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Phthalocyanine-Peptide Conjugates for Epidermal Growth Factor Receptor Targeting¹

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Abstract

Four phthalocyanine (Pc)-peptide conjugates designed to target the epidermal growth factor receptor (EGFR) were synthesized and evaluated *in vitro* using four cell lines: human carcinoma A431 and HEp2, human colorectal HT-29, and kidney Vero (negative control) cells. Two peptide ligands for EGFR were investigated: EGFR-L1 and -L2, bearing 6 and 13 amino acid residues, respectively. The peptides and Pc-conjugates were shown to bind to EGFR using both theoretical (Autodock) and experimental (SPR) investigations. The Pc-EGFR-L1 conjugates **5a** and **5b** efficiently targeted EGFR and were internalized, in part due to their cationic charge, whereas the uncharged Pc-EGFR-L2 conjugates **4b** and **6a** poorly targeted EGFR maybe due to their low aqueous solubility. All conjugates were non-toxic ($IC_{50} > 100 \mu M$) to HT-29 cells, both in the dark and upon light activation ($1 J/cm^2$). Intravenous (iv) administration of conjugate **5b** into nude mice bearing A431 and HT-29 human tumor xenografts resulted in a near-IR fluorescence signal at ca. 700 nm, 24 h after administration. Our studies show that Pc-EGFR-L1 conjugates are promising near-IR fluorescent contrast agents for CRC, and potentially other EGFR over-expressing cancers.

Introduction

Colorectal cancer (CRC) is currently the second leading cause of cancer-related deaths among both men and women in the US.¹ Colon cancer typically develops over several years and in a linear fashion from adenomatous polyps to carcinoma. Routine colon screening, detection and removal of polyp adenomas and early stage cancer reduces the incidence of CRC; however, since the disease lacks outward signs or symptoms, most cancers are detected at a stage where they may become terminal.² Current methods of detection for CRC include flexible sigmoidoscopy, standard colonoscopy, radiography, and computer

¹Abbreviations: Pc, phthalocyanine; CRC, colorectal cancer; CLE, confocal laser endomicroscopy; EGFR, epidermal growth factor receptor; PDT, photodynamic therapy; DMF, dimethylformamide; DMSO, dimethylsulfoxide; THF, tetrahydrofuran; DBN, 1,5-diazabicyclo(4.3.0)non-5-ene; DMAE, dimethylaminoethanol; TFA, trifluoroacetic acid; DIEA, *N,N*-diisopropylethylamine; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HATU, 2-(1H-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; TEA, triethylamine; Fmoc, Fluorenylmethyloxycarbonyl; TIS, triisopropylsilane; HOBt, 1-hydroxybenzotriazole; PBS, phosphate buffered saline; FBS, fetal bovine serum; DMEM, Dulbecco's modified eagle's medium; ER, endoplasmic reticulum.

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Supporting Information Available: NMR spectra, MS, HPLC chromatograms, phototoxicity and dark toxicity plots, subcellular localization microscopy. This material is available free of charge via the internet at <http://pubs.acs.org>.

tomography (CT) colonoscopy, which identify large adenoma lesions but frequently miss small adenomas (< 5 mm) and flat lesions, two early stages of CRC.² Improvements in the early detection of small adenomas and flat lesions could prevent the development of malignant tumors, decreasing mortality and overall health care cost. New detection methods currently being employed to accomplish this goal include chromoendoscopy, narrow band imaging, and blue light auto-fluorescence.³ Chromoendoscopy utilizes an absorptive or contrast dye, such as methylene blue, to stain the mucosa for standard white light colonoscopy, enhancing tissue characterization, differentiation, and diagnosis of small adenomas.^{2,3} Narrow band imaging uses a blue and green filter to narrow the white colonoscopic light to illuminate the mucosa, and blue light auto-fluorescence involves a UV light source to generate mucosal auto-fluorescence. In addition, confocal laser endomicroscopy (CLE) has been employed to image the mucosa, with fluorescein, acriflavine, or cresyl violet as the fluorescent dyes. Drawbacks of these dyes are their poor selectivity for CRC, residual toxicity, and emission wavelengths in the visible region of the optical spectrum.⁴ On the other hand, phthalocyanines (Pcs) are tetrapyrrolic macrocycles with extended π -conjugated systems that typically emit at long wavelengths (> 670 nm) with relatively high fluorescence quantum yields.^{5,6} Advantages of near-IR fluorescence for bioimaging applications include low Raman scattering cross-sections associated with the use of low energy excitation photons, larger Raman-free observation windows and reduced absorption and fluorescence from other compounds.⁷ Pcs have been extensively investigated in the last decades for a variety of applications, including as colorant dyes, catalysts, sensors, and as photosensitizers for photodynamic therapy (PDT) of cancers.⁸⁻¹⁰ PDT involves the administration of a photosensitizer followed by activation with red light to produce cytotoxic oxygen species that destroy malignant cells.^{11,12} Due to their low dark toxicity, high photostability and ability for preferential accumulation within tumor tissue, Pcs are promising cancer diagnostic and treatment agents. Furthermore, conjugation of Pcs with peptide ligands directed at specific receptors over-expressed in cancer cells, such as the human epidermal growth factor receptor (EGFR), is an attractive strategy for increasing their biological efficacy.¹³⁻¹⁷ EGFR is over-expressed in CRC, including small cancers (< 5 mm) and the flat, dysplastic, aberrant crypt foci that are believed to precede cancer development.¹⁸⁻²⁰ Among the EGFR-targeting biomolecules recently reported for selective delivery of cytotoxic drugs to the tumor sites,²¹⁻²⁵ two small peptides with sequences LARLLT (designated EGFR-L1)²⁶ and YHWYGYTPQNVI (designated EGFR-L2)²⁷ are particularly attractive due to their readily availability, low immunogenicity, ease of conjugation to various molecules, and reported superior EGFR-targeting ability. EGFR-L1 was selected from computational screening of an EGFR peptide ligand virtual library, and shown to target EGFR both *in vitro* (in EGFR over-expressing H1299 cells) and *in vivo* (in H1299 tumor-bearing mice following intravenous (iv) administration).²⁶ On the other hand, EGFR-L2 was identified from screening of a phage display peptide library and also shown to bind to EGFR both *in vitro* (SMMC-7721 cells) and *in vivo* (SMMC-7721 tumor bearing mice following iv injection).²⁷ In our continuing investigation of tumor-selective fluorescent imaging and PDT agents, we have recently shown that a Pc conjugated to a bifunctional nuclear localizing sequence and cell penetrating peptide containing 32 amino acid residues, via either a short (5-atom) or a PEG (20-atom) linker, displays higher fluorescence quantum yield and increased cellular uptake compared with unconjugated Pc.²⁸ We now report the synthesis, photophysical, and biological evaluation of Pc conjugates to either EGFR-L1 or EGFR-L2 peptide ligands.

Experimental Section

1. Chemistry

All reagents and solvents were purchased from commercial sources and used directly without further purification. Silica gel 60 (230×400 mesh) and C18 (200×400), both from Sorbent Technologies, were used for column chromatography. Analytical thin-layer chromatography (TLC) was carried out using polyester backed TLC plates 254 (precoated, 200 μm) from Sorbent Technologies. NMR spectra were recorded on AV-400 LIQUID Bruker spectrometer (400 MHz for ¹H, 100 MHz for ¹³C). The chemical shifts are reported in δ ppm using the following deuterated solvents as internal references: Acetone-*d*₆ 2.05 ppm (¹H), 29.92 ppm (¹³C); DMF-*d*₇ 8.03 ppm (¹H), 163.15 ppm (¹³C); Pyridine-*d*₅ 7.58 ppm (¹H), 135.91 ppm (¹³C). HPLC analyses were carried on a Dionex system equipped with a P680 pump and UVD340U detector. Absorption spectra were measured on a UV-vis NIR Scanning Spectrometer, using UV-3101PC SHIMADZU (Cell positioned)–CPS-260 lamp and emission spectra were obtained on a Fluorolog® - HORIBA JOBINVYON (Model LFI-3751) spectrofluorimeter. MALDI-TOF mass spectra were recorded on a Bruker ProFlex III mass spectrometer using dithranol as the matrix or Bruker UltrafleXtreme (MALDI-TOF/TOF) using 4-chloro- α -cyanocinnamic acid as the matrix; high resolution ESI mass spectra were obtained on an Agilent Technologies 6210 Time-of-Flight LC/MS. The melting points (mp) were determined using MEL-TEMP electrothermal instrument. HPLC separation was carried out on a Waters system including a 2545 quaternary gradient module pump, 2489 UV/Visible detector, and a fraction collector III. Analytical HPLC was carried out using a XBridge C₁₈ 300Å, 5 μm, 4.6 × 250 mm (Waters, USA) column using a stepwise gradient. Semipreparative HPLC was carried out using a XBridge C₁₈ 300Å, 5 μm, 10 × 250 mm (Waters, USA) column using a stepwise gradient. The solvent system for peptides consisted of millipure water and HPLC grade acetonitrile, while it consisted of millipure water and HPLC grade methanol for the EGFR-L1 conjugates. Pcs **1a,b** were synthesized as previously described.²⁹

Peptide synthesis and conjugations—Applied Biosystems Pioneer, Peptide Synthesis System was used to synthesize peptide sequences. Each peptide was synthesized using Fmoc-PAL-PEG-PS on 0.2 mmol scale using Fmoc strategy of solid-phase peptide synthesis. A 4-fold excess of the L-Fmoc protected amino acids were coupled using HOBt and TBTU as the activating agents. The peptide sequences, prepared using this methodology, were: LARLLT (EGFR-L1) and GYHWYGYTPQNVI (EGFR-L2). Removal of the Fmoc group from the last amino acid was the final step for each synthesis. This was followed by washing the peptide several times with DMF/dichloromethane and dried under high vacuum for 24 h. The Pc-peptide conjugates were synthesized as previously described.²⁸ In summary, resin containing either GYHWYGYTPQNVI or LARLLT was dissolved in DMF and soaked for 2 h. The Pcs were dissolved in DMF, the base and coupling reagents (HOBt and TBTU) added to the Pc solutions. The activated mixture was transferred into the reaction vessel containing the resin and left to shake for 4 days. The resin was washed under vacuum several times using DMF, then methanol and finally dichloromethane. A cleavage cocktail consisting of TFA/phenol/TIS/H₂O 88:5:2:5 was added with constant shaking for 4 h. The solution was washed with TFA (2 × 2 mL) into a flask and concentrated under vacuum. Cold diethyl ether was added to the residue and the mixture centrifuged. The Pc-EGFR-L1 conjugates were purified using reverse-phase HPLC using a Waters system including a 2545 quaternary gradient module pump, 2489 UV/Visible detector, and a fraction collector III. Analytical HPLC was carried out using a XBridge C₁₈ 300 Å, 5 μm, 4.6 × 250 mm (Waters, USA) column with a stepwise gradient. Semipreparative HPLC was carried out using a XBridge C₁₈ 300 Å, 5 μm, 10 × 250 mm (Waters, USA) column with a stepwise gradient. The solvent system consisted of Millipure water and

HPLC grade methanol (30:70 → 0:100). The purity of the conjugates was > 95% as determined by HPLC.

ZnPc 2a—A mixture of Pc **1a** (80.0 mg, 0.096 mmol) and 1,4-dioxane-2,6-dione (18.0 mg, 0.16 mmol) was dissolved in DMF (1.0 mL) and the solution stirred overnight at room temperature. Water (5.0 mL) was added to the solution to precipitate the product. The solid was filtered under vacuum and washed with water and hexane. The solid was dried under vacuum for 2 days to afford the pure blue solid (80.4 mg, 86.6%), mp 235 – 236 °C. ¹H NMR (Pyridine-*d*₅): δ 10.78-10.71 (s, 1H, –COOH), 10.17 - 9.41 (m, 7H, Ar-H), 8.44 - 8.23 (m, 6H, Ar-H), 8.01 - 7.71 (m, 3H, Ar-H), 6.04 (br, 1H, N-H), 4.55 (d, J = 12.4 Hz, 2H, CH₂O), 4.47 (d, J = 10.5 Hz, 2H, CH₂O), 1.73 – 1.59 (m, 27H, C(CH₃)₃). ¹³C NMR (Pyridine-*d*₅): δ 168.3, 168.2, 156.6, 155.9, 155.8, 154.7, 154.3, 154.0, 152.9, 150.6, 150.4, 150.1, 149.8, 142.4, 140.1, 140.0, 137.8, 137.7, 136.2, 136.0, 135.8, 135.5, 134.3, 134.8, 131.54, 128.46, 128.3, 124.8, 124.2, 124.0, 123.7, 123.4, 122.8, 122.5, 121.4, 120.6, 120.5, 120.4, 120.2, 119.4, 118.1, (Ar-C) 73.1, 70.5 (OCH₂), 35.8, 32.4 (Ar-C, C(CH₃)₃). MS (MALDI-TOF) *m/z* 968.319 [M+H]⁺, calcd for C₅₄H₅₀N₉O₅Zn 967.323. UV-vis (DMF): λ_{max} (log ε) 346 nm (5.04), 614 nm (4.80), 680 nm (5.50).

