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***In vitro* Cellular Uptake and Dimerization of Signal Transducer and Activator of Transcription-3 (STAT3) Identify the Photosensitizing and Imaging-Potential of Isomeric Photosensitizers Derived from Chlorophyll-a and Bacteriochlorophyll-a**

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Abstract

Among the photosensitizers investigated, both ring-D and ring-B reduced chlorins containing the *m*-iodobenzyloxyethyl group at position-3 and a carboxylic acid functionality at position-17² showed highest uptake by tumor cells and light-dependent photo reaction that correlated with maximal tumor-imaging [positron emission tomography (PET) and fluorescence] and long-term photodynamic therapy (PDT) efficacy in BALB/c mice bearing Colon26 tumors. However, among the ring-D reduced compounds, the isomer containing 1'-*m*-iodobenzyloxyethyl group at position-3 was more effective than the corresponding 8-(1'-*m*-iodobenzyloxyethyl) derivative. All photosensitizers showed maximum uptake by tumor tissue 24h after injection and the tumors exposed with light at low fluence and fluence rates (128 J/cm², 14 mW/cm²) produced significantly enhanced tumor eradication than those exposed at higher fluence and fluence rate (135 J/cm², 75mW/cm²). Interestingly, dose-dependent cellular uptake of the compounds and light-dependent STAT3 dimerization have emerged as sensitive rapid indicators for PDT efficacy *in vitro* and *in vivo* and could be used as *in vitro/in vivo* biomarkers for evaluating and optimizing the *in vivo* treatment parameters of the existing and new PDT candidates.

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Supporting Information Available: ¹H and ¹³C-NMR spectra of all compounds, HPLC profiles of compounds 4, 7, 12 and 23. Experimental details of known compounds with spectroscopic data of compounds 10, 18, 21 and 22. This material is available free of charge on the Internet at <http://pubs.acs.org>.

Introduction

Molecular imaging can be used for early detection and localization of lesion, and “real time” monitoring of efficacy of therapeutic treatments^{1–3}. In recent years, PET technology has been included in drug development, and contributed to the understanding of drug action, drug dose regimens and treatment strategies⁴. Such studies will eventually provide the means to accomplish “personalized medicine” by monitoring individual response to drug delivery. For diagnostic imaging, the half-life of the radionuclide must be long enough to carry out the chemistry of the desired radiopharmaceutical and allow its accumulation in the target tissue in the patient while the drug clears from the non-target organs. Radiometals for pharmaceuticals used in PET and gamma scintigraphy range in half-life from about 10 min (⁶²Cu) to several days (⁶⁷Ga). For example, heart or brain perfusion-based radiopharmaceuticals require shorter half-lives, since they reach the target quickly whereas tumor-targeted compounds often take longer to reach the target and to be accumulated for gaining an optimal target-to-background ratios⁵.

In PET imaging, ¹⁸F-FDG (¹⁸F-fluoro-2-deoxyglucose) is widely used in the clinic for the assessment of glucose metabolism in the heart, lungs and the brain.^{6–8} However, ¹⁸F-FDG-imaging suffers from several limitations such as the reduced specificity for tumor cells due to FDG uptake in inflammatory lesions, short half-life etc. The longer half-life (4 days) of ¹²⁴I- product(s) compared to the 90 minutes to ¹⁸F-FDG has number of additional advantage: (i) patients do not have to go through another injection after surgery to view its effectiveness, (ii) imaging centers do not need to order a large number of vials (iii) due its long half-life it can be transported over long distances and therefore could reduce the number of highly expensive cyclotrons, and finally (iv) not only does this mean an increased benefit to the end user (the patient), it also provides a significant benefit to insurance companies.

The design of a radiopharmaceutical agent requires optimizing the balance between specific *in vivo* targeting (e.g., cancerous tumor), clearance from non-target tissues/organs as well as time-dependent application in line with the decay properties of the radionuclide⁹. Several considerations need to be made when designing selective radiolabeled drugs. These include efficient drug delivery, optimization of relative accumulation of the drug at the target over non-target sites, maximizing the residence time of the radioactivity at target sites, and prevention of structural alteration of the drug¹⁰. Because of the multiple parameters that must be considered, developing effective radiopharmaceuticals for combined imaging and therapy of cancer is a challenging undertaking that is not simply solved by attaching a radionuclide in any fashion to a non-radiolabeled targeting compound. At the very least, the radionuclide should not interfere with pharmacokinetics, binding specificity or retention by cancer cells. The selection of the radionuclides, and the chemical strategies for radiolabeling of molecules are critical elements for developing safe and improved imaging/therapeutic agents¹¹.

For the last several years one of the objectives of our laboratory has been to use porphyrin-based compounds for the treatment of cancer by PDT^{12,13}. The accumulation of certain porphyrins and related tetrapyrrolic systems is higher in malignant tumors than in most normal tissues and that has been the main reason to use these molecules as PS. Some tetrapyrrole-based compounds have been effective in treating lesions at different organ sites, including skin, lung, bladder, head & neck and esophagus¹⁴. The precise mechanism(s) of PDT and post-PDT reactions are still largely unknown; however, data from *in vitro* and *in vivo* studies suggested that localized oxidative damage mediated by photosensitizer (PS)-specified photoreactions determine direct cell killing, loss of vascular function, and post-PDT immune reactions, all of which play significant roles in PDT outcome^{15–20}. Superficial

lesions, or those that are endoscopically accessible, e.g. endobronchial or esophageal tumors, are easily treated. However, the majority of malignant lesions are too deep to be reached by light of the wavelength required to trigger singlet oxygen production by the current generation of PSs. Although the technology to deliver therapeutic light to deep lesions via optical fibers “capped” by a terminal diffuser is well developed, a deep lesion is by definition not visible from the skin surface and the precise PDT of deep tumors, thus far, has been impractical. However nuclear imaging guided implantation of optical fibers within deep tumors would greatly enhance PDT.

Based on a SAR study of a series of alkyl ether analogs of pyropheophorbide-*a*, a relatively long wavelength absorbing PS, the 3-(1-hexyloxyethyl) derivative (HPPH; Scheme 1)^{21,22}, has been developed in our laboratory that proved to be tumor-avid and is currently in Phase I/II multi-center human clinical trials^{23,24}. Initially, we investigated the utility of HPPH as a “vehicle” for delivering the mono- or di-bisaminoethanethiols (N₂S₂ ligand)²⁵ combined with technetium-99m to tumors. The *in vivo* biodistribution data indicated that the tumor/non-tumor uptake ratio of the conjugates correlated with time and tumor size. Its clearance from tumor was slower than most of the non-tumor tissues. However, the short 6-h half-life of ^{99m}Tc was incompatible with a 24-hour PS uptake time, suggesting that longer-lived isotopes, such as ¹¹¹In or ¹²⁴I, could provide a useful scanning agent. Among a series of radiolabeled PSs^{25–28} investigated so far the ¹²⁴I- labeled methyl 3-(*m*-iodobenzyloxyethyl)pyropheophorbide-*a* was found to be an effective PET/PDT imaging agent

In our attempt to develop improved PDT agents we have shown that (i) overall lipophilicity and (ii) the presence and position of various substituents in a variety of tetrapyrrolic systems related to pyropheophorbides, purpurinimides and bacteriopurpurinimides have significant impact on cellular uptake, intracellular localization, photoreaction and PDT efficacy. Though the ¹²⁴I-labeled PS **3** (Scheme 1) containing 3-(1'-*m*-iodobenzyloxyethyl) group at position-3 of the molecule showed promising tumor imaging (PET) ability, we were interested to determine whether an alternative position of the iodobenzyl group on the macrocycle or reduction of the Bring in place of the D-ring would change the cell biology of the PS, such as cellular uptake and retention, that could then be exploited for designing tumor-specific PS delivery. To do so, we synthesized a series of related analogs by (i) modifying the methyl ester group of the propionic ester side chain, (ii) introducing the (*m*-iodobenzyloxyethyl) group at position-8 (instead of position-3), (iii) prepare the corresponding ring-B reduced isomer (instead of ring-D) and investigate their biological consequences of these both *in vitro* and *in vivo* (Figure 1). We discovered that the position of the iodobenzyl group affects efficacy in uptake and retention of the PSs, whereas the carboxylic group at position-17² determines not only quantitative amount of PS internalization but also the site of long-term intracellular retention. Interestingly, the mechanism of internalization and subcellular deposition of the PS radically changed by conjugating polyethyleneglycol (mol. wt. 5000) at position-17².

Results and Discussions

Chemistry

The iodinated PS **1** containing the methyl ester functionality and the corresponding ¹²⁴I-analog **3** were prepared by following the methodology developed in our laboratory²⁶ (Scheme 1). To investigate the effect of polyethyleneglycol (PEG) moiety in cellular uptake and PDT efficacy the PEG analog **7** was synthesized in excellent yield. The corresponding isomer **11** containing a (1'-*m*-iodobenzyloxyethyl) group at position-8 and the corresponding carboxylic acid derivative **12** were prepared from the methyl pyropheophorbide-*a* **8** by following the methodology depicted in Scheme 2. During the preparation of the desired

compound, methyl-8-vinylpyropheophorbide-*a* **16** a by-product in minor quantity was also isolated and the possible mechanism for the formation of the by-product **16** is shown in Scheme 2. To investigate the impact of ring-B vs. ring-D reduced isomers in tumor imaging and PDT efficacy, the desired labeled analogs containing either a propionic ester or carboxylic acid side chain were synthesized from methyl bacteriopyropheophorbide-*a* **17**, which in turn was obtained from *Rb. sphaeroides*.²⁹ The reaction sequences followed for the preparation of the desired compounds are shown in Scheme 3. In brief, two approaches were used for the preparation of the intermediate **20**. In the first approach, the bacteriochlorin **17** was reacted with sodium borohydride and the resulting hydroxy ethyl derivative **18** was reacted with HBr gas, the intermediate bromo- analog was not isolated and, after drying under vacuum (inert atmosphere), was immediately reacted with 3-iodobenzyl alcohol resulting in bacteriochlorin **19**. For the preparation of ring-B reduced chlorin **20**, bacteriochlorin **19** was subjected to ferric chloride oxidation and the desired analog was isolated in 70–75% yield. In the second approach, chlorin **21** in a sequence of reaction gave the corresponding 3-(m-iodobenzyloxyethyl) analog **20** corresponding 3-(m-iodobenzyloxyethyl) analog **20** (Scheme 3). Starting from **20**, the ¹²⁴I- labeled analogs either as methyl ester **26** or carboxylic acid **27** were synthesized by following the methodology discussed for other isomers (Schemes 1). The structures of all the intermediate and the final products were confirmed by NMR, mass spectrometry and elemental analyses. The purity was ascertained by HPLC. Radio-labeling efficiency and specificity of the labeled analogs both as methyl ester and carboxylic acid derivatives was 30–32 % and the specific activity was greater than 1 Ci/μmol.

