–10 per cage under a light-dark (12 h/12 h) cycle with *ad libitum* access to water and food. Experimental manipulations were performed under the inhalation anesthesia induced by 4% and maintained by 2.3% mixture of isofluorane in oxygen administered through facial masks. Experiments were carried in compliance with the bioethical principles adopted by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), and Ethical Committee for Animal Experiments of the North Stockholm region N66_13 and Latvian Animal Protection Ethics Committee of the Latvian Food and Veterinary Service, permission Nr 99. 4T1luc2 cells (Perkin Elmer) in 50 μl of serum free DMEM (Dulbecco's Modified Eagle's Medium, HyClone) were injected into the mammary fat pads of 9 week old BALB/C mice. Cells were implanted at two sites per mouse in serial five-fold dilutions from a starting concentration of $6.25*10^5$ cells per 50 μl. After the experiments, mice were humanely sacrificed by cervical dislocation.

2.4. BLI of 4T1luc2 cells expressing luciferase in vivo

BALB/c mice implanted with varying concentrations of cells (n = 12, one cell dose per two mice) were injected i.p. with 100 μl of the solutions of Cycluc (0.5–1 mM) or D-luciferin (100 mM). Mice were allowed to run freely for 10 min to allow sufficient time for distribution of the substrates. Thereafter, mice were anaesthetized using 2.5% isoflurane in an inhalation chamber. I*n vivo* bioluminescence was monitored using the IVIS Spectrum (Perkin-Elmer). Photon flux (p/s) was measured using the Living Image 4 software (Perkin Elmer).

3. Results and discussion

First of all, we assessed the *in vivo* performance of Cycluc compared to D-Luc in detecting 4T1luc2 cells in a mouse model, where it has previously been reported to provide the same peak photon flux at concentrations 20–200 fold lower than the standard D-Luc dose [12]. Of note, Cycluc was reported to give a 100-fold enhancement of the total flux compared to D-Luc when they were used in an equimolar concentration based on the concentration 0.5 mM optimal for Cycluc [12]. This enhancement is irrelevant to an ordinary basic user, who will not risk applying D-luciferin at a concentration lower than the recommended dose.

To test the actual benefit offered by Cycluc compared to D-Luc, we built *in vivo* calibration curves for quantification of Luc

expressing cells using as substrates D-Luc and Cycluc (Fig. 1). Their dosage per 20 g mouse was: D-Luc, 100 μmol based on the standard protocol for *in vivo* luminescent imaging by Perkin Elmer; Cycluc, 50 nmol based on the data presented by Evans MS et al. [12]. The average total flux per mole of substrates calculated based on the total flux signal per mole in all 4T1luc2 cell measurements (Fig. 1A)

