Published in final edited form as:

Dyes Pigm. 2016 December ; 135: 127–133. doi:10.1016/j.dyepig.2016.05.007.

Hydrophobic resorufamine derivatives: potent and selective red fluorescent probes of the endoplasmic reticulum of mammalian cells

Sahishna Phaniraj, Zhe Gao, Digamber Rane, and Blake R. Peterson*

Department of Medicinal Chemistry, The University of Kansas, Lawrence, KS 66045

Abstract

The endoplasmic reticulum (ER) of eukaryotic cells plays critical roles in the processing of secreted and transmembrane proteins. Defects in these functions are associated with a wide range of pathologies. To image this organelle, cells are often treated with fluorescent ER-Tracker dyes. Although these compounds are selective, existing red fluorescent probes of the ER are costly glibenclamide derivatives that inhibit ER-associated sulphonylurea receptors. To provide simpler and more cost-effective red fluorescent probes of the ER, we synthesized amino analogues of the fluorophore resorufin. By varying the polarity of linked substituents, we identified hexyl resorufamine (HRA) as a novel hydrophobic (cLogD (pH 7.4) = 3.8) red fluorescent (Ex. 565 nm; Em. 614 nm in ethanol) molecular probe. HRA is exceptionally bright in organic solvents (quantum yield = 0.70), it exclusively localizes to the ER of living HeLa cells as imaged by confocal microscopy, it is effective at concentrations as low as 100 nM, and it is non-toxic under these conditions. To examine its utility, we used HRA to facilitate visualization of small molecule-mediated release of a GFP-GPI fusion protein from the ER into the secretory pathway. HRA represents a potent, selective, and cost-effective probe for imaging and labeling the ER.

Graphical abstract

*Corresponding author. brpeters@ku.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The endoplasmic reticulum (ER) generally contributes at least half of all membranes of animal cells. Processes essential for cellular maintenance and survival occur on and in this organelle. The surface of the ER captures ribosomes involved in translation of membrane-bound and secreted proteins, whereas the single internal space, termed 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 such as hypoxia, oxidants or reductants, glucose deprivation, altered calcium regulation, viral infection, and expression of aberrant proteins can cause unfolded proteins to accumulate in the ER.² The resulting stress causes pathologies including neurodegenerative disease, stroke, heart disease, diabetes, and cancer. Consequently, agents that modulate ER stress are of substantial therapeutic interest.^{3–6}

Fluorescent molecular probes^{7, 8} have been developed for imaging the ER in living mammalian cells. Small molecules that accumulate selectively in this organelle are generally amphipathic and moderately lipophilic.⁹ As shown in Figure 1, these compounds include ER Tracker Blue-White DPX (**1**, Ex. 374 nm; Em. 430–640 nm),¹⁰ ER Tracker Green (**2**, Ex. 504 nm; Em. 511 nm), ER Tracker Red (**3**, Ex. 587 nm; Em. 615 nm), BODIPY Nile Red (**4**, Ex. 490 nm; Em. 565–635 nm),¹¹ ER Thermo Yellow (**5**, Ex. 559 nm; Em. 581 nm),³⁶ and fluorinated hydrophobic rhodols (**6**, Ex. 512 nm; Em. 532 nm).¹² Some of these compounds are thought^{9, 13, 14} to selectively accumulate in the ER because of the unique cholesterol-poor lipid composition of ER membranes.¹⁵ Although ER membranes have a surface area comparable to or greater than the plasma membrane, only 0.5–1% of the total cellular cholesterol is found in the ER, compared with the 30–40% of the total cellular cholesterol contained in the plasma membrane.^{16,17} Membranes of the golgi apparatus, mitochondria, and lysosomes lie between these two extremes.^{15, 18} Other fluorescent ER probes, such as ER Tracker Green (**2**) and ER Tracker Red (**3**, Figure 1), link the BODIPY fluorophore to glibenclamide. This compound binds and inhibits sulphonylurea receptors of ATP-sensitive potassium channels that are abundant on the cytosolic face of ER membranes.¹⁹ A potential drawback of these glibenclamide derivatives is that binding to these channels may alter ER function by perturbing calcium homeostasis.²⁰ Other drawbacks of many commercially

available probes of the ER relate to their structural complexity and associated high cost, typically in the range of thousands of dollars per milligram.

We report here the synthesis of hexyl resorufamine (HRA, **7**) as a novel and highly specific ER-targeted molecular probe. This probe can be prepared in only two steps from the commercially available fluorophore resorufin. The design of HRA was inspired by our recent syntheses of structurally related fluorinated hydrophobic rhodols such as **6** that selectively accumulate in this organelle.¹² HRA represents a cost-effective, potent, non-toxic, red fluorescent molecular probe that can be useful for imaging of this critical organelle by fluorescence microscopy.

