Extended Rhodamine Photosensitizers for Photodynamic Therapy of Cancer Cells

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Abstract: Extended thio- and selenorhodamines with a linear or angular fused benzo group were prepared. The absorption maxima for these compounds fell between 640 and 700 nm. The extended rhodamines were evaluated for their potential as photosensitizers for photodynamic therapy in Colo-26 cells. These compounds were examined for their photophysical properties (absorption, fluorescence, and ability to generate singlet oxygen), for their dark and phototoxicity toward Colo-26 cells, and for their co-localization with mitochondrial-specific agents in Colo-26 and HUT-78 cells. The angular extended rhodamines were effective photosensitizers toward Colo-26 cells with 1.0 J cm⁻² laser light delivered at $\lambda_{\text{max}} \pm 2$ nm with values of EC₅₀ of $(2.8 \pm 0.4) \times 10^{-7}$ M for sulfur-containing analogue **6-S** and $(6.4 \pm 0.4) \times 10^{-8}$ M for selenium-containing analogue **6-Se**. The linear extended rhodamines were effective photosensitizers toward Colo-26 cells with 5 and 10 J cm⁻² of broad-band light (EC₅₀'s $\leq 2.4 \times 10^{-7}$ M.)

KEYWORDS: Photodynamic therapy, photosensitizers, extended rhodamines.

1. Introduction

Photodynamic therapy (PDT) is a treatment for cancer that utilizes a photosensitizer that is targeted to the tumor and light directed to the tumor-localized photosensitizer to generate cytotoxic reactive oxygen species.¹ Ideally, the photosensitizer absorbs light in the 600-800-nm window where penetration of light in tissue is optimal. In addition, the photosensitizer should display minimal toxicity in the absence of light and should not photosensitize the patient to light in general.

The rhodamine dyes are cationic dyes based on the xanthylium core. The rhodamines and other cationic dyes have been examined as photosensitizers for PDT due to their ability to target the mitochondria of transformed cells and to absorb light.^{2,3} Rhodamine-123, in particular, targeted the mitochondria and was also a chemotherapeutic agent in the dark.^{4,5} Rhodamine-123 and other members of the rhodamine/rosamine family absorb wavelengths of light too short for effective penetration of tissue (λ_{max} 500-550 nm). Furthermore, these molecules generate singlet oxygen ($^1\text{O}_2$) and other reactive oxygen species inefficiently due to the lack of a heavy atom in the chromophore.

Heavy atoms have been introduced to the rhodamine core in two different ways. Bromination of the xanthylium nucleus gave brominated rhodamines that generated $^1\text{O}_2$ more efficiently, but still absorbed light in the 500-550-nm window.⁶⁻⁹ We have prepared rhodamine derivatives with selenium replacing oxygen in the xanthylium core. Not only did this substitution give quantum yields for the generation of $^1\text{O}_2$ [$\Phi(^1\text{O}_2)$] of up to 0.87 for the selenorosamine **TMR-Se** (Chart 1), but also gave a longer-wavelength absorption maximum (λ_{max}) of 580 nm.¹⁰ We have recently described the synthesis of selenorhodamines **1-3** (Chart 1) as the first rhodamine photosensitizers

with values of $\lambda_{\text{max}} > 600$ nm that maintain their ability to target mitochondria in cancer cells and are useful as photosensitizers for PDT.^{11,12}

Chart 1. Structures of **TMR-Se** and selenorhodamines **1-3**.

Extending values of λ_{max} to even longer wavelengths while still maintaining high values of $\Phi(^1\text{O}_2)$ and the ability to localize in the mitochondria of cancer cells should provide even better photosensitizers for PDT. One possible approach to longer-wavelength-absorbing rhodamine-related dyes is the incorporation of additional fused benzene rings. This approach has been successful with fluorescein-related molecules¹³⁻¹⁵ and has given chromophores with longer wavelengths of absorption and emission with such as examples **4** [λ_{max} (H₂O) 542 nm, λ_{EM} 629 nm]¹⁴ and **5** [λ_{max} (H₂O) 536 nm, λ_{EM} 733 nm]¹⁴ shown in Chart 2. It should be possible to prepare rhodamine-related structures with similar additional fused rings as well as derivatives that substitute sulfur and selenium for the xanthene oxygen atom. Herein, we report our initial biological evaluation of extended chalcogenorhodamine chromophores **6-E** and **7-E** (E = S or Se, Chart 2) as photosensitizers for PDT.

Chart 2. Extended fluorescein molecules **4** and **5** and extended rhodamine analogues **6-E** and **7-E**.

2. Results and Discussion

2.1 Synthesis of Selenorhodamines **6-E** and **7-E**.

The key intermediates to the extended rhodamine dyes **6-E** and **7-E** are the corresponding extended xanthenes **8-E** and **9-E**. As shown in Scheme 1, xanthenes **8-E** and **9-E** can be prepared by the cyclization of diaryl chalcogenides **10-E** and **11-E**, respectively. The addition of bis-3-dimethylaminophenyl dichalcogenides **12-E**¹⁶ to 1-lithio-6-dimethylamino-2-naphthamide **13** or 3-lithio-6-dimethylamino-2-naphthamide **14**, respectively, in turn gives the diaryl chalcogenides **10-E** and **11-E**. The regioselectivity of deprotonation of **15** to give **13** and **14** will determine the efficiency of the synthesis.

Scheme 1. A synthetic approach to the xanthone precursors to extended rhodamines **6-E** and **7-E**.

In our synthetic approach to **TMR-Se** and selenorhodamines **1** and **2**, directed metalations of 4-dimethylamino-substituted benzamide derivatives with *s*-BuLi/TMEDA gave 2-lithio-4-dimethylamino-benzamides in excellent yield, which then reacted with diaryl dichalcogenides to give xanthone precursors.¹⁶ A similar approach with naphthamide **15** leads to precursors to xanthenes **8-E** from 1-lithio-2-naphthamides **13** and to **9-E** from 3-lithio-2-naphthamides **14**. However, the regiochemical preference for metalation with *s*-BuLi/TMEDA is not clear. The directed metalation of 2-substituted naphthalenes in the literature has varying regioselectivity depending upon the specific substrate, directing group, base, and solvent conditions.¹⁷⁻²¹

Amide **15** was subjected to directed metalation with *s*-BuLi and TMEDA at -78 °C and the resulting mixture of anions was quenched with dichalcogenides **12-E** (Scheme 2). Lithiation at the 1-position appeared to be favored by a ratio of 2.5:1 as determined by ¹H NMR spectroscopy following quenching of the anion mixture with either disulfide **12-S** or diselenide **12-Se** to give a mixture of **10-S** and **11-S** in 76% isolated yield and a mixture of **10-Se** and **11-Se** in 60%

isolated yield (Scheme 2). At this stage, the mixture of regioisomers **10-E/11-E** was not readily separable by chromatography and the mixture was treated with POCl₃ and Et₃N in refluxing acetonitrile to give the extended xanthenes **8-E** and **9-E**. From the mixture of regioisomers **10-E/11-E**, the two isomeric xanthenes were separable by silica chromatography and **8-S** was isolated in 42% yield and **9-S** in 25% isolated yield while **8-Se** was isolated in 45% yield and **9-Se** was isolated in 21% yield (Scheme 2).

Scheme 2. Synthesis of extended xanthenes **8-E** and **9-E** from naphthamide **15**.

