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Design and synthesis of an ER-specific fluorescent probe based on carboxylesterase activity with quinone methide cleavage process

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ABSTRACT

CEs are important enzymes that catalyze the hydrolysis of prodrugs. In this Letter, we present a new mechanistic ER-specific fluorescent probe **1** based on CE activity. Permeation of **1** into cells and subsequent hydrolytic activation by CEs causes spontaneously quinone methide cleavage, resulting in bright red fluorescence in ER with high specificity. Probe **1** was developed for CE activity imaging and inhibitor screening at the cellular level.

In mammals, CEs (EC 3.1.1.1) are expressed in numerous tissues.[1] CEs hydrolyze various prodrugs, including the anti-influenza prodrug oseltamivir, the anti-thrombogenic agent clopidogrel, the cholesterol reduction drug lovastatin, and the β-blocker esmolol.[2] The hydrolytic activity of CEs has important roles in drug protection and metabolism,[3] yet fluoresces probes for these enzymes to study these important processes at the cellular level are insufficient. In addition, the development of inhibitors of CEs has many potential uses, including increasing the drug lifetime and improving, delaying, controlling, and specifically expressing the action of the parent drug using a prodrug.

CE-activated fluorescent probes that are commercially available, mainly ester-protected fluorescein derivatives such as fluorescein diacetate, carboxy fluorescein diacetate, and calcein-AM,

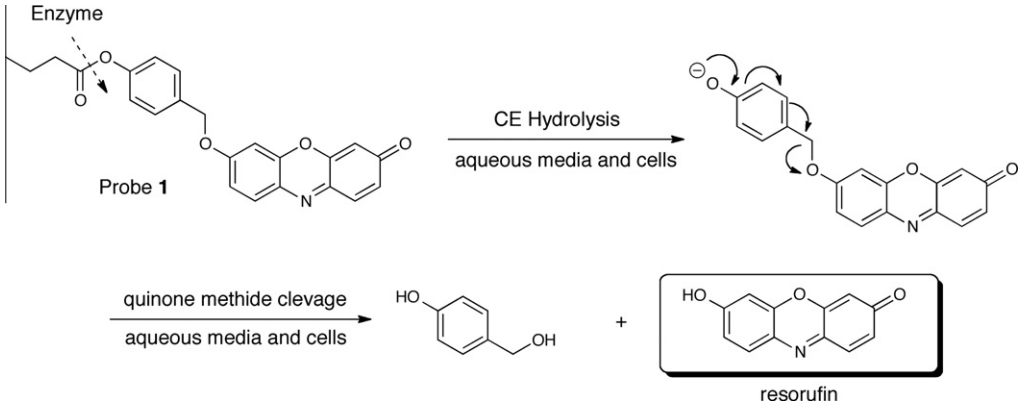

Figure 1. Schematic illustration of the fluorescence revealing by quinone methide-type cleavage.

Abbreviations: CE, carboxylesterase; ER, endoplasmic reticulum; TBSCl, *tert*-butyldimethylchlorosilane; DMF, *N,N*-dimethylformamide; TBAF, tetrabutyl ammonium fluoride; THF, tetrahydrofuran; TBP, tributylphosphine; ADDP, 1,1′-(azodicarbonyl)dipiperidine.

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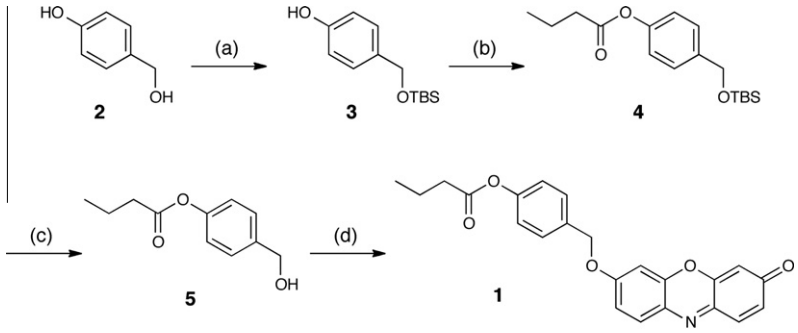

(a) NaH, TBSCl, DMF, 95.6 %, (b) $CH_3CH_2CH_2COCl$, C_5H_5N, 73.3 %,
(c) TBAF, THF, 80.1 %, (d) Resolufin, TBP, ADDP, THF, 89.0 %

Figure 2. Synthetic scheme of probe **1**.

