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Multicolor Imaging of Endoplasmic Reticulum-Located Esterase As a Prodrug Activation Enzyme

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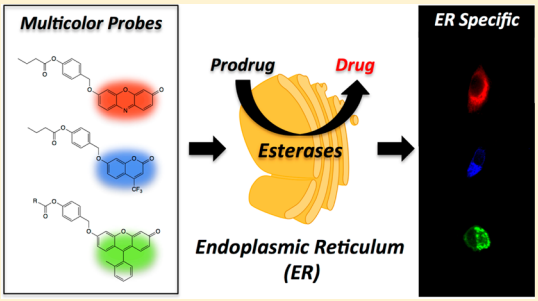
Supporting Information

ABSTRACT: The carboxylesterase families of enzymes are key participants in phase I drug metabolism processes. Carboxylesterase families 1 and 2 are of particular clinical relevance. These enzymes produce endoplasmic reticulum localization signals, are primarily localized in the endoplasmic reticulum, and hydrolyze a wide range of ester-containing prodrugs into an activated form. In order to detect enzymes belonging to both families, we developed an optical multicolor imaging technique, which provides a distinct color window for multicolor imaging. This technique required the design and synthesis of three new mechanistic colored probes that fluoresce red, green, or blue and are based on the quinone methide cleavage process. These activity-based probes allow rapid and clear visualization with high specificity against the endoplasmic reticulum in cultured cells based on endoplasmic reticulum localized esterases including both families of carboxylesterase enzymes.



KEYWORDS: *Prodrug, carboxylesterase, fluorescent probe, quinone methide cleavage*

Carboxylesterases (CEs) metabolize numerous exogenous and endogenous ester-containing compounds and play a role in a broad range of biological processes. CEs are a key participant in phase I drug metabolism processes by catalyzing the hydrolysis of various prodrugs. This is a well-established strategy for improving the physicochemical, biopharmaceutical, or pharmacokinetic properties of pharmacologically potent compounds.[1,2] Another interesting bioactive phenomenon, recently reported by Pezacki et al., is that carboxylesterase 1 is a host factor involved in the hepatitis C virus life cycle.[3] The CEs are classified into five families (CES1, CES2, CES3, CES4, and CES5) according to the homology of their amino acid sequence.[4] The hydrolytic abilities of the CES1 and CES2 families are important for the bioconversion of ester-containing prodrugs. Both families contain the HXEL (His-X-Glu-Leu) sequence at the C-terminal, which can bind with the KDEL (Lys-Asp-Glu-Leu) sequence in the endoplasmic reticulum (ER) protein retention receptor. This binding reaction is essential for retention of the protein on the luminal site of the ER. Ester-containing prodrugs that pass through the cell membrane and ER membrane by simple diffusion are therefore hydrolyzed to their activated forms by CES1 and/or CES2 family enzymes in the ER. The activation of prodrugs by these enzymes provides a possible means for overcoming various barriers in drug formulation and delivery such as poor aqueous solubility, chemical instability, insufficient oral absorption, rapid presystemic metabolism, inadequate brain penetration, toxicity, and local irritation.[5−8] However, despite their importance in drug activation, biological activity imaging of CEs in the ER at the cellular level has yet to be fully established. Our group has reported an ER-localized CE-specific fluorescence probe, **1**, for detecting CE activity using the quinone methide cleavage process, which is a 1,6-elimination reaction, as shown in Figure 1A. The fluorogenic chemical transformation of **1** triggered by CEs in the ER occurs through two reactions, ester hydrolysis and quinone methide cleavage, which are spontaneous and irreversible in cells. After hydrolysis, released resorufin (EX, 571 nm; EM, 585 nm) from **1** specifically stained ERs with bright red fluorescence in a human fibrosarcoma cell line, HT-1080, and kidney cells of the African green monkey, COS-1.[9] Previous research on the location of CEs in human cells showed that CEs are located in the ER[10] and cytoplasm.[11−13] Interestingly, probe **1** is an ER-specific dye, whereas commercially available ester-protected fluorescein derivatives activated by CEs also dye the cytoplasm.

