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Visualization of Peroxynitrite-Induced Changes of Labile Zn$^{2+}$ in the Endoplasmic Reticulum with Benzoresorufin-based Fluorescent Probes

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KEYWORDS: labile zinc, zinc fluorescent sensor, unfolded protein response, fluorescence imaging, benzoresorufin.

ABSTRACT: Zn$^{2+}$ plays essential roles in biology, and the homeostasis of Zn$^{2+}$ is tightly regulated in all cells. Subcellular distribution and trafficking of labile Zn$^{2+}$, and its interrelation with reactive nitrogen species, are poorly understood due to the scarcity of appropriate imaging tools. We report a new family of red-emitting fluorescent sensors for labile Zn$^{2+}$, ZBR1-3, based on a benzoresorufin platform functionalized with dipicolylamine or picolylamine-derived metal binding groups. In combination, the pendant amines and fluorophore afford an [N,O] binding motif that resembles that of previously reported fluorescein-based sensors of the Zinpyr family, reproducing well their binding capabilities and yielding comparable $K_d$ values in the subnanomolar and picomolar range. The ZBR sensors display up to 8.4-fold emission fluorescence enhancement upon Zn$^{2+}$ binding in the cuvette, with similar responses obtained in live cells using standard wide-field fluorescence microscopy imaging. The new sensors localize spontaneously in the endoplasmic reticulum (ER) of various tested cell lines, allowing for organelle-specific monitoring of zinc levels in live cells. Study of ER zinc levels in neural stem cells (NSC) treated with a peroxynitrite generator, Sin-1, revealed an immediate decrease in labile Zn$^{2+}$ thus providing evidence for a direct connection between ER stress and ER Zn$^{2+}$ homeostasis.

Introduction

Zinc is a d-block element essential for all living organisms.$^{1,2}$ Zn$^{2+}$ ions play various key roles in biological systems including enzymatic catalysis, stabilization of protein structures and modulation of interactions between macromolecules.$^3$ The percentage of genes coding for zinc-bound proteins is estimated to be 10%, consistent with the value found in the known proteome.$^4,5$ The total concentration of Zn$^{2+}$ in mammalian cells is estimated to be in the range of 100 to 500 µM$^6$ the largest fraction is tightly bound to metalloproteins, whereas a smaller portion is loosely bound to various readily exchangeable molecules.$^5$ Cytosolic pools of labile Zn$^{2+}$ participate in signaling pathways associated with different physiological and pathological events.$^4,6$ The intracellular sources of these pools of exchangeable zinc, however, remain unclear in many systems.

Zinc and reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite (ONOO$^-$) have physiological roles in the nervous system and are implicated in neurological dysfunction.$^{7,13}$ Exposure of neurons to high concentrations of exogenous NO leads to the formation of peroxynitrite and causes Zn$^{2+}$ release from intracellular stores. In turn, higher concentrations of labile Zn$^{2+}$ induce mitochondrial dysfunction and increased production of reactive oxygen species, ROS, thus resulting in amplification of the apoptotic signaling pathway contributing to neurodegeneration.$^{12}$

High concentrations of NO and peroxynitrite also have an effect on the endoplasmic reticulum (ER), inducing ER stress and activating a cellular stress response named unfolded protein response (UPR).$^{14,15}$ A number of neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, prion disease, Huntington’s disease, frontotemporal dementia, amyotrophic lateral sclerosis, and Ehlers–Danlos syndrome are all characterized by the accumulation and aggregation of misfolded proteins in the ER.$^{16,17}$ Previous studies demonstrated that deletion of zinc transporters and zinc deficiency in the ER up-regulates the UPR.$^{18,19}$

The temporal, spatial, and functional characteristics of the interplay between RNS, ROS, and labile Zn$^{2+}$ in neurodegeneration are still far from clear. New approaches are needed to track the source and destination of these species in live cells and tissues. In particular, convenient tools that allow for easy visualization of the fluctuations of labile Zn$^{2+}$ in specific organelles and its translocation to or from the cytosol may shed some light on the role of Zn$^{2+}$ in these pathologies. Neural stem cells (NSC) are a class of cells that can proliferate through symmetric division and differentiate into neurons and glial cells through asymmetric division.$^{20}$ Owing to their ability to self-renew and produce neurons and glial cells, NSCs are a powerful resource to study synaptic plasticity and neuronal disorders.$^{21-28}$ In this work we employ NSCs to investigate the interrelation between RNS and zinc homeostasis with potential implications for understanding neurodegenerative processes.

Over the last two decades, our group has developed a large number of fluorescein-based fluorescent sensors for mobile zinc with apparent dissociation constants spanning the range from sub-nanomolar to sub-millimolar.$^{27}$ Among these, the Zinpyr$^{27}$ (ZP) family is a series of probes containing dipicolylamine (DPA) or DPA-analog metal binding moieties with dissociation constants in the nanomolar range.$^{27,29,30}$ which have proved to be particularly useful for the imaging of Zn$^{2+}$ distribution, uptake, and translocation in different types of cells.$^{23,24,34}$ Fluorescein-based sensors, however, are limited by their high energy absorptions and small Stokes shifts. Cellular auto-fluorescence in the corresponding spectral window leads to a high background signal that reduces the distinguishable fluorescence turn-on of probes associated with Zn$^{2+}$ binding, compared to the response obtained in the cuvette. For biological imaging, sensors emitting in the red or near infrared region are thus desirable.