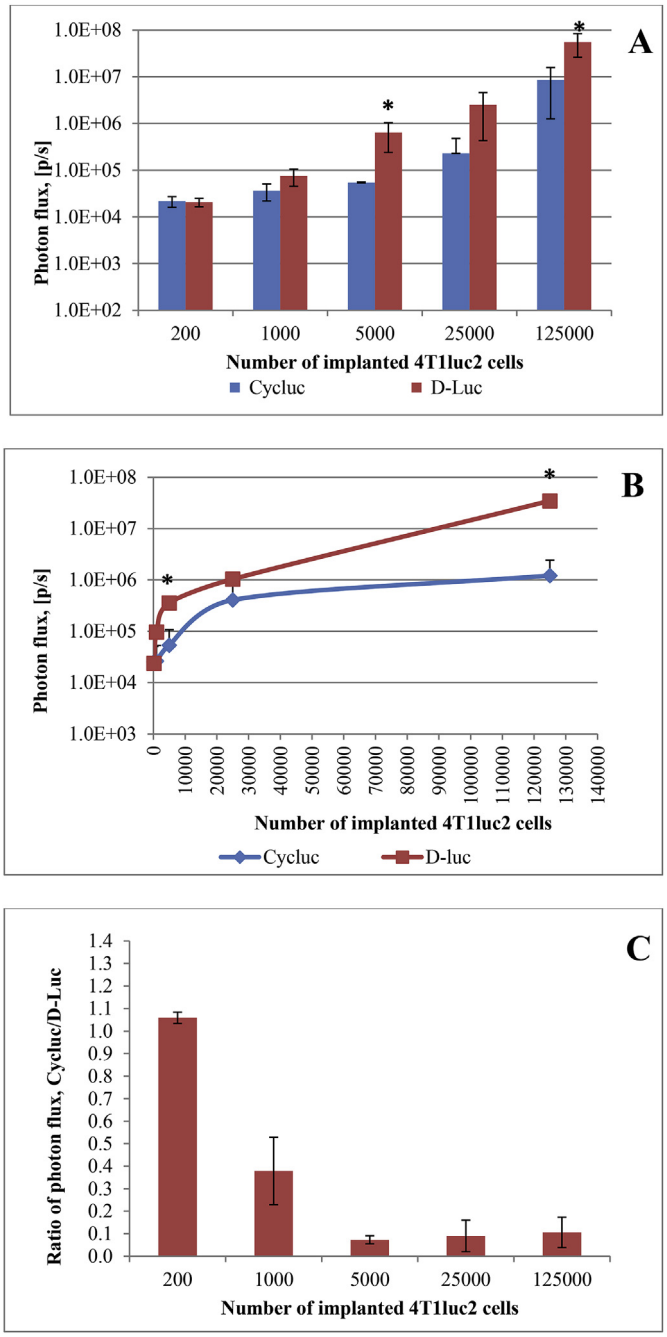

Fig. 1. Bioluminescence of murine mammary gland adenocarcinoma 4Tluc2 cells stably expressing firefly luciferase (Perkin Elmer) after implantation into syngenic BALB/c mice. From 200 to 1.25×10^5 4T1luc2 cells were implanted into mammary fat pads of BALB/c mice (20 g), intraperitoneally injected with 10 μmol of D-Luc (100 μl of 100 mM solution) or 50 nmol of Cycluc (100 μl of 0.5 mM solution), 10 min later anaesthetized by inhalation, and monitored by bioluminescent imaging (Spectrum, Perkin Elmer) (A); Dependence of photon flux generated by D-Luc and Cycluc substrates on the number of injected 4T1luc2 cells (B); Ratio of total flux generated by D-Luc to that of Cycluc at different number of implanted 4T1luc2 cells (C). Results from 2 to 4 implantation experiments. *, $p < 0,05$. Pair-wise comparisons were by Mann-Whitney test.

constituted 4,12E+5 p/s for D-Luc, and 5,77E+7 p/s for Cycluc. The average ratio of Cycluc to D-Luc enhancement was equal to 140 which corroborated earlier findings [12]. However, no enhancement was demonstrated for the measurements done at the substrate concentrations relevant for the experimental practice (Fig. 1A).

Total photon flux depends on the number of injected 4T1luc2 cells. However, for D-Luc, signal grew with increasing cell numbers, while for Cycluc the growth slowed down at 5000, and reached a plateau at 10000 cells (Fig. 1A and B). Due to this, Cycluc/D-Luc photon flux ratio also depended on the number of photon-emitting cells, significantly decreasing with the increasing cell numbers (Fig. 1C). On average, Cycluc was 6–10 times less efficient in generating photon flux than D-Luc.

In part, this could be due to the Cycluc solubility and/or peculiarities of its pharmacokinetics limiting delivery of the substrate to the tumor after the intraperitoneal delivery. To avoid this possible interference in the substrate comparison, we performed the same experiment in the oocytes of *Xenopus laevis*. The oocytes are giant cells about 1.2 mm in diameter, a live test-tube for short-term mechanistic studies of gene expression, apoptosis, metabolism, and hormone signaling [13,14]. A number of applications in oocytes employs BLI, including measurements of calcium levels in *X. laevis* [15], of ATP in human oocytes [16], of biotin in sea urchin oocytes [17], and transgene expression in porcine ovarian follicles [18]. Oocytes were microinjected into the nucleus with a Luc-expressing vector pVaxLuc, grouped 8 to 10 per well of the cell culture plate in medium supplied with 5 mM solution of Cycluc, or 100 mM of D-Luc, as concentrations relevant for the experimental practice. The concentrations of the cofactors magnesium (approx 2 mM) and ATP (approx 1 mM) inside oocytes as other living cells are saturating relative to the $K_{M(ATP)}$ of luciferase (25–250 μM) [19]. Therefore, the reaction is independent of their concentration, and their addition into the medium is not required. Bioluminescent imaging allowed detection of photon flux from 1 to 2 oocytes (Fig. 2A). The average total photon flux generated by the Luc-expressing oocytes treated with Cycluc was two times lower than by the oocytes treated with the equimolar amount D-Luc (Fig. 2B). Furthermore, the photon flux did not decrease with the decrease of concentration of Cycluc in the incubation buffer from 5 to 0,05 mM. This could only be explained by the high affinity of Cycluc-Luc binding to the enzyme, due to which active sites of all luciferase molecules were occupied by Cycluc already at concentrations as low as 0,05 mM, and remained occupied due a low substrate turnover. Also it was found that at the lowest concentrations of Cycluc 180 min after addition of substrate no signal was detected probably due to its full conversion.

