Redox-Triggered Self-Assembly of Gadolinium-Based MRI Probes for Sensing Reducing Environment

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Supporting Information

ABSTRACT: Controlled self-assembly of small molecule gadolinium (Gd) complexes into nanoparticles (GdNPs) is emerging as an effective approach to design activatable magnetic resonance imaging (MRI) probes and amplify the $r_1$ relaxivity. Herein, we employ a reduction-controlled macrocyclization reaction and self-assembly to develop a redox activated Gd-based MRI probe for sensing a reducing environment. Upon disulfide reduction at physiological conditions, an acyclic contrast agent 1 containing dual Gd-chelates undergoes intramolecular macrocyclization to form rigid and hydrophobic macrocycles, which subsequently self-assemble into GdNPs, resulting in a ~60% increase in $r_1$ relaxivity at 0.5 T. Probe 1 has high $r_1$ relaxivity (up to 34.2 mM$^{-1}$ s$^{-1}$ per molecule at 0.5 T) upon activation, and also shows a high sensitivity and specificity for MR detection of thiol-containing biomolecules.

INTRODUCTION

Over the past few decades, MRI has become one of the most powerful diagnostic tools available in both clinical and research settings. It can provide noninvasive, multidimensional visualization of the entire body with high spatial resolution.¹ However, compared to other molecular imaging modalities, MRI has relatively poor sensitivity, and therefore contrast agents (CAs) are often used to enhance imaging contrast between pathological and normal tissues. Currently, more than 40% of clinical MRI protocols include the use of CAs.²

Gd-based CAs are able to shorten the longitudinal relaxation time ($T_1$) of water protons to produce “positive” (bright) contrast and are most commonly used in MRI diagnosis. However, currently clinically used CAs possess relatively low contrast efficacy ($r_1$), and large amounts of CAs have to be administered to achieve sufficient contrast, which is often a safety concern. In addition, they are generally nonspecific and not capable of sensing biochemical activity.³ As a result, the design of molecular probes with higher relaxivity is crucial for MR molecular imaging. “Smart” or “activatable” MR probes are particularly attractive because they modulate their MR properties (relaxivity) leading to signal amplification upon molecule target interaction.³⁻⁵ These smart probes can greatly improve MR detection of specific molecular events arising from either receptor binding (i.e., RIME, receptor-induced magnetization enhancement)⁶⁻⁹ or molecular activation (i.e., pH, light, metal ions, and enzymes).¹⁰⁻¹⁴ A number of environmentally responsive MRI probes¹⁵⁻²¹ capable of specifically reporting on their local physiological or biochemical conditions (e.g., pH, redox, ions) have been actively explored, providing wide applications in diagnosis.

Thiol redox chemistry is central to the control of cell fate and is associated with various abnormal biochemical processes.¹⁷ Alternations in the levels of thiol-containing biomolecules (biothiols), including glutathione (GSH), cysteine (Cys), and homocysteine (Hcy), are directly linked with many diseases (e.g., cancer, AIDS, liver damage, psoriasis, and Alzheimer’s disease).²² Therefore, various methods for monitoring of their levels have been developed, including thiol-sensitive optical probes,²³⁻²⁵ inorganic quantum dots,²⁶ and luminescent semiconductors.²⁷ Recently, a few thiol-responsive Gd-based MRI probes have also been reported.²⁸⁻³⁴ These probes typically have a small value of $r_1$ relaxivity (2.77 mM$^{-1}$ s$^{-1}$)³¹ or show a decrease in $r_1$ relaxivity after thiol-activation that leads to a darker contrast.²⁸,²⁹,³¹ Here we report MR probes that give an increasing, high value (>10 mM$^{-1}$ s$^{-1}$) of $r_1$ relaxivity upon biothiol activation, resulting in a brighter contrast.