In our continuing effort to improve the tools available for the study of labile Zn$^{2+}$ in memory formation and neurodegeneration, we herein report the design of a new series of probes based on a benzoresorufin fluorophore with a DPA or DPA-analog metal binding group. The resulting red-emitting probes display a conserved [N,O] zinc-binding motif similar to that offered by ZP analogues (Fig. 1), which endows them with similar dissociation constants to their green-emitting counterparts. We employed the new probes to study zinc homeostasis in NSCs and demonstrate that the peroxynitrite-induced changes of labile Zn$^{2+}$ in the endoplasmic reticulum with benzoresorufin-based fluorescent probes. 
material and methods

Synthetic Materials and Methods. All synthetic procedures were performed under a nitrogen atmosphere unless otherwise specified. 4-Chloro-2-methylresorcinol, 6-bromo-8-(bromomethyl)-5-oxo-benzo[a]phenoxazin-9-yl acetate and (2-picolyl)-(pyrazin-2-yl)methyaline were synthesized according to reported procedures. All other reagents were purchased from commercial sources and used as received. Solvents were purified and degassed by standard procedures. NMR spectra were acquired on Bruker Avance 400 spectrometers. 1H NMR chemical shifts are reported in ppm relative to SiMe4 (δ = 0) and were referenced internally with respect to residual protons in the solvent (δ = 7.26 for CDCl3, 3.31 for CD2DOD, 2.50 for DMSO-d6). 13C NMR chemical shifts are reported in ppm relative to SiMe4 (δ = 0) and were referenced internally with respect to the solvent signal (δ = 77.16 for CDCl3, 49.00 for CD3OD, 39.52 for DMSO-d6). Low-resolution mass spectra were acquired on an Agilent 1100 Series LC/MSD Trap spectrometer, using electrospray ionization. High-resolution mass spectrometry (HRMS) was conducted by staff at the MIT Department of Chemistry Instrumentation Facility on a Bruker Daltonics APEXIV 4.7 Tesla FT-ICR-MS.

Synthesis of 4-Chloro-6-nitroso-2-methylresorcinol, 1b. A solution of 4-chloro-6-nitrosoresorcinol (2.00 g, 12.6 mmol) in ethanol (12.5 mL) was cooled to 0 °C and treated with a solution of KOH (0.991 g, 17.6 mmol) in water (4.2 mL), followed by isomyl nitrite (2.0 mL, 14.9 mmol) added dropwise. After addition was complete, the solution was allowed to warm to room temperature and stirred for 1.5 h. The mixture was acidified to pH ~2 by addition of 1 M HCl and stirring was continued at room temperature, open to air, for 1 h. The yellow precipitate was collected by filtration, washed with a 1:1 mixture of ethanol:water (2x10 mL) followed by water (30 mL), and dried under vacuum to give 4-chloro-6-nitroso-methylresorcinol as a bright yellow powder (1.95 g, 82%). 1H NMR (400 MHz, MeOH-d4, room temperature): δ 7.70 (s, 1H), 1.89 (s, 3H). 13C(1H) NMR (100.6 MHz, MeOH-d4) 181.8, 158.5, 144.8, 137.8, 119.3, 115.2, 8.4. ESI-MS (m/z): [M+H+] calcd for C10H8NO3, 188.0; found 188.1.

Synthesis of 10-Chloro-9-hydroxy-8-methyl-5-benzo[a]phenoxazone, 2b. A suspension of 4-chloro-6-nitroso-2-methylresorcinol (0.750 g, 4.00 mmol) in n-butanol (7.5 mL) was mixed with a solution of 1,3-dihydroxynaphthalene (0.640 g, 4.00 mmol) in n-butanol (7.5 mL) and heated to 50 °C. Concentrated sulfuric acid (1.5 mL) was added dropwise to the warm reaction mixture open to air, and heating was continued for additional 1 h. The suspension was then allowed to stand at room temperature overnight. The solid was collected by centrifugation and the pellet was washed with a 1:1 mixture ethanol: n-butanol (2x10 mL), followed by 1:1 ethanol:water (5 mL), and dried under vacuum to give resorufin 2b as a brown-red powder (1.03 g, 80% yield). The crude product was used in the next step without further purification.
measurements were conducted in aqueous buffer containing 50 mM PIPES (pH 7.0) and 100 mM KCl except the pKᵢ titrations. The buffer solutions were pretreated with Chelex resin (Bio-Rad) to remove residual metal ion in the solution. The pH measurements were made using a Mettler Toledo FE20 pH meter. UV-visible spectra were acquired on a Cary 1E spectrophotometer using quartz cuvettes (1 cm path length). Fluorescence spectra were acquired on a QuantaMaster 4 Photon Technology International fluorometer. All measurements were conducted at 25.0°C, maintained by a circulating water bath. Extinction coefficients were determined in the 1-10 µM range in buffer solutions at pH 7.0 with the presence of 10 µM EDTA for the metal-free form or with 20 µM ZnCl₂ for the metal-bound form of the sensor. Fluorescence quantum yields were determined using 1-5 µM sensor in buffer solution at pH 7.0, exciting at the maximum excitation of the corresponding sensor. Due to poor solubility in aqueous solution, the extinction coefficients and quantum yield of ZBR3 were obtained in the 0.4-2 µM range. The quantum yield calculation was standardized to resorufin, with a reported quantum yield of 0.74 at pH 9.5 at the excitation of 572 nm. Fluorescence emission spectra were integrated from 550 to 800 nm.