Multicolor imaging is used for the in vitro microscopic imaging of specific enzyme activity. These in vitro microscope studies are generally performed using combinations of commercially available red, green. and blue fluorescent probes. The images are taken separately, one color at a time, using an appropriate filter set, and then the colors are merged during postprocessing to construct a multicolor image. Multicolor imaging of CES activity in the ER at the cellular level is clearly of interest and could be accomplished based on our previous research.[9] To develop probes for multicolor imaging of CE activity in the ER, TFMU (EX, 385 nm; EM, 502 nm) and

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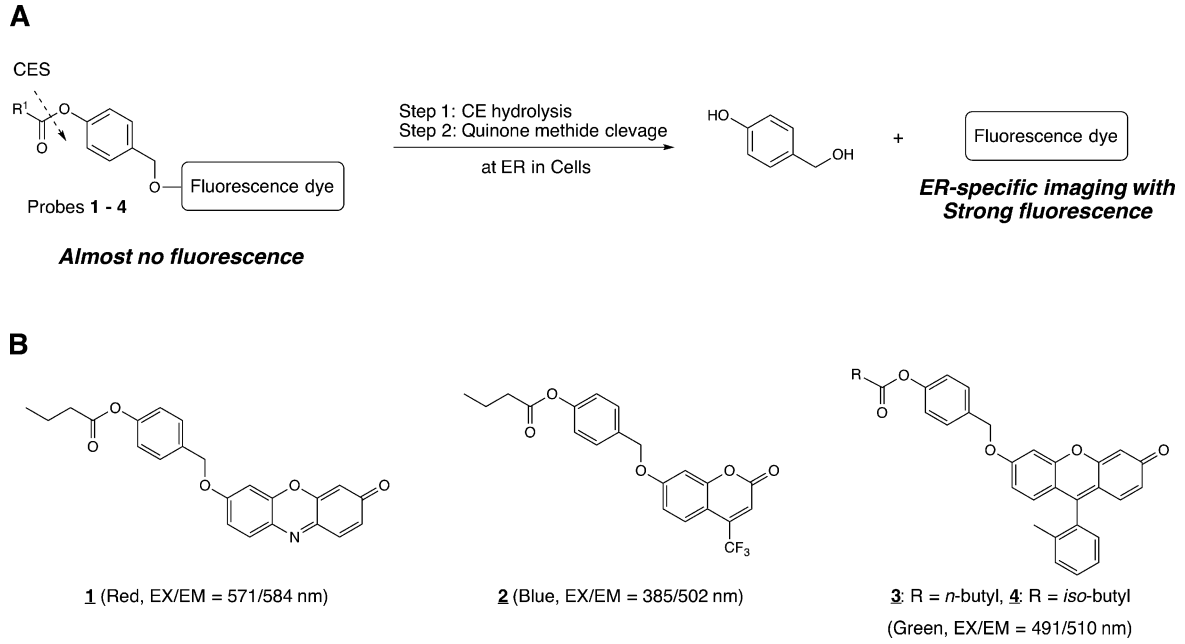

Figure 1. Schematic illustration of ER-specific imaging using the strong fluorescence provided by the probes following quinone methide cleavage (A). Structure and fluorescence properties of probes **1**–**4** (B).