The ¹H NMR spectra of the extended xanthenes **8-E** and **9-E** (Supplementary Data) allowed the unambiguous assignment of structure. The number of aromatic protons with coupling constants consistent with an *ortho*-coupling partner (~7-9 Hz) define the structure. Xanthenes **8-E** have six protons with *ortho*-coupling partners while xanthenes **9-E** have four.

Scheme 3. Synthesis of extended rhodamine dyes **6-E** and **7-E**.

The addition of phenylmagnesium bromide to a stirred suspension of **8-E** or **9-E** in THF (16 h at reflux) followed by work up with 10% aqueous HPF₆ gave dyes **6-E** and **7-E**, respectively, in 84-95% isolated yield (Scheme 3). The dyes **6-E** and **7-E** were sparingly soluble in most organic solvents, which made the acquisition of high-quality ¹³C and any ⁷⁷Se NMR spectra difficult.

However, the ^1H and ^{13}C NMR spectra (Supplementary Data) were consistent with expected structures and the extended rhodamine dyes all passed combustion analysis and high-resolution mass spectral analysis. The solubility of the dyes **6-E** and **7-E** also limited an accurate experimental determination of values of the *n*-octanol/water partition coefficient ($\log P$).

2.2. Photophysical properties.

Absorption spectra for **6-E** and **7-E** are shown in Figure 1 and values of λ_{max} and the molar extinction coefficient (ϵ) in MeOH and in CH_2Cl_2 are compiled in Table 1. The extended rhodamines have values of λ_{max} in the 630 to 700-nm range. Fluorescence emission maxima (λ_{FL}) with excitation at 532 nm are also compiled in Table 1. Fluorescence quantum yields (Φ_{FL}) were determined from the steady-state fluorescence spectra for **6-E** and **7-E** (TMR-Se was used as a reference standard with known fluorescence quantum yield, $\Phi_{\text{FL}} = 0.009$ in MeOH).¹⁰ Dyes **6-S** and **7-S** had values of Φ_{FL} of 0.47 and 0.18, respectively, while the dyes **6-Se** and **7-Se** were weakly fluorescent with identical values of Φ_{FL} of 0.009. Quantum yields for the generation of $^1\text{O}_2$ [$\Phi(^1\text{O}_2)$] by **6-E** and **7-E** were measured by time-resolved spectroscopy at 1270 nm of $^1\text{O}_2$ luminescence in air-saturated MeOH.²² While values of $\Phi(^1\text{O}_2)$ could be quantified for **6-S** and **6-Se** with excitation at 532 nm, only a weak 1270-nm signal was detected from **7-S** and **7-Se**, which could not be resolved to an accurate value of $\Phi(^1\text{O}_2)$. Values of $\Phi(^1\text{O}_2)$ for **7-S** and **7-Se** are listed as <0.05 to place an upper boundary on the value and to acknowledge that weak, but detectable, amounts of $^1\text{O}_2$ are being produced.

Figure 1. Electronic absorption spectra of extended rhodamines a) **6-E** and b) **7-E** in dichloromethane.

Table 1. Absorption maxima ($\lambda_{\max}$) and molar extinction coefficients (ϵ), fluorescence emission maxima (λ_{FL}) and quantum yields for fluorescence (Φ_{FL}) in MeOH, and quantum yields for the generation of singlet oxygen [$\Phi(^1\text{O}_2)$] in MeOH for extended rhodamines **6-E** and **7-E**.

compd	$\lambda_{\max}$, nm	ϵ , $\text{M}^{-1} \text{cm}^{-1}$	λ_{FL} , nm	$\Phi_{\text{FL}}^{a,b}$	$\Phi(^1\text{O}_2)^{a,b}$
6-S	630 (MeOH)	6.1×10^4	715	0.47 ± 0.01	0.15 ± 0.02
	641 (CH_2Cl_2)	6.5×10^4			
6-Se	647 (MeOH)	6.4×10^4	717	0.009 ± 0.001	0.65 ± 0.03
	658 (CH_2Cl_2)	6.9×10^4			

7-S	661 (MeOH)	4.7×10^4	775	0.18 ± 0.01	$< 0.05^c$
	689 (CH ₂ Cl ₂)	4.9×10^4			
7-Se	671 (MeOH)	4.7×10^4	781	0.009 ± 0.001	$< 0.05^c$
	700 (CH ₂ Cl ₂)	5.3×10^4			

^a Excitation at 532 nm. ^b Error limits are $\pm$ SD. ^c A weak signal was detected at 1270 nm indicating some ¹O₂ was being produced, but < 0.05 places an upward boundary on the yield.

2.3. Phototoxicity of dyes **6-E** and **7-E**.

The angular dyes **6-S** and **6-Se** have values of λ_{max} of 630 and 647 nm, respectively, in MeOH, which is in the 600-nm–800-nm window where penetration of light in tissue is optimal.¹ Both **6-S** and **6-Se** generate singlet oxygen upon irradiation, which is an important cytotoxic agent in PDT.¹ We surveyed the phototoxicity of **6-S** and **6-Se** toward Colo-26 cells (a murine colon carcinoma cell line) using a tunable dye laser delivered at 642 ± 2 nm for **6-S**, 660 ± 2 nm for **6-Se**, 690 ± 2 nm for **7-S**, and 700 ± 2 nm for **7-Se**. Colo-26 cells were treated with a range of concentrations of extended rhodamines **6-S** and **6-Se** (1×10^{-8} to 1×10^{-6} M) and toxicity was examined in the dark or with 1.0 J cm^{-2} of light delivered at a fluence rate of $\sim 3.2 \text{ mW cm}^{-2}$ and cell viability was evaluated 48 h after exposure. Solubility issues precluded the use of higher concentrations of dyes **6-E** (or **7-E**) in dark and phototoxicity studies. Both **6-S** and **6-Se** show minimal dark toxicity with concentrations up to 1×10^{-6} M (Figure 2). The extended thiorhodamine **6-S** (Figure 2a) is less phototoxic than **6-Se** (Figure 2b), which is consistent with results comparing other thiorhodamines with their selenium-containing counterparts.^{11,12,23} The effective concentration to give 50% cell kill (EC₅₀) with 1.0 J cm^{-2} of light from a laser source was $(2.8 \pm 0.4) \times 10^{-7}$ M for **6-S** and $(6.4 \pm 0.4) \times 10^{-8}$ M for **6-Se**.

Figure 2. Dark toxicity (●) and phototoxicity (▲) of a) **6-S** and d) **6-Se** toward Colo-26 cells. Phototoxicity was measured following irradiation from 1.0 J cm⁻² of light from a tunable dye laser delivered at ~3.2 mW cm⁻² (642 ± 2 nm for **6-S** and 660 ± 2 nm for **6-Se**). Error bars are ± SD. Values of EC₅₀ (± SD) in a) and b) were determined by a sigmoidal does-response (variable slope) analysis.

The linear extended rhodamines **7-S** and **7-Se** show greater dark toxicity than the angular extended rhodamines **6-S** and **6-Se** at 1.0 μM (Figure 3). Irradiation with 1.0 J cm⁻² of light from a tunable dye laser (690 ± 2 nm for **7-S** and 700 ± 2 nm for **7-Se**) gave minimal if any phototoxicity and the phototoxicity from the tunable dye laser is essentially identical to the dark toxicity. This is consistent with the poorer quantum yields for the generation of ¹O₂ with **7-S** and **7-Se** relative to **6-S** and **6-Se** (Table 1) when the dyes were excited with a 532-nm laser.