We have previously described an activatable Gd-based CA containing a disulfide that undergoes reduction-triggered polymerization and self-assembly to generate enhanced $r_1$ relaxivity after activation, by taking advantage of the increased molecular tumbling time ($T_2$) arising from in situ self-assembly of GdNPs.³⁵"
The mechanism of polymerization is based on the reduction-triggered disulfide cleavage to yield 1,2-aminothiol, which further condenses with 2-cyanobenzothiazole (CBT) in another monomer to produce oligomers. A potential limitation of this system is the competition from the reaction between CBT in the monomer and the endogenous free cysteine—that stops polymerization and self-assembly. On the other hand, the $r_1$ relaxivity of the probe after reduction activation is just $\sim 8.3 \text{ mM}^{-1} \text{s}^{-1}$ (Table 1), which is smaller than that of other reported GdNPs-based CAs. The lower $r_1$ value is presumably due to the flexibility of the linear oligomer of the scaffold after polymerization. In order to effectively outcompete free cysteine, we have optimized the self-assembly strategy by switching the concentration-dependent intramolecular polymerization to concentration-independent intramolecular macrocyclization chemistry, showing high efficacy to self-assemble fluorescence and PET probes into nanoparticles for imaging of enzyme activity in biological systems. This optimized self-assembly system can also be applied to develop the first example of caspase-3/7 activatable Gd-based MRI probe by introducing a Gd-DOTA amide chelate. The $r_1$ relaxivity following activation is $\sim 15.6 \text{ mM}^{-1} \text{s}^{-1}$ per molecule at 1.5 T, which is around 2-fold higher than that of polymerization-based probe 1a ($\sim 8.3 \text{ mM}^{-1} \text{s}^{-1}$). This suggests that, with the replacement of the flexible linear oligomer with a macrocyclic scaffold after activation, the CAs with more hydrophobic and rigid macrocyclic scaffold can further enhance the $r_1$ relaxivity following activation according to the Slomon-Bloembergen-Morgan (SBM) theory. To further investigate the capability of the macrocyclization chemistry mediated self-assembly approach for activatable MRI probes with improved MR properties, we develop new redox activated CAs containing two Gd-DOTA amide chelates and a biothiol sensitive disulfide bond. The newly designed CAs upon reduction activation have high $r_1$ relaxivities per molecule (up to $\sim 34.2 \text{ mM}^{-1} \text{s}^{-1}$ at 0.5 T) and are specific to biothiols and capable of detecting GSH concentration with high sensitivity.

### RESULTS AND DISCUSSION

#### Design of Redox Activated Gd-Based CAs.

The conceptual design in Figure 1 was demonstrated with a biocompatible
intramolecular macrocyclization system derived from reaction between 2-cyano-6-hydroxyquinoline (CHQ) and free cysteine.40

Macrocycles are an important class of molecules which exhibit profound chemical and biological properties derived from the preorganized ring structures.45 In comparison to their acyclic analogues, macrocycles inherently possess a lower number of rotatable bonds, and are more conformationally restricted. Topologically, macrocycles also have larger surface areas.46,47

Scheme 1. Synthesis of Probes 1 and 1-Oa

*(a) (I) N-Boc-N-Fmoc-t-Lys-OH, t-BuO-Cl, NMM, THF, 0 °C to r.t., 72%; (II) 20% TFA/DCM, r.t., 2 h, 98%; (III) S-Trt-N-Boc-n-Cys-OH, HBTU, DIPEA, THF, 3 h, 82%; (b) 5% Piperidine/DMF, r.t., 10 min, 63%; (c) Tri-(t-BuO)-DOTA-NHS, DMF, DIPEA, 2 h, 75%; (d) (I) Py·HCl, 200 °C, 2 h, 35%; (II) K2CO3, KI, DMF, BocNH(CH2)3Cl, 80 °C, overnight, 90%; (III) 20% TFA/DCM, 99%; (e) (I) S-Trt-N-Boc-t-Cys-Lys-(N-Fmoc)-OH, HBTU, DIPEA, DMF, 3 h, 91%; (II) 5% Piperidine/DMF, r.t., 10 min, 65%; (f) (I) Tri-(t-BuO)-DOTA-COOH, HBTU, DIPEA, DMF, 3 h, 90%; (II) TFA/TIPS/DCM (95/2.5/2.5), r.t., 3 h, 85%; (g) TCEP, DIPEA, MeOH, r.t. 1 h, 79% for two steps; (j) PBS buffer (pH 7.4), Ar, r.t., 24 h, 43% yield with 45% recovery of 11-II; (j) GdCl3, NaHCO3, r.t. overnight, 93%; (k) MnO2, DIPEA, DMF/MeOH, 67%.*
All these characteristics make macrocycles attractive scaffolds for drug and material design. For example, well-defined macrocycles can assemble to robust organic nanotubes or supramolecular barrels driven by noncovalent interactions. We envisioned that if two Gd-chelates were simultaneously incorporated into the acyclic precursor, the increasing number of Gd-chelates can result in a higher value of MR relaxivity upon formation of Gd-based macrocycle. Furthermore, the self-assembled GdNPs from these macrocycles will further increase $r_1$ relaxivity compared to that of the acyclic precursor (small molecule) with an identical Gd concentration.

To prove this concept, we designed probe 1 containing a disulfide that could be activated by reduction (Figure 1). The application of thiol/disulfide redox couple is well explored for biotiol sensing. The established chemical reaction is highly biocompatible, ensuring sufficient reaction to form more rigid and hydrophobic macrocycles and effectively self-assemble into GdNPs. The introduction of two Gd-DOTA amide chelates to the same molecule should increase the accumulation of Gd$^{3+}$ ions in the GdNPs to further enhance $r_1$ relaxivity of the activation product.