2. Experimental section

General

¹H NMR and ¹³C NMR spectra were acquired on Bruker DRX-400 or Avance AVIII 500 MHz instruments. ¹⁹F NMR was acquired on a Bruker Avance VIII HD 400 MHz instrument. Chemical shifts (δ) are reported in parts per million (ppm) and are referenced to the central solvent peaks of DMSO-*d*₆ (δ 2.50 (¹H), 39.52 (¹³C)) or methanol-*d*₄ (δ 3.31 (¹H), 49.0 (¹³C)). Coupling constants are in Hertz (Hz). Absorbance spectra were obtained on an Agilent 8452A diode array spectrometer. Fluorescence spectra were acquired with a Perkin-Elmer LS55 Fluorescence Spectrometer. High Resolution mass spectra were obtained at the Mass Spectrometry Laboratory at the University of Kansas on a Micromass LCT Premier time of flight mass spectrometer. All reactions were performed under an inert atmosphere (argon or nitrogen) in flame-dried or oven-dried glassware, or in a glass microwave vial (Biotage, LLC). All anhydrous solvents were either purchased from Sigma Aldrich or dried over a Glass Contour solvent system (Pure Process Technology, LLC). Thin-layer chromatography (TLC) was performed using commercial aluminum backed silica plates (TLC Silica gel 60 F254, Analytical Chromatography). Visualization was accomplished by UV irradiation. Flash chromatography was carried out on normal phase using silica gel (230–400 mesh) or by reverse phase on a Combiflash purification system (HP C18 gold column, 50 g). All reagents were purchased from TCI, Sigma Aldrich or Alfa Aesar. Molar extinction coefficients (ϵ) were calculated from Beer's Law plots of absorbance λ_{max} versus concentration (data shown in Figure S1). Linear least squares fitting of the data (including a zero intercept) was used to determine the slope (corresponding to ϵ). Values ($\text{M}^{-1} \text{cm}^{-1}$) were calculated as follows: Absorbance = ϵ [concentration (M)] L, where L = 1 cm. Relative quantum yields (Φ) were determined by the method of Williams,²¹ where the fluorophores were excited at 510 nm, and the integrated fluorescence emission (530 nm to 800 nm) was quantified (concentrations of 1 nM to 10 nM). Rhodamine 6G (Φ = 0.95 in ethanol) provided the standard (data shown in Figure S1).^{22–24}

3-Oxo-3H-phenoxazin-7-yl trifluoromethanesulfonate, resorufin triflate, **9**

Resorufin (**8**, 500 mg, 2.34 mmol) was dissolved in anhydrous DMF (12 mL) in a flask wrapped in aluminum foil to protect from light. This flask was cooled to 4 °C in an ice bath and treated with sodium hydride (118 mg, 2.81 mmol), added in a single portion. This mixture was stirred at 4 °C for 30 min. *N*-Phenyl-bis(trifluoromethanesulfonimide) (1.0 g,

2.81 mmol) was added, and the reaction mixture was stirred at 4 °C for 10 min. The reaction mixture was warmed to 22 °C (room temperature) and stirred at room temperature for 24 h. The reaction was monitored by TLC (eluent: ethyl acetate and hexanes). The reaction was quenched with aqueous HCl (1N, 20 mL) and extracted with ethyl acetate (150 mL). The organic layer was further washed with saturated aqueous NaCl (2 X 50 mL), dried over anhydrous MgSO₄, and concentrated to dryness. The crude reaction mixture was purified by silica gel chromatography (eluent: ethyl acetate/hexanes) to yield **9** as a yellow solid (488 mg, 60%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.00 (m, 1H), 7.84 (m, 1H), 7.60–7.53 (m, 2H), 6.87 (m, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆): δ 185.8, 149.9, 149.8, 149.2, 144.2, 135.2 (2 carbons) 132.9, 131.6, 118.6, 118.2 (q, *J* = 321.4 Hz, CF₃), 110.3, 106.4; ¹⁹F NMR (376 MHz, DMSO-*d*₆) δ -72.5; HRMS (ESI) *m/z* calcd for C₁₃H₇F₃NO₅S [M + H]⁺: 345.9997, found: 345.9996.