Figure 3. a) Action spectrum of unfiltered Curelight broad-band light source overlaid with absorption spectra of **7-S** and **7-Se**. Dark toxicity (●) and phototoxicity with 5 J cm⁻² (▲) or 10 J cm⁻² (■) of light from Curelight source of b) **7-S** and c) **7-Se** toward Colo-26 cells. Error bars are ± SD. Values of EC₅₀ (± SD) and LD₅₀ (± SD) in b) and c) were determined by a sigmoidal dose-response (variable slope) analysis.

The chromophores associated with dyes **7-S** and **7-Se** absorb light over a 250- to 300-nm section of the 500-nm-to-800-nm window. We also examined the phototoxicity of dyes **7-S** and **7-Se** toward Colo-26 cells using a Curelight broad band source that emits light in this range as shown in Figure 3a. With the broad-band light source delivered to 5.0 and 10 J cm⁻² at a fluence rate of 47.6 mW cm⁻², phototoxicity greater than dark toxicity was observed with both **7-S** and **7-Se**. Dye **7-S** gave an EC₅₀ of $(1.4 \pm 0.1) \times 10^{-7}$ M with 5 J cm⁻² of broad-band light and $(9.0 \pm 0.9) \times 10^{-8}$ M with 10 J cm⁻² of broad-band light (Figure 3b). Dye **7-Se** gave an EC₅₀ of $(2.4 \pm 0.9) \times 10^{-7}$ M with 5 J cm⁻² of broad-band light and $(1.8 \pm 0.3) \times 10^{-7}$ M with 10 J cm⁻² of broad-band light (Figure 3c).

The ratio of dark toxicity to phototoxicity as an approximation of the therapeutic ratio for the photosensitizers is perhaps a better measure of photosensitizer effectiveness. Dark toxicity is expressed as the LD₅₀ or the concentration resulting in 50% cell kill in the dark. The angular extended selenorhodamine **6-Se** has an LD₅₀ of $> 1.0 \times 10^{-6}$ M as shown in Figure 2b. The LD₅₀/EC₅₀ ratio is an approximation of therapeutic index and, for **6-Se**, this ratio is >15 with 1.0 J cm⁻² of laser light. For photosensitizers **7-S** and **7-Se**, dark toxicity is much greater than for dyes **6-E**, with LD₅₀/EC₅₀ ratios of 3.3 and 5.1 observed for **7-S** with 5 and 10 J cm⁻² of broad-band light, respectively, and LD₅₀/EC₅₀ ratios of 8.3 and 11 observed for **7-Se** with 5 and 10 J cm⁻² of broad-band light, respectively.

2.4. Subcellular localization of dyes 6-E and 7-E.

Rhodamines such as rhodamine-123 target the mitochondria of cancer cells and show increased uptake in cancer cells relative to normal epithelial cells presumably due to the increased mitochondrial membrane potential in cancer cells.^{24,25} The cellular localization of dyes **6-E** and **7-E** in Colo-26 cells was examined using imaging flow cytometry as shown in Figure 4. The co-localization of the mitochondrial specific agents MitoTracker GreenTM (MTG) and MitoTracker RedTM (MTR) was shown in Colo-26 cells incubated with both agents. A mean bright detail similarity score of 1.64 ± 0.62 for 3158 Colo-26 cells was determined indicating a high degree of co-localization²⁶ of these two agents. LysoTracker GreenTM (LYS) targets the lysosomes and a bright detail similarity score of 0.69 ± 0.26 for 2437 Colo-26 cells was determined in a comparison of LYS (5×10^{-8} M) and MTR (1×10^{-8} M) suggesting these agents are not co-localized and target different cellular sites.

Figure 4. a) Representative examples of MTG/**6-E**- or **7-E**-stained Colo-26 cells as a bright field image (BF), MTG fluorescence, **6-E**- or **7-E** fluorescence, and a merged image of MTG/**6-E**- or **7-E** fluorescence with Image Stream flow cytometry. b) The average similarity coefficient determined by Image Stream flow cytometry of all Colo-26 cells for each pair of agents (MTR, MTG, LYS, **6-E**- or **7-E**) is shown; error bars represent SD. c) A histogram of the pixel-by-pixel statistical analysis of each Colo-26 cell ($n = 3730$) analyzed, in which the y-axis is number of cells and the x-axis is the similarity coefficient between MTG and **6-E** or **7-E**.

Colo-26 cells were next incubated for 15 min with a dye solution consisting of MTG (5×10^{-7} M) and 2×10^{-7} M **6-E** or **7-E**. Figure 4a shows a representative example of imaging by flow cytometry¹¹ of MTG/**6-E**- or **7-E**-stained Colo-26 cells as a bright field image, MTG fluorescence, dye fluorescence, and a merged image of MTG/**6-E**- or **7-E**. Similarity scores are shown in Figure 4b which were determined by the histograms of Figure 4c showing MTG/**6-E**- or **7-E** bright detail similarity scores.²⁶ Dyes **6-S**, **6-Se**, and **7-S** gave similarity scores with MTG of 1.20 to 1.77, which is indicative of a high degree of co-localization of the dye and

MTG.²⁶ In contrast, the similarity score of **7-Se** with MTG was 0.72, which suggests reduced co-localization of the two dyes.

Figure 5. a) Representative examples of MTG/**6-E**- or **7-E**-stained HUT-78 cells as a bright field image (BF), MTG fluorescence, **6-E**- or **7-E** fluorescence, and a merged image of MTG/**6-E**- or **7-E** fluorescence with Image Stream flow cytometry. b) The average similarity coefficient determined by Image Stream flow cytometry of all HUT-78 cells for each pair of agents (MTR, MTG, LYS, **6-E**- or **7-E**) is shown; error bars represent SD. c) A histogram of the pixel-by-pixel statistical analysis of each HUT-78 cell (n = 3730) analyzed, in which the y-axis is number of cells and the x-axis is the similarity coefficient between MTG and **6-E** or **7-E**.

We examined the cellular localization of dyes **6-E** and **7-E** in a second cell line using imaging flow cytometry. HUT-78 cells are erythrodermic cutaneous T cell lymphoma cells and are characterized by hyperpolarized mitochondria. HUT-78 cells were incubated for 15 min with a dye solution consisting of MTG (5×10^{-7} M) and 2×10^{-7} M **6-E** or **7-E**. Figure 5a shows a representative example of imaging by flow cytometry¹¹ of MTG/**6-E**- or **7-E**-stained HUT-78

cells as a bright field image, MTG fluorescence, dye fluorescence, and a merged image of MTG/**6-E**- or **7-E**. Bright detail similarity scores for MTG/**6-E**- or **7-E** comparisons are shown in Figure 5b and the histograms providing them are shown in Figure 5c. Dyes **6-S**, **6-Se**, and **7-S** gave similarity scores with MTG of 2.15 to 2.82, which is indicative of a very high degree of co-localization of the dye and MTG.²⁶ Again, the similarity score of **7-Se** with MTG was much lower at 0.68 and suggests that **7-Se** may have a different site of localization.

Figure 6. Representative example by Image Stream flow cytometry of a) LYS/MTR-stained Colo-26 cell with LYS fluorescence, MTR fluorescence, and a merged image of LYS/MTG fluorescence; b) LYS/MTR-stained HUT-78 cell with LYS fluorescence, MTR fluorescence, and a merged image of LYS/MTG fluorescence; c) MTG/**7-E**-stained Colo-26 cell with MTG fluorescence, **7-Se** fluorescence, and a merged image of MTG/**7-Se** fluorescence; d) MTG/**7-E**-stained HUT-78 cell with MTG fluorescence, **7-Se** fluorescence, and a merged image of MTG/**7-Se** fluorescence.

