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Synthesis and Comparative Evaluation of Photoswitchable Magnetic Resonance Imaging Contrast Agents

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ABSTRACT: A series of spiropyran (SP)-based magnetic resonance imaging (MRI) contrast agents have been synthesized and evaluated for changes in relaxivity resulting from irradiation with visible light. Both electron-donating and electron-withdrawing substituents were appended to the SP ring in order to study the electronic effects on the photochromic and relaxivity properties of these photoswitchable MRI contrast agents. Photoswitches lacking an electron-withdrawing substituent isomerize readily between the merocyanine and SP forms, while the addition of a nitro group prevents this process. Complexes capable of isomerizing were demonstrated to effect a change in the relaxivity of the appended gadolinium complex.

INTRODUCTION

Magnetic resonance imaging (MRI) represents one of the most common diagnostic tools in modern medicine, and it is widely used to explore the structural features in living systems because of its strengths for noninvasive and three-dimensional imaging with high spatial resolution.1 To further the advantages of MRI, there is interest in developing activatable contrast agents that can increase the MRI contrast between target tissues and their surroundings as a result of the agent’s response to specific biological processes.2,3 An early example was galactopyranosyl-substituted 1-hydroxyethyl-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic acid, which is an analogue of DOTA.4 The number of coordination sites for water changes when β-galactosidase enzymatically cleaves the galactopyranose moiety. In recent decades, much effort has been dedicated on the development of target-specific and activatable contrast agents. Kikuchi reported pH-responsive polymers achieved by the alteration of the molecular-tumbling rate.5 Herges reported a light-activatable MRI contrast agent based on an intramolecular light-driven coordination-induced spin state switch.6 Our lab previously developed a redox-sensitive spironaphthoxazine-based MRI contrast agent based on an intramolecular light-driven coordination-induced spin state switch.6

Photochromic compounds are a large class of compounds that can respond to light reversibly, and spiropyran (SP) is one of the most interesting subtypes.16 The photochromic behavior of SPs has been well-studied (Scheme 1a). The compound appears colorless or pale yellow in its closed-ring (SP) isomeric form, while an open-ring [merocyanine (MC)] isomer is generated with an optical absorption peak at 550−600 nm after UV irradiation or incubation in the dark.16 This new absorbance peak is due to the transformation of the photoswitch from its orthogonal configuration (SP form) to the planar one (MC form).17 In 2007 and 2009, we reported nitro- and dinitro-substituted SP-based contrast agents, where difference in the hydration number and a significant relaxivity change were observed before and after light irradiation.18,19 The responsiveness of the SP-based contrast agent to light encouraged further investigation of the relation between the chemical and photochromic properties. Although a comprehensive study in 2016 discussed the electronic effects on the photochromic behavior of free SPs, the chemical and photochromic properties could be significantly altered by...
conjugation with a Gd-coordinated DO3A ligand. As such, it is of great value to understand the electronic effects of substituents on photoswitchable MRI contrast agents. Herein, we report our recent efforts on the synthesis and detailed mechanistic investigation of light-sensitive SP-based MRI contrast agents in which the indoline substituents are varied in their ability to donate or withdraw electron density (Scheme 1b).

Scheme 1. (a) Photochromic Behavior of SPs; (b) Photochromic Behavior of Photoswitchable MRI Contrast Agents

a. Photochromic behavior of spiropyrans

b. This work

Scheme 2. Synthesis of Complex 12

Scheme 3. Synthesis of Complexes 23 and 24
RESULTS AND DISCUSSION

Synthesis of SPs Appended to DO3A. Photoswitchable MRI contrast agents were synthesized as illustrated in Scheme 2. Commercially available 4-methoxyphenol 1 was used as the starting material and afforded dihydroxymethylation product 2 in the presence of 37% formaldehyde aqueous solution and CaO, which was further oxidized into aldehyde 3 by MnO2. Indole 4 was treated with CH3I and a base to generate intermediate 6. Spirocyclic intermediate 7 was prepared in 79% yield after the reaction of 3 and 6 in refluxing EtOH. The benzylic hydroxyl group of this spirocycle 7 was converted to benzylic iodide 8 by successively chlorinating and displacing with iodide under Finkelstein conditions. The amination proceeded smoothly in the presence of Cs2CO3 and heating in acetonitrile with the t-butyl ester of DO3A (9). After deprotection and coordination to gadolinium, complex 12 was obtained and characterized by mass spectroscopy. The gadolinium content was determined by using a microwave plasma-atomic emission spectrometer (MP-AES). With a similar pathway, we also succeeded in making complexes 23 and 24 from NO2- and OCH3-substituted indolium salts, respectively (Scheme 3).

