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An Activatable Photosensitizer Targeted to γ-Glutamyltranspeptidase**

Mayumi Chiba[†], Yuki Ichikawa[†], Mako Kamiya, Toru Komatsu, Tasuku Ueno, Kenjiro Hanaoka, Tetsuo Nagano, Norbert Lange, Yasuteru Urano*

Abstract: *We adopted a spirocyclization-based strategy to design γ-glutamyl-hydroxymethyl selenorhodamine green (gGlu-HMSeR) as a photo-inactive compound that would be specifically cleaved by the tumor-associated enzyme γ-glutamyltranspeptidase (GGT) to generate a potent photosensitizer, HMSeR. gGlu-HMSeR takes a colorless, spirocyclic structure, and does not show marked phototoxicity to low-GGT-expressing cells or normal tissues upon irradiation with visible light. In contrast, HMSeR predominantly takes an open colored structure, and generates reactive oxygen species upon irradiation. Thus, the γ-glutamyl group serves as a tumor-targeting moiety for photodynamic therapy (PDT), switching on tumor-cell-specific phototoxicity. To validate this system, we employed chick chorioallantoic membrane (CAM), a widely used model for preliminary evaluation of drug toxicity. Photoirradiation after gGlu-HMSeR treatment resulted in selective ablation of implanted tumor spheroids, without damage to healthy tissue. gGlu-HMSeR is the first activatable photosensitizer targeting an aminopeptidase, a class of enzymes overexpressed in various cancers, for highly tumor-selective PDT. The design strategy should be readily adaptable to target other peptidases by substituting the γ-glutamyl moiety.*

Photosensitizers are compounds that produce reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) upon light irradiation. This ability is utilized in photodynamic therapy (PDT), which employs photosensitizers and light irradiation to induce cell death at the targeted region, such as a tumor. Compared to other cancer therapies, such as surgery, chemotherapy and radiotherapy, PDT can be repeated, is less invasive, and allows precise targeting of an illuminated area. However, non-specific photodamage to skin or healthy tissue remains a significant issue[1]. This is because most of the photosensitizers currently used for clinical PDT are based on porphyrin[2], which mediates phototoxicity regardless of whether it is distributed in tumor or normal tissues (i.e., it is an always-on photosensitizer). To overcome this drawback, there is a need for activatable photosensitizers whose photosensitizing ability is switched on by a tumor-specific biomarker, enabling targeted activation. 5-Aminolevulinic acid (5-ALA), a precursor of the porphyrins, is already used clinically as an activatable photosensitizer for PDT[3]. 5-ALA itself lacks photosensitizing activity, but it serves as a substrate for biosynthesis of protoporphyrin IX (PpIX) through multiple steps of the heme cycle PpIX is accumulated in tumor cells, and as it is an efficient photosensitizer, PDT with 5-ALA leads to tumor-specific cell death.

Here, we aimed at developing a novel activatable photosensitizer that would be rapidly activated by a tumor-specific peptidase after topical administration. Topical administration is desirable to minimize the dose and to reduce the risk of side effects associated with systemic administration, without the need for a wash-out process. Also, by employing single-step activation with a peptidase, we expected that faster activation would be achieved than with 5-ALA, where a sequence of enzyme reactions is required to generate PpIX. A protease-triggered photosensitizing beacon has been reported as an activatable photosensitizer[4], but no aminopeptidase-targeting activatable photosensitizer has yet been described. We anticipated that a suitable choice of target enzyme would extend the range of treatable cancers.