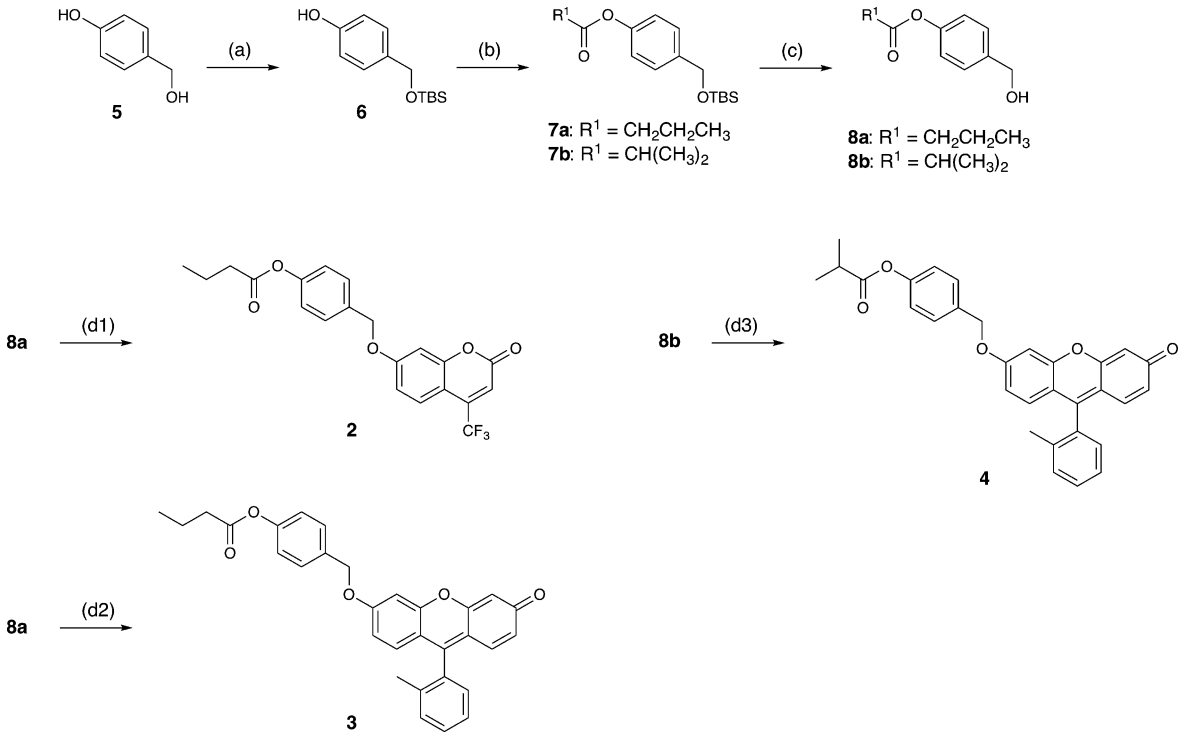

Figure 2. Synthetic scheme for probes **2**–**4**.

2MeTG (EX, 491 nm; EM, 510 nm)[14−16] were chosen as the blue and green fluorophores, respectively. The fluorophores resorufin, TFMU and 2MeTG each have distinct excitation and emission wavelengths, excellent fluorescence quantum yields, and low pH-dependent change in fluorescence, making them suitable for multicolor imaging at the cellular level.

Here we describe the design, synthesis, and photochemical and biological properties of fluorescence probes **1**–**4**. The probes were used to image CEs in three human cell lines, as shown in Figure 1B. The results show that this approach can provide multicolor imaging of CES1 and CES2 activity as prodrug activators in the ER. The substrate specificities of CES1 and CES2 are significantly different: CES1 has a broad substrate specificity, whereas CES2 does not accept bulky acyl groups as substrate.[17] All the probes contain a large alcohol group, making these probes likely CES2 substrates.

Probes **1**–**4** are compatible for use as fluorescent dyes in cells as they exhibit appropriate membrane permeability. Waring[18] reported that, to achieve good membrane permeation, a logD value of >1.7 is required for compounds with a molecular

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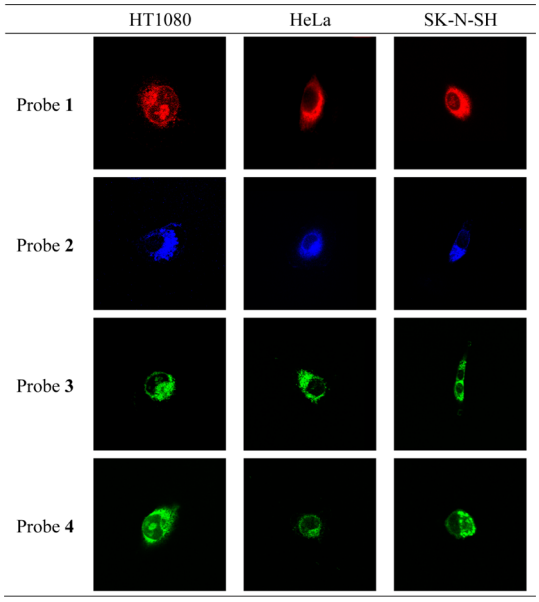

