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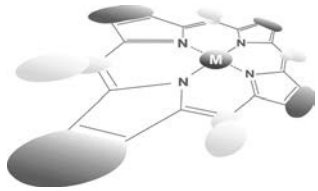

Synthesis and cellular studies of PPIX-homing peptide conjugates

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Dedicated to Professor Emanuel Vogel in memoriam

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ABSTRACT: Five amphiphilic protoporphyrin IX-peptide conjugates bearing the sequences ATWLPPR, AAhexPQRRSARLSA and cERGDPhe conjugated *via* the propionic side chains, were synthesized and evaluated *in vitro* using two cell lines: human carcinoma HEp2 and human leukemia HL-60. All conjugates were found to have low dark- and photo-toxicities in both cell lines, and 3 to 10-fold higher accumulation was observed within HL-60 *vs.* HEp2 cells, depending on the nature of the peptide sequence. The preferential subcellular sites of localization for all conjugates were found to be the lysosomes in HEp2 cells, and the mitochondria in HL-60 cells, suggesting different mechanisms of cellular internalization.

KEYWORDS: porphyrin, peptide, PDT, toxicity, cell uptake.

INTRODUCTION

Photodynamic therapy (PDT) is a form of photochemotherapy (PCT) that involves light activation of a tumor-localized photosensitizer, which produces reactive oxygen species, such as $^1O_2$, that lead to selective photooxidative destruction of tumor cells [1]. Two protoporphyrin IX (PPIX) derivatives, Photofrin® and Visudyne™ (Fig. 1), are FDA-approved for the PDT treatment of various cancers, including lung, melanoma, bladder and Barrett's esophagus, and in the latter case, for the "wet-form" of age-related macular degeneration. Since both drugs are mixtures of compounds and not tumor-targeted, a considerable amount of research in the last decade has centered on the investigation of targeted sensitizers of defined structure, and with improved selectivity for tumor tissues. On the other hand, the aminolevulinic acid (5-ALA)-induced intracellular accumulation of PPIX, as a consequence of ferrochelatase's limited activity, is also used for the PDT treatment of keratoses, basal cell carcinoma and malignant gliomas [2].

Targeted therapies are useful for the specific delivery of drugs into tumor cells, and have been shown to improve the biodistribution and biological efficacy of photosensitizers [3]. The general strategy involves the non-covalent or covalent attachment of drugs to targeting moieties or vectors that specifically bind tumor antigens or over-expressed enzymes or receptors on cancer cells. Examples of targeting moieties are monoclonal antibodies, proteins, peptides, oligonucleotides, and glycosides [4]. In particular, the use of homing peptides to target integrins, a family of cell surface proteins responsible for cell–cell and cell–matrix adhesion with a major role in the survival of endothelial cells, has been used for the specific delivery of cytotoxic drugs [5]. Moreover, studies have shown that the tumor vasculature can be targeted by homing peptides containing an RGD (Arg–Gly–Asp) sequence, which bind to β and v3αβ integrins on angiogenic endothelial cells [6]. Both linear and cyclic peptides containing the RGD sequence have been designed or isolated from phage display experiments as ligands for the v3 integrin. Cyclic peptides containing the RGD sequence have revealed higher specificity for integrins and increased stability towards enzymatic hydrolysis compared with the linear counterparts [7]. *In vitro* studies have shown that photosensitizer-homing

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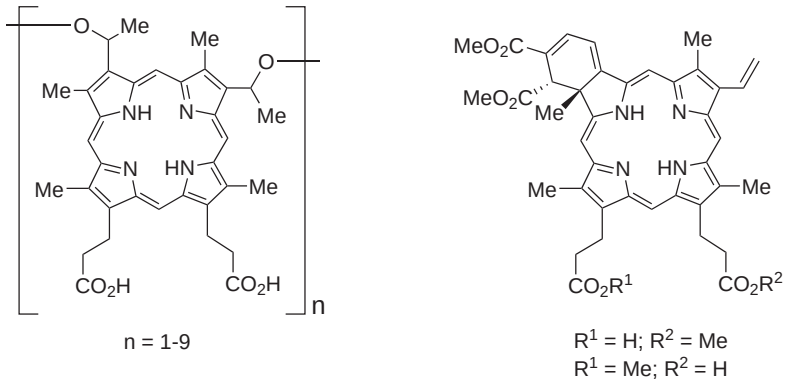


Fig. 1. Structures of Photofrin® (left) and Visudyne™ (right)

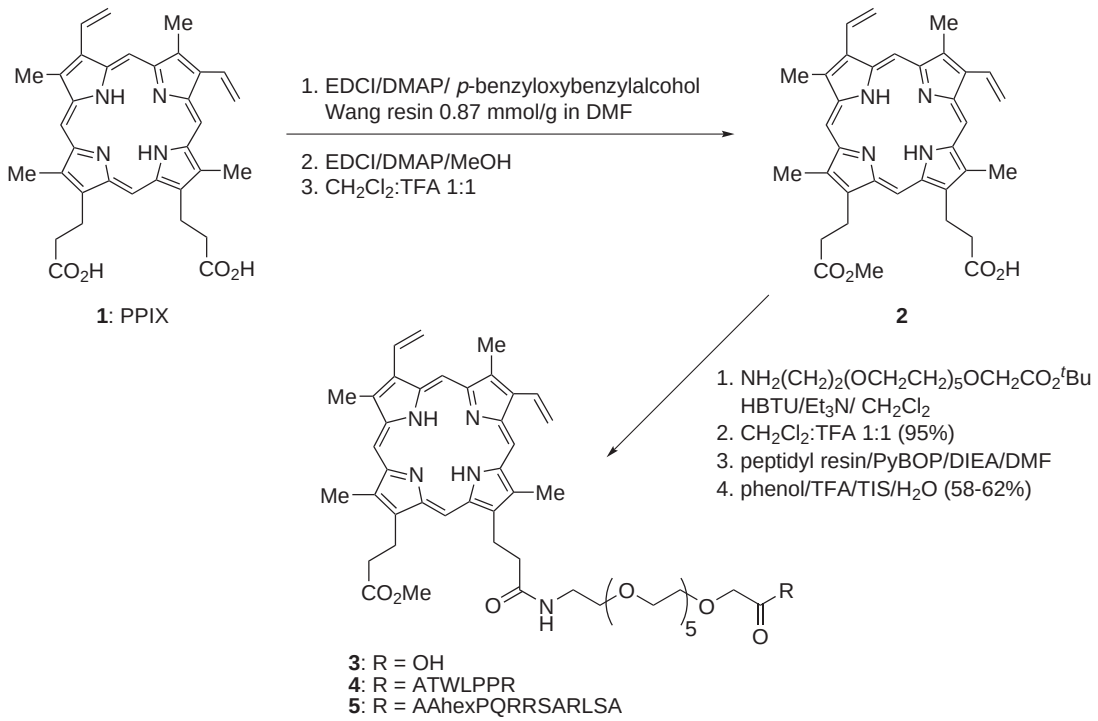
peptide conjugates are selectively delivered within cell lines over-expressing αvβ3 integrins, heterodimeric glycoproteins, and they retain their PDT efficacy [8].

Several PPIX conjugates to homing peptides and other targeting molecules including folic acid [9] have been reported to date [4]. A PPIX-cyclic RGDfk peptide conjugate was found to have higher tumor accumulation and retention than free PPIX, both *in vitro* using integrin positive SiHa cells, and *in vivo* in a mouse CaNT tumor model [10]. Herein we report the synthesis and characterization of a series of conjugates of PPIX to homing peptides and a vascular endothelial growth factor (VGEF). Homing peptide sequences include the cyclic ERGDPhe sequence, the VEGF sequence ATWLPPR and the PQRRSARLSA sequence. The last sequence is found in the HMGN2 protein, a highly conserved nucleosomal protein involved in unfolding higher-order chromatin structure and to facilitate the transcriptional activation of mammalian genes [11]. The 31 amino acid synthetic peptide (KDEPQRRSARLSAKPAPPKPEPKPK-KAPAKK) from the HMGN2 fragment containing the PQRRSARLSA sequence was shown to be readily internalized by human leukemia HL-60 and breast cancer MDA-MB-435 cells and to localize in the nuclei, both *in vitro* and *in vivo*, following intravenous injection into tumor-bearing nude mice [11].

