Red-shifted luciferase–luciferin pairs for enhanced bioluminescence imaging

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Red-shifted bioluminescent reporters are desirable for biological imaging. We describe the development of red-shifted luciferins based on synthetic coelenterazine analogs and corresponding mutants of NanoLuc that enable bright bioluminescence. One pair in particular showed superior in vitro and in vivo sensitivity over commonly used bioluminescence reporters. We adapted this pair to develop a bioluminescence resonance-energy-based Antares reporter called Antares2, which offers improved signal from deep tissues.

There is enormous interest in harnessing bioluminescence for ultrasensitive bioassays, drug screening, and optical imaging. The photon fluxes of bioluminescent reporters are determined by the quantum efficiencies and catalytic rates of bioluminescence reactions. The slow catalysis of common luciferases often results in bioluminescence emission that is several orders of magnitude lower than that of fluorescence. A recent advance was the development of NanoLuc luciferase, which, when paired with a synthetic furimazine substrate, generates intense blue bioluminescence much higher than that of other popular reporters. However, the in vivo performance of NanoLuc is poor, particularly in deep tissues, since photons shorter than 600 nm interact strongly with mammalian tissues. Because a significant portion of Firefly luciferase (FLuc) emission is longer than 600 nm, FLuc–d-luciferin remains among the top choices for deep-tissue bioluminescence imaging. Although both FLuc and d-luciferin have recently been engineered for longer wavelength emission, this desirable spectral shift is often offset by a substantially reduced total intensity. Only a very few examples, such as the d-luciferin analogs CycLuc1 and AkaLumine-HCl, can modestly enhance FLuc bioluminescence at limited substrate concentration ranges.

It has been thought that an improved bioluminescence reporter may be derived by red-shifting the emission of NanoLuc. Early studies reported red-shifted coelenterazine (CTZ) analogs for Oplophorus luciferase, the ancestor of NanoLuc; however, the intensities of these analogs were low, and it is unclear whether the spectral shift could be extended to NanoLuc. Recent studies directly tested NanoLuc with several synthetic CTZ analogs, but no appreciable red-shift was noted. Herein, we present novel luciferase–luciferin pairs based on other CTZ analogs and correspondingly re-engineered NanoLuc mutants for substantially brighter and red-shifted bioluminescence.

We prepared several CTZ analogs, including diphenylerazine (DTZ; which extends the CTZ conjugation system through an aromatic ring at C-8) and selenoterazine (STZ; which has a selenium heteroatom at C-8) (Supplementary Table 1). When assayed with NanoLuc, DTZ and STZ caused 44- and 71-nm red shifts, respectively, with reduced brightness compared with furimazine (Supplementary Table 1).

We next engineered NanoLuc for enhanced activity toward either DTZ or STZ by simultaneously randomizing residues I44, I54, and I138 (Supplementary Fig. 2), since these positions have been reported to modulate the substrate preference of NanoLuc. Screening this library did not yield any useful mutants. We subsequently screened another library with full randomization at residues L18, D19, R162, and C164 based on the proximity of these residues to a putative substrate-binding site (Supplementary Fig. 3 and Supplementary Fig. 4). We also introduced random mutations across the gene using error-prone PCR. From this, we identified a NanoLuc mutant (NanoLuc-L18Q/D19A/S28T/C164S) that displayed enhanced activity toward DTZ and STZ (Supplementary Fig. 2). Additionally, we identified another NanoLuc mutant (yeLuc0.8 or NanoLuc-L18Q/D19A/S28T/C164S) that displayed enhanced activity toward STZ. Further evolution of yeLuc0.8 with three additional rounds of error-prone PCR and bacterial-colony-based screening resulted in a variant with six additional mutations (yeLuc or yeLuc0.8-F1L/A14D/V27L/Q69R/R112Q/L142R). This new mutant showed an 11.5-fold overall enhancement of STZ bioluminescence over that of NanoLuc.

In summary, we presented a pair of red-shifted bioluminescent reporters that are derived from the NanoLuc platform and are developed using a balanced strategy of directed evolution and screening. These reporters have improved in vitro and in vivo bioluminescence properties over FLuc–d-luciferin and NanoLuc for deep-tissue imaging applications.

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Recently, NanoLuc was fused to fluorescent proteins for BRET-based reporters, and this fusion resulted in the two most red-shifted variants, Antares and ReNL\textsuperscript{17,18}. The absorbance of CyOFP1 in Antares overlaps better with teLuc–DTZ emission than with NanoLuc–furimazine (Supplementary Fig. 7). By replacing NanoLuc with teLuc in Antares, we derived a BRET-based Antares2 reporter emitting 3.8 times more photons above 600 nm than Antares and 65 times more than FLuc–α-luciferin (Fig. 1b and Table 1).