Procedure for Buchwald-Hartwig amination

Resorufin triflate (**9**, 1.0 equiv), Pd₂(dba)₃ (0.1 equiv), BINAP (0.12 equiv), cesium carbonate (2.5 equiv) and amine (1.2 equiv) were weighed in a flame-dried Ar-flushed microwave vial (5 mL) in a glove box. The vial was sealed and anhydrous 1,4-dioxane (0.05 mM) was added. The sealed vial was placed in an oil bath preheated to 100 °C and stirred for 2.5 h. Completion of reaction was confirmed by TLC. The vial was cooled to room temperature and quenched by addition of trifluoroacetic acid (5 equiv). The crude reaction mixture was subjected to reverse phase purification (gradient of acetonitrile/water both containing 0.1% TFA) to yield resorufamines as reddish-brown solids.

7-(Hexylamino)-3H-phenoxazin-3-one, HRA, **7**

Following the procedure for Buchwald-Hartwig amination, triflate **9** (34.5 mg, 0.1 mmol), Pd₂(dba)₃ (9.15 mg, 0.01 mmol), BINAP (7.47 mg, 0.012 mmol), cesium carbonate (81.45 mg, 0.25 mmol) and hexylamine (20 μL, 0.15 mmol) were weighed in a 5 mL microwave vial. 1,4-Dioxane (2 mL) was added and the reaction mixture was heated at 100 °C for 2.5 h to yield **7** (28 mg, 94%). ¹H NMR (400 MHz, methanol-*d*₄): δ 7.92 (d, *J* = 9.1 Hz, 1H), 7.79 (d, *J* = 9.5 Hz, 1H), 7.43–7.29 (m, 1H), 7.15 (dd, *J* = 9.1, 2.5 Hz, 1H), 7.04–6.90 (m, 2H), 3.62 (t, *J* = 7.2 Hz, 2H), 1.79 (p, *J* = 7.3 Hz, 2H), 1.59–1.23 (m, 6H), 1.02–0.84 (m, 3H); ¹³C NMR (126 MHz, methanol-*d*₄): δ 170.4, 162.5, 152.5, 149.2, 141.4, 136.5, 135.0, 133.3, 125.5, 120.6, 103.1, 96.1, 46.0, 32.6, 29.6, 27.7, 23.6, 14.3. HRMS (ESI) *m/z* calcd for C₁₈H₂₁N₂O₂ [M + H]⁺: 297.1603, found: 297.1604.

7-((2-Methoxyethyl)amino)-3H-phenoxazin-3-one, **10**

Following the procedure for Buchwald-Hartwig amination, triflate **9** (34.5 mg, 0.1 mmol), Pd₂(dba)₃ (9.15 mg, 0.01 mmol), BINAP (7.47 mg, 0.012 mmol), cesium carbonate (81.45 mg, 0.25 mmol) and 2-methoxyethylamine (13 μL, 0.15 mmol) were weighed in a 5 mL microwave vial. 1,4-Dioxane (2 mL) was added and the reaction mixture was heated at 100 °C for 2.5 h to yield **10** (24 mg, 88%). ¹H NMR (400 MHz, methanol-*d*₄): δ 7.87 (d, *J* = 9.1 Hz, 1H), 7.76 (d, *J* = 9.5 Hz, 1H), 7.38–7.29 (m, 1H), 7.15–7.09 (m, 1H), 6.98 (d, *J* = 2.4 Hz, 1H), 6.88 (q, *J* = 2.3, 1.9 Hz, 1H), 3.80–3.61 (m, 4H), 3.41 (s, 3H); ¹³C NMR (126 MHz, methanol-*d*₄) δ 170.3, 163.1, 152.4, 149.2, 141.4, 136.6, 135.0, 133.3, 125.5, 120.6,

103.1, 96.8, 71.0, 59.2, 45.9. HRMS (ESI) m/z calcd for $C_{15}H_{15}N_2O_3$ $[M + H]^+$: 271.1083, found: 271.1083.

Cell culture and confocal microscopy

HeLa cells, obtained from ATCC (CCL-2), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with FBS (10%), penicillin (100 units/L), and streptomycin (100 μ g/L). Cells were added to an 8-well cover glass slide (Ibidi μ -Slide, 300 μ L, 20,000 cells/well) and allowed to proliferate for 24 h prior to addition of compounds. Compounds in DMSO stock solutions were serially diluted 1000-fold with complete media (final concentration of 0.1% DMSO) prior to addition to cells. Cells were treated with compounds at 37 °C. Living cells were imaged, without any additional washing steps, using a Leica SPE2 confocal laser-scanning microscope fitted with a 63X objective. ER-Tracker Blue-White DPX (100 nM) was excited with a 405 nm laser and emitted photons were collected from 425 nm to 500 nm. Resorufamines were excited with a 532 nm laser and emitted photons were collected from 545 nm to 700 nm. EGFP was excited with a 488 nm laser and emitted photons were collected from 500–600 nm. To image the fixed cells shown in Figure S2 (panel B) of the supporting information, HeLa cells were treated with aqueous paraformaldehyde (4%) for 30 min, washed three times with PBS, further incubated for 15 min in PBS containing Triton X-100 (0.5%), washed an additional three times with PBS prior to addition of the fluorescent ER probes.