Figure 6 shows a comparison of LYS emission, MTR emission, and an overlay of LYS/MTR emission in Colo-26 cells (Figure 6a) and HUT-78 cells (Figure 6b) compared to MTG emission, **7-Se** emission, and the overlay of MTG/**7-Se** emission in Colo-26 cells (Figure 6c) and HUT-78

cells (Figure 6d). The distribution pattern of **7-Se** in both cell lines suggests lysosomal localization rather than mitochondrial localization.

2.11. Discussion of biological results.

The extended rhodamine dyes **6-E** and **7-E** absorb at much longer wavelengths ($\lambda_{\text{max}} = 640\text{--}700\text{ nm}$) than **TMR-Se** ($\lambda_{\text{max}} = 580\text{ nm}$)¹⁰ and other selenorhodamine derivatives **1-3** (Chart 1) with constrained amino groups ($\lambda_{\text{max}} = 600\text{--}626\text{ nm}$).^{11,12} While the 9-phenyl substituents of **6-E** and **7-E** may not be the optimal groups for effective photosensitizers (dark toxicity and phototoxicity, solubility, cellular uptake, formulation), dyes **6-E** and **7-E** illustrate that the extended rhodamine structures are a viable framework for the design of photosensitizers. Dyes **6-E** may be better suited for use with a laser light source while dyes **7-E** may be better suited for a broad-beam light source. Dye **6-Se** has an EC_{50} of $6.4 \times 10^{-8}\text{ M}$ with 1 J cm^{-2} of $660 \pm 2\text{ nm}$ light from a tunable dye laser, which is similar to the EC_{50} of $3 \times 10^{-8}\text{ M}$ with 1 J cm^{-2} of $630 \pm 2\text{ nm}$ with selenorhodamine dye **3** with $\text{X} = \text{S}$.¹² Selenorhodamine **3** with $\text{X} = \text{S}$ has been optimized for performance as a photosensitizer in Colo-26 cells.

Chart 3. Structures of the phenothiazine dye methylene blue and the benzophenothiazine nile blues.

The extended rhodamine dyes **6-E** and **7-E** share structural similarity with the phenothiazine methylene blue dyes and the benzophenothiazine nile blue dyes (Chart 3). The phenothiazine and benzophenothiazine dyes have been useful as photosensitizers for PDT and their wavelengths of absorption are similar to those of extended rhodamines **6-E** and **7-E**.¹ The subcellular sites of localization of the methylene blue and nile blue dyes suggest that the

mitochondria need not be the site of initial localization. Based on values of $\log P$, early workers described the methylene blue derivatives as targeting the mitochondria,²⁷ but subsequent work suggested that initial localization in the lysosomes was followed by photochemical release and relocation in the mitochondria.²⁸ Similarly, some nile blue derivatives ($X = I$ in Chart 3, as one example) target the lysosomes initially and are then released upon irradiation to relocate in the mitochondria,²⁹ while other derivatives ($X = H$ in Chart 3, as one example) have mitochondrial, lysosomal, and cytoplasmic sites of localization.³⁰ The Image Stream flow cytometry of Colo-26 and HUT-78 cells stained with dyes **6-E** and **7-E** suggest that **6-S**, **6-Se** and **7-S** show a high degree of mitochondrial localization in both Colo-26 (Figure 4) and HUT-78 cells (Figure 5), but dye **7-Se** does not appear to target the mitochondria in either Colo-26 (Figure 4) or HUT-78 cells (Figure 5). The localization of derivatives of **6-E** and **7-E** with different substituents in the 9-position will be of interest in the design of improved photosensitizers.

3. Conclusions

The extended chalcogenorhodamine dyes **6-E** and **7-E** provide the first rhodamine photosensitizers with $\lambda_{\max} > 640$ nm. Both **6-S** and **6-Se** are effective photosensitizers *in vitro* toward Colo-26 cells with values of EC_{50} of 2.8×10^{-7} M and 6.4×10^{-8} M, respectively, with only 1.0 J cm^{-2} of laser light delivered at $\lambda_{\max} \pm 2$ nm. The longer wavelengths of absorption give **6-S** and **6-Se** greater potential for use *in vivo*. The extended chalcogenorhodamine dyes **7-S** and **7-Se** are photosensitizers *in vitro* toward Colo-26 cells using broad-band light with values of EC_{50} of 9.0×10^{-8} M and 1.8×10^{-7} M, respectively, with 10 J cm^{-2} of broad-band light.

4. Experimental

4.1 General Methods

Di-3-*N,N*-dimethylaminophenyl dichalcogenides **12-E** were prepared by literature methods.¹⁶ Fluorescence quantum yields (Φ_F) were measured as previously described.¹⁰ Quantum yields for the generation of singlet oxygen [$\Phi(^1O_2)$] were measured as previously described.²² Reactions were run under dry argon. Tetrahydrofuran was distilled from sodium benzophenone ketyl prior to use. Concentration *in vacuo* was performed on a Büchi rotary evaporator. NMR spectra were recorded on an Inova 500 instrument (500 MHz for 1H , 125 MHz for ^{13}C) with residual solvent signal as internal standard. Infrared spectra were recorded on a Perkin-Elmer FTIR instrument. UV-vis near-IR spectra were recorded on a Perkin-Elmer Lambda 12 spectrophotometer or on a Shimadzu UV-3600 spectrophotometer in quartz cuvettes with a 1-cm path length. Melting points were determined with a Büchi capillary melting point apparatus and are uncorrected. All compounds tested have a purity of at least 95%, which was determined from NMR spectra or by elemental analyses for C, H, and N (Atlantic Microlab, Inc., Norcross, GA). Experimental values of C, H, and N are within 0.4% of theoretical values.

4.1.1. *N,N*-Diethyl 2-(6-Dimethylamino)naphthamide (**15**).

Iodomethane (12.0 mL, 192 mmol) was added dropwise to a stirred solution of 6-amino-2-naphthoic acid (4.50 g, 24.0 mmol) and sodium carbonate (15.3 g, 144 mmol) in DMF (200 mL). The resulting mixture was heated at 110 °C for 16 h before being cooled to 60 °C and DABCO (4.05 g, 36.0 mmol) added. Heating was then resumed at 100 °C for 4 h. After cooling to ambient temperature, water (150 mL) was added, and the product was extracted with diethyl ether (3 × 50 mL). The combined organic extracts were washed with water (3 × 100 mL), dried over anhydrous $MgSO_4$, and concentrated. The crude product was recrystallized from CH_2Cl_2 /hexanes to give 5.46 g (99%) of methyl 6-(dimethylamino)-2-naphthoate as a brown solid, mp 150–152 °C (lit.³¹ mp 148–149 °C): 1H NMR (500 MHz, $CDCl_3$) δ 8.44 (s, 1 H), 7.93