Figure 3. Confocal laser scanning microscopy fluorescence images of three cell lines stained with probes **1**−**4**.

weight of 350−400, a logD value of >3.1 is required for compounds with a molecular weight of 400−450, and a logD value of >3.4 is required for compounds with a molecular weight of 450−500. This has been demonstrated using Caco-2 cell data from AstraZeneca. Probes **1**−**4**, which have molecular weights of 389, 406, 479, and 479 and ClogD values of 4.87, 4.03, 8.10 and 7.95, respectively, between pH 7 to 8, have good membrane permeability.[19] The synthesis of probes **2**−**4** is presented in Figure 2. The fluorescence spectra of probes **1**−**4** are provided in the Supporting Information.

To be useful as CES activity multicolor imaging tools in cells, the emission of probes **1**−**4** should not overlap the excitation conditions of the parent fluorophores resorufin, TFMU and 2MeTG. To observe the emission spectra of each fluorophore and probes **1**−**4** in PBS (−), the emission spectra were measured. Probes **1**−**4** showed almost no emission. For synthesis details, refer to Supporting Information.

We investigated the release of each fluorophore (TFMU and 2MeTG) from probes **2**−**4** activated by porcine liver esterase in vitro. The release of the fluorescence signal of each fluorophore was clearly observed when probes **2**−**4** were coincubated with the esterase. Plots of fluorescence intensity vs incubation time (see Supporting Information) show pseudo-first order kinetics for all the probes. The results indicate that the esterase shows appropriate specific activity (5.91, 3.51, and 4.03 units/mg protein) against probes **2**−**4**, respectively. These results and comparable results using probe **1**[9] show that the enzyme catalyzes probes **1**−**4** to release each fluorophore via a two-step reaction: ester hydrolysis and spontaneous quinone methide cleavage. These two reactions are not dependent on the fluorophore structure under the conditions used. Therefore, differences in the structure of the fluorophore do not affect the quinone methide reaction in aqueous media. For enzyme kinetics details, refer to Supporting Information.

Finally, to demonstrate the utility of probes **1**−**4** for multicolor imaging of CEs in ERs in three human cell lines,

