Published in final edited form as:

Bioconjug Chem. 2007 ; 18(2): 379–388. doi:10.1021/bc0602578.

Peptide-based pharmacomodulation of a cancer-targeted optical imaging and photodynamic therapy agent

Klara Stefflova[†], Hui Li[‡], Juan Chen^{‡,¶}, and Gang Zheng^{‡,¶,§,*}

[†]Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

[‡]Department of Radiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

[¶]Ontario Cancer Institute, University of Toronto, MaRS Center, TMDT 5-363, 101 College St., Toronto, Ontario M5G 1L7, Canada

[§]Joey and Toby Tanenbaum/Brazilian Ball Chair in Prostate Cancer Research

Abstract

We designed and synthesized a folate receptor-targeted, water soluble, and pharmacomodulated photodynamic therapy (PDT) agent that selectively detects and destroys the targeted cancer cells while sparing normal tissue. This was achieved by minimizing the normal organ uptake (e.g., liver and spleen) and by discriminating between tumors with different levels of folate receptor (FR) expression. This construct (Pyro-peptide-Folate, **PPF**) is comprised of three components: 1) Pyropheophorbide *a* (Pyro) as an imaging and therapeutic agent, 2) peptide sequence as a stable linker and modulator improving the delivery efficiency, and 3) Folate as a homing molecule targeting FR-expressing cancer cells. We observed an enhanced accumulation of **PPF** in KB cancer cells (FR+) compared to HT 1080 cancer cells (FR-), resulting in a more effective post-PDT killing of KB cells over HT 1080 or normal CHO cells. The accumulation of **PPF** in KB cells can be up to 70% inhibited by an excess of free folic acid. The effect of Folate on preferential accumulation of **PPF** in KB tumors (KB vs. HT 1080 tumors 2.5:1) was also confirmed *in vivo*. In contrast to that, no significant difference between the KB and HT 1080 tumor was observed in case of the untargeted probe (Pyro-peptide, **PP**), eliminating the potential influence of Pyro's own nonspecific affinity to cancer cells. More importantly, we found that incorporating a short peptide sequence considerably improved the delivery efficiency of the probe – a process we attributed to a possible peptide-based pharmacomodulation – as was demonstrated by a 50-fold reduction in **PPF** accumulation in liver and spleen when compared to a peptide-lacking probe (Pyro-K-Folate, **PKF**). This approach could potentially be generalized to improve the delivery efficiency of other targeted molecular imaging and photodynamic therapy agents.

Keywords

In vivo imaging; photodynamic therapy; peptide-based pharmacomodulation; folate receptor; delivery efficiency

INTRODUCTION

When a porphyrin-like molecule is activated by light, it relaxes to its ground state in three ways – through nonradiative decay, by emitting a photon, or transferring the energy, producing

*To whom correspondence should be addressed: E-mail: gang.zheng@uhnres.utoronto.ca. Phone: 1-416-581-7666. Fax: 1-416-581-7667.

reactive oxygen species, mainly singlet oxygen (1). The detectable outcomes are fluorescence (2) and phototoxicity (3), making the porphyrin-based photosensitizer (PS)^a a perfect candidate for image-guided therapy.

Curing cancer is a complicated goal and the success of treatment is often short-term owing to the difficulty in clearing-out all the cancer cells. Being able to clearly identify the cancer cells shortly before or during the treatment would most likely increase the success of the therapy, making imaging and therapy a beneficial union (4-7). We have utilized the natural connection of near-infrared (NIR) fluorescence imaging – a sensitive and accessible means of *in vivo* cancer detection (2,8,9) – with photodynamic therapy (PDT) – a promising cancer therapy using a laser to excite a tumor-associated photosensitizer that produces short lived cytotoxic singlet oxygen (10,11). Although many target-specific NIR imaging and PDT agents are being developed, the common limit remains: these agents are mostly lost in organs involved in drug clearance, generating an unwanted toxicity and elevated background (12). Here we propose a novel construct designed for improving the delivery efficiency of NIR imaging and PDT agents to the desired cancer cell targets with reduced normal tissue retention. It is composed of three functional modules (Scheme 1). The first is a fluorescent photosensitizer Porphyrinophorbide *a* (Pyro) for NIR imaging (with a long-wavelength absorption at 665 nm and emission at 675 nm and 720 nm) and PDT (over 50% singlet oxygen yield) (13,14). This semisynthetic photosensitizer, obtained by three steps from *Spirulina* algae, has minimal dark toxicity and its derivative Photochlor® is in phase I/II clinical trial (15).