(dd, 1 H, $J = 1.5, 9.0$ Hz), 7.79 (d, 1 H, $J = 9.0$ Hz), 7.63 (d, 1 H, $J = 9.0$ Hz), 7.17 (dd, 1 H, $J = 2.5, 9.5$ Hz), 6.88 (d, 1 H, $J = 2.5$ Hz), 3.94 (s, 3 H), 3.10 (s, 6 H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 167.6, 150.0, 137.5, 130.9, 130.3, 125.9, 125.7, 125.2, 123.0, 116.2, 105.3, 51.8, 40.4; IR (film on NaCl) 2949, 1714, 1624, 1508, 1436, 1384, 1290 cm^{-1} ; HRMS (ESI, HRDFMagSec) m/z 230.1174 (calcd for $\text{C}_{14}\text{H}_{15}\text{NO}_2 + \text{H}^+$: 230.1176).

n-Butyllithium (1.25 M, 8.37 mL, 10.5 mmol) was added dropwise to a stirred solution of diethylamine (1.17 mL, 11.3 mmol) in THF (30 mL) at -78°C . The resulting mixture was stirred for 1 h then transferred to a stirred solution of methyl 6-(dimethylamino)-2-naphthoate (2.0 g, 8.72 mmol) in THF (50 mL) at ambient temperature. The resulting solution was heated at 45°C for 30 min and then cooled to ambient temperature. A solution of saturated NH_4Cl (50 mL) was added and products were extracted with CH_2Cl_2 (3×150 mL). The combined organic extracts were dried over anhydrous MgSO_4 and concentrated. The crude product was recrystallized from CH_2Cl_2 /hexanes to give 2.29 g (97%) of **15** as a yellow solid, mp $99\text{--}100^\circ\text{C}$: ^1H NMR (500 MHz, CDCl_3) δ 7.72 (s, 1 H), 7.71 (d, 1 H, $J = 9.0$ Hz), 7.65 (d, 1 H, $J = 8.5$ Hz), 7.37 (dd, 1 H, $J = 2.0, 8.5$ Hz), 7.18 (dd, 1 H, $J = 2.5, 9.0$ Hz), 6.90 (d, 1 H, $J = 2.5$ Hz), 3.69–3.26 (m, 4 H), 3.07 (s, 6 H), 1.40–1.06 (m, 6 H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 171.5, 148.8, 134.9, 130.1, 128.8, 125.8, 125.5, 125.4, 124.2, 116.4, 105.4, 43.3, 40.2, 39.1, 13.4; IR (film on NaCl) 2970, 2890, 2750, 1627, 1506, 1420, 1382 cm^{-1} ; HRMS (ESI, HRDFMagSec) m/z 271.1805 (calcd for $\text{C}_{17}\text{H}_{22}\text{N}_2\text{O} + \text{H}^+$: 271.1805).

4.1.2. Metallation of **15** and quenching with disulfide **12-S**.

s-Butyllithium (0.87 M, 4.46 mL, 3.88 mmol) was added dropwise to a stirred solution of naphthamide **15** (1.0 g, 3.70 mmol) and TMEDA (606 μL , 4.07 mmol) in THF (75 mL) at -78°C . A solution of disulfide **12-S**¹⁶ (2.25 g, 7.39 mmol) in THF (30 mL) at -78°C was

immediately added, and the resulting mixture was stirred at -78 °C for 3 h and then warmed to ambient temperature with stirring overnight (12 h). A solution of saturated NH₄Cl (50 mL) was added, and products were extracted with CH₂Cl₂ (3 × 150 mL). The combined organic extracts were dried over anhydrous MgSO₄ and concentrated. The crude product was purified via chromatography on SiO₂ eluted with 0.5:9.5 Et₂O:CH₂Cl₂ (*R*_f = 0.5) to give 1.18g (76%) of a mixture of **10-S** and **11-S**, which was used without further purification or separation. HRMS (EI, HRDFMagSec) *m/z* 421.2182 (calcd for C₂₅H₃₁N₃OS: 421.2182).

4.1.3. Metallation of **15** and quenching with diselenide **12-Se**.

s-Butyllithium (1.07 M, 3.63 mL, 3.88 mmol), TMEDA (610 µL, 4.07 mmol), naphthamide **15** (1.0 g, 3.70 mmol), and diselenide **12-Se**¹⁶ (2.95 g, 7.40 mmol) in THF were treated as described for the preparation of **10-S/11-S**. The crude product was purified via column chromatography on SiO₂ (0.5:9.5 Et₂O:CH₂Cl₂ (*R*_f = 0.5)) to give 1.04 g (60%) of the **10-Se/11-Se** mixture as a yellow oil, which was used without further purification or separation. HRMS (ESI, HRDFMagSec) *m/z* 470.1690 (calcd for C₂₅H₃₁N₃O⁸⁰Se + H⁺: 470.1705).

4.1.4. Preparation of extended xanthenes **8-S** and **9-S**.

Phosphorus oxychloride (1.14 mL, 12.2 mmol) was added dropwise to a solution of Et₃N (1.79 mL, 12.2 mmol) and the mixture of **10-S** and **11-S** (429 mg, 1.02 mmol) in CH₃CN (25 mL). The resulting mixture was heated at reflux for 12 h and then cooled to 0 °C. A solution of 1 M NaOH (10 mL) was added slowly, and the resulting mixture was poured into a stirred solution of cold 1 M NaOH (200 mL), stirred for 6 h, and products were extracted with CH₂Cl₂ (3 × 250 mL). The combined organic extracts were dried over anhydrous MgSO₄ and concentrated. The mixture of xanthenes was purified by chromatography on SiO₂ eluted with 0.5:9.5 CH₃CN:CH₂Cl₂ (*R*_f = 0.6 and 0.7) to give the individual thioxanthenes **8-S** and **9-S**.

Xanthone **8-S** was recrystallized from CH₂Cl₂/hexanes to give 150 mg (42%) of **8-S** as a yellow solid, mp >260 °C: ¹H NMR (500 MHz, CDCl₃) δ 8.47 (d, 1 H, *J* = 9 Hz), 8.49 (d, 1 H, *J* = 9 Hz), 8.20 (d, 1 H, *J* = 9.5 Hz), 7.59 (d, 1 H, *J* = 9.0 Hz), 7.18 (dd, 1 H, *J* = 2.5, 9.0 Hz), 6.90 (d, 1 H, *J* = 2.5 Hz), 6.85 (dd, 1 H, *J* = 2.5, 9.5 Hz), 6.73 (d, 1 H, *J* = 2.0 Hz), 3.16–3.09 (m, 12 H); ¹³C NMR (75.5 MHz, CDCl₃) δ 178.8, 151.9, 150.2, 138.2, 136.6, 136.2, 130.8, 125.3, 124.8, 124.5, 124.4, 120.9, 118.9, 115.3, 112.0, 106.6, 105.4, 40.3, 40.0; IR (film on NaCl) 2925, 1614, 1587, 1507, 1406, 1370, 1335 cm⁻¹; HRMS (EI, HRDFMagSec) *m/z* 348.1291 (calcd for C₂₁H₂₀N₂OS⁺: 348.1291).

Xanthone **9-S** was recrystallized from CH₂Cl₂/hexanes to give 89 mg (25%) of an orange solid, mp >250 °C: ¹H NMR (500 MHz, CDCl₃) δ 8.97 (s, 1 H), 8.46 (d, 1 H, *J* = 9.0 Hz), 7.89 (d, 1 H, *J* = 9.5 Hz), 7.68 (s, 1 H), 7.16 (dd, 1 H, *J* = 2.5, 9.0 Hz), 6.70 (dd, 1 H, *J* = 2.5, 9 Hz), 6.78 (d, 1 H, *J* = 2.5 Hz), 6.60 (m, 1 H); 3.12 (s, 6 H), 3.11 (s, 6 H); ¹³C NMR (75.5 MHz, CDCl₃) δ 179.4, 152.3, 150.3, 139.7, 136.6, 132.6, 131.5, 131.1, 130.6, 124.8, 123.9, 120.3, 118.6, 116.3, 110.9, 105.1, 102.9, 40.4, 40.1; IR (film on NaCl) 1613, 1591, 1500, 1441, 1389, 1371, 1336, 1286 cm⁻¹; HRMS (ESI, HRDFMagSec) *m/z* 349.1369 (calcd for C₂₁H₂₀N₂OS + H⁺: 349.1370).