**Cell Cultures and Staining Procedures.** HeLa and macrophage 246.7 cells were incubated in Dulbecco’s Modified Eagle Medium (DMEM, Gibco), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂. NSCs were originally isolated from the hippocampus of adult rats as previously described, and they were maintained in DMEM/F12 medium (GIBCO) with 20 ng/ml FGF2 (Pepro Tech) and N: supplement (GIBCO) at 37°C in a humidified atmosphere with 5% CO₂. Cells were plated in 35 mm glass-bottom culture dishes with 14 mm opening (MatTek) 24-48 h before imaging. All the cells used were at the passage number from 5 to 15 and experiments were repeated using a minimum of two separate frozen cell stocks. A confluence level of 50-70% was reached at imaging. Cells were incubated with sensors at 37°C for 30 min before mounting to the microscope. The growth medium was replaced with fresh DMEM containing 5 µM sensor (from a 1 mM stock solution in DMSO) before imaging. The organelle-specific dyes Hoechst 33258 (Alrich, final concentration 5 µM), MitoTracker Green (Invitrogen, final concentration 0.1-0.5 µM) and ER-Tracker Blue-White DPX (Invitrogen, final concentration 1-5 µM) were incubated for 30 min before imaging. Golgi-specific staining BacMam 2.0 (Invitrogen, final concentration 10 particles per cell (PPC)) was incubated for 24 h to transduce the cells. Cells were rinsed with sterile PBS buffer (2 x 2 mL), and then with dye-free DMEM without serum to remove excess unbound sensors. Cells were bathed in 2 mL dye-free DMEM without serum before mounting on the microscope. To measure Zn²⁺-induced fluorescence changes, cells were treated with 25 µM ZnCl₂ and 50 µM sodium pyridine-2-pyridinethiol-1-oxide (DMSO). A portion of 100 µM N,N,N′,N′-tetrakis(2-pyridylmethyl) ethylendiamine (TPEN) or tris(2-pyridylmethyl)amine (TPA) was applied to the Petri dish to reverse the fluorescence response induced by zinc. For experiments involving Sin-1, a 10 mM working solution of the reagent (Sigma) was freshly prepared by dissolving solid Sin-1 in 1× phosphate buffered saline (PBS) (Corning Cellgro). After initial images were acquired, 20 µl of the Sin-1 working solution was applied to the petri dish containing 2 mL of dye-free DMEM/F12 medium to obtain a final concentration of 100 µM.
Fluorescence Microscopy. Fluorescence imaging experiments were performed using a Zeiss Axiovert 200M inverted epifluorescence microscope with a Hamamatsu EM-CCD digital camera C9100 and a MS200 XY Piezo Z stage (Applied Scientific Instruments, Inc.). An X-Cite 120 metal halide lamp (EXFO) was used as the light source. Zeiss standard filter sets 49, 38 HE, and 43 HE were employed for imaging Hoechst 33258/ER-Tracker, Mitotracker Green/BacMam 2.0 Golgi staining and ZBR sensors. The microscope was operated with Velocity software (version 6.01, Improvision). The exposure time for acquisition of fluorescence images was kept constant for each series of images at each channel. Images corresponding to colocalization studies were deconvoluted using Velocity restoration algorithms. The Pearson’s correlation values were obtained in Velocity to evaluate the colocalization level of ZBR sensors with specific organelle trackers. Z-sectioned images were obtained at 0.3-μm intervals in a 20 μm range to discern possible effects of organelle fluorescence overlap along the z-axis. The quantification of fluorescence intensity was analyzed using ImageJ (version 1.45s, NIH). The whole cell was selected as region of interest (ROI). The integrated fluorescence from the background region was subtracted from the integrated fluorescence intensity of the cell body region. The relative change of fluorescence intensity was plotted for comparison.

Pearson’s Correlation = \[ \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}} \]

Cytotoxicity of ZBR probes. The cytotoxicity of ZBR probes was evaluated by the MTT assay. Solutions of the sensors were freshly prepared in DMSO before use. HeLa cells were plated in a 96-well plate (1,200 cells per well) in 200 μL DMEM media and incubated for 24 h. The cells were treated with ZBR sensors at various concentrations for an incubation period of 24 h at 37 °C, with 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL in PBS), and incubated for 4 h. After removing the medium, 100 μL DMSO was added to dissolve the violet crystals, and then the absorbance of the purple formazan dye was recorded at 570 nm using a BioTek Synergy HT multi-detection microplate plate reader. For each condition, three independent experiments were carried out in triplicate. The reported percentage of cell survival values was normalized to 10 μL DMSO 24 h-treated control cells.

Study of pH-Dependent Fluorescence. The apparent pK_s values were measured by plotting the integrated fluorescent intensity of the emission spectrum against pH recorded in the 12 to 2 range. A 5 μM (1 μM for ZBR3) solution of ZBR sensors (10 mL) containing 100 mM KCl and 1 mM EDTA was adjusted to pH 12 by KOH. The pH was adjusted in 0.5 decrements by addition of the appropriate amounts of 6, 1, 0.1, 0.01 N HCl until reaching pH 2. The absorption and emission spectra at each pH value were recorded. The volume of the added acid was controlled so that the final change in volume was less than 1%. Emission spectra were integrated from 550 to 800 nm. The integrated emission spectral areas were normalized, plotted against pH value, and fitted to the nonlinear expression to calculate the pK_s value.

\[ \Delta F = \frac{\Delta F_{1{\text{max}}}}{(1 + 10^{(pH - pK_{a1})})} + \frac{\Delta F_{2{\text{max}}}}{(1 + 10^{(pH - pK_{a2})})} \]