Before evaluating these compounds for biological efficacy, the structures of ring-B vs. ring-D reduced isomers were confirmed by ¹H NMR. The striking features observed between the ring-D and ring-B reduced chlorins are shown in Figure 2. In brief, the ¹³C-H proton observed as an ABX pattern at δ 5.37 ppm in ring-D reduced chlorin **1**, appeared as a singlet at 5.82 ppm in ring-B reduced chlorin **20** suggesting a regioselective oxidation of ring-D, which was further confirmed by the presence of triplets at δ 4.25 and 3.33 ppm, each integrating for two protons and were assigned to 17¹- and 17²-CH₂ protons of the propionic ester side chain, whereas in ring-D reduced chlorin, these protons were observed at δ 2.51, 2.35 and 2.05 ppm respectively. The *meso*- region of both the chlorins showed interesting features. For example, the 5-H proton appeared at δ 9.62 for chlorin **1** was observed at 9.21 ppm for the ring-B reduced isomer **20**. The 20- *meso* proton, which appeared at δ 8.46 ppm in D-ring reduced chlorin **1** was observed at δ 8.65 in isomer **20**. Compared to ring-D reduced isomer, the resonances of **20** at δ 9.01 ppm integrated for a single proton assigned to 10- *meso* proton, appeared at δ 9.21 ppm in chlorin **1** possibly due to higher electron density at these positions (see Supporting Material).

The course of reaction for the preparation of the intermediates and final products for the synthesis of the isomeric structures **1**, **11** and **20** (or the corresponding carboxylic acid **4**, **12**, and **23** respectively) could be followed by monitoring changes in the absorption spectrum. Following interesting differences between the electronic spectra of the ring-B and ring-D reduced isomers were observed: As shown in Figure 3, the isomers **4** and **12** containing an iodobenzyloxyethyl group at position-3 or position-8 of the chlorin system showed almost identical electronic absorption spectra (long wavelength absorption 660 nm (CH₂Cl₂), however the ring-B reduced chlorin **23** exhibited the long wavelength absorption at λ_{max} 670 nm. No significant difference in singlet oxygen yields was detected between the ring-B and ring-D reduced isomers and ranged from 0.45 to 0.48.

The rate of photobleaching of isomers **4**, **12** and **23** was also investigated in 17% bovine calf serum (BCS) in equimolar concentration. As can be seen from figure 3B, all isomer showed almost 50% photobleaching on exposing the photosensitizers with light (75 mW/cm²) for

20–22 min. Interestingly, on further irradiation, the rate of photobleaching of **12** (ring-D reduced chlorin containing the 1'-benzyloxyethyl group at position-8) was slightly slower than the other two isomers, suggesting its increased photostability.

Biological Studies

The initial functional assessment of the PSs (cellular uptake, intracellular localization, STAT3 dimerization and PDT efficacy) was carried out in the cell culture model of human basal cell carcinoma (BCC1). The relative PDT efficacies determined in these cells were then evaluated in the mouse colon adenocarcinoma cell line (Colon26) grown *in vitro* and as subcutaneous tumors in BALB/c mice.

Correlation between cellular uptake, STAT3 dimerization and cell killing

The cell biological properties of the iodinated PS **1** was evaluated as a function of derivatization at the position 17² to carboxylic acid (PS **4**) or conjugated with PEG (polyethylene glycol, mol.wt., 5000) (PS **7**). The same cellular characterization determined the impact of the iodobenzyl group added to position 8 (PS **12**) or B-ring reduction (PS **23**) in the carboxylic acid PS derivatives. Incubation of BCC1 cells with these PSs for 30 min allowed assessment of immediate uptake (Fig. 4A). The cellular distribution of the PS fluorescence indicated that PS **4** was efficiently taken up and deposited into mitochondrial compartment. Both, the B-Ring reduction (PS **23**) and placement of the iodobenzyl group to position-8 (PS **12**) in the carboxylic acid derivatives lowered the cellular uptake by 2-fold and 4-fold, respectively, relative to PS **4**. The subcellular localization was identical for all three PSs and was not altered as a function of continued incubation of the cells in PS-free medium for 4 to 24 h (data not shown). However, as found for other porphyrin-based PSs, cell-associated PS **4**, **12**, and **23** was lost with a ~4–5-fold higher rate when the cells that were cultured in medium containing 10% serum compared to cells cultured in serum-free medium (data not shown). Strikingly different was the >100-fold lower uptake of the methylester derivative, PS **1**, and the PEGylated PS **7** relative to PS **4** (Fig. 4A). The quantitative difference in uptake and retention was also evident following incubation of the cells with the PSs for 2 h, although the overall level of cell-associated PSs was enhanced (data not shown).

Treatment of the PS-incubated cells with therapeutic light mediated photoreactions that were strictly proportional to the level of PS as determined by fluorescence. The photoreactions were manifested in the level of STAT3 crosslinking, loss of EGFR protein, and activation of the stress MAPK, p38 (Fig. 4B). The same PDT treatment also led to a PS dose-dependent cell killing as determined by the fraction of the cells surviving 24 h post PDT incubation (Fig. 4C). Under the conditions used in Fig. 4, the cellular uptake of PS **1** and PS **7** was barely detectable by fluorescent microscopy and the low levels of PS were ineffective in mediating photoreaction that were quantifiable by the biochemical markers. By increasing the concentrations of the PSs in cell treatments, we detected that the two PSs displayed fundamentally different modes of uptake and subcellular localization compared to the carboxylic acid-containing PSs. A time course analysis indicated that within 30 min incubation period, a low-level of PS **1** was accumulated in the mitochondrial compartment yielding a pattern as observed for PS **4** (Fig. 5A, left panel). However, during a subsequent 4 h incubation period in PS-free medium, a major fraction of the cell-associated PS fluorescence was redistributed and retained at new subcellular sites that include the lysosomal compartment, whereas PS **4** maintained the initial mitochondrial localization (Fig. 5A, right panel). The uptake of PS **1**, as PS **4**, was subject to competition with serum protein with ~5-fold lower fluorescence recorded for cells incubated with PS **1** in presence of 10% fetal calf serum (data not shown). The opposite uptake properties in presence of serum proteins were detected for PS **7**. The PEGylated PS **7** was synthesized to identify whether

this hydrophilic version of the PS will reduce hepatic clearance and, thus, enhance the availability for uptake by tumor *in vivo*. Short-term incubation of basal cell carcinoma (BCC1) cells with PS **7** indicated that PS **7** uptake was very low, with levels below microscopic detection (Fig. 4A). Only at higher input concentrations and prolonged incubation, i.e., 24 h, could an increased and time-dependent cellular accumulation be observed (Fig. 5B, right panel). Unexpectedly, the cellular uptake was several-fold higher in presence of serum proteins (Fig. 5B, right panel). The subcellular localization, regardless of the incubation conditions, was exclusively the endosomal/lysosomal compartment. Since PS **7**, unlike other lysosomally targeted PSs, such as HPPH-Gal³⁰, did not show any binding to cell surface internalization, we concluded that the uptake of PS **7** was not through the route of first binding to the plasma membrane and followed by endocytosis but largely through fluid phase pinocytosis.

The differential functions of the PSs are reproducible in murine Colon26 tumor model

The relative activities of the PSs determined in BCC1 cells were confirmed in the mouse tumor cell lines Colon26. The comparative study involved two separate sets of experiments. In the first set, Colon26 cells were incubated for 24 h in serum-containing culture medium with either PS **1**, **4**, or **7**. The duration of PS treatment was extended to 24 h in order to enhance intracellular accumulation of the PSs, in particular PS **1** and **7**. Following the photoreaction at increasing light doses and culture in growth medium for additional 24 h, the surviving Colon26 cells were evaluated by MTT assay (Fig. 6A). The relative PDT efficacy of the PSs followed the patterns determined in BCC1 cells (Fig. 4C) with PS **4** as the most effective PS. The second experimental set determined the efficacy of PS **4** with the other isomers, PS **12** and PS **23** (Fig. 6B). All three isomers showed comparable *in vitro* efficacy that in part was due to the application of higher PS concentration. Measurement of PS uptake by Colon26 cells showed comparable values (PS **4** $\geq$ **23** > **12**) as found in BCC1 cells (Figures 4C & 6C). Based on *in vitro* finding, the relative activity of the PSs in mediating Colon26 tumor treatment *in vivo* was determined. PS **1**, **4** and **7** were administered at a dose of 1.0 $\mu\text{mol/kg}$ to BALB/c mice bearing subcutaneous Colon26 tumors. After 24 h, the tumors were exposed to light (128 J/cm², 14mW/cm²). The animals were monitored for 60 days for recurring tumor growth (Fig. 7A). In line with the relative activities determined in cell culture, PS **4** gave 50% tumor cure whereas PS **1** was significantly less effective and only 20% cure mice were tumor-free on day 60. Under these treatment parameters PS **7** was ineffective. We interpret that the substantial *in vivo* activity of PS **1** to be the result from progressive PS **1** accumulation in the tumor during the 24 h period post injection and may involve the mode of cellular uptake detected in cultured cells (Fig. 5A). A second series of *in vivo* tumor treatments tested the activity of PS **23** and PS **12** relative to PS **4** (Fig 7B). PS **4** and PS **23** showed relative efficacy that matches those detected for these two PSs *in vitro* (Figs. 4C and 6B).