We further evaluated the bioluminescence of our new reporters using transiently transfected Human Embryonic Kidney (HEK) 293T cells. Under all conditions, the brightness of DTZ with teLuc or Antares2 was two to three orders of magnitude higher than that of FLuc–α-luciferin (Fig. 1c–f; Table 1; Supplementary Figs. 8 and 9). These enhancements were more dramatic than the results observed in protein assays because of the high levels of teLuc and Antares2 in live cells (Supplementary Fig. 10) and better cell permeability of DTZ compared with that of α-luciferin. teLuc and Antares2 also displayed improvements over NanoLuc, Antares, and ReNL (Table 1). teLuc emitted sustained bioluminescence with a half-life of >2 h in both intact cells and cell lysates, whereas yeLuc showed flash-type

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**Figure 1** | Bioluminescent reporters based on synthetic substrates and re-engineered luciferases. (a) Chemical structures of diphenylterazine (DTZ) and selenoterazine (STZ). (b) Bioluminescence emission of purified luciferases (1 nM) with their corresponding luciferin substrates (30 μM). The spectra were normalized to peak emission of FLuc–α-luciferin. (c–f) Representative pseudocolored images (c,e) and quantifications (d,f) of luciferase-expressing HEK 293T cells in the presence of various luciferins. Images were acquired without a filter (c) or with a 695 ± 25-nm NIR emission filter (e). Panels d and f are quantification results for panels c and e, respectively. All values were normalized to the intensities of FLuc–α-luciferin (50 μM) under the same imaging conditions. The graphs show mean values and individual data points of three independent measurements.
we intraperitoneally injected HEK 293T cells expressing individual luciferases near the right dorsolateral trapezium region of unshaved BALB/c mice. For comparison, we also subcutaneously administered cells transfected with an empty vehicle to the same mice at the left dorsolateral thoracolumbar region. These injections were followed up with an additional subcutaneous luciferin injection. At a 0.1 mM substrate concentration, teLuc–DTZ was \( \approx 54 \) -fold brighter than FLuc–d-luciferin and \( \approx 7.5 \)-fold brighter than NanoLuc–furimazine (Fig. 2a,b and Table 1). The brightness of Antares2 was comparable to that of teLuc, and no background bioluminescence was observed from sites injected with vehicle-transfected cells. To further evaluate the reporters for imaging deep-tissue targets, we used hydrodynamic transfection to express luciferase genes within the internal organs of mice. After intraperitoneal injections of 0.3 µmol individual luciferin substrates, teLuc–DTZ generated \( \approx 52 \)-fold higher emission than FLuc–d-luciferin (Fig. 2c,d and Table 1). With 3.3 µmol of each substrate (a dose recommended for AkaLumine–HCl), teLuc–DTZ was \( \approx 32 \)-fold brighter than FLuc–d-luciferin and \( \approx 15 \)-fold brighter than FLuc–AkaLumine–HCl. Moreover, teLuc with 3.3 µmol DTZ was still \( \approx 12 \)-fold brighter than FLuc with 10 µmol d-luciferin (equivalent to the standard \( \approx 150 \) mg/kg FLuc imaging condition). DTZ injections into untransfected BALB/c mice did not yield any background emission (Fig. 2c). When used for deep-tissue imaging, Antares2 shows an additional 35–90% signal increase over teLuc. Furthermore, the bioluminescence resulting from intraperitoneally injected DTZ displayed extended kinetics (Supplementary Fig. 14), which suggests that both teLuc and Antares2 are suitable for time-lapse BLI. To further demonstrate the use of these new bioluminescent reporters to detect low-abundance targets, we intravenously injected luciferase-expressing cells into unshaved BALB/c mice. Despite the fact that \( \approx 5 \times 10^5 \) FLuc-expressing cells were only marginally detectable, \( \approx 1 \times 10^5 \) teLuc- or Antares2-labeled cells generated signals well above the background (Supplementary Fig. 15).