Metal Selectivity and Dissociation Constant. Metal selectivity was determined by comparing the fluorescence emission spectrum of a 5.0 μM solution of the sensor in aqueous buffer at pH 7.0, before and after treatment with NaCl, CaCl_2, MgCl_2, MnCl_2, FeSO_4 (freshly prepared), CoCl_2, NiCl_2, CuCl_2, CdCl_2, or HgCl_2 stock solution in water, for a final cation concentration of 50 μM. Fluorescence was then recorded after subsequent addition of ZnCl_2 for a total concentration of 50 or 500 μM. In each case, the integrated fluorescence emission spectra were normalized with respect to that of the metal-free control spectrum, arbitrarily assigned as unity. The dissociation constant for Zn^2+ of ZBR sensors was determined with Zn^2+/Ca^2+-EDTA or Zn^2+-EDTA buffering system, which affords concentrations of free Zn^2+ in the nanomolar or picomolar range. Excitation was provided at 525 nm for ZBR1, 530 for ZBR2 and 535 for ZBR3. The response was quantified by integrating the emission intensity from 550 to 800 nm and normalizing. The plot of response versus [Zn] was fitted to the equation R=B[Zn]/(K_a + [Zn]), where R is the integrated fluorescence response and B is 1 for normalized data.

Results and Discussion

Probe Design and Synthesis

Previous work with functionalized fluoresceins in our laboratory has led to the development of a large number of green-emitting probes with dissociation constants covering six orders of magnitude, from sub-millimolar to sub-nanomolar. This careful tuning was achieved by rational modification of the metal-binding group and minor alteration of the appended fluorescein platform. Seeking to develop probes that have lower energy excitation and emission wavelengths, are more amenable to live-cell imaging experiments that require prolonged observation, and are suitable for multicolor multianalyte microscopy experiments, we adopted the benzoresorufin fluorophore as the design platform. We sought to retain the [N,O] coordination motif offered by ZP and ZPP sensors (Fig. 1) in order to produce probes with similar zinc-binding properties, thus allowing us to translate previous research on green fluorophor-based probes into red-emitting sensors with predictable Zn^2+ dissociation constants. The benzophenoazoxane (benzoresorufin) molecule presents an oxygen atom poised to participate in zinc binding similar to that offered by the hydroxyl-xanthene core of fluorescein dyes. The general synthetic scheme for the ZBR probes is depicted in Scheme 1. Assembly of the fluorophore starts with condensation of an appropriate 2-alkyl substituted resorcinol (1a or 1b) and 1,3-dihydroxynaphthalene under acidic conditions, followed by acylation of the resulting benzoresorufin in a mixture of acetic anhydride and pyridine. This esterification step aids in the purification of the fluorophores and increases their solubility in non-polar solvents, thus facilitating further manipulation in the subsequent steps. The acylated methyl-substituted benzoresorufins 2a,b were subjected to radical bromination conditions resulting in the halogenation of both benzylic and quinoid positions in good yields. Occasional precipitation of the ring-brominated intermediates in the reaction mixture resulted in sluggish rates for the second, benzylic, bromination. Adjustment of the reaction temperature helped avoid this problem. With bromomethyl intermediates 4a and 4b in hand, reaction with the appropriate picolyl amine derivative 5a or 5b yielded compounds ZBR1-3 in moderate yields. The dipicolylamine-containing analogue, ZBR3, was characterized crystallographically in its metal-free form (Fig. S7 and Table S1), showing the [N,O] metal binding pocket in a geometry resembling that of ZP1.
Table 1. Spectroscopic Properties of ZBR Derivatives

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<th>Probe</th>
<th>Absorption(^a)</th>
<th>Excitation(^b)</th>
<th>Emission(^c)</th>
<th>(K_c) (nM)</th>
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<td>ZBR1</td>
<td>478, 1.93(6)</td>
<td>530, 2.64(1)</td>
<td>514</td>
<td>525</td>
<td>625, 0.067(6)</td>
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<tr>
<td>ZBR2</td>
<td>480, 1.69(9)</td>
<td>524, 2.56(1)</td>
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<td>530</td>
<td>630, 0.069(6)</td>
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<tr>
<td>ZBR3</td>
<td>480, 1.33(4)</td>
<td>535, 1.93(8)</td>
<td>530</td>
<td>535</td>
<td>623, 0.342(7)</td>
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\(^a\)Measurements were performed in 50 mM PIPES buffer at pH 7.0 with 100 mM KCl. \(^b\)Resorufin (\(\Phi_{res} = 0.74\) at pH 9.5) was used as a standard for quantum yield determination. Standard deviations in the last significant digit are shown in parentheses. \(^c\)Limited solubility of ZBR3 under the titration conditions precluded the accurate determination of the apparent \(K_c\); an estimated upper limit is provided based on the range of [Zn\(^{2+}\)] at which fluorescence saturation was observed.

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fully deprotonated form (Fig. 4A). At pH 7, absorption in this wavelength range is significantly weaker. Furthermore, at acidic pH (pH=2) the broad band centered at 590 nm completely disappears and another weak absorption band appears at 490 nm. The fluorescence response of ZBR1 in the range of pH 2-12 was monitored as well (Fig. 4B). The maximum emission intensity of metal-free probe was observed at pH 7. Reduced emission at high pH is most likely due to the full quenching effect of the pendant amine on the fluorophore, which in turn is abolished at neutral pH values as the amine becomes partially protonated. At acidic pH, the fluorophore itself becomes protonated and the emission efficiency decreases, and effect previously documented for non-functionalized resorufin.37 Non-linear fits of the emission intensity as a function of pH afforded two apparent pK\textsubscript{a} values: pK\textsubscript{a1}=5.35 ± 0.05 and pK\textsubscript{a2}=8.15 ± 0.05, respectively. The fitting for ZBR2 gave the pK\textsubscript{a} values as pK\textsubscript{a1}=4.4 and pK\textsubscript{a2}=7.3 (Fig. S10). The incorporation of the chloro substituent has an effect on the apparent pK\textsubscript{a}. In particular, the first pK\textsubscript{a}, corresponding to deprotonation of the resorufin fluorophore, is reduced by almost one unit owed to the electron withdrawing effect of the Cl atom.

