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Design and Development of Enzymatically Activatable Photosensitizer Based on Unique Characteristics of Thiazole Orange

Yuichiro Koide,[†,‡] Yasuteru Urano,[†,§] Akira Yatsushige,[†,‡] Kenjiro Hanaoka,[†,‡] Takuya Terai,[†,‡] and Tetsuo Nagano*[,†,‡]

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan, and CREST, JST, and PRESTO, JST, Japan Science and Technology Agency, 3-5 Sanbancho, Chiyoda, Tokyo 102-0075, Japan

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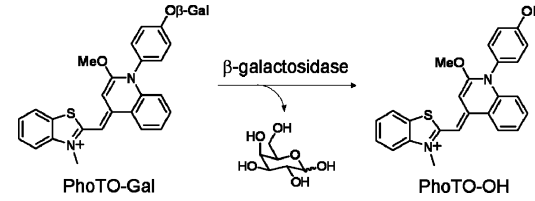
Nucleic acid binding fluorescent probes, such as Hoechst, DAPI, and SYTO dyes, are widely used for DNA visualization, DNA quantification, and contrasting cell nuclei for fluorescence microscopy of living cells.[1] These compounds, having cationic character and a planar aromatic system, interact noncovalently with negatively charged double-stranded DNA or RNA by way of intercalation or minor groove binding. Thiazole orange (TO), which is composed of a benzothiazole ring covalently linked to a quinoline ring through a monomethine bridge, is a widely used dye for nucleic acids, allowing detection of DNA and RNA in gels, flow cytometry, or microscopy.[2] Although the fluorescence of free TO is extremely low in aqueous solution, when TO binds to nucleic acids, the viscosity of the dye's local environment is markedly increased, resulting in a drastic increase in fluorescence.[3] This is thought to arise from restriction of rotation around the methine bond between the two heterocyclic systems.[4,5] The strong fluorescence of nucleic acid-bound dye and almost nonfluorescence of unbound dye provide excellent contrast for imaging and detection.

This dramatic fluorescence difference between the unbound and bound states was recently utilized by Waggoner et al. They developed a novel fluorescence imaging technique for cell surface proteins by synthesizing a sulfonated TO derivative and by screening for peptides which, when displayed on the cell surface, specifically bind the derivative.[6] However, we thought that a more versatile approach to developing fluorescent probes would be to change the structure of TO itself by utilizing a specific chemical or enzymatic reaction. In addition, we focused on the fact that TO also has photosensitizing ability, which changes dynamically in the same way as its fluorescence.[7,8] We thought that these two properties could be utilized to create novel enzymatically activatable photosensitizers whose activation would be signaled by increased fluorescence.

Initially, we focused on the intracellular distribution and fluorescence intensity of TO derivatives. In living cells, it is reported that TO and its derivatives nonspecifically stain cellular components including nucleus, nucleolus, and mitochondria, but subtle structural changes of the dyes result in divergent organelle staining patterns and differences of fluorescence emission which appear to be unrelated to fluorescence induced by DNA-binding *in vitro*.[9,10] We speculated that attaching bulky and hydrophilic structures to the dyes might block their binding to biomolecules, especially to DNA in the cells, where it is separated by the nuclear membrane, extensively folded, and associated with chromatin protein, thereby decreasing the fluorescence emission. To examine this hypothesis, we synthesized a TO derivative, PhoTO-OH (Scheme 1), including

