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Novel photoactivatable substrates for *Renilla* luciferase imaging *in vitro* and *in vivo*

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Abstract

To develop a photoactivatable bioluminescence imaging technique, a set of high and efficient photoactivatable substrates for *Renilla* luciferase has been well designed and synthesized.

Surprisingly, all of them could release the free luciferin that presented robust bioluminescent signals *ex vivo* and in living animals after UV irradiation at 365 nm.

Abstract graphic

A set of photoactivatable substrates for *Renilla* luciferase activity imaging was well developed with robust bioluminescent signals *in vitro* and *in vivo* after UV irradiation.

Light-directed activation of caged compounds has become a powerful tool for tracking molecular and cellular dynamics in the complex biological systems with high spatial and temporal precision.¹ Caging is often made possible by introduction of photolabile protecting groups into biologically active molecules, the function of which can be restored upon photoillumination to remove the protective groups.² This strategy has been successfully used

† Electronic Supplementary Information (ESI) available: Chemical synthesis

to photo-control both ions³, nuclei acids^{4, 5}, neurotransmitters⁶ and proteins⁷. In line with this strategy, a lot of photoactivatable fluorophores or probes have also been reported, including new photoactive dyads as efficient tools to monitor incorporation of cholic acid and cholesterol,⁸ a turn-on fluorescent probe designed by linking a protein-targeting core with a photoactivatable alkene-appended tetrazole,⁹ a photoactivatable two-photon probe developed for spatiotemporal bioimaging applications,¹⁰ caged salicylaldehyde hydrazone derivatives served as efficient photoactivatable solid-state fluorophores,¹¹ as well as a large palette of photoactivatable probes synthesized for proteins labelling in living cells.¹² However, due to the short emission wavelength, autofluorescence and low penetration ability, these photoactivatable fluorophores or probes are limited to *ex vivo* fluorescence imaging and cannot be applied for *in vivo* studies.¹³ To overcome these limitations, the present study turns to photoactivatable bioluminescence imaging techniques *in vivo*. It should be noted that some efforts on developing photoactivatable bioluminescence imaging system have been made, however, rare of them could successfully applied in *in vivo* bioluminescence imaging.^{14, 15}

Bioluminescence imaging (BLI), a consistently sensitive, convenient, steady and non-invasive imaging technique, has been employed for monitoring various biological phenomena such as protein–protein interactions,¹⁶ enzyme activities,¹⁷ bacterial burden¹⁸ and tumours growth.¹⁹ Among approximately 30 different bioluminescence systems, the coelenterazine-luciferase system is one of the most commonly applied.²⁰ Moreover, compared with systems of firefly luciferin–luciferase and bacterial bioluminescent systems, it is the simplest bioluminescence system without any cofactors except luciferin and luciferase.²¹ The coelenterazine (CTZ) is one of the known widespread luciferin, which could be catalysed by various marine luciferases from *Renilla*, *Periphylla*, *Oplophorus*, *Metridia*, *Gaussia*, etc.²² By introducing protecting groups at the 3-carbonyl position of the imidazopyrazinone moiety, the CTZ can be caged which temporarily prohibit its recognition with *Renilla* luciferase.²³ Along this line, we could develop caged bioluminescent substrates by inducing photolabile protecting groups at the reaction site. There are various photolabile protecting groups such the coumarin group,²⁴ the o-nitrobenzyl group,²⁵ the ketoprofen group²⁶ and the p-hydroxyphenacyl group.²⁷ The 2-nitrobenzyl group is the most widely used photolabile protecting group for application in synthesis.

Herein, we designed and synthesized a series of novel and photoactivatable coelenterazine derivatives (CCTZD) with three different substituents (a-c, Scheme 1) and their bioluminescent properties were also further investigated in buffer, cells and living animals by using *Renilla* luciferase as a reporter enzyme. Surprisingly, all of them could release the free luciferin which could emit a bioluminescent signal in the presence of *Renilla* luciferase and O₂ after UV irradiation (365 nm). Therefore, these caged coelenterazine derivatives could serve as novel photoactivatable substrates for bioluminescence imaging.