The fluorescence response of the three ZBR sensors to a variety of biologically relevant metal ions was investigated. As shown in Figs. 5 and S13-14, the treatment of ZBR1 and the other ZBR sensors with alkali and alkaline-earth metals has no effect on the fluorescence whereas paramagnetic transition metal ions such as Mn\textsuperscript{2+}, Fe\textsuperscript{3+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+} and Cu\textsuperscript{2+} quench the emission. Iron and manganese, however, may be displaced by zinc, leading to a partial restoration in the fluorescence.

**Figure 3.** Integrated emission intensity of 1 µM ZBR1 vs. [Zn\textsuperscript{2+}]\textsubscript{free} in aqueous buffer at 25 °C (100 mM KCl, 50 mM PIPES, pH 7.0). λ\textsubscript{ex} = 525 nm.

**Figure 4.** Effect of pH on a 5 µM solution of ZBR1 (100 mM KCl, 25 °C). (A) Representative UV-Vis absorption spectra at different pH values. (B) Plot of the normalized integrated emission intensity vs. pH. The pK\textsubscript{a} values were obtained from fitting the experimental data (red circles) to a nonlinear model (continuous line).

**Figure 5.** Metal selectivity of ZBR1 in aqueous solution at pH 7.0. (50 mM PIPES, 100 mM KCl). For each sample, 5 µM ZBR1 was mixed with 50 µM metal ion of interest (light gray) and then subsequently treated with 50 µM ZnCl\textsubscript{2} (dark gray). The integrated fluorescence (550 to 800 nm) after each addition was normalized to the fluorescence of the metal-free sensor. λ\textsubscript{ex} = 525 nm.
**Live Cell Imaging of ZBR sensors.** The cytotoxicity of the ZBR sensors, a critical factor to be considered for their utilization in live cell imaging, was assessed by MTT assays in HeLa cells. Fig. S15 shows the cell viability data for HeLa cells treated with ZBR probes, suggesting that these cells are not damaged following a 24 h treatment with 1 µM ZBR1, ZBR2, or ZBR3. Decreased cell survival (88 ± 5%) occurred following 24 h incubation with 5 µM ZBR1, but no significant cell loss was noticed compared to the control. These results indicate that low micromolar concentrations of ZBR sensors are essentially nontoxic over at least a 24 h period and can be applied to biological studies that require lengthy sensor incubation times.

The ability of the ZBR sensor family to track zinc ions in living cells was investigated. HeLa cells (Fig. 6) were incubated with 5 µM ZBR1 for 30 min at 37 °C before imaging. Because of the low level of endogenous fluorescence from the cells in the red region, in addition to the low quantum yields of the metal-free probes, there is imperceptible background signal before the addition of exogenous zinc (Fig. 6d). The intracellular fluorescence increased in response to addition of exogenous zinc as the 1:2 complex Zn<sup>2+</sup>/pyrithione (2-mercaptopyridine-N-oxide) (Fig. 6e). Integration of the fluorescence signal over the cell body indicated an ~6-fold increase when excess Zn<sup>2+</sup> is present. The signal decreased substantially after treatment with 50 µM of the chelator TPA (Figs. 6c, f). The Zn<sup>2+</sup> response of ZBR2 and ZBR3 was also tested and quantified (Figs. S16 and S17). These results prove that the ZBR sensor family is cell-permeable and can be efficiently used to image intracellular labile Zn<sup>2+</sup> within living cells.

Because the localization of small-molecule sensors is affected by a number of factors, the subcellular localization of the ZBR sensor family was investigated in different cell lines including HeLa, RAW 246.7, and NSCs. For example, co-incubation of HeLa cells with ZBR1 and either a Hoechst 33258, MitoTracker Green, or Bac-

**Figure 6.** Fluorescence microscopy of live HeLa cells incubated with 5 µM ZBR1 and 5 µM Hoechst 33258 at 37°C for 30 min. A) Bright-field transmission image. B) Nuclear staining by Hoechst 33258. C) Quantification of Zn<sup>2+</sup>-induced ZBR1 fluorescence response. D) ZBR1 fluorescence without addition of exogenous Zn<sup>2+</sup>. E) ZBR1 Fluorescence 5 min after treatment with 25 µM Zn<sup>2+</sup>/pyrithione (1:2). F) ZBR1 Fluorescence 5 min after addition of 50 µM TPA. (mean±SD, N = 22). Scale bar = 25 µm.

**Figure 7.** Colocalization analysis of ZBR1 with organelle-specific markers in HeLa cells incubated with 5 µM ZBR1, 1 µM ER-Tracker Green and 0.2 µM MitoTracker Green for 30 min, and then treated with 25 µM Zn<sup>2+</sup>/pyrithione (1:2) on the microscope stage. A) Bright-field image. B) ZBR1. C) MitoTracker Green. D) ER-Tracker E) Overlay of ZBR1 and ER-Tracker. F) Overlay of ZBR1 and MitoTracker Green. Scale bar = 25 µm.