ZnPc 2b—A mixture of Pc **1b** (50.0 mg, 0.06 mmol) and 1,4-dioxane-2,6-dione (11.3 mg, 0.1 mmol) was dissolved in DMF (0.5 mL) and the solution stirred overnight at room temperature. The mixture was purified as **2a** above to afford the pure blue solid (53.5 mg, 92.2%), mp 249 – 250 °C. ¹H NMR (Pyridine-*d*₅): δ 10.84-10.77 (s, 1H, –COOH), 10.01 - 9.35 (m, 8H, Ar-H), 8.50 - 8.21 (m, 5H, Ar-H), 8.10 - 7.90 (m, 1H, Ar-H), 7.54 – 7.41 (m, 2H, Ar-H), 6.74 (br, 1H, N-H), 4.69 (d, J = 5.4 Hz, 2H, CH₂O), 4.60 (d, J = 5.6 Hz, 2H, CH₂O), 1.75 – 1.64 (m, 27H, C(CH₃)₃). ¹³C NMR (Pyridine-*d*₅): δ 173.9, 168.43, 168.38, 160.0, 159.7, 154.8, 154.7, 154.6, 154.5, 154.3, 153.9, 153.8, 153.5, 153.4, 153.0, 150.6, 150.4, 150.1, 140.8, 139.4, 139.2, 137.0, 136.8, 134.2, 134.0, 131.0, 127.7, 124.7, 122.0, 120.8, 120.7, 120.3, 119.8, 112.5, 111.6 (Ar-C) 72.5, 69.9 (OCH₂), 35.8, 31.7 (Ar-C, C(CH₃)₃). MS (MALDI-TOF) *m/z* 968.314 [M+H]⁺, calcd for C₅₄H₅₀N₉O₅Zn 968.323. UV-vis (DMF): λ_{max} (log ε) 351 nm (4.86), 611 nm (4.58), 679 nm (5.33).

ZnPc 3a—Pc **2a** (30.0 mg, 0.032 mmol) was dissolved in DMF (400 ZL). Et₃N (4.0 mg, 0.041 mmol), HOBT (4.6 mg, 0.034 mmol) and tert-butyl-12-amino-4,7,10-trioxadecanoate (10.8 mg, 0.039 mmol) were added to the reaction mixture. EDCI (5.3 mg, 0.034 mmol) was then added in one portion. The reaction solution was stirred for 3 days at room temperature, diluted using ethyl acetate (10 mL) and washed subsequently with water (20 mL × 2). The organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated and the crude product purified on silica column eluted with mixed solvents of DCM/methanol (98:2 → 96:4) to afford a blue solid (30.0 mg, 76.5%). ¹H NMR (DMF-*d*₇): δ 9.60 - 9.01 (m, 7H, Ar-H), 8.45 - 8.15 (m, 5H, Ar-H), 8.00 – 7.77 (m, 3H, Ar-H), 7.63 – 7.49 (m, 2H, Ar-H), 4.27 – 4.15 (m, 4H, CH₂O), 3.62 – 3.57 (m, 3H, CH₂O), 3.54 – 3.47 (m, 9H, CH₂O), 3.42 – 3.37 (m, 2H, CH₂O), 2.44 – 2.39 (m, 2H, CH₂CO), 1.82 – 1.78 (m, 27H, C(CH₃)₃), 1.40 (s, 9H, C(CH₃)₃). ¹³C NMR (DMF-*d*₇): δ 171.7, 170.5, 168.8, 168.7, 156.8, 156.2, 155.6, 155.5, 155.2, 155.1, 155.0, 154.7, 154.6, 154.4, 154.3, 154.20, 154.16, 154.0, 153.4, 153.3, 153.2, 152.4, 152.1, 152.04, 151.96, 142.3, 140.03, 139.98, 139.9, 139.6, 137.64, 137.57, 137.5, 137.2, 135.10, 135.06, 143.5, 131.7, 131.6, 130.1, 129.4, 128.7, 128.6, 128.4, 123.6, 123.5, 123.3, 122.6, 122.4, 122.3, 122.0, 120.3, 120.1, 120.0, 119.8, 119.6, 119.0, 118.9, 117.5 (Ar-C), 80.8 (O-C(CH₃)₃), 72.4, 72.1, 71.20, 71.18, 71.15, 71.1, 71.0, 70.5, 70.32, 70.30, 67.2 (OCH₂), 39.64, 39.61, (COCH₂), 32.7, 32.6 (Ar-C, C(CH₃)₃), 28.5 (N-C(CH₃)₃). MS (MALDI-TOF) *m/z* 1170.497 [M-^tBu+H]⁺, calcd for C₆₃H₆₆N₁₀O₉Zn 1170.431. The protected Pc conjugate was dissolved in a mixture of DCM/TFA (4 mL/4 mL) and stirred at 0° C for 3 h. The solvent was evaporated, the residue

treated with 2N NaOH (2 mL) and then extracted by ethyl acetate (15 mL). The product was dried under vacuum to afford blue product (24.0 mg, 89.3%), mp 191 – 192 °C. ^1H NMR (DMF- d_7): δ 10.15-10.13 (s, 1H, -COOH), 9.59 - 9.08 (m, 7H, Ar-H), 8.42 - 8.10 (m, 5H, Ar-H), 7.95 - 7.82 (m, 3H, Ar-H), 7.61 - 7.52 (m, 2H, Ar-H), 4.25 - 4.11 (m, 4H, CH₂O), 3.79-3.57 (m, 12H, CH₂O), 3.41-3.37 (m, 2H, CH₂O), 2.51-2.48 (m, 2H, CH₂CO), 1.79 (s, 27H, C(CH₃)₃). ^{13}C NMR (Pyridine- d_5): δ 173.7, 170.5, 168.7, 156.9, 156.2, 155.4, 154.5, 153.7, 152.6, 145.6, 142.6, 140.2, 139.8, 137.6, 135.2, 134.6, 131.7, 128.7, 123.5, 122.7, 122.4, 120.0, 119.1, 117.7 (Ar-C), 72.5, 72.2, 71.3, 70.4, 67.8 (OCH₂), 35.5 (COCH₂), 32.6 (Ar-C, C(CH₃)₃). MS (MALDI-TOF) m/z 1170.485 [M]⁺, calcd for C₆₃H₆₆N₁₀O₉Zn 1170.431. UV-vis (DMF): λ_{max} (log ϵ) 349 nm (4.90), 612 nm (4.61), 678 nm (5.35).

ZnPc 3b—Pc 2b (30.0 mg, 0.032 mmol) was dissolved in DMF (400 ZL). Et₃N (4.0 mg, 0.041 mmol), HOBT (4.6 mg, 0.034 mmol) and tert-butyl-12-amino-4,7,10-trioxadodecanoate (10.8 mg, 0.039 mmol) were added to the reaction solution and the solution was stirred for 20 min. EDCI (5.3 mg, 0.034 mmol) was added to the reaction solution in one portion. The reaction was then treated as **3a** above to afford a blue solid (32.2 mg, 82.3%). ^1H NMR (Acetone- d_6): δ 9.87 - 9.77 (m, 1H, Ar-H), 9.07 - 7.40 (m, 16H, Ar-H), 3.99 (s, 2H, CH₂O), 3.87 (s, 2H, CH₂O), 3.58 - 3.54 (m, 2H, CH₂O), 3.45 (s, 8H, CH₂O), 3.38 (s, 2H, CH₂O), 3.22 (s, 2H, CH₂O), 2.36 - 2.33 (m, 2H, CH₂CO), 1.88 - 1.78 (m, 27H, C(CH₃)₃), 1.37 (s, 9H, C(CH₃)₃). ^{13}C NMR (DMF- d_7): δ 171.8, 170.7, 169.3, 169.2, 162.4, 160.8, 160.4, 155.0, 154.8, 154.3, 154.2, 141.4, 140.0, 139.8, 137.6, 136.7, 136.4, 134.5, 128.6, 125.2, 123.4, 123.0, 121.4, 121.3, 120.9, 120.0, 112.8, 111.7 (Ar-C), 81.0 (O-C(CH₃)₃), 72.6, 72.4, 71.5, 71.43, 71.36, 71.3, 70.6, 67.8 (OCH₂), 32.8 (Ar-C, C(CH₃)₃), 28.7 (N-C(CH₃)₃). MS (MALDI-TOF) m/z 1227.682 [M+H]⁺, 1170.495 [M-^tBu+H]⁺, calcd for C₆₇H₇₅N₁₀O₉Zn 1227.501, C₆₃H₆₆N₁₀O₉Zn 1170.431. The protected Pc conjugate was then deprotected as **3a** above to afford a blue solid (25.3 mg, 88.3%), mp 161 - 163 °C. ^1H NMR (DMF- d_7): δ 10.42-10.33 (s, 1H, COOH), 9.57 - 9.32 (m, 6H, Ar-H), 9.09 - 8.75 (m, 1H, Ar-H), 8.55 - 8.20 (m, 4H, Ar-H), 8.15 - 8.00 (m, 2H, Ar-H), 7.99 - 7.81 (m, 1H, Ar-H), 7.65 - 7.50 (m, 2H, Ar-H), 4.36 (d, J = 7.2 Hz, 2H, CH₂O), 4.28 (d, J = 5.1 Hz, 2H, CH₂O), 3.65 - 3.57 (m, 12H, CH₂O), 3.41 - 3.37 (m, 2H, CH₂O), 2.55 - 2.48 (m, 2H, CH₂O), 1.85 - 1.74 (m, 27H, C(CH₃)₃). ^{13}C NMR (DMF- d_7): δ 173.7, 170.6, 169.2, 169.1, 160.64, 160.58, 160.25, 160.18, 155.3, 155.22, 155.16, 155.1, 155.0, 154.9, 154.8, 154.7, 154.3, 154.2, 1154.15, 154.11, 153.0, 153.9, 153.4, 153.3, 153.2, 153.1, 141.5, 141.4, 140.1, 140.0, 139.8, 137.7, 137.64, 137.55, 137.5, 136.5, 136.2, 134.8, 134.7, 134.6, 128.3, 128.2, 126.2, 125.0, 123.3, 123.2, 122.8, 122.0, 121.2, 121.11, 121.06, 120.8, 120.7, 119.9, 119.8, 112.7, 111.6, (Ar-C), 72.4, 72.1, 71.28, 71.25, 71.14, 71.10, 70.4, 68.3, 67.7 (OCH₂), 39.7, 36.8 (COCH₂), 32.6 (Ar-C, C(CH₃)₃). MS (MALDI-TOF) m/z 1170.508 [M]⁺, calcd for C₆₃H₆₆N₁₀O₉Zn 1170.431. UV-vis (DMF): λ_{max} (log ϵ) 350 nm (4.65), 610 nm (4.41), 676 nm (5.19).

ZnPc conjugate 4b—Resin (60.0 mg) containing 0.0052 mmol GYHWYGYTPQNV I was transferred into a reaction vial. DMF was added (5:1, DMF/resin) and soaked for 2 h. The resin was washed four times with DMF. Pc **2b** (10.0 mg, 0.010315 mmol) was weighed into a vial and DMF (200 μL) added. Then HOBT (1.4 mg, 0.010315 mmol), TBTU (3.3 mg, 0.010315 mmol) and DIEA (5.39 μL , 0.030946 mmol) were added to the Pc solution. The mixture was stirred for 30 min, transferred into the vial containing the resin and left to shake for 3 days. The resin was washed under vacuum using DMF (till DMF was clear), followed by methanol and finally DCM. A cleavage cocktail - TFA/phenol/TIS/H₂O (88:5:2:5) - was added and shaken constantly for 4 h. The solution was then washed with TFA (2 \times 2 mL) into a 50 mL flask under vacuum. Cold diethylether was added to the residue and the mixture centrifuged. The precipitate was then sonicated in water and centrifuged several times to give the title compound (9.2 mg, 35.0%), mp 211 - 212 °C. ^1H NMR (DMF- d_7): δ

9.51-9.20 (m, 5H, Ar-H), 8.45-8.10 (m, 9H, Ar-H), 7.80-7.75 (m, 2H, Ar-H), 7.70-7.50 (m, 2H, Ar-H), 7.35-7.20 (m, 24H, Ar-H), 7.07-6.91 (m, 14H, Ar-H), 6.80-6.70 (m, 4H, Ar-H), 6.65-6.53 (m, 9H, Ar-H), 4.68-4.30 (m, 12H, CH₂NH), 4.24-3.65 (m, 4H, CH₂O/CH₂NH), 3.45-3.30 (m, 4H, CH₂O/CH₂NH), 2.25-2.04 (m, 2H, CH₂CO), 2.01-1.75 (m, 21H, C(CH₃)₃/CH₂), 1.33 (s, 5H, CH₃), 1.26 (s, 2H, CH₂), 1.18-1.10 (m, 4H, CH₂), 1.00-0.94 (m, 8H, CH₂), 0.85-0.75 (m, 18H, CH₃). MS (MALDI-TOF) *m/z* 2545.04 [M]⁺, calcd for C₁₃₁H₁₄₈N₂₈O₂₃Zn 2545.0563. UV-vis (DMF): λ_{max} (log ε) 351 nm (4.44), 610 nm (4.07), 677 nm (4.87).