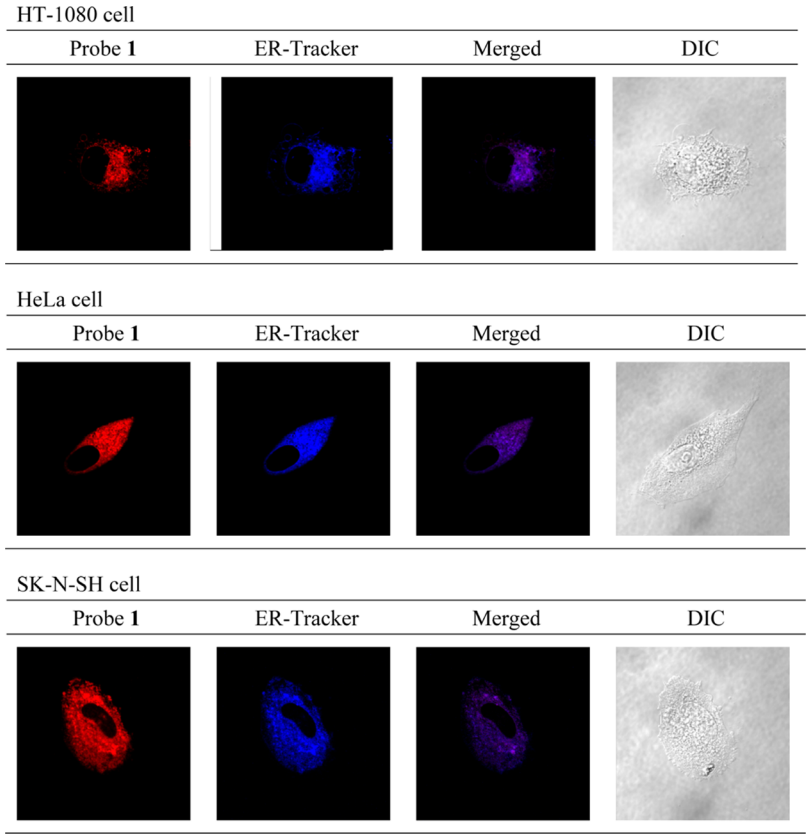

Figure 4. Fluorescence images of probe **1**, ER-Tracker, merged fluorescence image, and differential interference contrast microscopy (DIC) images of three kinds of cell lines.

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we characterized the dynamics between localization and enzyme activity. We introduced probes **1**−**4** into the cells across the cell membrane and the ER membranes. The imaging potential of **1**−**4** based on CE activity was evaluated in cell culture of a human fibrosarcoma cell line, HT-1080, a human neuroblastoma cell line, SK-N-SH, and a human epithelial carcinoma cell line, HeLa. Each cell line was incubated with 5 μM probe **1**, **2**, **3** or **4** for 30 min in the presence of Hoechst 33258 as a nuclear fluorescence dye. Note that probe **2** is excited at the same wavelength as Hoechst 33258. Differential interference contrast microscope images and fluorescence images were taken using confocal laser scanning microscopy. The fluorescence signal of probes **1**−**4** indicated the intracellular accumulation of resorufin, TFMU, and 2MeTG around the nucleus, which was dyed with Hoechst 33258; typical images showing the reticular structure of the ER are shown in Figure 3. These images indicated that probes **1**−**4** had high selectivity for the ERs of all the cell lines tested and provided bright fluorescence. These results show that the cell enzyme transformations and imaging of ERs by probes **1**−**4** do not depend on the fluorophore structures and the ester structures. We thus succeeded in optically separating the fluorescence signal from probes **1**−**4**, allowing us to visualize esterase activity in the ER. On the basis of these results, many other fluorophores with different structures may prove useful in imaging studies and provide the possibility of additional fluorescence colors.

Next, to conclusively determine the staining areas of these activity-based probes, the three human cell lines were stained simultaneously with probe **1** and a commercially available ER-specific fluorescence dye, ER-Tracker Blue-White DPX dye (EX, 374 nm; EM, 430 nm). Probe **1** was used as a typical resorufin red fluorophore probe that possesses different fluorescence excitation and emission wavelengths from ER-Tracker. Figure 4 summarizes the results of the staining areas of both dyes in the three cell lines. The staining area obtained using probe **1** is identical to that obtained using ER-Tracker at the same time. Imaging with probe **1** substantiated the conclusion that these probes stain the ER with high specificity. Clearly, probes **1**−**4** hold promise as ER-specific fluorescent probes for esterase activity containing CEs. However, the amount of fluorescence from these probes is highly dependent on the expression level of the esterases. The probes should be required to have high specificity for the CEs. Finally, we used multicolor imaging of highly expressed esterase activity located in the ERs using probes **1**−**4** to hydrolyze various prodrugs. The methods can be used in the ERs of different human cell types to image esterase activity containing CEs.

CE enzymes are one of the major determinants of the metabolism and disposition of numerous prodrugs hydrolyzed and activated in the ER. Thus, elucidating the mechanism governing CE enzyme activity using small molecules will significantly impact rational drug design and the future development of prodrugs. These activity-based probes could provide a basis for the design and development of the physicochemical, biopharmaceutical, or pharmacokinetic properties of pharmacologically potent compounds as novel therapeutics when activated by CEs. We envision the application of these activity-based probes in the quantitation of CE activity, the functional analysis of CEs, and the screening of CE inhibitors at the cellular level.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedures and characterization of compounds, in vitro enzyme assays, and cell culture assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CE, carboxylesterase; ER, endoplasmic reticulum; EX, excitation wavelength; EM, emission wavelength; TFMU, 4-trifluoromethylumbelliferone; 2MeTG, 2-methyl TokyoGreen; PBS, phosphate buffered saline

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