As shown in Scheme S1, we synthesised the caged bioluminescent substrates in a facile and efficient method. Under argon, to a solution of a mixture containing coelenterazine derivatives and one of caged groups such as 2-nitrobenzyl, 4,5-dimethoxy-2-nitrobenzyl and 1-(2-nitrophenyl)ethyl in anhydrous DMF was added K₂CO₃ and then stirred at 30 °C. Then, the mixture was added to water and the product was extracted with ethyl acetate. The

organic layer was dried with MgSO_4 and concentrated in vacuo. The crude product was further purified by chromatography on silica gel. The details for the preparation of the compounds and their ^1NMR , ^{13}C NMR and ESI-HRMS spectra can be found in the Supporting Information.

After obtaining the products, the bioluminescence imaging of the caged luciferin derivatives was evaluated. These compounds which were pro-substrates did not display bioluminescent signals before UV exposure, indicating they were efficiently blocked by the three different cages. After UV illumination, they could release the free luciferin which could generate a bioluminescent signal measured by an IVIS kinetic imaging system. These compounds at same concentration were assayed in buffer. After UV irradiation at 365 nm (0–12 min), the buffer containing *Renilla* luciferase was added and the bioluminescent signal was immediately recorded (Fig. 1 and S1). As shown in Fig. 1A, there was no bioluminescent signal observed without UV excitation. The light emission intensities are enhanced with the increased time of UV irradiation within a certain fixed range (6 min) and then reached a plateau. And the three different compounds appeared to be the similar bioluminescent signals. Furthermore, different concentrations of these compounds were also estimated upon the same time of UV irradiation at 365 nm. We can see from Fig. 1B that the bioluminescent intensities increased with increasing concentrations of these compounds after 6 min of UV light excitation. Overall, these results displayed that these caged coelenterazine derivatives could emit bioluminescent signals after photolysis.

To test the effect of metal ions of these photoactivatable bioluminescent substrates, we treated these compounds with various representative cations (Na^+ , K^+ , Fe^{3+} , Al^{3+} , Pa^{2+} , Ba^{2+} , Fe^{2+} , Sn^{2+} , Ca^{2+}) in buffer. After UV irradiation (365 nm) and the addition of *Renilla* luciferase, the bioluminescent signal was recorded. As shown in Fig. 2, we can see that the above common ions displayed little or negligible luminescence alterations. Hence, we did not perform further research.

Previous results demonstrated that these compounds had good chemical properties. Then the cytotoxicity of these caged coelenterazine derivatives were evaluated using a CCK-8 assay. After the ES-2-Rluc cells were incubated in 96-well plates for 24 h, the culture medium was removed and serial dilutions of these caged luciferins in complete medium were added. After 14 h incubation and 12 min UV irradiation (365 nm) to the caged substrates, the solution of CCK-8 was added. Finally, a microplate reader was used to record the absorbance signals at 450 nm. There was no obvious cytotoxicity observed, demonstrating that these compounds have good biocompatibility with living cells (Fig. S2).

To further validate the properties of the three caged coelenterazine derivatives and determine their photolytic effectiveness, the bioluminescence imaging at the cellular level was assayed. ES-2-Rluc cancer cell lines which were constitutive expression of *Renilla* luciferase were passed and plated in 96-well plates. After 24 h incubation, the culture media were removed and 60 μM of the three caged coelenterazine derivatives in buffer were added. Then, the solution was removed after incubating 1 h and ES-2-Rluc cells were treated with some normal saline. The bioluminescent signal was collected at once by an IVIS kinetic imaging system equipped with a cooled CCD camera after different time of UV irradiation at 365

nm. Similarly, at various concentration of the three caged coelenterazine derivatives were also incubated with ES-2-Rluc cells for 1 h. Then normal saline was added after removing the buffer. After 6 min of UV light irradiation, the bioluminescent intensity was recorded (Fig. 3 and S3). There was almost no luminescence observed in the caged coelenterazine derivatives in cell without UV excitation (Fig. 3A). But upon UV illumination, the caged luciferins were photoactivated and produced bioluminescent signals. Moreover, the bioluminescent signal from ES-2-Rluc cells was also proportionally dependent on the time of UV irradiation within 6 min and then got to the peak. As shown in Fig. 3B, we can see that light emission intensities were enhanced with increased the caged coelenterazine derivatives concentration and the bioluminescent imaging spectra were similar to those of caged coelenterazine derivatives. Overall, these interesting results further indicated that the caged groups could be cleared by UV light (365 nm) and then the caged coelenterazine derivatives were activated in living cells.

