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Synthesis of Tumor-Avid Photosensitizer-Gd(III)DTPA Conjugates: Impact of the Number of Gadolinium Units in T₁/T₂ Relaxivity, Intracellular localization and Photosensitizing Efficacy

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Abstract

To develop novel bifunctional agents for tumor imaging (MR) and photodynamic therapy (PDT) certain tumor-avid photosensitizers derived from chlorophyll-a were conjugated with variable number of Gd(III)aminobenzyl DTPA moieties. All the conjugates containing three or six gadolinium units showed significant T₁ and T₂ relaxivities. However, as a bifunctional agent, the 3-(1'-hexyloxyethyl)pyropheophorbide-a (HPPH) containing 3Gd(III) aminophenyl DTPA was most promising with possible applications in tumor-imaging and PDT. Compared to HPPH, the corresponding 3- and 6Gd(III)aminobenzyl DTPA conjugates exhibited similar electronic absorption characteristics with a slightly decreased intensity of the absorption band at 660 nm. However, compared to HPPH, the excitation of the broad "Soret" band (near 400 nm) of the corresponding 3Gd(III)aminobenzyl-DTPA analogs showed a significant decrease in the fluorescence intensity at 667 nm, which was further diminished by increasing the number of Gd(III)units.

INTRODUCTION

Magnetic resonance imaging (MRI) is a medical imaging technique (1) commonly used in radiology to visualize the internal structure and function of the body. MRI provides greater contrast between the different soft tissues of the body than computed tomography (CT), making it especially useful in neurological (brain), musculoskeletal, cardiovascular, and oncological (cancer) imaging (2). However, MR imaging has some practical disadvantages. For example, benign tumors may be difficult to distinguish from malignant lesions. The fibrocystic disease of the breast, fibroadenomas, sclerosing adenosis, atypical hyperplasia, lobular carcinoma in situ (LCIS), and breast papillomas can all produce contrast enhancement patterns that are hard to distinguish from malignant processes. In addition, some benign processes such as proliferative dysplasia, inflammation, wounds, and benign tumors can enhance in a similar fashion to malignant tumors (3). These shortcomings of MRI in measuring tissue properties offer opportunities that may be satisfied with information provided by optical fluorescence imaging with near-infrared light (4). Optical techniques offer the potential to contribute greatly to the expansion of clinical multimodality techniques. Their ability to image structural, functional and molecular information at different spatial and temporal scales makes them very attractive. In this case, the multimodality approach can be used as the combination of multiple

Supporting Information Available: The ¹H NMR spectra of selected compounds and the intracellular localization profiles of the conjugates. This material is available free of charge via the Internet at <http://pubs.acs.org>.

optical techniques in one instrument and/or the fusion of an optical technique with another well-established clinical modality, such as PET, CT or MRI. (5,6).

Clinically, optical imaging modality has been employed to enable or improve PDT (7). Most of the porphyrin-based photosensitizers generally fluoresce and the fluorescent nature of these porphyrins has been exploited for the detection of early-stage cancers in the lung, bladder and other sites (8). In addition, for treatment of early disease or for deep seated tumors the fluorescence can be used to guide the activated light. Most of the porphyrin-based compounds are not optimal fluorophores for tumor detection for several reasons, especially a small shift between the long-wavelength absorption and emission bands, which make it technically difficult to separate the fluorescence from excitation wavelength (9). However, certain pyropheophorbide-a analogs (e. g., HPPH) produce very high fluorescence quantum yields and exciting them at their long wavelength absorption (665 nm) produces two emission bands; a strong peak at 670 nm, and a weaker band at 720 nm. This characteristic of HPPH has been used for *in vivo* fluorescence imaging of canine and other tumors (10). In recent years, integrating the strengths of each imaging modality has become a major research area. The research in our laboratory has also been focused in designing certain porphyrin-based compounds as “Multifunctional Agents” for tumor imaging and photodynamic therapy (PDT) (11-15).

We have previously shown that HPPH (16-21) a tumor-avid photosensitizer (currently undergoing Phase I/II human clinical trials) on conjugating with Gd(III)aminobenzyl DTPA [Gd(III)ADTPA] can be used for both tumor MR imaging and PDT (22,23). In our initial study, among the compounds investigated, the conjugates with two Gd(III)ADTPA moieties showed greater tumor-contrast than the corresponding mono- Gd(III)ADTPA derivative. However, poor solubility of these agents in various clinically acceptable formulations restricted their use for detailed *in vivo* studies, including toxicity. To investigate the effect of increased number of Gd(III)ADTPA moieties in tumor imaging/PDT and also to improve water solubility, we decided to extend this approach further and a series of tumor-avid long-wavelength absorbing chlorophyll-a analogs (pyropheophorbide-a and purpurinimide) containing three- or six Gd (III)ADTPA moieties with variable lipophilicity were synthesized. For developing “bifunctional agents” with longer wavelength absorption than HPPH. ($\lambda_{\max}$: 660 nm) a related conjugate containing a highly tumor-avid purpurinimide ($\lambda_{\max}$: 700nm) (24) was also prepared. In this manuscript the synthesis, photophysical properties, T1/T2 relaxivity and *in vitro* photosensitizing efficacy of a series of novel photosensitizer-Gd(III)ADTPA conjugates are discussed. The second part of our study (see the following paper in this issue) is focused in selecting the optimal “Multifunctional Agent” for tumor-imaging (MRI, fluorescence imaging) and PDT. *In vivo* PDT response is discussed in terms of long-term cure rate and monitoring of the vascular response *via* immunohistochemistry. Finally, the biodistribution and organ specific toxicity of the lead compound (C-14 labeled) at variable time points is also discussed.

EXPERIMENTAL PROCEDURES

Chemistry

The synthetic intermediates and the final products were characterized by NMR (400 MHz), mass spectrometry (EIMS and HRMS) and elemental analyses. ^1H NMR spectra were recorded on a Bruker AMX-400 spectrometer. Chemical shifts are expressed in δ ppm. All photo physical experiments were carried out using spectroscopic grade solvents. The reactions were monitored by TLC and/or spectrophotometrically. Column chromatography was performed either over Silica Gel 60 (70-230 mesh) or neutral Alumina (Brockmann grade III, 50 mesh). UV-visible spectrums were recorded on Varian Cary 50 Bio UV-visible spectrophotometer using dichloromethane as solvent unless otherwise specified. Fluorescence spectra were

recorded on a Varian Cary Eclipse Fluorescence spectrophotometer with an excitation wavelength in the “Soret” band region between 410 – 425 nm.