In summary, we have synthesized CTZ analogs with modifications at the C-8 position and re-engineered NanoLuc luciferase to catalyze these new substrates, and these analogs exhibit brighter and more red-shifted bioluminescence. Although the water solubility of these CTZ analogs is worse than that of \( \delta \)-luciferin, the corresponding bioluminescence reactions with low amounts of the CTZ analogs are much brighter than FLuc with \( \delta \)-luciferin at saturated concentrations. In particular, teLuc–DTZ is one of the brightest bioluminescent systems and shows robust performance in vitro, in cellulo, and in live mice. teLuc–DTZ will streamline a variety of applications to afford high sensitivity and reproducibility and expand the scope of BLI by allowing the use of less complicated instrumentation to track less abundant targets with higher spatiotemporal resolution. Moreover, our study demonstrates the feasibility of coengineering CTZ-consuming luciferases and substrates for improved bioluminescence. Increased detection sensitivity is not the only advantage of teLuc–DTZ. The small sizes of NanoLuc and its derivatives teluc and yeLuc (19 kDa), compared with that of FLuc (61 kDa) and some other luciferases, facilitate their use as fusion reporters and in the assembly of viral vectors with limited packaging capacities. Moreover, NanoLuc forms a \( \beta \)-barrel structure that can be genetically and structurally manipulated, such as for splint and fragment complementation\(^{19} \). Furthermore, the bioluminescence of NanoLuc is independent of Mg\(^{2+}\), Ca\(^{2+}\), and ATP\(^5\). All these factors suggest that teLuc may be an excellent scaffold for the development of bioluminescent biosensors compatible with other popular optogenetic tools\(^{20} \). We fused teLuc with CyOFP1 to derive Antares2, and this further improved bioluminescence detection in deep tissues. Antares2 is an excellent bioluminescent reporter when the molecular size of

### Table 1 | Photoluminescence properties of various luciferase–luciferin pairs

| Protein\(^b\) | HEK 293T cells\(^c\) | Mice | 0.3 µmol | 3.3 µmol |
|---------------|------------------|------|----------|----------|
|               | Total | >600 nm | Intact | (total) (695/50 nm) | Lysate | Subcutaneously injected cells\(^d\) | Hydrodynamic transfection\(^e\) |
| NanoLuc + furimazine | 456  | 19     | 43.5  | 0.7      | 277  | 5   | 167   | 7.2 | ND\(^f\) | ND\(^f\) |
| teLuc + DTZ    | 502  | 19     | 113   | 13       | 733  | 55  | 317   | 54  | 52   | 133 |
| yeLuc + STZ    | 527  | 19     | 13    | 3.7      | 78   | 21  | 6.5   | 1.8 | 3.5  | ND\(^f\) |
| FLuc + o-luciferin | 563  | 61     | 1     | 1        | 1    | 1   | 1     | 1   | 1    | 4.2\(^g\) |
| FLuc + AkaLumine-HCl | 677  | 61     | 3.4   | 8.7      | 2.3  | 10  | 11    | 1.3 | ND\(^f\) | 9.3 |
| Antares + furimazine | 459, 583 | 71.8 | 26   | 13       | 159  | 130 | 100   | ND\(^f\) | ND\(^f\) | ND\(^f\) |
| Antares2 + DTZ | 501, 583 | 70.5 | 30   | 17       | 180  | 137 | 112   | 26  | 64   | 105 |

\(^a\)Intensity values normalized to FLuc–o-luciferin under comparable experimental conditions. \(^b\)10 µM substrates and 100 µM proteins. Values are based on intensities integrated over the first 10 min post-substrate injection. \(^c\)50 µM substrates and 2,000 cells with an average transfection efficiency of \(\approx 70\%\). \(^d\)Subcutaneous injection of 2 million HEK 293T cells and 100 µl of 100 µM of each substrate. \(^e\)Intraperitoneal injection of each substrate. All intensity values are normalized to FLuc and 0.3 µmol o-luciferin. \(^f\)Not determined. \(^g\)The relative intensity increased to 11.7 when 10 µmol o-luciferin was intraperitoneally injected.
Figure 2 | Bioluminescence imaging of luciferase–luciferin pairs at superficial sites and in deep tissues of live mice. (a, b) Representative bioluminescence images (a) and quantitative analysis (b) of BALB/c mice with subcutaneously injected luciferase-expressing HEK 293T cells and 100-µl luciferin substrates at the indicated concentrations. The group numbers in panel b are aligned with those in panel a. Two injection sites (one for luciferase-expressing cells and one for empty vehicle controls) for each mouse are illustrated with red arrows. Intensity values were normalized to the intensity of Fluc–o-luciferin (0.1 mM) acquired under the same condition. The data shown are the mean ± s.d. of three independent measurements; colored circles represent individual data points. (c, d) Representative bioluminescence images (c) and quantitative analysis (d) of BALB/c mice, to which luciferase-coding plasmids were hydrodynamically delivered through tail vein injection, and luciferase substrates were intraperitoneally (i.p.) injected at 12 h postplasmid injection. Intensity values were normalized to the intensity of Fluc–o-luciferin (0.3 µmol). Data are shown as mean ± s.d.; colored circles represent individual data points (n = 4 for teLuc, Antares, and Antares2 with 3.3 µmol substrates; and n = 3 for all other groups). Unpaired two-tailed t-tests were used to compare teLuc–DTZ and Antares2–DTZ with Fluc–o-luciferin or Antares–furimazine (*1, P = 0.0015; *2, P = 0.0002; *3, P < 0.0001; *4, P = 0.0042; *5, P < 0.0001; *6, P < 0.0001), indicating the existence of a significant enhancement by teluc and Antares2.