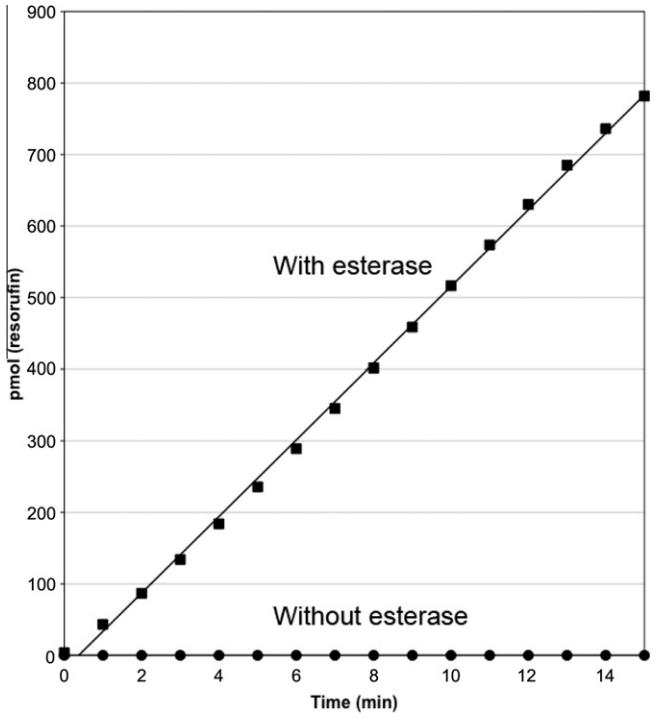

Figure 3. Esterase-assisted probe activation. Hydrolysis of probe **1** with and without porcine liver esterase at 37 °C.

have been applied to studies of living cell labeling, cell viability, and cytotoxicity for a variety of cells. After permeation of protected fluorescein derivatives into cells, the ester bonds can be hydrolyzed by CEs. Hydrolysis often results in direct conversion of the protected fluorescein derivatives into fluorescent forms that are retained and dye the cell cytoplasm green.

Here we describe the design, synthesis, photochemical, and biological properties, and cell imaging of resorufin releasing fluorescence probe **1** in Figure 1. The fluorogenic chemical transformation of **1** triggered by the CEs in cells is through a tandem reaction, ester hydrolyzation, and quinone methide-type cleavage reaction, which are spontaneous and irreversible at physiological temperature in aqueous media (also see Fig. 1). Resorufin is the parent fluorescent used widely in cell imaging, and has an emission spectrum at 585 nm to long wavelength excitation at 571 nm. The major benefits of long wavelength excitation include much reduced fluorescence of the background obtained from cells. Probe **1** is used as a fluorescent dye in cells as it has appropriate membrane permeability. Waring reported that a log *D* value of >1.7 is required for compounds of molecule weight 350–400 for good membrane permeation.[4] The designed probe **1**, which has a molecular weight of 389 and a *c* log *D* value of 4.87 at pH 7–8,[5] will have good membrane permeability.

Details of the synthetic scheme of **1** are outlined in Figure 2. In the final step, Mitsunobu reaction conditions, using compound **5**[6] and resorufin, yielded the respective product **1**.[7] The overall four-step synthesis had a 50.0% yield from starting material. To be a CE activity imaging tool in cells, probe **1** should not have emission spectra under resorufin excitation conditions at 571 nm. To observe the emission spectra of resorufin and **1** in PBS(-) at 571 nm, these emission spectra were measured and indicated no emission spectra from **1**.[8]

We investigated the release of resorufin from **1** activated by the porcine liver esterase in vitro and observed clearly the release of the fluorescence signal of resorufin when probe **1** was co-incubated with the esterase (Fig. 3). In that reaction, the enzyme show appropriate activity, 0.05 unit/mg protein, against **1**.[9] These results show that the enzyme transformation of **1** to release resorufin is through a tandem reaction, ester hydrolyzation, and spontaneous quinone methide cleavage, at 37 °C in phosphate buffer at pH 8.0.