Scheme 1. Design of PhoTO-Gal



a single modifiable phenyl functional group and its derivatives. PhoTO-OH emitted bright fluorescence in living cells, but cells loaded with PhoTO-OH derivatives bearing bulky structures emitted relatively weak fluorescence. Notably, PhoTO-OH and its derivatives were localized preferentially in the nucleolus and inner membrane, but weak illumination induced relocation to the whole nucleus, with an increase of fluorescence. The ratios of relocation to the nucleus and fluorescence increase of PhoTO-OH were larger than those of the other PhoTO-OH derivatives examined (Figure S1). Such photoactivation in living cells has been reported for other DNA-binding molecules, and Andrei et al. suggested that it may reflect a slow binding to DNA with concomitant displacement of histones and/or chromatin proteins from their association with DNA.[11] Therefore, we concluded that bulky substituents might decrease both the fluorescence in living cells and the photosensitizing ability. Thus, we combined PhoTO-OH with a bulky and hydrophilic β-galactoside moiety to synthesize an enzymatically activatable photosensitizing prodrug, PhoTO-Gal, which was expected to show a dramatic increase of fluorescence and photoactivity in living cells after hydrolysis by β-galactosidase.

We confirmed by HPLC that PhoTO-Gal is a substrate of β-galactosidase, which exclusively generates PhoTO-OH (Figure S2), and then we evaluated the fluorescence intensity of PhoTO-Gal and PhoTO-OH in cultured cells by fluorescence microscopy (Figure 1). PhoTO-OH-loaded HeLa cells showed strong fluores-

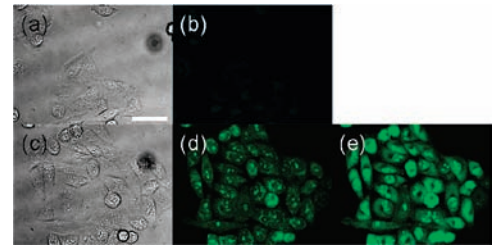


Figure 1. Brightfield and fluorescence images of HeLa cells and relocation of dyes after photoillumination. HeLa cells were stained with 1 μM PhoTO-Gal (a, b) or 1 μM PhoTO-OH (c−e). Fluorescence images were captured after the dyes had been loaded (b, d) and again after 9 mJ/cm^2 of light exposure (e). Scale bar represents 50 μm.

[†] The University of Tokyo.
[‡] CREST, JST.
[§] PRESTO, JST.

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cence, while PhoTO-Gal-loaded HeLa cells showed almost no fluorescence. Moreover, to assess the effectiveness of PDT under these conditions, the cells were illuminated with light, and incubated for an additional 4 h. Cell survival was then determined with Calcein AM and EthD-1 (Figure 2). Although HeLa cells incubated with

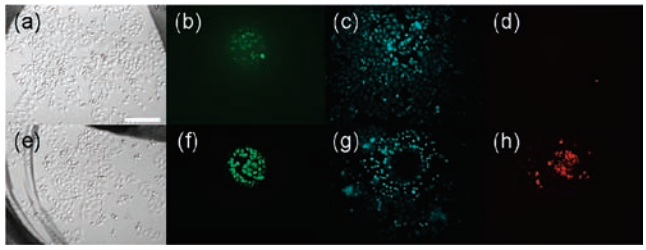

Figure 2. Fluorescence images of HeLa cells stained with 2 μM Calcein-AM and 4 μM EthD-1 after 4 J/cm^2 of light irradiation following treatment with 1 μM PhoTO-Gal (a−d) or 1 μM PhoTO-OH (e−h). (a, e) Brightfield images before light irradiation. (b, f) Fluorescence images of light-irradiated area. For easy identification of the irradiated area, the scale of fluorescence intensity of image (b) was decreased as compared with that of image (f). (c, g) Fluorescence images of Calcein-AM, staining live cells. (d, h) Fluorescence images of EthD-1, staining dead cells. Scale bar represents 200 μm.

PhoTO-Gal were all alive after light illumination, in the case of PhoTO-OH, cell death occurred in the light-irradiated area. The major mechanism of the cell death appeared to be apoptosis (Figure S5).