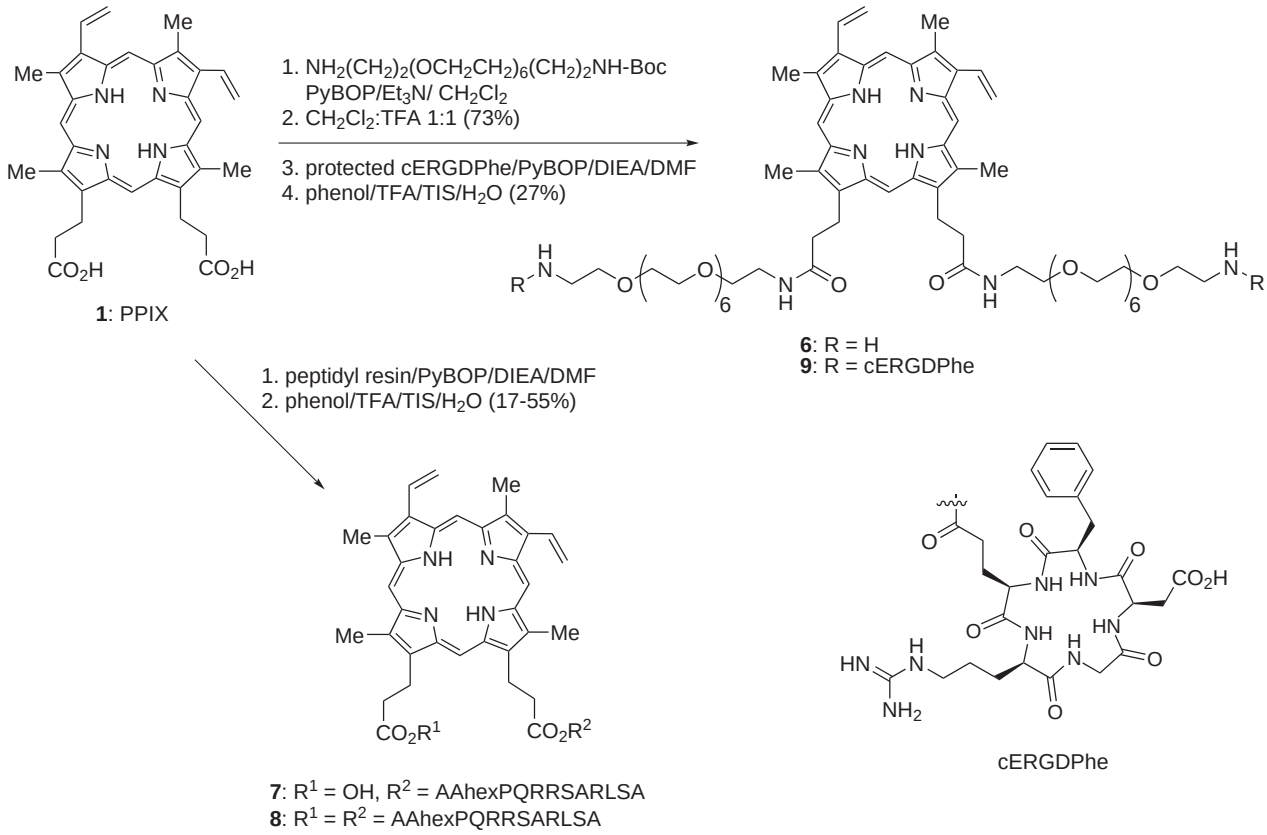
RESULTS AND DISCUSSION

Synthesis

The PPIX-peptide conjugates **4**–**9** were synthesized as shown in Schemes 1 and 2, from commercially available PPIX (**1**). The targeting peptide sequences conjugated to the propionic acid chains of PPIX are ATWLPPR, AAhex-PQRRSARLSA and the cyclic peptide cERGDPhe. Our previous studies on *meso*-tetraphenylporphyrin (TPP)-peptide conjugates suggest that the incorporation of a low molecular weight polyethylene glycol (PEG) linker reduces intramolecular interactions between the porphyrin and the peptide moiety, favoring extended conformations for the conjugates [12, 13]. In addition, one or two PEG groups generally increase the tumor cell uptake and water-solubility of porphyrin macrocycles



Scheme 1. Synthesis of PPIX-peptide conjugates **4** and **5**



Scheme 2. Synthesis of PPIX-peptide conjugates **7**, **8** and **9**

[14]. In this series of PPIX conjugates, the peptides were either linked directly to the propionic acid side chains of PPIX (in **7** and **8**) or *via* a short PEG linker (in **4**, **5** and **9**). The mono and di-peptide PPIX conjugates were synthesized in solution- or solid-phase using standard conjugation methodologies [15].

As shown in Scheme 1, the PPIX-mono-peptide conjugates **4** and **5** were synthesized by selective esterification of PPIX in solid phase support [16] following by conjugation of the remaining propionic acid group, after cleavage from the solid support under acidic conditions. The free carboxylic group of PPIX mono-methyl ester **2** (as regioisomeric mixture), was conjugated to a low molecular weight PEG linker *via* amide bond formation using HBTU and Et_3N [13]. Acid hydrolysis of the terminal *tert*-butyl ester using a 1:1 mixture of CH_2Cl_2/TFA provided the free carboxylic acid **3** in 95% yield. Porphyrin **3** was activated as the benzotriazole ester with PyBOP and conjugated on solid support to the corresponding peptide sequence *via* amide bond formation. After deprotection and cleavage from the solid support, PPIX-mono-peptide conjugates **4** and **5** were purified by reversed HPLC and isolated in 58 and 62% yields, respectively.

The conjugation of activated PPIX benzotriazole ester to the peptide sequence AAhexPQRRSARLSA on solid support (Scheme 2) gave a mixture of the mono- and di-peptide conjugates **7** and **8** in 17 and 55% yields, respectively, after deprotection and cleavage from the solid support, followed by reversed-phase HPLC purification. The PPIX-di-peptide conjugate **9** containing the cERGDPhe cyclic peptide sequence, was synthesized from di-pegylated PPIX **6** containing terminal amino groups. Porphyrin **6** was prepared in 73% overall yield, by conjugation of both the propionic acid chains of PPIX with commercially available monoamino-protected $NH_2CH_2CH_2(OCH_2CH_2)_6CH_2CH_2NH$-Boc, following by cleavage of the Boc group using CH_2Cl_2/TFA 1:1. Reaction of the activated δ–benzotriazole ester of the glutamic acid residue on the protected ERGDPhe cyclic peptide sequence, in solution phase, with porphyrin **6**, followed by deprotection of the arginine and aspartic acid residues using a 5:88:2:5 mixture of phenol/TFA/TIS/H_2O afforded the corresponding PPIX-di-cERGDPhe conjugate **9** in 27% yield.

All PPIX conjugates were characterized by NMR, MS and UV-vis spectrophotometry (see Supporting information).

Cell studies

Time dependent cellular uptake. The cellular uptake of PPIX-peptide conjugates **4**, **5**, **7**, **8** and **9** was investigated in a time dependent manner at a concentration of 10 μM in both human squamous cell carcinoma HEp2 and human myeloid leukemia HL-60 cells (Fig. 2). The conjugation

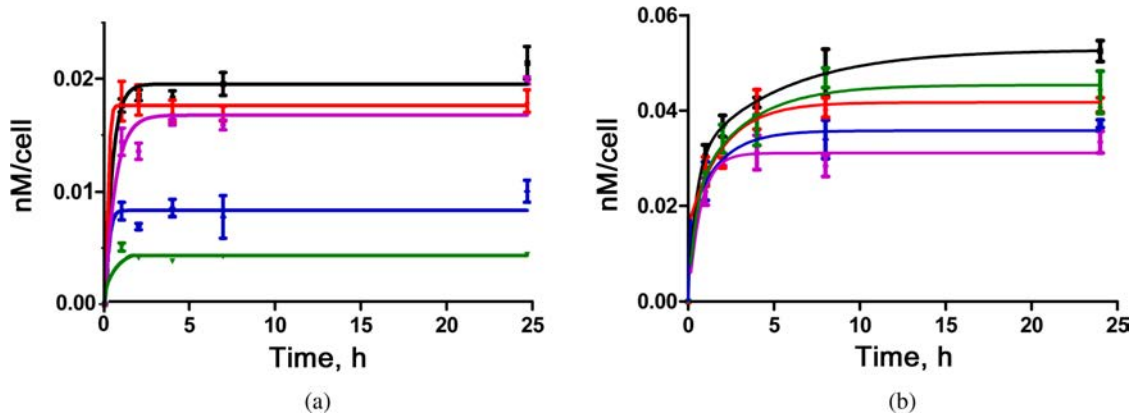


Fig. 2. Time-dependent uptake of PPIX-peptide conjugates **4** (blue), **5** (purple), **7** (black), **8** (red), and **9** (green) at 10 μM by (a) HEp2 cells and (b) HL-60 cells

of the peptide sequences *via* the propionic acid chains of PPIX produced amphiphilic conjugates that are believed to show enhanced affinity for lipid/aqueous interfaces and increased cellular uptake compared with hydrophilic molecules, which generally show decreased interactions with the lipid bilayer cell membrane [15, 17].