The most important application for **1** is as a CE activity imaging tool in cells. To test this capability, we tried to transfer **1** into cells across membranes, and the imaging potential of **1** based on CE activity was then evaluated in cell culture using a human fibrosarcoma cell line, HT-1080, and kidney cells of the African green monkey, COS-1. Following incubation of both cell lines with **1** (50 μM) for 120 min and Hoechst 33258 as the nuclear fluorescence dye, phase-contrast microscopy images and fluorescence images were taken by a fluorescence microscope.[10] The fluorescence signal of **1** indicated intracellular accumulation of resorufin around the nucleus which was dyed by Hoechst 33258, as shown in Figure 4. To specify the fluorescence dyeing area of **1**, the ERs of both cell lines were dyed with **1** and ER-Tracker Blue–White DPX, a highly selective dye for the ER. The results show that the fluorescence dyeing areas of **1** and ER-Tracker complementally overlapped, as shown in Figure 5. These images indicated that **1** had good selectivity against ERs of both cell lines with bright red fluorescence.

Clearly, probe **1** is feasible as an ER-specific fluorescent probe based on CE activity: in particular, the selectivity for ER dyeing was high, whereas ester-protected fluorescein derivatives, which were activated by CEs, showed dyeing ability for cytoplasm. Previous research on the location of CEs in human cells show that CEs are located in ER[11] and cytoplasm.[12] These results showed that probe **1** can enhance the importance and usefulness of CE activity

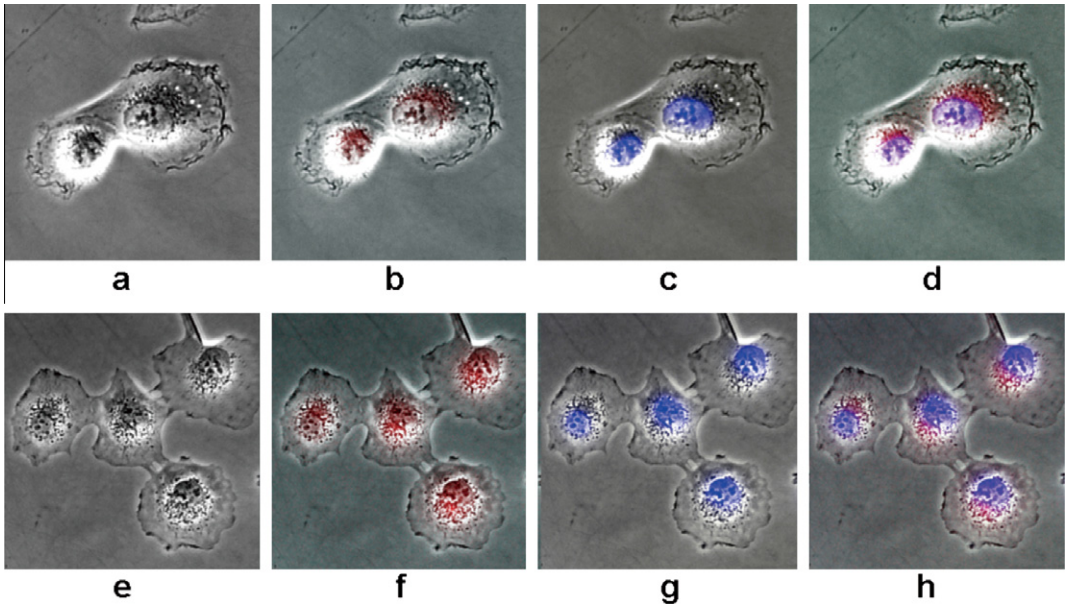

Figure 4. Images of HT1080 cells (a–d) and COS-1 cells (e–h). The phase-contrast images are a and e. The images of 1, in red, are b and f. The images of Hoechst 33258, in blue, are c and g. The overlaid images of 1 and Hoechst 33258 are d and h.