**Synthesis and Characterization of Gd-based CAs.** The general synthesis of probe 1 is shown in Scheme 1, which afforded two isomers (1-I and 1-II) with a ratio of 1:2 on HPLC. Both isomers showed the same UV/vis absorption and fluorescence spectra (Supporting Information Figure S2 and Table S1), and HRMS characterization gave the same molecular weight (Supporting Information Figure S1). 2-D NMR spectroscopy revealed an identical NMR pattern of the precursors (11-I and 11-II) of both isomers. Interestingly, both isomers can undergo spontaneous structural isomerization to each other. When diastereomerically pure 1-I and 1-II were incubated in PBS buffer (pH 7.4) at r.t., they could gradually isomerize and the ratio of 1-I and 1-II in both solutions stabilized at around 2 after 60 h based on the HPLC analysis (Figure 2A and B). We estimated the isomerization rate constants of 1-I and 1-II at the first 25 h of incubation (Figure 2C and D). The pseudo-first-order rate constants are 0.0133 h$^{-1}$ for 1-I and 0.0296 h$^{-1}$ for 1-II. These results demonstrate that isomer 1-I is thermodynamically more stable than 1-II in PBS buffer, and the kinetics of transforming 1-I to 1-II is about 2-fold slower than that of transforming 1-II to 1-I. Furthermore, the spontaneous isomerization of 1-I and 1-II in solution is pH-dependent (see Supporting Information Figure S3). A much slower isomerization process was observed in aqueous solution with a lower pH value (<5.5). Since the chiral center at the C-4 position of thiazoline (4-(-S) or 4-(R)) is reported to easily racemize/epimerize via a proposed keto–enol tautomerism process under basic condition (Supporting Information Figure S4), the same mechanism is likely responsible for the epimerization of 1-I and 1-II. Indeed, the enolization-based racemization of L-luciferin has been proposed for the biosynthesis of firefly $\beta$-luciferin. Based on the structure of precursor 11-II, which contains a 4-(S) chiral center at the thiazoline linker, we therefore assigned isomer 1-I the same 4-(S) chirality as that of 11-II, and 1-I the 4-(R) configuration (Scheme 1). To further verify this assumption, we used MnO$_2$ to oxidize thiazoline to eliminate the chiral center at C-4 position (Scheme 1). As expected, both isomers 1-I and 1-II were converted to the same oxidation compound 1-O that contains a thiazole linker, with an identical peak on HPLC analysis. 1-O was stable in PBS buffer without any isomerization.

To evaluate whether disulfide reduction of probe 1 could trigger intramolecular cyclization, isomer 1-I was treated with reducing agent TCEP at pH 3 to afford reduced intermediate 2-I (Figure 3A). Subsequent adjustment of pH value to 7.4 resulted in intramolecular cyclization to give two macrocyclic isomers 3-I and 3-II as observed before (Supporting Information Figure S5).

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**Figure 2.** Spontaneous isomerization of 1-I and 1-II in PBS solution. (A) Isomer 1-I (100 $\mu$M) is slowly converted to 1-II, and gives rise to a final ratio of ~2:1 (1-I: 1-II) after 60 h incubation at r.t. (B) Isomer 1-II (100 $\mu$M) is slowly converted to 1-I, and gives rise to a final ratio of ~2:1 (1-I: 1-II) after 72 h incubation at r.t. (C, D) Plots of the HPLC data estimate the pseudo-first-order rate constant for isomerization of 1-I (C) and 1-II (D) at the first 25 h incubation time (1-I: $k_1$ = 0.0133 h$^{-1}$; 1-II: $k_2$ = 0.0296 h$^{-1}$).
The ratio of 3-I to 3-II is about 7:1 based on the HPLC analysis. Similarly, treatment of 1-II with TCEP followed by adjustment of pH value to 7.4 provided the same macrocyclic products 3-I and 3-II but at a ratio of 1:4 (Figure 3B). This difference may arise from different chirality at the C-4 position of thiazoline linker in 1-I and 1-II and their different epimerization rates. Macrocyclization of 1-O provided just one product 3-O based on HPLC analysis (Figure 3C). Furthermore, incubation of 1-I, 1-II, and 1-O in lysates of human breast cancer cell line MDA-MB-468 gave the same macrocyclic products as those in the buffer (Figure 3D). Similar results were obtained when incubating 1-I and 1-II in HeLa cell lysates (Supporting Information Figure S5). These results suggest that the reduction-triggered macrocyclization of our designed probes can take place efficiently in a complex cellular environment.