ZnPc conjugate 5a—Resin (25.7 mg, 0.0052 mmol) containing LARLLT, was transferred into a reaction vial. DMF was added (5:1, DMF/resin) and left to soak for 4 h, after which it was washed four times with DMF. Pc **3a** (10.0 mg, 0.010315 mmol) was weighed into a 2.0 mL vial and DMF (200 μL) added. DIEA (6.4 ZL, 0.036745 mmol) was added to the solution and stirred for 1 h. Then HOBt (1.8 mg, 0.013321 mmol), HATU (4.0 mg, 0.010521 mmol) were added to the Pc solution. The mixture was added to the resin and left to shake for 4 days and cleaved as described above for Pc **4b**. The product was purified using reverse-phase HPLC eluted by water/methanol (30:70 → 0:100) to afford a blue solid (7.6 mg, 79.2%), mp 151–152 °C. ¹H NMR (DMF-*d*₇): δ 10.18, 10.13 (s, 1H, N-H), 9.67-9.20 (m, 5H, Ar-H), 8.55-8.45 (m, 3H, Ar-H), 8.25-8.15 (m, 2H, Ar-H), 8.13-8.05 (m, 1H, Ar-H), 7.97-7.86 (m, 5H, Ar-H), 7.85-7.79 (m, 2H, Ar-H), 7.61-7.47 (m, 5H, Ar-H), 7.20 (s, 1H, Ar-H), 7.13 (s, 1H, Ar-H), 4.39-4.01 (m, 26H, CH₂O/CH₂NH), 3.77-3.70 (m, 3H, CH₂O), 3.56-3.50 (m, 12H, CH₂O), 3.43-3.31 (m, 3H, CH₂O), 2.55-2.41 (m, 2H, CH₂CO), 2.01-1.92 (m, 3H, CH₂), 1.85-1.71 (m, 27H, C(CH₃)₃/7H, CH₂), 1.69-1.62 (m, 3H, CH₂), 1.43-1.35 (m, 4H, CH₂), 1.21-1.15 (m, 4H, CH₂), 0.94-0.87 (m, 20H, CH₃). ¹³C NMR (DMF-*d*₇): δ 175.5, 175.3, 174.10, 174.07, 173.7, 173.4, 170.5, 168.8, 168.7, 160.3, 159.9, 158.7, 156.8, 156.2, 155.6, 155.3, 155.1, 154.4, 152.4, 152.1, 140.0, 137.9, 137.6, 135.1, 134.5, 131.7, 128.6, 123.4, 122.6, 122.4, 120.0, 119.0, 117.5, 116.1 (Ar-C), 72.3, 72.0, 71.3, 71.2, 71.0, 70.9, 70.3, 68.2, 68.0, 66.3 (OCH₂), 60.1, 55.2, 54.6, 54.0, 53.5, 51.8, 41.9, 41.3, 41.2, 39.5, 37.2 (CH₂) 32.6 (Ar-C, C(CH₃)₃), 29.4, 26.5, 25.6, 25.5, 24.0, 23.8, 23.5, 22.3, 22.0, 21.8, 20.7, 17.5 (CH₃). MS (MALDI-TOF) *m/z* 1837.907 [M+H]⁺, calcd for C₉₄H₁₂₅N₂₀O₁₅Zn 1837.892. MS-MS (MALDI-TOF-TOF) *m/z* 1839.90 [PcPEG-LARLLT+H]⁺, 1822.88 [PcPEG-LARLLT-NH₂+H]⁺, 1794.88 [PcPEG-LARLLT-CONH₂+H]⁺, 1723.042 [PcPEG-LARLL-NH₂+H]⁺, 1580.75 [PcPEG-LARL-CO+H]⁺, 1339.56 [PcPEG-LA-NH₂+H]⁺, 1268.52 [PcPEG-L-NH₂+H]⁺, 1240.53 [PcPEG-L-CO+H]⁺, 1155.44 [PcPEG-NH₂+H]⁺, calcd for C₉₄H₁₂₅N₂₀O₁₅Zn 1837.892, C₉₄H₁₂₄N₁₉O₁₅Zn 1822.882, C₉₃H₁₂₄N₁₉O₁₄Zn 1794.887, C₉₀H₁₁₇N₁₈O₁₃Zn 1721.834, C₈₃H₁₀₆N₁₇O₁₁Zn 1580.755, C₇₂H₈₃N₁₂O₁₀Zn 1339.565, C₆₉H₇₈N₁₁O₉Zn 1268.528, C₆₈H₇₈N₁₁O₈Zn 1240.533, C₆₃H₆₇N₁₀O₈Zn 1155.444. UV-vis (DMF): λ_{max} (log ε) 348 nm (4.79), 612 nm (4.56), 680 nm (5.33).

ZnPc conjugate 5b—Resin (25.7 mg, 0.0052 mmol) containing LARLLT, was transferred into a reaction vial. DMF was added (5:1, DMF/resin) and left to soak for 4 h, after which it was washed four times with DMF. Pc **3b** (10.0 mg, 0.010315 mmol) was weighed into a 2.0 mL vial and DMF (200 μL) added. DIEA (6.4 ZL, 0.036745 mmol) was added to the solution and stirred for 1 h. Then HOBt (1.8 mg, 0.013321 mmol), HATU (4.0 mg, 0.010521 mmol) were added to the Pc solution. The mixture was added to the resin and left to shake for 4 days. It was then cleaved and purified using reverse-phase HPLC eluted by water/methanol (30:70 → 0:100) to afford a blue solid (8.1 mg, 84.4%, mp 167 – 168 °C). ¹H NMR (DMF-*d*₇): δ 10.40, 10.35 (s, 1H, N-H), 9.67-9.20 (m, 5H, Ar-H), 8.57-8.45 (m, 3H, Ar-H), 8.30-8.25 (m, 2H, Ar-H), 8.13-8.05 (m, 2H, Ar-H), 7.97-7.86 (m, 3H, Ar-H), 7.85-7.79 (m, 2H, Ar-H), 7.61-7.47 (m, 5H, Ar-H), 7.18 (s, 1H, Ar-H), 7.12 (s, 1H, Ar-H), 4.37 (d, *J* = 7.6, 4H, CH₂O), 4.29-4.21 (m, 9H, CH₂NH), 3.77-3.70 (m, 16H, CH₂NH),

3.65-3.57 (m, 12H, CH₂O), 3.54-3.43 (m, 2H, CH₂NH), 3.37-3.29 (m, 3H, CH₂NH), 2.61-2.56 (m, 2H, CH₂CO), 2.01-1.92 (m, 2H, CH₂), 1.91-1.71 (m, 27H, C(CH₃)₃/7H, CH₂), 1.69-1.58 (m, 5H, CH₂), 1.39-1.36 (m, 4H, CH₂), 1.17-1.15 (m, 4H, CH₂), 0.95-0.86 (m, 20H, CH₃). ¹³C NMR (DMF-*d*₇): δ 175.5, 175.3, 174.1, 174.0, 173.7, 173.6, 173.4, 170.6, 169.2, 169.1, 160.2, 159.9, 158.8, 154.6, 154.2, 153.7, 140.2, 139.8, 136.7, 136.3, 128.6, 128.4, 122.8, 122.2, 121.4, 120.9, 119.3, 116.3 (Ar-C), 72.4, 72.2, 71.3, 71.1, 71.0, 70.4, 68.2, 68.1, 66.3 (OCH₂), 60.1, 55.2, 54.6, 54.0, 53.5, 51.8, 41.9, 41.34, 41.29, 39.7, 37.2 (CH₂) 32.6 (Ar-C, C(CH₃)₃), 29.4, 26.5, 25.7, 25.6, 25.5, 24.0, 23.8, 23.5, 22.4, 22.0, 21.8, 20.7, 17.6 (CH₃). MS (MALDI-TOF) *m/z* 1837.925 [M+H]⁺, calcd for C₉₄H₁₂₅N₂₀O₁₅Zn 1837.892. MS-MS (MALDI-TOF-TOF) *m/z* 1839.90 [PcPEG-LARLLT + H]⁺, 1822.88 [PcPEG-LARLLT-NH₂+H]⁺, 1794.88 [PcPEG-LARLLT-CONH₂+H]⁺, 1721.83 [PcPEG-LARLL-NH₂+H]⁺, 1580.75 [PcPEG-LARL-CO+H]⁺, 1339.56 [PcPEG-LA-NH₂+H]⁺, 1268.52 [PcPEG-L-NH₂+H]⁺, 1240.53 [PcPEG-L-CO+H]⁺, 1155.44 [PcPEG-NH₂+H]⁺, calcd for C₉₄H₁₂₅N₂₀O₁₅Zn 1837.892, C₉₄H₁₂₄N₁₉O₁₅Zn 1822.882, C₉₃H₁₂₄N₁₉O₁₄Zn 1794.887, C₉₀H₁₁₇N₁₈O₁₃Zn 1721.834, C₈₃H₁₀₆N₁₇O₁₁Zn 1580.755, C₇₂H₈₃N₁₂O₁₀Zn 1339.565, C₆₉H₇₈N₁₁O₉Zn 1268.528, C₆₈H₇₈N₁₁O₈Zn 1240.533, C₆₃H₆₇N₁₀O₈Zn 1155.444. UV-vis (DMF): λ_{max} (log ε) 352 nm (4.79), 610 nm (4.52), 677 nm (5.29).

ZnPc conjugate 6a—Resin (60.0 mg, 0.0052 mmol) containing GYHWYGYTPQNVI was transferred into a reaction vial. DMF was added (5:1, DMF/resin) and left to soak for one hour, after which it was washed four times with DMF. Pc **3a** (12.1 mg, 0.010315 mmol) was weighed into a 2.0 mL vial and DMF (200 μL) added. Then HOBt (1.4 mg, 0.010315 mmol), HATU (3.9 mg, 0.010315 mmol) and DIEA (5.39 μL, 0.030946 mmol) were added to the Pc solution. The mixture was stirred for five minutes, transferred into the reaction chamber containing the resin and left to shake for 3 days. Cleavage from solid support and purification proceeded as described above for Pc **4b** to afford a blue solid (11.5 mg, 40.5%, mp 208 – 209 °C). ¹H NMR (DMF-*d*₇): δ 10.40, 10.35 (s, 1H, COOH), 9.51-9.20 (m, 6H, Ar-H), 8.45-8.10 (m, 10H, Ar-H), 7.80-7.75 (m, 2H, Ar-H), 7.70-7.50 (m, 2H, Ar-H), 7.45-7.20 (m, 30H, Ar-H), 7.15-6.95 (m, 14H, Ar-H), 6.90-6.78 (m, 4H, Ar-H), 6.74-6.65 (m, 9H, Ar-H), 4.75-4.10 (m, 16H, CH₂O/CH₂NH), 4.04-3.65 (m, 7H, CH₂O/CH₂NH), 2.25-2.04 (m, 6H, CH₂CO), 2.01-1.75 (m, 21H, C(CH₃)₃/CH₂), 1.43 (s, 4H, , CH₂), 1.31 (s, 3H, CH₂), 1.21-1.15 (m, 4H, CH₂), 1.05-1.00 (m, 8H, CH₂), 0.94-0.85 (m, 19H, CH₃). MS (MALDI-TOF) *m/z* 2748.17 [M]⁺, calcd for C₁₄₀H₁₆₅N₂₉O₂₇Zn 2748.1723. MS-MS (MALDI-TOF-TOF) *m/z* 2751.19 [PcPEG-GYHWYGYTPQNVI]⁺, 2733.25 [PcPEG-GYHWYGYTPQNVI-NH₂]⁺, 2702.25 [Pc-PEGGYHWYGYTPQNVI-CONH₂]⁺, 2165.86 [PcPEG-YHWYGYT-NH₂-OH]⁺, 1670.78 [PcPEG-GYHW-CONH₂]⁺, 1490.70. [PcPEG-GYH-CONH₂]⁺, 1346.53 [Pc-PEG-GY-CONH₂]⁺, 1226.34 [PcPEG-G]⁺, 1155.44 [PcPEG-NH₂+H]⁺, calcd for C₁₄₀H₁₆₅N₂₉O₂₇Zn 2748.17, C₁₄₀H₁₆₄N₂₈O₂₇Zn 2733.16, C₁₃₉H₁₆₄N₂₈O₂₆Zn 2705.17, C₁₁₅H₁₂₃N₂₁O₁₉Zn 2165.86, C₉₀H₉₅N₁₇O₁₂Zn 1669.66, C₇₉H₈₅N₁₅O₁₁Zn 1483.58, C₇₃H₇₈N₁₂O₁₀Zn 1346.53, C₆₅H₇₀N₁₂O₉Zn 1226.47, C₆₃H₆₇N₁₀O₈Zn 1155.44. UV-vis (DMF): λ_{max} (log ε) 345 nm (3.84), 612 nm (4.25), 680 nm (4.60).