**Peroxynitrite-induced released labile Zn<sup>2+</sup> in the ER.** Previous studies have shown that peroxynitrite induces ER stress and triggers the release of labile Zn<sup>2+</sup> from intracellular stores in neuronal cells. The source of released labile Zn<sup>2+</sup>, however, is still under debate owing to the lack of readily applicable organelle-specific sensors for this ion. With our new ER-specific fluorescent probes for labile Zn<sup>2+</sup> in hand, we sought to investigate further the interplay between peroxynitrite and Zn<sup>2+</sup> homeostasis in the endoplasmic reticulum of NSCs and evaluate the ER as a possible source of the released Zn<sup>2+</sup>.

Fluorescence imaging of NSCs treated with ZBR1 showed accumulation of endogenous Zn<sup>2+</sup> in the ER without the addition of exter-
n-eral ionophores. The metal content of the growth media for NSCs contains 2 μM total Zn$^{2+}$ (information supplied by GIBCO and confirmed by independent measurements in our lab). The use of Zn$^{2+}$-depleted preparations as growth medium (e.g. DMEM, with an estimated Zn$^{2+}$ concentration in the nM range) led to a significantly lower ER-localized signal. We treated the cells with 3-morpholininosydnonimine (Sin-1), a reagent that releases NO and superoxide simultaneously at physiological pH$^+$, which results in the generation of peroxynitrite in-situ with a half-life of 14-26 min in common buffers. Images of Zn$^{2+}$-induced ZBR1 fluorescence in NSCs at different time periods after the addition of 100 μM Sin-1 revealed a loss of signal intensity, consistent with zinc release. As indicated in Figs. 9 and 10, in the first 15 min after addition of peroxynitrite there is a statistically significant decrease (89 ± 4% versus 105 ± 7%, Fig. 10) in the fluorescence intensity of ZBR1. On the other hand, treatment with vehicle (PBS) showed no decrease of fluorescence in the same time period (Fig. 10). The time scale of the observed changes is consistent with the kinetics of decomposition of Sin-1, thus indicating a rapid effect of the reactive species on the Zn$^{2+}$ levels within the ER. Exogenous Zn$^{2+}$/pyrithione (1:2) followed by TPA was applied at the end of the experiments to confirm that ZBR1 is still localized in the cells and that the change of fluorescence intensity is indeed produced by a decrease in labile Zn$^{2+}$ and not by diffusion of the dye out of the organelle, photo-bleaching, or loss of cell viability. To rule out direct interaction between Sin-1 and ZBR1 or the sensor-Zn$^{2+}$ complex, the effect of Sin-1 on the absorption and emission spectra of ZBR1 in its metal-free and -bound form was tested in the cuvette. The results showed that 100 μM Sin-1 has no significant effect on the emission of ZBR1 or the sensor-Zn$^{2+}$ complex (Fig. S24). Taken together, these results suggest that the decrease in fluorescence intensity from ZBR1 is due to the mobilization of zinc from the ER in response to RNS-induced stress.

**Summary and Conclusions.** We prepared and characterized a new family of red-emitting fluorescent sensors for mobile Zn$^{2+}$ based on a benzoresorufin fluorophore functionalized with pyridine- and pyrazine-containing metal binding groups. The new probes, with a conserved [N,O] metal binding motif, have apparent binding affinities comparable to those measured for fluorescein-based counterparts ZPP1 and ZP1, which contain similar binding groups. These results indicate that the benzoresorufin platform can serve as the basis for the design of new red-emitting sensors with predictable binding properties, based upon simple extrapolation of trends in coordinating properties observed for hydroxy-xanthene containing probes such as fluorescein-based sensors. The sub-nanomolar dissociation constant of ZBR1 and ZBR2 makes them suitable for detecting changes in intracellular labile Zn$^{2+}$ with little background interference. The new ZBR sensors spontaneously localize in the ER of various tested cell lines and make them valuable for the investigation of zinc trafficking into and out of this organelle. This property confers them with the potential utility for investigating zinc in ER stress and various related phenomena such as UPR. We provide direct observation of the depletion of labile zinc induced by peroxynitrite in the ER of neural stem cells. Further studies will be aimed at characterizing the destination of released Zn$^{2+}$ under ER stress and the possible downstream signaling pathways thereby triggered.

**Figure 8.** 3D-view representations of co-localization analysis within NSCs. A) ER-Tracker fluorescence. B) ZBR1 fluorescence. C) Overlay of ZBR1 and ER-Tracker fluorescence. See Fig. S20 for 2-D images of NSCs. Scale grid = 13 μm.

**Figure 9.** Representative images of Sin-1-induced ZBR1 fluorescence change in NSCs. A) Bright-field image of NSCs. B) Nuclear staining by Hoechst 33258. C) Fluorescence image of ZBR1 before the addition of 100 μM Sin-1. D) 5 min after the addition of Sin-1. E) 10 min after the addition of Sin-1. F) 15 min after the addition of Sin-1. G) 5 min after the addition of 25 μM Zn$^{2+}$/pyrithione (1:2). H) 5 min after the addition of 50 μM TPA. Scale bar =25 μm.
ASSOCIATED CONTENT
Supporting Information. X-ray crystallographic data in cif format, NMR spectroscopic data, absorption and emission profiles, pH profiles, metal selectivity plots, cytotoxicity data, images of Zn²⁺-induced response of all probes, co-localization studies in RAW 264.7 cells and NSCs, absorption and emission profiles of the probes in the presence of Sin-1. This material is available free of charge via the Internet at http://pubs.acs.org.*

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(2) Takeda, A. Brain Res. Rev. 2000, 34, 137.
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SUPPORTING INFORMATION for

Visualization of Peroxynitrite-Induced Changes of Labile Zn²⁺ in the Endoplasmic Reticulum with Benzoresorufin-based Fluorescent Probes