**Nanocharacterization of Self-Assembled GdNPs.** We further confirmed the formation of GdNPs after macrocyclization of probe 1 upon reduction. DLS showed the particles with a mean diameter of 100–300 nm from both isomers 1-I and 1-II upon TCEP reduction in water at pH 7.4 (Supporting Information Figure S6). SEM (Figure 4A and B) and TEM (Figure 4C) images revealed the shapes of separated GdNPs with diameters ranging from 100 nm to a few hundred nanometers. The presence of Gd in the particles was confirmed by energy-dispersive X-ray (EDX) spectroscopy (Supporting Information Figure S6). The formation of nanoparticles from the macrocyclic products of 1-O was also observed based on the DLS analysis (Supporting Information Figure S6).

**Characterization of MR Properties.** Water proton relaxivities for isomers 1-I and 1-II and the oxidized 1-O before and after disulfide reduction were measured on 0.5, 1.5, and 3 T clinical MRI scanners (Supporting Information Figure S7). The relaxivity values of all these probes were summarized in Table 1. Before disulfide reduction, all the three di-Gd-chelate based CAs exhibited higher relaxivity per molecule at all three magnetic field strengths compared to single-Gd chelate containing agents such as Dotarem or our previously reported probe 1a.15 The larger relaxivities are in line with their larger molecular size and also more Gd-chelates in the probes.57 Upon disulfide reduction, they all produced enhanced relaxivities, which resulted in a 1.5-, 1.6-, and 1.4-fold increase in r₁ for 1-I, 1-II, and 1-O at 0.5 T, respectively. These results indicate that, after macrocyclization, the relaxivities are further enhanced owing to the formation of GdNPs. When measured at a higher field strength (1.5 T), similar increases in r₁ were also observed for 1-I (1.4-fold), 1-II (1.5-fold), and 1-O (1.4-fold), and even at 3T, more than 1.2-fold increase in r₁ was observed for 1-II. Among all three probes, 1-O gave the highest r₁ values per molecule upon activation at all three field strengths: 34.2 (0.5 T), 31.8 (1.5 T), and 21.0 mM⁻¹ s⁻¹ (3 T). The value of 1-O at 1.5 T is ~8 times
greater than that of Dotarem and ~4 times greater than that of our previous probe 1a upon activation (Table 1), which demonstrates that the self-assembly of CAs with more Gd chelates can effectively increase the $r_1$ relaxivity.

$T_1$-weighted MR images of a series of solutions containing Dotarem, 1-I, 1-II, and 1-O in PBS buffer were obtained at 0.5 T (Figure 5) using a fast spin–echo pulse sequence with 74 ms. As expected, the solution of di-Gd probes 1-I, 1-II, and 1-O are clearly brighter than that of Dotarem at the same Gd concentration (800 μM). Moreover, upon disulfide reduction, the solutions containing di-Gd probes in PBS buffer exhibited much higher signal intensity than those before reduction. These results confirm that higher $r_1$ associated with the self-assembled GdNPs can result in faster $T_1$ relaxation, leading to a significant enhancement in MR contrast.

MR Detection of Reducing Environment. We first evaluated the capability for MR detection of GSH levels by measuring the $T_1$ value of representative probe 1-II solution (200 μM) upon incubation with varying concentrations of GSH (0–10 mM) in PBS buffer at 0.5 T. As shown in Figure 6A, the $T_1$ value of the solution reduced gradually as the GSH concentration was increased. At GSH concentration as low as 10 μM, a significant reduction in $T_1$ value ($\Delta T_1 = -17 \pm 4$ ms) was observed for 1-II, and at ≥1 mM GSH, 1-II was extensively activated in solution after 6 h, leading to the maximum reduction in $T_1$ value ($\Delta T_1 = -74 \pm 5$ ms). A plot of the change of relaxation rate ($\Delta (1/T_1)$) in solution shows a GSH level-dependent increase of relaxation rate for 1-II (Figure 6B). These results indicate that 1-II has a good sensitivity for MR detection of GSH levels. Since the GSH is present at millimolar levels in cells, the sensitivity of 1-II is sufficient for MR detection of cellular reducing environment.

The selectivity of our CAs for biothiols against other representative natural amino acids was next examined according to $T_1$ value measurement (Figure 6C). GSH, Cys, and Hcy, the three major biothiols, all caused remarkable reduction in the $T_1$ value like TCEP. No apparent response to other amino acids including sulfur-containing methionine was observed in the test system. The response to Cys is of importance, suggesting that 1-II can efficiently outcompete free endogenous Cys in biological samples for macrocyclization. As shown in Figure 6C, the incubation of 1-II (200 μM) in healthy HeLa cell lysates resulted in a similar $T_1$ value ($T_1 = 178 \pm 4$ ms) as that observed with GSH (1 mM) activation in PBS buffer, which was inhibited by a thiol scavenger, N-ethylmaleimide (NEM). Therefore, 1-II was specific to biothiols over other analytes existing in cells.