Compound No. 1—HPPH (100.0 mg, 0.157 mmol), amine **A** (97.8 mg, 0.235 mmol), EDCI (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride); (60.2 mg, 0.314 mmol) and DMAP (4-dimethylaminopyridine); (38.36 mg, 0.314 mmol) were taken in a dry round-bottom flask (100 ml). Dry dichloromethane (30 ml) was added and the reaction mixture was stirred at room temperature for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over silica gel using 1-3% methanol/dichloromethane mixture as eluent to give product **1**. Yield: 130.0 mg (80.0%). UV-vis (λ_{max} , dichloromethane): 318, 412, 506, 537, 604 & 660 nm. ¹HNMR (400MHz, CDCl₃): δ 9.78 (s, 1H, H-5), 9.41 (s, 1H, H-10), 8.54 (s, 1H, H-20), 5.96-5.91 (m, 2H, CH₃CHOhexyl, NH), 5.33 (d, 1H, CH-15¹, J = 19.6 Hz), 5.14 (d, 1H, CH-15¹, J = 19.6 Hz), 4.53 (q, 1H, H-17, J = 7.2 Hz), 4.32 (m, 1H, H-18), 3.71-3.67 (m, 2H, -OCH₂-hexyl), 3.63-3.60 (m, 2H, 8-CH₂CH₃), 3.52 (s, 3H, 7-CH₃), 3.39 (s, 3H, 2-CH₃), 3.27 (s, 3H, 12-CH₃), 2.67 (m, 1H, CH-17²), 2.37 (m, 1H, H-17²), 2.26 (m, 1H, H-17¹), 2.15-2.13 (m, 9H, 3CH₂-chain & CH₃CHOhexyl), 1.97 (m, 1H, H-17¹), 1.92 (t, 6H, 3CH₂-chain, J = 7.6 Hz), 1.80 (d, 3H, 18-CH₃, J = 7.2 Hz), 1.73 (m, 2H, CH₂hexyl), 1.66 (t, 3H, 8-CH₂CH₃, J = 7.6 Hz), 1.44 (m, 2H, CH₂hexyl), 1.30 (s, 27H, 3CO₂^tBu), 1.24 (m, 4H, 2CH₂hexyl), 0.78 (t, 3H, CH₃hexyl, J = 6.8Hz), 0.04 (brs, 1H, NH), -1.68 (brs, 1H, NH). EIMS: 1035 (MH⁺). HRMS: Calculated for C₆₁H₈₇N₅O₉: 1033.6503, found: 1034.6564 (MH⁺).

Compound No. 2—Compound **1** (73.0 mg, 0.07 mmol) was stirred with 70% Trifluoroacetic acid (TFA) / dichloromethane (DCM, 5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace of TFA. To this crude preparation were added, amino-benzyl-DTPA-penta-*tert*-butyl ester (219.0 mg, 0.282 mmol), EDCI (67.0 mg, 0.352 mmol) and DMAP (43.0 mg, 0.352 mmol). Dry dichloromethane (30 ml) was added and the reaction mixture was stirred at RT for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over alumina G (III) using 1-3% methanol/dichloromethane mixture as eluent to give product **2**. Yield: 130.0 mg (58.47%). UV-vis (λ_{max} , dichloromethane): 319, 411, 506, 537, 604, 661. ¹HNMR (400MHz, CDCl₃): δ 9.74 (splitted s, 1H, H-5), 9.36 (splitted s, 1H, H-10), 8.54 (splitted s, 1H, H-20), 8.17 (brs, 1H, NH), 7.74 (m, 1H, Ph-DTPA), 7.61-7.56 (m, 2H, Ph-DTPA), 7.43 (m, 4H, Ph-DTPA), 7.12 (m, 5H, Ph-DTPA), 6.96 (brs, 1H, NH), 5.96 (m, 1H, CH₃CHOhexyl), 5.29 (d, 1H, CH-15¹, J = 19.2 Hz), 5.11 (d, 1H, CH-15¹, J = 19.2 Hz), 4.47 (m, 1H, H-17), 4.26 (m, 1H, H-18), 3.64 (m, 2H, & OCH₂hexyl), 3.54 (m, 2H, 8-CH₂CH₃), 3.45-3.38 (m, 30H, 15CH₂-DTPA), 3.36 (s, 3H, 7-CH₃), 3.32 (s, 3H, 2-CH₃), 3.22 (s, 3H, 12-CH₃), 3.12 (m, 3H, CH-DTPA), 2.84-2.70 (m, 19H, 9CH₂-DTPA & CH-17²), 2.59 (m, 6H, 6CH₂-benzyl), 2.47-2.41 (m, 8H, 3CH₂-chain, CH-17² & CH-17¹), 2.19-2.15 (m, 9H, 3CH₂-chain, CH₃CHOhexyl), 2.06 (d, 3H, 18-CH₃, J = 7.6 Hz) 2.00 (m, 1H, CH-17¹), 1.74 (m, 4H, 2CH₂-hexyl), 1.66 (t, 3H, 8-CH₂CH₃, J = 7.2 Hz), 1.60 (m, 135H for 15 CO₂^tBu), 1.26 (m, 4H, 2CH₂-hexyl), 0.77 (m, 3H, CH₃-Ohexyl), 0.55 (brs, 1H, NH), -0.24 (brs, 1H, NH). HRMS: Calculated for C₁₇₂H₂₆₇N₁₇O₃₆: 3149.053, found: 3150.10 (MH⁺).

Compound No. 3—Compound **2** (110.0 mg, 0.034 mmol) was stirred with 70% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. The crude preparation thus obtained was dissolved in pyridine (10 ml) and while stirring, GdCl₃·6H₂O (77.9 mg, 0.21 mmol) in 1 ml of water was added slowly and the resultant mixture was stirred for 16 hr. The reaction mixture was

concentrated to dryness under high vacuum. The residue was washed with water (10 ml $\times$ 3), acetone (10 ml $\times$ 3) and finally dried under high vacuum using P₂O₅ as drying agent. Yield: 75.0 mg (77.55%). UV-vis (λ_{max} , MeOH): 620, 408, 504, 537, 604 & 660 nm. Elemental analysis: Calculated for C₁₁₃H₁₅₁Gd₃N₁₇O₃₆: C, 48.55; H, 5.44; Gd, 16.88; N, 8.52; O, 20.61, found: C, 48.68; H, 5.49; N, 8.57.