2. Spectroscopic studies

All absorption spectra were measured on UV-VIS NIR Scanning Spectrometer UV-3101PC SHIMADZU (Cell positioned) equipped with a CPS-260 lamp. The DMF solvent used was HPLC grade and it was the solvent of choice since it dissolved all the Pcs relatively good. Stock solutions (1000 ZM, 1.0 mL) of all Pcs were prepared and the dilutions were prepared by spiking 20 – 80 ZL of the corresponding stock solution into 10.0 mL of solvent. Emission spectra were obtained on a Fluorolog[®]-HORIBA JOBINVYON (Model LFI-3751) spectrofluorimeter. The optical densities of the solutions used for emission studies ranged

between 0.04 – 0.05 at excitation wavelengths. All experiments were carried out within 4 h of solution preparation at room temperature (23–25 °C) with 10 mm path length spectrophotometric cell. The fluorescent quantum yields (Φ_f) were determined using a secondary standard method.³⁰ By comparison with ZnPc ($\Phi_f = 0.17$) as a reference, the values of fluorescence were obtained in DMF solvent.³¹

3. Computational and SPR studies

Docking of EGFR-L1, L2 peptides and their conjugates to EGFR protein extracellular domain was performed using AUTODOCK 4 software.^{32, 33} EGFR crystal structure was obtained from a protein databank (PDB code: 1nql).³⁴ Solvent molecules were removed from the pdb file. Polar hydrogen atoms were added to the structure. Three dimensional structures of peptides and their conjugates, EGFR-L1, EGFR-L2, **4b**, **5b** and **6a** were generated using InsightII (Accelrys Inc., Sandiego, CA). Structures were subjected to 300 K and molecular dynamics (MD) followed by simulated annealing MD.³⁵ The final structure from simulated MD was energy minimized and used for docking studies. A grid box with dimensions of 128×128×128 Å³ was used for calculations. For EGFR-L1 peptide and **5b** docking a grid box was created with amino acid residue on EGFR Asn134 as center of the grid box, and for EGFR-L2, **4b** and **6a** docking a grid box was created near the EGF binding site on EGFR.^{26, 27} For **4b**, **5b** and **6a**, the zinc atom was included in addition to the atom types of the peptide/protein for grid calculations. In all these ligand molecules rotatable bonds were allowed to rotate during docking calculations. For docking, 50 runs with 10 million energy evaluations were carried out using Lamarkian genetic algorithm. Docking calculations were performed on Linux cluster on high performance supercomputers at LSU Baton Rouge. Docked structures were listed in increasing order of energy, and low energy clusters were used as the most probable binding models. The energy reported is for the lowest energy docked structure from a cluster of conformations. Structures from low energy docking were displayed and analyzed using PyMol software.

The surface plasmon resonance (SPR) experiments were performed using Biacore X100 (GE Health Sciences) at 25 °C.^{36, 37} Immobilization of EGFR (obtained from Leinco Technologies, St. Louis, MO), was performed by standard amine coupling procedure on a CM5 chip. The carboxyl groups on the sensor chip were activated by a solution containing 0.2 M N-ethyl-N-(dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccinimide (35 ZL solution, flow rate 5 ZL/min). Running buffer consisted of 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% Tween at pH 7.5. Regeneration buffers were 50% acid cocktail and glycine 100 mM at pH 2.5. The EGF ligand was obtained from Abcam, Inc. (Cambridge, MA).

4. Cell Studies

All tissue culture medium and reagents were purchased from Invitrogen (Carlsbad, CA). Human carcinoma HEp2 cells, human epidermoid carcinoma A431, human colorectal adenocarcinoma HT-29, and cercopithecus aethiops kidney Vero cells, were purchased from ATCC. HT-29 cells were cultured and maintained in McCoy's 5a Medium Modified supplemented with 10% FBS and 1% antibiotic (Penicillin Streptomycin). HT-29 cells were infected with a lentivirus containing the enhanced green fluorescent protein (eGFP; virus purchased from Biogenova, Ellicott City, MD). Green fluorescent cells were sorted by flow cytometry and expanded to generate a line termed "HT-29 eGFP". Both A431 and Vero cells were cultured and maintained in ATCC formulated DMEM supplemented with 10% FBS and 1% antibiotic (Penicillin Streptomycin). HEp2 cells were cultured and maintained in 50:50 ATCC formulated DMEM/Advanced MEM containing 10% FBS and 1% antibiotic (Penicillin Streptomycin). The cells were split twice weekly to maintain a sub-confluent stock. All compound solutions were filter-sterilized using a 0.22 µm syringe filter. A 32 mM

stock solution was prepared for each Pc by dissolving in DMSO containing 5% Cremophor EL (as a nonionic emulsifier), to avoid compound precipitation upon dilution into media. From this solution, a 400 μM stock was also prepared in desired medium and filter-sterilized using a 0.22 μm syringe filter.

4.1. Time-Dependent Cellular Uptake—HEp2 cells and A431 cells were plated at 7,500 per well in a Costar 96 well plate and allowed to grow for 48 h. Vero cells were plated at 7,500 per well in a Costar 96 well plate and allowed to grow for 24 h. HT-29 cells were plated at 7,500 per well in a Costar 96 well plate and allowed to grow for 96 h. Pc stock solutions (32 mM) were diluted to 10 μM Pc solutions in media and added to the cells at different time periods of 0, 1, 2, 4, 8, and 24 h. Uptake of the compounds was stopped by removing loading medium and washing once with PBS. Cells were solubilized by adding 0.25% Triton X-100 in PBS. The Pc concentration was determined by reading its fluorescence emission at 650/700 nm (excitation/emission) using a BMG FLUOstar plate reader (Cary, NC). Cell number was quantified using CyQuant reagent.

4.2. Dark Cytotoxicity—The cells were plated and allowed to grow as described above. Pc stock solutions (32 mM) were diluted to concentrations of 25, 50, 100 and 125 μM in medium, and added to cells for 24 h. The loading medium was removed and medium containing Cell Titer Blue was added to determine the toxicity of the compounds (viable cells were measured fluorescently at 570/615 nm); untreated cells were considered 100% viable and cells treated with 0.2% saponin as 0% viable.

4.3. Phototoxicity—The cells were plated and allowed to grow as described above. Pc stock solutions (32 mM) were diluted to concentrations of 6.25, 12.5, 25, 50 and 100 μM in medium and added to cells for 24 h. The loading medium was removed and fresh medium was added. The conjugates were exposed to light for 20 min using a light system (Newport) for a light dose of $\sim 1 \text{ J/cm}^2$. The plates were chilled at 5 $^{\circ}\text{C}$ using a cooling block. Water was used as a filter for IR radiation. The plates were then incubated for another 24 h followed by removing medium and adding medium containing Cell Titer Blue to determine the toxicity of the compounds.

4.4. Microscopy—The cells were inoculated in a glass-bottom 6-well plate (MatTek) and allowed to grow for 48 h. The cells were then exposed to 10 μM for each Pc for 6 h. Organelle tracers were obtained from Invitrogen and used at the following concentrations: LysoSensor Green 50 nM, MitoTracker Green 250 nM, ER Tracker Blue/white 100 nM, and BODIPY FL C5 ceramide 1 μM . Images were acquired using a Leica DMRXA microscope with 40 \times NA 0.8dip objective lens and DAPI, GFP and Texas Red filter cubes (Chroma Technologies).

5. In Vivo uptake studies

For the animal studies, Nu/nu mice were purchased from Charles Rivers Laboratories at 6 weeks of age. After approximately 2 weeks of quarantine, mice were implanted with tumor cells subcutaneously in the upper flank. For these injections, each cell line was cultured to approximately 75% confluence, then dissociated with trypsin, and concentrated by centrifugation. Since the two tumor lines were found to grow at different rates in nude mice, 1×10^6 A431 cells and 2×10^6 HT-29 eGFP cells were implanted in a volume of 100 μL . The injection material consisted of 4 parts DMEM and 1 part MatriGel basement membrane matrix (BD biosciences). Tumors were allowed to develop until palpable (approximately 6 days for A431 cells and 9 days for HT-29 eGFP cells), after which mice were imaged for time = 0, then injected through the tail vein with 20 μL of a 10 mM solution of Pc **5b** in 10% DMSO and 5% Cremophor EL in PBS, for a dose of $\sim 10 \text{ mg/kg}$. The mice were then

observed for acute adverse responses to the injected Pc and returned to their box until imaged. Prior to imaging at selected time points after Pc administration, mice were anesthetized with isoflurane gas to effect then imaged individually for 30 s at excitation 630 nm and emission 700 nm (x630/m700) in a Kodak In Vivo FX whole animal imager. All animal experiments were conducted by adherence to a protocol approved by the LSU IACUC committee.

To rule out toxicity related to the concentration of DMSO (10%) and Cremophor EL (5%) used to solubilize the conjugates, an additional viability assay was performed *in vitro* to estimate cancer tissue exposure *in vivo* (see Supporting Information for details). Addition of DMSO and Cremophor EL to culture media at either blood volume or extracellular fluid volume dilutions showed no effect on cell viability over a 96 h period suggesting it is unlikely that these agents resulted in toxicity *in vivo* after dilution and distribution.

To quantify relative fluorescence within the image of the tumor region, a region-of-interest (roi) was drawn around the tumor and the mean pixel intensity (mpi) of this roi was divided by the mpi's of 3 adjacent skin areas to obtain a percent of tumor fluorescence over adjacent regions. In order to visually distinguish one roi as more fluorescent than adjacent skin, we found that the roi needed to be at least 120%.

At 24 and 96 h following iv injection of Pc **5b**, the HT-29 tumor tissue was harvested from mice and flash frozen. The tissue was kept in 5 mL of acetone/methanol (5:1) at -20 °C overnight, then crushed repeatedly using a mortar, filtered and the organic solvents removed under reduced pressure. The residues were analyzed by MS-MALDI-TOF and UV-Vis spectrophotometry. Frozen sections of tumor were cut to 10 μm thickness and mounted on glass slides. Tissues were immediately imaged for both eGFP indicating regions of HT-29 tumor and near-IR indicating Pc fluorescence using Chroma 41017 (450–490 nm excitation, 500–550 nm emission) and Omega Optical 140-2 (570–645 nm excitation, 668–723 nm emission) filter sets respectively.

Results and Discussion

1. Synthesis and characterization

The Pc-peptide conjugates described in this study were designed to specifically target EGFR. The synthetic route to conjugates **4b**, **5a**, **5b** and **6a** is shown in Scheme 1. Pcs **1a,b** were prepared by statistical condensation of 3- or 4-(*p*-*N*-Boc-aminophenoxy)phthalonitrile and 4-*tert*-butylphthalonitrile (in 1:3 ratio) in DMAE at 140 °C for 5 h, and in the presence of Zn(II) acetate and DBN, followed by TFA deprotection of the Boc groups, as we have previously described.²⁹ Reaction of Pcs **1a,b** with diglycolic anhydride in DMF gave the corresponding α- or β-substituted carboxy-terminated Pcs **2a,b**, respectively, in 87–92% yields.^{28, 38} The coupling of Pcs **2a,b** with commercially available *tert*-butyl protected PEG using HOBt, EDCI and DIEA, following by deprotection of the *tert*-butyl group afforded the Pcs **3a,b** in 68–73% overall yields;³⁸ lower yields were obtained when TBTU was used in place of EDCI, due to a more difficult purification of the target Pc-PEG compounds. Solid-phase conjugation of Pcs **2b**, **3a** and **3b** to the two peptide sequences LARLLT²⁶ (EGFR-L1) and GYHWYGYTPQNV²⁷ (EGFR-L2) using DIEA, HOBt and TBTU or HATU in DMF and at room temperature, gave the targeted Pc-peptide conjugates **4b**, **5a**, **5b** and **6a** in 20–80% yields, after deprotection and cleavage from the solid support, followed by reversed-phase chromatographic purification. A glycine residue was added to the N-terminus of the EGFR-L2 peptide in order to increase the conjugation reaction yields.^{38, 39}

EGFR over-expression has been found in a variety of human cancers, including breast, ovarian, prostate, pancreatic, and colorectal;⁴⁰ for this reason EGFR has been an important

target for cancer treatment.^{13–17} Monoclonal antibodies (mAbs), such as cetuximab and trastuzumab, and tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib, are HER1/EGFR-targeted agents currently in clinical development, or already approved, for use in several countries. Peptides EGFR-L1 and EGFR-L2 were designed to act as a substitute for the natural ligand EGF, which has reported mitogenic and neoangiogenic activity, and has been shown to specifically target EGFR over-expressing tumor cells both *in vitro* and *in vivo*.^{26, 27} Advantages of using small peptide ligands as target units are their easy synthesis and coupling to fluorophores, and their low immunogenicity and high binding affinity for the biological target. We have previously conjugated a Pc macrocycle to a lysine-rich bifunctional peptide sequence containing 32 amino acid residues, via a similar short (5-atom) and a long (20-atom) PEG linker, and observed that the PEG linker increased cellular uptake into human HEP2 cells, and decreased cytotoxicity of the Pc conjugate.²⁸ On the other hand, the short linker Pc conjugate showed higher fluorescence quantum yield, probably as a result of its lower conformational flexibility compared with the PEG group. In the present study we investigated a short (5-atom) and a low molecular weight PEG (13-atom) linkers between the Pc and the peptide ligand; the smaller PEG group has the advantages of being commercially available and potentially less flexible than the penta(ethylene glycol) previously used. In addition, the PEG linker is believed to favor an extended conformation for conjugates **5a,b** and **6a**,³⁸ which might favor EGFR target binding, and to increase their aqueous solubility compared with conjugate **4b** (*vide infra*).

