A Water-Soluble Coelenterazine for Sensitive *In Vivo* Imaging of Coelenterate Luciferases

To the editor:

Luciferases have played an integral role in bioluminescence imaging as useful tools in monitoring various biological processes.[1–3] The most commonly used luciferase is from the American firefly *Photinus pyralis* (Fluc), which uses beetle D-luciferin as a substrate.[4,5] Other promising luciferases for *in vivo* imaging are from coelenterates such as the sea pansy *Renilla reniformis* (Rluc) and the marine copepod *Gaussia princeps* (Gluc).[6–10] Both of these luciferases catalyze the oxidative decarboxylation of their substrate coelenterazine (CTZ), resulting in emission of blue light.[1] Because Fluc and Gluc/Rluc utilize different substrates, they can be combined as dual reporters for monitoring two distinct biological processes.[11,12] Firefly, *Gaussia*, and *Renilla* luciferases are being used as reporters for the *in vivo* monitoring of numerous biological processes in a variety of fields, including immunology, oncology, virology, neuroscience, and gene and cell therapy.[1–3]

The major drawback to using coelenterate luciferases as reporters for *in vivo* imaging has been the unavailability of a CTZ variant that is soluble in aqueous solution, making the use of alcohol essential in animals. The ensuing toxicity/solubility *in vivo* limits the injected dose (typically up to 100 μg), leading to lower sensitivity. Here, we show that a water-soluble coelenterazine (s-CTZ) that was recently synthesized and is commercially available yields up to 100-fold greater sensitivity as compared with native CTZ for *in vivo* imaging of *Renilla* and *Gaussia* luciferases. This s-CTZ is very useful for monitoring *in vivo* biological processes in which high sensitivity is required, such as detecting few circulating cells, early tumor metastasis, and apoptosis.[7,10,13,14]

We first compared the bioluminescence reaction kinetics for coelenterate luciferases using either CTZ or s-CTZ. We determined the light output for Gluc and Rluc samples over time using a spectrophotometer (**Supplementary Methods** online). Both substrates showed similar flash-type bioluminescence reactions for Gluc and Rluc (**Supplementary Figure S1a**). Recently, we characterized a Gluc variant (GlucM43I) that catalyzes a glow-type bioluminescence reaction in the presence of a detergent such as Triton X-100 (ref. 15). We compared the reaction kinetics of this variant as well as wild-type Gluc in the presence of Triton X-100 using CTZ and s-CTZ. The substrates exhibited similar glow-type reactions with GlucM43I and enhanced stable light output with wild-type Gluc in the presence of Triton X-100 (**Supplementary Figure S1a**). We also investigated whether s-CTZ has any influence on the characteristics of light emission by Gluc or Rluc. We analyzed their emission spectra using either CTZ or s-CTZ and observed that both luciferases exhibited their characteristic peaks around 480 nm, indicating that the water-soluble substrate had no effect on the light emission of coelenterate luciferases (**Supplementary Figure S1b**). These data suggest that the s-CTZ yields bioluminescence characteristics similar to those of native CTZ.

We then determined the effect of native and water-soluble CTZ concentrations on Gluc and Rluc light production. Gli36 human glioma cells were transduced with a lentivirus vector to express either Rluc or Gluc (**Supplementary Methods**). Equal aliquots of conditioned medium (for Gluc) or cell lysates (for Rluc) were analyzed with increasing doses of both CTZs using a luminometer. As expected, increasing the substrate dose increased the signal from Gluc in a roughly linear manner, whereas Rluc reached a plateau at around 2 μg/ml. Interestingly, s-CTZ yielded over 10-fold higher activity for Gluc and fourfold for Rluc at all concentrations tested, as compared with CTZ (**Supplementary Figure S2a**). We also compared the sensitivity of the water-soluble CTZ to the native substrate in detecting viability of mammalian cells. Different numbers of Gli36 glioma cells stably expressing Gluc or Rluc were plated in 96-well plates.