the reporter is not important and when CyOF1 does not cause spectral crosstalk. Overall, our work provides several robust bioluminescent reporters, including teLuc and Antares2, which are expected to have broad applications in the fields of chemistry, biology, and biomedicine.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.-w.A. conceived and supervised the entire project; H.-w.A. and M.M.M.-G. supervised the mouse imaging experiments; H.-W.Y. performed synthetic, protein engineering, and in vitro and in vivo characterization experiments; H.-W.Y. and O.K. performed the mouse imaging experiments. A.J. assisted H.-W.Y. in completing compound synthesis. D.C. assisted in the mouse imaging experiments. H.-W.Y. and H.-w.A. analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Materials and general methods. Synthetic DNA oligonucleotides were purchased from Integrated DNA Technologies. Restriction endonucleases were purchased from Thermo Scientific Fermentas. Accura high-fidelity DNA polymerase and EconoTaq DNA polymerase were purchased from Lucigen. Products of PCR and restriction digestion were purified by gel electrophoresis and Syd Laboratories Gel Extraction columns. Plasmid DNA was purified using Syd Laboratories Miniprep columns. DNA sequences were analyzed by Retrogen. d-luciferin was purchased from Thermo Fisher Scientific. Coelenterazine was purchased from Gold Biotechnology. Furimazine was purchased from Promega. AkaLumine was purchased from Wako Chemicals USA and acidified with one equivalent of HCl to derive AkaLumine–HCl. All other chemicals were purchased from Sigma-Aldrich, Fisher Scientific, or VWR and used without further purification. Varian Inova 500 with a 5-mm triple resonance (1H/13C/15N) triple-axis gradient probe at the UCR ACIF NMR Facility was used to record all NMR spectra. Chemical shift (δ) is given in parts per million relative to 1H (7.24 p.p.m.) and 13C (77.33 p.p.m.) for CDCl3 and 1H (2.50 p.p.m.) and 13C (39.5 p.p.m.) for DMSO-d6. Splitting patterns are reported as s (singlet), bs (broad singlet), d (doublet), t (triplet), dd (doublet of doublets), and m (multiplet). Coupling constant (J) is given in Hz. ESI-MS was run on an Agilent LC-TOF system by direct infusion. A Gilson PLC 2020 Purification System coupled with an Agela Venusil XBP C18 HPLC Column (10 μM, 100 Å, 10 × 150 mm) was used for preparative reverse-phase HPLC purifications. BALb/c mice obtained from the Jackson Laboratory were used for in vivo experiments. Animals were maintained and treated in standard conditions that complied with all relevant ethical regulations, and all animal procedures were approved by the UCR Institutional Animal Care and Use Committee. Images were analyzed using the Fiji image analysis software11, Microsoft Excel and GraphPad Prism were used to analyze data and prepare figures. Other general information is available from the Life Sciences Reporting Summary.

Synthesis of diphenylterazine and selenoterazine. Methods for preparing DTZ and STZ are described in Supplementary Note 2.