Transient transfection of HeLa cells

The plasmid Str-KDEL_SBP-EGFP-GPI²⁵ (pIRESneo3 backbone) was a gift from Franck Perez (Addgene Plasmid #65294). To form a DNA complex, this plasmid DNA (3 μ g) was incubated (30 min, room temperature) with the DNA transfection reagent X-tremeGENE HP (9 μ L, Roche) in serum free DMEM medium (300 μ L). HeLa cells in DMEM were seeded onto an 8-well cover glass slide (Ibidi μ Slide, 300 μ L, 10,000 cells/well). After incubation at 37 °C for 16 h, the DNA complex (30 μ L) was added to each well. After further incubation at 37 °C for 48 h, the cells were washed, further treated with probes, and analyzed by confocal microscopy.

Assays of cellular toxicity

HeLa cells were seeded on a 48-well plate in complete DMEM at 20000 cells/300 μ L/well 16 h prior to treatments. All compounds were serially diluted in DMSO and added to media to achieve a 1:1000 dilution factor (0.1% DMSO in each well). The original media was removed from all wells by aspiration and replaced with treatment media (330 μ L) at the concentrations indicated. Plates were incubated for 48 h at 37 °C and cells were analyzed in triplicate. Following this incubation period, the media was aspirated and wells were washed with PBS (phosphate-buffered saline, pH 7.4). Wells were further treated with trypsin EDTA solution (50 μ L) at 37 °C for 5 min followed by complete DMEM (100 μ L). The total cell count for each well was determined by flow cytometry (Accuri C6) using light scattering to identify populations of live cells. Counts of viable cells for each treatment were used to generate dose-response curves that were fitted by non-linear regression (log inhibitor vs. response variable slope 4-parameter model (bottom constraint < 10%, GraphPad Prism 6) to determine IC₅₀ values.

3. Results and discussion

To develop new molecular probes of the ER, we investigated the use of resorufin (**8**, Scheme 1) as a starting material. This compound has several attractive features as a building block: it is relatively inexpensive (~ \$60/g), red fluorescent (Ex. = 572 nm; Em. = 585 nm), and has a high extinction coefficient ($56,000 \text{ M}^{-1} \text{ cm}^{-1}$) and quantum yield (0.74) when deprotonated ($\text{pK}_a = 5.8$).²⁶ Moreover, O-alkyl derivatives of resorufin,^{27, 28} and the resorufin N-oxide termed resazurin (Alamar blue),²⁹ have been employed in a variety of bioassays.⁸ However, to our knowledge, only one analogue has been described in a peer-reviewed journal that replaces the phenol of resorufin with a nitrogen atom (in the form of the azide).³⁰ We previously used Buchwald-Hartwig amination chemistry to convert the fluorophore Pennsylvania Green^{31–33} to *N*-alkyl rhodols such as **6**, and we envisioned using a similar approach to access *N*-alkyl resorufamines for studies of the subcellular distribution of these structurally related compounds.

As shown in Scheme 1, resorufin (**8**) was converted to the triflate (**9**)³⁴ to provide a reactive building block. This triflate was used to prepare resorufamines by reaction with alkylamines under palladium-catalyzed conditions. Because ER Tracker Blue-White DPX is a highly hydrophobic ($\text{cLogD (pH 7.4)} = 4.0$) membrane-associated probe, similar in hydrophobicity to **6** ($\text{cLogD (pH 7.4)} = 3.9$), we reasoned that novel probes that bind ER membranes should be of comparable hydrophobicity. Additionally, we hypothesized that the inclusion of straight chain alkanes might favor interactions with phospho-lipids that are prevalent in ER membranes. Correspondingly, a hydrophobic hexyl substituent was installed to provide the similarly hydrophobic hexyl resorufamine (HRA, $\text{cLogD (pH 7.4)} = 3.8$). As a control, by installing the same 2-methoxyethyl substituent found in **6**, we also prepared the structurally related 2-methoxyethyl resorufamine (**10**), but this compound was predicted to be substantially more polar ($\text{cLogD (10, pH 7.4)} = 1.5$).