Comparative PET imaging ability of Photosensitizers **3** and **6**

As evident from the tissue culture experiments and *in vivo* tumor treatments, PS **4** displayed the highest PDT efficacy followed by PS **23** and **12**. We have previously shown that the ¹²⁴I- labeled methyl ester derivative of PS **1**, PS **3** showed a great potential for imaging tumors in BALB/c mice bearing Colon26 tumors. In the light of the new finding regarding the activity of PS **4**, and our goal to develop a “Multifunctional Agent” based on optimized PS structures for efficient tumor-imaging and therapy, we compared the tumor-imaging and biodistribution of PS **3** with the corresponding ¹²⁴I-labeled form of PS **4**, PS **6**. Similar to the methyl ester derivative²⁶, PS **6** showed significant tumor uptake at 24, 48 and 72h post injection (Fig. 8). Compound PS **27** showed similar uptake profile as compared PS **6** (data not shown). The corresponding methyl-ester derivative of compound PS **27** and its ¹²⁴I-label form, PS **26** also exhibited similar uptake profile as compared to compound PS **3**

(data not shown). With both analogs, the maximal tumor-uptake was observed at 24 h post injection as determined by region of interest (ROI) calculations. However, for imaging, 72 h post injection for compounds PS **4**, **6** and compound PS **27** (data not shown) produced the best contrast, as the compounds cleared more rapidly from liver, spleen and intestine than from the tumor (Figure. 8). The accumulation of label in the thyroid due to release of free ^{124}I could be blocked by injecting cold potassium iodide in water 24 h prior to injecting the labeled photosensitizer (data not shown).

Comparative *in vivo* biodistribution of PS **3**, PS **6** and ^{18}F -FDG

Among the isomers investigated for PDT efficacy, PS **4**, the pyropheophorbide-*a* containing *m*-iodobenzyloxyethyl at position-3 of the ring-D reduced chlorin system and with carboxylic acid group at 17²- position, was found to be most effective. Interestingly, PS **1**, the derivative bearing ester functionality, despite its lower PDT efficacy, showed remarkable tumor imaging ability²⁶. For determining the relative tumor-uptake and other organ uptake, we compare the *in vivo* biodistribution of both ^{124}I following injection of PS **3** and **6** into BALB/c mice bearing Colon26 tumors. Specifically, a set of 9 tumor-bearing mice were injected with each compound (50 μCi), and 3 mice per group were imaged after 24, 48 and 72 h for 30 min with microPET. Following imaging, the mice were sacrificed and specific radioactivity in individual organs (% injected dose per gram) were determined (Fig. 9A and B). Both PSs showed similar biodistribution and clearance from all the organs with the notable exception of slightly higher spleen-uptake of PS **1**. ^{124}I -labelled PS **27** (B-ring isomer) also demonstrated similar tumor uptake as compared to PS **6** (Figure 9A) but showed significantly lower spleen uptake (data not shown).

Among the PET imaging agents, ^{18}F -FDG is a current clinical standard, with the several limitation of short-term uptake processes. To assess the usefulness of PS **3** or PS **6** as alternative PET agents to FDG, we also determined the biodistribution of ^{18}F -FDG (isotope half life 110 min) in BALB/c mice bearing Colon26 tumors. In brief, ^{18}F -FDG (150 μCi) was injected i. v. into 3 tumor-bearing mice, imaged by a PET scanning at 2h post injection. Mice were then sacrificed and the specific radioactivity in the various organs was measured (Fig. 10). The results indicated that substantially different organ distribution than PS **3** and PS **6**, with higher uptake in muscle and heart relative to tumor.

Isomers **4**, **12** and **23** showed a direct correlation between tumor-uptake and fluorescence imaging

For investigating the fluorescence imaging potential of the isomers, the PS **4**, **12** and **23** were injected intravenously (1.0 $\mu\text{mol/kg}$) in BALB/c mice (3 mice/group) bearing Colon26 tumors. For best visualization, the tumors were grown subcutaneously on the right shoulder to a size of 4–5 mm in diameter. The fluorescence in intact animals was imaged using the Nuance camera system at 24, 48 and 72 h post-injection. The images were then analyzed by generating false color images representing fluorescence intensity of the drug at the tumor site. Among all the isomers investigated, the PS **4** (ring-D isomer) and **23** (ring-B isomer) showed similar drug uptake at 24, 48 and 72 h post injection (p.i.) (Figure 11a and 11c). This could explain the similar *in vivo* photosensitizing efficacy of the two PS. Both PSs also showed similar clearance rates at 48 and 72 h PI (Figure 11d). PS **12** showed lower tumor uptake at 24 h compared to PSs **4** and **23** (Figure 11b), which is in agreement with the low accumulation in cultured cells (Fig. 4) and tumor phototoxicity exhibited by PS **12** (Fig. 7).

To study the potential of these agents as tumor imaging agents, the most effective compounds PS **4** and **23** were injected into mice bearing colon 26 tumors at the therapeutic dose of 1 $\mu\text{mol/kg}$. Twenty four hours post injection the animals were sacrificed, the primary tumor removed surgically and imaged for fluorescence. The skin beneath the tumor was also

exposed to light to see if any fluorescence was associated with skin mass. The results clearly showed the tumor avid nature of compounds of PS **4** and **23** (Fig 12). Most of the fluorescence was displayed from the tumor with little fluorescence associated with the skin. This might be useful to identify tumor mass from normal tissue mass during surgical removal of tumors.

Conclusions

In summary, the results obtained from a series of iodinated PSs suggests that a carboxylic acid group at position 17² favors a significant increase in cellular uptake by diffusion and deposition into the mitochondrial compartment whereby increasing *in vitro/in vivo* PDT efficacy. In contrast, the same PS but with a methylester at position 17² displays low level cellular uptake and is subjected to a yet to defined intracellular redistribution. Linking PEG moiety (mol. Wt: 5,000) to position-17² changes the PS to a hydrophilic molecule that assumes a behavior common to plasma constituents that is to be taken up by cells through fluid phase pinocytosis and deposited in the endosomal/lysosomal compartment. Interestingly, the isomer **12** in which the *m*-iodobenzoyloxyethyl group was substituted at position-8 showed limited PDT efficacy. However the isomer **23** in which the *m*-iodobenzoyloxy was present at position-3, but instead of ring-D (PS **4**), the ring-B was reduced, was equally effective. The function/structure analysis of these photosensitizers demonstrated the close relationship of PS concentration in cells, PS-mediated photoreaction resulting in STAT3 dimerization and PDT efficacy. These activities defined *in vitro* proved to a large extent to be predictive for the PS's action *in vivo* and also confirmed activities reported for other photosensitizers³¹. The ¹²⁴I- analogs of the effective PS also showed a great potential to function as tumor-imaging agent. PS **1** and **4** were successfully applied to image tumors by PET and fluorescence. Detailed toxicological studies of these compounds in a good laboratory practice (GLP) facility following the United States Food and Drug Administration (US-FDA) guidelines are currently in progress.

Experimental

Chemistry

All chemicals were of reagent grade and used as such. Solvents were dried using standard methods. Reactions were carried out under nitrogen atmosphere and were monitored by thin-layer chromatography and/or UV-visible spectroscopy. Preparative scale thin-layer chromatography was carried out on 20×20 cm glass plates coated with Merck G 254 silica gel (2 mm thick). ¹H & ¹³C-NMR spectra were recorded on Bruker AMX 400-MHz or Varian 400 NMR spectrometer at 303 K in CDCl₃ or ~10% of CD₃OD in CDCl₃ or DMSO-d₆. Proton chemical shifts(δ) are reported in parts per million (ppm) relative to CDCl₃ (7.26 ppm) or TMS (0.00 ppm). Coupling constants (J) are reported in Hertz (Hz) and s, d, t, q, p, m and br refer to singlet, doublet, triplet, quartet, pentet, multiplet and broad respectively. Mass spectral data (Electrospray ionization, ESI by fusion) were obtained from Biopolymer facility, RPCI. The high resolution Mass spectrometry analysis were performed at Mass spectrometry facility, Michigan State University, East Lansing, MI. CHN analysis were done at Midwest Microlab LLC., Indianapolis, IN. UV-visible spectrum were recorded on Varian Cary 50 Bio UV-visible spectrophotometer using CH₂Cl₂/MeOH as solvent. Melting points were uncorrected and were measured on a Thomas/Bristonline microscopic hot stage apparatus. In general most of the products obtained after column or preparative chromatography were crystallized/precipitated from dichloromethane/hexane. The purity of the compounds was confirmed by HPLC and all compounds were >95%. The HPLC profiles of the final products are included in the "Supporting Material" section.