The second component is a folate moiety, serving as a tumor homing molecule that guides the photosensitizer into folate receptor (FR)-overexpressing cancer cells (16,17) via receptor-mediated endocytosis (18). FRs are mainly overexpressed on ovary, breast, colon, lung, nose, prostate, and brain cancer cells and activated macrophages (19) but have limited expression on normal cells (e.g. kidney, intestine, lung) with restricted accessibility for blood-circulating drugs (20).

The third component is a short peptide sequence GDEVDGSGK inserted between the photosensitizer and folate. It serves multiple purposes: A) it is a stable and hydrophilic linker that prevents the separation of folate and Pyro and enhances water solubility, B) it separates the Pyro from the Folate to avoid the hindrance of FR-targeting (20), C) it serves as a pharmacomodulator for better delivery efficiency and decreased normal tissue toxicity, and D) it is possible to exchange it with other peptide sequences for targeting subcellular organelles (21-23).

There have been many attempts to enhance the photosensitizer's efficacy by targeting "cancer fingerprints" (24) through association with various vehicles (25,26) like proteins (e.g. BSA targeting scavenger receptors on macrophages (27), transferrin (28) or LDL (29)), tumor-selective monoclonal antibodies (30), saccharides (31), aptamers (32) or other small molecule ligands (e.g. short peptides or peptidomimetics) (22,33-35). By attaching Pyro to Folate, an easy-to-conjugate, small, soluble, and non-immunogenic tumor homing molecule (20) targeting cancer cells overexpressing FR (9,36,37), we expected to enhance the cancer-specificity of Pyro. To potentiate this specificity, we have inserted a short peptide sequence to serve as a spacer, a solubilizer, and a pharmacomodulator (38). As a spacer, it makes the Folate more accessible to FR and, being small and hydrophilic, it decreases its retention in the excretion organs making it more suitable for *in vivo* applications.

^aAbbreviations: BSA, bovine serum albumin; DCM, Dichloromethane; DIPEA, Diisopropylethylamine; DMSO, Dimethylsulfoxide; EDTA, Ethylenediaminetetraacetic acid; MALDI-ToF, Matrix Assisted Laser Desorption/Ionization - Time of Flight mass spectrometry; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NHS, N-Hydroxysuccinimide; NIR, near-infrared; NMP, 1-Methyl-2-pyrrolidinone; PBS, Phosphate-buffered saline; PDT, photodynamic therapy; PS, photosensitizer; TEAA, Triethyl amine and Acetic acid; TFA, Trifluoroacetic acid; TIS, Triisopropylsilane.

In this paper, we described the synthesis and characterization of such agent, Pyro-GDEVDSGSK-Folate (**PPF**, Figure 1). We have demonstrated, both *in vitro* and *in vivo*, that all three functional modules act in synergy and thus significantly improve the delivery efficiency, reducing the potential toxicity of the probe.

