Activatable Photosensitizer for Targeted Ablation of lacZ-Positive Cells with Single-Cell Resolution

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Supporting Information

ABSTRACT: To achieve highly selective ablation of lacZ-positive cells in a biological milieu in vivo, we developed an activatable photosensitizer, SPiDER-killer-βGal, targeted to β-galactosidase encoded by the lacZ reporter gene. Hydrolysis of SPiDER-killer-βGal by β-galactosidase simultaneously activates both its photosensitizing ability and its reactivity to nucleophiles, so that the phototoxic products generated by light irradiation are trapped inside the lacZ-positive cells. The combination of SPiDER-killer-βGal and light irradiation specifically killed lacZ-positive cells in coculture with cells without lacZ expression. Furthermore, β-galactosidase-expressing cells in the posterior region of cultured Drosophila wing discs and in pupal notum of live Drosophila pupae were selectively killed with single-cell resolution. This photosensitizer should be useful for specific ablation of targeted cells in living organisms, for example, to investigate cellular functions in complex networks.

INTRODUCTION

Ablation of specific types of cells is a powerful approach to study cellular functions in complex networks, such as the nervous system, or during biological development. Conditional cell ablation in a spatiotemporally controlled manner has already been achieved by employing the combination of light irradiation and photosensitizers. One approach is based on the genetically encodable photosensitizer proteins, such as KillerRed or MimiSOG, which produce free radicals or singlet oxygen (¹O₂) upon light irradiation, leading to cell death. Since the expression of these photosensitizer proteins is genetically controllable, it is relatively easy to achieve selective ablation of target cells. Small-molecule-based photosensitizers are alternative tools for conditional cell ablation, since in general they show higher phototoxicity, and larger amounts of photosensitizers can be accumulated intracellularly due to the small molecular size. To control the subcellular localization or selectivity toward target cells, these small-molecule photosensitizers can be used in combination with protein-tags or enzyme activities that are selectively expressed in target cells. Small-molecule-based photosensitizers targeting β-galactosidase encoded by the lacZ reporter gene. The photosensitizing ability of some of these photosensitizers is precisely controlled by the characteristic spirocyclization reaction of selenorhodol or selenorhodamine derivatives bearing a hydroxymethyl group; these compounds are colorless and non-phototoxic but are converted to a colored and phototoxic form after reaction with the enzyme (Figure S1a). An advantage of this approach is that the phototoxicity is strongly amplified by enzymatic turnover. However, these photosensitizers cannot achieve target cell ablation with single-cell resolution, because of the leakage of phototoxic products from the target cells during prolonged incubation. Therefore, an activatable photosensitizer capable of inducing cell death with single-cell resolution is still needed.

Here, we describe the development of an activatable photosensitizer capable of ablating lacZ-expressing target cells with single-cell resolution. The design is based on our recently reported activatable fluorescent probes for β-galactosidase, SPiDER-βGal (Figure S1b), which are capable of selective labeling of lacZ-expressing cells with single-cell resolution based on precise control of their fluorescence emission and...
cell-permeability via application of intramolecular spirocyclization and quinone methide chemistry. We demonstrated that our newly developed activatable photosensitizer exhibits simultaneous activation of its photosensitizing ability and its reactivity to nucleophiles, so that the phototoxic products are trapped inside the lacZ-positive cells, and β-galactosidase-
expressing cells were selectively ablated with single-cell resolution in cell culture, ex vivo and in vivo.

**RESULTS AND DISCUSSION**

**Design, Synthesis, and In Vitro Evaluation of a New Activatable Photosensitizer.** We designed a new activatable photosensitizer, SPiDER-killer-βGal, by incorporating a fluoromethyl group as a leaving group at the 4-position of our previously reported activatable photosensitizer HMDESeR-βGal11 (Figure 1a). We anticipated that SPiDER-killer-βGal would exist in a non-phototoxic, spirocyclic, cell-permeable form before reaction with the enzyme, but the enzyme-catalyzed hydrolysis reaction would generate a quinone methide intermediate, which would be trapped selectively in lacZ(+) cells by intracellular nucleophiles to form colored, phototoxic adducts. We first synthesized SPiDER-killer-βGal and 4-CH<sub>2</sub>OH-HMDESeR as a model reaction product (Schemes S1 and S2) and examined their photochemical properties (Figure 1a). Measurement of the absorption spectra of SPiDER-killer-βGal over pH 2−12 indicated that the pK<sub>acid</sub> value (the pH value at which the extent of spirocyclization is sufficient to reduce the absorbance of the compound to one-half of the maximum)15 was 5.4, suggesting that SPiDER-killer-βGal would mainly exist in its colorless, non-phototoxic form at the physiological pH of 7.4 (Figure 1b, Figures S2 and S3). On the other hand, pK<sub>acid</sub> and the pK<sub>base</sub> of the phenolic hydroxyl group of 4-CH<sub>3</sub>OH-HMDESeR were calculated to be 10.3 and 4.8, respectively, indicating that it would mainly exist as the open, phototoxic form at pH 7.4 (Figure 1b, Figure S2). The open form of 4-CH<sub>3</sub>OH-HMDESeR is a potent producer of singlet oxygen (1O<sub>2</sub>) upon light irradiation at 532 nm (Φ<sub>Δ</sub> = 0.36) (Table 1, Figure S3). Further, SPiDER-killer-βGal was efficiently converted to 4-CH<sub>3</sub>OH-HMDESeR upon reaction with β-galactosidase in vitro, and the reaction was accompanied by recovery of the absorption in the visible region and the ability to produce 1O<sub>2</sub> upon light irradiation (Figure 1c,d, Figure S4 and Table S1: kinetic parameters). In addition, SDS-PAGE analysis of the reaction solution in the presence of bovine serum albumin (BSA) revealed that a quinone methide intermediate is produced upon enzyme activation and binds to BSA to produce a fluorescent adduct (the enzyme reaction product of SPiDER-killer-βGal emits faint red fluorescence; Figure 1e). These results indicated that reaction of SPiDER-killer-βGal with β-galactosidase results in significant activation of photosensitizing ability and labeling ability, supporting the idea that SPiDER-killer-βGal can work as an effective photosensitizer for ablating cells with single-cell resolution.