Synthesis of 3-devinyl-3-{1'-(*m*-iodobenzyoxy)-ethyl} pyropheophoride-a (4)

Methyl 3-devinyl-3-{1'-(*m*-iodobenzyoxy)-ethyl}pyropheophoride-a **1** (150 mg, 0.19 mmol) obtained following the procedure developed by our group²⁶ was reacted with 240 mg (10.04 mmol) aqueous LiOH in THF, and the reaction was stirred at room temperature overnight. The reaction mixture was neutralized with 2% AcOH in H₂O, and compound was extracted with CH₂Cl₂. The organic layer was washed with H₂O, dried over Na₂SO₄, concentrated, and precipitated with hexanes to yield proposed compound (yield: 80%), mp: 138–140°C. UV-visible, λ_{max} (MeOH/CH₂Cl₂), nm (ϵ): 663 nm (4.75×10^4), 605 (6.81×10^3), 539 (6.81×10^3), 506 (6.61×10^3), 411 (9.52×10^4). ¹HNMR (CDCl₃; 400MHz): δ 9.62, 9.21 and 8.46 (three s, each for 1H, meso-H), 7.67 (s, 1H, ArH), 7.558 (d, $J = 8.0$, 1H, ArH), 7.183 (m, 1H, ArH), 6.962 (m, 1H, ArH), 5.867 (q, $J = 6.8$, 1H, 3¹-H), 5.16 (d, $J = 18.6$, 1H, 13²-CH₂), 4.97 (d, $J = 18.6$, 1H, 13²-CH₂), 4.62–4.50 (m, 1H, 18H), 4.49–4.26 (m, 2H, OCH₂Ar), 4.20–4.00 (m, 1H, 17H), 3.46 (q, $J = 8.0$, 2H, 8-CH₂CH₃), 3.35–2.99 (m, all 3H, for 3 $\times$ ring CH₃), 2.40–2.73, 2.38–2.18 (m, 4H, 17¹ and 17² -H), 2.08 (s, 3H, 3²-CH₃), 1.61 (d, $J = 7.6$, 3H, 18-CH₃), 1.48 (s, 3H, 8-CH₂CH₃), 0.07 (brs, 1H, NH), –1.82 (brs, 1H, NH). ¹³C NMR (CDCl₃; 400MHz): δ 196.95, 171.71, 160.67, 155.35, 151.04, 149.13, 145.13, 141.38, 140.87, 138.51, 137.85, 137.11, 136.91, 135.4, 132.99, 130.34, 128.41, 127.94, 127.35, 106.16, 104.13, 97.93, 94.62, 93.00, 72.19, 70.37, 51.74, 50.19, 48.14, 31.24, 29.97, 24.73, 23.29, 19.58, 17.59, 14.21, 12.03, 11.53, 11.26, 1.25. MS for C₄₀H₄₁N₄O₄I: 768.22 (calcd), 769.2(found, MH⁺). Anal. Calcd for C₄₀H₄₁N₄O₄I: C, 62.50; H, 5.38; N, 7.29, Found: C, 62.59; H, 5.42, N, 7.20.

Synthesis of 3-devinyl-3-{1'-(*m*-trimethylstannylbenzyoxy)-ethyl} pyropheophoride-a (5)

The solution of methyl 3-devinyl-3-{1'-(*m*-iodobenzyoxy)-ethyl}pyropheophoride-a carboxylic acid (**4**) (50 mg, 0.065 mmol) in dry THF were added hexamethydistannane (0.1 ml, 0.48 mmol) and bis(triphenylphosphine) palladium(II)dichloride (10 mg, 0.014 mmol), and the reaction mixture was stirred at room temperature for overnight. The solvent was evaporated. The crude product was purified by preparative plates. 4% methanol/dichloromethane was used as the eluting solvent to yield compound **5**. The compound obtained after evaporating the solvents was crystallized from dichloromethane/hexane in 75% yield (39 mg). mp>320°C. UV-visible, λ_{max} (MeOH/CH₂Cl₂), nm (ϵ): 663 (4.65×10^4), 606 (9.20×10^3), 537 (9.42×10^3), 506 (9.64×10^3), 411 (9.52×10^4). ¹HNMR (CDCl₃; 400MHz): δ 9.74, 9.50, and 8.53 (all s, 1H, meso-H), 7.50 (m, 2H, ArH), 7.40 (m, 2H, ArH), 6.01(q, $J = 6.4$, 1H, 3¹-H), 5.27 (d, $J = 17.6$, 1H, 13²-CH₂), 5.10 (d, $J = 19.6$, 1H, 13²-CH₂), 4.78 (dd, $J = 5.4$, 11.9, 1H, OCH₂Ar), 4.60 (dd, $J = 1.7, 12.0$, 1H, OCH₂Ar), 4.50 (m, 1H, 18-H), 4.28 (m, 1H, 17-H), 3.70 (q, $J = 7.8$, 2H, 8-CH₂CH₃), 3.64, 3.35 and 3.16 (m, all 3H, for 3 $\times$ ring CH₃), 2.36–2.76, 2.50–2.62, and 2.20–2.34 (m, 4H, 17¹ and 17² -H), 2.14 (m, 3H, 3²-CH₃), 1.81 (d, $J = 5.6$, 3H, 18-CH₃), 1.71 (t, $J = 7.6$, 3H, 8-CH₂CH₃), 0.50 (brs, 1H, NH), 0.19 (s, 9H, *tert*-butyltin), –1.70 (brs, 1H, NH). ¹³C NMR (CDCl₃; 400MHz): δ 197.39, 172.07, 160.96, 155.59, 151.08, 149.12, 145.21, 141.32, 140.68, 138.46, 137.63, 137.03, 136.49, 135.49, 133.11, 130.21, 130.01, 128.37, 127.80, 127.29, 114.66, 105.88, 104.21, 97.77, 94.44, 93.00, 72.08, 70.30, 51.72, 50.21, 48.03, 31.47, 31.11, 30.32, 29.75, 24.52, 23.14, 19.49, 17.44, 14.24, 11.95, 11.33, 11.09, 1.05. Mass calcd for C₄₃H₅₀N₄O₄Sn 806.3, found: 807.5(MH⁺). HRMS, Calcd for C₄₃H₅₁N₄O₄Sn (MH): 807.2932, Found 807.2995.

Synthesis of Peglate 3-devinyl-3-{1'-(*m*-iodobenzyoxy)ethyl}pyropheophoride-a (7)

Methyl 3-devinyl-3-{1'-(*m*-iodobenzyoxy)-ethyl}pyropheophoride-a carboxylic acid (**4**) (50 mg, 0.065 mmol), PEG (390 mg, 0.078 mmol), DCCI (13.8 mg, 0.072 mmol) and DMAP (7.93 mg, 0.065 mmol) were dissolved in anhydrous DMF (4 mL). The reaction mixture was stirred at room temperature for overnight. The solvent was evaporated. The crude product was purified by preparative plates. 4% methanol/dichloromethane was used as the eluting solvent to yield compound **7**. It was then crystallized from dichloromethane/hexane

in 51% yield (191 mg). mp>320°C. UV-visible, λ_{max} (CH₂Cl₂), nm (ϵ): 664 (4.64×10^4), 607 (9.22×10^3), 535 (9.43×10^3), 507 (9.66×10^3), 412 (9.53×10^4). ¹HNMR (CDCl₃; 400 MHz): δ 9.77, 9.76 (two s, 1H, 5-H), 9.54, and 8.59 (each s for 1H, 5- and 10- meso-H), 7.75, 7.63, 7.07, 6.89 (each m for 1H, ArH), 7.40 (m, 2H, ArH), 5.96 (q, $J = 6.3$, 1H, 3¹-H), 5.28 (d, $J = 17.7$, 1H, 13²-CH₂), 5.12 (d, $J = 17.6$, 1H, 13²-CH₂), 4.68 (dd, $J = 5.4$, 11.3, 1H, OCH₂Ar), 4.57 (m, 2H, 1H for OCH₂Ar, 1H for 17-H), 4.35 (m, 1H, 18-H), 4.04-3.14 (broad and high peak, many protons, including all protons from PEG group – NHCH₂CH₂(OCH₂CH₂)_nOCH₃, another 11H for 2-CH₃, 7-CH₃, 12-CH₃, and 8-CH₂CH₃), 2.43 (m, 2H, 17¹-H), 2.17 (m, 2H, 17²-H), 1.82 (d, $J = 5.5$, 3H, 3-CH₃), 1.70 (t, $J = 7.6$, 3H, 18-CH₂CH₃), 1.25 (brs, 1H, NH), –1.73 (brs, 1H, NH). HRMS, Calcd for C₂₆₇H₄₉₆N₅O₁₁₆I: 5756.2111, Found 5756.2132.