4.1.5. Preparation of extended xanthenes **8-Se** and **9-Se**.

Phosphorus oxychloride (931 μL, 9.99 mmol), Et₃N (1.39 mL, 9.99 mmol) and the mixture of **10-Se** and **11-Se** (390 mg, 0.832 mmol) in CH₃CN (25 mL) were treated as described for the preparation of **8-S** and **9-S**.

Xanthone **8-Se** was recrystallized from CH₂Cl₂/hexanes to give 135 mg (41%) of a yellow solid, mp >260 °C: ¹H NMR (500 MHz, CDCl₃) δ 8.54 (d, 1 H, *J* = 9 Hz), 8.53 (d, 1 H, *J* = 9 Hz), 7.97 (d, 1 H, *J* = 9.5 Hz), 7.59 (d, 1 H, *J* = 9.0 Hz), 7.19 (dd, 1 H, *J* = 2.0, 9.0 Hz), 6.89 (d, 1 H, *J*

= 2.5 Hz), 6.83 (dd, 1 H, J = 2, 9.5 Hz), 6.81 (d, 1 H, J = 2.5 Hz), 3.12 (s, 6 H), 3.11 (s, 6 H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 180.9, 151.8, 150.1, 136.4, 136.2, 135.9, 132.2, 126.7, 126.2, 124.8, 122.7, 120.1, 115.5, 112.0, 107.8, 106.4, 40.3, 40.0; IR (film on NaCl) 2932, 1606, 1582, 1507, 1407, 1372, 1329 cm^{-1} ; HRMS (EI, HRDFMagSec) m/z 396.0742 (calcd for $\text{C}_{21}\text{H}_{20}\text{N}_2\text{O}^{80}\text{Se}^+$: 396.0742).

Xanthone **9-Se** was recrystallized from CH_2Cl_2 /hexanes to give 66 mg (20%) of a yellow solid, mp >250 °C: ^1H NMR (500 MHz, CDCl_3) δ 9.02 (s, 1 H), 8.50 (d, 1 H, J = 9.0 Hz), 7.87 (d, 1 H, J = 9.0 Hz), 7.75 (s, 1 H), 7.15 (dd, 1 H, J = 2.5, 9.0 Hz), 6.75 (J = 2.5, 9.0 Hz), 6.73 (d, 1 H, J = 2.5 Hz), 6.69 (d, 1 H, J = 2.5 Hz), 3.11 (s, 6 H), 3.09 (s, 6 H); ^{13}C NMR (75.5 MHz, CDCl_3) δ 181.3, 152.2, 150.3, 136.8, 136.7, 132.8, 132.0, 131.2, 129.5, 125.2, 125.0, 123.0, 120.2, 116.2, 111.1, 107.9, 102.8, 40.4, 40.0; IR (film on NaCl) 2949, 1610, 1588, 1498, 1438, 1389, 1328 cm^{-1} ; HRMS (EI, HRDFMagSec) m/z 396.0733 (calcd for $\text{C}_{21}\text{H}_{20}\text{N}_2\text{O}^{80}\text{Se}^+$: 396.0735).

4.1.6. Preparation of extended rhodamine **6-S**.

Phenylmagnesium bromide (1.0 M in THF, 2.87 mL, 2.87 mmol) was added to a stirred solution of **8-S** (100 mg, 0.297 mmol) in THF (5 mL), and the resulting mixture was heated at reflux for 16 h and then cooled to ambient temperature. The reaction was quenched by the addition of glacial acetic acid (2 mL) and the resulting mixture was poured into 100 mL of aqueous 10% HPF_6 . The resulting mixture was stirred 12 h and the precipitate was collected via filtration, then washed with water (50 mL) and diethyl ether (100 mL). The crude product was recrystallized from MeOH to give 145 mg (90%) of **6-S** as a dark purple solid, mp 182–183 °C: ^1H NMR (500 MHz, CD_2Cl_2) δ 8.49 (d, 1 H, J = 9.5 Hz), 7.64–7.71 (m, 3 H), 7.57 (d, 1 H, J = 9.0 Hz), 7.52 (d, 1 H, J = 9.0 Hz), 7.35–7.41 (m, 3 H), 7.32 (d, 1 H, J = 9.0 Hz), 7.28 (d, 1 H, J =

9.0 Hz), 7.11 (dd, 1 H, $J = 2.5, 9.5$ Hz), 6.97 (d, 1 H, $J = 2.0$ Hz), 3.35 (s, 6 H), 3.25 (s, 6 H); ^{13}C NMR (75.5 MHz, CD_3CN) δ 161.3, 154.6, 154.0, 145.0, 137.5, 137.1, 136.7, 130.3, 130.0, 129.7, 129.0, 128.3, 126.9, 124.7, 121.6, 119.7, 118.5, 117.4, 110.9, 107.3, 106.6, 41.2, 40.5; λ_{max} (MeOH) 630 nm ($\epsilon = 6.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); λ_{max} (CH_2Cl_2) 641 nm ($\epsilon = 6.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); HRMS (ESI, HRDFMagSec) m/z 409.1726 (calcd for $\text{C}_{27}\text{H}_{25}\text{N}_2\text{S}^+$: 409.1733). **Anal.** Calcd for $\text{C}_{27}\text{H}_{25}\text{N}_2\text{S}\cdot\text{PF}_6$: C, 58.48; H, 4.54; N, 5.05. Found: C, 58.54; H, 4.65; N, 5.07.

4.1.7. Preparation of extended rhodamine 6-Se.

Phenylmagnesium bromide (1 M in THF, 1.26 mL, 1.26 mmol) and **8-S** (50 mg, 0.126 mmol) in THF (5 mL) were treated as described for the preparation of **6-S**. The crude product was purified by recrystallization from MeOH, to give 72 mg (95%) of **6-Se** as a dark purple solid, mp 187–186 °C: ^1H NMR (500 MHz, CD_2Cl_2) δ 8.28 (d, 1 H, $J = 9.0$ Hz), 7.69–7.63 (m, 3 H), 7.58 (d, 1 H, $J = 10.0$ Hz), 7.51 (s, 1 H), 7.45 (d, 1 H, $J = 9.5$ Hz), 7.37–7.33 (m, 2 H), 7.31 (d, 1 H, $J = 8.5$ Hz), 7.28 (d, 1 H, $J = 9.0$ Hz), 7.00 (d, 1 H, $J = 9.5$ Hz), 6.89 (s, 1 H), 3.32 (s, 6 H), 3.24 (s, 6 H); ^{13}C NMR (75.5 MHz, CD_3CN) δ 162.7, 154.1, 153.9, 147.2, 141.8, 138.7, 138.6, 137.3, 130.8, 130.1, 129.8, 129.6, 128.5, 127.8, 125.7, 122.2, 121.9, 117.5, 110.9, 109.9, 107.1, 41.3, 40.5; λ_{max} (MeOH) 647 nm ($\epsilon = 6.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); λ_{max} (CH_2Cl_2) 658 nm ($\epsilon = 6.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); HRMS (ESI, HRDFMagSec) m/z 457.1178 (calcd for $\text{C}_{27}\text{H}_{25}\text{N}_2^{80}\text{Se}^+$: 457.1177). **Anal.** Calcd for $\text{C}_{27}\text{H}_{25}\text{N}_2\text{Se}\cdot\text{PF}_6$: C, 53.92; H, 4.19; N, 4.66. Found: C, 54.16; H, 4.27; N, 4.63.