Spectroscopic studies of the resorufamines HRA and **10** revealed high fluorescence in organic solvents such as ethanol and octanol (Figure 2). However, their fluorescence was substantially weaker in aqueous buffers, particularly with HRA, which showed a very broad absorbance spectrum in phosphate buffered saline (PBS), suggesting aggregation of this more hydrophobic fluorophore in aqueous solution (Figure 2, panel A). Measurement of extinction coefficients, and quantum yields relative²¹ to rhodamine 6G (data shown in Figure S1 of the supporting information), revealed that both HRA (Abs. $\lambda_{\text{max}} = 565 \text{ nm}$; Ex. $\lambda_{\text{max}} = 614 \text{ nm}$, $\epsilon = 34,300 \text{ M}^{-1} \text{ cm}^{-1}$, $\Phi = 0.70$, in ethanol) and **10** (Abs. $\lambda_{\text{max}} = 565 \text{ nm}$; Ex. $\lambda_{\text{max}} = 605 \text{ nm}$, $\epsilon = 31,600 \text{ M}^{-1} \text{ cm}^{-1}$, $\Phi = 0.63$, in ethanol) exhibit excellent spectral properties for fluorescence microscopy, and since their emission is red-shifted compared to resorufin, they should be highly orthogonal to many blue and green fluorophores commonly used to study cellular biology.

To examine effects on living cells, the human cervical carcinoma cell line HeLa was treated with HRA and **10** for 30 min, followed by imaging by confocal laser scanning microscopy. As shown in Figure 3, these experiments revealed accumulation of both of these compounds in tubular subcellular structures. However, probe **10** required at least a 10-fold higher concentration than HRA to generate comparable cellular fluorescence, and consistent with

its greater fluorescence in PBS, the background signal in media was much higher for **10** than HRA. In contrast, even at concentrations as low as 100 nM, HRA exhibited substantially higher fluorescence in these compartments, and its low background fluorescence in aqueous media obviated the need to wash cells prior to imaging. The tubular structures labeled by these compounds were identified as the ER by colocalization with the spectrally orthogonal organelle marker ER Tracker Blue-White DPX.¹⁰ These studies demonstrated that HRA has particular promise as a novel molecular probe of the ER, since it specifically accumulates in this organelle in living cells with high potency comparable to ER Tracker Blue-White DPX. Moreover, in fixed HeLa cells, HRA was superior to ER Tracker Blue-White DPX as a fluorescent probe (images shown in Figure S2, supporting information). For fixation, cells were treated with paraformaldehyde, followed by dilute Triton X-100. Under these conditions, HRA labeled putative ER membranes of fixed cells to a much greater extent than ER Tracker Blue White DPX. This compatibility of HRA with cellular fixation protocols suggests another potential advantage of this novel fluorescent probe as a tool for studies of the ER.

As shown in Figure 4, we further examined the cytotoxicities of HRA and **10** towards HeLa cells treated with these compounds for 48 h. As a toxic positive control, the tubulin-binding plant natural product colchicine³⁵ was used, with cellular viability measured by flow cytometry. As expected, colchicine was highly toxic (IC_{50} = 6 nM) under these conditions. Interestingly probe **10** (IC_{50} = 1 μ M) was at least 10-fold more toxic than HRA (IC_{50} > 10 μ M), illustrating another potential advantage of HRA over **10** as a molecular probe of the ER. Beneficially, even after continuous treatment for 48 h at concentrations (100 nM) that can be used to image the ER by confocal microscopy, HRA was non-toxic.

To further explore HRA as a probe of the ER, we investigated its utility as a counterstain for cells expressing enhanced green fluorescent protein (EGFP). To express EGFP in HeLa cells, we transiently transfected this cell line with a previously reported²⁵ plasmid construct termed Str-KDEL_SBP-EGFP-GPI that can be used to synchronize secretory protein traffic in populations of cells. This construct encodes two different proteins bearing signal peptides that direct them into the ER: one is the protein streptavidin (Str) fused to the ER retention signal sequence KDEL; the second is a streptavidin binding peptide (SBP) fused to EGFP linked to a C-terminal peptide that will be modified with a GPI membrane anchor. If expressed alone, the signal sequence of the SBP-EGFP-GPI protein will cause its translocation into the ER for eventual secretion to the plasma membrane, where the attached GPI lipids will result in localization of EGFP on the cell surface. However, coexpression with the Str-KDEL protein in the ER results in binding of Str to SBP, which traps the EGFP fusion protein in this organelle. Release of the EGFP fusion protein from the ER-localized Str protein can be accomplished by addition of the small molecule biotin to the cell culture media as a competitor. As shown in Figure 5, we found that transient transfection of HeLa cells with Str-KDEL_SBP-EGFP-GPI resulted in green fluorescence predominantly localized to the ER as imaged by colocalization with HRA. Moreover, addition of biotin to these transfected cells for 1 h triggered secretion of EGFP from the ER to the cellular plasma membrane, which could be observed as a substantial change in protein subcellular localization (Figure 5, compare panels A and B). These experiments confirmed that HRA is

spectrally orthogonal to EGFP for studies by confocal microscopy, and it can be used as an ER counterstain in these types of experiments. Consequently, HRA may be useful for image-based screens for molecules that affect protein transport to and from the ER.