MATERIALS AND METHODS

The coupling reagents HOBt (1-Hydroxybenzotriazole) and HBTU (*O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate) were purchased from ACROS and Fluka respectively. KB cells (human epidermal cancer cells; folate receptor positive), HT 1080 cells (human fibrosarcoma cells; folate receptor negative), and CHO (chinese hamster ovary cells; folate receptor negative) were purchased from ATCC. "Complete MEM" (for HT 1080 and KB cells) consists of 85% Minimum Essential Medium (MEM), 10% Fetal Bovine Serum, 2% of 1.5g/L Sodium Bicarbonate, 1% of 200mM L-glutamine, 1% of 0.1mM Non-essential Amino Acids, and 1% of 1mM Sodium Pyruvate and "MEM containing 0.8% BSA" consists of MEM supplemented with 8g/L of Bovine Serum Albumin. "Complete F12K" (for CHO cells) consists of 85% Ham's F12K medium, (F12K), 10% Fetal Bovine Serum, 2% of 1.5g/L Sodium Bicarbonate and 1% of 200mM L-glutamine and "F12K containing 0.8% BSA" consists of F12K supplemented with 8g/L of Bovine Serum Albumin. Compounds **3** (**PP**), **4** (**PPF**), and Pyro-K-Folate (**PKF**) prepared for *in vivo* experiments were dissolved in 1μL DMSO and 20μL PBS, filtered through a 0.22μm filter and further diluted with saline to the 120-150μL volume. For the *in vitro* experiments, compound **4** (**PPF**) and Pyro-K-Folate (**PKF**) was first dissolved in DMSO (no more than 0.5% of total volume) then diluted with 0.1% Tween-80 in DNA-water, filtered through a 0.22μm filter and further diluted with MEM containing 0.8% BSA (HT 1080 and KB cells) or F12K containing 0.8% BSA (CHO cells).

All compounds were purified by HPLC (Waters 600 Controller with a 2996 Photodiode Array Detector). The eluents were A=0.1M TEAA (pH 7) and B=acetonitrile and the method used for all injections was 90% of A and 10% of B to 100% of B in 45 minutes with flow 1.5 mL/min on a Zorbax 300SB-C3 column. MALDI-ToF was measured on an Applied Biosystems Voyager DE Mass Spectrometer and fluorescence was measured on a Perkin Elmer LS50B Luminescence Spectrometer. We acquired confocal images using a Leica TCS SP2 Confocal Microscope and Xenogen images at the Bioluminescence Molecular Imaging Core facility at UPenn on an IVIS Xenogen Imager. The laser for PDT treatment was tuned to 670nm with a fluence rate of 20mW/cm². Mice were euthanized according to guidelines established by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Synthesis of PPF

The peptide *Fmoc-GD(O-2-PhiPr)E(O-2-PhiPr)VD(O-2-PhiPr)GS(Trt)GK(Mtt)* (**1**) was synthesized by manual SPPS using commercially available Fmoc amine protected amino acids as building blocks and Sieber resin (cleavable by 2 % TFA) as a solid phase, all purchased from Novabiochem®. After the last Fmoc group cleavage, the resin was washed with NMP and Pyro-acid was coupled to the N-terminal glycine to give *Pyro-GD(O-2-PhiPr)E(O-2-PhiPr)VD(O-2-PhiPr)GS(Trt)GK(Mtt)-Sieber resin* (**2**). The molar ratio of Pyro/HOBt/HBTU to the peptide (**1**) was 3/3/3:1 and the reaction was done in a shake flask with a 12 hour coupling time using dry NMP as a solvent. The resin was then washed with NMP, capped by 0.3M Acetylimidazol in NMP for 15 minutes and washed again by an excess of NMP, DCM and dry methanol, transferred from the shake flask and dried. Compound **2** (37.7mg, 11.5μmol) was cleaved from the resin and deprotected in one step by 2% TFA / 5% TIS / DCM for 1 hour to yield *Pyro-GDEVDSGSK(NH₂)* (**3**) with the ε-NH₂ group of the C-terminal lysine exposed. The compound was precipitated from the cleavage solution by dry ether and prepurified by a few cycles of ether precipitation-DMSO dissolving. Compound **3** (10.9mg, 6.8μmol) was dried

under high vacuum and, without further purification, used in the next reaction. For *in vivo* experiments, compound **3** (**PP**) was purified by HPLC. The structure was confirmed by MALDI-ToF (Supporting information, Figure 1A, mass calculated: 1377.64, found: 1377.95). Crude **PP** (9.3mg, 5.8 μ mol) was dissolved in 100 μ l of dry 1% DIPEA / DMSO and reacted for 2 hours with Folate-NHS (4.7mg, 6 μ mol) dissolved in 100 μ l of dry DMSO to give Pyro-GDEVDSGK-Folate (**4**, **PPF**). The reaction was concentrated by precipitation with ether and directly separated by HPLC. Purified compound **4** (11.7mg, 5.5 μ mol) was dried under high vacuum and stored at -20 °C. The purity was checked by HPLC and MALDI-ToF (mass calculated: 1800.76, found: 1799.79).