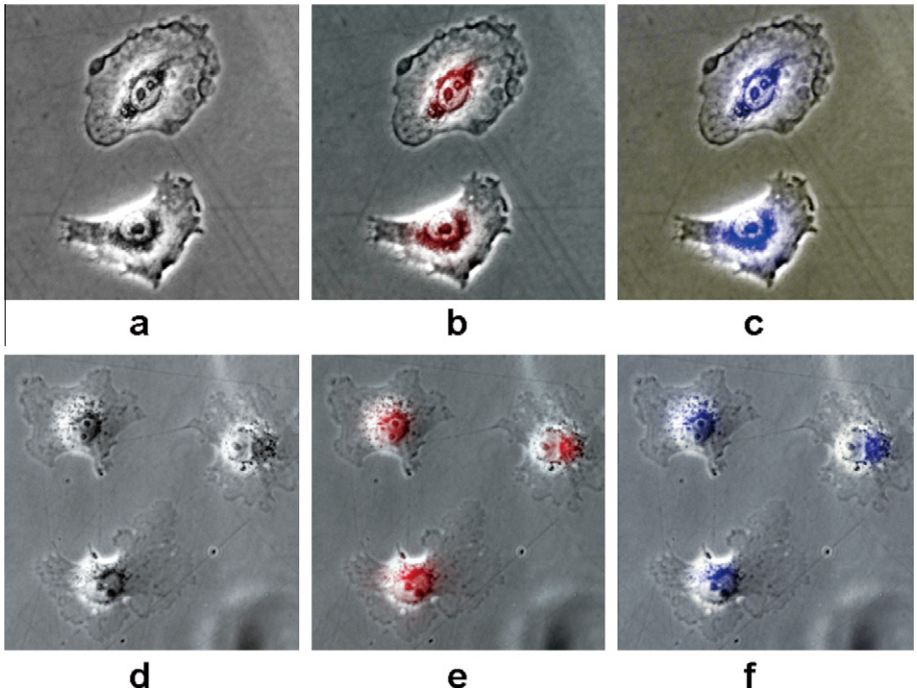

Figure 5. Images of HT1080 cells (a–c) and COS-1 cells (d–f). The phase-contrast images are a and d. The images of 1, in red, are b and e. The images of ER-Tracker Blue–White, in blue, are c and f.

imaging, functional analysis of CEs, and inhibitor screening at the cellular level.

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References and notes

1. (a) Satoh, T.; Hosokawa, M. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 257; (b) Satoh, T.; Hosokawa, M. *Chem. Biol. Interact.* **2006**, *162*, 195.
2. Wheelock, C. E.; Nakagawa, Y. *J. Pestic. Sci.* **2010**, *35*, 215.
3. Imai, T.; Hosokawa, M. *J. Pestic. Sci.* **2010**, *35*, 229.
4. Waring, M. J. *Expert Opin. Drug Disc.* **2010**, *5*, 235.
5. Advanced Chemistry Development Inc., Structure Design Suite Ver. 12.
6. Briggs, A. D.; Camplo, M.; Freeman, S.; Lundströmb, J.; Brian, G. P. *Tetrahedron* **1996**, *52*, 14937.
7. Synthesis of **1**. To a solution of benzyl alcohol derivative **5** (107 mg, 0.55 mmol) in dry THF 200 mL and the mixture was added ADDP (831 mg, 3.3 mmol), TBP (814 μL, 3.3 mmol), and resorufin (141 mg, 0.66 mmol). After stirring the