Wei Lin, Daniela Buccella, and Stephen J. Lippard*

Table of Contents

Figure S1 ¹H NMR spectrum of ZBR1 2
Figure S2 ¹³C{¹H} NMR spectrum of ZBR1 2
Figure S3 ¹H NMR spectrum of ZBR2 3
Figure S4 ¹³C{¹H} NMR spectrum of ZBR2 3
Figure S5 ¹H NMR spectrum of ZBR3 4
Figure S6 ¹³C{¹H} NMR spectrum of ZBR3 4
Figure S7 Molecular structure of ZBR3 5
Table S1 Crystal intensity, collection and refinement data 6
Figure S8 Absorption and emission spectra of ZBR2 7
Figure S9 Absorption and emission spectra of ZBR3 7
Figure S10 Plot of the normalized integrated emission intensity of ZBR2 with pH 8
Figure S11 Plot of the normalized integrated emission intensity of ZBR3 with pH 8
Figure S12 Integrated emission intensity of ZBR2 vs. [Zn²⁺]free in aqueous buffer 9
Figure S13 Metal selectivity of ZBR2 in PIPES buffer solution 9
Figure S14 Metal selectivity of ZBR3 in PIPES buffer solution 10
Figure S15 Cytotoxicity of the ZBR sensors 10
Figure S16 Representative images of ZBR2 response to exogenous Zn²⁺ in HeLa cells 11
Figure S17 Representative images of ZBR3 response to exogenous Zn²⁺ in HeLa cells 11
Figure S18 Representative images of co-localization analysis of ZBR1 with organelle-specific dyes in HeLa cells 12
Figure S19 Representative images of co-localization analysis of ZBR1 with ER tracker in RAW 246.7 cells 13
Figure S20 Representative images of co-localization analysis of ZBR1 with ER tracker in NSCs 14
Figure S21 Representative images of co-localization analysis of ZBR2 with ER tracker in RAW 246.7 cells 15
Figure S22 Representative images of co-localization analysis of ZBR3 with ER tracker in RAW 246.7 cells 16
Figure S23 Extended view of co-localization analysis of ZBR1 with ER tracker and Mito tracker in HeLa cells 17
Figure S24 Effect of Sin-1 on the absorption response and fluorescence emission of ZBR1 and ZBR1-Zn²⁺ complex 19
Figure S1. $^1$H NMR spectrum of ZBR1

Figure S2. $^{13}$C{$^1$H} NMR spectrum of ZBR1
Figure S3. $^1$H NMR spectrum of ZBR2

Figure S4. $^{13}$C\{$^1$H\} NMR spectrum of ZBR2
Figure S5. $^1$H NMR spectrum of ZBR3

Figure S6. $^{13}$C [$^1$H] NMR spectrum of ZBR3
**Figure S7.** Molecular structure of ZBR3. ORTEP with 50% probability thermal ellipsoids.
Table S1. Crystal intensity, collection, and refinement data

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**Figure S8.** Absorption (A) and normalized fluorescence emission (B) spectra of ZBR2 in the absence and in the presence of excess Zn$^{2+}$. Spectra were acquired on 5 µM solutions of ZBR2 in 100 mM KCl, 50 mM PIPES, pH 7.0 at 25 °C, after the addition of 10 µM ZnCl$_2$, followed by 50 µM EDTA. Excitation was performed at 530 nm.

**Figure S9.** Absorption (A) and normalized fluorescence emission (B) spectra of ZBR3 in the absence and in the presence of excess Zn$^{2+}$. Spectra were acquired on 3 µM solutions of ZBR3 in 100 mM KCl, 50 mM PIPES, pH 7.0 at 25 °C, after the addition 10 µM ZnCl$_2$. Excitation was performed at 535 nm.
Figure S10. Plot of normalized integrated emission of ZBR2 vs. pH. The pKₐ values were obtained from fitting the experimental data to a nonlinear model (continuous line).

Figure S11. Plot of normalized integrated emission of ZBR3 vs. pH. The pKₐ values were obtained from fitting the experimental data to a nonlinear model (continuous line).
Figure S12. Integrated emission intensity of 1 µM ZBR2 vs. [Zn$^{2+}$]$_{\text{free}}$ in aqueous buffer at 25 °C (100 mM KCl, 50 mM PIPES, pH 7.0). Excitation was provided at 530 nm.

Figure S13. Metal selectivity of ZBR2 in PIPES buffer solution at pH 7.0. 5 µM ZBR2 was mixed with 50 µM of the metal ion of interest (gray) and then subsequently treated with 50 µM (dark gray) or 500 µM (light gray) ZnCl$_2$. Emission spectra was integrated from 550 nm to 800 nm. The integrated fluorescence after each addition was normalized to the fluorescence of the metal-free sensor. $\lambda_{\text{ex}} = 530$ nm.
Figure S14. Metal selectivity of ZBR3 in PIPES buffer solution at pH 7.0. 5 µM ZBR3 was mixed with 50 µM of the metal ion of interest (gray) and then subsequently treated with 50 µM (dark gray) or 500 µM (light gray) ZnCl₂. Emission spectra were integrated from 550 nm to 800 nm. The integrated fluorescence after each addition was normalized to the fluorescence of the metal-free sensor. λₑₓ = 535 nm.

Figure S15. Cytotoxicity of the ZBR sensors applied to HeLa cells treated with different concentrations of ZBR1 (red), ZBR2 (black) and ZBR3 (green) for 24 h, measured by MTT assay. The data points represent the averages of three independent trials.
Figure S16. Representative images of ZBR2 response to exogenous Zn\textsuperscript{2+} in HeLa cells. Cells were incubated with 5 µM ZBR2 and 5 µM Hoechst 33258 at 37 °C for 30 min. A) Bright-field transmission image. B) Nuclear image stained by Hoechst 33258. C) Fluorescence image without addition of exogenous Zn\textsuperscript{2+}. D) Fluorescence image after addition of 25 µM Zn\textsuperscript{2+}/pyrithione (1:2). E) Fluorescence image 5 min after treatment with 50 µM TPA. Scale bar = 25 µm.

Figure S17. Representative images of ZBR3 response to exogenous Zn\textsuperscript{2+} in HeLa cells. Cells were incubated with 5 µM ZBR2 and 5 µM Hoechst 33258 at 37 °C for 30 min. A) Bright-field transmission image. B) Nuclear image stained by Hoechst 33258. C) Fluorescence image without addition of exogenous Zn\textsuperscript{2+}. D) Fluorescence image after addition of 25 µM Zn\textsuperscript{2+}/pyrithione (1:2). E) Fluorescence image 5 min after treatment with 50 µM TPA. Scale bar = 25 µm.
**Figure S18.** Representative images of co-localization analysis of ZBR1 with organelle-specific dyes in HeLa cells. Cells were incubated with 5 μM ZBR1, 5 μM Hoechst 33258 for 30 min. Golgi specific staining BacMam 2.0 was incubated at 10 PPC for 24 h to transduce HeLa cells. HeLa cells were then treated with 25 μM Zn\(^{2+}\)/pyrithione (1:2) on the microscope stage. A) Bright-field image. B) Nuclear image stained by Hoechst 33258. C) Overlay of ZBR1 and Hoechst 33258. D) Zoom-in of image C. E) BacMam 2.0 for Golgi staining. F) ZBR1. F) Overlay of ZBR1 and BacMam 2.0. H) Zoom-in of image G. Scale bar = 25 μm or 8 μm (for D and H).
**Figure S19.** Representative images of co-localization analysis of ZBR1 with ER-Tracker in RAW 246.7 cells. Cells were incubated with 5 µM ZBR1, 1 µM ER-Tracker for 30 min. Cells were then treated with 25 µM Zn\(^{2+}\)/pyrithione (1:2) on the microscope stage. 
Figure S20. Representative images of co-localization analysis of ZBR1 with ER-Tracker in NSCs. Cells were incubated with 5 μM ZBR1, 1 μM ER-Tracker for 30 min. NSCs were then treated with 25 μM Zn^{2+}/pyrithione (1:2) on the microscope stage. A) Bright-field image. B) Fluorescence image by ER-Tracker. C) Fluorescence image by ZBR1. D) Overlay of ZBR1 and ER-Tracker. E) Zoom-in of image D. F) 2D-view of Bright-field image from integrated images. G) 2D-view of fluorescence image of ER-Tracker from integrated images. H) 2D-view of fluorescence image of ZBR1 from integrated images. I) Overlay of ZBR1 and ER-Tracker. J) Zoom-in of image I. Scale bar = 26 μm or 6 μm (for E and J).
**Figure S21.** Representative images of co-localization analysis of ZBR2 with ER-Tracker in RAW 246.7 cells. Cells were incubated with 5 µM ZBR2, 1 µM ER-Tracker for 30 min. RAW 246.7 cells were then treated with 25 µM Zn²⁺/pyrithione (1:2) on the microscope stage. A) Bright-field image. B) Fluorescence image of ER-Tracker. C) Fluorescence image of ZBR2. D) Overlay of ZBR2 and ER-Tracker. Scale bar = 25 µm.
Figure S22. Representative images of co-localization analysis of ZBR3 with ER-Tracker in RAW 246.7 cells. Cells were incubated with 5 μM ZBR3, 1 μM ER-Tracker for 30 min. RAW 246.7 cells were then treated with 25 μM Zn²⁺/pyrithione (1:2) on the microscope stage. A) Bright-field image. B) Fluorescence image of ER-Tracker. C) Fluorescence image of ZBR3. D) Overlay of ZBR3 and ER-Tracker. Scale bar = 25 μm
Figure S23. Colocalization analysis of ZBR1 with organelle-specific markers in HeLa cells incubated with 5 µM ZBR1, 1 µM ER tracker and 0.2 µM MitoTracker Green for 30 min, and then treated with 25 µM Zn²⁺/pyrithione (1:2) on the microscope stage. A) Overlay of ZBR1 and ER-Tracker. B) Zoom-in of panel A. C) Overlay of ZBR1 and MitoTracker Green. D) Zoom-in of panel C. Scale bar = 25 µm (A and C), or 12 µm (B D and D).
Figure S24. Effect of Sin-1 on the absorption and fluorescence emission of ZBR1 and ZBR1-$\text{Zn}^{2+}$ complex. Spectra were acquired in 100 mM KCl, 50 mM PIPES, pH 7.0 at 25 °C. A) Absorption spectra of 5 µM ZBR1 after the sequential addition of 100, 200 µM, 1 mM Sin-1 and 20 µM ZnCl$_2$. B) Emission spectra of 5 µM ZBR1 after the sequential addition of 100, 200 µM, 1 mM Sin-1 and 20 µM ZnCl$_2$. C) Absorption spectra of 5 µM ZBR1 after the addition of 20 µM ZnCl$_2$, 100, 200 µM and 1 mM Sin-1. D) Emission spectra of 5 µM ZBR1 after the addition of 20 µM ZnCl$_2$, 100, 200 µM and 1 mM Sin-1. Excitation wavelength was provided at 525 nm. Emission data were normalized to the response of the maximum fluorescence.