Photochromic Analysis. We investigated the photochromic properties of complexes 12, 23, and 24 (Figure 1). Both complexes 12 and 24 turned from yellow to purple after the coordination step with Gd, and both exhibited significant absorbance peaks at 510 and 545 nm, which indicated isomerization of the SP groups from the closed-ring SP to open-ring MC form and that the generated MC form was stabilized by Gd3+34. It is hypothesized that the stabilization is attributed to the newly formed interaction between phenolate oxygen and Gd3+. With visible light irradiation of the compound in aqueous solution for 1.5 min, 53 and 88% absorbance decrease at 510 and 545 nm was observed for complexes 12 and 24, respectively. The absorbance changes suggested that the SP form for the SPs was regenerated from the MC form. Complex 23 remained yellow even after coordination and exhibited no significant peak above 500 nm in the absorbance spectrum. Furthermore, no absorbance change was observed after visible light irradiation. These results indicated that complex 23 might not be responsive to light; furthermore, there was a minimal absorbance difference above 400 nm before and after coordination for 23. The electron-withdrawing nitro group on the indoline side of the SP appears to inhibit ring-opening. On the other hand, no significant absorbance above 500 nm was observed for free photoswitchable molecules without the Gd-DO3A complex.17 This suggested that the MC form could be stabilized through the coordination of Gd3+ and the phenolate anion.

Fluorescence measurements were also conducted for complexes 12 and 24 under different excitation wavelengths (Figure 2). The excitation wavelengths were chosen based on the MC absorbance peaks. Fluorescence peak decreases at 664 nm (complex 12) and 663 nm (complex 24) were observed after visible light irradiation, which further supports the conclusion drawn from the absorbance spectral experiments that the open-ring MC form can be isomerized to the closed-ring SP form after visible light irradiation.

Fluorescence spectra were also acquired for complexes 12 (510 nm) and 24 (545 nm) in nanopure water (pH = 7.4) at a concentration of 80 μM [Gd3+].

Figure 1. Absorbance spectra of complexes 12, 23, and 24 in nanopure water (pH = 7.4) at a concentration of 50 μM [Gd3+].

Figure 2. Fluorescence spectra using MC peak wavelength excitation of complexes 12 (510 nm) and 24 (545 nm) in nanopure water (pH = 7.4) at a concentration of 80 μM [Gd3+].
of the PET effect and the enhancement of SP fluorescence intensity.

Reversibility determination was performed for complexes 12 and 24 (Figure 4). New absorbance peaks appeared at 440 and 480 nm for complexes 12 and 24, respectively, after subsequent incubation in the dark at room temperature, which did not overlap with the original absorbance peak prior to light irradiation. We hypothesized that a block to reversibility, such as a twisted MC structure or intermediate, may have occurred.

Relaxivity and Statistical Analysis. The effect of irradiation on the longitudinal ($r_1$) relaxivity of complexes 12, 23, and 24 in an aqueous solution was evaluated as shown in Table 1. The $r_1$ values of the three compounds were $5.29 \pm 0.11$, $2.09 \pm 0.09$, and $2.79 \pm 0.05$ mM$^{-1}$ s$^{-1}$, respectively, under the dark conditions in water, pH = 7.4. Complex 12 exhibited larger relaxivity than complexes 23 and 24. This may be because the MC isomer being more stabilized for complex 12 under the dark conditions. Relaxivities for all complexes were determined statistically through a single gamma-generalized linear-mixed model with the identity link function (GGLMM-ID).

Based on the results of absorbance and fluorescence spectral experiments, the effect of light on the relaxivity properties of complexes 12 and 24 was investigated. The relaxivity change of complex 12 was larger than that of 24 after visible light irradiation. We propose that the methoxy group leads to a higher electron density on the indoline ring of complex 24, generating a stronger electrostatic interaction between Gd$^{3+}$ and indoline rings. The resulting closer distance between GdDO3A and the indoline "cap" makes it more difficult for water to access Gd$^{3+}$, inhibiting the relaxivity enhancement.