Compound No. 5—Acid **4** (100.0 mg, 0.143 mmol), amine **A** (89.1 mg, 0.214 mmol), EDCI (54.9 mg, 0.28 mmol) and DMAP (34.9 mg, 0.28 mmol) were taken in a dry round bottom flask (RBF, 100 ml). Dry dichloromethane (30 ml) was added to it and the reaction mixture was stirred at RT for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, the organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over silica gel using 1-2% methanol/dichloromethane mixture as eluent to give product **5**. Yield: 134.0 mg (85.3%). UV-vis (λ_{max} nm, dichloromethane): 318, 411, 506, 536, 604 & 661. ¹HNMR (400MHz, CDCl₃): δ 9.76 (splitted s, 1H, H-5), 9.38 (splitted s, 1H, H-10), 8.55 (s, 1H, H-20), 6.02 (d, 1H, CH₃CHOOTEG, J = 6.4 Hz), 5.99 (brs, 1H, NH), 5.34 (d, 1H, CH-15¹, J = 20.0 Hz), 5.15 (d, 1H, CH-15¹, J = 20.0 Hz), 4.58 (q, 1H, H-17, J = 6.8 Hz), 4.33 (m, 1H, H-18), 3.88-3.75 (m, 4H, 2CH₂-O-TEG), 3.70-3.62 (m, 6H, 3CH₂-O-TEG), 3.55 (m, 2H, 8-CH₂CH₃), 3.47-3.44 (m, 2H, CH₂-O-TEG), 3.40 (s, 3H, 7-CH₃), 3.39 (s, 3H, 2-CH₃), 3.29 (s, 3H, 12-CH₃), 3.27 (s, 3H, CH₃-O-TEG), 2.69 (m, 1H, CH-17²), 2.39 (m, 1H, CH-17²), 2.31 (m, 1H, CH-17¹), 2.14 (m, 8H, 4CH₂-chain), 2.00 (m, 1H, CH-17¹), 1.92 (m, 7H, 2CH₂-chain, CH₃CHOTEG), 1.82 (d, 3H, 18-CH₃, J = 7.2 Hz), 1.68 (t, 3H, 8-CH₂CH₃, J = 7.6 Hz), 1.31 (s, 27H, 3CO₂^tBu), 0.42 (brs, 1H, NH), -1.69 (brs, 1H, NH). EIMS: 1097 (MH⁺). Calculated for C₆₂H₈₉N₅O₁₂: 1095.6507, found: 1096.6571 (MH⁺).

Compound No. 6—Compound **5** (100.0 mg, 0.091 mmol) was stirred with 80% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. To this crude preparation were added, amino-benzyl-DTPA-penta-*tert*-butyl ester (285.0 mg, 0.365 mmol), EDCI (105.0 mg, 0.54 mmol) and DMAP (66.8 mg, 0.54 mmol). Dry dichloromethane (30 ml) was added and the reaction mixture was stirred at RT for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, the organic layer was separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over alumina Gr(III) using 1-3% methanol/dichloromethane mixture as eluent to give product **6**. Yield: 250.0 mg (85.3%). UV-vis (λ_{max} nm, dichloromethane): 319, 411, 506, 537, 606, 661. ¹HNMR (400MHz, CDCl₃): δ 9.70 (splitted s, 1H, H-5), 9.44 (splitted s, 1H, H-10), 9.37 (brs, 1H, NH), 9.20 (brs, 1H, NH), 8.56 (m, 1H, NH), 8.47 (s, 1H, H-20), 7.77 (m, 1H, Ph-DTPA), 7.59 (m, 2H, Ph-DTPA), 7.44 (m, 2H, Ph-DTPA), 7.10 (m, 6H, Ph-DTPA), 6.81 (m, 1H, Ph-DTPA), 5.97 (m, 1H, CH₃CHOTEG), 5.20 (m, 2H, CH₂-15¹), 4.60 (m, 1H, H-17), 4.22 (m, 1H, H-18), 3.81-3.64 (m, 4H, 2CH₂-OTEG), 3.57-3.50 (m, 4H, 8-CH₂CH₃, CH₂-OTEG), 3.60 (m, 6H, 3CH₂OTEG), 3.38 (s, 30H, 15CH₂-DTPA), 3.35 (s, 3H, 7-CH₃), 3.23 (m, 6H, 12-CH₃, OCH₃-TEG), 3.04 (m, 3H, 3CH-DTPA), 2.70 (m, 19H, 9CH₂-DTPA, CH-17²), 2.55 (m, 7H, 3CH₂-benzyl & CH-17²), 2.32 (t, 6H, 3CH₂-chain, J = 6.8 Hz), 2.23 (m, 1H, CH-17¹), 2.10 (d, 3H, CH₃CH-OTEG, J = 6.4 Hz), 2.01 (m, 1H, CH-17¹), 1.77 (d, 3H, 18-CH₃, J = 7.2 Hz), 1.68 (t, 3H, 8-CH₂CH₃, J = 7.6 Hz), 1.59 (t, 6H, 3CH₂-chain, J = 6.4 Hz), 1.44 (m, 135H, 15CO₂^tBu), -1.75 (brs, 1H, NH). HRMS: Calculated for C₁₇₃H₂₆₉N₁₇O₃₉: 3211.077, found: 3212.20 (MH⁺).

Compound No. 7—Compound **6** (226.0 mg, 0.07 mmol) was stirred with 80% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. The crude mixture thus obtained was dissolved in pyridine (10 ml) and while stirring, GdCl₃.6H₂O (156.9 mg, 0.422 mmol) in 1 ml of water was added

slowly and the resultant mixture was stirred for 16 hr. The reaction mixture was concentrated to dryness under high vacuum. The residue was washed with water (10 ml $\times$ 3), acetone (10 ml $\times$ 3) and finally dried under high vacuum using P₂O₅ as drying agent. Yield: 165.0 mg (82.5%). UV-vis (λ_{max} nm, MeOH): 320, 408, 505, 537, 605 and 660 nm. Elemental analysis: Calculated for C₁₁₃H₁₄₉Gd₃N₁₇O₃₉: C, 47.77; H, 5.29; Gd, 16.60; N, 8.38; O, 21.96, found: C, 47.85; H, 5.30; N, 8.43.

Compound No. 9—Acid **8** (82.0 mg, 0.118 mmol), amine **A** (73.6 mg, 0.177 mmol), EDCI (45.3 mg, 0.236 mmol) and DMAP (28.8 mg, 0.236 mmol) were taken in a dry RBF (100 ml). Dry dichloromethane (30 ml) was added and the reaction mixture was stirred at RT for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, the organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over silica gel using 1-2% methanol/dichloromethane mixture as eluent to give product **9**. Yield: 90.0 mg (69.8%). UV-vis (λ_{max} , dichloromethane): 365, 418, 509, 545 and 699 nm. ¹HNMR (400MHz, CDCl₃): δ 9.75 (splitted s, 1H, meso-H), 9.64 (s, 1H, meso-H), 8.55 (s, 1H, meso-H), 6.25 (m, 1H, CONH), 5.79 (q, 1H, CH₃CHObutyl, J = 6.4 Hz), 5.34 (m, 1H, H-17), 4.52 (t, 2H, -NCH₂butyl, J = 7.2 Hz), 4.42 (m, 1H, H-18), 3.84 (s, 3H, ring-CH₃), 3.70-3.59 (m, 4H, -OCH₂-butyl, 8-CH₂CH₃), 3.32 (s, 3H, ring-CH₃), 3.17 (s, 3H, ring-CH₃), 2.61 (m, 1H, CH-17²), 2.43 (m, 1H, H-17²), 2.27 (m, 1H, H-17¹), 2.20 (t, 6H, 3CH₂-chain, J = 7.2 Hz), 2.06 (m, 3H, CH₃CHObutyl), 2.01 (t, 6H, 3CH₂-chain, J = 7.6 Hz), 1.82 (m, 1H, H-17¹), 1.75 (d, 3H, 18-CH₃, J = 6.0 Hz), 1.68 (t, 3H, 8-CH₂CH₃, J = 7.6 Hz), 1.62 (m, 8H, 4CH₂butyl), 1.34 (s, 27H, 3CO₂^tBu), 1.10 (t, 3H, CH₃-obutyl, J = 7.2 Hz), 0.87 (t, 3H, CH₃-Nbutyl, J = 7.2 Hz), 0.40 (brs, 1H, NH), -0.06 (brs, 1H, NH). EIMS: 1092 (MH⁺). HRMS: Calculated for C₆₃H₉₀N₆O₁₀: 1090.6718, found: 1091.6823 (MH⁺).