Synthesis of Methyl 3-devinyl-8-{1'-(*m*-iodobenzyoxy)ethyl}-pyropheophoride-a (11)

Methyl 3-devinyl-3-ethyl-7, 8-dihydroxy-pyropheophoride-a **10** (100 mg, 0.17 mmol) was reacted with 3-Iodobenzyl alcohol in refluxing toluene, and a few drops of HCl was added. The reaction mixture was neutralized with NaHCO₃ in H₂O, and compound was extracted with CH₂Cl₂. The organic layer was washed with H₂O, dried over Na₂SO₄. The crude product was purified by preparative plates, using 5 % methanol/dichloromethane as the eluting solvent, two bands were separated and identified as follows: the slower moving band (minor product) was characterized as methyl 3-devinyl-3-ethyl-8-vinyl- pyropheophoride-a **16**, the faster moving band (major product, 54 mg, 40% yield) was identified as the desired product **11**, mp: 130–132°C. UV-visible, λ_{max} (CH₂Cl₂), nm (ϵ): 656 (4.69×10^4), 599 (8.22×10^3), 539 (8.14×10^3), 506 (9.91×10^3). ¹HNMR (CDCl₃; 400MHz): δ 9.98, 9.26, 8.48 (all s, 1H, meso-H), 7.71 (d, $J = 4.0$, 1H, ArH), 7.59 (d, $J = 8.0$, 1H, ArH), 7.20 (t, $J = 8.2$, 1H, ArH), 7.05 (t, $J = 8.2$, 1H, ArH), 5.78 (q, 6.8, 1H, 8¹-H), 5.28 (d, $J = 19.8$, 1H, 13²-CH₂), 5.13 (d, $J = 19.8$, 1H, 13²-CH₂), 4.68 (d, $J = 13.0$, 1H, OCH₂Ar), 4.56 (d, $J = 11.6$, 1H, OCH₂Ar), 4.41–4.48 (m, 1H, 18-H), 4.22–4.31 (m, 1H, 17-H), 3.84 (q, $J = 8.0$, 2H, 3-CH₂CH₃), 3.62, 3.59, 3.30 and 3.28 (all s, all 3H, for 173-COOCH₃ and 3×ring CH₃), 2.61–2.75, 2.44–2.58, and 2.17–2.35 (m, 4H, 17¹ and 17²-H), 2.12 (t, $J = 4.8$, 3 H, 8²-CH₃), 1.79 (t, $J = 10.4$, 3H, 18-CH₃), 1.73 (t, $J = 7.6$, 3H, 3-CH₂-CH₃), 0.41 (brs, 1H, NH), –1.74 (brs, 1H, NH). ¹³C NMR (CDCl₃; 400MHz): δ 196.42, 173.69, 172.16, 160.78, 153.90, 149.58, 148.86, 142.67, 142.19, 141.33, 138.03, 137.24, 136.83, 131.44, 130.68, 129.55, 128.08, 127.38, 119.98, 114.96, 106.67, 105.97, 96.22, 94.65, 92.77, 72.55, 70.20, 51.90, 50.24, 48.22, 31.11, 30.08, 25.39, 23.32, 19.61, 18.70, 17.17, 12.72, 12.41, 11.61, 11.17. MS for C₄₁H₄₃O₄N₄I: 782.23 (calcd), 805.1 (found, M+23). Anal. Calcd for C₄₁H₄₃O₄N₄I: C, 62.90; H, 5.54; N, 7.16. Found: C, 62.80; H, 5.52; N, 7.11.

Synthesis of 3-devinyl-3-ethyl-8-{1'-(*m*-iodobenzyoxy)-ethyl}-pyropheophoride-a (12)

Methyl 3-devinyl-8-{1'-(*m*-iodobenzyoxy)-ethyl}-pyropheophoride-a **11** was reacted with aqueous LiOH by following the method discussed for the preparation of 3-devinyl-3-{1'-(*m*-iodobenzyoxy)-ethyl}pyropheophoride-a carboxylic acid **4** and **12** was obtained in 70% yield, mp: 171–172°C. UV-visible, λ_{max} (CH₂Cl₂), nm (ϵ): 655 (4.65×10^4), 600 (8.03×10^3), 538 (8.00×10^3), 506 (9.63×10^3). ¹HNMR (CDCl₃; 400MHz): δ 9.90, 9.16 and 8.42 (all s, 1H, meso-H), 7.70 (s, 1H, ArH), 7.55 (d, $J = 7.8$, 1H, ArH), 7.21 (d, $J = 8.0$, 1H, ArH), 6.962 (t, $J = 8.0$, 1H, ArH), 5.73 (q, $J = 8.0$, 1H, 8¹-H), 4.95–5.36 (m, 2H, 13²-CH₂), 4.57–4.70 (m, 1H, 18H), 4.44–4.54 (d, $J = 10.4$, 1H, OCH₂Ar), 4.43–4.33 (m, 1H, OCH₂Ar), 4.20–4.24 (m, 1H, 17H), 3.75 (q, $J = 8.0$, 2H, –CH₂CH₃), 3.56, 3.25, 3.22 (all s, all 3H, for 3×ring CH₃), 2.47–2.71, 2.20–2.37 (m, 4H, 17¹ and 17² –H), 2.09 (s, 3H, 8²-CH₃), 1.78 (s, 3H, 18-CH₃), 1.67 (s, 3H, 3-CH₂CH₃), 0.85 (brs, 1H, NH), –1.72 (brs, 1H, NH). ¹³C NMR (CDCl₃; 400MHz): δ 197.25, 173.94, 172.50, 160.65, 154.17, 149.36, 148.67, 142.64, 142.01, 140.84, 138.01, 137.28, 137.02, 136.86, 136.60, 131.53, 130.05, 129.80, 129.12, 127.61, 127.22, 114.49, 105.38, 95.81, 94.19, 92.62, 72.27, 69.94, 51.59, 51.42, 50.01,

30.79, 29.79, 29.57, 24.87, 22.85, 19.23, 16.71, 11.89, 11.16. Mass for $C_{40}H_{41}O_4N_4I$: 768.22 (calcd), 769.4 (found, MH^+). Anal. Calcd for $C_{40}H_{41}O_4N_4I$: C, 62.50; H, 5.38; N, 7.29. Found: C, 62.39; H, 5.45; N, 7.20.

Synthesis of Methyl 3-(1'-(3-Iodobenzyloxy)ethyl-3-deacetyl-bacteriopyropheophorbide-a (19)

Compound **18**²⁹ (100.0 mg, 0.17 mmol, 1.0 equiv) was dissolved in dry CH_2Cl_2 (15 mL), and HBr gas was bubbled into the solution for 2 min. The reaction mixture was stirred for 5 min at room temperature. The solvent was evaporated under high vacuum, the resulting crude was dried, and the entire crude was dissolved in dry CH_2Cl_2 (15 mL). A 50.0 mg portion of dry K_2CO_3 was added to this mixture, followed by addition of 3-iodobenzyl alcohol (0.25 mL). The resulting reaction mixture was stirred for 30 min at room temperature and then worked up. Purification of the crude mixture was done by column chromatography (silica gel, 50% ethyl acetate in hexanes). Yield: 89 mg, 65%. 1H NMR (400 MHz, $CDCl_3$): δ 8.52/8.53, 8.23, 8.04 (all s, 1H, meso-H), 7.72 (d, 1H, ArH), 7.62 (d, J = 8.0 Hz, 1H, ArH), 7.25 (s, 1H, ArH), 7.04 (t, 1H, ArH), 5.68–5.73 (m, 1H, 3^1 -H), 4.96 (d, J = 19.6 Hz, 1H, 13^1 -H), 4.79 (d, J = 19.6 Hz, 1H, 13^1 -H), 4.60–4.63 (m, 1H, OCH_2 Ar), 4.45–4.50 (m, 1H, OCH_2 Ar), 4.14–4.19 (m, 2H, 7-H + 18-H), 4.00–4.02 (m, 1H, 17-H), 3.88–3.90 (m, 1H, 8-H), 3.61 (s, 3H, $COOCH_3$), 3.35 (s, 3H, 12- CH_3), 3.14 (s, 3H, 2- CH_3), 2.44–2.59 (m, 2H, 81-H + 171-H), 2.21–2.43 (m, 4H, 171-H + 171- CH_2 , + 81-H), 2.01 (d, J = 8.0 Hz, 3H, 3^1 - CH_3), 1.65–1.77 (doublets, 6H, 18- CH_3 + 7- CH_3), 1.12 (t, 3H, 8^1 - CH_3). ^{13}C NMR ($CDCl_3$; 400MHz): δ 195.56, 173.54, 170.95, 170.85, 170.21, 161.53, 155.22, 147.83, 147.81, 140.78, 140.74, 140.70, 140.62, 138.48, 138.41, 137.37, 137.36, 136.95, 136.84, 136.74, 136.02, 135.99, 133.67, 130.12, 127.13, 127.09, 118.22, 118.18, 108.74, 99.24, 99.21, 95.00, 94.94, 94.35, 93.82, 93.80, 71.72, 71.49, 70.10, 70.00, 54.34, 54.30, 51.65, 50.43, 50.41, 49.89, 49.06, 47.38, 30.78, 30.21, 30.19, 29.88, 24.01, 23.90, 22.78, 22.63, 22.54, 22.51, 11.26, 10.88, 10.84, 10.80. MS (ESI): m/z 785.4 ($M + H^+$). HRMS (ESI): calcd for $C_{41}H_{46}IN_4O_4$: 785.2564; found, 785.2587 (MH^+). UV-vis (CH_2Cl_2 , λ_{max} , nm, ϵ): 719 (3.58×10^4), 659 (1.21×10^4), 602 (4.71×10^3), 517 (2.55×10^4), 486 (6.69×10^3), 456 (2.81×10^3), 382 (4.59×10^4), 354 (8.63×10^4).

