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Synthesis of Fluorophores that Target Small Molecules to the Endoplasmic Reticulum of Living Mammalian Cells**

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Abstract

The endoplasmic reticulum (ER) plays critical roles in the processing of secreted and transmembrane proteins. To deliver small molecules to this organelle, we synthesized fluorinated hydrophobic analogues of the fluorophore rhodol. These cell-permeable fluorophores are exceptionally bright, with quantum yields of ~0.8, and specifically accumulate in the ER of living HeLa cells, as imaged by confocal laser scanning microscopy. To target a biological pathway controlled by the ER, we linked a fluorinated hydrophobic rhodol to 5-nitrofur-2-acylaldehyde. In contrast to an untargeted nitrofur warhead, delivery of this electrophilic nitrofur to the ER by the rhodol resulted in cytotoxicity comparable to the ER-targeted cytotoxin eeyarestatin I, and specifically inhibited protein processing by the ubiquitin-proteasome system. Fluorinated hydrophobic rhodols represent outstanding fluorophores that enable delivery of small molecules for targeting of ER-associated proteins and pathways.

Keywords

endoplasmic reticulum; cytotoxin; proteasome; fluorescence; chemical biology

The endoplasmic reticulum (ER), an organelle found in all eukaryotes, generally contributes at least half of all membranes of animal cells. These membranes spread throughout the cytosol to define a network of tubes and sacs that enclose a single internal space termed the lumen. Numerous processes essential for cellular maintenance and survival occur on and in the ER. The external surface of the ER captures ribosomes involved in translation of membrane-bound and secreted proteins, whereas the lumen stores intracellular calcium, regulates folding and posttranslational processing of proteins in the secretory pathway, and is a major site of cellular lipid biosynthesis.^[1] Stressful conditions can cause unfolded proteins to accumulate in the ER.^[2] These stressors include hypoxia, oxidants or reductants, glucose deprivation, altered calcium regulation, viral infection, and expression of aberrant proteins. This triggers an unfolded protein response (UPR) in the ER that initiates complex

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Dedicated to our dear colleague and friend Professor Dr. Lester A. Mitscher (1931-2015)

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signaling pathways. These pathways can promote adaptive responses, or when stress is excessive, cellular death.^[3] Pathologies associated with ER stress include neurodegenerative disease, stroke, heart disease, diabetes, and cancer.^[4-7]

Because of the importance of the ER in disease, modulators of targets in this organelle are of substantial interest as potential therapeutics and probes. A number of small molecules are known to induce ER stress,^[2, 4] including eeyarestatin I (**1**, Figure 1).^[8-14] This agent (**1**) blocks the ubiquitin-proteasome system (UPS) by accumulating in the ER and inhibiting the protein p97.^[8-9, 12-13, 15] This protein is involved in the translocation of misfolded polypeptides from the ER to the cytosol for eventual degradation by the proteasome. Inhibition of p97 triggers a build-up of misfolded proteins, causing apoptosis in some cancer cell lines.^[10, 16-17] Previous SAR studies^[12] of eeyarestatin I (**1**) demonstrated that the 5-nitrofuranyl-2-acrylaldehyde hydrazone functions as an electrophilic warhead^[18-19] that interacts with the p97 protein complex, whereas the aromatic urea moiety principally localizes this warhead to ER membranes to gain selectivity for ER-membrane-bound p97 over the cytosolic form of this protein.

We report here the synthesis of novel analogues of the fluorophore rhodol (**2**, Figure 1) that uniquely accumulate in the ER of mammalian cells. Analogues such as **3** (Figure 1) incorporate fluorine atoms at the 2' and 7'-positions to enhance photophysical properties, and substitute the polar carboxylate of rhodol with a hydrophobic methyl group to favor association with cellular membranes. Analogue **4** (Figure 1) was designed to include a 5-nitrofuranyl-2-acrylaldehyde hydrazone, analogous to the electrophilic warhead found in eeyarestatin I (**1**), to target the p97 protein of the ER.

To synthesize these compounds, the fluorophore Pennsylvania Green (**5**, Scheme 1),^[20-21] prepared from 2,7-difluoro-3,6-dihydroxyxanthene-9-one,^[22-23] was transformed into iodoarene **6**. This iodoarene was converted to rhodols **3** and **8** via Buchwald-Hartwig cross-coupling with microwave irradiation. This approach is similar to methods used to prepare other rhodols^[24] and rhodamines^[25] from triflates. Rhodol **3** is a unique fluorinated hydrophobic (cLogP = 3.9) analogue of other previously reported^[24, 26-30] rhodol fluorophores. The related rhodol **4** was derived from deprotection of **8**, preparation of the rhodol-hydrazide **9**, and condensation with **10** to install the warhead, whereas the nitrofuranyl derivative **12** was prepared as an untargeted control compound.

Rhodols are generally fluorescent in a wide variety of solvents.^[24, 26] To examine the photophysical properties of rhodol **3**, we obtained absorbance and fluorescence emission spectra of this compound in PBS, ethanol, and octanol, and we measured its extinction coefficient and quantum yield relative^[31] to rhodamine 6G (data shown in Figure S1 of the supporting information). As shown in Figure 2, its spectral properties in ethanol (Abs. λ_{max} = 512 nm; Em. λ_{max} = 532 nm, ϵ = 73,500 M⁻¹ cm⁻¹, Φ = 0.85) make it a potentially outstanding probe for cellular studies.

Confocal laser scanning microscopy was used to examine the subcellular distribution of rhodol **3** in human HeLa cervical carcinoma cells. As shown in Figure 3, these experiments revealed accumulation of **3** in distinct tubular structures. These structures were identified as

the ER by colocalization with the spectrally orthogonal organelle marker ER Tracker Blue-White^[32] (Figure 3). Although the mechanism of the selectivity of fluorophores such as ER-Tracker Blue-White for the ER is not completely understood, QSAR^[33-34] and microscopy^[35] studies of related probes suggest that these types of hydrophobic amphipathic compounds preferentially associate with cholesterol-poor ER membranes.^[36]

Similar colocalization results were obtained with cells treated with rhodol-nitrofurane **4**, indicating that the ER-targeting ability of the rhodol was unaffected by attachment of the warhead (images are shown in Figure S2 of the supporting information). To examine the cytotoxicities of eeyarestatin I (**1**), **4**, **9** and **12**, HeLa cells were treated with these compounds for 48 h and cellular viability measured by flow cytometry. The rhodol-nitrofurane **4** (IC₅₀ = 2.7 μM) and eeyarestatin I (**1**, IC₅₀ = 2.0 μM) were similar in potency, suggesting that the ER-targeting motifs of both of these compounds are effective at delivering the nitrofurane to this organelle (Figure 4). As controls, rhodol-hydrazide **9**, lacking the cytotoxic warhead, showed no appreciable toxicity (IC₅₀ > 50 μM), whereas the nitrofurane hydrazone warhead **12** alone was less toxic (IC₅₀ = 13 μM) than the ER-targeted compounds.

Eeyarestatin I (**1**) manifests toxicity by inhibiting p97 on ER membranes.^[4] This protein is required for maturation of the transcription factor Nrf1, which activates genes that encode subunits of the proteasome of the UPS.^[37] To characterize inhibitors of the UPS, cells are often transfected with fluorescent proteins fused to a ubiquitin degradation signal.^[38-39] The fused ubiquitin of these reporters results in rapid trafficking to and degradation by the proteasome,^[40] whereas inhibitors of the UPS,^[4] such as eeyarestatin I (**1**),^[41] prevent degradation of these reporter proteins and restore cellular fluorescence.

By targeting the nitrofurane to the ER, we hypothesized that the rhodol-nitrofurane **4** might selectively inhibit the UPS. To test this hypothesis, we constructed a spectrally orthogonal fluorescent reporter comprising an optimized G76V mutant of ubiquitin (Ub^{G76V})^[42] fused to the cyan fluorescent protein cerulean. This mutant of ubiquitin is efficiently degraded by the proteasome when fused to fluorescent proteins.^[42] As shown in Figure 5, in the absence of inhibitors, HeLa cells transfected with the Ub^{G76V}-cerulean reporter showed no appreciable cellular fluorescence, consistent with rapid degradation of this protein by the proteasome. However, when these cells were treated for 12 h with the p97 inhibitor eeyarestatin I (**1**), transfected cells, in contrast to adjacent non-transfected cells, became highly cyan fluorescent. Similarly, when treated with rhodol-nitrofurane **4**, cerulean was highly expressed by transfected cells, demonstrating that this compound is a specific inhibitor of the UPS. By contrast, the nitrofurane control **12**, either at 5 μM or at an elevated concentration of 20 μM, did not activate the reporter, thus demonstrating that targeting of this moiety to the ER is critical for inhibition of this pathway.

The results presented here demonstrate that fluorinated hydrophobic rhodols represent outstanding fluorophores that can be used to deliver small molecule cargo to the ER. When this cargo was an electrophilic nitrofurane warhead, delivery to the ER resulted in inhibition of the UPS, with concomitant effects on cellular proliferation. Given the widespread interest in targeting ER-associated proteins involved in cancer^[4, 43] neurodegeneration,^[44] and other

diseases,^[7] these compounds may provide useful tools for imaging and modulation of biological pathways associated with this critical organelle.

Supplementary Material

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Figure 1.

Structures of the ER-targeted p71 inhibitor eeyarestatin I (**1**), the fluorophore rhodol (**2**), a fluorinated hydrophobic analogue of rhodol (**3**), and a related derivative (**4**) linked to an electrophilic 5-nitrofuran warhead.

Figure 2.

Photophysical properties and spectra of rhodol 3. Panel A: Absorbance (Abs., acquired at 10 μ M) and normalized fluorescence emission (Em., acquired at 10 nM, Ex. 500 nm) spectra in aqueous phosphate-buffered saline (PBS, pH 7.4), absolute ethanol (EtOH), and *n*-octanol (Oct.).

Figure 3.

Confocal laser scanning and differential interference contrast (DIC) micrographs of living HeLa cells treated with ER-tracker Blue-White DPX (0.1 μM, 0.5 h) and rhodol **3** (0.5 μM, 0.5 h). The fluorescence emission of ER-tracker Blue-White DPX can be observed in the upper left panel and the fluorescence emission of the spectrally orthogonal **3** can be observed in the upper right panel. Colocalization of these two fluorophores is shown in yellow in the lower left panel

Figure 4.
Cytotoxicity of ER-targeted and untargeted compounds towards HeLa cells after 48 h in culture.

Figure 5.

Confocal laser scanning and DIC micrographs of living HeLa cells transiently transfected with the cyan fluorescent protein Ub^{G76V}-cerulean. The spectrally orthogonal rhodol-nitrofurane **4** can be observed in the yellow channel. Cells were treated with compounds for 12 h prior to imaging.

Scheme 1.

Synthesis of the fluorinated rhodol **3**, rhodol-hydrazide **9**, rhodol-nitrofuran **4**, and the untargeted nitrofuran control **12**.