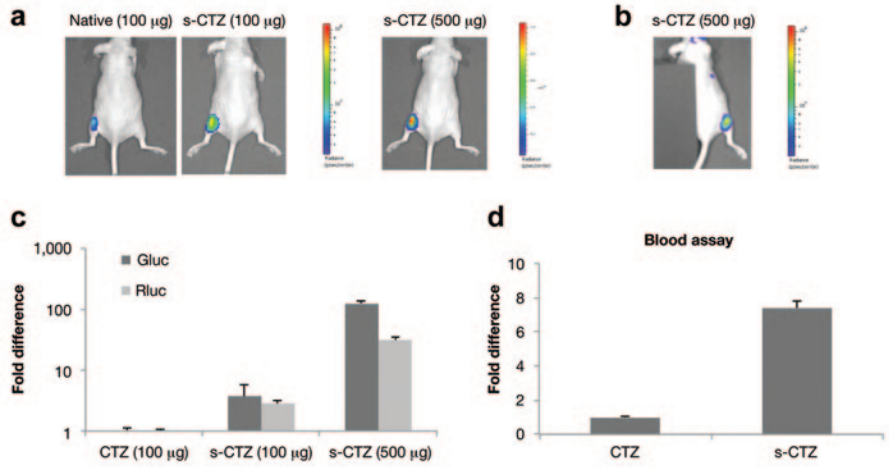

Figure 1 Sensitive *in vivo* imaging of coelenterate luciferases using water-soluble coelenterazine (s-CTZ). A total of 500,000 Gli36-Gluc or Gli36-Rluc cells were injected into the muscle of the left and right leg of nude mice, respectively ($n = 4$). (**a**) The mice were injected with 100 μg (4 mg/kg) of native CTZ and imaged using a cooled charge-coupled device camera. Four hours later, when signal from the previous imaging session dropped to background levels, the mice were injected with 100 μg of s-CTZ and imaged again. After another 4 hours, they were imaged after injection of 500 μg of s-CTZ. (**b**) A typical mouse in which the Gluc-expressing tumor is covered so as to visualize the signal from the Rluc-expressing tumor. (**c**) Quantitation of tumor-associated signals in **a**. (**d**) Blood was collected from the mice, and 5-μl aliquots (in triplicates) were assayed for Gluc activity using 100 μl (50 μg/ml) of either s-CTZ or CTZ and a luminometer. Data are presented as fold differences (in which the signals obtained from native CTZ are set to 1) ± SD ($n = 4$).

Twenty-four hours later, an aliquot of the conditioned medium (for Gluc) or total cell lysate (for Rluc) was assayed using both CTZs. Again, we observed over 10-fold higher photon output with Gluc and fourfold for Rluc using s-CTZ, as compared with the native CTZ substrate. These data collectively suggest that the water-soluble CTZ could be more sensitive for detecting coelenterate luciferases *in vitro* and *in vivo* (**Supplementary Figure S2b**).

Finally, we determined the usefulness of s-CTZ for *in vivo* bioluminescence imaging in deep tissues. We injected Gli36-Gluc and Gli36-Rluc cells into the leg muscle of nude mice ($n = 4$). One week later, mice were injected intravenously with 100 μg (4 mg/kg body weight) of CTZ and imaged immediately using a cooled charge-coupled device (CCD) camera. Four hours later, when signal from the previous imaging session had dropped to background levels, mice were injected with s-CTZ and imaged using the same protocol. Interestingly, the water-soluble CTZ showed 10-fold higher photon counts for Gluc and fourfold for Rluc, as compared with the native CTZ at the same dose, showing that s-CTZ is more sensitive in detecting coelenterate luciferases *in vivo* (**Figure 1a–c**). We also determined the effect of increasing the amount of s-CTZ on Gluc and Rluc detection in deep tissues. We injected the same mice mentioned above with 500 μg (20 mg/kg) of water-soluble CTZ and imaged them using a CCD camera. Tumors expressing Gluc resulted in a >120-fold increase in photon counts as compared with tumors imaged with native CTZ (**Figure 1a–c**). Similarly, Rluc-expressing tumors yielded a 30-fold increase in bioluminescence signal intensity (**Figure 1a–c**). Importantly, mice did not exhibit any sign of toxicity at this higher dose of s-CTZ. These results collectively demonstrate that the water-soluble CTZ is a sensitive substrate for *in vivo* imaging of coelenterate luciferase and can be used at a higher dose, overcoming one of the primary limitations of native CTZ, which is its insolubility in water.

Recently, we demonstrated that Gluc can be used as a blood reporter for *ex vivo* monitoring of *in vivo* biological processes such as gene transfer, viral replication, and circulating cells tracking.[10] To evaluate the sensitivity of s-CTZ to detect Gluc in the blood, we collected blood samples from mice bearing Gli36-Gluc intramuscular tumors and analyzed 5-μl aliquots (in triplicates) with 100 μl (50 μg/ml) of either CTZ or s-CTZ, using a luminometer. As compared with the native form, the water-soluble CTZ showed a bioluminescence signal more than eightfold higher, demonstrating a greater sensitivity for Gluc detection in the blood (**Figure 1d**).