Since our probe will produce an enhanced MR contrast upon reductive activation, it would be more suitable for detection of pathology associated with higher cellular GSH levels as compared with normal tissues such as malignant and drug resistant tumors. In cells with oxidative stress that have lower GSH levels (e.g., Parkinson’s disease), our approach would result in a decrease in the MR contrast relative to healthy cells; in this case, other previously reported MRI probes that show a decrease in the $r_1$ relaxivity after thiol-activation may be applied in order to generate a positively increased contrast. One potential limitation of our current MRI probe is its irreversibility after activation, and in the future a reversible MRI probe may be designed to image the dynamics of the cellular reducing environment if assembled nanoparticles can be disassembled and then reassembled.

**CONCLUSIONS**

In summary, we have described the development of novel redox activated Gd-based CAs using an optimized macrocyclization chemistry-mediated self-assembly approach. These CAs can be activated by disulfide reduction to form rigid and hydrophobic macrocycles, which can further assemble into GdNPs, resulting in enhanced relaxivity (~60% amplified at 0.5 T). The developed CAs containing two Gd-chelates exhibit a high value of $r_1$ (up to 34.2 mM$^{-1}$ s$^{-1}$ per molecule at 0.5 T) upon activation, and also show high specificity and sensitivity to...
biothiols with MR detection, indicating a feasible approach for sensing a reducing environment.

- **EXPERIMENTAL PROCEDURES**

**Materials and Methods.** All chemicals were purchased from commercial sources (such as Aldrich, Fluka, Anaspec, and Novabiochem). Analytical TLC was performed with 0.25 mm silica gel 60F plates with fluorescent indicator (254 nm). Plates were visualized by ultraviolet light. The 1H and 13C NMR spectra were taken on a Bruker 400 MHz magnetic resonance spectrometer. Data for 1H NMR spectra are reported as follows: chemical shifts are reported as \( \delta \) in units of parts per million (ppm) relative to chloroform-d (\( \delta 7.26, s \)); multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broadened); coupling constants are reported as a J value in Hertz (Hz); the number of protons (n) for a given resonance is indicated nH, and based on the spectral integration values. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and high-resolution mass spectrometry (HRMS) analyses were performed at the Mass Spectrometry Facility of Stanford University. Higher-performance liquid chromatography (HPLC) was performed on a Dionex HPLC System (Dionex Corporation) equipped with a GP50 gradient pump and an inline diode array UV–vis detector. A reversed-phase C18 (Phenomenex, 5 μm, 10 × 250 mm or Dionex, 5 μm, 4.6 × 250 mm) column was used with a MeCN (B)/H2O (A) gradient mobile phase containing 0.1% trifluoroacetic acid (TFA) at a flow of 1 or 3 mL/min for the analysis. Scanning electron microscope (SEM) micrographs were obtained on a FEI Nova NanoSEM microscope. Transmission electron microscopy (TEM) micrographs were obtained on a JEM 1230 Electron Microscope. Dynamic light scattering (DLS) experiment was operated on a 90 plus particle size analyzer (Brookhaven Instruments Corporation). Inductively coupled plasma mass spectrometry (ICP-MS) measurement was conducted on Nu Plasma Attom equipment.

**Synthesis of Gd^{3+} Probes 1 and 1-O (Scheme 1).** Synthesis of Intermediate 5. Isobutyl chloroformate (\( \text{t-BuO}-\text{COCl} \)) (156 μL, 1.3 mmol) was added to a solution of Nα-Boc-Nε-Fmoc-L-Lys-OH (515 mg, 1.1 mmol) and N-methylmorpholine (NMM) (143 μL, 1.3 mmol) in dry THF (2.0 mL) at room temperature (r.t.). After 15 min, the reaction was warmed to 60 °C for 2 h. The solvent was removed under vacuum. The residue was dissolved in ethyl acetate (EtOAc) (50 mL) and washed with water (2 × 10 mL) and brine (10 mL). The organic layer was dried over anhydrous Na2SO4, and EtOAc was removed under vacuum. The residue