Synthesis of Methyl-3-deacetyl-3-(1'-*m*-iodobenzyloxyethyl)-7,8-dihydrophyloerythrin (20)

Compound **19** (50.0 mg, 0.06 mmol, 1.0 equiv) was dissolved in dichloromethane (50 mL). To this mixture was added slowly a nitromethane (4 mL) solution of $FeCl_3 \cdot 6H_2O$ (68.9 mg, 0.25 mmol, 4.0 equiv). The resulting reaction mixture was stirred at room temperature for 30 min, quenched by addition of 20 mL of methanol, and washed with water three times. The organic layer was separated and dried over anhydrous Na_2SO_4 , and solvent was removed under vacuum. The product obtained after evaporating the solvents was purified by preparative plates (silica gel, eluting solvent: 2% acetone in dichloromethane). Yield: 35.0 mg, 70%. UV-vis (CH_2Cl_2 , λ_{max} , nm, ϵ): 670 (4.69×10^4), 613 (5.54×10^3), 540 (5.11×10^3), 513 (9.57×10^3), 413 (7.70×10^4). 1H NMR (400 MHz, $CDCl_3$): δ 9.21, 9.00/8.99, 8.65 (all s, 1H, meso-H), 7.75 (d, 1H, ArH), 7.62 (d, J = 8.0 Hz, 1H, ArH), 7.25 (s, 1H, ArH), 7.04 (t, J = 8.0 Hz, 1H, ArH), 5.91 (m, 1H, 3^1 -H), 5.44 (s, 2H, 13^1 -H), 4.64–4.67 and 4.50–4.54 (m, 2H, OCH_2 Ar), 4.43–4.45 and 4.21 (m, 2H, 7-H + 8-H), 3.83 (t, J = 8.0 Hz, 2H, 17^1 - CH_2), 3.74 (s, 3H, $COOCH_3$), 3.55 (s, 3H, 12- CH_3), 3.42 (s, 3H, 2- CH_3), 3.21 (s, 3H, 18- CH_3), 2.91 (t, J = 8.0 Hz, 2H, 17^2 - CH_2), 2.44–2.49 (m, 1H, 8^1 -H), 2.14–2.17 (m, 4H, 8^1 -H + 3^1 - CH_3), 1.80/1.90 (distorted d, 3H, 7- CH_3), 1.16 (t, 3H, 81- CH_3). ^{13}C NMR ($CDCl_3$; 400MHz): δ 195.99, 195.98, 173.11, 172.15, 172.06, 166.25, 154.24, 146.54, 145.24, 145.22, 142.43, 140.79, 140.69, 139.77, 139.61, 139.31, 137.31, 136.98, 136.87, 136.75, 136.38, 136.31, 135.19, 135.13, 133.54, 130.12, 127.19, 127.12, 121.73, 121.72, 115.51, 97.08, 97.05, 96.72, 96.68, 94.49, 94.38, 93.85, 93.77, 71.97, 71.71, 70.18, 70.06, 55.55, 55.51, 51.82, 51.81, 48.96, 48.95, 48.90, 36.08, 30.35, 30.30, 24.34, 24.19, 23.36,

23.23, 11.66, 11.64, 11.22, 11.19, 11.18, 11.17, 11.04, 11.00. MS (ESI): m/z 783.4 ($M + H^+$). HRMS (ESI): calcd for $C_{41}H_{44}IN_4O_4$ 783.2407; found, 783.2410 (MH^+). Anal. Calcd for $C_{41}H_{43}IN_4O_4$: C, 62.91; H, 5.54; N, 7.16. Found: C, 62.85; H, 5.57; N, 7.01.

Synthesis of 3-deacetyl-3-(1'-*m*-iodobenzoyloxyethyl)-7,8-dihydrophyloerythrin (23)

Compound **20** (40 mg, 0.05 mol, 1equiv) was dissolved in degassed THF (10 mL). Degassed water and a solution of $LiOH \cdot H_2O$ (50 mg, 1.19 mmol, 23 equiv) in methanol (10 mL, 1:1 v/v) were added. The entire reaction mixture was then stirred under nitrogen atmosphere at room temperature for 2 h, and the resulting mixture was diluted with dichloromethane (20 mL). It was then washed with water (3×200 mL). The organic layer was separated and dried over Na_2SO_4 , and the solvent was removed under reduced pressure. The residue obtained was purified by preparative TLC (silica gel, 5% methanol in CH_2Cl_2) and crystallized from dichloromethane/hexane. Yield: 22 mg, 55%. UV-vis (CH_2Cl_2 , λ_{max} , nm, (ϵ)): 670 (4.69×10^4), 613 (5.54×10^3), 540 (5.11×10^3), 513 (9.57×10^3), 413 (7.70×10^4). 1H NMR (400 MHz, $CDCl_3$): δ 9.21, 8.64 (all s, 1H, meso-H), 7.73 (d, 1H, ArH), 7.60 (d, 1H, ArH), 7.24 (s, 1H, ArH), 6.99–7.05 (m, 1H, ArH), 5.87–5.88 (m, 1H, 3^1 -H), 5.40 (s, 2H, 13^1 -H), 4.48–4.62 and 4.48–4.53 (m, 2H, OCH_2Ar), 4.40–4.43 and 4.19–4.21 (m, 2H, 7-H + 8-H), 3.86 (m, 2H, 17^2 - CH_2), 3.53 (s, 3H, 12 - CH_3), 3.30 (s, 3H, 2 - CH_3), 3.22 (s, 3H, 18 - CH_3), 2.95 (t, 2H, 17^1 - CH_2), 2.43–2.47 (m, 1H, 8^1 -H), 2.12–2.17 (m, 4H, 8^1 -H + 3^1 - CH_3), 1.75–1.89 (d, 3H, 7 - CH_3), 1.13 (m, 3H, 8^1 - CH_3). MS (ESI): m/z 769.2 ($M + H^+$). HRMS (ESI): calcd for $C_{40}H_{42}IN_4O_4$ 769.2251; found, 769.2271 (MH^+).

Synthesis of Methyl 3-{1'-(*m*-trimethylstannylbenzyloxy)ethyl}-pyropheophorbide-a (25)

To a solution of **20** (50 mg, 0.06 mmol, 1.0 equiv) in dry DMF (10 mL) were added hexamethylditin (83 mg, 0.25 mmol, 4.0 equiv) and $Pd_2(dba)_3 \cdot CHCl_3$ (6.6 mg, 0.006 mmol, 0.1 equiv.) and the reaction mixture was stirred at 50° for 4 hours. After removal of the solvent under vacuum to dryness, the crude mixture was purified preparative plates using 5% acetone/ CH_2Cl_2 to give **25**. Yield = 21 mg, 40% UV-vis (CH_2Cl_2 , λ_{max} , nm, (ϵ)): 669, 613, 540, 513, 413 1H NMR (400 MHz, $CDCl_3$): δ 9.17, 9.06, 8.65 (all s, 1H, meso-H), 7.26–7.51 (m, 4H, ArH), 5.92–5.97 (m, 1H, 3^1 -H), 5.53 (s, 2H, 13^1 -H), 4.69–4.77 and 4.56–4.62 (m, 2H, OCH_2Ar), 4.42–4.45 and 4.22 (m, 2H, 7-H + 8-H), 3.75 (overlapped, 5H, 2H of 17^1 - CH_2 , 3H of $COOCH_3$), 3.56 (s, 3H, 12 - CH_3), 3.42 (s, 3H, 2 - CH_3), 3.18 (s, 3H, 18 - CH_3), 2.88 (t, $J = 8.0$ Hz, 2H, 17^2 - CH_2), 2.51 (m, 1H, 8^1 -H), 2.14–2.17 (m, 4H, 8^1 -H + 3^1 - CH_3), 1.80/1.89 (d, $J = 8.0$ Hz, 3H, 7 - CH_3), 1.16 (t, 3H, 8^1 - CH_3), 0.25 (s, 9H, $Sn(CH_3)_3$). ^{13}C NMR ($CDCl_3$; 400MHz): δ 196.05, 173.14, 172.24, 172.13, 166.18, 154.37, 146.55, 145.26, 142.56, 142.46, 142.34, 139.84, 139.79, 139.35, 137.68, 137.62, 137.46, 136.35, 136.24, 135.72, 135.62, 135.53, 135.50, 135.26, 135.19, 133.51, 128.25, 128.19, 128.17, 128.04, 121.60, 115.50, 96.96, 96.92, 96.72, 96.66, 94.02, 93.86, 71.74, 71.39, 71.28, 71.23, 55.55, 55.46, 51.81, 48.94, 36.12, 30.36, 30.30, 24.43, 24.20, 23.33, 23.19, 22.44, 11.64, 11.24, 11.19, 11.15, 10.96, 10.92, -9.58, -9.59. MS (ESI): m/z 821.5 ($M + H^+$). HRMS (ESI): calcd. for $C_{44}H_{52}N_4O_4Sn$ 821.3089; found, 821.3078 (MH^+).

3-Deacetyl-3-(1'-*m*-trimethylstannylbenzyloxyethyl)-7,8-dihydrophyloerythrin (24)

The solution of (**23**) (9.0 mg, 0.01 mmol, 1.0 equiv) in dry THF were added hexamethydistannane (35.0 μ l) and bis(triphenylphosphine) palladium(II)dichloride (3.2 mg, 0.004 mmol, 0.4 equiv), and the reaction mixture was stirred at room temperature for overnight. The solvent was evaporated. The crude product was purified by preparative plates. 4% methanol/dichloro-methane was used as the eluting solvent to yield compound (**24**). Yield: 6.0 mg (75%), UV-visible, λ_{max} (CH_2Cl_2), nm (ϵ): 670, 613, 541, 513, 412 1H NMR ($(C_4D_8)O$; 400MHz): δ 9.23, 9.11/9.03, 8.70 (all s, 1H, meso-H), 7.48 (d, 1H, ArH, $J = 7.6$ Hz), 7.39–7.34 (m, 2H, ArH), 7.32–7.23 (m, 1H, ArH), 6.03–5.98 (m, 1H, 3^1 -H), 5.25 (s, 2H, 13^1 -H), 4.60–4.72 (m, 2H, OCH_2Ar), 4.42–4.45 and 4.17–4.18 (broad m,

2H, 7-H+ 8-H), 3.68 (broad m, 2H, 17²-CH₂), 3.47 (s, 3H, 12-CH₃), 3.40 (s, 3H, 2-CH₃), 3.15 (s, 3H, 18-CH₃), 2.83 (t, 2H, 171-CH₂, J= 8.0 Hz), 2.68 (broad m, 5H, 2H of 8¹-H + 3 H of 3¹-CH₃), 2.11/2.12 (distorted D, 3H, 7-CH₃), 1.18 (m, 3H, 81-CH₃), 0.20 & 0.18 (split s, 9H, Sn(CH₃)₃). MS(ESI): *m/z* 807.3 (M + H⁺). HRMS (ESI): calcd for C₄₃H₅₁N₄O₄Sn (MH): 807.2932, Found 807.2920.