Synthesis of PKF

The Fmoc-Lys was coupled to the Sieber resin after activation with HOBt/HBTU in a ratio 1/1/1:3 (Fmoc-Lys/HOBt/HBTU : Sieber resin) in NMP. After the Fmoc group cleavage, the resin was washed with NMP and Pyro-acid was coupled to the N-terminus to give Pyro-K (Mtt)-Sieber resin. The molar ratio of Pyro/HOBt/HBTU to the NH₂-K(Mtt)-Sieber resin was 3/3/3:1 and the reaction was done in a shake flask with a 12 hour coupling time using dry NMP as a solvent. The resin was then washed with NMP, capped by 0.3M Acetylimidazol in NMP for 15 minutes and washed again by an excess of NMP, DCM and dry methanol, transferred from the shake flask and dried. Pyro-K(Mtt)-Sieber resin was cleaved from the resin and Mtt group deprotected in one step by 2% TFA / 5% TIS / DCM for 1 hour to yield Pyro-K- ϵ NH₂. The compound was precipitated from the cleavage solution by dry ether and dried under high vacuum. This crude compound (21mg, 31.7 μ mol) was dissolved in 300 μ l of dry 1% DIPEA / DMSO and reacted for 2 hours with Folate-NHS (31.3mg, 40 μ mol) dissolved in 200 μ l of dry DMSO to give Pyro-K-Folate (**PKF**). The reaction was concentrated and directly separated by HPLC. Purified **PKF** (29.8mg, 27.5 μ mol) was dried and the purity was checked by HPLC and MALDI-ToF (Supporting information, Figure 1B, mass calculated: 1084.50, found: 1084.18).

Confocal microscopy

KB and HT 1080 cells were grown in 4-well Lab-Tek chamber slides (Naperville, Illinois) at a density of 50,000 cells/well and grown for 24 hours in complete MEM, then rinsed with HBSS (Hank's balanced salt solution) and incubated with 50 μ M **PPF** in 300 μ l of MEM containing 0.8% BSA at 37°C for 5 hours. Cells were repeatedly rinsed with HBSS after the incubation and fixed by 1% formaldehyde in PBS for 20 minutes prior to scanning with confocal microscopy. The settings for confocal microscopy were as follows: 640-800 nm detector slit for detection of Pyro fluorescence, with zoom=2, expander=3, resolution 512 $\times$ 512, 633nm excitation wavelength, 40 $\times$ objective, 100% power, 851.1 gain, and -28.5 offset.

Flow cytometry experiments

KB, HT 1080, and CHO cells were seeded in T25 flasks (each flask 10⁶ cells) and grown for 1 day in complete MEM (KB cells and HT 1080 cells) or complete F12K (CHO cells) medium. The cells were incubated for 12 hours with 5 μ M **PPF** or **PKF** in MEM containing 0.8% BSA (KB and HT 1080 cells) or F12K containing 0.8% BSA (CHO cells) 1.3mL/flask alone or with 5mM folic acid added, and at the end of incubation the medium was aspirated and cells washed 3 times with 2mL of PBS. The cells were detached by 0.25% Trypsin-EDTA, fixed by 1% formaldehyde (methanol free) in PBS, resuspended in 1 mL of PBS and immediately analyzed on a BD LSRII machine at the Flow Cytometry Laboratory, Abramson Cancer Center at the University of Pennsylvania focusing on Pyro fluorescence (exc. 633 nm, em. 695 nm). The gain was constant for all three samples and set so that the control cells had a fluorescent intensity between 1-100 AU.