**Selective Ablation of lacZ-Positive Cells in Cell Culture.** To confirm that SPiDER-killer-βGal can selectively induce death of lacZ(+) cells, we applied SPiDER-killer-βGal to two cultured cell lines, one expressing and one not expressing β-galactosidase (HEK/lacZ(+) cells and HEK293 cells, respectively), and evaluated the cell viability after light irradiation. We found that SPiDER-killer-βGal induced dose-dependent cell death of HEK/lacZ(+) cells in the concentration range up to 10 μM, while it had no effect on HEK293 cells (Figure 2a). We also confirmed that SPiDER-killer-βGal showed essentially no cytotoxicity in the absence of light irradiation (i.e., no dark toxicity) under our experimental conditions (Figure S5).

Next, to examine whether SPiDER-killer-βGal can achieve cell ablation with single-cell resolution, we applied SPiDER-killer-βGal to a coculture of HEK/lacZ(+) cells and HEK293 cells. HEK/lacZ(+) cells and HEK293 cells were pre-stained with CellTracker Blue and CellTracker Green, respectively. The coculture was incubated with SPiDER-killer-βGal and then irradiated with 561 nm laser light under a confocal microscope. We found that HEK/lacZ(+) cells selectively started to form blebs, an indicator of apoptosis, followed by cell shrinkage and rupture after photoirradiation (Figure 2b, Figure S6). On the other hand, HEK293 cells showed no marked change in cell shape and remained intact after light irradiation. We confirmed that selective cell death of HEK/ lacZ(+) cells occurs similarly in a coculture in which only HEK293 cells were pre-stained with CellTracker Blue, and HEK/lacZ(+) cells were unstained, indicating that the cell death was not induced by the staining with CellTracker Blue (Figure 7a). When the cocultured cells were incubated without light irradiation or without SPiDER-killer-βGal, neither HEK/lacZ(+) nor HEK293 cells showed any marked change in cell shape (Figure 2b, Figure S7b−d). Further, when our previously reported activatable photosensitizer HMDESeR-βGal was applied to the coculture, death of both HEK/lacZ(+) cells and HEK293 cells was induced, in marked contrast to the case of SPiDER-killer-βGal (Figure S8). These
results confirm that SPiDER-killer-βGal can induce selective death of β-galactosidase-expressing cells at the single-cell level, which is not possible with previously reported small-molecular photosensitizers.

**Selective Ablation of lacZ-Positive Cells in Cultured Tissue and In Vivo.** To investigate whether SPiDER-killer-βGal works in tissue, we next applied it to live cultured tissues of *Drosophila melanogaster*, which is one of the most widely used model organisms for genetic studies.16,17 We used *en-lacZ* wing discs from third instar larvae, the epithelial precursors of a part of the adult thorax including the wing, in which β-galactosidase is expressed only in the posterior region (Figure 3a). After incubation with SPiDER-killer-βGal, whole wing discs were exposed to irradiation with a Xe lamp at 550 nm. After culture for 4 h, the wing discs were stained with Calcein-AM as an indicator of live cells. Consistent with restricted expression of β-galactosidase to the posterior region, green fluorescence of Calcein-AM was detected outside the posterior regions, suggesting that SPiDER-killer-βGal was activated and induced cell death selectively in the posterior region (Figure 3b, upper). Cell death in the posterior region was not observed when the tissue was incubated without SPiDER-killer-βGal and/or without light irradiation (Figure 3b, lower; Figure S9). These results indicate that SPiDER-killer-βGal can induce cell death with high target selectivity not only in cultured cells but also in cultured tissue.