All Pcs were characterized by MS, NMR, UV-Vis and fluorescence spectroscopy; MALDI-TOF was used to confirm the amino acid sequence in the Pc-peptide conjugates (see Supporting Information, Figures S22–S52). The spectroscopic properties for Pcs **3a,b**, **4b**, **5a,b** and **6a** are summarized in Table 1 and Figure S18 of the Supporting Information). All Pcs showed strong Q absorption bands between 677 – 680 nm in DMF, and emissions between 680 – 683 nm in the same solvent, with fluorescence quantum yields in the range 0.10–0.13 and Stokes' shifts of 2 – 4 nm, characteristic of this type of compound.^{28, 29} All Pc-peptide conjugates are soluble in polar organic solvents, such as DMSO and DMF, up to 1.0 mM concentrations, however precipitation was observed upon dilution into aqueous solutions. Therefore, Cremophor EL, a non-ionic solubilizer and emulsifier, was added to all Pc stock solutions used in the biological and EGFR-binding experiments; the cell studies were conducted in PBS/DMSO/Cremophor (94:1:5) and the mice studies in PBS/DMSO/Cremophor (85:10:5). No toxic effects were observed *in vitro* nor *in vivo* from these amounts of DMSO and Cremophor EL (see Figure S57 of the Supporting Information).

The solubility of the Pcs decreased in the order Pc-PEG (**3a,b**) > Pc-EGFR-L1 (**5a,b**) > Pc-EGFR-L2 (**6a**) > Pc-EGFR-L2 (**4b**), due to the high hydrophobicity of the longer EGFR-L2 sequence (containing 11 hydrophobic amino acids and only two polar), compared with EGFR-L1 (containing 4 hydrophobic amino acids, one polar and one cationic). Indeed, the uncharged Pc conjugates **4b** and **6a** showed decreased solubility in polar protic solvents such as methanol, compared with the positively charged Pc conjugates **5a,b**. The least soluble was the Pc-EGFR-L2 conjugate **4b** bearing a short 5-atom linker.

2. Docking and binding studies

To model the interaction of peptides EGFR-L1 and EGFR-L2 and their Pc conjugates with EGFR docking studies were carried out using Autodock.^{32, 33} EGFR-L1 is known to bind to a pocket away from the EGF binding pocket in EGFR,²⁶ while EGFR-L2 binds to the EGF binding pocket.²⁷ Hence, for EGFR-L1 grid box for docking calculations were around Glu71, Asn134, Gly177. The low energy docked structure (docking energy of -6.06 kcal/mol) of EGFR-L1 is shown in Figure 1a. The Leu4 backbone carbonyl and Thr6 side chain

hydroxyl groups form hydrogen-bonding interaction with EGFR. The RLLT sequence of the peptide acquired a β -turn conformation when bound to the receptor EGFR.

The low energy docked structure of EGFR-L2 peptide is shown in Figure 1b. EGFR-L2 peptide binds to EGF binding pocket with docking energy of -5.96 kcal/mol. Tyr1 and Ile12 residues from the peptide formed hydrogen-bonding interactions with the EGFR stabilizing the peptide-receptor interaction. Overall, the peptide did not have any particular secondary structure as shown in the Figure 1b. Peptide was bound in the cavity of EGF binding pocket of EGFR. These results indicate that both peptides EGFR-L1 and EGFR-L2 can bind to EGFR.

To evaluate the effect of conjugation on binding to EGFR and the linker size, docking studies were performed on Pc conjugates **4b**, **5b** and **6a**. In Pc-EGFR-L2 **4b** the peptide is conjugated via a short (5-atom) linker, in **6a** via 13-atom PEG linker, while in Pc-EGFR-L1 **5b** the peptide is conjugated via 13-atom PEG linker. Docking of **4b** and **6a** were similar to that of EGFR-L2 (see Figure S55 of the Supporting Information). However, the docking energies obtained for conjugates **4b** and **6a** were lower (with a docking intermolecular energy of -17 kcal/mol for **4b** and -8 kcal/mol for **6a**) than that of peptide EGFR-L2 alone (-6 kcal/mol). Conjugates **4b** and **6a** were docked to the EGF binding site, with the Pc macrocycle extending outside the EGF binding pocket and anchoring near the hydrophobic region around amino acid residues Tyr89, Tyr93 and Phe148 of EGFR. On the other hand, Pc-EGFR-L1 conjugate **5b** showed lower docking energy (-12 kcal/mol) compared with the peptide alone (-6 kcal/mol). The peptide part of **5b** was bound to a groove near amino acids Lys56, Asn79, Glu181 and Lys185 of EGFR, which are away from the binding site of EGF. The Pc part of conjugate **5b** was anchored to the other face around Pro171 of EGFR (see Figure S56 of the Supporting Information). These results suggest that the Pc-peptide conjugates should bind to EGFR with even higher affinity than the peptides alone. A surface plasmon resonance (SPR) binding assay^{36, 37} was performed to confirm binding of the peptides and Pc conjugates **5b** and **6a** to EGFR (see Table S1 and Figure S58 of the Supporting Information). EGFR was immobilized on the chip surface and the relative binding of different ligands was analyzed. The binding of high affinity ligand EGF to EGFR was clearly seen in SPR. Other ligands and Pc conjugates reported in this study showed relatively lower affinity to EGFR. Conjugate **6a** was found to have the highest affinity for EGFR, followed by **5b**. The peptides alone bound with lower affinities than the Pc conjugates, and EGFR-L2 showed higher affinity for EGFR than EGFR-L1, in agreement with the docking studies. The Pc-PEG **3b** was also observed to bind to EGFR but with lower affinity than the Pc-peptide conjugates.

3. Cell culture

Four cell lines with different EGFR expressions were used to investigate the cytotoxicity, uptake and subcellular distribution of Pc-peptide conjugates **4b**, **5a**, **5b** and **6a**: human squamous cell carcinoma HEP2,^{28, 29, 38, 39} human epidermoid carcinoma A431,⁴¹⁻⁴³ cercopithecus aethiops kidney Vero (as negative control),⁴⁴ and human colorectal adenocarcinoma HT-29 cells.⁴⁵⁻⁴⁷ The model human HEP2 cells are often used in the investigation of peptide-fluorophore conjugates, while the human A431 cells are a positive control for high EGFR expression (~1-3 million EGFR per cell), and the African green monkey Vero cells are a negative control (lowest expression of EGFR). The human colorectal adenocarcinoma HT-29 cells over-express EGFR, although to a lower degree (~9000 EGFR per cell) than the A431 cells.⁴⁵⁻⁴⁷

Cytotoxicity—The dark- and photo-cytotoxicity for Pc-PEG **3a** and Pc-peptide conjugates **4b**, **5a,b** and **6a** were evaluated in all cell lines at concentrations up to 125 μ M, using the

Cell Titer Blue Assay, and the results are summarized in Table 2 and shown in Figures S20 and S21 of the Supporting Information. None of the Pcs was found to be toxic in the dark, with determined IC_{50} values for all Pcs above 125 μM , the highest concentration investigated. Upon exposure to a low light dose (1 J/cm^2), only Pc **5a** bearing the EGFR-L1 peptide was found toxic to A431, HEp2 and Vero cells ($IC_{50} = 16, 17$ and $47 \mu\text{M}$, respectively). In human carcinoma HEp2 cells, Pc conjugate **5a** was significantly more phototoxic than **5b** ($IC_{50} \sim 100 \mu\text{M}$), in agreement with our recent observations showing that α -substituted Pcs tend to be more phototoxic than the corresponding β -substituted Pcs.²⁹ On the other hand, none of the Pcs were phototoxic to human HT-29 cells, suggesting that this type of conjugate could potentially be used for imaging of colorectal tumors. Low toxicity is an important feature of potential new imaging agents to be employed in conjunction with CLE. The Pc-EGFR-L1 conjugate **5a** was significantly more phototoxic than the corresponding Pc-EGFR-L2 conjugate **6a**, which might be a result of its higher uptake into cells due to its cationic charge (*vide infra*). The presence of the PEG linker did not have an effect on the observed cytotoxicity of the conjugates, although it increased their aqueous solubility.

Time-dependent uptake—The time-dependent cellular uptake for all Pcs was performed at the non-toxic concentration of 10 μM in all cell lines, and the results are shown in Figure 2. There were marked differences in the cellular uptake of the Pc-EGFR-L1 conjugates compared with the Pc-EGFR-L2 conjugates, which might be due to their different overall charge, solubility and tendency for aggregation. While the highly hydrophobic and uncharged Pc-EGFR-L2 conjugates **4b** and **6a** were poorly taken up by all cell lines, in particular by A431 and HT-29 cells which over-express EGFR, the positively charged Pc-EGFR-L1 conjugates **5a** and **5b** accumulated within cells to a much higher extent. Cells with higher EGFR expression showed higher uptake of the Pc conjugates, about 4-fold increase in A431 vs Vero cells, and a 3- or 2-fold increase in HT-29 colorectal and HEp2 cancer cells, respectively. This result shows that Pc conjugates **5a** and **5b** can indeed target cancer cells over-expressing EGFR, in particular CRC cells. It is interesting to compare the very different Pc uptake into Vero cells, with low expression of EGFR; Pc conjugate **5b** accumulated the fastest, followed by **5a** and precursor Pc-PEG **3a**. The Pc-EGFR-L2 conjugates **4b** and **6a** were taken up by Vero cells to a much lower extent than the Pc-EGFR-L1 conjugates **5a,b**, and **6a** bearing a PEG linker accumulated ~ 2 -fold higher than **4b**. These results show that the Pc macrocycle has a natural tendency to accumulate within cells, with or without over-expression of EGFR, and that the PEG-containing compounds tend to be taken up to a higher extent. The conjugation of the Pc to a small cationic peptide sequence (EGFR-L1) changes the uptake kinetics, as seen in Figure 2b, and as previously observed.³⁹ In EGFR over-expressing cells, Pc conjugates **5a** and **5b** clearly were taken up the most of all Pcs investigated. For example, in A431 cells, conjugates **5a** and **5b** accumulated ~ 15 -fold more than Pc-PEG **3a** and than the Pc-EGFR-L2 conjugates **4b** and **6a** (Figure 2a). Similarly in HT-29 and HEp2 cells, **5a** and **5b** were taken up to a significant higher extent than Pcs **3a**, **4b** and **6a**. These results indicate that the peptide sequence has a marked effect on the cell targeting and uptake ability of the Pc conjugates. Autodock and SPR investigations show that peptides EGFR-L1 and EGFR-L2, and Pc conjugates **4b**, **5b** and **6a** bind to EGFR (*vide supra*). Although Pc-EGFR-L2 conjugates can bind to EGFR with high specificity and be retained at the cell surface rather than internalized, their low uptake into Vero cells with low expression of EGFR suggests that their low solubility and high tendency for aggregation are responsible for their observed low cellular uptake. Indeed, the least soluble Pc **4b** accumulated the least within cells compared with all Pcs investigated, although this conjugate gave the lowest docking energy (-17 kcal/mol) of all molecules investigated. On the other hand, the Pc-EGFR-L1 conjugates bearing an arginine residue and overall +1 charge were readily taken up by all cells, as we have previously observed for

porphyrin-peptide conjugates bearing 1 to 4 positively charged residues.⁴⁸ Positively charged molecules have been observed to have enhanced ability for crossing negatively charged plasma membranes, in particular those containing arginine due to the unique ability of the protonated guanidinium group to form bidentate hydrogen bonds.^{48–52}

Of all Pc-peptide conjugates investigated, **5b** accumulated the fastest within all cell lines, followed by **5a**, which was found in the highest amount within A431 and HT-29 cells, at times > 8 h after Pc exposure. Because of its rapid and efficient uptake into cells and low cytotoxicity, Pc conjugate **5b** was chosen for further investigation in animal studies (*vide infra*).