For the present purpose, we drew upon our previous studies of activatable fluorescent probes for cancer imaging, which are based on hydroxymethylrhodamine derivatives[5]. By utilizing these fluorescent scaffolds, we have developed a series of activatable fluorescent probes targeting a variety of proteases, based on a strategy of conjugating the fluorescent moieties to specific substrates of the enzymes. For example, gGlu-HMRG (γ-glutamyl hydroxymethylrhodamine green) is a probe for γ-glutamyl transpeptidase (GGT), a cell-surface enzyme that is overexpressed in some types of cancer, such as ovarian, lung and prostate cancers[6]. With this probe, we have demonstrated rapid and sensitive in vivo imaging of intraperitoneally disseminated GGT-positive tumor nodules in mouse models[5a]. Moreover, we have confirmed the usefulness of gGlu-HMRG to visualize breast cancer[7], oral cancer[8], and head and neck cancer[9] in freshly resected clinical specimens for intraoperative margin assessment. On the other hand, we also took advantage of a recent finding that a xanthene-based fluorophore can be converted to a photosensitizer simply by substituting the oxygen atom at the 10 position with a selenium atom; this results in a reduction of the fluorescent emission and an increase of $^1O_2$ productivity upon light irradiation[10]. Indeed, we recently developed HMDESeR-βGal (hydroxymethyldiethylselenorhodol- β-galactopyranoside) as an

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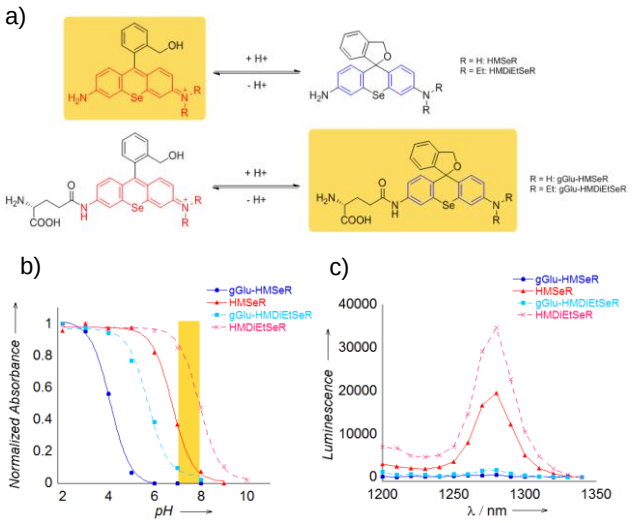

Figure 1. a) Chemical structures of novel photosensitizers. Predominant structures at physiological pH values are highlighted in orange. b) pH dependency of the absorbance of the dyes. c) Luminescence spectra of $^1O_2$ generated by excitation with a 532 nm laser (10 μM in phosphate-buffered saline, pH 7.4).

activatable photosensitizer by replacing the oxygen atom of HMDER-βGal, our previously developed fluorescent probe for β-galactosidase[11], with a selenium atom. In HMDESeR-βGal, photosensitizing ability is blocked by intramolecular spirocyclization, but specific hydrolysis of the βGal moiety in β-galactosidase-expressing cells opens the spirocyclic ring and activates the photosensitizing ability. However, this strategy has not yet been applied to develop an activatable photosensitizer targeting peptidases (such as GGT), which are an important category of enzymes overexpressed in various cancers.

Therefore, we aimed here to use a similar design strategy to develop a novel photosensitizer activatable by GGT. We initially designed two candidate scaffolds, i.e., hydroxymethyl selenorhodamine green (HMSeR), a seleno analogue of HMRG[5a], and hydroxymethyl *N*,*N*-diethyl selenorhodamine (HMDiEtSeR), a seleno analogue of HMDiEtR[5b], since HMRG and HMDiEtR show slightly different spirocyclization properties. We also prepared their γ-glutamyl derivatives (gGlu-HMSeR and gGlu-HMDiEtSeR) (Figure 1a).

We first examined the photochemical properties of HMSeR and HMDiEtSeR (Table 1), and confirmed that the pH-dependent change of absorption spectrum seen with the parental compounds (HMRG and HMDiEtR) was retained (Figure 1a, 1b, S1). The calculated pK_{cycl} values (pH value at which the extent of spirocyclization is sufficient to reduce the absorbance of the compound to one-half of the maximum absorbance) were 6.8 and 7.9, respectively, indicating that 20% of HMSeR and 76% of HMDiEtSeR exist in the colored xanthene form at the physiological pH of 7.4 (Table S1). Also as expected, γ-glutamyl derivatives of HMSeR and HMDiEtSeR showed little $^1O_2$ production upon 532 nm laser irradiation (Figure 1c). We also confirmed that gGlu-HMSeR and gGlu-HMDiEtSeR were stable in buffer and efficiently converted to HMSeR and HMDiEtSeR upon reaction with GGT in vitro, and the reaction was accompanied with recovery of absorption in the visible region and ability to produce $^1O_2$ upon laser irradiation (Figure 2a-c, Figure S2, S3).