To find out if this is the case, we measured the K_m and V_{max} values for Cycluc and D-Luc as the substrates of Luc of *P. pyralis*. The experiment was performed in physiological conditions applied for *in vivo* imaging, which implied the use of phosphate buffered saline (pH7.4). K_m for D-Luc and Cycluc, the initial enzymatic reaction velocity was recorded as the average of triplicate sample readings and the Michaelis constant was calculated (Fig. 3). From a double reciprocal plot, the K_m(D-luc) was found to be 0.35 mM with the 95% confidence interval of ±0,07 mM (Fig. 3a). The K_m values were 10-times higher than K_m(D-Luc) values determined for this and other luciferases under the conditions optimized for *in vitro* assays (Tris-acetate buffer, pH 7.8). The increase of K_m (decrease of affinity to the enzyme) was attributable to a partial inhibition of Luc by inorganic phosphate [21]). In these physiological conditions, V_{max} for D-Luc was equal to 0.65×10^6. Under the same conditions, luciferase affinity to Cycluc was very high: K_m(Cycluc) was equal to ~ 0.05 μM. However, the V_{max}(Cycluc) was only about 1% of V_{max}(D-Luc) (0.63×10^4 vs 0.65×10^6, respectively) (Fig. 3b). This confirmed what we have suspected from the comparison of D-Luc

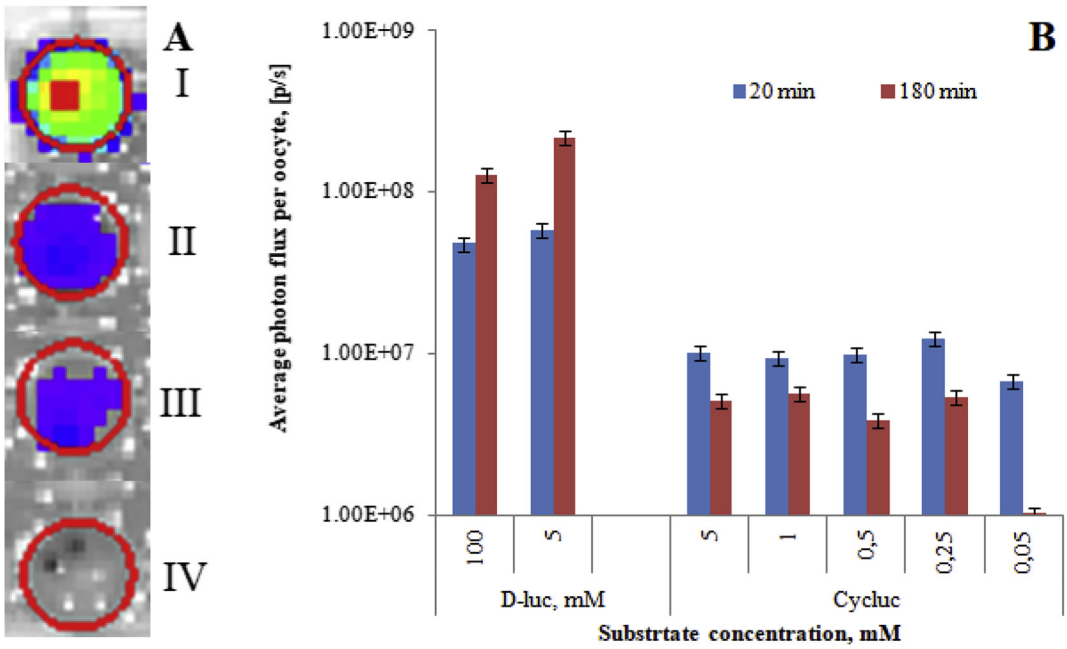

Fig. 2. Bioluminescence in oocytes of *X laevis* (n = 10) microinjected with Luc-expressing plasmid (pVaxLuc) [20] registered using Spectrum imager (Perkin-Elmer). Typical images from two oocytes microinjected into the nucleus with pVaxLuc, placed into the oocyte culture medium, and supplemented with substrates: D-Luc at 5 mM (field AI), Cycluc at 5 (AII), 0,5 (AIII), 0,05 (AIV) mM; bioluminescence was registered every 20 min during the next 3 h using Spectrum (Perkin Elmer); Average photon flux per oocyte 20 and 180 min after the. start of incubation (B). Data represent average photon flux [p/s], per oocyte ± STDEV. Independent assays gave concordant results.