MR Imaging. The effect of visible light on the MR properties of complexes 12 and 24 was evaluated by MRI (Figure 5). $T_1$-weighted imaging was performed before and after 1.5-min-long visible light irradiation. After the irradiation, both complexes 12 and 24 exhibited a lower signal intensity, which indicates that the longitudinal relaxation time increases after visible light irradiation for both cases.

CONCLUSIONS

In this work, we have synthesized three SP-based MRI contrast agents. Carefully optimized conditions were required for the challenging nucleophilic substitution of the secondary amine to the benzyl iodides to provide SP-based DO3A ligands 11, 21, and 22 leading to complexes 12, 23, and 24. Compounds 12 and 24 exhibited photoswitching behavior upon irradiation with visible light, whereas compound 23, with a nitro-

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Table 1. $r_1$ Relaxivity before and after Visible Light Irradiation of Complexes 12, 23, and 24

| contrast agents | $r_1$ in the dark (mM$^{-1}$ s$^{-1}$) | $r_1$ light (mM$^{-1}$ s$^{-1}$) | $r_1$ change (%) | $p$-value |
|-----------------|-------------------------------------|--------------------------------|-----------------|-----------|
| complex 12      | $5.29 \pm 0.11$                     | $4.57 \pm 0.10$                | $13.4 \pm 1.7$  | $2.2 \times 10^{-13}$**  |
| complex 23      | $2.09 \pm 0.09$                     |                                |                 |           |
| complex 24      | $2.79 \pm 0.05$                     | $2.53 \pm 0.05$                | $9.3 \pm 1.6$   | $9.8 \times 10^{-4}$***  |

The relaxivity was determined using five gradient concentration sample solutions. Each concentration was measured three times with independently prepared solutions. Two-tail unpaired t-test was performed with complexes 12 and 24 before/after visible light irradiation. ***$p$-value less than 0.001
substituted indoline ring, did not. This effect probably originates from the reduced basicity of the indoline nitrogen. The differential behavior of 12 and 24 compared to the free photoswitches lacking the pendant Gd complex suggests that the MC form is stabilized by coordination of Gd\(^{3+}\) and the phenolate oxygen anion. Conjugation to the chelated gadolinium also prevented PET in 12 and 24 and allowed the SP form to exhibit fluorescence, suggesting close interaction with the nitrogen lone pair. This result is consistent with relaxivity measurements on 12 and 24, which demonstrate little change between the open and closed forms of the photoswitches. Although photoswitching between the SP and MC forms produces a dramatic structural change, both forms have strong Lewis basic interactions with the gadolinium complex, resulting in relatively small changes in relaxivity. This work represents the first example of studies on the electronic effect of substituents on the light-responsive MIRI contrast agents. Further work on the evaluation of other photoswitch-based MIRI contrast agents is underway.

**EXPERIMENTAL SECTION**

**General Experimental Methods.** All reagents were purchased from commercial sources and used without further purification unless stated otherwise. Solvents were dried over an activated alumina solvent system or purchased anhydrous where required. Reactions requiring anhydrous conditions were performed under argon; glassware was flame-dried under vacuum immediately prior to use and allowed to cool under reduced pressure; liquid reagents, solutions, or solvents were added via a syringe through rubber septa; solid reagents were reduced pressure; liquid reagents were added under an atmosphere of argon. Reactions were monitored by TLC on silica gel [230–400 mesh (40–63 µm)], unless otherwise stated. Accurate mass measurements were recorded in the positive ESI mode in CH\(_2\)OH or CH\(_3\)CN.

Extracts were concentrated in vacuo using both a rotary evaporator and a rotavap. The product filtrate and washes were filtered and washed with cold water. After drying under high vacuum, the product was obtained (4.76 g, 72%). The solid 2 was obtained as a brown oil (131.5 mg, 98% for 2 steps).