4.1.8. Preparation of extended rhodamine 7-S.

Phenylmagnesium bromide (1 M in THF, 2.87 mL, 2.87 mmol) and **9-S** (100 mg, 0.207 mmol) in THF (5 mL) were treated as described for preparation of **6-S**. The crude product was recrystallized from MeOH to give 145 mg (91%) of **7-S** as a dark purple solid, mp 204–205 °C:

^1H NMR (500 MHz, CD_2Cl_2) δ 7.99 (m, 2 H), 7.67–7.70 (m, 4 H), 7.49 (d, 1 H, $J = 9.5$ Hz), 7.41 (dd, 2 H, $J = 1.5, 8.0$ Hz), 7.22 (dd, 1 H, $J = 2.0, 9.0$ Hz), 7.15 (d, 1 H, $J = 2.0$ Hz), 6.94 (dd, 1 H, $J = 2.5, 9.5$ Hz), 6.89 (d, 1 H, $J = 2.5$ Hz), 3.36 (s, 6 H), 3.25 (s, 6 H); ^{13}C NMR (75.5 MHz, CD_3CN) δ 162.5, 155.7, 153.9, 147.7, 138.8, 138.5, 138.4, 136.8, 133.9, 133.3, 133.2, 130.5, 129.7, 126.4, 123.4, 121.3, 120.5, 118.6, 117.1, 107.4, 103.0, 41.6, 40.7; λ_{max} (MeOH) 661 nm ($\epsilon = 4.71 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); λ_{max} (CH_2Cl_2) 689 nm ($\epsilon = 4.90 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); HRMS (ESI, HRDFMagSec) m/z 409.1734 (calcd for $\text{C}_{27}\text{H}_{25}\text{N}_2\text{S}^+$: 409.1733). **Anal.** Calcd for $\text{C}_{27}\text{H}_{25}\text{N}_2\text{S}\cdot\text{PF}_6$: C, 58.48; H, 4.54; N, 5.05. Found: C, 58.23; H, 4.60; N, 4.93.

4.1.9. Preparation of extended rhodamine 7-Se.

Phenylmagnesium bromide (1 M in THF, 2.53 mL, 1 M in THF, 2.53 mmol) and **9-Se** (100 mg, 0.253 mmol) in THF (10 mL) were treated as described for the preparation of **6-S**. The crude product was recrystallized from MeOH to give 128 mg (84%) of **7-Se** as a dark purple solid, mp 213–214 °C: ^1H NMR (500 MHz, CD_2Cl_2) δ 8.04 (s, 1 H), 8.00 (s, 1 H), 7.72–7.60 (m, 4 H), 7.50 (d, 1 H, $J = 10.0$ Hz) 7.40–7.32 (m, 3 H), 7.18 (d, 1 H, $J = 9.0$ Hz), 6.86 (d, 1 H, $J = 9.5$ Hz), 6.79 (s, 1 H), 3.34 (s, 6 H), 3.24 (s, 6 H); ^{13}C NMR (75.5 MHz, CD_3CN) δ 164.0, 155.2, 154.1, 148.7, 140.6, 140.5, 138.6, 138.1, 133.6, 132.9, 130.4, 130.2, 129.6, 126.3, 124.3, 123.4, 122.2, 118.4, 116.9, 111.2, 103.1, 41.6, 40.7; λ_{max} (MeOH) 671 nm ($\epsilon = 4.71 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); λ_{max} (CH_2Cl_2) 700 nm ($\epsilon = 5.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); HRMS (ESI, HRDFMagSec) m/z 457.1176 (calcd for $\text{C}_{27}\text{H}_{25}\text{N}_2^{80}\text{Se}^+$: 457.1177). **Anal.** Calcd for $\text{C}_{27}\text{H}_{25}\text{N}_2\text{Se}\cdot\text{PF}_6$: C, 53.92; H, 4.19; N, 4.66. Found: C, 54.11; H, 4.24; N, 4.64.

4.2. Determination of Singlet Oxygen Yields from Singlet Oxygen Luminescence Spectroscopy.

Time-resolved detection of the long-lived $^1\text{O}_2$ emission at 1270 nm was used to distinguish signal from singlet oxygen.²² A SPEX 270 M spectrometer (Jobin Yvon) equipped with a Hamamatsu IR-PMT coupled to an Infinium oscilloscope (Hewlett-Packard) was used for recording singlet oxygen phosphorescence decay. The monochromator was tuned to 1270 nm. The second harmonic (532 nm) from a nanosecond pulsed Nd:YAG laser (Lotis TII, Belarus) operating at 20 Hz was used as the excitation source. Additional long-pass filters (a 950LP filter and a 538AELP filter, both from Omega Optical) were used to attenuate the scattered light and fluorescence from the samples. The samples (methanol solutions of the compounds in quartz cuvettes) were placed in front of the spectrometer entrance slit.

4.3 Growth of Colo-26 and HUT-78 Cells.

Colo-26 cells were grown in RPMI 1640, 1X with L-glutamine medium. The medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HUT-78 cells (ATCC, Manassas, VA) were grown in RPMI 1640, 1X with L-glutamine medium. The medium was supplemented with 10% FBS, 1% HEPES buffer, and 1% penicillin-streptomycin.

4.4. Phototoxicity and Dark Toxicity Studies with Colo-26 Cells

Colo-26 cells were harvested, plated 10,000 to a well in a 96-well plate (0.32 cm² on a flat bottom plate), and incubated for 24 h. Extended rhodamines **6-E** and **7-E** were added from stock solutions of known concentration. All plates were incubated 1 h in the dark after dye addition then either kept in the dark or irradiated with a tunable dye laser at 642 ± 2 nm for **6-S**, 660 ± 2 nm for **6-Se**, 690 ± 2 nm for **7-S**, and 700 ± 2 nm for **7-Se** at a fluence rate of 3.2 mW cm⁻² to various light doses. New medium was added to each well before they were placed in the incubator (37 °C, 5% CO₂). After a 48-h incubation, a sulforhodamine B assay^{32,33} was performed on the plates. The absorbance of each well was read on an EL800 BioTek plate

reader at 570 nm to give fraction cell viability after data manipulation. Cells were similarly treated with dyes **7-E** and were then incubated 1 h in the dark after dye addition and then either kept in the dark or irradiated with 5 J cm^{-2} or 10 J cm^{-2} of unfiltered light from a Curelight broad band source (Photocure, Oslo, Norway) at a fluence rate 47.6 mW cm^{-2} . After a 48-h incubation, cell viability was again determined with a sulforhodamine B assay.^{28,29}

4.5. Flow Cytometry Studies

Image Stream flow cytometry was run on an Image Stream Mark II instrument using channels 2 (480-560 nm detection) and 5 (642-745 nm detection) with MTG excitation at 488 nm and excitation of dyes **6-E** and **7-E** at 561 nm. Samples were made using 5×10^5 Colo-26 cells or 5×10^5 HUT-78 cells in 0.5 mL medium. Each photosensitizer was run at a concentration of $2 \times 10^{-7} \text{ M}$ with and without $5 \times 10^{-7} \text{ M}$ MTG. Cells were also run with $5 \times 10^{-7} \text{ M}$ MTG alone ($5 \times 10^{-7} \text{ L}$ of $1 \times 10^{-3} \text{ M}$ MTG stock in DMSO). All samples were incubated 15 min, centrifuged, and the medium was removed. Samples were re-suspended in Hanks PBS (60 μL), placed on ice, and analyzed. Co-localization was determined in each individual cell using the IDEAS similarity feature, which is a log-transformed Pearson's correlation coefficient of the intensities of the spatially correlated pixels within the whole cell, of the MTG and **6-E** or **7-E** images, MTG and MTR images, or LYS and MTR images, respectively. The similarity score is a measure of the degree to which two images are linearly correlated.