Construction of plasmids and libraries. Polymerase chain reaction (PCR) with various synthetic oligonucleotide pairs (see Supplementary Table 2) was used to amplify all genetic elements. The gene for NanoLuc was purchased from Integrated DNA Technologies as a gBlock and further amplified with oligos XhoI-NL-F and NL-R-HindIII. The product was digested with XhoI and HindIII restriction enzymes and then ligated into a predigested, compatible pBAD/His B plasmid. To create a gene library with randomization at randomization at residues 18, 19, 162, and 164, a similar multistep overlap PCR strategy was used. XhoI-NL-F and L18D19-NNK-R, L18D19-NNK-F and R162C164-NNK-R were used to create two fragments, which were next assembled by using XhoI-NL-F and NL-R-HindIII. The resultant gene fragment was also treated with XhoI and HindIII and ligated into a predigested pBAD/His B plasmid. To introduce random mutations across the NanoLuc gene, Taq DNA polymerase was used in all reactions with 0.2 mM MnCl2 added to promote amplification errors. To create mammalian expression plasmids, HindIII-NL-F-Koz and NL-R-XhoI (or NL-R-164H and NL-R-164S) were used to amplify NanoLuc and NanoLuc mutants. The products were treated with HindIII and XhoI restriction enzymes and ligated into a predigested compatible pcDNA3 plasmid (Life Technologies). The Firefly luciferase (FLuc) gene and the FLuc2 gene were amplified from a pGL2-GAL4-UAS-Luc plasmid (Addgene cat. no. 33020) and pGL4.17 (Promega), respectively, by using FLuc-F and FLuc-R or Luc2-F-HindIII-Kozak and Luc2-Myc-R, and inserted into pcDNA3 between HindIII and XhoI sites. Ant-HindIII-F-koz and Ant-XhoI-R were used to amplify a fragment containing the Antares gene from pNCS-Antares (Addgene cat. no. 74279). The product was digested with HindIII and XhoI and then ligated into a predigested pcDNA3 plasmid as mentioned above. To replace the NanoLuc fragment in Antares with teLuc, oligo pairs Ant-HindIII-F-koz and Te19DtoS_R, Te19DtoS_F and Te85DtoN_R, Te85DtoN_F and Te164CtoH_R, Te164CtoH_F and Antares_R-HindIII were used to amplify four individual fragments from pNCS-Antares. The resultant four fragments were used as templates and assembled via PCR reactions by using oligo pairs Ant-HindIII-F-koz and Ant-XhoI-R. The product was digested with HindIII and XhoI, purified by agarose gel electrophoresis, and ligated into a predigested pcDNA3 plasmid to give pcDNA3-Antares2. To construct a bacterial expression plasmid for Antares2, oligos Antares_F_XhoI and Antares_R_HindIII were used to amplify the whole gene from pcDNA3-Antares2, which was subsequently digested with XhoI and HindIII and inserted into a compatible, predigested pBAD/HisB plasmid. To construct mammalian expression plasmids with C-terminal Myc tags, the aforementioned forward primers for the construction of pcDNA3 plasmids were individually paired with NL-Myc-R, teLuc-Myc-R, yeLuc-Myc-R, FLuc-Myc-R, Luc2-Myc-R, or Antares-Myc-R to amplify the corresponding luciferase genes, which were further extended using Myc-R-XhoI paired with the corresponding forward primers. To build bicistronic plasmids containing a self-cleaning porcine teschovirus-1 P2A peptide (GSGATNSLKLQAGDVEENPGP), oligos P2A-FLuc-F and FLuc-R were used to amplify the FLuc gene; and teLuc-P2A-R, NLuc-P2A-R, and P2A-ext-R were paired with the corresponding forward primers to amplify teLuc or NanoLuc. These fragments were further assembled using overlap PCR to create NanoLuc-P2A-FLuc or teLuc-P2A-FLuc. The products were digested with HindIII and XhoI, purified by agarose gel electrophoresis, and ligated into a predigested pcDNA3 plasmid. All ligation products were used to transform Escherichia coli DH10B electrocompetent cells, which were next plated on LB agar plates supplemented with ampicillin (100 μg/mL). Additional l-arabinose (0.02%, w/v%) was supplemented to induce protein expression for direct bioluminescence imaging of bacterial colonies.

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Library screening. DH10B cells containing NanoLuc mutants were plated on LB agar plates supplemented with ampicillin (100 µg/mL) and l-arabinose (0.02%, w/v%) and incubated at 37 °C overnight to form bacterial colonies. Agar plates were left at room temperature for another 6 h, and this was followed by bioluminescence imaging using a luminescence dark box (Stanford Photonics) equipped with a Pixis 1024B CCD camera (Princeton Instruments). Digital images were acquired after spraying ~100 µL of 200 µM substrates to each agar plate, and next the images were processed with the Fiji image analysis software to derive bioluminescence intensities of individual colonies. For each compound, the brightest 50 colonies from a total of ~20,000 colonies were chosen and inoculated in 5 mL liquid LB broth containing ampicillin (100 µg/mL) and l-arabinose (0.02%, w/v%). After overnight growth at 37 °C and 250 r.p.m., the cultures were moved onto a shaker at room temperature for another 6 h. Cells were next diluted with assay buffer (1 mM CDTA, 0.5% Tergitol NP-40, 0.05% Antifoam 204, 150 mM KCl, 100 mM MES, pH 6.0, 1 mM DTT, and 35 mM thiourea) to OD600 of 0.1. Next, bioluminescence activities of individual samples were measured in white 96-well plates (Costar 3912) on a Synergy Mx Microplate Reader (BioTek) after directly injecting substrates (final concentration of 30 µM). Kinetics were followed for 1 s signal integration every 60 s for a total of 40 min. Mutants showing exceptionally high bioluminescence activities and extended kinetics were chosen for sequencing, protein preparation, and other additional characterization.