General method for the preparation of ¹²⁴I-labeled photosensitizers 6, 26 and 27

The trimethyltin analog (**5**, **25** or **24** respectively) (60 µg) dissolved in 5% acetic acid in methanol (50 µl) was added to a tube containing dried Na¹²⁴I (60 ng). To this solution, 10 µl of N-chlorosuccinimide (1mg/mL in Methanol) was added. The reaction mixture was incubated at room temperature for 8 minutes. The reaction mixture was loaded on to a HPLC column (Symmetry C18 5µm, 4.6 × 150 mm). Methanol and water (88:12) was used as eluant. The flow rate was 1 mL/min. The UV detector was set at 254 nm. The labeled product was collected and the solution was evaporated under nitrogen gas flow at 80° C. The product was dissolved in 10% Ethanol/saline solution for *in-vivo* experiments. The radiochemical yield was 30% and the specific activity was greater than 1 Ci/µmol. The purity was ascertained by HPLC.

HPLC analyses of the final products

HPLC Analyses of compounds **4**, **12**, **23** were performed using a Waters Delta 600 System consisting of the 600 Controller, 600 Fluid Handling Unit and 996 Photodiode Array Detector equipped with a EMD LichroCart RP8, reverse phase C8 column, 5 micron particle size with dimensions of 4 × 250 mm. Compounds were eluted at a flow rate of 1.5 ml/min using a gradient program: Solvent A – Methanol, Solvent B – water; gradient profile: 0 minutes- 75% A, 25% B, linear grade to 90%A, 10% B from 0 to 10 minutes, maintained at this composition until 20 minutes, then linear grade to 100% A. The retention time and percent purity of each compound was as follows: **4**, 10.13 min; **12** 10.48 and **23**, 9.94 min. The component percentages are based on the area counts of the peaks from the 408 nm channel.

Cell cultures

A subclonal line of human basal cell carcinoma cells (Dicker et al, 2002, J. Invest Dermatol 118: 859–865) (provided by Dr. Tak-Wah Wang) were cultured in DMEM containing 10% fetal calf serum. PS uptake was determined in subconfluent cultures established in 24- or 6-well culture plates. The cells were washed twice with serum-free DMEM and cells and then incubated in DMEM containing the concentration of fetal calf serum and photosensitizers as indicated in the specific experiments. After incubation period ranging from 30 to 24 h, the cells were washed with serum-free DMEM. Cell-associated PS was visualized by fluorescent microscopy 640X on an inverted fluorescence microscope (Zeiss Axiovert 200) with Axio camera. Filter for HPPH fluorescence was Ex 410/40 nm and Em 675/750 nm. Internalization of PS was achieved by incubating PS-treated cells in serum-free or serum-containing medium for 4 to 24 h at 37°. PS amounts in cells were quantified by extraction in Solvable™ (PerkinElmer Life and Analytical Sciences) and measurement of fluorescence (Ex 415 nm; Em 667 nm) in a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA).

Photoreaction was performed by illuminating PS-treated cell cultures in serum-free medium at 37° (within a tissue culture incubator) with 665 nm light of an argon-pumped dye laser for 9 min to a total fluence of 3 J/cm². Depending upon the experimental design, cells were either extracted immediately after light treatment (identification of signaling reaction) or incubated at 37° for 24 h (evaluation of PDT-mediated cell kill).

Cell analyses

Survival was defined as the percent viable cells recovered after 24h PDT and incubation in full growth medium. Cells were released by trypsin treatment and trypan blue-excluding cells counted in a hemocytometer. For protein analysis, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer and subjected to protein analyses as described^{8,9}. Briefly, protein extracts (20 µg) were separated on 6% to 10% SDS-polyacrylamide gels. On all gels, reference protein markers for loading, molecular size detection and cross-comparison among gels were included. After separation, proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech). Nonspecific interactions were blocked by incubating the membranes with PBS-0.1% Tween 20–5% skim milk. Membranes were then reacted overnight at 4°C with one of the primary antibodies: STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA), p38 P-p38, and EGFR (Cell Signaling Technologies, Inc., Danver, MA). The immune complexes were visualized by reacting with peroxidase-coupled secondary antibodies and enhanced chemiluminescence detection (ECL) (Pierce Chemicals, Rockford IL). ECL images were recorded on X-ray films by various lengths of exposure to ensure recovery of signals within the linear range of digital scanning. The net pixel values of each band were integrated by using the ImageQuant TL program (Amersham Biosciences). The cross comparison of separate analyses and immunoblots relied on co-separated reference markers. STAT3 crosslinking was expressed by the percent conversion of monomeric STAT3 into the dimer form I of the STAT3 cross-linked complexes³¹.

To quantify uptake PS by Colon26 cells, the cells were seeded in 6-well plates with 0.33×10^6 cells per well in 2 ml of growth medium. The cells were incubated overnight at 37°C. Four concentrations (100, 200, 400, 800 nM) of the photosensitizers were added to triplicate wells and incubated at 37°C for 4 h. Triplicate wells without photosensitizers were included to determine the baseline autofluorescence. The cells were then harvested by trypsinization, neutralized with cold medium containing 2% FCS, transferred to 5ml flow cytometry tubes and centrifuged for 5 minutes at 500 g at 4°C to prevent efflux of the photosensitizers. The supernatant was removed, the cell pellet resuspended with 0.25ml cold PBS containing 2% FCS, placed on ice in the dark and immediately analyzed by flow cytometry. Data was acquired with Cell Quest on an LSRII flow cytometer (Beckton and Dickinson), using an excitation wavelength of 633 nm and detecting fluorescence emission at 730 ± 45 nm of 10,000 events and analyzed with FCS Express (Denovo software). Histograms were generated and data was expressed as the mean fluorescence of triplicate samples after subtracting autofluorescence.

The photosensitizing activity was determined in Colon26 cells using the colorimetric MTT assay. Colon26 cells were seeded in 96-well plates at a density of 3×10^3 cells/well in RPMI with 10% fetal calf serum. After overnight incubation, the PSs were added at the indicated concentrations and incubated at 37°C for 24h in the dark. Before light treatment, the medium was replaced with drug-free complete medium. Cells were then illuminated (0–4 J/cm²) with 665 nm for compounds **1**, **4** and **7**; 660 nm for compound **12** and 674 nm for compound **23** light from an argon-pumped dye laser at a fluence rate of 3.2 mW/cm². After PDT the cells were incubated for further 48h at 37°C in the dark. During the last 4h of incubation, 10 µL of a 4.0 mg/mL solution in PBS of 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) (Sigma, St. Louis, MO) was added to each well. After 4h, the MTT and medium were removed and 100 µL DMSO was added to solubilize the formazan crystals. Absorbances were read on a microtiter plate reader (BioTek Instruments, Inc., ELx800 Absorbance Microplate Reader) at 570 nm. The results were plotted as the survival rate of treated cells against different light doses for each compound tested. Each experiment was done with 4 replicate wells and repeated 3 times independently to get accurate photosensitizing efficacy.

Animal Models

The photosensitizers were evaluated in mice (BALB/c, 10 mice/group) bearing Colon26 tumors, implanted subcutaneously and were allowed to grow to an approximate diameter of 5–7 mm. All animal procedures were carried out in accordance to guidelines approved by the Institute Animal Use and Care Committee.

PET Imaging

Mice were imaged in the micropet FOCUS 120, a dedicated 3D small-animal PET scanner (Concorde Microsystems Inc.) at State University of New York at Buffalo (South Campus) under the Institutional Animal Care and Use Committee (IACUC) guidelines. All BALB/c mice ($n = 10$ for each compound) tumored with Colon 26 cells were injected via the tail vein with 52–122 μCi of **PS 1**, **6** and **27** respectively. After 24, 48 and 72h postinjection, the mice were anesthetized by inhalation of isoflurane/oxygen, placed head first, prone for imaging, and the acquisition time was set for 30 min. No efforts were made to block the radioiodine uptake by thyroid or stomach, and surprisingly, in only a few cases was a little bit of radioiodine uptake observed in the thyroid, and that too was cleared by 72h. Some tumored mice were also imaged using ^{18}F -FDG radiotracer 24 h prior to radioactive ^{124}I imaging. These mice were administered 150–200 μCi of ^{18}F -FDG via the tail vein and were scanned for 20 min in the fashion described above, beginning 45 min after FDG injection. For biodistribution studies The mice were injected with 15–50 μCi of compounds **PS 1**, **6** and **27** respectively via tail vein, and three mice each at 24, 48 and 72h time interval were sacrificed, and body organs (tumor, heart, liver, spleen, kidney, lung, muscle, etc.) were removed immediately. After weighing, the amount of radioactivity in the tumor (300–400 mg), body organs, and blood was measured by a γ -well counter. Radioactivity uptake was calculated as the percentage of the injected dose per gram of the tissue (%ID/g). Statistical analyses and data (%ID/g vs time point) were plotted using GraphPad Prism 5.

In vivo PDT efficacy and the assessment of response

Before PDT treatment, Nair was used to remove all hair or surrounding the tumor site. When tumor reached 4–5 mm in diameter, the mice were injected with compounds **1**, **4**, **7**, **12** and **23** at a dose of 1 $\mu\text{mol/kg}$. At 24 h post-injection, the mice were restrained in plastic plexiglass holders without anesthesia, treated with a laser light (665 nm for compounds **1**, **4** and **7**; 660 nm for compound **12** and 674 for compound **23**, 128J/cm², 14 mW/cm²). The power was monitored during the entire treatment. Post-PDT, the mice were observed daily for tumor re-growth or tumor cure. Visible tumors were measured using two orthogonal measurements L and W (perpendicular to L) and the volumes were calculated using the Microsoft Excel formula $V = L \cdot W^2/2$ and recorded. Mice were considered cured if there was no palpable tumor by day 60–90. Once the tumor reached 400 mm³ the mice were euthanized due to tumor burden as per RPCI IACUC rules.