As described above, relocation and fluorescence enhancement were observed upon relatively weak illumination in the case of PhoTO-OH, but not PhoTO-Gal (Figure 1 and Figure S3). Therefore, it is likely that PhoTO-OH was translocated to nuclei, where it caused DNA breakage and cell death under illumination.[7]

We next applied PhoTO-Gal to *lacZ* gene-transfected HEK293 *lacZ*(+) cells and untransfected *lacZ*(−) cells. β-Galactosidase, encoded by *lacZ* gene from *E. coli*, is a widely used reporter enzyme in molecular biology.[12] As expected, a bright fluorescence image was captured in HEK293 *lacZ*(+) cells incubated with PhoTO-Gal, while only weak fluorescence was observed for *lacZ*(−) cells (Figure 3). After PDT as described above, PhoTO-Gal-loaded

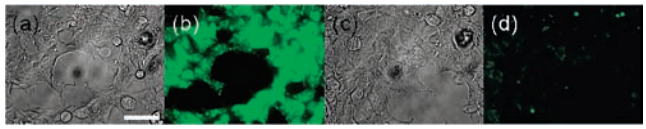

Figure 3. Brightfield and fluorescence images of 1 μM PhoTO-Gal-loaded HEK293 *lacZ*(+) cells (a, b) and HEK293 *lacZ*(−) cells (c, d). Scale bar represents 50 μm.

lacZ(+) cells were killed only in the light-irradiated area. However, almost all of the PhoTO-Gal-loaded *lacZ*(−) cells were alive after irradiation (Figure 4). During this phototreatment, relocation of the dye to the nucleus was observed only in *lacZ*(+) cells (Figure S4). These results demonstrate that PhoTO-Gal is activated to exhibit fluorescence and photosensitization specifically in β-galactosidase-expressing cells.

In conclusion, a novel design strategy for controlling the fluorescence and photosensitizing ability of TO has been developed and employed to obtain a β-galactosidase-activatable photosensitizer, PhoTO-Gal, in which fluorescence is simultaneously activated. By using PhoTO-Gal, we achieved reporter enzyme expression-specific cell ablation in cell culture. Application of this approach to other enzyme/substrate pairs is expected to yield a range of activatable photosensitizing prodrugs with different activating

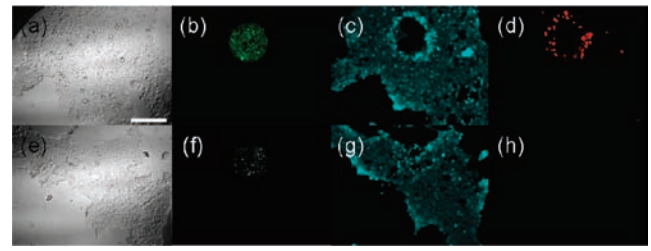

Figure 4. Fluorescence images of HEK293 *lacZ*(+) (a−d) and *lacZ*(−) (e−h) cells stained with 2 μM Calcein-AM and 4 μM EthD-1 after 4 J/cm^2 of light irradiation following treatment with 1 μM PhoTO-Gal. (a, e) Brightfield images before light irradiation. (b, f) Fluorescence images of light-irradiated area. (c, g) Fluorescence images of Calcein-AM, staining live cells. (d, h) Fluorescence images of EthD-1, staining dead cells. Scale bar represents 200 μm.

switches, and this in turn might allow highly refined photodynamic therapy (PDT)[13−15] by utilizing enzymes that are highly expressed in cancers.[16] It might also be applied in antibody- or gene-directed enzyme prodrug therapy (ADEPT or GDEPT)[17,18] in which enzymatic or gene targeting affords high selectivity for tumors over normal tissues. Such activatable photosensitizers, which are converted to active form specifically in the cancerous environment, should permit highly specific PDT, without the side effect of prolonged light sensitivity.

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Supporting Information Available: Synthesis, HPLC charts, experimental details and characterization of PhoTO-Gal and PhoTO-OH, and experiments using living cells. These materials are available free of charge via Internet at http://pubs.acs.org.

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