Compound No. 10—Compound **9** (80.0 mg, 0.073 mmol) was stirred with 70% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. To this crude mixture were added, amino-benzyl-DTPA-penta-*tert*-butyl ester (286.0 mg, 0.366 mmol), EDCI (84.4 mg, 0.44 mmol) and DMAP (53.7 mg, 0.44 mmol). Dry dichloromethane (30 ml) was added and the reaction mixture was stirred at RT for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, the organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over alumina G (III) using 1-3% methanol/dichloromethane mixture as eluent to give product **10**. Yield: 140.0 mg (59.57%). UV-vis (λ_{max} , dichloromethane): 365, 418, 509, 546, 699 nm. ¹HNMR (400MHz, CDCl₃): δ 9.75 (splitted s, 1H, meso-H), 9.63 (splitted s, 1H, meso-H), 9.33 (brs, 1H, NH), 8.60 (splitted s, 1H, meso-H), 7.61 (m, 2H, Ph-DTPA), 7.58 (m, 1H, Ph-DTPA), 7.37 (m, 3H, Ph-DTPA), 7.12 (m, 5H, Ph-DTPA), 6.84 (m, 1H, Ph-DTPA), 5.76 (m, 1H, CH₃CHObutyl), 5.39 (m, 1H, H-17), 4.45 (m, 3H, H-18 & NCH₂butyl), 3.82 (s, 3H, ring-CH₃), 3.65 (m, 4H, 8-CH₂CH₃ & OCH₂butyl), 3.38 (m, 22H, 11CH₂-DTPA), 3.31 (m, 11H, 4CH₂-DTPA & ring-CH₃), 3.17 (s, 3H, ring-CH₃), 3.03 (m, 3H, CH-DTPA), 2.84-2.61 (m, 19H, 9CH₂-DTPA & CH-17²), 2.47 (m, 8H, 6CH₂-benzyl & CH-17² & CH-17¹), 2.20 (m, 6H, 3CH₂-chain), 2.04 (d, 3H, CH₃CHObutyl, J = 6.8 Hz), 1.96 (m, 6H, 3CH₂-chain), 1.84 (m, 1H, CH-17¹), 1.73 (s, 3H, 17-CH₃), 1.67 (t, 3H, 8-CH₂CH₃, J = 7.2 Hz), 1.60 (m, 4H, 2CH₂-Obutyl), 1.41 (m, 135H for 15 CO₂^tBu), 1.37 (m, 4H, 2CH₂-N-butyl), 1.03 (t, 3H, CH₃-Obutyl, J = 6.8 Hz), 0.86 (t, 3H, CH₃-N-butyl, J = 6.8 Hz), -0.09 (brs, 1H, NH). HRMS: Calculated for C₁₇₄H₂₇₀N₁₈O₃₇: 3206.104, found: 3207.250 (MH⁺).

Compound No. 11—Compound **10** (130.0 mg, 0.04 mmol) was stirred with 70% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. The crude preparation thus obtained was dissolved in pyridine (10 ml) and while stirring, GdCl₃.6H₂O (90.3 mg, 0.243 mmol) in 1 ml of water was

added slowly and the resultant mixture was stirred for 16 hr. The reaction mixture was concentrated to dryness under high vacuum. The residue was washed with water (10 ml $\times$ 3), acetone (10 ml $\times$ 3) and finally dried under high vacuum using P₂O₅ as drying agent. Yield: 80.0 mg (69.9%). UV-vis (λ_{max} , MeOH): 364, 415, 546 and 700 nm. Elemental analysis: Calculated for C₁₁₄H₁₅₀Gd₃N₁₈O₃₇: C, 48.28; H, 5.33; Gd, 16.63; N, 8.89; O, 20.87, found: C, 48.14; H, 5.40; N, 8.93.

Compound No. 12—HPPH (100.0 mg, 0.157 mmol), Di-*tert*-butyl iminodiacetate (77.0 mg, 0.314 mmol), EDCI (60.2 mg, 0.314 mmol) and DMAP (38.36 mg, 0.314 mmol) were taken in a dry RBF (100 ml). Dry dichloromethane (30 ml) was added and the reaction mixture was stirred at room temperature for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, the organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over silica gel using 1-1.5% methanol/dichloromethane mixture as eluent to give product **12**. Yield: 120.0 mg (88.3%). UV-vis (λ_{max} nm, dichloromethane): 317, 411, 506, 538, 605 and 660 nm. ¹HNMR (400MHz, CDCl₃): δ 9.80 (s, 1H, H-5), 9.51 (s, 1H, H-10), 8.55 (s, 1H, H-20), 5.94 (q, 1H, CH₃CHOhexyl, J = 6.4 Hz), 5.33 (d, 1H, CH-15¹, J = 20.0 Hz), 5.17 (d, 1H, CH-15¹, J = 20.0 Hz), 4.52 (q, 1H, H-17, J = 7.6 Hz), 4.41 (m, 1H, H-18), 4.04 (m, 2H, CH₂chain), 3.75 (m, 2H, -OCH₂-hexyl), 3.67 (s, 3H, 7-CH₃), 3.62 (m, 2H, 8-CH₂CH₃), 3.42 (s, 3H, 2-CH₃), 3.37 (m, 2H, CH₂chain), 3.29 (s, 3H, 12-CH₃), 2.77 (m, 1H, CH-17²), 2.46 (m, 1H, CH-17²), 2.16 (m, 1H, CH-17¹), 2.13 (m, 3H, & CH₃CHOhexyl), 1.97 (m, 1H, CH-17¹), 1.84 (d, 3H, 18-CH₃, J = 7.2 Hz), 1.78 (m, 2H, CH₂hexyl), 1.72 (t, 3H, 8-CH₂CH₃, J = 7.6 Hz), 1.49 (s, 9H, CO₂^tBu), 1.46 (m, 2H, CH₂hexyl), 1.45 (s, 9H, CO₂^tBu), 1.25 (m, 4H, 2CH₂hexyl), 0.8 (t, 3H, CH₃hexyl, J = 6.8Hz), 0.42 (brs, 1H, NH), -1.7 (brs, 1H, NH). EIMS: 865 (MH⁺) (25).