In human HEp2 cells higher uptake was observed for conjugates **5**, **7**, and **8** containing the more hydrophilic AAhexPQRRSARLSA peptide sequence bearing three arginine residues, than for conjugates **4** and **9** that contain the ATWLPPR and cERGDPhe sequences, respectively, bearing only one arginine residue. We have previously observed that the cellular uptake of porphyrin conjugates bearing short peptide sequences, increases with increasing number of arginine residues [12]. Although pegylated porphyrin conjugates tend to accumulate to a larger extent within cells than the non-pegylated derivatives [13, 14], conjugates **4**, **5**, and **9** bearing a PEG linker exhibited relatively lower uptake compared with **7** and **8** bearing a three carbon spacer, due to the nature of the peptide sequence. Interestingly, the pegylated PPIX-peptide conjugate **9** containing two zwitterionic cERGDPhe and two PEG linkers was the least accumulated within HEp2 cells. As expected, in HL-60 cells a significantly higher cellular uptake was observed for all PPIX conjugates compared with that in HEp2 cells. The highest increase in uptake was observed for conjugate **9** (about 10-fold) followed by conjugate **4** (about 5-fold); all other conjugates showed about a 3-fold increase in uptake in HL-60 *vs.* HEp2 cells. Therefore, the differences observed in the cellular uptake for these PPIX conjugates are mostly attributed to the nature of the peptide sequence. It is interesting to note that PPIX conjugate **7**, containing only one AAhexPQRRSARLSA peptide, accumulated the most of all conjugates in both cell lines, while its corresponding di-peptide derivative, conjugate **8**, accumulated to a lesser extent, maybe due to its higher hydrophilicity. All conjugates showed similar uptake kinetics in both cell lines, accumulating more rapidly in the first hour after exposure to cells, and reaching a plateau after 2 h in HEp2 cells, and 4–8 h in HL-60 cells.

Cytotoxicity. The dark cytotoxicity and phototoxicity (1 J/cm^2) of PPIX-peptide conjugates **4**, **5**, **7**, **8** and **9** were evaluated in both HEp2 and HL-60 cells exposed to increasing concentrations of each conjugate for 24 h, and the results obtained are summarized in Table 1 (the IC_{50} values were calculated from dose-response curves). All conjugates were found to have low cytotoxicity in the dark in both cell lines (IC_{50} > 100 μM), with the exception of conjugate **8** which was moderately toxic to HEp2 cells, with determined IC_{50} = 74 μM. Upon exposure to low light dose (1 J/cm^2), conjugate **7** was the most toxic to the HL-60 cells, with IC_{50} = 7 μM. Conjugate **7** bearing the tri-cationic PQRRSARLSA sequence also had the highest cellular uptake in both cell lines. Both the amount of photosensitizer accumulated within cells and its subcellular distribution affect its phototoxicity, and consequently determine its biological efficacy [18].

Table 1. Cytotoxicity (Cell Titer Blue assay, light dose ~1 J/cm^2) in human HEp2 and HL-60 cells

Compound	Human carcinoma HEp2 cells			Human leukemia HL-60 cells		
	Dark toxicity (IC_{50}, μM)	Phototoxicity (IC_{50}, μM)	Ratio	Dark toxicity (IC_{50}, μM)	Phototoxicity (IC_{50}, μM)	Ratio
4	>100	>10	>10	>100	>20	>5
5	>100	>10	>10	>100	>20	>5
7	>100	>10	>10	>100	>20	>5
8	>73.8	>10	>7.4	>100	>20	>5
9	>100	>10	>10	>100	7.1	>14

Intracellular localization. The subcellular localization of all PPIX-peptide conjugates was investigated in both HEp2 and HL-60 cells at a 10 μM concentration using fluorescence microscopy. Figures 3–7 show the fluorescent pattern observed for all PPIX conjugates in HEp2 cells and its overlay with the organelle specific fluorescent probes LysoSensor Green (lysosomes), Mitotracker Green (mitochondria), $DiOC_6$ (ER), and BODIPY FL C_5-ceramide (Golgi complex). Similar figures were obtained using HL-60 cells (see Supporting information). In HEp2 cells all conjugates collected in vesicles, as shown by the punctuate pattern observed in Figs 3–7, some of which correlated with the cell lysosomes. When Cremophor EL was used as a nonionic emulsifier in the incubation media, conjugates **4** and **9** were also found in the ER. Although it has been reported that the PQRRSARLSA sequence has the ability to target and accumulate in the nuclei of tumor endothelial cells [11], no nuclear localization was observed for conjugates **5**, **7**, and **8** containing this peptide sequence. This could be a result of an endocytic mechanism of cellular uptake for these conjugates, thus favoring their localization within endosomal vesicles [15]. On the other hand, in HL-60 cells all PPIX-peptide conjugates were found in mitochondria (see Figs S14–S18 of the Supporting information), suggesting a different mechanism of cellular uptake in this cell line. In addition, conjugates **4** and **5** were also found in lysosomes, and conjugate **7** in the ER of HL-60 cells.

EXPERIMENTAL

General

Unless otherwise indicated, all commercially available starting materials were used directly without further purification. Reactions under anhydrous conditions were performed in dried and distilled solvents under an argon atmosphere. All reactions were monitored by TLC using Sorbent Technologies 0.25 mm silica gel plates with or without UV indicator (60F–254). Silica gel Sorbent Technologies 32–63 μm was used for flash column chromatography. ^{1}H and ^{13}C NMR were obtained on a ARX-300 Bruker spectrometer. DQCOSY, NOESY and TOCSY spectra were obtained on a Varian Inova-500 spectrometer. Chemical shifts (δ) are given in ppm relative to $CDCl_3$ (7.26 ppm, ^{1}H) unless otherwise indicated. Electronic absorption spectra were measured on a Perkin Elmer Lambda 35 UV-vis spectrophotometer. Mass spectra were obtained at the LSU Mass Spectra Facility. HPLC separation and analysis were carried out on a Dionex system including a P680 pump and a UVD340U, detector. Semi-preparative HPLC was carried out using a Luna C_{18} 100 Å, 5 μm, 10 × 250 mm (Phenomenex, USA) column and a stepwise gradient; analytical HPLC was carried out using a Delta Pak C_{18} 300 Å, 5 μm, 3.9 × 150 mm (Waters, USA) column and a stepwise gradient.

Synthesis

Peptide synthesis. Peptide sequences were prepared on an automated peptide synthesizer (Applied Biosystems Pioneer, Peptide Synthesis System, USA) in a 0.2 mmol scale, using the Fmoc strategy of solid-phase peptide synthesis. A 4-fold excess of the Fmoc-protected amino acids were coupled to the PAL-PEG-PS resin using PyBOP as the activating agent. The peptide sequences prepared using this methodology were ATWLPPR and AAhexPQRRSARLSA. After the final coupling reaction and the successive removal of the Fmoc group, the resin was washed with DMF and isopropyl alcohol, and then dried under vacuum. The dried resin containing the protected amino acid sequences were used in the coupling reaction to the PPIX derivatives. Protected cyclic peptide cERGDPhe was synthesized following a protocol described in the literature [19]. The PPIX monomethyl ester **2** was synthesized in solid support using a NovaPEG Wang resin (0.87 mmol/g) and as described in the literature [16]. The spectroscopic characterization for this compound is in agreement with that reported in the literature [16].