Luciferase expression and purification. Luciferases were expressed and purified as His6-tagged fusion proteins. DH10B cells containing corresponding pBAD plasmids were grown in 5 mL LB starter cultures containing ampicillin (100 µg/mL) at 37 °C and 250 r.p.m. overnight. Each saturated overnight culture was diluted 100-fold into 2YT media containing the appropriate antibiotics and grown under the same conditions. When OD600 reached 0.7–0.9, the expression culture was induced with l-arabinose (0.2%, w/v) and incubated at room temperature with shaking at 250 r.p.m. for another 16 h. Cells were harvested by centrifugation at 4,700 r.p.m. for 15 min and lysed by sonication. The resulting cell lysate was clarified by centrifugation at 18,000 r.p.m. for 30 min at 4 °C. The supernatant was incubated with Ni-NTA agarose beads (Pierce) at 4 °C for 2 h. Agarose beads loaded to a plastic column were sequentially washed with 10 mL of wash buffer 1 (pH 8.0, 50 mM Tris HCl, 20 mM Imidazole, 300 mM NaCl, 1 mM DTT) and 10 mL of wash buffer 2 (pH 8.0, 50 mM Tris HCl, 50 mM Imidazole, 300 mM NaCl, 1 mM DTT). Proteins were buffer exchanged into Tris–HCl (50 mM, pH 7.4) containing 1 mM DTT using Thermo Scientific Snakeskin dialysis tubing and next concentrated using 3 kDa Amicon Ultra Centrifugal Filters (EMD Millipore). Protein concentrations were determined using the Pierce Coomasie Bradford Protein Assay Kit (Thermo Fisher). For storage, glycerol was added to a final concentration of 50% (v/v), and the resultant mixtures were kept at −20 °C.

In vitro bioluminescence characterization. A Synergy Mx Microplate Reader (BioTek) was used for in vitro bioluminescence characterizations. For kinetics measurements, no emission filter or monochromator was used. 50 µL of luciferin substrates in the assay buffer (1 mM CDTA, 0.5% Tergitol NP-40, 0.05% Antifoam 204, 150 mM KCl, 100 mM MES pH 6.0, 1 mM DTT, and 35 mM thiourea) was injected into the wells of white 96-well plates containing 50 µL of pure enzymes in the same assay buffer. The final concentrations of all enzymes and substrates were 100 pM and 30 µM, respectively. Measurements were taken every 60 s postinjection (1 s integration and 10 s shaking during intervals). FLuc bioluminescence assays were performed similarly, except the assay buffer contained 30 mM MOPS, pH 7.0, 1.5 mM ATP, and 5 mM MgSO4. To derive values for apparent Michaelis constants (Km), substrate concentrations varied from 0.78 to 50 µM, and peak bioluminescence intensities at individual substrate concentrations were used to fit the Michaelis–Menten equation. To convert relative arbitrary unit (RLU) to the number of photons, the instrument was calibrated by determining the chemiluminescence of 50–800 nM luminol (QY = 1.23%) in the presence of 100 mM horseradish peroxidase and 2 mM hydrogen peroxide in 0.1 M K2CO3 aqueous solution for a total volume of 200 µL. To determine the quantum yields of bioluminescent reactions, 0.01 nmol of each luciferin in 50 µL PBS was injected into 150 µL PBS containing 0.5 nmol of the corresponding luciferase. 1.5 mM ATP and 5 mM Mg were added to the reaction between FLuc and d-luciferin. Signals were integrated until the substrates were completely consumed. Integrated total photos were divided by the total numbers of substrate molecules to derive the quantum yields of bioluminescence reactions. The validation of our results was confirmed by measuring the quantum yields of the Renilla luciferase mutant RLuc8 in the presence of CTZ (QY = 6.9%) and FLuc in the presence of d-luciferin (QY = 41 ± 7.4%) for individual enzymes were determined using the equation: kcat = Imax/(QY×[E]), where Imax is the maximal luminescence intensity from the fitting of the Michaelis–Menten equation, and [E] is the enzyme concentration. A Tecan M1000 Pro Plate Reader was used to record emission spectra. 50 µL of individual substrates (60 µM) in assay buffers were injected into 50 µL of 2 mM pure enzymes, and the bioluminescence spectra were collected with 0.1 s integration and 1 nm increments from 400 to 750 nm.