4. Conclusion

We synthesized and investigated *N*-alkyl resorufamines as a novel fluorescent chemotype. These compounds were designed based on structural similarity to fluorinated hydrophobic rhodol fluorophores¹² that are known to accumulate in the ER of mammalian cells. Two resorufamines bearing hexyl (HRA) and 2-methoxyethyl (**10**) substituents could be readily prepared in only two steps from the commercially available fluorophore resorufin, making them highly cost-effective molecular probes. The fluorescence emission of these compounds is red-shifted compared to resorufin, making them spectrally orthogonal to many blue and green fluorophores, but they retain high quantum yields. Although both HRA and **10** accumulate in the ER of HeLa cells, HRA proved to be a superior molecular probe, with low background cellular fluorescence, high potency, and low toxicity. These advantages of HRA are correlated with its higher hydrophobicity, as evidenced by its calculated (ChemAxon software) octanol-water distribution coefficient (cLogD, pH 7.4) of 3.8. This high hydrophobicity is very similar to ER-associated rhodol **6** (cLogD (pH 7.4) = 3.9) and ER Tracker Blue-White DPX (cLogD (pH 7.4) = 4.0). In contrast the calculated distribution coefficient of resorufamine **10** (cLogD (pH 7.4)) is 1.5, indicating substantially greater polarity, which limits its association with membranes and increases its background fluorescence in aqueous media. These differences in polarity are also evident in major changes in the absorbance spectra of HRA in organic solvents compared with PBS, where a substantial broadening, likely due to aggregation, is observed. Taken together, the cellular and physicochemical properties of HRA are consistent with its association with the unique membranes of the ER. Consequently, its mechanism of subcellular localization is likely to be similar to that of other hydrophobic probes such as ER Tracker Blue-White DPX. However, when compared with ER Tracker Blue-White DPX, HRA exhibited better cellular staining of cells that were fixed with paraformaldehyde and Triton X-100, suggesting it may have a broader range of potential applications. Based on its favorable photophysical properties, low cost of production, high potency, and low toxicity, HRA has potential as an important new red fluorescent molecular probe of the ER.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the NIH (P20-GM103638) and the KU Cancer Center for financial support. We thank the KU Molecular Probes Core laboratory for assistance with chemical synthesis.