### 2-Hydroxy-3-(hydroxymethyl)-5-methoxybenzaldehyde (3).

To a 100 mL round bottom flask were added 4-methoxyphenol 1 (4.48 g, 36 mmol), HCHO (37 wt % in H\(_2\)O, 6.4 mL, 90 mmol), CaO (1.02 g, 18 mmol), and H\(_2\)O (30 mL). The mixture was stirred in the dark for 8 days. Glacial AcOH (4 mL) was then added and the reaction was heated until all the solid was dissolved. After cooling to ambient temperature, the reaction was placed in a freezer at −30 °C overnight. The precipitated pale yellow solid was then filtered and washed with cold water. After drying under high vacuum, the product was obtained (4.76 g, 72%). The solid 2 (921 mg, 5 mmol) was then dissolved in acetonitrile (50 mL), followed by the addition of MnO\(_2\) (2.16 g, 25 mmol). The reaction was stirred at room temperature for 18 h. After filtration and further purification by flash column chromatography, the product 3 was obtained as a yellow solid (243.7 mg, 27%). \(^1\)H NMR (599 MHz, CDCl\(_3\)): \(\delta\) 10.99 (s, 1H), 9.88 (s, 1H), 7.23 (s, 1H), 6.96 (s, 1H), 4.75 (s, 2H), 3.83 (s, 3H), 2.36 (s, 1H). \(^{13}\)C NMR (151 MHz, CDCl\(_3\)): \(\delta\) 196.5, 153.9, 152.8, 130.9, 123.9, 120.0, 114.5, 60.8, 56.1.

### (6-Methoxy-1′,3′,3′-trimethylspirol[chromene-2,2′-indo[1,2-b]pyrrole]-8-yl)methanol (7).

To a 50 mL round bottom flask were added 2,3,3-trimethyl-3H-indole 4 (398.1 mg, 2.5 mmol), CH\(_3\)I (0.23 mL, 3.75 mmol), and CH\(_3\)CN (15 mL). The mixture was then stirred at 83 °C for 16 h. After cooling to room temperature, the solvent was removed. The residue was dissolved in CHCl\(_3\) and hexane, which was sonicated for 30 min. After filtration, the iodide 5 was obtained (669.3 mg, 89%). The mixture of 5 (301.2 mg, 1 mmol) and H\(_2\)O (10 mL) was placed in an ice bath. KOH (101.0 mg, 1.8 mmol) was then added. The reaction was warmed to room temperature and stirred for 30 min. Upon completion, the reaction was extracted by ether (3 × 10 mL) and washed by brine. The combined organic phase was then dried over anhydrous Na\(_2\)SO\(_4\). After concentrating, compound 6 was obtained (122.8 mg, 71%). The mixture of 6 (71.8 mg, 0.41 mmol), 3 (75.5 mg, 0.41 mmol), and EtOH (4 mL) was reacted at 80 °C for 16 h. Upon the completion of the reaction, the product 7 was obtained (101.9 mg, 79%) after flash column chromatography (hexane/EtOAc = 5:1). \(^1\)H NMR (599 MHz, CDCl\(_3\)): \(\delta\) 7.15 (t, \(J = 7.5\) Hz, 1H), 7.07 (d, \(J = 7.2\) Hz, 1H), 6.88–6.80 (m, 2H), 6.69 (s, 1H), 6.58 (s, 1H), 6.50 (d, \(J = 7.7\) Hz, 1H), 5.76 (d, \(J = 10.2\) Hz, 1H), 4.50 (dd, \(J = 13.3, 5.8\) Hz, 1H), 4.34 (dd, \(J = 13.1, 7.7\) Hz, 1H), 3.76 (s, 3H), 2.68 (s, 3H), 1.98 (d, \(J = 7.3\) Hz, 1H), 1.31 (s, 3H), 1.19 (s, 3H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)): \(\delta\) 153.2, 147.9, 146.1, 136.8, 129.6, 127.8, 127.7, 121.6, 119.9, 119.6, 119.3, 114.6, 111.0, 107.1, 104.4, 61.8, 56.0, 51.4, 29.1, 25.9, 20.4. AMM (ESI-TOF) m/z: calcd for C\(_{22}\)H\(_{28}\)NO\(_4\)\(^+\) [M + H\(^+\)], 338.1751; found, 338.1741.

### Tri-tert-butyl 2,2′,2″-(10-((6-Methoxy-1′,3′,3′-trimethylspirol[chromene-2,2′-indo[1,2-b]pyrrole]-8-yl)methyl)-4,7,10-tetraazaacyclododecane-1,4,7-triyl)triacetate (10).