Mammalian cell culture and transfection. HEK 293T (purchased from ATCC and tested for mycoplasma by PCR), which is one of the most widely used and readily transfectable cell lines, was used for all tissue culture transfections. HEK 293T cells were cultured at 37 °C with 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfection mixtures were prepared with 3 µg of plasmid DNA and 9 µg of PEI (polylethlenimine, linear, MW 25 kDa) in DMEM and incubated for 20 min at room temperature. The medium was first aspirated, and subsequently the transfection mixtures were added to cells at 70% confluency on 35 mm culture dishes seeded 1 d before transfection. Incubation lasted for 3 h at 37 °C. Fresh DMEM containing 10% FBS was next used to replace the transfection mixtures. After incubation for another 24 h at 37 °C in a CO2 incubator, the medium was removed and cells were collected and resuspended in Dulbecco’s phosphate-buffered saline (DPBS).

Evaluation of the cytotoxicity of luciferins. Cell viability was determined using RealTime-Glo MT Cell Viability Assay (Promega) after incubation of HEK 293T cells with individual luciferin substrates for 24 h at 37 °C. Cell morphology was further
Bioluminescence measurements in HEK 293T cells and cell lysates. The number and density of cells in DPBS suspension were determined using a hemocytometer. Cells were next diluted in DPBS to gain the needed numbers in each 100 µL solution. To use the luminescence dark box to directly image cells, we added luciferase-expressing HEK293T cells (2,000 cells per well with ~70% transfection efficiency) and the corresponding luciferin substrates into wells of a white-wall, 96-well plate. Bioluminescence was imaged using a Pixis 1024B cooled CCD camera equipped with a 50-mm f/0.95 lens at 1 m post-substrate addition. The camera exposure time was 1 s, and the field of view was 6 × 6 inches. A 695BP50 (Omega Optical) filter was used to acquire far-red emission. All images were analyzed using the Fiji image analysis software. Cell lysates were prepared by sonication. Without further separation, luciferin substrates in 100 µL assay buffers were added to initiate bioluminescence reactions.

Western blot. To examine the protein levels of tested luciferases in mammalian cells, we transfected HEK 293T cells with mammalian expression plasmids harboring C-terminal, c-Myc-tagged luciferase genes. Cell lysates were prepared using CellLytic M Cell Lysis Buffer (Sigma-Aldrich) supplemented with cOmplete Protease Inhibitor Cocktail (Promega). Cell lysates were clarified by centrifugation at 14,000 × g for 10 min. The Bradford assay was used to determine total protein concentrations in the lysates. 10 µg of total proteins were loaded to each lane and resolved on a 12% SDS–PAGE gel. Proteins were next transferred to a nitrocellulose membrane at 40 V for 3 h. The membrane was blocked with 5% bovine serum albumin (BSA) in 1× PBS with Tween 20 (PBST) for 1 h and then incubated with either an anti-c-Myc (Santa Cruz Biotechnology, sc-40; 1:3,000 dilution) or an anti-β-actin antibody (ThermoFisher Scientific, PA1-183; 1:10,000 dilution) were added to initiate bioluminescence reactions.

Bioluminescence imaging of HEK 293T cells at superficial sites on live mice. BALB/c mice on a 37 °C electronic heat pad were anesthetized using 2% isoflurane in 100% oxygen with a flow of 0.5 l/min. We subcutaneously injected 2 million HEK 293T cells transfected with luciferase genes and resuspended in 100 µL PBS into the right dorsolateral thoracolumbar region. After cells were settled for 5 min, the corresponding luciferase substrates with indicated concentrations in 100 µL PBS were also subcutaneously injected to each site. Mice were subsequently imaged with a 30 s exposure per frame for a total of 5 min using a luminescence dark box (Stanford Photonics) equipped with a Pixis 1024B cooled CCD camera. The Fiji image analysis software was used to analyze images and integrate bioluminescence intensities over common regions of interest encompassing all injected cells.

Bioluminescence imaging of intravenously injected HEK 293T cells. At 48 h after transfection, 1 million HEK 293T cells expressing luciferases were trypsinized, pelleted, and resuspended in 100 µL PBS. Cells expressing teLuc, Antares, or Antares2 were combined with the same number of cells expressing FLuc and injected into the tail vein of BALB/c mice placed in a restrainer. Mice were recovered for 5 h, anaesthetized, intraperitoneally injected with 3.3 µmol DTZ or furimazine, and immediately injected with a 1 min exposure per frame over a course of 10 min. The Fiji image analysis software was used to process images and derive integrated intensities.