In vivo optical fluorescence imaging

In vivo fluorescence imaging was accomplished by using a Illumination Dual Light System (Lighttools Research, Encinitas, CA), designed for small animal studies. True-Color fluorescence images were obtained using dielectric long-pass filters (Chroma Tech) and a digital color camera (Optronics, Magnafire SP, Olympus America). Wavelength- resolved spectral imaging was carried out by using a Nuance multispectral imaging system (CRI, Inc., Woburn, MA) comprising an optical head that includes Varispec liquid crystal tunable filters (LCTFs, with a bandwidth of 20 nm and a scanning wavelength range of 400–720 nm), an optical coupler, and a high-resolution scientific-grade CCD camera, along with image acquisition and analysis software. The tunable filter was automatically stepped in 10-nm increments from 550 to 720 nm while the camera captured images at each wavelength

with a constant exposure. The 27 resulting TIFF images were loaded into a single data structure in memory, forming a spectral stack with a spectrum at every pixel. Autofluorescence spectra and PS fluorescence spectra were manually selected from the spectral image using the computer mouse to select appropriate regions. Spectral unmixing algorithms (available from CRI, Inc.) were applied to create the unmixed images of pure autofluorescence and pure PS fluorescence signal, as shown in Figure 11 and 12. All the images were re analyzed using Image J software to show false color fluorescence reflectance (FRI) images

Following an IACUC-approved protocol, the BALB/c mice were subcutaneously injected with 1×10^6 Colon 26 cells in 50 μ L of serum free complete RPMI-1640 media (into the right posterior axilla), and tumors were grown until they reached 4–5 mm in diameter. The day before PS injection, all hairs were removed from the inoculation site and the mice were imaged under anesthesia (IP injection of ketamine and xylazine mixture). In a dark box, illumination was provided by fiber optic lighting at 540/40x nm (green color), and a long-pass filter was used to reject scattering and to pass Stoke-shifted PS fluorescence at 667 nm and 676nm respectively. Under similar conditions, mice were imaged at 24, 48, and 72 h post-injection of the PS's 4, 12 and 23 (at therapeutic dose 1 μ mol/kg).

After whole-body imaging, the tumor was surgically removed from the original site (axilla) and placed on a shaved region of skin away from the original site as a means to image the skin flap (minus the tumor). Imaging was repeated as described before.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PS	Photosensitizer
PDT	Photodynamic Therapy
PET	Positron Emission Tomography
STAT3	Signal transducer and activation of transcription 3
ROI	Region of Interest
NMR	Nuclear magnetic resonance
HRMS	High resolution mass spectrometry
FDG	fluorodeoxyglucose
SAR	structure/function relationship

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Figure 1.

Various approaches of structural modification of iodinated PS: (**X**). modification of methyl ester functionality; (**Y**) effect of the position of (1'-m-iodobenzoyloxyethyl) substituent and (**Z**) effect of ring-B vs. ring-D reduced PSs in biological efficacy.

Figure 2.
¹H NMR spectra of ring-D vs. ring-B reduced chlorins **1** and **20** respectively

Figure 3. (A) Electronic absorption spectra of isomers **4**, **12** and **23** at equimolar concentration (6.5 μM) in 17% bovine calf serum (BCS). (B) Rate of photobleaching of bacteriochlorins **4**, **12** and **23** in 17% BCS. The photosensitizers at equimolar concentration (6.5 μM) were irradiated with light (light dose: 75 mW/cm^2) and the spectra were taken at variable time points. The normalized long wavelength absorption values were plotted with time showing a comparative rate of photobleaching of the photosensitizers by reactive oxygen species (mainly singlet oxygen) produced *in-situ*.

Figure 4.

PS-specific uptake, photoreaction and post-PDT survival. Replicate 24-well culture plates of BCC1 cells were incubated for 30 min in serum-free DMEM containing 200 nM (A) or for 2 h with serum-free DMEM containing 2-fold serially diluted preparations (B&C) of the PSs indicated at the top. The cells were washed free of PS and imaged at 640X magnification under phase contrast and HPPH fluorescence (A). The plates treated with the serially diluted PSs were illuminated for 9 min with 665 nm light yielding a fluence of 3 J/cm². The cell monolayers in one cultures plate were immediately extracted. Equal aliquots of the extracts were analyzed on western blots for the level of immune detectable STAT3, EGFR and phosphorylated and total p38 MAPK (B). The oxidative crosslinking of STAT3 was determined by densitometric scanning of the bands representing monomeric and homodimeric complex I. The percent conversion of STAT3 to the dimeric form is indicated at the top of panel “B”. The second culture plate was changed to DMEM containing 10% fetal calf serum immediately after light treatment and cultured for an additional 24 h period. The number of surviving, trypan blue-excluding cells were counted in a hemocytometer and expressed relative to the number of cells in the control (C).

Figure 5.

Specific patterns of uptake and subcellular distribution of the PSs. **A**, BCC1 cells were incubated for 30 min in serum-free DMEM containing 3.2 μM PS 1 or 0.2 μM PS 4. The cells were washed and images of the cell monolayers were recorded at 640X magnification under phase contrast or HPPH fluorescence. The cultures were incubated in PS- and serum-free DMEM for an additional 4 h period. The cells were imaged again under identical condition as before. **B**, BCC1 cells were incubated for 24 h in either serum-free DMEM or DMEM with 10% fetal calf serum, both containing 3.2 μM PS 7. The cells were washed free of PS and imaged a 640X magnification as the cells in A.

Figure 6.

(**A**) *In vitro* photosensitizing efficacy of PS **1**, **4** and **7**. (**B**) PS **4**, **12** and **23**. Colon26 cells were incubated with 0.3 μM (**A**) and 0.0625 μM (**B**) of PSs respectively in growth medium for 24 h. The cells were treated at the peak absorption wavelength with variable light doses and cultured for additional 48h post-treatment. (**C**) PS uptake of compounds **4**, **12** and **23** in Colon26 cells 24 h post incubation was determined by flow cytometry.

Figure 7.

(A) Comparative *in vivo* photosensitizing efficacy of PS **1**, PS **4**, and PS **7** and (B) PS **4**, **12** and **23** in BALB/c mice bearing Colon26 tumors (10 mice/group). The mice were injected with photosensitizers (1.00 $\mu\text{mol/kg}$) and the tumors were exposed to laser light at 665 nm for compound **1**, **4**, and **7**; 674 nm for compound **23** and 660 nm for compound **12** (light dose: 128J/cm², 14 mW/cm²) at 24h post injection. At day 60, photosensitizers **4** and **23** gave 70% and 60% tumor response respectively (7/10 and 6/10 mice were tumor free), whereas compound **12** showed limited efficacy.

Figure 8.

Whole-body PET images of a BALB/c mouse bearing Colon26 tumor with ¹²⁴I- PS **6** (A) taken at 24, 48 and 72 h post-injection. The maximum uptake of the PS was observed at 24h postinjection. However, the best contrast was obtained at 72h for compound PS **6** postinjection.

Figure 9. Comparative biodistribution of ^{124}I following injection of PS **3** (A) and PS **6** (B) (100 μCi) in BALB/c mice bearing Colon 26 tumors (3 mice/time point) at 24, 48 and 72 h post-injection. Replacing the methyl ester functionality in PS **3** with carboxylic acid functionality (PS **6**) did not make much difference in uptake of the PS in tumor and other organs, except the spleen, where the uptake was significantly reduced.

Figure 10. Biodistribution of ^{18}F -FDG in BALB/c mice (3 mice/group) bearing Colon26 tumors at 2 h post-injection. The tumor uptake ratio is also presented in tabulated form.

Figure 11.

Whole body fluorescence reflectance images (FRI) of representative BALB/c mice implanted with C-26 tumors on the shoulder with compounds **4**, **12** and **23** respectively at variable time points with a therapeutic dose ($1 \mu\text{mol kg}^{-1}$). (A): 24 h p.i.; (B): 48 h p.i. (C) 72 h p.i. (D) Average Fluorescent Intensity (AFU) of 3 mice $\pm$ SD of a ROI (20 mm diameter) over the tumor in AU, arbitrary units.

Figure 12.

Whole body fluorescence reflectance images (FRI) of representative BALB/c mice implanted with Colon26 tumors on the shoulder with compounds 4 and 23 at 24 h post-injection (dose: $1 \mu\text{mol kg}^{-1}$). $\lambda_{\text{ex}} = 665$ and 674 nm for PS 4 and 23 respectively; $\lambda_{\text{em}} = 710$ nm). (A) PS 4; (B) PS 23; tumor was removed and skin was flapped back to expose the residual tumor. The two PSs 4 and 23 exhibiting the highest *in vivo* phototoxicity were injected intravenously into mice bearing colon 26 tumors to confirm that the recorded fluorescence coincided with the actual tumor mass. After 24 h, the tumors were surgically removed from the primary tumor site and imaged tumor was imaged (Figure 13). The results clearly indicate that most of the fluorescence was confined to the tumor with only low fluorescence associated with the skin section.

Scheme 1.
Modification of methyl ester functionality.

Scheme 2.

Synthesis of 8-(1'-*m*-iodobezilyloxyethyl)pyropheophorbide **11** and **12** and the 8-Vinyl analog **16**.

Scheme 3.

Synthetic approaches for the preparation of ring-B reduced 3-(1'-*m*-iodobenzoyloxyethyl) chlorins from bacteriopheophorbide-*a*.