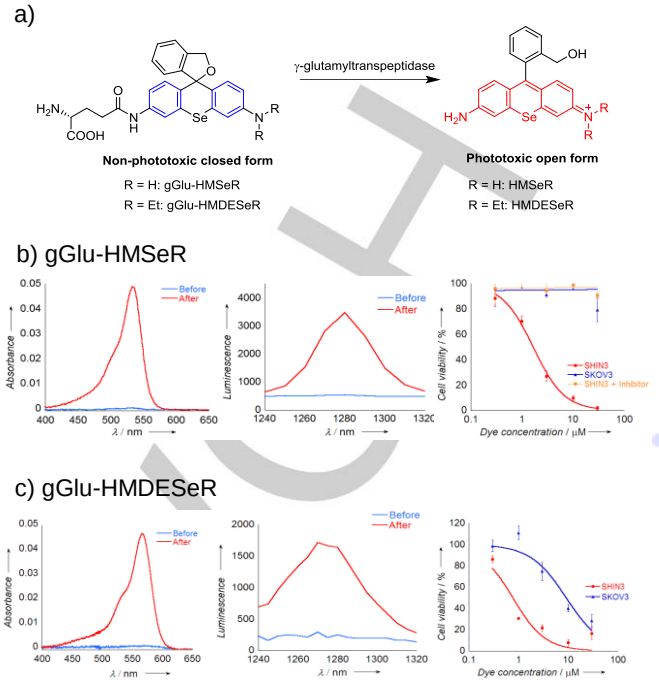

Figure 2. a) Chemical structure of our novel photosensitizers. b-c) (left) Absorption spectra of gGlu-HMSeR, gGlu-HMDESeR before and after addition of GGT (5 units). (middle) Luminescence spectra of $^1O_2$ generated by excitation with a 532 nm laser (10 μM in phosphate-buffered saline, pH 7.4), before and after addition of GGT (5 units). (right) Viability assay of cultured SHIN3 and SKOV3 cells loaded with gGlu-HMSeR or gGlu-HMDESeR. Cells were pre-incubated with photosensitizer in the presence or absence of GGT inhibitor (100 μM GGsTop) for 4 hours, followed by light irradiation (510-550 nm, 50 mW/cm^2, 1 min). CCK-8 assay was performed 24 h post irradiation to measure cell viability. Error bars for S.D. (n = 4).

Table 1. Photochemical properties of HMSeR and HMDiEtSeR derivatives. λ_{abs} and λ_{em} values were measured in 100 mM sodium phosphate buffer, pH 2.0. The relative fluorescence quantum yield was measured in 100 mM sodium phosphate buffer (pH 7.4) using rhodamine B in ethanol (Φ_{fl} = 0.65) as a standard. n.d.; not detectable. The relative $^1O_2$ quantum yield was measured in PBS (pH 7.4) using Rose bengal in PBS (Φ_{Δ} = 0.75) as a standard.

	λ_{abs} [nm]	λ_{em} [nm]	Φ_{fl}	Φ_{Δ}	pK_{cyc}
HMSeR	534	562	0.002	0.40	6.8
gGlu-HMSeR	450, 521, 551	n.d.	n.d.		4.1
HMDiEtSeR	567	602	0.007	0.74	7.9
gGlu-HMDiEtSeR	453, 551, 588	n.d.	n.d.		5.8