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References and Notes

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1. Detty, M. R.; Gibson, S. L. Wagner, S. J. *J. Med. Chem.* **2004**, *47*, 3987-3915.
2. Johnson, L. V.; Walsh, M. L.; Bockus, B. J.; Chen, L. B. *J. Cell. Biol.* **1981**, *88*, 526-535.
3. Davis, S.; Weiss, M. J.; Wong, J. R.; Lampidis, T. J.; Chen, L. B. *J. Biol. Chem.* **1985**, *260*, 13844-13850.
4. Lampidis, T. J.; Bernal, S. D.; Summerhayes, I. C.; Chen, L. B. *Cancer Res.* **1983**, *43*, 716-720.
5. Bernal, S. D.; Lampidis, T. J.; McIsaac, R. M.; Chen, L. B. *Science* **1986**, *222*, 169-172.
6. Pal, P.; Zeng, H.; Durocher, G.; Girard, D.; Li, T. C.; Gupta, A. K.; Giasson, R.; Blanchard, L.; Gaboury, L.; Balassy, A.; Turmel, C.; Laperriere, A.; Villeneuve, L. *Photochem. Photobiol.* **1996**, *63*, 161-168.
7. Gorman, A.; Killoran, J.; O'Shea, C.; Kenna, T.; Gallagher, W. M.; O'Shea, D. F. *J. Am. Chem. Soc.* **2004**, *126*, 10619-10631.
8. Kessel, D.; Woodburn, K. *Br. J. Cancer* **1995**, *71*, 306-310.
9. Ogata, M.; Inanami, O.; Nakajima, M.; Nakajima, T.; Hiraoka, W.; Kuwabara, M. *Photochem. Photobiol.* **2003**, *78*, 241-247.

10. Ohulchanskyy, T.; Donnelly, D. J.; Detty, M. R.; Prasad, P. N. *J. Phys. Chem. B* **2004**, *108*, 8668-8672.
11. Hill, J. E.; Linder, M. K.; Davies, K. S.; Sawada, G. A.; Morgan, J.; Ohulchanskyy, T. Y.; Detty, M. R. *J. Med. Chem.* **2014**, *57*, 8622-8634.
12. Kryman, M. W.; Davies, K. S.; Linder, M. K.; Ohulchanskyy, T. Y.; Detty, M. R. *Bioorg. Med. Chem.* **2015**, *23*, 4501-4507.
13. Lavis, L. D.; Raines, R. T. *ACS Chem. Biol.* **2014**, *9*, 855-866. (b) Grimm, J. B.; English, B. P.; Chen, J. J.; Slaughter, J. P.; Zhang, Z. J.; Revyakin, A.; Patel, R.; Macklin, J. J.; Normanno, D.; Singer, R. H.; Lionnet, T.; Lavis, L. D. *Nature Meth.* **2015**, *12*, 244-250.
14. Yang, Y.; Lowry, M.; Escobedo, J. O.; Sibrian-Vazquez, M.; Wong, L.; Showalter, C. M.; Jensen, T. J.; Fronczek, F. R.; Warner, I. M.; Strongin, R. M. *PNAS* **2008**, *105*, 8829-8834.
15. Sibrian-Vazquez, M.; Escobedo, J. O.; Lowry, M.; Fronczek, F. R.; Strongin, R. M. *J. Am. Chem. Soc.* **2012**, *134*, 10502-10508.
16. Brennan, N. K.; Donnelly, D. J., Detty, M. R. *J. Org. Chem.* **2003**, *68*, 3344-3347.
17. Chen, C.W.; Beak, P. *J. Org. Chem.* **1986**, *51*, 3325-3334.
18. Gay, R.; Hauser, C. R. *J. Am. Chem. Soc.* **1967**, *89*, 2297-2303.
19. Coll, G.; Morey, J.; Costa, A.; Saa, J. M. *J. Org. Chem.* **1988**, *53*, 5345-5348.
20. Jastrzebski, J. T.; Arink, A. M.; Kleijn, H.; Braam, T. W.; Lutz, M.; Spek, A. L.; van Koten, G. *J. Am. Chem. Soc.* **2013**, *135*, 13371-13378.
21. Comins, D.; Brown, J. D. *J. Org. Chem.* **1984**, *49*, 1078-1083.
22. Ohulchanskyy, T. Y.; Roy, I.; Goswami, L. N.; Chen, Y.; Bergey, E. J.; Pandey, R. K.; Oseroff, A. R.; Prasad, P. N. *Nano Lett.* **2007**, *7*, 2835-2842.

23. Holt, J. J.; Gannon, M. K.; Tomblin, G.; McCarty, T. A.; Page, P. M.; Bright, F. V.; Detty, M. R. *Bioorg. Med. Chem.* **2006**, *14*, 8635-8643.
24. Johnson, L. V.; Walsh, M. L.; Bockus, B. J.; Chen, L. B. *J. Cell. Biol.* **1981**, *88*, 526–535.
25. Davis, S.; Weiss, M. J.; Wong, J. R.; Lampidis, T. J.; Chen, L. B. *J. Biol. Chem.* **1985**, *260*, 13844–13850.
26. Riddell, J. R.; Wang, X.-Y. Hans Minderman, H.; Gollnick, S. O. *J. Immunol.* **2010**, *184*, 1022–1030.
27. Noodt, B. B.; Rodal, G. H.; Wainwright, M.; Peng, Q.; Horobin, R.; Nesland, J. M.; Berg, K. *Int. J. Cancer* **1998**, *75*, 941-948.
28. Millish, K. J.; Cox, R. D.; Vernon, D.; Griffiths, J.; Brown, S. B. *Photochem. Photobiol.* **2002**, *75*, 392-397.
29. Lin, C. W.; Shulk, J. R.; Kirley, S. D.; Bachelder, C. M.; Flotte, T. J.; Sherwood, M. E.; Cincotta, L.; Foley, J. W. *Photochem. Photobiol.* **1993**, *58*, 81-91.
30. Lin, C-W.; Shulok, J. R.; Kirley, S. D.; Cincotta, L.; Foley, J. W. *Cancer Res.* **1991**, *51*, 2710-2719.
31. Adcock, W.; Wells, P. R. *Aust. J. Chem.* **1965**, *18*, 1351-1364.
32. Houghton, P.; Fang, R.; Techatanawat, I.; Steventon, G.; Hylands, P. J.; Lee, C. C. *Methods* **2007**, *42*, 377-387.
33. Vichai, V.; Kirtikara, K. *Nature Protocols* **2006**, *1*, 1112-1116.

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