Cell viability (MTT) assay

KB, HT 1080, and CHO cells were seeded in clear 96-well plates at a density of 50,000 cells/well in 250 μ L of complete MEM (KB and HT 1080 cells) or complete F12K (CHO cells) and grown for 24 hours at 37°C. Cells were subsequently rinsed with HBSS and incubated with no or 0.5, 5 or 15 μ M **PPF** in MEM containing 0.8% BSA (KB and HT 1080 cells) or F12K containing 0.8% BSA (CHO cells) for 24 hours. Cells were then rinsed with HBSS, 100 μ L/well of complete MEM was added, and the cells were either kept in the dark or treated with different light doses (5 or 10 J/cm²) by a 670nm laser with 20mW/cm² fluence rate. After incubation for 24 hours at 37°C, the cells were incubated for 2 hours with a 0.5 mg/mL solution of MTT in complete MEM/complete F12K, that was disposed afterwards and replaced with 100 μ L of equal parts of DMSO and 70% isopropanol in 0.1M HCl. Absorbance at 570nm was measured. Data shown are based on 3 different experiments and the results were expressed as mean $\pm$ standard error.

In vivo imaging

Nude mice were inoculated subcutaneously with 10⁷ KB cells above the right leg and 10⁷ HT 1080 cells above the left leg and the tumors were grown for about 7 days. First, three mice were initially scanned with a Xenogen IVIS imager with a Cy 5.5 filter (λ_{exc} =615-665nm, λ_{em} =695-770nm) and then intravenously injected with 50nmol (left), 100nmol (middle), and 150nmol (right mouse) of **PPF** 60-180 μ L/mouse into the tail vein. The mice were imaged 5 minutes, 6 hours, and 24 hours after injection. In order to compare the biodistribution of **PP** and **PPF**, two double tumor mice were intravenously injected with 30nmol of **PPF** or 30nmol of **PP** and imaged. These mice were sacrificed 26-30 hours after the injection and the organs (KB tumor, HT 1080 tumor, muscle, heart, adrenal, kidney, spleen, and liver) were imaged directly after dissection and used for biodistribution. In order to compare the biodistribution of **PP**, **PPF** and **PKF**, the first mouse was injected with 30nmol of **PPF**, the second with 30nmol of **PP**, and the third with 15nmol of **PKF** in two different experiments. The Xenogen images were obtained with the same settings (small binning, 2s exposure time) and were compared using max=20,000, min=3,000 for **PPF** and **PP** and max=10,000, min=1,500 for **PKF**. The mice were sacrificed 26-30 hours after *iv*, the organs were scanned by Xenogen and used for biodistribution.

Semi-quantitative *ex vivo* organ biodistribution

The mice were euthanized 26-30 hours after drug injection and the dissected organs were washed in PBS and scanned by Xenogen in a clear 24-well plate. The organs were weighed and disintegrated in DMSO : MeOH (2 : 3). After settling, the fluorescence was measured (excitation 650nm, emission 670nm) and divided by weight for each organ. This ratio of fluorescence vs. weight was normalized with respect to muscle and these semi-quantitative values plotted in graphs comparing either **PP** and **PPF** (these data are based on three different experiments and the results expressed as mean $\pm$ standard error) or **PP**, **PPF** and **PKF**. Statistically significant differences between KB tumor and other organs of **PPF** mouse are indicated with an asterisk ($P < 0.05$).

RESULTS AND DISCUSSION

Synthesis and characterization of Pyro-GDEVDSGK-Folate (**PPF**)

First, Fmoc-GDEVDSGK was synthesized according the standard solid phase peptide synthesis (SPPS) protocol using a Sieber resin as a solid phase (**1**). The photosensitizer Pyro acid was then coupled to the N-terminus of the immobilized peptide (Figure 1A) to give Pyro-GDEVDSGK-Sieber resin (**2**), which was cleaved from the solid phase and deprotected with 2% TFA. The Folate-NHS was then coupled in a solution reaction to the ϵ -NH₂ group of C-

terminal lysine of Pyro-GDEVDSGSGK (**PP**, **3**) and the product Pyro-GDEVDSGSGK-(Folate) (**PPF**, **4**) was characterized by MALDI-ToF (mass calculated: 1800.76, found: 1799.79, Figure 1B) after purification by HPLC (Figure 1C, absorbance at 410nm).