Porphyrin 3. Porphyrin **2** (50 mg, 0.086 mmol) was dissolved in 50 mL of CH_2Cl_2. To this solution Et_3N (26 mg, 0.258 mmol) and HBTU (32.6 mg, 0.086 mmol) were added. The mixture was stirred at room temperature for 10 min, then $NH_2CH_2CH_2(OCH_2CH_2)_5OCH_2CO_2{}^tBu$ (37.7 mg, 0.095 mmol) was added [13]. The reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated under vacuum and the target porphyrin was isolated by flash column chromatography on silica gel using $CHCl_3$/MeOH for elution. Yield 58.8 mg, 72%. Protected porphyrin **2** (50 mg) was dissolved in 4 mL of a mixture of CH_2Cl_2/TFA 1:1. The mixture was stirred at room temperature for 4 h, the solvent evaporated under vacuum and the residue triturated with ethyl ether. The green residue was dried under vacuum. Yield 44.7 mg (95%). ^{1}H NMR (300 MHz; $CDCl_3$): δ_H, ppm -4.74 (2H, s, pyrrole-N*H*), 1.27–2.03 (4H, m, CH_2CONH), 3.37–3.61 (36H, m, OCH_2-PEG, CH_2NH-PEG, CH_3-pyrrol), 3.81–4.02 (4H, m, CH_2-pyrrol, COOCH_3), 4.24 (2H, s CH_2CO), 6.11–6.17 (2H, m, CH_2 vinyl), 6.24–6.33 (2H, m, CH_2 vinyl), 8.02–8.17 (2H, m, C*H* vinyl), 9.54 (1H, s, C*H* ar pyrrol), 9.59 (1H, s, C*H* ar pyrrol), 9.66 (1H, s, C*H* ar pyrrol), 9.74 (1H, s, C*H* ar pyrrol). MS (HR ESI): *m/z* 897.4498 (calcd. for $[M + H]^+$ 897.4523).

General procedure for syntheses of PPIX-peptide conjugates on solid support. Peptidyl resin (0.0165 mmol) was introduced into a glass synthesizer, swelled in DMF for 1 h, and then washed with DMF (2 × 5 mL). To the peptidyl resin was added 500 μL of a solution containing 0.033 mmol of pegylated PPIX **3**, 0.099 mmol of DIEA and 0.033 mmol of PyBOP. The reaction mixture was

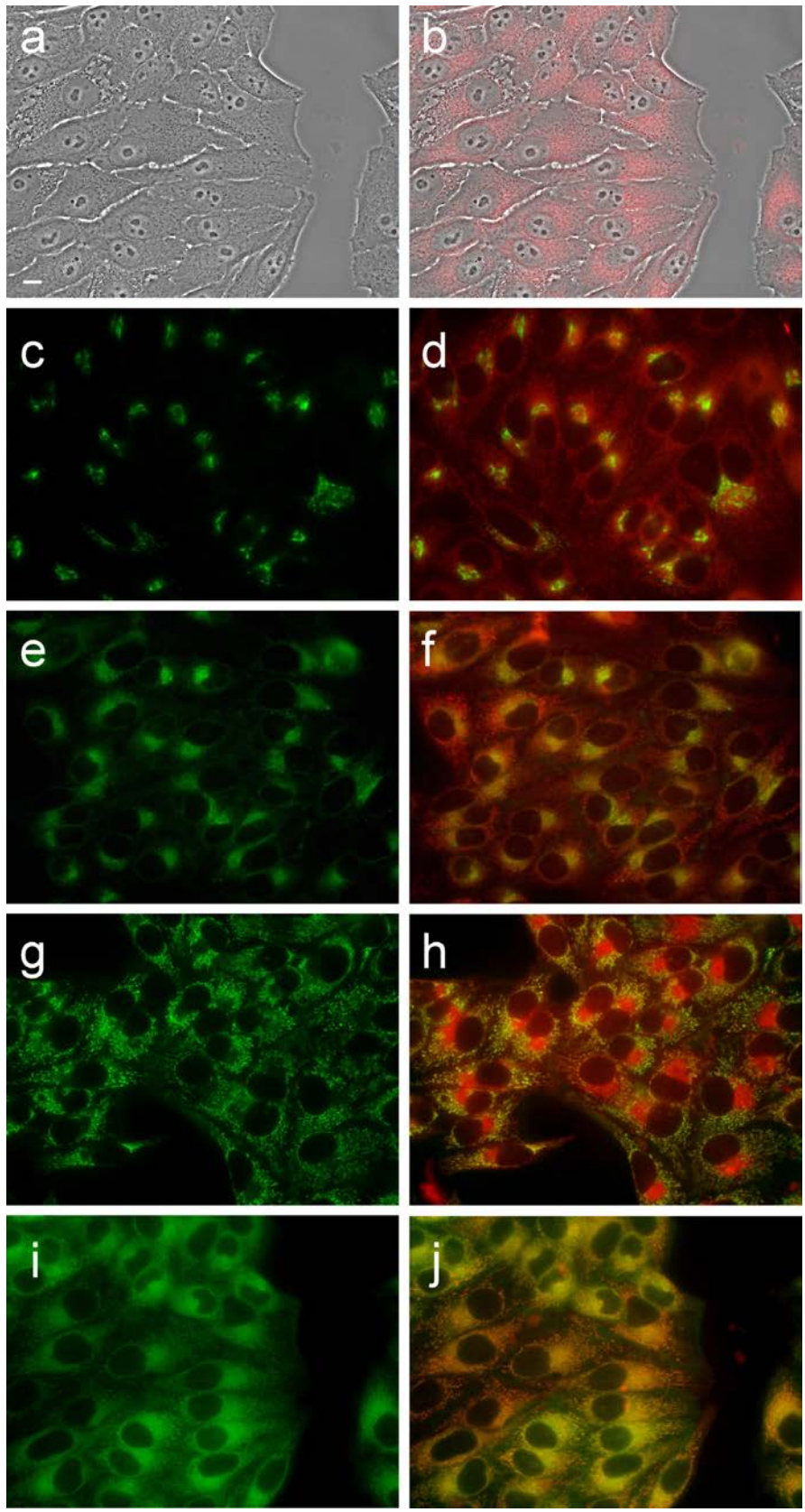


Fig. 3. Subcellular localization of PPIX-peptide conjugate **4** in HEp2 cells at 10 μM for 24 h. (a) Phase contrast, (b) overlay of conjugate **4** fluorescence and phase contrast, (c) BODIPY Ceramide fluorescence, (e) LysoSensor Green fluorescence, (g) MitoTracker Green fluorescence, (i) $DiOC_6$ fluorescence, (d), (f), (h) and (j) overlays of organelle tracers with conjugate **4** fluorescence. Scale bar: 10 μm