To a flame-dried round bottom flask were added 7 (102.9 mg, 0.3 mmol) and DCM (6 mL). SOCl\(_2\) (4 drops) was then added at 0 °C. After reacting for 30 min, the reaction was quenched by the saturated NaHCO\(_3\) solution (5 mL) and extracted with DCM (3 × 10 mL). The combined organic phase was then dried over anhydrous Na\(_2\)SO\(_4\) and concentrated. The residue was dissolved in acetonitrile (10 mL), followed by the addition of KI (199.2 mg, 1.2 mmol). Upon the completion of reaction after 22 h, the mixture was concentrated and dissolved in DCM. After filtration of the insoluble solid, the filtrate was concentrated and dried without further purification, affording compound 8 as a brown solid (131.5 mg, 98% for 2 steps).

### Compounds 3, 7, and 10 were purchased from commercial sources and used without further purification.
1H) for 2 steps). AMM (ESI-TOF) m/z: calcd for C_{47}H_{71}N_{6}O_{10}^{+} [M + H]^+), 368.2156; found, 368.2156.

**Synthesis of Compound 15.** To a round bottom flask were added 1,2,3,3-tetramethyl-5-nitro-3-methoxybenzaldehyde (50 mg) was added. TFA (2 mL) was then added. The mixture was stirred for another 30 min at room temperature. The solvent was evaporated in vacuo. The solid was dissolved in MeOH (0.2 mL), followed by the addition of nanopure water (2 mL) and Gd(OTf)₃ (2.0 mg equiv based on the yield of previous step). The pH of the solution was adjusted to 5.8–6.0 with 0.1 M NH₄OH and the solution was stirred at room temperature for 24 h. After the reaction, 5 g of Chelex 100 was added and the solution was stirred for another 30 min at room temperature. The solvent was collected using a 50 mL polypropylene conical tube after filtering out the solid residues and dried in a lyophilizer for 3 d to yield product 12 as a yellow solid. The final products were characterized with mass spectroscopy. AMM (ESI-TOF) m/z: calcd for complex 12 [M + H]^+, 821.2504; found [M + H]^+, 821.2513 and other gadolinium isotope patterns.

(5′,6-Dimethoxy-1′,3′,3′-trimethyl-5′-nitrospirol[cromene-2,2′-indolin]-8-yl)methanol (16). To a round bottom flask were added 5-methoxy-1,2,3-tetramethyl-3H-indol-1-ium (14) (82.8 mg, 0.25 mmol), 2-hydroxy-3-(hydroxymethyl)-5-methoxybenzaldehyde (Synthesis of Compound 15) (56.6 mg, 0.11 mmol) and DCM (6 mL). SOCl₂ (3 drops) was then added. The mixture was stirred at 80 °C for 18 h. After cooling to room temperature, the product 16 was obtained (79.9 mg, 87%) by flash column chromatography (hexane/ EtOAc = 4:1). AMM (ESI-TOF) m/z: calcd for complex 16 [M + H]^+, 866.2334; found [M + H]^+, 866.2336 and other gadolinium isotope patterns.
further purification, affording compound 18 as a brown solid (68.9 mg, 66% for 2 steps). Compound 18 (34.0 mg, 0.07 mmol) was dissolved in CH3CN (1 mL). Tri-tert-butyl 2,2′,2″- (1,4,7,10-tetraazaacyclododecane-1,4,7-triyl)triacetate 9 (24.4 mg, 0.05 mmol) and Na2CO3 (10.0 mg, 0.1 mmol) were then added. The mixture was reacted at 80 °C for 17 h. Upon the completion, the product 20 was obtained as a brown oil (30.6 mg, 75%) by flash column chromatography (DCM/MeOH = 30:1).1H NMR (599 MHz, CDCl3): δ 6.59 (s, 1H), 6.81 (d, J = 10.5 Hz, 1H), 6.73–6.64 (m, 2H), 6.56 (s, 1H), 6.37 (d, J = 7.5 Hz, 1H), 5.73 (d, J = 10.1 Hz, 1H), 3.80 (s, 3H), 3.75 (s, 3H), 3.48 (d, J = 12.5 Hz, 1H), 3.32 (d, J = 12.5 Hz, 1H), 3.15–1.98 (m, 25H), 1.51–1.40 (m, 27H), 1.22 (s, 3H), 1.15 (s, 3H).13C NMR (201 MHz, CDCl3): δ 173.8, 173.7, 172.9, 153.9, 152.8, 147.2, 142.2, 138.3, 129.7, 123.1, 119.8, 116.2, 112.5, 111.0, 109.9, 106.7, 82.9, 82.6, 82.3, 77.3, 56.2, 56.0, 55.9, 55.6, 51.1, 29.4, 28.1, 28.03, 28.02, 27.95, 20.4. AMM (ESI-TOF) m/z: calcd for C45H27NiO8+ [M + H]+, 864.5481; found, 864.5491.