Accumulation of Pyro-GDEVDSGSGK-Folate (**PPF**) inside FR positive and negative cells monitored by confocal microscopy and flow cytometry

To confirm the preferential uptake of **PPF** by folate receptor overexpressing tumor cells, KB cells (human epidermal cancer cells; folate receptor positive, FR+) and HT 1080 cells (human fibrosarcoma cells; folate receptor negative, FR-) were incubated with 50 μ M **PPF** for 5 hours and the fluorescence of fixed cells was then monitored by confocal microscopy (Figure 2). By comparing Figure 2B and 2D it is clear that **PPF** accumulates more in KB cells (FR+) (Figure 2B) than in HT 1080 cells (FR-) (Figure 2D).

In order to establish the FR delivery pathway as the main uptake mechanism for **PPF**, the inhibition of **PPF** uptake was monitored by flow cytometry using an excess of free folic acid in KB, HT 1080 and CHO (chinese hamster ovary) cells. As expected, there is a minimal influence of folic acid on the uptake of **PPF** into HT 1080 and CHO cells (both FR-) in contrast to KB cells, where the uptake was inhibited up to 70% (Figure 3). These results suggest that: A) the main delivery pathway for **PPF** into the FR+ cells is *via* folate receptor-mediated endocytosis, B) **PPF**'s uptake can be inhibited up to 70% by folic acid in the case of KB cells (FR+), and C) the remaining uptake of **PPF** to KB cells and the lower uptake of **PPF** to the HT 1080 and CHO cells can be attributed to the nonspecific tumor affinity of the Pyro moiety.

Selective PDT-induced cell killing as a result of FR-targeting

To compare the post-PDT viability reduction for cell lines with different levels of FR expression, KB tumor cells (FR+), HT 1080 tumor cells (FR-) and normal CHO cells (FR-) were incubated with 0.5, 5 and 15 μ M **PPF** for 24 hours and were then either kept in the dark or treated by PDT with 5 or 10 J/cm² light doses using a 670nm tunable laser with a fluence rate of 20mW/cm². The MTT study (Figure 4) shows that the viability of KB cells drops below 10% when incubated with 0.5 μ M **PPF** and treated with 5 J/cm² light dose. Achieving the same cell viability reduction in CHO cells requires both a 10-fold concentration (5 μ M) in combination with a 2-fold light dose (10 J/cm²). Interestingly, using the combination of highest drug dose (15 μ M) and light dose (10 J/cm²) does not effectively kill the HT 1080 cells, suggesting an even lower accumulation of **PPF** or a possible PDT-resistance of the HT1080 cells. This study further supports the FR-mediated PDT activity of **PPF** towards FR+ cancer cells.

In vivo discrimination between KB (FR+) and HT 1080 (FR-) tumors

To validate the FR-targeting specificity of **PPF** *in vivo*, nude mice bearing an HT 1080 tumor (FR-) on the left flank and a KB tumor (FR+) on the right flank were used for *in vivo* imaging studies. Three double tumor mice were intravenously injected with 50, 100, and 150nmol of **PPF** and scanned by Xenogen imager (Figure 5, using a Cy 5.5 filter) before the drug injection (prescan, Figure 5A) and after the drug injection at three different time points (5 min, 6 hrs and 24 hrs). Five minutes after injection (Figure 5B), **PPF** is distributed rapidly throughout the body, carried by the blood stream. At 6 hrs post-injection (Figure 5C) KB tumor accumulates significantly more **PPF** than HT 1080 and accumulation in normal organs is considerably reduced. At 24 hrs post-injection (Figure 5D), the uptake of **PPF** in normal organs as well as the HT1080 (FR-) tumor for "50nmol" and "100nmol" mouse drops almost to the prescan level in contrast to a high fluorescent signal that remains in the KB tumor. These *in vivo* imaging studies clearly show that: A) **PPF** accumulates preferentially in KB tumor with respect to HT1080 tumor and other normal organs, B) it is clear from the saturated image of

the “150nmol” mouse (Figure 5D) that **PPF** also accumulates in HT 1080 tumor, and C) **PPF** is not toxic at the 150nmol drug dose and is cleared quickly out of normal tissues.