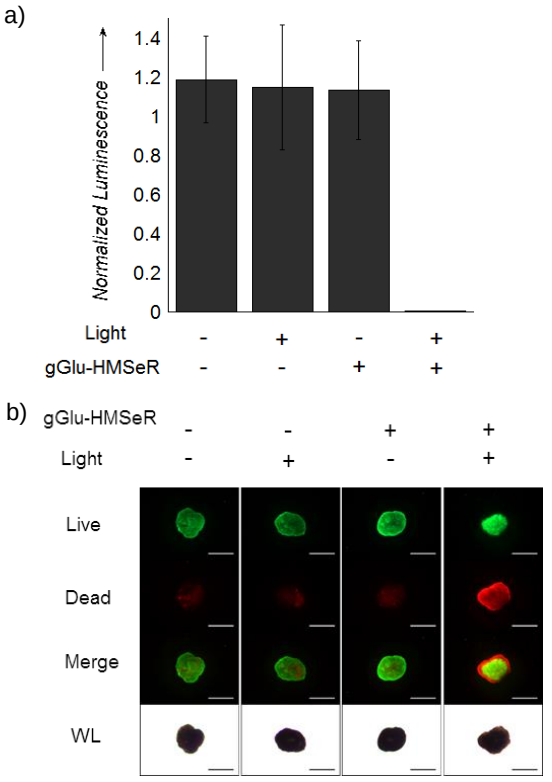

Figure 3. a) Viability of tumor spheroids after PDT. Normalized luminescence intensity was calculated by dividing the intensity by that on day 1. Each value shows the mean ±S.D. (n = 8). b) Live/Dead fluorescence staining of tumor spheroid (A549-luc-C8 lung cancer cells) with Calcein-AM (for live cells, green) and EthD-1 (for dead cells, red) at 24 h after PDT treatment (50 mW/cm^2 LED photoirradiation at 510-550 nm for 30 min). Scale bars: 500 μm.

In order to examine whether gGlu-HMSeR and gGlu-HMDiEtSeR could induce cell death in a GGT activity-dependent manner, we applied both compounds to two cultured cell lines with different GGT activities (SHIN3 cells with high GGT activity and SKOV3 cells with low GGT activity)[12], and evaluated the cell viability after light irradiation (Figure 2b, 2c). We found that gGlu-HMDiEtSeR showed phototoxicity to both cell lines at 5 μM or more (Figure 2c), indicating that cell death was induced regardless of GGT activity. On the other hand, gGlu-HMSeR induced dose-dependent cell death only in SHIN3 cells, but not in SKOV3 cells. Also, the death of SHIN3 cells was strongly suppressed in the presence of a GGT inhibitor (Figure 2b). The difference in behavior between gGlu-HMDiEtSeR and gGlu-HMSeR can be explained by the difference in their pK_{cycl} values. At the physiological pH of 7.4, 2.5% of gGlu-HMDiEtSeR exists in its open form (pK_{cycl} = 5.8), which can mediate non-specific phototoxicity. But, in the case of gGlu-HMSeR, only 0.1% exists in the open form (pK_{cycl} = 4.1) (Table S1), therefore background phototoxicity is strongly suppressed. As for HMSeR, its pK_{cycl} value is 6.8, so that about 20% of the molecules exist in the open form at pH 7.4. Although a higher proportion would be desirable for efficient induction of cell death after activation, it should be noted that HMSeR accumulates in acidic organelles such as lysosomes (ca. pH 5; Figure S4). In this environment, more than 90% of HMSeR would exist in its open form (Table S1). Therefore, we decided to focus on gGlu-HMSeR for further evaluation as a candidate activatable photosensitizer.

We thus tested the performance of gGlu-HMSeR with spheroids (three-dimensional clusters of cultured cells), which may be a better model for evaluating the effective depth of treatment with gGlu-HMSeR than two-dimensional cell cultures[13].