**Synthesis of Compound 24.** Compound 20 (50 mg) was dissolved in DCM (0.2 mL), and TFA (2 mL) was then added to the solution. The solution was stirred at room temperature for 24 h. The solvent was evaporated in vacuo. The residue was taken up in methanol (3 × 5 mL) and each time the resulting solution was evaporated to dryness to give product 22 as a red solid. The product was dissolved in MeOH (0.2 mL), followed by the addition of nanopure water (2 mL) and Gd(OTf)3 (2.0 mmol) to achieve 3H, 1.15 (s, 3H). 13C NMR (201 MHz, CDCl3): δ 173.8, 173.7, 172.9, 153.9, 152.8, 147.2, 142.2, 138.3, 129.7, 123.1, 119.8, 116.2, 112.5, 111.0, 109.9, 106.7, 82.9, 82.6, 82.3, 77.3, 56.2, 56.0, 55.9, 55.6, 51.1, 29.4, 28.1, 28.03, 28.02, 27.95, 20.4. AMM (ESI-TOF) m/z: calcd for C45H27NiO8+ [M + H]+, 864.5481; found, 864.5491.

**Spectroscopic Analysis.** Solutions were prepared by dissolving the SP-based GdDO3A complex in nanopure water. The Gd content was determined by using a MP-AES (4210 MP-AES, Agilent Technologies, Malaysia). The solution was carefully adjusted to pH = 7.4 with 0.1 M NH4OH and the pH of the previous step). The pH of the solution was adjusted to 5.8−6.0 with 0.1 M NH4OH and the solution was stirred at room temperature for 24 h. After the reaction, 5 g of Chelex 100 was added and the solution was stirred for another 30 min at room temperature. The sample was collected using a 50 mL polypropylene conical tube after the completion, the product 22 was obtained as a brown oil.

**Relaxivity Measurements.** A series of aqueous sample solutions (0.2 mL each) with gradient gadolinium (complex 12: 0, 20, 39, 59, and 78 μM; complex 23: 0, 21, 42, 62, and 83 μM; and complex 24: 0, 27, 55, 109, and 136 μM) concentration were prepared and incubated in the dark for 18 h. T₁ relaxation time was measured on a 1.5 T Minispec relaxometer (Bruker) at 37 °C. The T₁ relaxation measurements were performed before and after irradiation with visible light for 1.5 min. Relaxivities for all complexes were determined statistically through a single GGLMM-ID.

**Statistical Analysis.** The gamma distribution was used to account for the heteroscedasticity with constant coefficient of variation in the data. Mixed models allow accounting for correlation of r_i in light off/on repeated measurement situation as well as correlation within the same trial. Furthermore, they allow us to account for unobserved nuisance factors such as light positioning from the tube to tube or correlated concentration errors within the same trial. A random intercept was fitted for each Minispec tube ID and a random slope for each trial. Fixed effects included concentration, SP, and light status (on/off). Bonferroni-corrected Wald Z tests were performed on the fitted model to obtain p-values for the change in relaxivity for complexes 12 and 24 when exposed to light.

**MR Imaging.** T₁-weighted MR images of complexes 12 and 24 were taken on a Biospec 7 T (300 MHz) system (Bruker, Billerica, MA) in an aqueous solution at room temperature. Aqueous solutions of complex 12 and 24 were prepared and stored in the dark for 18 h before imaging. Images were taken before and after irradiation with visible light for 1.5 min. Pulse sequence: MSME, TR = 50 ms, and TE = 15 ms. Relaxivities for all complexes were measured before and after light irradiation.
Author Contributions
M.G. and B.S. contributed equally.

Notes
The authors declare no competing financial interest.

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