Bioluminescence imaging in deep tissues of live mice. Hydrodynamic transfections were performed on BALB/c mice as described elsewhere. Briefly, 20 µg of each luciferase-expressing plasmid in sterilized saline (volume equivalent to 9% body-weight of the treated mouse) was injected into restrained mice via the tail vein over 4–8 s. Mice were allowed to recover on heat pads and were monitored until their breathing rate returned to normal. Bioluminescence images were acquired at 12 h post-injection. In Antares or Akalumine-HCl the indicated dose was dissolved in 100 µL PBS and intraperitoneally injected into FLuc-transfected mice. Prior to intraperitoneal injections of CTZ analogs to teLuc, Antares, or Antares2-transfected mice, the indicated dose of DTZ or furimazine was dissolved in a 100 µL solution containing 8% glycerol, 10% ethanol, 10% hydroxypropyl-β-cyclodextrin, and 35% PEG 400 in water. To inject 3.3 µmol DTZ or furimazine, the total volumes were increased to 500 µL. The luminescence dark box (Stanford Photonics) equipped with a Pixis 1024B cooled CCD camera was again used to image anesthetized mice with a 1 min exposure per frame over a course of 10 min. The Fiji image analysis software was used to process images and derive integrated intensities.

Statistical analysis. Unpaired two-tailed t-tests were used to determine all P values. No statistical methods were used to predetermine the sample size. No sample was excluded from data analysis, and no blinding was employed. Animals were randomly assigned to receive various treatments. Unless otherwise indicated, data are shown as mean ± s.d., and error bars in figures represent s.d.

Data availability statement. The gene sequences for teLuc, yeLuc, and Antares2 have been deposited to GenBank under the accession
numbers KX963378, KX963379, and KY474379, respectively. The plasmids for teLuc (100026) and Antares2 (100027) have been deposited to Addgene. Source data for Figures 1 and 2 are available online. Materials, associated protocols, and other supporting data are available from the corresponding author upon request.

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22. Ando, Y. et al. Photochem. Photobiol. 83, 1205–1210 (2007).
23. Loening, A.M., Dragulescu-Andrasi, A. & Gambhir, S.S. Nat. Methods 7, 5–6 (2010).
24. Ando, Y. et al. Nat. Photonics 2, 44–47 (2008).
25. Liu, F., Song, Y. & Liu, D. Gene Ther. 6, 1258–1266 (1999).
## Experimental design

### 1. Sample size
Describe how sample size was determined. 

See Methods, "Statistical analysis" subsection. No statistical methods were used to pre-determine the sample size, because no existing data were available for power analysis. We used $n=3$ for in vitro experiments, $n=3$ or 4 for in vivo experiments. Unpaired two-tailed t-tests were used to evaluate the significance of differences between groups.

### 2. Data exclusions
Describe any data exclusions.

See Methods, "Statistical analysis" subsection. No sample was excluded from data analysis.

### 3. Replication
Describe whether the experimental findings were reliably reproduced.

See Methods and legends of figures. All experiments were replicated at least 3 times.

### 4. Randomization
Describe how samples/organisms/participants were allocated into experimental groups.

See Methods, "Statistical analysis" subsection. Samples and animals were randomly assigned to receive various treatments.

### 5. Blinding
Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

See Methods, "Statistical analysis" subsection. No blinding was employed.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

### 6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- [ ] The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- [ ] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [ ] A statement indicating how many times each experiment was replicated
- [ ] The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- [ ] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- [ ] The test results (e.g. $P$ values) given as exact values whenever possible and with confidence intervals noted
- [ ] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- [ ] Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

See Methods, “materials and general methods” subsection.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

See "Data availability" statement. Materials are available from the corresponding author upon request.

Information provided under Methods, “Western Blot" subsection. We used common antibodies, and validation information is available from manufacturers’ website.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Information provided under Methods, "Western Blot" subsection. We used common antibodies, and validation information is available from manufacturers’ website.

10. Eukaryotic cell lines
a. State the source of each eukaryotic cell line used.

Methods, "mammalian cell culture and transfection" subsection

b. Describe the method of cell line authentication used.

Not authenticated in our lab, but the cell lines were directly purchased from ATCC.

Methods, "mammalian cell culture and transfection" subsection
c. Report whether the cell lines were tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by iCLAC, provide a scientific rationale for their use.

Justification provided under "Methods, 'mammalian cell culture and transfection’ subsection”

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

See Methods, "materials and general methods” subsection.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study does not involve human subjects.