Compound No. 13—Compound **12** (120.0 mg, 0.139 mmol) was stirred with 70% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. To this crude preparation were added, amine **A** (144.2 mg, 0.34 mmol), EDCI (106.6 mg, 0.556 mmol) and DMAP (67.8 mg, 0.556 mmol). Dry dichloromethane (30 ml) was added and the reaction mixture was stirred at RT for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over silica gel using 2-6% methanol/dichloromethane mixture as eluent to give product **13**. Yield: 190.0 mg (88.3%). UV-vis (λ_{max} , dichloromethane): 318, 411, 506, 537, 605 and 660 nm. ¹HNMR (400MHz, CDCl₃): δ 9.78 (s, 1H, H-5), 9.52 (s, 1H, H-10), 8.3 (s, 1H, H-20), 8.27 (brs, 1H, NH), 8.17 (brs, 1H, NH), 6.59 (brs, 1H, NH), 5.90 (m, 1H, CH₃CHOhexyl), 5.34 (d, 1H, CH-15¹, J = 20.0 Hz), 5.15 (d, 1H, CH-15¹, J = 20.0 Hz), 4.53 (q, 1H, H-17, J = 6.0 Hz), 4.36 (m, 1H, H-18), 3.77 (m, 2H, -OCH₂-hexyl), 3.69 (m, 2H, 8-CH₂CH₃), 3.67 (s, 3H, 7-CH₃), 3.61 (m, 4H, 2CH₂chain), 3.36 (s, 3H, 2-CH₃), 3.26 (s, 3H, 12-CH₃), 2.77 (m, 1H, CH-17²), 2.66 (m, 1H, CH-17²), 2.52 (m, 1H, CH-17¹), 2.23 (m, 12H, 6CH₂-chain), 2.11 (d, 3H, CH₃CHOhexyl, J = 6.4 Hz), 2.07 (m, 6H, 3CH₂-chain), 1.95 (m, 6H, 3CH₂-chain), 1.93 (m, 1H, CH-17¹), 1.81 (d, 3H, 18-CH₃, J = 7.2 Hz), 1.75 (m, 2H, CH₂hexyl), 1.71 (t, 3H, 8-CH₂CH₃, J = 8.0 Hz), 1.43 (m, 2H, CH₂hexyl), 1.41 (s, 27H, 3CO₂^tBu), 1.32 (s, 27H, 3CO₂^tBu), 1.24 (m, 4H, 2CH₂hexyl), 0.77 (t, 3H, CH₃hexyl, J = 6.8Hz). EIMS: 1548 (MH⁺). HRMS: Calculated for C₈₇H₁₃₁N₇O₁₇: 1545.9601, found: 1545.9574 (M⁺).

Compound No. 14—Compound **13** (100.0 mg, 0.064 mmol) was stirred with 80% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. To this crude preparation were added, amino-benzyl-DTPA-penta-*tert*-butyl ester (503.5 mg, 0.64 mmol), EDCI (123.9 mg, 0.64 mmol) and DMAP (78.8 mg, 0.64 mmol). Dry *N,N*-dimethylformamide (15 ml) was added and reaction mixture

was stirred at RT for 16 hr under N₂ atm. The reaction mixture was concentrated under vacuum, dichloromethane added (100 ml), washed with brine solution, the organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over alumina G (III) using 1-3% methanol/dichloromethane mixture as eluent to give product **14**. Yield: 250.0 mg (70.0%). UV-vis (λ_{max} , dichloromethane): 318, 413, 507, 539, 606 & 660. Elemental analysis: Calculated for C₃₀₉H₄₉₁N₃₁O₇₁: C, 64.25; H, 8.57; N, 7.52; O, 19.67, found: C, 64.30; H, 8.59; N, 7.56.

Compound No. 15—Compound **14** (225.0 mg, 0.038 mmol) was stirred with 70% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. The crude preparation thus obtained was dissolved in pyridine (10 ml) and while stirring, GdCl₃·6H₂O (173.7 mg, 0.46 mmol) in 1 ml of water was added slowly and the resultant mixture was stirred for 16 hr. The reaction mixture was concentrated to dryness under high vacuum. The residue was washed with water (10 ml × 3), acetone (10 ml × 3) and finally dried under high vacuum using P₂O₅ as drying agent. Yield: 170.0 mg (86.7%). UV-vis (λ_{max} , MeOH): 320, 411, 507, 539, 606 and 660 nm. Elemental analysis: Calculated for C₁₈₉H₂₅₁Gd₆N₃₁O₇₁: C, 45.07; H, 5.02; Gd, 18.73; N, 8.62; O, 22.55, found: C, 45.15; H, 5.10; N, 8.58.

Compound No. 16—Acid **4** (150.0 mg, 0.214 mmol), Di-*tert*-butyl iminodiacetate (105.0 mg, 0.429 mmol), EDCI (82.3 mg, 0.429 mmol) and DMAP (52.0 mg, 0.429 mmol) were taken into a dry RBF (100 ml). Dry dichloromethane (30 ml) was added and the reaction mixture was stirred at RT for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, the organic layer was separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over silica gel using 1-1.5% methanol/dichloromethane mixture as eluent to give product **16**. Yield: 165.0 mg (82.9%). UV-vis (λ_{max} , dichloromethane): 318, 411, 506, 536, 605 and 661 nm. ¹HNMR (400MHz, CDCl₃): δ 9.75 (splitted s, 1H, H-5), 9.52 (splitted s, 1H, H-10), 8.53 (s, 1H, H-20), 6.01 (m, 1H, CH₃CHOTEG), 5.31 (d, 1H, CH-15¹, J = 20.0 Hz), 5.13 (d, 1H, CH-15¹, J = 20.0 Hz), 4.50 (q, 1H, H-17, J = 7.2 Hz), 4.36 (m, 1H, H-18), 4.02 (m, 2H, CH₂chain), 3.85 (m, 2H, CH₂-O-TEG), 3.79 (m, 2H, CH₂-O-TEG), 3.73 (m, 4H, 3CH₂-O-TEG), 3.68 (s, 3H, 7-CH₃), 3.66 (m, 2H, 8-CH₂CH₃), 3.55 (m, 2H, CH₂-O-TEG), 3.39 (s, 3H, 2-CH₃), 3.27 (s, 6H, 12-CH₃ & OCH₃-TEG), 2.75 (m, 1H, CH-17²), 2.44 (m, 1H, CH-17²), 2.41 (m, 1H, CH-17¹), 2.16 (m, 1H, CH-17¹), 2.14 (d, 3H, CH₃CHOTEG, J = 6.4 Hz), 1.81 (d, 3H, 18-CH₃, J = 7.6 Hz), 1.71 (t, 3H, 8-CH₂CH₃, J = 7.6 Hz), 1.44 (splitted s, 9H, CO₂^tBu), 1.06 (splitted s, 9H, CO₂^tBu), 0.39 (brs, 1H, NH), -1.80 (brs, 1H, NH). EIMS: 927 (MH⁺). HRMS: Calculated for C₅₂H₇₁N₅O₁₀: 925.5201, found: 926.5288 (MH⁺).