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**Figure 6.** MR detection of reducing environment with 1-II. (A) \( T_1 \) values (0.5 T) of 1-II (200 μM) in PBS buffer (10×, pH 7.4) following incubation with varying concentrations of GSH at 37 °C for 6 h. (B) Plot of the change of relaxation rate (\( \Delta (1/T_1) \)) calculated from a) versus GSH concentrations between 0–10 mM. \( \Delta (1/T_1) = 1/T_1 \) (with GSH) – 1/T1 (without GSH). The inset is a linear fitting line in the region of 10–100 μM GSH. (C) \( T_1 \) values (0.5 T) of 1-II (200 μM) in PBS buffer (10×, pH 7.4) following incubation with different agents at 37 °C overnight. 1 mM of TCEP, various biothiols (GSH, Cys, and Hcy) and representative natural amino acids (Gly, Glu, Lys, His, Met, Ser, Phe) were used for the studies. HeLa cell lysates (10 million cells per mL PBS buffer) with or without 2 mM NEM pretreatment were studied. \( T_1 \) value (0.5 T) for each solution was measured with Bruker Minispec (mq20 NMR analyzer) at 37 °C, using the standard inversion recovery program. Data represent mean values ± standard deviation; \( n = 4 \).
was purified by silica gel chromatography (hexane:EtOAc = 1:5:1) to give a white solid in 72% yield, which was directly dissolved in dry dichloromethane (DCM) (8 ml). Trifluoroacetic acid (TFA) (2 ml) was then added to remove the Boc group. After removal of TFA under vacuum, the residue was dissolved in dry THF (15 ml), to which S-Trt-N-Boc-o-Cys-OH (1.05 equiv), O-benzotriazol-1-yl-N,N,N′,N′-tetramethyl-uroniumhexafluoro-phosphate (HBTU, 1.1 equiv), and N,N-disopropylethylamine (DIPEA, 3 equiv) were added. After reaction at r.t. for 2 h, THF was removed, and compound 4 was obtained as a light yellow solid in 82% yield after purification by silica gel chromatography (Hexane:EtOAc = 1:1). The following deprotection of the Fmoc group in compound 4 with 5% piperidine in dimethylformamide (DMF) at r.t. for 10 min produced intermediate 5 in 63% yield after HPLC purification. 1H NMR (400 MHz, CD3OD) δ 8.60 (s, 1H), 7.99 (d, J = 9.0 Hz, 1H), 7.77 (d, J = 8.9 Hz, 1H), 7.34 (m, 6H), 7.26 (m, 6H), 7.20 (m, 3H), 4.55 (dd, J = 9.6, 4.3 Hz, 1H), 3.96 (t, J = 6.8 Hz, 1H), 2.88–2.76 (m, 2H), 2.54 (m, 2H), 2.05 (s, br, 1H), 1.83–1.24 (m, 14H). 13C NMR (101 MHz, DMSO-d6) δ 171.57, 171.18, 159.52 (TFA), 155.79, 148.43, 144.93, 139.83, 137.28, 135.77, 129.65, 128.71, 127.44, 125.40, 121.63 (TFA), 118.63, 115.70, 114.19, 112.21, 79.26, 66.61, 54.16, 54.02, 44.34, 31.74, 27.83, 27.29, 23.00. MS: calcd. for C16H16N2O4SO2 [(M+H)+]: 479.3. LC-MS found: m/z 479.7.

**Synthesis of Intermediate 6.** To a solution of 5 (0.2 mmol) in dry DMP (3 ml) was added tri-tert-butylation 2,2′,2′′-(10-(2-(2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7,10-tetrazacyclodecane-1,4,7,10-tetraacetate (Tri-(t-BuO)-DOTA-NHS) (0.21 mmol) and DIPEA (0.4 mmol). Two hours later, EtOAc (30 ml) was added, and the solution was washed with water (2 × 10 ml) and brine (10 ml). The organic layer was dried over anhydrous Na2SO4 and EtOAc was removed under vacuum. The residue was purified by HPLC to give 6 as white powder in 75% yield. 1H NMR (400 MHz, DMSO-d6) δ 10.47 (s, 1H), 8.74 (s, 1H), 8.54 (s, 1H), 8.29 (m, 1H), 8.16 (d, J = 8.8 Hz, 1H), 7.79 (d, J = 8.9 Hz, 1H), 7.29–7.22 (m, 15H), 7.09 (m, 1H), 4.41 (s, br, 1H), 4.13 (s, br, 1H), 4.03 (m, 1H), 3.93 (s, br, 2H), 3.64–3.24 (m, 11H), 3.20–2.64 (m, 12H), 2.34 (s, br, 2H), 1.76 (s, 1H), 1.60 (m, 1H), 1.51–1.22 (m, 40H). 13C NMR (101 MHz, DMSO-d6) δ 171.60, 171.10, 170.22, 162.96, 159.68, 159.34 (TFA), 155.78, 148.40, 144.93, 139.94, 137.28, 135.68, 129.74, 128.67, 128.44, 128.15, 127.41, 125.41, 125.13 (TFA), 118.44, 115.52, 114.16, 112.59, 112.02, 83.97, 81.74, 79.24, 66.61, 55.25, 54.99, 54.17, 53.94, 51.50, 48.90, 48.49, 36.40, 34.41, 32.12, 31.38, 29.30, 28.70, 28.23, 23.40. MS: calcd. for C16H15N2O4S2 [(M+H)+]: 834.3. LC-MS found: m/z 834.3.