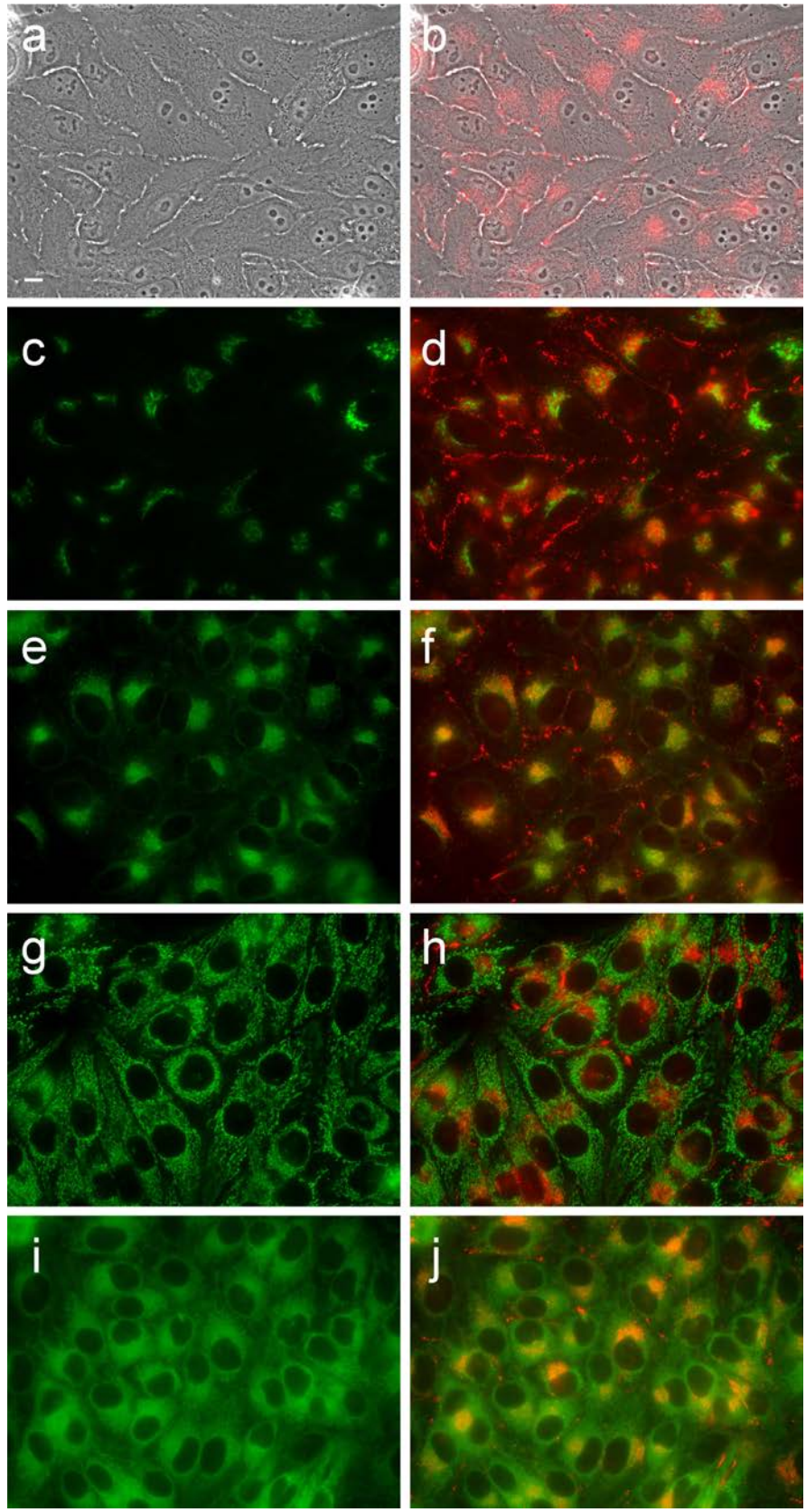


Fig. 4. Subcellular localization of PPIX-peptide conjugate **5** in HEp2 cells at 10 μM for 24 h. (a) Phase contrast, (b) overlay of conjugate **5** fluorescence and phase contrast, (c) BODIPY Ceramide fluorescence, (e) LysoSensor Green fluorescence, (g) MitoTracker Green fluorescence, (d), (f), and (h) overlays of organelle tracers with conjugate **5** fluorescence. Scale bar: 10 μm

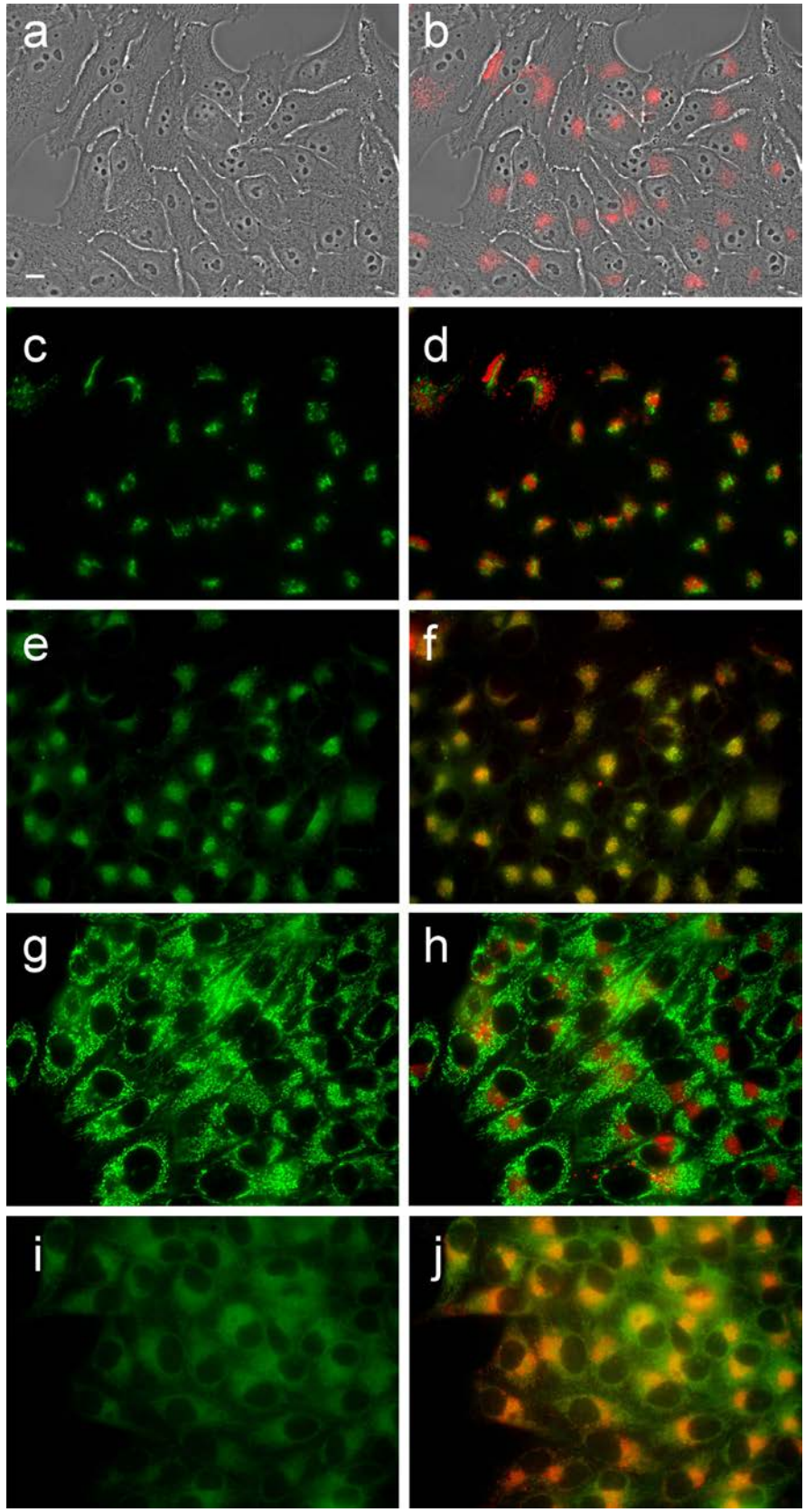


Fig. 5. Subcellular localization of PPIX-peptide conjugate **7** in HEp2 cells at 10 μM for 24 h. (a) Phase contrast, (b) overlay of conjugate **7** fluorescence and phase contrast, (c) BODIPY Ceramide fluorescence, (e) LysoSensor Green fluorescence, (g) MitoTracker Green fluorescence, (i) $DiOC_6$ fluorescence, (d), (f), (h) and (j) overlays of organelle tracers with conjugate **7** fluorescence. Scale bar: 10 μm