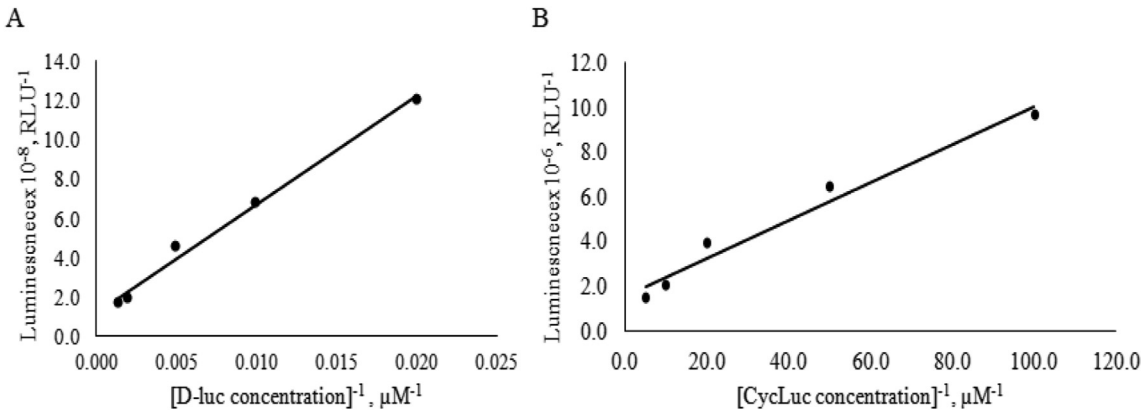

Fig. 3. Double reciprocal plot of luminescence vs substrate concentration dependence in the reaction of luciferase oxidation of D-Luc (A) and Cycluc (B) in PBS, pH 7.4, ATP concentration was constant and equal to 1.2 mM, luciferase concentration was 1.6 nM.

and Cycluc activities in *Xenopus* oocytes. The flash intensity is generally used to determine the luciferase catalytic parameters and specific activity, and the glow intensity is crucial for bioanalytical applications of the enzyme [22]. Despite partial Luc inhibition, the time dependence of light intensity typical to firefly luciferases was retained: substrate addition was followed by a bright flash of light that rapidly decreased to a semi-stable glow. Glow intensity for D-Luc constituted approximately 10%, and for Cycluc, 1% of the flash intensity reproducing earlier observations [10] (data not shown). Thus, under the given conditions, Cycluc acted more like a Luc inhibitor than a Luc substrate. It is worth noting that many potentially attractive luciferin analogues are strong inhibitors for firefly luciferases [10,23]. This could be solved by genetic modification of luciferase as in the case of Ultra-Glo (Promega), or series of mutant luciferases [24–26] which are resistant to inhibition during high-throughput screening assays. Matched mutant luciferase-luciferin pairs have been created [3,4,27] with the most recent allowing registration of single cells genetically labelled with mutant Luc in freely moving animals, from mice to marmosets (Acaluc; [11]). *In vivo* applications of the novel substrates with the parental firefly luciferase are limited due to their suboptimal recognition, and/or inhibitory properties, resulting in a low quantum yield. This needs to be taken into account in *in vivo* experiments with biological objects, proteins, tumor cells or microorganisms, labelled with the parental “wild-type” luciferases.

4. Conclusions

Many chemical modifications of D-luciferin have been tested lately in order to enhance/improve its performance in BLI to be able to detect single labelled cells. However, careful *in vivo* and *in vitro* analysis of these substrates reveal serious drawbacks in their utility with the original wild-type enzymes. In this paper, we demonstrated that despite promising results obtained with Cycluc at low

concentrations, it yields significantly weaker signal than D-luciferin in head-to-head comparison. This drawback is explained by high affinity of Cycluc to firefly luciferase (K_m 0.05 μM) reflecting highly efficient substrate/enzyme binding. This, in turn, leads to its slow turn-over strongly decreasing the total amount of photons emitted by luciferase, as reflected by a low V_{max}. Further improvements of substrate design are needed to produce luciferin variants which would have K_m values of D-luciferin, but higher V_{max}, allowing more efficient measurements of the activity of firefly luciferase both *in vivo* and *in vitro*.

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Transparency document

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