**Synthesis of Intermediate 10.** A solution of 9 (0.11 mmol) in MeOH was added dropwise to a solution of 6 (0.1 mmol), tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 0.1 mmol), and DIPEA (0.6 mmol) in MeOH under Ar. After reaction at r.t. for additional 30 min, the solution was directly purified by HPLC to afford 10 in 60% yield. 1H NMR (400 MHz, CD3OD) δ 8.51 (s, 1H), 8.33 (d, J = 8.5 Hz, 1H), 7.95 (d, J = 9.4 Hz, 2H), 7.76 (d, J = 8.9 Hz, 1H), 7.68 (d, J = 8.4 Hz, 1H), 7.52 (dd, J = 9.3, 2.7 Hz, 1H), 7.47–7.16 (m, 16H), 5.34 (t, J = 9.5 Hz, 1H), 4.49 (m, 4H), 4.38 (m, 1H), 4.28–2.89 (m, 59H), 2.52 (m, 2H), 2.08 (m, 3H), 1.95–1.28 (m, 47H). 13C NMR (101 MHz, CD3OD) δ 173.07, 172.56, 171.30, 171.18, 171.06, 166.46, 165.17 (TFA), 160.85, 160.47, 159.74, 159.51, 156.42, 149.55, 146.49, 147.17, 138.88, 136.75, 136.62, 130.87, 130.49, 130.21, 129.54, 127.99, 126.92, 125.01, 124.09, 123.82, 120.45, 118.17, 117.75, 115.28 (TFA), 112.33, 105.84, 81.72, 79.89, 79.24, 66.99, 66.06, 54.64, 54.11, 51.93, 59.29, 38.97, 36.25, 35.01, 33.46, 31.67, 31.33, 28.94, 28.73, 28.54, 27.63, 27.44, 23.45, 22.93. MS: calcd. for C16H14NO3S2 [(M–H–)−]: 2129.0. MALDI-MS found: m/z 2129.2.

**Synthesis of Intermediate 11 (11-I and 11-II).** Compound 10 was treated with a solution of TFA/DCM/TIPSH (95/2.5/2.5, 5 ml) to remove all of the protected groups. After stirring at r.t. for 3 h, the solvent was removed under vacuum, and the residue was dissolved in MeOH (5 ml). 2-(Ethylidisulfanyl)-pyridine ((EtS)S-Py) (1.5 equiv) was then added, and the mixture was stirred at r.t. for another 1 h. After removal of MeOH under vacuum, cold Et2O was added. The precipitate was...
collected by centrifugation, followed by HPLC purification to give 11-II in 79% yield. In order to obtain 11-I for NMR analysis, we further incubated 11-II in PBS buffer (pH 7.4) under Ar at r.t. for additional 24 h to isomerize 11-II to 11-I. 11-I was obtained in 43% yield after HPLC purification.

11-I: $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.50 (s, 1H), 8.33 (d, $J$ = 6.5 Hz, 1H), 7.97 (dd, $J$ = 11.6, 9.3 Hz, 2H), 7.67 (d, $J$ = 8.5 Hz, 2H), 7.53 (dd, $J$ = 9.3, 2.7 Hz, 1H), 7.36 (d, $J$ = 2.6 Hz, 1H), 5.34 (t, $J$ = 9.4 Hz, 1H), 4.52 (m, 1H), 4.43–4.34 (m, 1H), 4.30 (m, 1H), 4.21 (t, $J$ = 5.9 Hz, 2H), 4.14–3.30 (m, 39H), 3.29–2.88 (m, 19H), 2.82 (q, $J$ = 7.3 Hz, 2H), 2.27–2.03 (m, 2H), 2.01–1.79 (m, 3H), 1.71 (m, 1H), 1.67–1.38 (m, 3H), 1.36 (t, $J$ = 7.3 Hz, 3H). $^{13}$C NMR (101 MHz, CD$_3$OD) $\delta$ 173.00, 171.53, 171.09, 167.70, 166.46, 161.56 (TFA), 160.87, 159.55, 159.41, 149.45, 144.01, 137.92, 136.72, 136.54, 130.79, 130.29, 130.10, 124.86, 124.11, 123.76, 121.18, 120.22, 118.28, 117.65, 115.37 (TFA), 112.18, 105.70, 79.21, 66.03, 55.05, 54.78, 54.09, 53.89, 53.72, 53.58, 53.34, 53.22, 52.88, 50.81, 50.00, 39.05, 39.00, 38.51, 36.23, 34.55, 31.80, 31.68, 31.47, 28.63, 23.22, 22.94, 13.47. MS: calculated for C$_7$H$_{20}$NgGd$_{2}$N$_{19}$O$_{19}$S$_4^+$ [(M+H)$^+$]: 1680.7; MALDI-MS found: m/z 1681.1.