mixture at rt for 30 min, the resulting mixture was concentrated and then the resulting residue was purified by column chromatography on silica gel (1:1 hexane–ethyl acetate). Obtained fractions containing probe **1** were concentrated and then the resulting residue was purified by column chromatography on silica gel (20:1 dichloromethane–methanol) to afford 191 mg (89.0%) of probe **1** and was recrystallized from CH_2Cl_2/hexane to give a light orange solid: mp 266–267 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.04 (t, 3H, J = 7.5, –CH_3), 1.78 (qt, 2H, J = 7.5, –CH_2–CH_3), 2.55 (t, 2H, –CO–CH_2–), 5.15 (s, 2H, benzyl position), 6.32 (d, 1H, J = 2.3), 6.83 (dd, 1H, J = 2.3, 9.7), 6.87 (d, 1H, J = 2.9), 7.00 (dd, 1H, J = 2.6, 8.9), 7.13 (d, 2H, J = 8.0), 7.41 (d, 1H, J = 9.8), 7.45 (d, 2H, J = 8.6), 7.71 (d, 1H, J = 9.2), ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.64 (–CH_2–CH_3), 18.43 (–CH_2–CH_2–), 36.19 (–CO–CH_2–), 70.25 (benzyl position), 101.03, 106.78, 114.22, 122.07, 128.51, 128.69, 131.63, 132.82, 134.28, 134.70, 145.58, 145.74, 149.77, 150.79, 162.46, 172.09 (–C=O, butyl ester), 186.33 (–C=O, resorufin), ESI-MS (Positive): m/z = 390 $[M+H]^+$. Anal. Calcd for $C_{23}H_{19}NO_5$: C, 70.94; H, 4.92; N, 3.60. Found: C, 70.92; H, 4.91; N, 3.54.

8. Photochemical characterization. The solutions of resorufin and probe **1** (10 μM) in PBS(-) containing 0.1% DMSO, respectively. The fluorescence emission spectra of these solutions were recorded with an excitation wavelength of 571 nm.
9. Enzyme assays. Probe **1** was prepared as a 1 mM solution in DMSO. Esterase (from porcine liver, purchased from SIGMA) was prepared as a 16.5 μg/mL solution in 200 mM Na phosphate buffer (pH 8.0). The assay were conducted by adding esterase solution (85 μL) to **1** (5 μL) and 200 mM Na phosphate buffer, pH 8.0 (100 μL) followed by incubation at 37 °C. The assays were followed by the monitoring fluorescence intensity change of resorufin. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of resorufin/min.
10. Fluorescence microscopy studies. The human fibrosarcoma cell line, HT-1080 (RCB1956), and kidney cells of the African green monkey, COS-1 (RCB0143) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. HT1080 and COS-1 cells were cultured, medium was exchanged for fresh medium, and then incubated at 37 °C in the presence of 50 μM of **1** for 120 min. After incubation, both cells were removed from the medium and under went formalin fixation. In this culture condition, probe **1** showed no significant cytotoxicity composed to the trypan blue method. The fluorescence signal of the cells was recorded using an BIOREVO BZ-9000 fluorescence microscope (KEYENCE Inc.) equipped with TRITC (exciter; 540/25 nm, emitter; 605/55 nm) and DAPI (exciter; 360/40, emitter; 460/50 nm) filter sets.
11. (a) Hosokawa, M. *Molecules* **2008**, *13*, 412; (b) Fukami, T.; Nakajima, M.; Maruichi, T.; Takahashi, S.; Takamiya, M.; Aoki, Y.; McLeod, H. L.; Yokoi, T. *Pharmacogenet. Genomics* **2008**, *18*, 911.
12. (a) Hennebelle, I.; Terret, C.; Chatelut, E.; Bugat, R.; Canal, P.; Guichard, S. *Anticancer Drugs* **2000**, *11*, 465; (b) Humerickhouse, R.; Lohrbach, K.; Li, L.; Bosron, W. F.; Dolan, M. E. *Cancer Res.* **2000**, *60*, 1189; (c) Takahashi, S.; Katoh, M.; Saitoh, T.; Nakajima, M.; Yokoi, T. *J. Pharm. Sci.* **2008**, *97*, 5434.