Next, to further investigate the in vivo applicability of SPiDER-killer-βGal at the single-cell level, we applied it to the epithelium of *Drosophila* pupal notum (Figure 4a). Expression of *lacZ*, H2B-ECFP (an expression marker), and VC3Ai (an apoptosis reporter detecting caspase-3-like protease activity)18 was induced in some cell populations of the pupal notum by heat-shock treatment (Figure S10). Then, a part of the pupal case was removed, and approximately 0.2−0.5 μL of a mixture of SPiDER-killer-βGal (5 mM) and Hoechst33342 (5 mM) in DMSO was injected into the abdomen. After 1.5 h of incubation, the pupal notum was laser-irradiated at 561 nm, and time-lapse imaging of Hoechst33342 (blue: nuclei), H2B-ECFP (green: expression marker of lacZ-expressing cells), and VC3Ai (red: marker of apoptotic cells) was carried out with a confocal microscope (Figure S11). The VC3Ai signal in lacZ-expressing cells started to appear at 3−4 h after light irradiation, indicating that apoptotic cell death was induced by the photosensitizer in combination with light irradiation.
(Figure 4b, upper; Figure S12a, Movie S1). After VC3Ai signal appearance, the nuclei of lacZ-expressing cells started to fragment, followed by delamination of the cells into the body cavity. As we performed imaging at the side of the midline of the pupal notum (Figure 4a), where sublethal caspase activity is known to be activated,19 cells closer to the midline are likely to be more sensitive to additional apoptotic stimuli, which may explain why lacZ-positive cells tended to die differently. Further, in the absence of SPIDER-killer-βGal injection and/or light irradiation, only a few VC3Ai signals or nuclear fragmentations were observed (Figure 4b, lower; cells undergoing spontaneous apoptosis can be seen in the time-lapse images with a 1 h time interval in Figure S12b–d). These results suggest that SPIDER-killer-βGal can induce cell death of lacZ-expressing cells in vivo.

To determine whether the cell death induced by SPIDER-killer-βGal was selective to lacZ-expressing cells, we carried out immunohistochemical staining of the pupal notum using antibody against the cleaved (or activated) form of Death caspase-1 (Dcp-1), a homologue of caspase-3,20 to detect apoptotic cells (Figure 4c). It was reported that, in apoptotic cells, where caspase-3 is strongly activated, nuclear fragmentation occurs, and intracellular proteins including fluorescent marker proteins tend to be degraded, diminishing the fluorescence signal of fluorescent proteins.21,22 In fact, among Dcp-1-positive cells, ECFP exhibited a normal nuclear distribution pattern or a fragmentation pattern, which is consistent with the requirement of Dcp-1 activation for nuclear fragmentation; some Dcp-1-positive cells seemed to have been fixed before the execution of nuclear fragmentation. Several isolated green fluorescent dots of ECFP were observed as a result of nuclear fragmentation and protein degradation, and they colocalized well with Dcp-1 staining. Therefore, we consider that the Dcp-1 staining observed in Figure 4c mainly originates from apoptotic lacZ-positive cells (ECFP-positive cells). Although there are a few Dcp-1-positive ECFP-negative cells (red-but-not-green cells), similar cells were also observed in control pupa notum without light irradiation or without SPIDER-killer-βGal injection and presumably represent cells undergoing spontaneous apoptosis during physiological development (Figure S13). More importantly, considering that cells without lacZ/ECFP expression did not show marked Dcp-1 staining, even when located adjacent to lacZ-expressing cells, it is clear that cell death induced by SPIDER-killer-βGal is highly selective for lacZ-expressing cells at the single-cell level in vivo.

## CONCLUSION

In conclusion, we have developed an activatable photosensitizer, SPIDER-killer-βGal, that exhibits dramatic activation of the photosensitizing ability upon reaction with the enzyme and is retained inside cells after activation due to reaction with intracellular nucleophiles. We confirmed that SPIDER-killer-βGal selectively killed β-galactosidase-expressing cells with single-cell resolution not only in cell cultures, but also in ex vivo and in vivo epithelium of Drosophila. To our knowledge, this is the first example of a small-molecular activatable photosensitizer capable of targeted ablation of lacZ-positive cells with single-cell resolution in vivo. We believe that this design strategy will also be applicable to develop activatable photosensitizers targeted to other enzymes, simply by replacing the substrate moiety. This should yield a range of versatile tools for studying cellular functions in complex networks, and also candidate agents for tumor-specific therapy.

## ASSOCIATED CONTENT

# Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.9b00678.

Materials and methods, synthetic procedures and characterization, detailed experimental procedures, spectroscopic data, and fluorescence imaging results (PDF) Movie S1: time-lapse fluorescence imaging of flip-out clones of live pupal notum injected with SPIDER-killer-βGal and Hoechst33342, followed by 561 nm light irradiation (AVI)

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### Notes

The authors declare no competing financial interest.

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