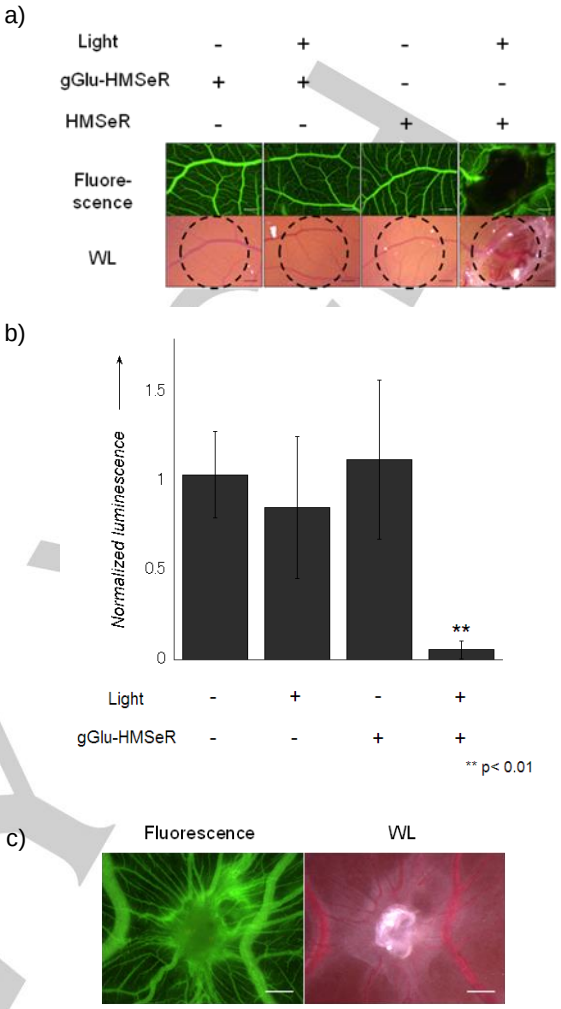

Figure 4. a) Evaluation of vessel occlusion on CAM after photoirradiation in the presence of gGlu-HMSeR and HMSeR. A 100 μM solution of photosensitizer in PBS (25 μL) was applied to CAM, which was then irradiated (532 nm LED: 50 mW/cm^2, 15 min). Fluorescence angiography was performed by intravenous injection of FITC-dextran (30 μL, 25 mg/mL). Black dashed lines represent the area where photosensitizer was applied. Light irradiation was done over the whole area of the picture. Scale bar, 500 μm (n = 3 or more). b) Viability of tumor spheroids (A549-luc-C8 lung cancer cells) implanted on CAM. After PDT (532 nm LED: 50 mW/cm^2, 30 min), cell viability was evaluated by incubation with D-luciferin. The luminescence intensity was normalized by dividing it by the corresponding intensity on day 1. Statistical significance was calculated by application of the Games-Howell test. Each value shows the mean ± S.D (n ≧10). c) Evaluation of vessel occlusion on CAM tumor model after PDT with gGlu-HMSeR. Scale bar, 500 μm (n = 3 or more).

We selected A549-Luc-C8 human lung adenocarcinoma cell line owing to its intrinsically high GGT activity (Figure S5) and its stable expression of luciferase for evaluating cell viability. The bioluminescence signal generated by externally added substrate (D-luciferin) was significantly decreased only when the spheroids were treated with gGlu-HMSeR followed by photoirradiation (Figure 3a). We also performed live/dead fluorescence staining of the spheroids (CalceinAM for live cells, EthD-1 for dead cells). Strong EthD-1 staining was observed at the surface of PDT-treated spheroids, whereas a strong green fluorescence signal of CalceinAM was observed from non-irradiated, or dye-free spheroids (Figure 3b). Probably due to low light penetration and limited photosensitizer uptake, the treatment efficacy seemed to be greatest at the surface of the spheroids. Nevertheless, the results

suggested that small tumor nodules could be targeted by gGlu-HMSeR and eradicated by PDT.

We would like to emphasize that the ability to suppress non-specific phototoxicity in normal tissues is a critical advantage of an activatable photosensitizer. In order to confirm that gGlu-HMSeR meets this requirement, we utilized chick chorioallantoic membrane (CAM), which is a widely used model for evaluating drug toxicity and angiogenesis inhibitors[14]. By using the CAM, it is possible to quantitatively evaluate the phototoxicity to normal tissue by scoring the level of vessel occlusion. Moreover, a tumor-bearing CAM model can be produced by xenografting tumor spheroids onto CAM, so that we can evaluate therapeutic efficacy for tumor and phototodamage to normal tissues simultaneously.