Subcellular Localization—The preferential sites of intracellular localization of Pc-peptide conjugates **4b**, **5a,b** and **6a** and Pc-PEG **3a** were evaluated using fluorescence microscopy, in the three human cell lines over-expressing EGFR, i.e. A431, HT-29 and HEP2 cells. The results are summarized in Table 3 and shown in Figures 3 and 4 for conjugate **5b** and in the Supporting Information, Figures S1–S13. Co-localization experiments were performed using the organelle-specific probes LysoTracker Green (lysosomes), ER Tracker Blue/White (ER), MitoTracker Green (mitochondria) and BODIPY Ceramide (Golgi). All conjugates localized in multiple sites within the cells. The Pc-EGFR-L1 conjugate **5b** was found in the lysosomes, mitochondria and Golgi of all cell lines; in addition its regioisomer **5a** was also observed in the ER, which might in part explain its higher phototoxicity compared with **5b**.^{53, 54} Pc-EGFR-L2 conjugates **4b** and **6a** were also found in lysosomes, Golgi and mitochondria. Lysosomal localization might result from an endocytic pathway of this type of molecules, as we have previously observed.^{28, 48}

We also investigated potential fluorescence from the Pc conjugates on the plasma membrane, in order to detect any non-internalized EGFR-bound conjugate. However, no fluorescence was detected by microscopy, indicating that the Pc conjugates do not localize on the plasma membrane. No co-localization was observed with fluorescent probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) that specifically labels the plasma membrane. This result indicates that the Pc-EGFR-L2 conjugates have poor EGFR targeting ability, probably because of their low solubility and high tendency for aggregation. On the other hand, the Pc-EGFR-L1 conjugates **5a** and **5b** efficiently target EGFR over-expressing cells and are internalized, to a level that is dependent on the degree of EGFR expression.

4. Mouse studies

Pc-peptide conjugate **5b** was chosen for further evaluation in mouse studies, due to its low cytotoxicity and rapid accumulation within cells *in vitro*. Following iv administration of 10 mg/kg of Pc **5b**, nude mice showed the emergence of tumor-selective fluorescence indicating that the conjugate was taken up into both A431 and HT-29 s.c. tumors. Fluorescence signal which exceeded the background of adjacent skin regions became apparent at 24 h in both tumor types (representative mice shown in Figure 5). Microscopically, **5b** deposition in the tumor tissue was not homogeneous but rather seemed to occur in a multifocal pattern perhaps reflecting regions of greater vascularity (Figure 6); however, sufficient Pc **5b** accumulated within the entire tumor to result in a near-IR fluorescence signal distinguishing tumor above adjacent normal tissue. Quantitatively, the signal in the HT-29 tumors declined from 24 to 96 h and could not be readily differentiated from adjacent regions without tumor at 96 h. Preliminary studies with the Kodak In Vivo FX imager, demonstrated that in order to visualize a fluorescence signal, that signal needed to be at least 1.2-fold greater than adjacent regions of the mouse. The fluorescence signal

emission in the A431 tumor continued to increase to 96 h (Figure 7) suggesting continued uptake of the Pc conjugate over a longer time period in cells over-expressing EGFR.

For purposes of comparison, the two tumor groups were not significantly different in tumor size (A431 = $133.3 \pm 28.7 \text{ cm}^3$ and HT-29 = $141.9 \pm 21.4 \text{ cm}^3$ at 96 h). Neither tumor demonstrated auto-fluorescence at the 630/700 nm spectra window at time 0 when compared with adjacent skin surfaces.

In vivo, tumor-associated fluorescence excitable at 630 and emitted at 700 nm was detectable at 24 h. Correspondence of these wavelengths to the Pc conjugate spectra indicates uptake of the conjugate by the two subcutaneous human tumor xenografts. While both HT-29 and A431 cells over-express EGFR, there is a vast difference in the degree of receptor expression, with A431 ~ 200 times higher EGFR expression than HT-29.⁴³⁻⁴⁵ If the Pc conjugate homed to the tumor tissue based solely on the presence of the EGFR ligand, we would not have expected to observe the comparable levels of fluorescence from both tumors at 24 h. Therefore, in addition to tumor attraction based on EGFR-peptide binding, our results suggest significant tumor homing by the Pc macrocycle itself, in agreement with the *in vitro* results (see Figure 2b) and previous observations.⁸ The extended fluorescence-time within the A431 tumor over the HT-29 tumor suggests that with greater quantities of EGFR, comes longer retention and/or greater capacity for selective uptake or re-uptake. At times prior to 24 h, we found no or weak signal, suggesting a prolonged time period of plasma circulation with gradual tumor accumulation. A similar pattern of tumor uptake was recently described in a xenograph breast cancer model imaged following administration of a chiral porphyrine,⁵⁵ which showed increasing signal over background up to 48 h post administration. On the other hand, chlorin e6-HSA nanoparticles were recently used for PDT of HT-29 tumor xenograph,⁵⁶ and shown to have higher tumor-targeting ability and accumulation than free chlorin e6 as a result of their prolonged blood circulation. Two other chlorin derivatives, HPPH-3Gd(II)ADTPA⁵⁷ and TCPCSO3H⁵⁸ were recently shown to accumulate within tumor-bearing mice, reaching maximum accumulation levels at 24 h post-administration.

We detected no signal in adjacent non-tumor regions indicating a high degree of tumor selectivity; however, we noted that normal gastrointestinal contents in the mice also fluoresced at the near-IR spectra window required for this Pc-conjugate. This background fluorescence is difficult to remove in mice since prolonged fasting is not feasible. For imaging of colon or urogenital malignancies, it would likely be necessary to remove fecal material prior to tumor detection.

In order to evaluate the *in vivo* stability of conjugate **5b**, the HT-29 tumors were harvested at 24 and 96 h following iv injection and extracted using acetone/methanol mixture. The UV-vis spectra of both the 24 and 96 h tumor extracts in methanol showed the characteristic Q band absorption of the Pc (Supporting Information, Figure S19), indicating that the Pc macrocycle has high stability *in vivo*, allowing for prolonged and selective accumulation within tumor tissue. MALDI-TOF MS indicated that at 24 h, intact conjugate **5b** was still present in the tumor extract, while at 96 h the major Pc species detected was Pc-PEG-LARL (Supporting Information, Figures S53 and S54). This result suggests that the EGFR-L1 peptide in Pc **5b** is slowly degraded within tumor tissue by proteolytic enzymes, mainly losing the two terminal amino acids 96 h after iv injection. This result is in agreement with our previous observations that this type of peptide conjugate can undergo metabolic degradation within tumor cells, with half life ~ 24 h.⁵⁹

Conclusions

Four Pc-peptide conjugates (**4b**, **5a**, **5b** and **6a**) were designed and synthesized to target EGFR, and investigated as potential fluorescence imaging agents for cancers over-expressing EGFR, such as CRC. Two peptide ligands for EGFR containing 6 (LARLLT) and 13 (GYHWYGYTPQNV) amino acid residues, were conjugated to the Pc via a short 5-atom, or a 13-atom PEG linker. The PEG group enhances the solubility of the Pc-peptide conjugates and tends to increase their cellular uptake. Using Autodock both peptide ligands were found to bind to EGFR, giving low energy (-6 kcal/mol) docking structures, while conjugation to the Pc gave even lower docking energies (-8 to -17 kcal/mol) due to additional interactions of the Pc macrocycle with hydrophobic residues on EGFR. SPR studies confirmed the binding of Pc-peptide conjugates to EGFR.

The short EGFR-L1 peptide conjugates **5a** and **5b** are positively charged and were efficiently internalized by all cell lines (A431, HT-29, HEp2 and Vero cells), localizing preferentially in lysosomes, Golgi and mitochondria. On the other hand, the 13-residue EGFR-L2 peptide produced highly hydrophobic Pc conjugates **4b** and **6a** that, mainly as a result of their poor water-solubility and high tendency for aggregation, poorly targeted EGFR at the plasma membranes and were poorly internalized. The amount of Pc-EGFR-L1 conjugates, **5a** and **5b**, taken up by cells was dependent on their degree of EGFR expression. While **5a** and **5b**, as well as Pc-PEG **3a** also accumulated within the low EGFR expressing Vero cells, increased uptake was observed with increasing EGFR expression in the human cell lines (A431 > HT-29 > HEp2). The observed uptake into low EGFR expressing cells indicates that the Pc macrocycle has a natural tendency to target and accumulate within cancer cells.

All conjugates were found to be non-toxic ($IC_{50} > 100 \mu\text{M}$) to both low- and over-expressing EGFR cells, with exception of conjugate **5a** that showed moderate phototoxicity toward A341, HEp2 and Vero cells ($IC_{50} = 16, 17$ and $47 \mu\text{M}$, respectively). None of the conjugates were toxic towards human colorectal HT-29 cells ($IC_{50} > 100 \mu\text{M}$). This result, in addition to the observed near-IR fluorescence emissions of all Pc conjugates at ca. 682 nm with quantum yields in the range 0.10–0.13, and enhanced uptake of **5a** and **5b** by cancer cells, makes these Pc-EGFR-L1 conjugates highly promising fluorescent contrast agents for CRC, and potentially other EGFR over-expressing cancers, in particular the least phototoxic ($IC_{50} > 100 \mu\text{M}$ at 1 J/cm^2) β -substituted Pc-peptide conjugate **5b**.

Conjugate **5b** was further investigated in nude mice bearing A431 and HT-29 human tumor xenografts. Twenty four hours after iv administration of **5b**, a clearly near-IR fluorescence signal (exc 630 nm/ em 700 nm) was seen over background adjacent tissues, in both tumor types. While the fluorescence signal decreased in HT-29 tumors after 24 h, it continued to increase in the A431 tumors up to 96 h, the longest time investigated. The MS analysis of tumor extracts 96 h after iv injection of Pc-peptide **5b** indicated partial degradation of the conjugate, by proteolytic enzymes, mainly leading to the cleavage of the last two amino acids of EGFR-L1.

Our studies show that Pc-peptide conjugates can be used for near-IR fluorescence imaging of cancers over-expressing EGFR, such as CRC. Due to the hydrophobic nature of the Pc macrocycle, a low molecular PEG linker and a polar or charged peptide ligand are required for adequate aqueous solubility and receptor targeting ability. In addition, a β -substituted Pc macrocycle appears to be the most suitable for imaging applications due to its lower phototoxicity compared with the corresponding α -substituted macrocycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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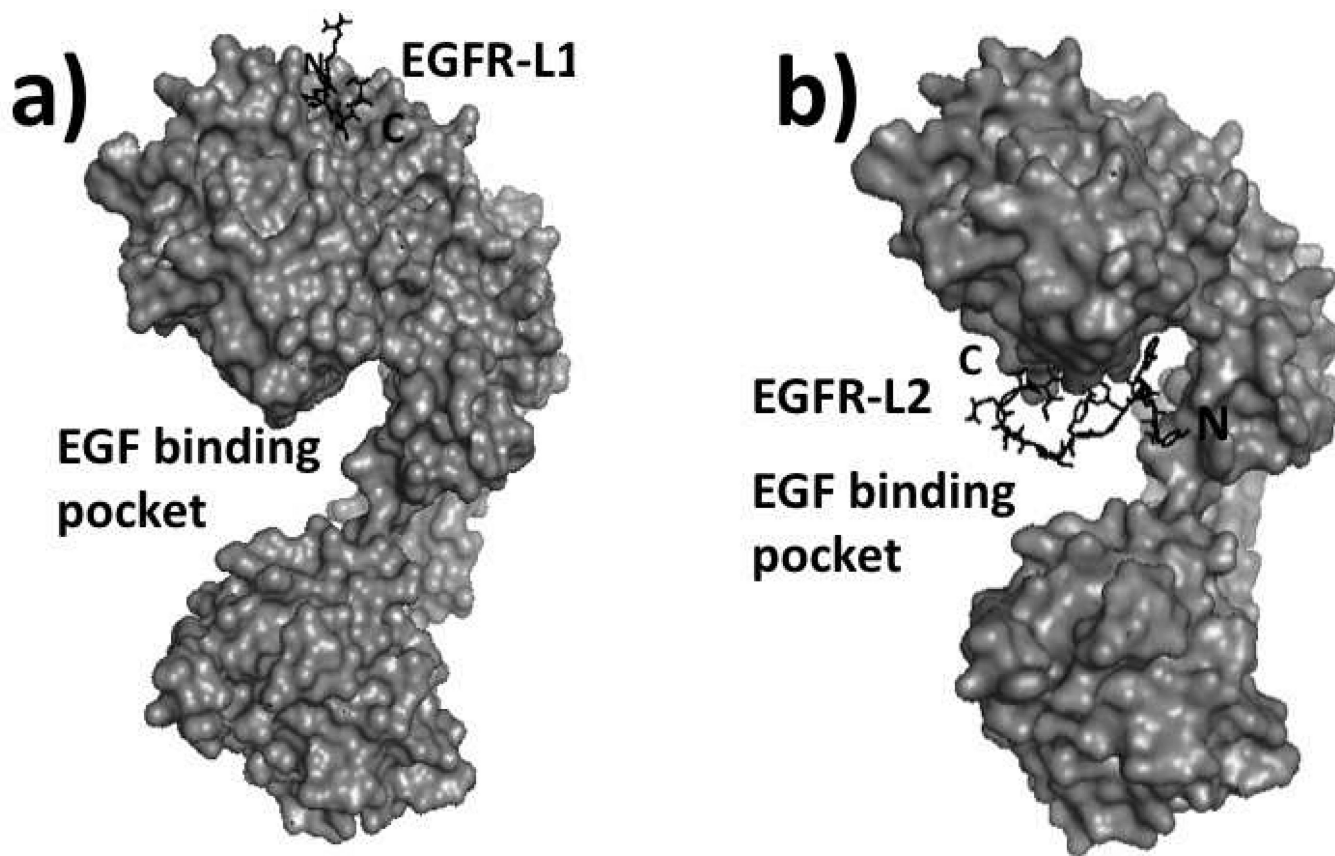


Figure 1. Low energy docked structure of (a) EGFR-L1 and (b) EGFR-L2 peptides with EGFR receptor. EGFR is shown in surface and the peptide is shown as dark sticks. The EGF binding pocket is shown.

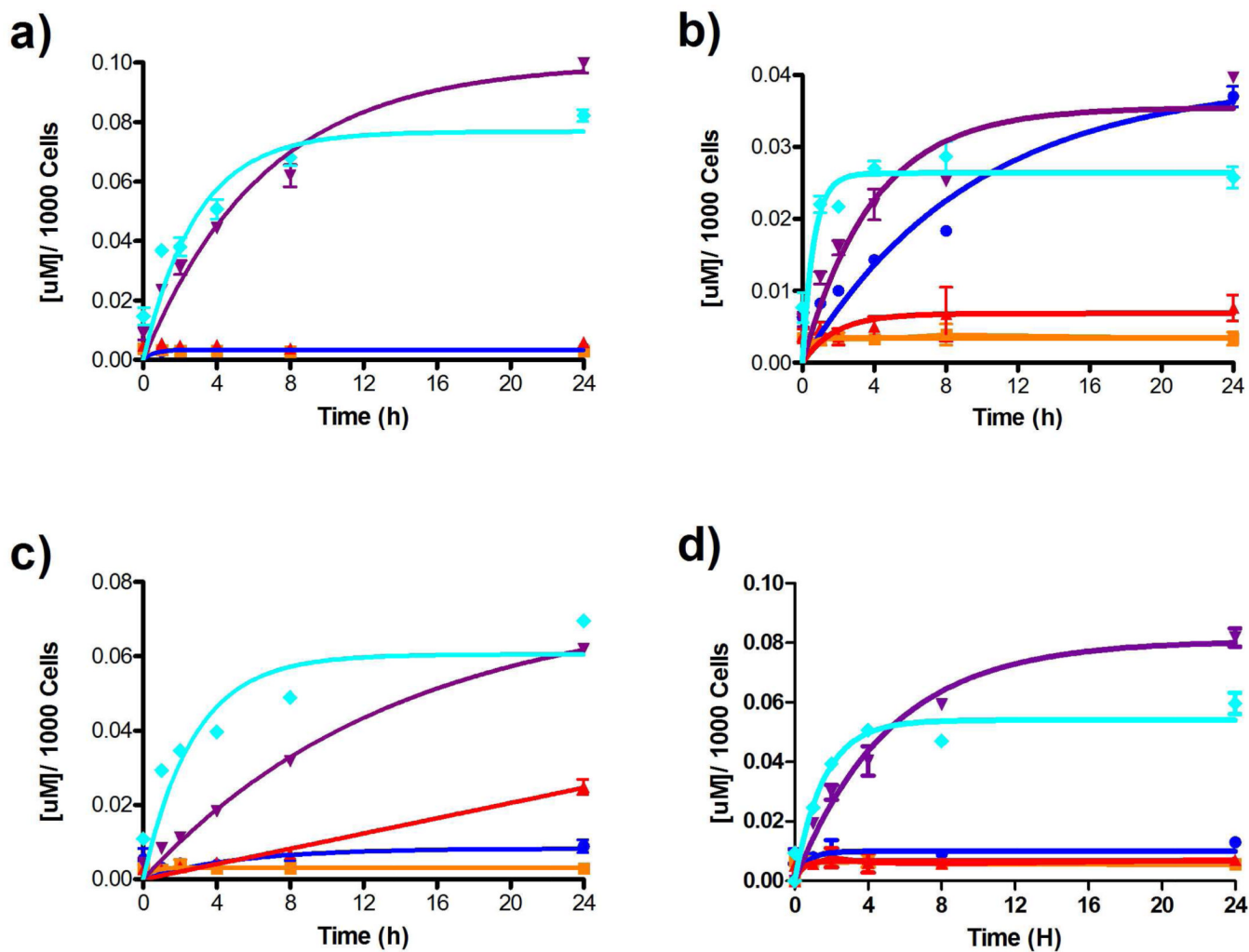


Figure 2. Time-dependent uptake of Pcs **3a** (blue), **4b** (orange), **5a** (purple), **5b** (turquoise) and **6a** (red) at 10 μM by (a) A431, (b) Vero, (c) HEP2 and (d) HT-29 cells.

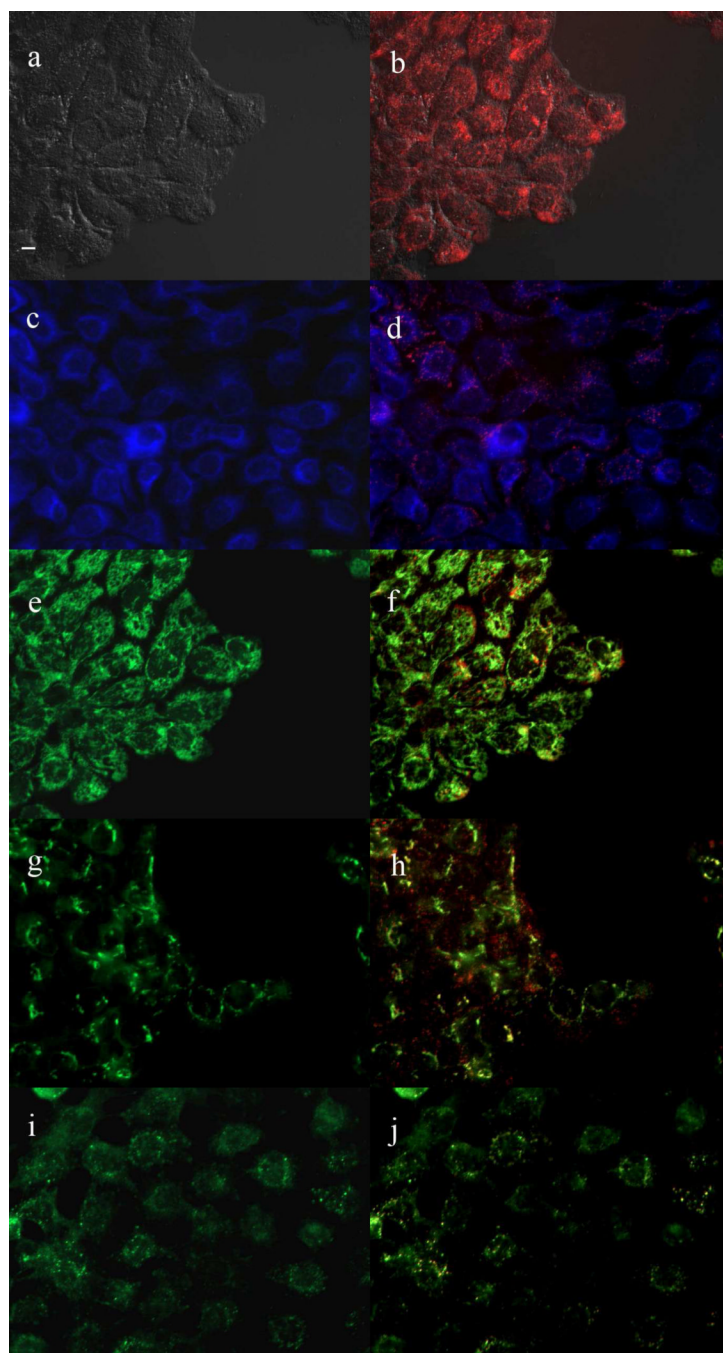


Figure 3. Subcellular fluorescence of Pc **5b** in A431 cells at 10 μM for 6 h. (a) Phase contrast, (b) Overlay of **5b** fluorescence and phase contrast, (c) ER Tracker Blue/White fluorescence, (e) MitoTracker green fluorescence, (g) BODIPY Ceramide, (i) LysoSensor green fluorescence, and (d, f, h, j) overlays of organelle tracers with **5b** fluorescence. Scale bar: 10 μm .

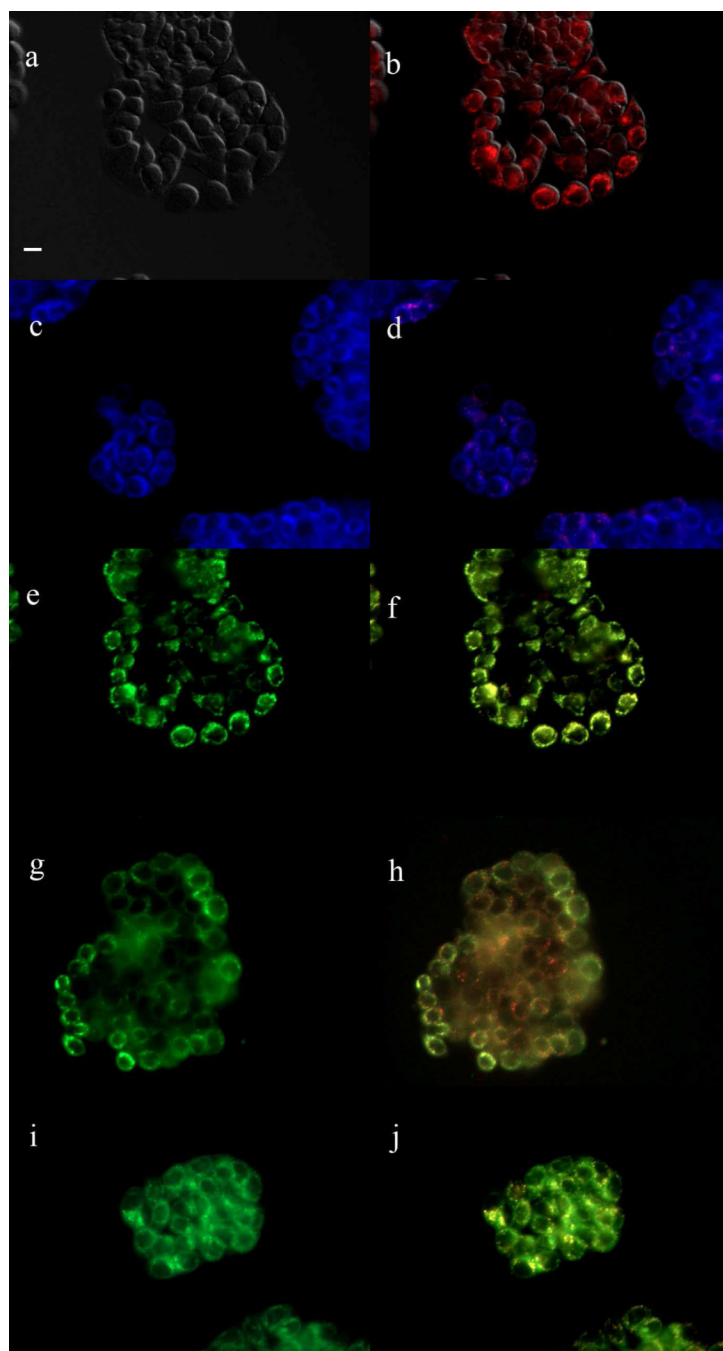


Figure 4. Subcellular fluorescence of Pc **5b** in HT-29 cells at 10 μ M for 6 h. (a) Phase contrast, (b) Overlay of **5b** fluorescence and phase contrast, (c) ER Tracker Blue/White fluorescence, (e) MitoTracker green fluorescence, (g) BODIPY Ceramide, (i) LysoSensor green fluorescence, and (d, f, h, j) overlays of organelle tracers with **5b** fluorescence. Scale bar: 10 μ m.