References

1. Vance JE. Phospholipid Synthesis and Transport in Mammalian Cells. *Traffic*. 2015; 16:1–18. [PubMed: 25243850]

2. Sovolyova N, Healy S, Samali A, Logue SE. Stressed to death - mechanisms of ER stress-induced cell death. *Biol Chem.* 2014; 395:1–13. [PubMed: 24002662]
3. Deshaies RJ. Proteotoxic crisis, the ubiquitin-proteasome system, and cancer therapy. *BMC Biol.* 2014; 12:94. [PubMed: 25385277]
4. Shore GC, Papa FR, Oakes SA. Signaling cell death from the endoplasmic reticulum stress response. *Curr Opin Cell Biol.* 2011; 23:143–149. [PubMed: 21146390]
5. Kim I, Xu W, Reed JC. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Disc.* 2008; 7:1013–1030.
6. Lin JH, Walter P, Yen TS. Endoplasmic reticulum stress in disease pathogenesis. *Annu Rev Pathol.* 2008; 3:399–425. [PubMed: 18039139]
7. Lavis LD, Raines RT. Bright building blocks for chemical biology. *ACS Chem Biol.* 2014; 9:855–866. [PubMed: 24579725]
8. Lavis LD, Raines RT. Bright ideas for chemical biology. *ACS Chem Biol.* 2008; 3:142–155. [PubMed: 18355003]
9. Colston J, Horobin R, Rashid-Doubell F, Pediani J, Johal KK. Why fluorescent probes for endoplasmic reticulum are selective: an experimental and QSAR-modelling study. *Biotech Histochem.* 2003; 78:323–332. [PubMed: 15473580]
10. Cole L, Davies D, Hyde J, Ashford AE. ER-Tracker dye and BODIPY-brefeldin A differentiate the endoplasmic reticulum and Golgi bodies from the tubular-vacuole system in living hyphae of *Pisolithus tinctorius*. *J Microscopy.* 2000; 197:239–249.
11. Yang Z, He Y, Lee JH, Chae WS, Ren WX, Lee JH, Kang C, Kim JS. A Nile Red/BODIPY-based bimodal probe sensitive to changes in the micropolarity and microviscosity of the endoplasmic reticulum. *Chem Comm.* 2014; 50:11672–11675. [PubMed: 25140835]
12. Meinig JM, Fu L, Peterson BR. Synthesis of Fluorophores that Target Small Molecules to the Endoplasmic Reticulum of Living Mammalian Cells. *Angew Chem Int Ed Engl.* 2015; 54:9696–9699. [PubMed: 26118368]
13. Terasaki M, Song J, Wong JR, Weiss MJ, Chen LB. Localization of endoplasmic reticulum in living and glutaraldehyde-fixed cells with fluorescent dyes. *Cell.* 1984; 38:101–108. [PubMed: 6432338]
14. Horobin R, Stockert J, Rashid-Doubell F. Uptake and localisation of small-molecule fluorescent probes in living cells: a critical appraisal of QSAR models and a case study concerning probes for DNA and RNA. *Histochem Cell Biol.* 2013; 139:623–637. [PubMed: 23542926]
15. van Meer G, de Kroon AIPM. Lipid map of the mammalian cell. *J Cell Sci.* 2011; 124:5–8. [PubMed: 21172818]
16. Lange Y, Ye J, Rigney M, Steck TL. Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol. *J Lipid Res.* 1999; 40:2264–2270. [PubMed: 10588952]
17. Maxfield FR, Mesmin B, Pipalia N, Lamlal T, Eliezer D. Intracellular cholesterol transport. *Faseb J.* 2011; 25
18. van Meer G, Voelker DR, Feigenson GW. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol.* 2008; 9:112–124. [PubMed: 18216768]
19. Zhou M, He HJ, Suzuki R, Liu KX, Tanaka O, Sekiguchi M, Itoh H, Kawahara K, Abe H. Localization of Sulfonylurea Receptor Subunits, SUR2A and SUR2B, in Rat Heart. *J Histochem Cytochem.* 2007; 55:795–804. [PubMed: 17438353]
20. Nelson TY, Gaines KL, Rajan AS, Berg M, Boyd AE. Increased cytosolic calcium. A signal for sulfonylurea-stimulated insulin release from beta cells. *J Biol Chem.* 1987; 262:2608–2612. [PubMed: 3029089]
21. Williams AT, Winfield SA. Relative Fluorescence Quantum Yields Using a Computer-controlled Luminescence Spectrometer. *Analyst.* 1983; 108:1067–1071.
22. Velapoldi RA, Tonnesen HH. Corrected Emission Spectra and Quantum Yields for a Series of Fluorescent Compounds in the Visible Spectral Region. *J Fluorescence.* 2004; 14:465–472.
23. Kubin RF, Fletcher AN. Fluorescence Quantum Yields of Some Rhodamine Dyes. *J Luminescence.* 1982; 27:455–462.

24. Snare MJ, Treloar FE, Ghiggino KP, Thistlethwaite PJ. The Photophysics of Rhodamine-B. *J Photochem.* 1982; 18:335–346.
25. Boncompain G, Divoux S, Gareil N, de Forges H, Lescure A, Latreche L, Mercanti V, Jollivet F, Raposo G, Perez F. Synchronization of secretory protein traffic in populations of cells. *Nat Methods.* 2012; 9:493–498. [PubMed: 22406856]
26. Bueno C, Villegas ML, Bertolotti SG, Previtali CM, Neumann MG, Encinas MV. The excited-state interaction of resazurin and resorufin with amines in aqueous solutions. Photophysics and photochemical reactions. *Photochem Photobiol.* 2002; 76:385–390. [PubMed: 12405144]
27. Burke MD, Thompson S, Weaver RJ, Wolf CR, Mayer RT. Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver. *Biochem Pharmacol.* 1994; 48:923–936. [PubMed: 8093105]
28. Kim SY, Hong JI. Chromogenic and fluorescent chemodosimeter for detection of fluoride in aqueous solution. *Org Lett.* 2007; 9:3109–3112. [PubMed: 17629289]
29. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem.* 2000; 267:5421–5426. [PubMed: 10951200]
30. Chen B, Lv C, Tang X. Chemoselective reduction-based fluorescence probe for detection of hydrogen sulfide in living cells. *Anal Bioanal Chem.* 2012; 404:1919–1923. [PubMed: 22868476]
31. Mottram LF, Boonyarattanakalin S, Kovel RE, Peterson BR. The Pennsylvania Green Fluorophore: A Hybrid of Oregon Green and Tokyo Green for the Construction of Hydrophobic and pH-Insensitive Molecular Probes. *Org Lett.* 2006; 8:581–584. [PubMed: 16468716]
32. Mottram LF, Maddox E, Schwab M, Beaufils F, Peterson BR. A concise synthesis of the Pennsylvania Green fluorophore and labeling of intracellular targets with O6-benzylguanine derivatives. *Org Lett.* 2007; 9:3741–3744. [PubMed: 17705395]
33. Woydziak ZR, Fu L, Peterson BR. Efficient and Scalable Synthesis of 4-Carboxy-Pennsylvania Green Methyl Ester: A Hydrophobic Building Block for Fluorescent Molecular Probes. *Synthesis-Stuttgart.* 2014; 46:158–164. [PubMed: 24914246]
34. Miller EW, Tulyathan O, Isacoff EY, Chang CJ. Molecular imaging of hydrogen peroxide produced for cell signaling. *Nat Chem Biol.* 2007; 3:263–267. [PubMed: 17401379]
35. Finkelstein Y, Aks SE, Hutson JR, Juurlink DN, Nguyen P, Dubnov-Raz G, Pollak U, Koren G, Bentur Y. Colchicine poisoning: the dark side of an ancient drug. *Clin Toxicol.* 2010; 48:407–414.
36. Arai S, Lee SC, Zhai D, Suzuki M, Chang YT. A molecular fluorescent probe for targeted visualization of temperature at the endoplasmic reticulum. *Sci Rep.* 2014; 4:6701. [PubMed: 25330751]