Compound No. 17—Compound **16** (140.0 mg, 0.151 mmol) was stirred with 70% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. To this crude preparation were added, amine **A** (188.2 mg, 0.453 mmol), EDCI (115.9 mg, 0.604 mmol) and DMAP (73.7 mg, 0.604 mmol). Dry dichloromethane (30 ml) was added and the reaction mixture was stirred at RT for 16 hr under N₂ atm. The reaction mixture was diluted with dichloromethane (100 ml), washed with brine solution, the organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over silica gel using 3-7% methanol/dichloromethane mixture as eluent to give product **17**. Yield: 210.0 mg (86.4%). UV-vis (λ_{max} , dichloromethane): 318, 411, 506, 536, 604 and 661 nm. ¹HNMR (400MHz, CDCl₃): δ 9.76 (splitted s, 1H, H-5), 9.53 (s, 1H, H-10), 8.63 (s, 1H, H-20), 8.31 (splitted s, 1H, CONH), 6.56 (splitted s, 1H, CONH), 6.00 (m, 1H, CH₃CHOTEG), 5.35 (d, 1H, CH-15¹, J = 20.0 Hz), 5.16 (d, 1H, CH-15¹, J = 20.0 Hz), 4.53 (q, 1H, H-17, J = 7.6 Hz), 4.36 (d, 1H, H-18, J = 10.4 Hz), 3.85-3.80 (m, 4H, 2CH₂-O-TEG), 3.74-3.71 (m, 6H, 2CH₂chain, CH₂-O-TEG), 3.67 (s, 3H, 7-CH₃), 3.66 (m,

4H, 8-CH₂CH₃, CH₂-O-TEG), 3.53 (m, 2H, CH₂-O-TEG), 3.42-3.39 (m, 5H, CH₂-O-TEG, 2-CH₃), 3.27-3.26 (m, 6H, 12-CH₃, OCH₃TEG), 2.75 (m, 1H, CH-17²), 2.67 (m, 1H, CH-17²), 2.52 (m, 1H, CH-17¹), 2.24-2.22 (m, 13H, 6CH₂-chain, CH-17¹), 2.14 (d, 3H, CH₃CHOTEG, J = 6.8 Hz), 2.09-2.04 (m, 6H, 3CH₂-chain), 1.96 (m, 6H, 3CH₂-chain), 1.81 (d, 3H, 18-CH₃, J = 7.2 Hz), 1.71 (t, 3H, 8-CH₂CH₃, J = 8.0 Hz), 1.41 (s, 27H, 3CO₂^tBu), 1.33 (s, 27H, 3CO₂^tBu), 0.39 (brs, 1H, NH), -1.79 (brs, 1H, NH). EIMS: 1610 (MH⁺). HRMS: Calculated for C₈₈H₁₃₃N₇O₂₀: 1607.9605, found: 1607.9650 (M⁺).

Compound No. 18—Compound **17** (100.0 mg, 0.06 mmol) was stirred with 80% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. To this crude preparation were added, amino-benzyl-DTPA-penta-*tert*-butyl ester (471.0 mg, 0.60 mmol), EDCI (115.9 mg, 0.60 mmol) and DMAP (73.8 mg, 0.60 mmol). Dry *N,N*-dimethylformamide (15 ml) was added and the reaction mixture was stirred at RT for 16 hr under N₂ atm. The reaction mixture was concentrated under vacuum, dichloromethane added (100 ml), washed with brine solution, the organic layer separated, dried over sodium sulfate and concentrated. The crude mixture was chromatographed over alumina G (III) using 1-3% methanol/dichloromethane mixture as eluent to give product **18**. Yield: 250.0 mg (69.0%). UV-vis (λ_{max} , dichloromethane): 319, 412, 508, 538, 606 and 661 nm. Elemental analysis: Calculated for C₃₀₉H₄₉₁N₃₁O₇₄: C, 63.72; H, 8.50; N, 7.46; O, 20.33, found: C, 63.67; H, 8.57; N, 7.46.

Compound No. 19—Compound **18** (215.0 mg, 0.0369 mmol) was stirred with 80% TFA/DCM (5.0 ml) at room temperature for 3 hr. The resultant mixture was concentrated and dried under high vacuum to remove trace TFA. The crude preparation thus obtained was dissolved in pyridine (10 ml) and while stirring, GdCl₃·6H₂O (164.6 mg, 0.44 mmol) in 1 ml of water was added slowly and resultant mixture was stirred for 16 hr. The reaction mixture was concentrated to dryness under high vacuum. The residue was washed with water (10 ml × 3), acetone (10 ml × 3) and finally dried under high vacuum using P₂O₅ as drying agent. Yield: 160.0 mg (85.0%). UV-vis (λ_{max} , MeOH): 320, 410, 507, 539, 606 and 661 nm. Elemental analysis: Calculated for C₁₉₀H₂₅₃Gd₆N₃₁O₇₄: C, 44.76; H, 5.00; Gd, 18.50; N, 8.52; O, found: C, 44.80; H, 5.07; N, 8.51.

Calculation of In Vitro Relaxivities

MRI acquisitions were performed using a General Electric 4.7T / 33 cm horizontal bore magnet (GE NMR instruments, Fremont, CA) incorporating AVANCE™ digital electronics (Bruker BioSpec platform with ParaVision® Version 3.0.2 acquisition software, Bruker Medical, Billerica, MA).

Each conjugate was diluted with phosphate-buffered saline (pH 7.4) to a concentration ranging from 0.02 mM to 0.10 mM and imaged at 25°C. T1 relaxation rates (R₁) were acquired utilizing a saturation recovery, spin-echo (SE) sequence with a fixed echo time (TE) = 10 ms and repetition times (TR) ranging from 75 to 8000 ms. Additional MR acquisition parameters were as follows: field of view (FOV) 32 × 32 mm, slice thickness = 1 mm, slices = 3, interslice gap = 2 mm, matrix = 192 × 192, number of averages (NEX) = 1. Signal intensities at each repetition time were sampled by taking the mean intensity within regions of interest (ROI's) using commercially available image processing software (Analyze 7.0, AnalyzeDirect, Overland, KS), and R₁ and S_{MAX} were calculated by nonlinear fitting of the equation:

$$S_{(\text{TR})} = S_{\text{MAX}} \left(1 - e^{-(R_1 \cdot \text{TR})} \right) + \text{Background Noise}$$

using Matlab's Curve Fitting Toolbox (Matlab 7.0, MathWorks Inc., Natick, MA). The T_1 relaxivity for each conjugate was then determined by obtaining the slope of the compound's molar concentration vs. R_1 via linear regression fitting of the data. Similarly, T_2 relaxation rates (R_2) were acquired using a multi-echo, Carr-Purcell-Meiboom Gill (CPMG) SE sequence with a fixed TR of 2500 ms and TE times ranging from 15 to 300 ms, NEX = 2. R_2 and S_{MAX} were calculated as described above using the equation:

$$S_{(TE)} = S_{MAX} \left(e^{-(R_2 * TE)} \right) + \text{Background Noise}$$

As before, the T_2 relaxivity was determined by obtaining the slope of concentration vs. R_2 via linear regression fitting of the data.