In summary, we showed that water-soluble CTZ, which is commercially available, is a sensitive substrate for *in vivo* bioluminescence imaging of coelenterate luciferases. s-CTZ proved to be more sensitive in detecting Gluc and Rluc expression in mammalian cells both in culture and *in vivo*. Because alcohol is not required, s-CTZ could be injected in mice at higher doses compared with native CTZ, yielding up to 100-fold more light output from tumors expressing Gluc or Rluc. Furthermore, s-CTZ yielded more than an eightfold increase in bioluminescence signal when Gluc was assayed in the blood. Together, these results suggest that s-CTZ is a more potent substrate in detecting subtle coelenterate luciferases *in vivo*, facilitating noninvasive monitoring of various biological processes.

SUPPLEMENTARY MATERIAL

Supplementary Methods.

Supplementary Figure S1. Time kinetics of Gluc and Rluc light production using s-CTZ.

Supplementary Figure S2. Sensitivity of s-CTZ in detecting coelenterate luciferases in culture.

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REFERENCES

1. Badr, CE and Tannous, BA (2011). Bioluminescence imaging: progress and applications. *Trends Biotechnol* **29**: 624–633.
2. Prescher, JA and Contag, CH (2010). Guided by the light: visualizing biomolecular processes in living animals with bioluminescence. *Curr Opin Chem Biol* **14**: 80–89.
3. Weissleder, R and Pittet, MJ (2008). Imaging in the era of molecular oncology. *Nature* **452**: 580–589.
4. Pichler, A, Prior, JL, Luker, GD and Piwnica-Worms, D (2008). Generation of a highly inducible Gal4®Fluc universal reporter mouse for *in vivo* bioluminescence imaging. *Proc Natl Acad Sci USA* **105**: 15932–15937.
5. Rabinovich, BA, Ye, Y, Etto, T, Chen, JQ, Levitsky, HI, Overwijk, WW *et al.* (2008). Visualizing fewer than 10 mouse T cells with an enhanced firefly luciferase in immunocompetent mouse models of cancer. *Proc Natl Acad Sci USA* **105**: 14342–14346.
6. Loening, AM, Dragulescu-Andrasi, A and Gambhir, SS (2010). A red-shifted *Renilla* luciferase for transient reporter-gene expression. *Nat Methods* **7**: 5–6.
7. Santos, EB, Yeh, R, Lee, J, Nikhamin, Y, Punzalan, B, Punzalan, B *et al.* (2009). Sensitive *in vivo* imaging of T cells using a membrane-bound *Gaussia princeps* luciferase. *Nat Med* **15**: 338–344.
8. Stefan, E, Aquin, S, Berger, N, Landry, CR, Nyfeler, B, Bouvier, M *et al.* (2007). Quantification of dynamic protein complexes using *Renilla* luciferase fragment complementation applied to protein kinase A activities *in vivo*. *Proc Natl Acad Sci USA* **104**: 16916–16921.
9. Tannous, BA, Kim, DE, Fernandez, JL, Weissleder, R and Breakefield, XO (2005). Codon-optimized *Gaussia* luciferase cDNA for mammalian gene expression in culture and *in vivo*. *Mol Ther* **11**: 435–443.
10. Wurdinger, T, Badr, C, Pike, L, de Kleine, R, Weissleder, R, Breakefield, XO *et al.* (2008). A secreted luciferase for *ex vivo* monitoring of *in vivo* processes. *Nat Methods* **5**: 171–173.
11. Bhaumik, S and Gambhir, SS (2002). Optical imaging of *Renilla* luciferase reporter gene expression in living mice. *Proc Natl Acad Sci USA* **99**: 377–382.
12. Wang, H, Cao, F, De, A, Contag, C, Gambhir, DD, Wu, JC, *et al.* (2009). Trafficking mesenchymal stem cell engraftment and differentiation in tumor-bearing mice by bioluminescence imaging. *Stem Cells* **27**: 1548–1558.
13. Chung, E, Yamashita, H, Au, P, Tannous, BA, Fukumura, D and Jain, RK (2009). Secreted *Gaussia* luciferase as a biomarker for monitoring tumor progression and treatment response of systemic metastases. *PLoS One* **4**: e8316.
14. Niers, JM, Kerami, M, Pike, L, Lewandrowski, G and Tannous, BA (2011). Multimodal *in vivo* imaging and blood monitoring of intrinsic and extrinsic apoptosis. *Mol Ther* **19**: 1090–1096.
15. Maguire, CA, Deliolanis, NC, Pike, L, Niers, JM, Tjon-Kon-Fat, LA, Sena-Esteves, M *et al.* (2009). *Gaussia* luciferase variant for high-throughput functional screening applications. *Anal Chem* **81**: 7102–7106.