Finally, we also applied these caged coelenterazine derivatives for BLI *in vivo*. All animal studies were approved by the Ethics Committee and IACUC of Qilu College of Medicine, Shandong University and were conducted in compliance with European guidelines for the care and use of laboratory animals.

To construct an ovarian cancer mouse model, ES-2-Rluc cells were implanted subcutaneously in the right armpit region of each mouse. When the solid tumours grew to reach a 1.2 cm diameter about 4 weeks and we mashed them into a homogenate. By grafting roughly the same amounts of the tumour homogenate, the new tumour xenografts of nude mice were obtained. After three weeks, two nude mice were injected with 4 mM (100 μ L) of the caged coelenterazine derivative 1 (CCTZD-1) in the tumour site. And one of them served as a control without UV, another was treated with 5 minutes UV irradiation at 365 nm. Then bioluminescence imaging was immediately performed and we made a total of three times. Compounds CCTZD-2 and CCTZD-3 were applied with the same operation. As shown in Fig. 4, no significant bioluminescence signals were observed in the control, indicating that the caged coelenterazine derivatives were stable *in vivo* and could not release the free luciferins. However, after 5 minutes of UV irradiation, the caged coelenterazine derivatives were photolyzed and imaging experiment showed bright bioluminescence signals *in vivo*. All three caged derivatives displayed similar imaging features. Compared with the control group, the bioluminescence imaging intensities of CCTZD-2 was greatly stronger about 12-fold. For CCTZD-3, the bioluminescent signal was enhanced by approximately 8-fold compared with the control group and the CCTZD-1 was about 5-fold. These interesting results confirmed that the three caged coelenterazine derivatives could release the free luciferins by photolysis *in vivo*. Therefore, they could serve as efficient bioluminescence substrates for BLI in living animals.

In summary, it is a notable strategy for repressive bioluminescence imaging to introduce photolabile protecting groups. We designed and evaluated a series of novel photoactivatable coelenterazine derivatives for bioluminescence imaging in buffer, cells and living animals. As a result, these caged coelenterazine derivatives could release the free luciferins which emitted realistic signals in the presence of *Renilla* luciferase and O₂ in buffer after UV irradiation (365 nm). In addition, there was a good positive correlation between the

concentrations and bioluminescent intensities, as well as the time of UV illumination (within 6 min) and the bioluminescent signal. Similar results were also obtained when the caged coelenterazine derivatives were treated with ES-2-Rluc cells. Surprisingly, the three caged luciferins could be used for bioluminescence imaging *in vivo*. In brief, all these findings suggested that these caged coelenterazine derivatives could serve as highly practical bioluminescent sensor for bioluminescence imaging *in vitro* and *in vivo* after UV excitation. We hope that these novel photoactivatable coelenterazine derivatives will offer new possibilities for real-time imaging the dynamics of the cellular functions or unknown biological phenomena *in vitro* and *in vivo* with high spatial and temporal precision.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(A) The comparison of bioluminescent intensities of caged luciferin derivatives (30 μ M) at the indicated time following UV irradiation. (B) The comparison of bioluminescent intensities of caged luciferin derivatives at different concentrations.

Fig. 2.

(A) Effects of various metal ions on the stability of caged coelenterazine derivatives. (B) The bioluminescence imaging of caged luciferin derivatives with different ions.

Fig. 3.

In cells imaging: (A) The comparison of bioluminescence intensities of caged luciferin derivatives (60 μM) with different time of UV irradiation. (B) The comparison of bioluminescence intensities of caged luciferin derivatives at different concentrations.

Fig. 4.

In vivo imaging: (A) The bioluminescent intensity of caged photoactivatable luciferins. (B) The relative intensity of caged photoactivatable luciferins. (C) The bioluminescence imaging of CCTZD-2.

Scheme 1.

The structure of caged coelenterazine derivatives that can release the substrates of *Renilla* luciferase.