Appendix. Supporting information

Additional supporting figures and NMR spectra can be found online.

Highlights

- Resorufin was used to synthesize resorufamines as a novel red fluorescent chemotype
- The resorufamine HRA localizes in the endoplasmic reticulum (ER) of mammalian cells
- HRA is a potent, selective, non-toxic, and cost-effective fluorescent probe of the ER

Figure 1.

Structures of fluorescent molecular probes that selectively accumulate in the ER of mammalian cells. Probes **1–6** have been previously reported. Hexyl resorufamine (HRA, **7**) is a novel fluorescent chemotype.

Figure 2.

Photophysical properties and spectra of the resorufamines HRA (panel A) and **10** (panel B) in aqueous phosphate-buffered saline (PBS, pH 7.4), ethanol (EtOH), and octanol (Oct.). Absorbance (Abs.) spectra were acquired at 10 μ M (1% DMSO). Fluorescence emission (Em.) spectra were acquired at 10 nM (Ex. 510 nm, 0.1% DMSO), with intensities normalized to the maximal fluorescence observed in ethanol.

(A) Treatment with HRA (100 nM) and ER Tracker Blue White DPX (100 nM)

(B) Treatment with **10** (1 μ M) and ER Tracker Blue White DPX (100 nM)

Figure 3.

Confocal laser scanning and differential interference contrast (DIC) micrographs of living HeLa cells treated with ER-tracker Blue-White DPX (100 nM, 0.5 h) and HRA (Panel A, 100 nM, 0.5 h) or **10** (Panel B, 1 μ M, 0.5 h). The fluorescence emission of ER-tracker Blue-White DPX can be observed in the upper left panels and the fluorescence emission of the spectrally orthogonal resorufamines can be observed in the upper right panels. Colocalization of the fluorophores is shown in yellow in the lower left panels. Scale bar = 25 microns.

Figure 4. Cytotoxicity of compounds towards HeLa cells, after 48 h in culture, as analyzed by flow cytometry.

(A) HeLa cells transfected with Str-KDEL / SBP-EGFP-GPI and treated (1 h) with HRA (100 nM)

(B) HeLa cells transfected with Str-KDEL / SBP-EGFP-GPI and treated (1 h) with HRA (100 nM) / Biotin (40 μ M)

Figure 5.

Panels A–B. Confocal laser scanning and differential interference contrast (DIC) micrographs of living HeLa cells. Cells were transiently transfected with a plasmid encoding both the Str-KDEL and SBP-EGFP-GPI proteins and treated with HRA (100 nM, 1 h) as a red fluorescent ER marker. In Panel B, the small molecule biotin (40 μ M) was additionally added for 1 h to release SBP-EGFP-GPI from the ER into the secretory pathway. The fluorescence emission of EGFP can be observed in the upper left panel and the fluorescence emission of the spectrally orthogonal HRA can be observed in the upper right panel. Colocalization of fluorophores is shown in yellow in the lower left panel. Scale bar = 25 microns.

Scheme 1.

The structure of resorufin and synthesis of resorufamine derivatives.