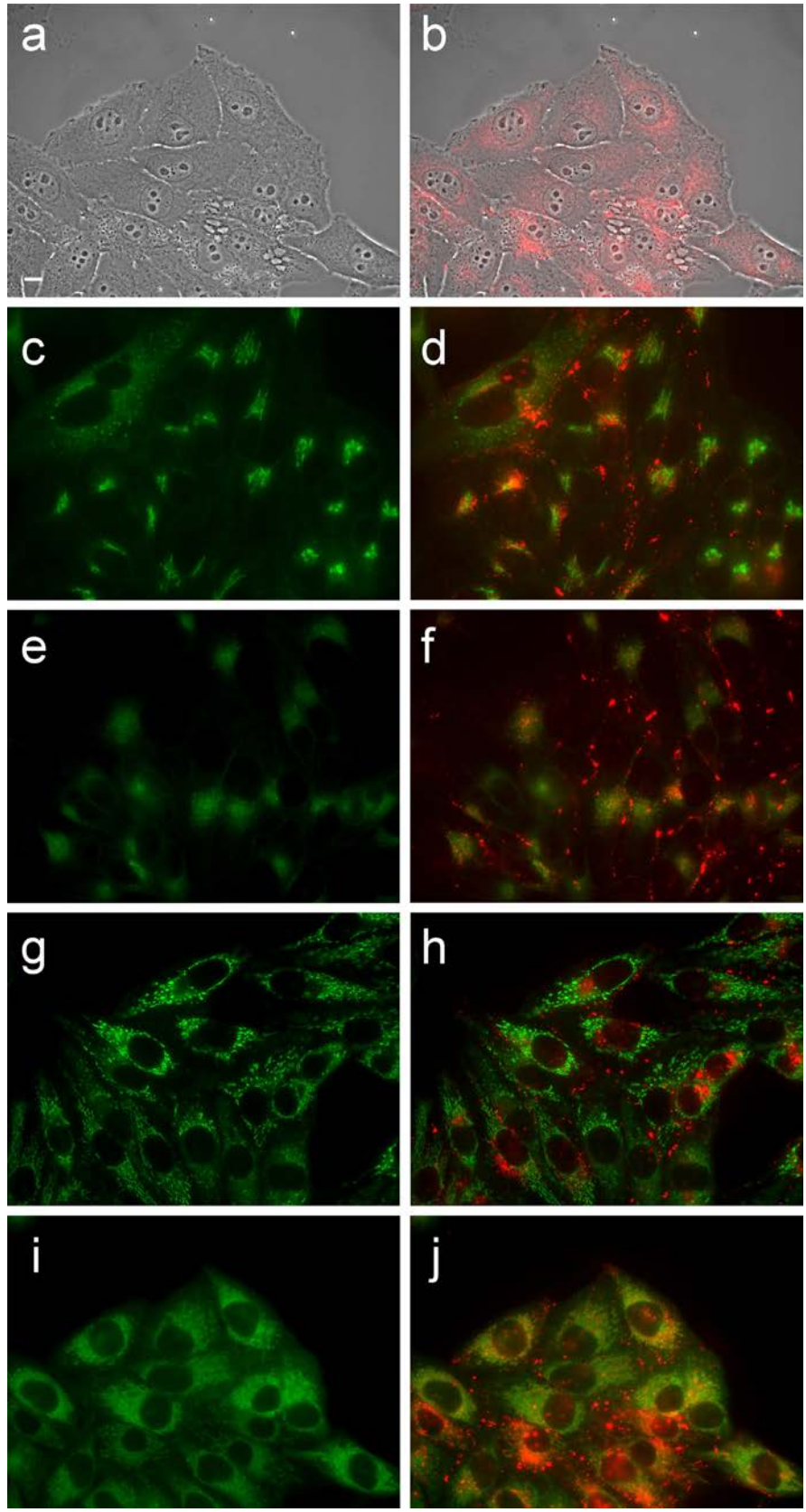


Fig. 6. Subcellular localization of PPIX-peptide conjugate **8** in HEp2 cells at 10 μM for 24 h. (a) Phase contrast, (b) overlay of conjugate **8** fluorescence and phase contrast, (c) BODIPY Ceramide fluorescence, (e) LysoSensor Green fluorescence, (g) MitoTracker Green fluorescence, (i) $DiOC_6$ fluorescence, (d), (f), (h) and (j) overlays of organelle tracers with conjugate **8** fluorescence. Scale bar: 10 μm

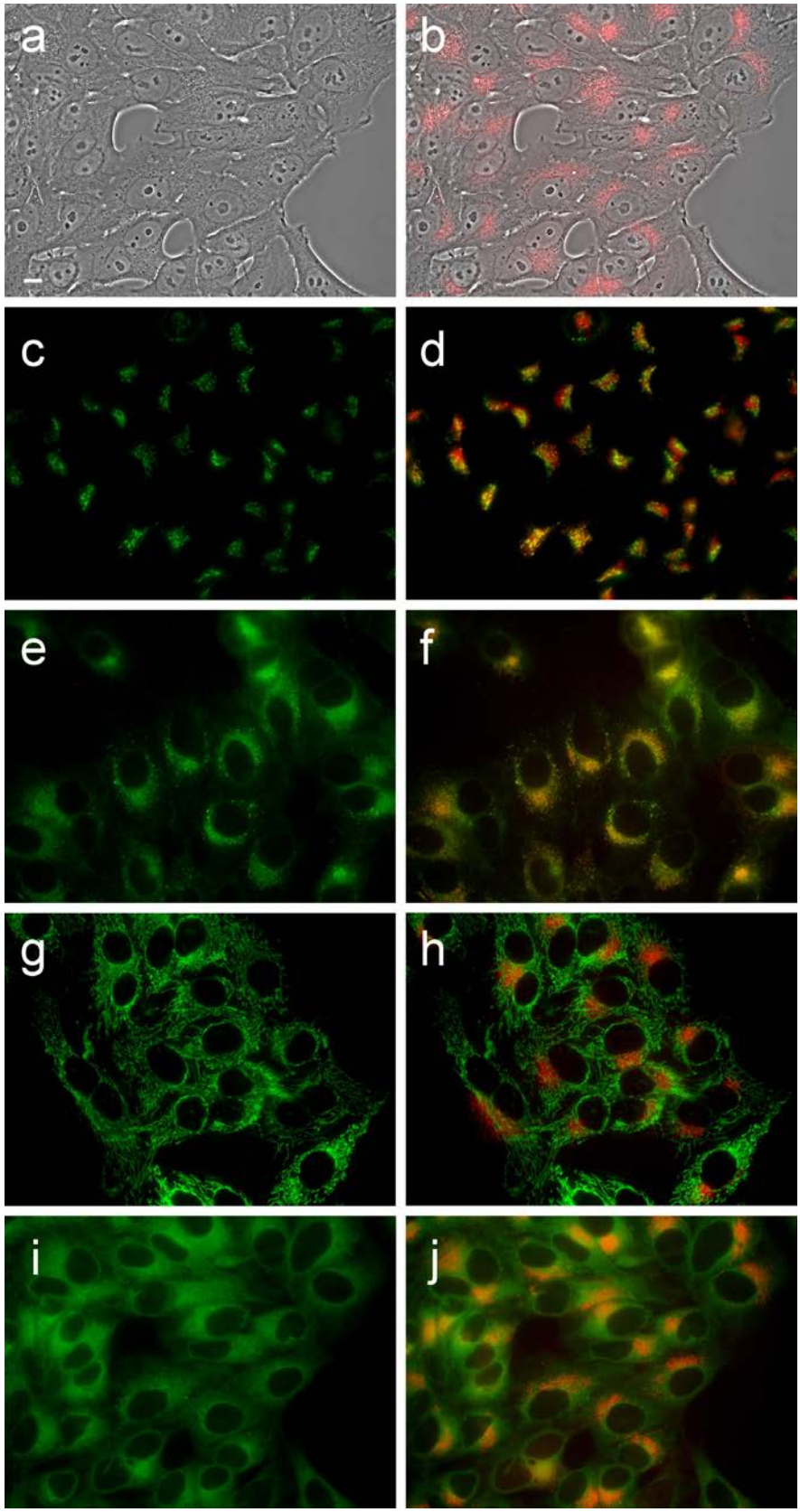


Fig. 7. Subcellular localization of PPIX-peptide conjugate **9** in HEp2 cells at 10 μM for 24 h. (a) Phase contrast, (b) overlay of conjugate **9** fluorescence and phase contrast, (c) BODIPY Ceramide fluorescence, (e) LysoSensor Green fluorescence, (g) MitoTracker Green fluorescence, (i) $DiOC_6$ fluorescence, (d), (f), (h) and (j) overlays of organelle tracers with conjugate **9** fluorescence. Scale bar: 10 μm

shaken overnight at room temperature and then filtered to give a dark purple resin. The resin was washed to remove un-reacted pegylated PPIX, first with DMF until the filtrate was colorless, then with dichloromethane and methanol, before being dried under vacuum. Cleavage and deprotection was carried out by treatment of the dried resin with 2 mL of a mixture of TFA/Phenol/TIS/H_2O, 88/5/2/5 at room temperature for 4 h. The resin was filtered, washed with TFA (3 × 2 mL), and the filtrates combined and evaporated under vacuum to give a green residue. Addition of cold diethyl ether yielded a green precipitate, which was washed repeatedly with diethyl ether and dried under vacuum. The purification of the PPIX-peptide conjugates was carried out by reversed-phase HPLC on a Luna C_{18} semi-preparative column (10 × 250 mm, 5 μm) (Phenomenex, USA) using a mixture of water/acetonitrile both containing 0.1% TFA, with a stepwise gradient. The fraction containing the conjugate was collected and lyophilized to yield pure conjugate. The purity of the peptides was >95% as obtained by HPLC on an analytical Delta Pak C_{18} (3.9 × 150 mm, 5 μm) column.