The role of folate and peptide modules in targeted delivery of PPF

In order to examine the impact of each module on delivery efficiency and target specificity, two analogues of **PPF** were synthesized. One is Pyro-GDEVDSGSK (**PP**) that lacks the folate moiety, the other is Pyro-K-Folate (**PKF**) with one lysine residue replacing the short peptide sequence (Figure 6).

A) The role of folate attachment for target-specific delivery of PPF *in vivo*

Since Pyro itself can serve as a delivery molecule (albeit with poor specificity), the *in vivo* imaging and biodistribution of the non-targeting Pyro-GDEVDSGSK (**PP**) can help to assess the true influence of folate as a tumor homing molecule. Thus, two double tumor-bearing mice in each experiment were injected with 30nmol of **PPF** or with 30nmol of **PP** (n=3). The image in Figure 7 (upper left) shows that around 6.5 hrs after injection of **PPF** (left) and **PP** (right), **PPF** accumulates significantly more in KB tumors than in HT1080 tumors, whereas **PP** accumulates comparably in both KB and HT 1080 tumors (see also Figure 8). The animals were sacrificed 26-30 hours after injection and the dissected organs were first imaged (Figure 7, lower left) and then homogenized. The images of the organs showed stronger fluorescence in the KB tumor from the **PPF** mouse, a weaker signal coming mainly from kidneys and livers of both the **PPF** and the **PP** mouse and almost no signal from other organs.

The uptake of **PPF** and **PP** was determined by extracting the homogenized organs with DMSO/MeOH, measuring the fluorescence at 670nm and correcting the signal for weight (Figure 7, right). By examining the biodistribution of **PPF** after normalizing with respect to muscle, the highest fluorescence signal was found in the KB tumor. This signal is only comparable with the signal from kidney, which is known as the major clearance pathway for folate bioconjugates. Moreover, the fluorescence signals in HT 1080 and other normal organs (adrenal, liver, heart, and spleen) were significantly lower (* P < 0.05) with the ratio of KB to HT 1080 on average 2.5 : 1. In contrast, the biodistribution of **PP** shows no significant difference between the HT 1080 tumors and KB tumors (or the rest of the organs), confirming the minimal role of Pyro on the **PPF**'s tumor selectivity and thus validating the importance of folate as a tumor homing molecule.

B) The role of peptide modulation in targeted delivery of the PPF

In order to clarify the role of peptide in the targeted delivery of **PPF**, Pyro-K-Folate (**PKF**) was synthesized by replacing the nine amino acid peptide sequence in **PPF** with a single lysine residue. The following investigations revealed the consequences of removing the peptide module. First, **PKF** showed the increased hydrophobicity and decreased water solubility when compared to **PPF** (HPLC, Supporting information, Figure 2). Next, the accessibility of folate moiety to FR in **PKF** vs. **PPF** was also investigated *in vitro* using flow cytometry. KB, HT 1080, and CHO cells were incubated with the same concentration (5μM) of **PKF** or **PPF**. It was observed that both **PPF** and **PKF** accumulate in HT 1080 and CHO cells (both FR-) with similar efficiency, whereas in KB cells (FR+) the efficiency of **PKF**'s uptake drops almost to 50% compared to that of **PPF**'s (Supporting information, Figure 3). This indicates that the nine amino acid linker in **PPF** increases the efficiency of the FR-mediated uptake probably by making the folate more accessible (Supporting information, Figure 4).