First, we used CAM as a normal tissue model by topically applying gGlu-HMSeR and HMSeR into plastic rings on the CAM, followed by irradiation at 532 nm with an LED. After 24 hours, vessel occlusion was evaluated by fluorescence angiography with FITC-dextran. As shown in Figure 4a, no marked phototoxicity was seen with gGlu-HMSeR, whereas vessel occlusion was potently induced in the presence of HMSeR. Next, we examined the PDT efficacy with a tumor-bearing CAM model in order to see whether gGlu-HMSeR can induce cell death in millimeter-sized tumors, since it is difficult to selectively ablate such tiny tumors with general photosensitizers via the EPR effect. A549-Luc-C8 spheroids were implanted onto CAMs, and after 2 days, the bioluminescence intensities of the spheroids were evaluated (day 1). Then, gGlu-HMSeR was topically applied by dropping a solution onto the tumor and its surrounding area on the CAM, followed by incubation for 30 minutes for activation, and then photoirradiation (532 nm LED, 50 mW/cm^2, 30 min). This PDT procedure was repeated 2 days later (day 3; two treatments in total) and the luminescence was measured on the following day (day 5). Luminescence intensities were normalized by the corresponding intensities on day 1. The luminescence intensities were significantly decreased only when the spheroids were pretreated with gGlu-HMSeR and then photoirradiated (Figure 4b). We also confirmed that CAM showed no marked vessel occlusion in the surrounding normal tissue after PDT with gGlu-HMSeR (Figure 4c), whereas significant vessel occlusion was seen when HMSeR was used (Figure S6). This result indicated that gGlu-HMSeR is specifically activated to HMSeR at the tumor, resulting in highly specific phototoxicity to GGT-expressing tumors without damage to surrounding tissue.

In conclusion, we have developed the first aminopeptidase-targeting activatable photosensitizer. This is important, because various peptidases are overexpressed in different types of tumors. Here, we focused on γ-glutamyl transpeptidase (GGT), which is overexpressed in ovarian, lung and prostate cancers, and employed a strategy based on precise control of intramolecular spirocyclization to design a probe, gGlu-HMSeR. GGT is a membrane-anchored enzyme expressed in cancer cells; therefore, we consider that gGlu-HMSeR is converted to HMSeR on the surface of the cells, and HMSeR is then internalized into the cells due to its greater hydrophobicity, and accumulates mainly in lysosomes. This is similar to the mechanism in the case of our previously reported GGT-targeting activatable fluorescent probe gGlu-HMRG[5a]. We confirmed that high-GGT-expressing cells were specifically killed by PDT with gGlu-HMSeR. Further, in a tumor-bearing CAM model, tumors were selectively ablated by PDT with gGlu-HMSeR, without damage to adjacent healthy tissues. Although penetration of gGlu-HMSeR into tumor spheroids was limited, it should be possible to overcome this issue by utilizing drug penetration-enhancing techniques such as ointments[15]. Since gGlu-HMSeR utilizes a simple enzymatic reaction for activation, sufficient amounts of photosensitizer to induce cell death could be accumulated at tumor sites within a short time, of the order of minutes. This rapid activation should enable gGlu-HMSeR to be applied topically to the tumor area, reducing the risk of photodamage to normal tissues, compared to systemic administration. We believe this is a promising approach to achieve highly tumor-specific PDT.

Keywords: activatable photosensitizer • γ-glutamyl transpeptidase• intramolecular spirocyclization •seleno-rhodamine

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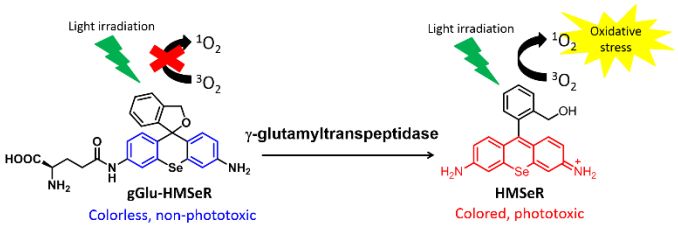

Specific destruction of cancer! We used an intramolecular spirocyclization strategy to design a photosensitizer activated by γ–glutamyltranspeptidase, which is overexpressed in cancer cells. Photodynamic therapy with gGlu-HMSeR/green-light irradiation in a chick chorioallantoic membrane model resulted in selective ablation of implanted tumor spheroids without damage to healthy tissue.

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