Determination of in vitro photobleaching

In vitro photobleaching (26) of each compound was performed using a continuous light source at 665 nm at a power of 500 mW and a dose rate of 75 mW/cm². Samples were prepared in 17% bovine calf serum. Individual, 2.5 mL samples in 17% bovine calf serum were irradiated in quartz cuvettes and monitored via a UV-Vis spectroscopy (Varian Cary Bio 50 spectrophotometer, Palo Alto, CA) at 665 nanometers until the peak had been reduced to a minimum of 70%.

Evaluation of phototoxicity viability

The compounds were tested for photodynamic efficacy at 4 μ M and variable light doses (at 665 nm for HPPH analogues and 705 nm for purpurinimides) after incubating for 24 h with (Colon26) cells seeded in 96 well plates. Viability was measured 48 h later using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay (26). The results were plotted as percent survival compared with the untreated dark control (no drug, no light) at each light dose (in J/cm²).

Determination of in vitro intracellular localization

Colon26 cells were seeded at 1×10^5 cells in 2 ml growth medium on poly-L-lysine coated glass coverslips in 6-well plates and cultured for 48 h to allow attachment and spreading. The photosensitizer (1.0 μ M) was incubated with the cells in the dark at 37°C for 4 h or 24 h. The cells were co-incubated with organelle specific dyes MitoTracker Green FM for 30 minutes at 0.5 μ M; LysoTracker Green at 1 μ M for 2 h and Bodipy Ceramide C5-albumin complexes at 5 μ M (27) according to manufacturer's instructions (Invitrogen). Prior to microscopy the cells were rinsed with PBS and mounted with PBS containing 1 μ M DAPI. Live cells were examined with a Zeiss Axiovert 200 inverted fluorescence with a Fluoarc mercury vapor shortarc lamp as light source. Fluorescent images from the same fields were captured successively in detector channels using suitable filter sets (Chromatechnology) for the PS and the organelle probes with an AxioCam MR-MRGrab frame grabber and a monochromatic CCD camera (Zeiss AxioCam MRm). Filter cubes contained 410/40 nm excitation, beamsplitter 505 and bandpass emission 675/50 nm for the PS; excitation bandpass 470/40 nm, beamsplitter 495, and bandpass emission 525/50 nm for MitoTracker Green and LysoTracker Green; excitation bandpass 565/30 nm, beamsplitter 585 and bandpass emission 620/60 nm for the Bodipy C5 ceramide and excitation 390/22 nm, beamsplitter 420 nm and bandpass emission 460/50 nm. Images were digitally processed with the Zeiss AxioVision LE v. 4.1 software.

Statistical Evaluation

In vitro PDT of the compounds was presented as the mean and standard deviation of three replicate experiments. *In vivo* PDT of the compounds was compared in Kaplan-Meier plots. GraphPad Prism 5.0 was utilized to compare tumor response curves

RESULTS AND DISCUSSION

Chemistry

For the preparation of photosensitizer-Gd(III) ADTPA conjugates, 3-(1'-hexyloxy ethyl)-3-devinylpyropheophorbide-a (HPPH) HPPH and 3-(1'-butyloxyethyl)3-devinyl-purpurin-18-N-butylbutylimide **8** were synthesized by following the methodologies developed in our laboratory (28). For the synthesis of HPPH-3Gd(III)ADTPA **3**, HPPH was reacted with aminotriester **1** which on treating with trifluoroacetic acid (TFA) and further reaction with aminobenzylDTPA butyl ester gave the desired conjugate. To investigate the effect of lipophilicity of the conjugate in tumor-imaging (MRI) and PDT, we synthesized a series of compounds with increasing/decreasing lipophilicity/hydrophilicity by modifying the position-17 of pyropheophorbide with alkyl ether (butyl or hexyl) or polyethylene glycol moieties. We started with compound **4** containing an ethylene glycol group at position-3, which on a sequence of reactions illustrated in Scheme-2 produced the corresponding 3Gd(III) aminobenzyl DTPA conjugate **7** in modest yield. For the synthesis of a bifunctional agent with longer wavelength absorption pyropheophorbide-a analogs (HPPH or compound **4**) was replaced with a highly effective photosensitizer **8** (developed on the basis of structure-activity relationships studies) exhibiting longer wavelength absorption near 700 nm, and the corresponding 3Gd(III)ADTPA conjugate **11** was synthesized. To further investigate the effect of increased Gd(III) units in MR imaging ability and the effect of overall lipophilicity in pharmacokinetic/pharmacodynamic characteristics pyropheophorbide-6Gd(III)aminobenzyl conjugates **15** and **19** containing a hexyl ether or an ethylene glycol group at position-3 were synthesized by following the methodology as depicted in Schemes 4 and 5 respectively. The structures of all the intermediates and the final products were confirmed by NMR, mass spectrometry and elemental analyses.

Effect of gadolinium chelates in absorption and fluorescence properties of HPPH

To investigate the effect of the number of Gd(III) units in absorption and fluorescence properties of the conjugates, HPPH and the corresponding conjugates **3** and **15** containing three- and six gadolinium chelates were dissolved in methanol, the electronic absorption spectra and fluorescence spectra were measured at equimolar concentrations. As can be seen from Figure 1 (A), subtle deviations were observed between the UV-Vis spectra of HPPH, **3** (HPPH-3Gd(III) chelate and **15** (HPPH-6Gd(III) chelate). The Soret band region in compound **15** was much broader. The long wavelength absorption intensity at 660 nm for conjugates **3** and **15** was slightly lower ($\epsilon = 35000$, 33000 respectively) than that observed for HPPH ($\epsilon = 45000$).

Compared to HPPH, the emission spectra (Figure 1B) of the metallated analogs **3** and **15** showed a significant decrease in fluorescence intensity. Excitations of these compounds in the "Soret" band region (410 – 425 nm) exhibited an emission peak at 667 nm and as can be seen from Figure 1 (B), compared to HPPH the corresponding 3Gd(DTPA) analog **3** produced a log decrease in fluorescence intensity, which further decreased by increasing the gadolinium units in conjugate **15**. A sharp decrease in fluorescence intensity indicates a possible interaction between the gadolinium chelates and the HPPH moiety and detailed photophysical studies of this phenomenon are currently in progress.

Effect of Gd(III)aminobenzyl DTPA moiety in photobleaching of HPPH

The singlet oxygen produced on exposing photosensitizer within a tumor to light is responsible for the destruction of tumor and also the photosensitizer (29,30). In order to achieve an optimal PDT response, it is extremely important to make the maximum use of the tumor oxygen supply and also to understand the photobleaching characteristics of the photosensitizer by the singlet oxygen produced *in situ*. In this study, HPPH and the corresponding gadolinium analogs **3** and **15** with 3 and 6 Gd(III)aminobenzyl DTPA units respectively were dissolved in 17% bovine calf serum (BCS) solution and irradiated with laser light at 665 nm. The absorption spectra were measured at regular intervals and the observed peak intensity values at λ_{max} 665 nm were plotted against the light exposure time.

As can be seen from the results summarized in Figure 2, the presence of Gd(III)aminobenzyl DTPA moieties in both conjugates **3** and **15** significantly reduced the rate of photobleaching of the HPPH. The longer stability of the Gd(III) containing photosensitizers provides an opportunity for an additional light treatment to those cancer patients, which did not produce satisfactory response after initial treatment without injecting more PDT agent.

T1 and T2 relaxivity of HPPH-Gd(III)aminobenzylDTPA conjugates

The T1 and T2 relaxivity values of the photosensitizer-Gd(III)ADTPA conjugates are summarized in Table 1.

Compared to Magnevist®, the relaxivity values were higher for compounds containing 3-or 6Gd-ADTPA moieties. However, the increase in relaxivity was non-linear with the increase in the number of Gd (III) units. The reason for the lack of linearity is attributed to the relative close proximity of Gd-ADTPA groups, which may be reducing the degree of relaxation enhancement by limiting the hydration of the molecule.

In selection of a lead candidate, T1 relaxivity was favored over T2, as an increase in T1 relaxation rate reduces saturation of signal, leading to brighter intensity in tumors, while an increase in T2 relaxation rate causes loss of signal intensity, producing poorer contrast. Although all compounds exhibited adequate T1/T2 ratios, compounds **15** and **19** displayed the highest T1/T2 ratios, but limited photosensitizing efficacy.

In vitro Photosensitizing Activity

To determine the effect of the nature of photosensitizer and the number of Gd(III)ADTPA moieties on photosensitizing efficacy, conjugates **3**, **7**, **11**, **15** and **19** were evaluated in RIF cells at various concentrations and light doses.

The results obtained at a fixed concentration of the conjugates (4.0 μM), but at variable light doses are shown in Figure 3. As can be seen, among all the derivatives both HPPH and purpurinimide linked with 3Gd(III)ADTPA moieties (**3** and **11** respectively) were more effective than those containing 6Gd(III)ADTPA moieties (**15** and **19** respectively). Interestingly, replacing the hexyl ether functionality at position-3 with PEG (**7** and **19**) reduced the *in vitro* efficacy.

Intracellular localization of HPPH vs. HPPH-3Gd(III)ADTPA Conjugate

Most of the porphyrin-based compounds can be observed intracellularly with a fluorescence microscope. It has been shown that photosensitizers localize to cytosolic targets such as Golgi, endoplasmic reticulum, mitochondria, lysosomes and membranes (31,32). Efforts have been made to investigate a correlation between the site(s) of localization and PDT activity of photosensitizers. Moan *et al.* (33) suggested that lysosomes are a primary localization site but not the photodynamic target. However, it has not been determined whether the lysosomes

repture after PDT damage and release the compound or whether some other cellular damage, caused by unobservable functions of the photosensitizer elsewhere in the cell, cause the lysosome to rupture and subsequently release the photosensitizer. We have previously shown that most of the effective PDT agents, including HPPH developed in our laboratory localize either in mitochondria or in lisosomes (34). We have also observed that besides the overall lipophilicity, the nature of the substituents introduced in photosensitizers also makes a remarkable difference in intracellular localization characteristics (35).

To determine the impact of Gd(III)ADTPA moieties in site of localization of HPPH, the intracellular localization of both the compounds was investigated by using various site-specific probes. Although some of the conjugates were also found in the mitochondra and lysosomes (see Figure 4 and the Supplementary Information), localization of the lead Gd-containing compound **3** appeared to be primarily in the Golgi apparatus as determined by its colocalization with a probe for that organelle. Addition of Gd alone may not account for the shift from mitochondria observed with HPPH to the Golgi, but may be partly due to the position at which the additional Moieties are attached, and a significant change in overall lipophilicity of the molecule. Primarily, photosensitizers which localize to the mitochondria are more effective at evoking cell death (34), however photosensitizers which localize to alternative cellular organelles also exhibit the ability to induce cell mortality upon light irradiation (36). Further studies to establish a correlation between the site(s) of localization and cell-death (apoptosis vs. necrosis) are currently in progress.

CONCLUSION

Our studies indicate that tumor-avid photosensitizers can be used as vehicles for delivering the MR imaging agents to tumors. Compared to Magnevist, a clinical standard, most of the conjugates showed enhanced tumor-imaging potential (T1/T2 relaxivity) which increased by increasing the number of Gd(III)ADTPA units. However, as a “Multifunctional Agent” for tumor-imaging (MR and fluorescence) and PDT, HPPH containing 3Gd(III)ADTPA showed the best PDT efficacy (in vivo results are presented in the following paper of this journal). Our approach has a great potential in developing dual-function agents for MR imaging and phototherapy with increased sensitivity by reducing false-positive and false-negative results in screening, diagnosis, treatment monitoring or image-guided intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme-1.

Scheme-2.

Scheme-3.

Scheme-4.

Scheme-5.

Figure 1. Comparative absorption (A) and fluorescence spectra (B) of HPPH and the corresponding 3 Gd(III)aminobenzyl DTPA **3** and 6Gd(III)aminobenzyl DTPA conjugate **15** in methanol (conc. 19.6 μM).

Figure 2.

Comparative photobleaching characteristics of HPPH and the corresponding 3 and 6Gd(III) aminobenzyl DTPA **3** and **15** respectively at (9.8 M) in 17% BCS. The solutions were exposed with a laser light (500 mW, 665 nm, 75 mW/cm²) and the absorption peak at 665 nm were recorded at variable time points.

Figure 3.

In vitro photosensitizing activity of photosensitizer-Gd(III)ADTPA conjugates (4.0 μ M) in RIF cells. The cells were incubated for 24h and then exposed to light at a dose of 3.2 mW/cm². Control cells were exposed to light only.

Figure 4.

False-color images showing subcellular localization of HPPH and compound **3** (red) in Colon26 cells after 4h (B) or 24 h incubations (A and C) respectively. Localization was determined by fluorescence microscopy by co-incubation with fluorescent organelle probes (green). Subcellular areas of co-localization in the overlay of compound **3** or HPPH with the probes for are yellowish orange. **A**, HPPH; **B** and **C**, compound **3**; **D**, MitoTracker Green; **E** and **F**, Bodipy ceramide C5-albumin complex; **G**, overlay of HPPH and MitoTracker Green; **H** and **I** overlays of compound **3** and Bodipy ceramide C5-albumin complex

Table 1

Comparative T1, T2 relaxivity values of the conjugates and Magnevist•

Compound	(mM • s) ⁻¹	
	T1	T2
3	13.50	81.37
15	24.72	66.54
11	14.14	85.32
7	13.58	40.11
Magnevist ®	4.33	5.10
19	25.09	69.19