Perhaps the most profound effect of peptide modulation on the targeted delivery of **PPF** was its role in the biodistribution pattern of **PPF** as depicted in Figure 8 and Supporting information, Figure 5. As expected, when we compare the *in vivo* biodistribution of **PPF** with **PP**, the difference in KB and HT 1080 tumor accumulation of **PPF** is visible at 1.5 hour (Figure 8-

column C) and is much more distinguishable at 6.5 hours (Figure 8-column D) post-injection. This is clearly due to the effect of FR-targeting by the folate moiety, which facilitates the preferential accumulation of **PPF** in the KB tumor (FR+), since folate-lacking **PP** does not distinguish between the FR+ and FR- tumors. However, what is notable is the common biodistribution pattern showing a low retention in the organs responsible for drug clearance for both **PPF** and **PP**. We attribute this finding to the peptide-based pharmacomodulation, meaning that the presence of a short peptide sequence significantly alters the pharmacokinetics and pharmacodynamics of the parent molecule. Such modulation becomes more obvious when we compare the biodistribution pattern of **PPF** with that of **PKF** (Figure 8-row 3 and Supporting information, Figure 5). Even though **PKF** immediately after the injection (Figure 8-3B) displays a distribution pattern very similar to that of **PPF** and **PP** (Figure 8-1B and 2B), this pattern is drastically changed only 1.5 hours post-injection (Figure 8-column C). At 6.5 hours it is clear that most **PKF** is retained by the excretion organs and exhibits extremely low tumor accumulation (Figure 8-3D). In contrast, both **PPF** and **PP** are delivered to the tumors with much higher efficiency even though there is a difference in FR-targeting specificity.

The *ex vivo* organ biodistribution corrected for weight and normalized for muscle (Supporting information, Figure 5) further confirmed this accumulation pattern. It identifies liver and spleen (followed by adrenal and kidney) as the organs accumulating most of the **PKF** and when comparing the relative fluorescence intensity with both **PPF** and **PP**, this accumulation is approximately 50-times higher for **PKF**. This pharmacomodulation highlights the positive impact of the peptide sequence on the targeted delivery of **PPF** by minimizing the nonspecific uptake by liver, spleen and other organs.

Future perspective

In principle, the target-specific, peptide-based pharmacomodulation approach described here could be applicable for improving the delivery efficiency of other cancer diagnostics and therapeutics. The versatility of this approach lies in its modular design in which any module could be easily replaced for a different purpose. For example, Pyro moiety could be replaced with another photosensitizer that is active at a longer wavelength absorption (e.g. bacteriochlorophylls (39) or naphthalocyanines (40)) or even a chemotherapeutic agent (e.g. docetaxel, etc.). The target specificity can also be modulated by exchanging the folate moiety for any small molecule ligand targeting other cancer signatures. Finally, the length and the sequence of the peptide linker could be fine tuned to optimize the pharmacomodulation, improving not only the solubility, the target accessibility but most importantly, also the delivery efficiency. In future, the peptide linker could also serve as an subcellular organelle-specific delivery vehicle when exchanged for different sequences.

Summary

We synthesized, characterized and evaluated a target-specific NIR imaging and PDT agent with improved target specificity and delivery efficiency. This agent is comprised of three modules: a fluorescent photosensitizer Pyro for NIR imaging and PDT, Folate for selective delivery, and a short peptide linker for pharmacomodulation. Although there were several previous attempts to link a dye (9) or a photosensitizer (9,37) to a folate moiety in order to specifically deliver the agent to cancer cells overexpressing FR, they were limited either to use *in vitro* or only to the imaging purposes. Here we report a novel, modular approach using the combination of a folate-mediated targeting and a peptide-based pharmacomodulation to improve the efficiency of targeted delivery of NIR imaging and PDT agents. This study created a fluorescent photosensitizer with excellent biodistribution pattern (folate receptor-specific tumor uptake and low accumulation in normal tissues), a crucial step toward lowering the toxicity and background signal, thus making it potentially applicable for detecting and treating cancers in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank André E. X. Brown for critical comments and suggestions on the manuscript. This work was supported by the DOD Breast Cancer Research Program DAMD17-03-1-0373 and the NIH grant U54 CA105008 (Penn NTROI).

References

1. Berg K, Selbo PK, Weyergang A, Dietze A, Prasmickaite L, