Subcellular Targeting

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

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In memory of Lester A. Mitscher

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 around 0.8, and they were found to 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-nitrofuran-2-acrylaldehyde. In contrast to an untargeted nitrofuran warhead, delivery of this electrophilic nitrofuran 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 are outstanding fluorophores that enable the delivery of small molecules for targeting ER-associated proteins and pathways.*

The endoplasmic reticulum (ER), an organelle found in all eukaryotes, generally contributes at least half of all of the membranes of animal cells. Membranes of the ER 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 the translation of membrane-bound and secreted proteins, whereas the lumen stores intracellular calcium, regulates the folding and post-translational processing of proteins in the secretory pathway, and is a major site of cellular lipid biosynthesis.[1] Stress conditions can cause unfolded proteins to accumulate in the ER.[2] 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 signaling pathways. These pathways can promote adaptive responses or, when the 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 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, which causes apoptosis in some cancer cell lines.[10,16,17] Previous structure–activity relationship (SAR) studies[12] of eeyarestatin I (**1**) demonstrated that the 5-nitrofuran-2-acrylaldehyde hydrazone functions as an elec-

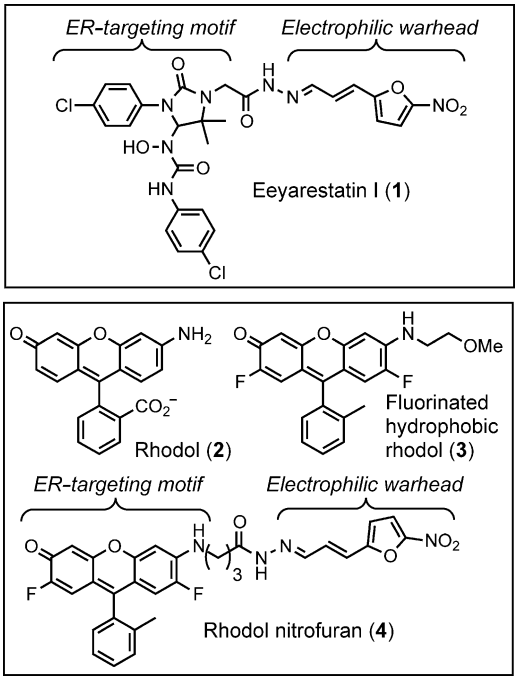

Figure 1. Structures of the ER-targeted p97 inhibitor eeyarestatin I (**1**), the fluorophore rhodol (**2**), a fluorinated hydrophobic analogue of rhodol (**3**), and a related derivative (**4**) linked to an electrophilic 5-nitrofuran warhead.

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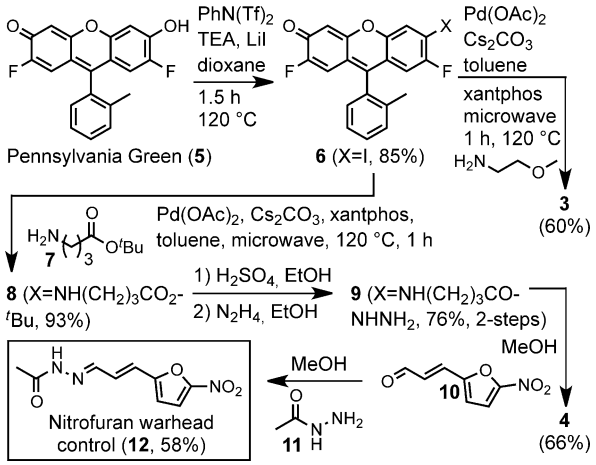
trophilic warhead[18,19] that interacts with the p97 protein complex, whereas the aromatic urea moiety principally localizes this warhead to ER membranes to gain selectivity for ER-membrane-bound p97 over the cytosolic form of this protein.

We report herein 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 the 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-nitrofuran-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 converted into iodoarene **6**. This iodoarene was transformed into rhodols **3** and **8**



Scheme 1. Synthesis of the fluorinated rhodol **3**, rhodol hydrazide **9**, rhodol nitrofuran **4**, and the untargeted nitrofuran control **12**.

through 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, calculated with ChemAxon MarvinView 6.2) analogue of other previously reported[24,26–30] rhodol fluorophores. The related rhodol **4** was derived through deprotection of **8**, preparation of the rhodol hydrazide **9**, and condensation with aldehyde **10** to install the warhead, whereas the analogous nitrofuran 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

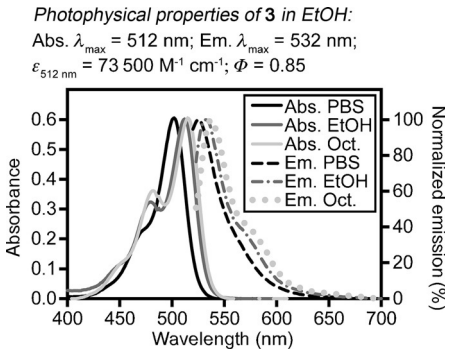


Figure 2. Photophysical properties and spectra of rhodol **3**. Absorbance (Abs., acquired at 10 μM) and normalized fluorescence emission (Em., acquired at 10 nM; Ex. at 500 nm) spectra are shown for aqueous phosphate-buffered saline (PBS, pH 7.4), absolute ethanol (EtOH), and *n*-octanol (Oct.).

properties in ethanol (Abs. $\lambda_{max} = 512$ nm; Em. $\lambda_{max} = 532$ nm, $\varepsilon = 73{,}500\,M^{-1}cm^{-1}$, $\Phi = 0.85$) make it a potentially outstanding probe for cellular studies.

Confocal laser scanning microscopy was used to examine the subcellular distribution of rhodol **3** in the human cervical carcinoma cell line HeLa. As shown in Figure 3, these

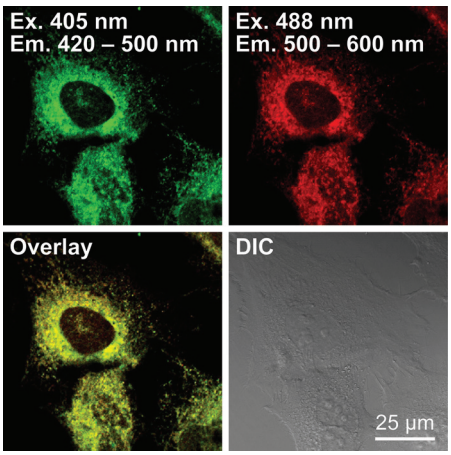


Figure 3. Confocal laser scanning and differential interference contrast (DIC) micrographs of living HeLa cells treated with ER-tracker Blue-White DPX (0.1 μM, 0.5 h) and rhodol **3** (0.5 μM, 0.5 h). The fluorescence emission of ER-tracker Blue-White DPX can be observed in green in the upper left panel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

experiments revealed the 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 in cells treated with rhodol nitrofuran **4**, thus indicating that the ER-targeting ability of the rhodol was unaffected by attachment of the warhead (see Figure S2 in the Supporting Information). To examine the cytotoxicities of eeyarestatin I (**1**), the rhodol derivatives **4** and **9**, and the untargeted nitrofuran **12**, HeLa cells were treated with these compounds for 48 h and cellular viability was measured by flow cytometry. The rhodol nitrofuran **4** ($IC_{50} = 2.7$ μM) and eeyarestatin I (**1**, $IC_{50} = 2.0$ μM) were similar in potency, thus suggesting that the ER-targeting motifs of both of these compounds are effective at delivering the nitrofuran to this organelle (Figure 4). As controls, rhodol hydrazide **9**, which lacks the cytotoxic warhead, showed no appreciable toxicity ($IC_{50} >$ 50 μM), whereas the nitrofuran hydrazone warhead **12** alone was less toxic ($IC_{50} = 13$ μM) than the ER-targeted compounds.

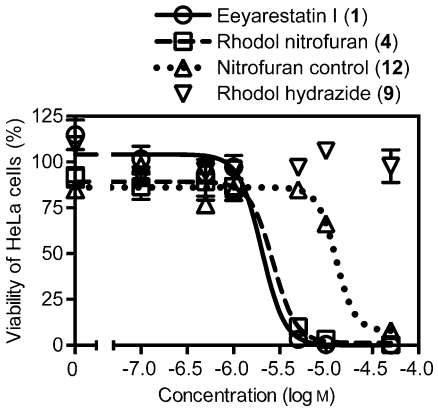


Figure 4. Cytotoxicity of ER-targeted and untargeted compounds towards HeLa cells after 48 h in culture. The half maximal inhibitory concentration (IC_{50}) values for **1**, **4**, and **12** are 2.0 μM, 2.7 μM, and 13 μM, respectively.

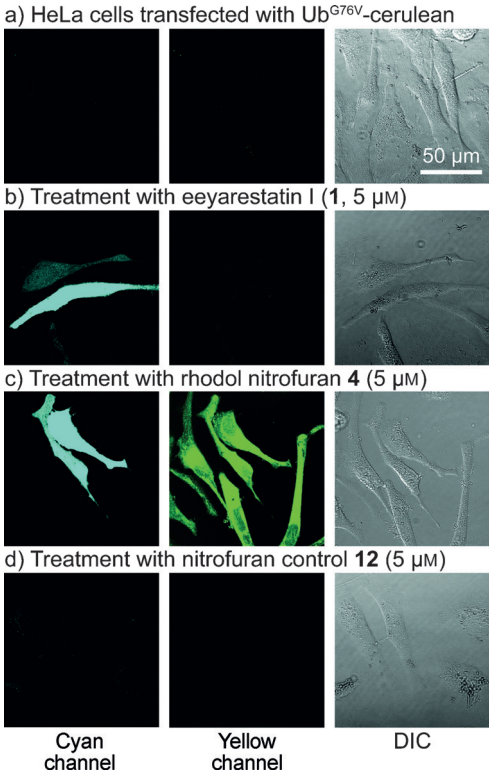


Figure 5. Confocal laser scanning and DIC micrographs of living HeLa cells transiently transfected with the cyan fluorescent protein Ub^{G76V}-cerulean. The spectrally orthogonal rhodol nitrofuran **4** was observed in the yellow channel. Cells were treated with the compounds for 12 h prior to imaging.

Eeyarestatin I 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,[41] prevent the degradation of these reporter proteins and restore cellular fluorescence.

By targeting the nitrofuran to the ER, we hypothesized that the rhodol nitrofuran **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, which is 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**, 5 μM), transfected cells but not the adjacent non-transfected cells became highly cyan fluorescent. Similarly, when treated with rhodol nitrofuran **4** (5 μM), cerulean was highly expressed by the transfected cells, thus demonstrating that this compound is a specific inhibitor of the UPS. By contrast, the nitrofuran 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 herein demonstrate that fluorinated hydrophobic rhodols are outstanding fluorophores that can be used to deliver small-molecule cargo to the ER. When this cargo was an electrophilic nitrofuran 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 the imaging and modulation of biological pathways associated with this critical organelle.

Keywords: chemical biology · cytotoxins · endoplasmic reticulum · fluorophores · proteasome

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