PPIX conjugate 4. Yield 26.8 mg (58%). UV-vis (methanol): λ_{max}, nm (ε, $M^{-1}.cm^{-1}$) 430 (136 932), 510 (7 926), 545 (7 174), 581 (5 522), 637 (3 434). ^{1}H NMR (500 MHz, d_6-DMSO): δ_H, ppm 0.08 (3H, s, C*H*$_3$-Leu), 0.25 (3H, s, C*H*$_3$-Leu), 0.78–0.98 (4H, m, δ C*H*$_2$ Thr, β C*H*$_2$ Leu), 1.09–1.14 (1H, m, δ C*H* Leu), 1.40 (3H, m, β C*H*$_3$ Ala), 1.71–1.91 (8H, m, β C*H*$_2$ Arg, δ C*H*$_2$ Arg, β C*H*$_2$ Pro, δ C*H*$_2$ Pro), 2.04–2.12 (6H, m, δ C*H*$_2$ Pro, CH_2CONH), 2.16–2.18 (2H, m, β C*H*$_2$ Pro), 2.32–3.0 (2H, m, δC*H*$_2$ Arg), 3.18–3.64 (51H, m, δ C*H*$_2$ Pro, δ C*H*$_2$ Pro, β C*H*$_2$ Trp, C*H*$_3$ pyrrole, COOC*H*$_3$ pyrrole, OC*H*$_2$-PEG, C*H*$_2$NH-PEG, C*H*$_2$CO), 5.30–5.35 (2H, m, C*H*$_2$ vinyl), 5.52–5.59 (2H, m, C*H*$_2$ vinyl), 6.01–7.17 (9H, m, C*H* ar Trp, N*H* Arg, N*H* Trp, N*H* Thr, ε C*H* Arg), 7.59–7.62 (1H, m, C*H* vinyl), 7.77–7.79 (1H, m, C*H* vinyl), 9.37 (1H, s, C*H* ar pyrrole), 9.43 (1H, s, C*H* ar pyrrole), 9.89 (1H, s, C*H* ar pyrrole), 9.96 (1H, s, C*H* ar pyrrole). MS (HR ESI): *m/z* 859.9697 (calcd. for $[M + H]^{2+}$ 859.9688).

PPIX conjugate 5. Yield 35.4 mg (62%). UV-vis (methanol): λ_{max}, nm (ε, $M^{-1}.cm^{-1}$) 418 (221 712), 510 (8 706), 545 (7 184), 582 (5 176), 638 (3 654). MS (HR ESI): *m/z* 711.7398 (calcd. for $[M]^{3+}$ 711.7371).

Porphyrin 6. Under an argon atmosphere, PPIX di-hydrochloride salt (0.1 g, 0.1563 mmol) was suspended in 100 mL of anhydrous CH_2Cl_2. To this suspension was added Et_3N (0.095 g, 0.938 mmol) and the mixture was heated at 35 °C until PPIX was completely dissolved. To the solution was added PyBOP (0.3438 mmol), the mixture was stirred at 35 °C for 5 min, then $NH_2CH_2CH_2(OCH_2CH_2)_6CH_2CH_2NH$-Boc (0.141 g, 0.3126 mmol) was added in one portion. The mixture was stirred at 35 °C for an additional 20 min, then at room temperature for 12 h. The organic phase was washed with water (3 × 100 mL), dried over Na_2SO_4, filtered and the solvent evaporated under vacuum. The target compound was isolated by flash column chromatography on alumina Grade V using chloroform/methanol 9:1 for elution. Yield 162 mg (74%). The Boc-protected pegylated PPIX (0.15 g, 0.1047 mmol) was dissolved in 8 mL of a mixture of CH_2Cl_2/TFA 1:1 and stirred at room temperature for 4 h. The solvent was evaporated under vacuum and the residue triturated with ethyl ether, then dried under vacuum. Yield 130 mg (98%). ^{1}H NMR (300 MHz; $CDCl_3$): δ_H, ppm 1.25–1.27 (4H, *J* = 1.25 N*H*$_2$), 2.92–3.72 (80H, m, OC*H*$_2$-PEG, C*H*$_2$NH-PEG, C*H*$_2$NHCO-PEG, C*H*$_2$CONH, C*H*$_3$pyrrole), 6.32–6.42 (4H, m, C*H*$_2$ vinyl), 7.2 (2H, broad s, N*H*CO-PEG), 7.61 (2H, broad s, C*H* vinyl), 8.14–8.25 (2H, m, C*H* vinyl), 10.40 (2H, s, C*H* ar pyrrole), 10.46 (1H, s, C*H* ar pyrrole), 10.72 (1H, s, C*H* ar pyrrole). MS (HR ESI): *m/z* 1263.7496 (calcd. for $[M + H]^+$ 1263.7486).

PPIX conjugate 7. Yield 8 mg (17%). UV-vis (methanol): λ_{max}, nm (ε, $M^{-1}.cm^{-1}$) 404.75 (61050), 508 (9 230), 546 (7 050), 580 (5 120), 638 (3 285). MS (HR ESI): *m/z* 1796.9993 (calcd. for $[M + H]^+$ 1796.9950).

PPIX conjugate 8. Yield 44 mg (55%). UV-vis (methanol): λ_{max}, nm (ε, $M^{-1}.cm^{-1}$) 408 (84 600), 510 (8 750), 545 (7 188), 582 (4 902), 638 (3 702). ^{1}H NMR (500 MHz, d_6-DMSO): δ_H, ppm 0.97 (6H, s, C*H*$_3$ Leu), 1.14 (6H, s, C*H*$_3$ Leu), 1.30 (6H, s, C*H*$_3$ Ala), 1.34 (6H, s, C*H*$_3$ Ala), 1.38–1.49 (4H, m, CH_2-C*H*$_2$-CH_2 AAhex), 1.68 (24H, s, β C*H*$_2$ Arg, γ C*H*$_2$ Arg), 2.15 (12H, s, β C*H*$_2$ Gln, β C*H*$_2$ Pro, γ C*H*$_2$ Pro), 2.29 (4H, s, C*H*$_2$-CONH AAhex), 2.56 (4H, s, C*H*$_2$-pyrrole), 2.96 (4H, s, C*H*$_2$-NHCO AAhex), 3.34 (3H, s, C*H*$_3$ pyrrole), 3.54 (9H, s, C*H*$_3$ pyrrole), 3.95–4.74 (36H, m, α C*H* Pro, α C*H* Gln, α C*H* Arg, α C*H* Arg, α C*H* Ser, α C*H* Ala, α C*H* Arg, α C*H* Leu, α C*H* Ser, α C*H* Ala, β C*H*$_2$ Ser), 6.69–6.72 (2H, m, C*H*$_2$ vinyl), 6.93–6.96 (2H, m, C*H*$_2$ vinyl), 7.25–7.86 (21H, m, N*H* AAhex, N*H* Pro, N*H* Gln, N*H* Arg, N*H* Arg, N*H* Ser, N*H* Ala, N*H* Arg, N*H* Leu, N*H* Ser, N*H* Ala, ε C*H* Arg), 8.01–8.02 (1H, m, C*H* vinyl), 8.94–9.02 (1H, m, C*H* vinyl), 10.97 (3H, s, C*H* ar pyrrole), 10.84 (1H, C*H* ar pyrrole). MS (HR ESI): *m/z* 3031.7564 (calcd. for $[M]^+$ 3031.7320).