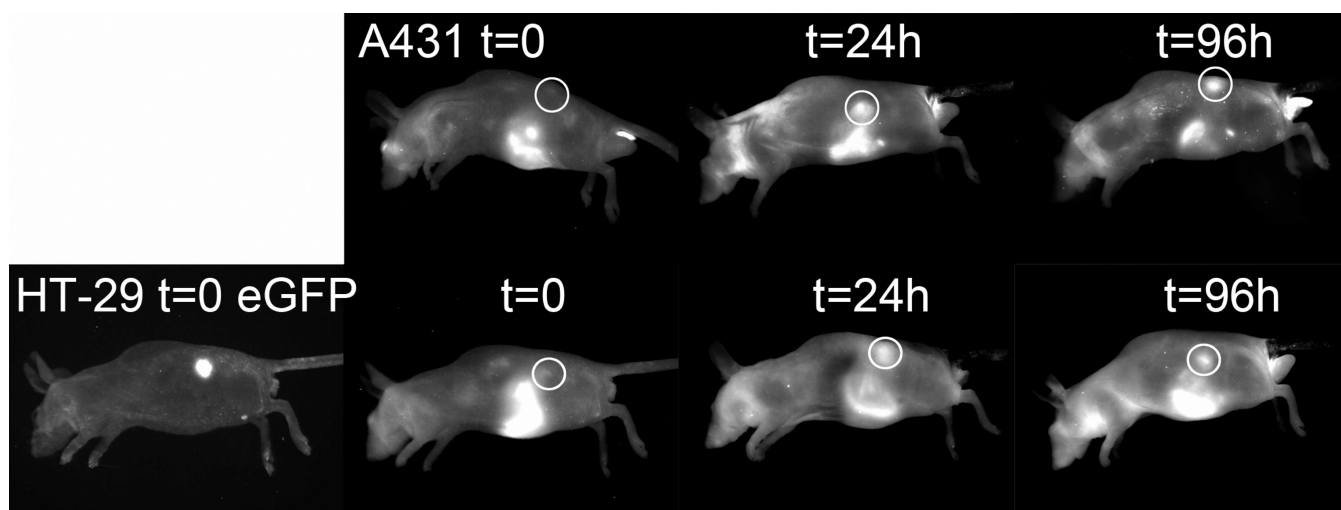


Figure 5. Fluorescent images (exc 630 nm/em 700 nm) of nude mice bearing s.c tumor implants of A431 (top) or HT-29 (bottom) cancer cells at various times following iv administration of Pc **5b**. The tumor positions are circled and the left panel of HT-29 mouse shows the eGFP tumor fluorescence (exc 490 nm/em 535 nm).

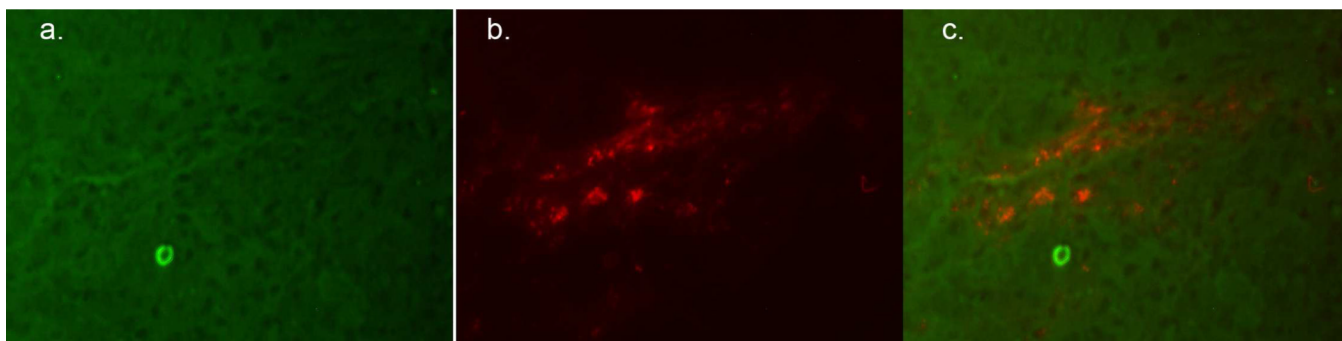


Figure 6. Deposition of Pc **5b** in the HT-29 tumor xenograph, 24 h following iv injection. Images show (a) eGFP indicating HT-29 tumor regions, (b) Pc **5b** fluorescence, and (c) overlap of tumor and Pc deposition.

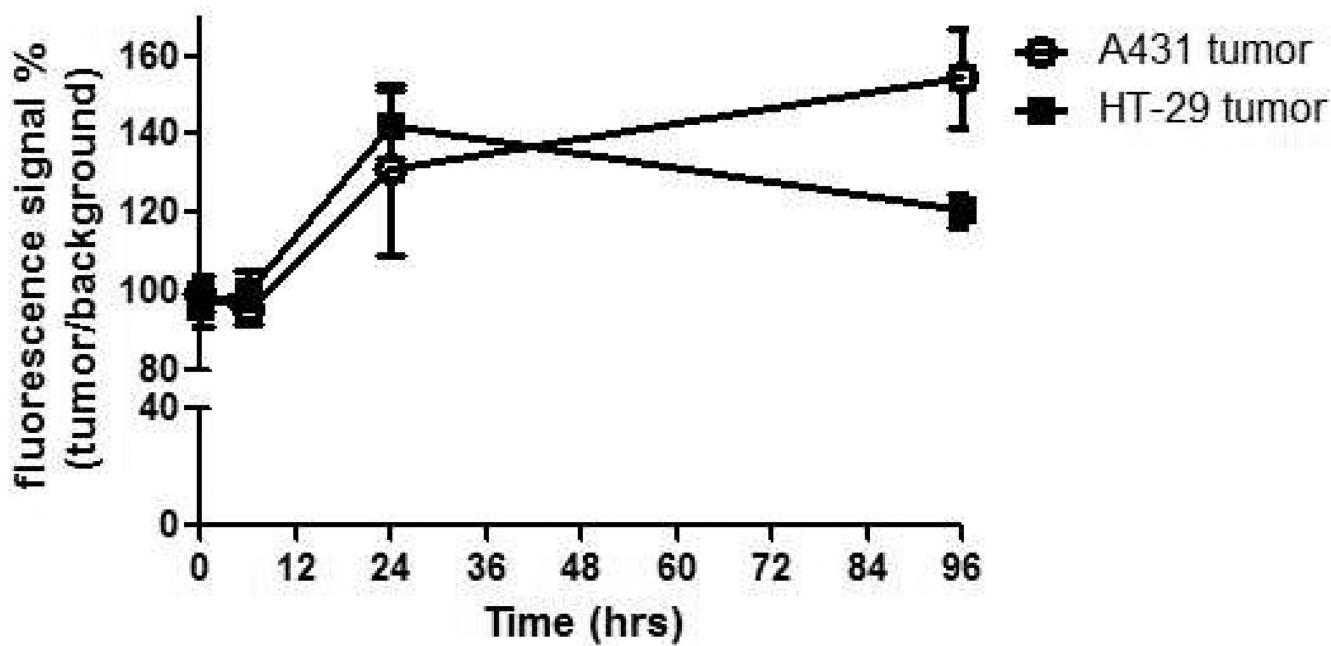
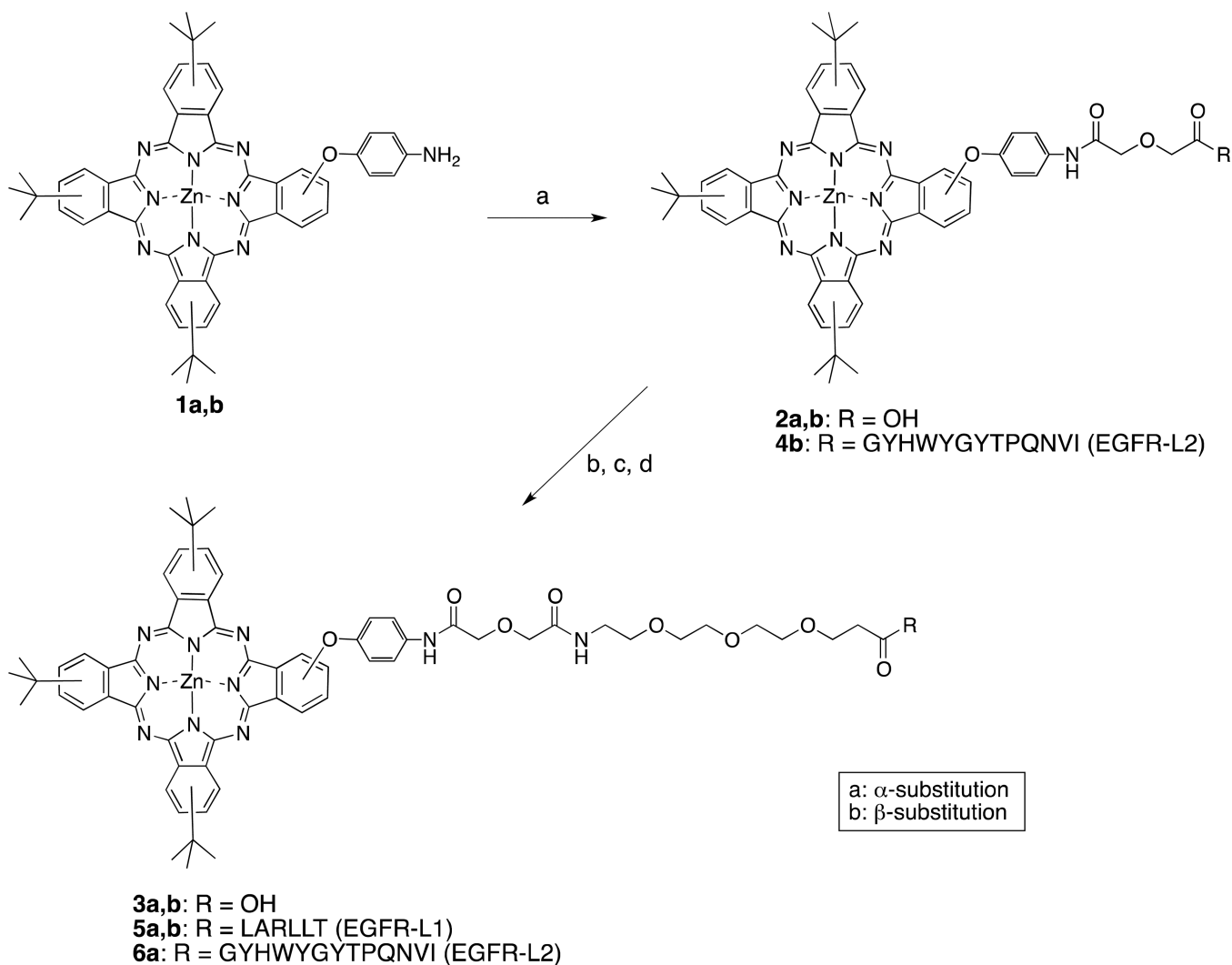


Figure 7. Emission signal of **Pc 5b** at 700 nm in the s.c. human tumor xenographs, over the adjacent (background) skin regions, expressed as a percent. To visually distinguish tumors by fluorescence, the tumor needs to be at least 120% of adjacent regions.

**Scheme 1^a**

^aConditions: (a) 1,4-dioxane-2, 6-dione, DMF, r.t., (87–92%); (b) *tert*-butyl-12-amino-4,7,10-trioxadecanoate, DIEA, HOBT, EDCI, DMF, 72 h, r.t., (77–82%); (c) TFA, dichloromethane, 4 h, (88.3–89.3 %); (d) DIEA, HOBT, TBTU, EGFR-L1 or EGFR-L2, DMF, 24 h, then TFA/H₂O/phenol/TIS 88/5/5/2 (20–80%).

Table 1

Spectroscopic Properties of Pcs in DMF.

Pc	Absorption (nm)	Emission ^c (nm)	Stokes' Shift	ϵ (M ⁻¹ cm ⁻¹) ^a	Φ_f ^b
3a	678	682	4	5.35	0.10
3b	677	680	3	5.19	0.13
4b	677	681	4	4.85	0.10
5a	680	683	3	5.33	0.12
5b	680	682	2	5.27	0.11
6a	680	683	3	4.60	0.13

^aExcitation at 670 nm.^bCalculated using ZnPc ($\Phi_f = 0.17$) as the standard.

Table 2

Dark and photo cytotoxicity for Pc conjugates using the Cell Titer Blue assay.

Compound	A431 cells	Vero cells	HEp2 cells	HT-29 cells
	IC ₅₀ (μM) Light/dark	IC ₅₀ (μM) Light/dark	IC ₅₀ (μM) Light/dark	IC ₅₀ (μM) Light/dark
3a	>100/>125	>100/>125	>100/>125	>100/>125
4b	>100/>125	>100/>125	>100/>125	>100/>125
5a	15.8/>125	47.0/>125	17.0/>125	>100/>125
5b	>100/>125	>100/>125	>100/>125	>100/>125
6a	>100/>125	>100/>125	>100/>125	>100/>125

Table 3

Main subcellular sites of localization for Pc conjugates in human cells.

Compound	A431 cells	HEp2 cells	HT-29 cells
3a	Lyso, Golgi, Mito	Lyso, Golgi	Lyso
4b	Lyso, Golgi, Mito	Lyso, Golgi, Mito	Lyso, Golgi, Mito
5a	Lyso, Golgi, Mito, ER	Lyso, Golgi, Mito, ER	Lyso, Mito
5b	Lyso, Golgi, Mito	Lyso, Golgi, Mito	Lyso, Golgi, Mito
6a	Lyso, Golgi, Mito	Lyso, Golgi, Mito	Lyso, ER