**Synthesis of 1 (I-I and II-I).** To a solution of 1 in water was carefully added a solution of 0.5 M NaHCO$_3$ to adjust pH value to 7. Then, a solution of GdCl$_3$ (5 equiv. to MnO$_2$) was added to cells using 1 mL of buffer per 10 million cells. The reaction mixture was stirred at r.t. overnight. After removal of MnO$_2$ by filtration, the solvent was removed under vacuum, and the residue was purified by HPLC to afford 1-O in 67% yield (>99% HPLC purity). MS: calculated for C$_{75}$H$_{96}$Ga$_{2}$N$_{30}$O$_{60}$S$_4^+$ [(M+H)$^+$]: 1988.5; MALDI-MS found: m/z 1988.6.

**MR Measurement of $T_1$ Relaxivity.** A series of solutions containing 1-I, 1-II, or 1-O at five different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mM) in PBS buffer (pH 7.4) were incubated with or without 2 mM TCEP at r.t. overnight. Then, the solutions were placed in 250 μL Eppendorf tubes, bundled together, and MR imaged at 0.5, 1.5, and 3T (Sigma HDx, GE Healthcare, Waukesha, Wisconsin) at 37 °C. The scanning procedure began with a localizer and then consisted of a series of inversion-prepared fast spin–echo scans, identical in all aspects (TR 4000 ms, TE minimum, BW 15.6 kHz, field of view 6 cm, slice thickness 3 mm, matrix 128 × 128, NEX 1) except for the inversion time (TI) which was varied as follows: 4000, 2400, 1000, 600, 400, 300, 200, 100, and 50 ms. This scanning procedure produced enough data to fit quantitative $T_1$ relaxation values to each voxel in the image. For quantitative data analysis, the acquired MR images were transferred as DICOM images to an offline system, and signal intensities were extracted from each of the 5 samples at each of the 10 TI times by manual region of interest (ROI) placement and voxel averaging within the ROI.

Signal intensity versus TI relationships were fit to the following exponential $T_1$ recovery model by nonlinear least-squares regression: SI (TI) = $S_0 [1−2×exp(−TI/T_1) + exp(−TR/T_1)]$. Relaxation rates ($R_1$) were determined as $1/T_1$. Longitudinal relaxivities ($r_1$, units of mM$^{-1}$ s$^{-1}$) were calculated as the slope of $R_1$ vs [Gd] after the determination of true Gd concentration of each sample by the ICP-MS measurement.

**MR Detection of GSH Concentration.** 1-II (200 μM) in PBS buffer (10×) was incubated with various concentrations of GSH (0, 1, 10, 50, 100, 250, 500 μM, and 1 and 10 mM) at 37 °C overnight. After incubation, the solutions were transferred into NMR tubes (5 mm O.D.), and the $T_1$ value for each incubation solution was acquired at 0.5 T with Bruker Minispec (mq20 NMR analyzer) at 37 °C, using the standard inversion recovery program.

**Cell Culture.** HeLa human cervix adenocarcinoma epithelial cells and MDA-MB-468 human breast adenocarcinoma epithelial cells from American Type Culture Collection were cultured in Dulbecco’s modified eagle medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 U mL$^{-1}$ penicillin, and 100 μg mL$^{-1}$ streptomycin (GIBCO).

**Incubation of 1-I, 1-II, and 1-O in Cell Lysates.** The culture medium for adherent MDA-MB-468 (or HeLa) cells was carefully removed and the cells were washed with cold PBS buffer twice. RIPA (RadioImmuno Precipitation Assay) buffer was added to cells using 1 mL of buffer per 5 million cells. Then the cells were kept on ice for 1 h. The cell lysates were centrifuged at 14 000 rpm for 15 min at 4 °C. The supernatant of the cell lysates was collected and incubated with each probe (100 μM) at r.t. overnight, and then injected into an HPLC system for analysis.

**MR Detection of Reducing Environment in Cell Lysates with 1-II.** Cultured HeLa cells were trypsinized, washed, and centrifuged to form cell pellets. PBS buffer (10×) was added to the cell pellets using 1 mL of buffer per 10 million cells. The cells were lysed with three repeated cycles of freezing and thawing procedure. The cell lysates were then gathered, centrifuged at 14 000 × g for 15 min at 4 °C. The supernatant of the cell lysate was collected and incubated with 1-II (200 μM) at 37 °C overnight. For the inhibition experiment,
the collected cell lysates were incubated with 2 mM N-ethylmaleimide at r.t. for 30 min. 1-II was added at a final concentration of 200 µM, and the solution was incubated at 37 °C overnight. After incubation, the solutions were transferred into NMR tubes (5 mm O.D.), and the T_1 value for each incubation solution was acquired at 0.5 T with Bruker Minispec (mq20 NMR analyzer) at 37 °C, using the standard inversion recovery program.

**ASSOCIATED CONTENT**

**Supporting Information**

Supporting figures and tables, and NMR, HRMS, and MALDI-MS spectra of compounds are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