PPIX conjugate 9. Protected cyclic peptide cERGDPhe was dissolved in 2 mL of anhydrous DMF, to this solution was added DIEA (30.7 mg, 0.2374 mmol), followed by the addition of PyBOP (51.4 mg, 0.0989 mmol). The mixture was stirred at room temperature 5 min, then porphyrin **6** (50 mg, 0.0396 mmol) was added in one portion and the reaction mixture stirred for an additional 24 h at room temperature. The PPIX-cERGDPhe protected conjugate was isolated by flash column chromatography on silica gel using CH_2Cl_2/methanol 4:1 for elution. Deprotection was carried out using a mixture TFA/Phenol/TIS/H_2O, 88/5/2/5 at room temperature for 4 h. The solvent was evaporated under vacuum and the residue triturated with ethyl ether, then dried under vacuum. Yield 23 mg (27%). UV-vis (methanol): λ_{max}, nm (ε, $M^{-1}.cm^{-1}$) 406 (78 648), 510 (10

088), 544 (8312), 582 (6520) 638 (4580). ^{1}H NMR (500 MHz, d_6-DMSO): δ_H, ppm 0.4–1.32 (6H, m, β–H-Glu, β,γ–H-Arg), 1.90–2.02 (2H m, β-*H*-Glu), 2.33–2.69 (6H, m, β-*H*-Asp, CH_2-pyrrole), 2.79–2.95 (4H, m, β–C*H*-Phe), 3.06–3.10 (4H, m, δ–C*H*-Arg), 3.15–3.19 (4H, m, CH_2NHCO-PEG), 3.27–3.22 (8H, m, CH_2NHCO-PEG, α–C*H* Gly), 3.37–3.61 (56 H, m, OCH_2-PEG, 12H; CH_3-pyrrole), 3.96–4.00 (2H, m, α–C*H*Glu), 4.05 (2H, dd, *J* = 15.2/7.7 Hz, α–N*H*Gly), 4.12–4.16 (2H, m, α–C*H*-Arg), 4.45 (2H, q, *J* = 7.2 Hz, α-C*H*-Phe), 4.60–4.65 (2H, m, α–C*H*-Asp), 5.32 (2H, m, CH_2 vinyl), 5.65 (2H, m, CH_2 vinylic), 6.77 (4H, s, NH_2-Arg), 7.13–7.26 (10H, m, C*H* -Phe ar), 7.42–7.45 (2H, m, ε–N*H*Arg), 7.60 (1H, m, C*H* vinyl), 7.62–7.65 (3H, m, N*H*-PEG, C*H* vinyl), 7.98–8.02 (4H, m, N*H*-Phe, N*H*-Asp), 8.05 (1H, d, *J* = 7.2 Hz, N*H*-Glu), 8.30 (1H, dd, *J* = 3.8/7.4 Hz, N*H*-Gly), 9.33 (1H, s, C*H* ar pyrrole), 9.37 (1H, s, C*H* ar pyrrole), 9.48 (1H, s, C*H* ar pyrrole), 9.75 (1H, s, C*H* ar pyrrole), 9.86 (1H, s, C*H* ar pyrrole). MS (HR ESI): *m/z* 812.7564 (calcd. for $[M]^{3+}$ 812.7544).

Cell studies

All tissue culture media and reagents were obtained from Invitrogen. Human HEp2 cells were obtained from ATCC and maintained in a 50:50 mixture of DMEM:Advanced MEM containing 5% FBS. HL-60 cells were obtained from ATCC and maintained in the ATCC-formulated Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 1% Penicillin-Streptomycin and 20% FBS. Both of the cells were sub-cultured biweekly to maintain sub-confluent stocks.

Cytotoxicity. The HL-60 and the HEp2 cells were plated and allowed 36–48 h to attach. The cells were exposed to increasing concentrations of PPIX-conjugate up to 100 μM and incubated overnight. The loading medium was then removed and the cells fed medium containing Cell Titer Blue (Promega) as per manufacturer's instructions. Cell viability was then measured by reading the fluorescence at 520/584 nm using a BMG FLUOstar plate reader. The signal was normalized to 100% viable (untreated) cells and 0% viable (treated with 0.2% saponin from Sigma) cells. For the suspension cells HL-60, the plates were centrifuged before the loading medium or PBS buffer was removed. For the phototoxicity experiments, the HL-60 and HEp2 cells were prepared as described above for the dark cytotoxicity assay and treated with conjugate concentrations of 0, 0.625, 1.25, 2.5, 5, and 10 μM. After compound loading, the medium was removed and replaced with medium containing 50 mM HEPES pH 7.4. The cells were then placed on ice and exposed to light from a 100 W halogen lamp filtered through a 610 nm long pass filter (Chroma) for 10 min. An inverted plate lid filled with water to a depth of 5 mm acted as an IR filter. The total light dose was approximately 1 J/cm^2. The cells were returned to the incubator overnight and assayed for viability as described above for the dark cytotoxicity experiment. For the suspension cells HL-60, the plates were centrifuged before the loading medium or PBS buffer was removed.

Time-dependent cellular uptake. Both HL-60 and HEp2 cells were plated at 10000 per well in a Costar 96 well plate and allowed to grow overnight. Conjugate stocks were prepared in water at a concentration of 10 mM and then diluted into medium to final working concentrations. The cells were exposed to 10 μM of each conjugate for 0, 1, 2, 4, 8, and 24 h. At the end of the incubation time the loading medium was removed and the cells were washed with PBS. For the suspension cells HL-60, the plates were centrifuged before the loading medium or PBS buffer was removed. The cells were solubilized upon addition of 100 μL of 0.25% Triton X-100 (Calbiochem) in PBS. To determine the conjugate concentration, fluorescence emission was read at 410/650 nm (excitation/emission) using a BMG FLUOstar plate reader. The cell numbers were quantified using the CyQuant reagent (Molecular Probes).

Microscopy. The HEp2 cells were plated on LabTek 2 chamber coverslips and incubated overnight, before being exposed to 10 μM of conjugate for either 1 or 18 h. For the co-localization experiments the cells were incubated for 18 h concurrently with conjugate and one of the following organelle tracers (Molecular Probes), for 30 min: MitoTracker Green 250 nM, LysoSensor Green 50 nM, $DiOC_6$ 5 μg/mL, and BODIPY FL C5 ceramide 1 μM. The slides were washed three times with growth medium and new medium containing 50 mM HEPES pH 7.4 was added. Fluorescent microscopy was performed using a Zeiss Labovert 200 M inverted fluorescent microscope fitted with standard FITC and Texas Red filter sets (Chroma). The images were aquired with a Zeiss Axiocam MRM CCD camera fitted to the microscope.

For the HL-60 cells, 10,000/100 μl cells were seeded in each vial in a 96 Costar microplate and allow to grow for 24 h. The cells were then exposed to 10 μM of each compound for 18 h. Organelle tracers were obtained from Invitrogen and used at the following concentrations: LysoSensor Green 50 nM, MitoTracker Green 250 nM, ER Tracker Green 100 nM, and BODIPY FL C5 ceramide 1 μM. The organelle tracers were diluted into medium and the cells were incubated concurrently with PPIX conjugate and tracers for 30 min. The cells were collected, centrifuged and washed with PBS. Fifty μl of washed cells were added to slide glass and gently covered by coverslips. The images were acquired using a Leica DMRXA microscope and DAPI, GFP and Cy5 filter cubes (Chroma Technologies).

CONCLUSION

Five new PPIX-peptide conjugates bearing both linear and cyclic homing peptides linked either directly or *via* a low molecular weight PEG group to the propionic side chains of PPIX, were synthesized in moderate yields

using solution- and solid-phase techniques. The peptides consisted of a VEGF sequence (ATWLPPR), a sequence from the HMGN2 nucleosomal protein (PQRRSARLSA), and a cyclic RGD-containing sequence (cERGDPhe) for targeting αvβ3 integrins, which are heterodimeric transmembrane glycoprotein receptors over-expressed in actively dividing endothelial cells. The PPIX conjugates containing the PQRRSARLSA peptide efficiently accumulated within human carcinoma HEp2 cells, probably due to the three arginine residues. In human leukemia HL-60 cells, 3 to 10-fold higher accumulation within cells was found for all conjugates. The most efficiently taken-up by both cell lines was conjugate **7**, bearing only one AAhexPQRRSARLSA sequence linked directly to one of the PPIX propionic side groups. The main sites of subcellular localization for these conjugates were found to be the lysosomes in HEp2 cells, possibly as a result from endocytosis, and the mitochondria in the HL-60 cells, suggesting different uptake mechanisms in these two cell lines. All PPIX conjugates showed low dark- and photo-toxicities towards both cell lines. The most photo toxic conjugate was 7(IC_{50} = 7 μM, 1 J/cm^2) to HL-60 cells.

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Supporting information

NMR of selected compounds, HPLC traces and microscopy images for conjugates (Figs S1–S18) are given in the supplementary material. This material is available free of charge *via* the Internet at http://www.worldscinet.com/jpp/jpp.shtml.

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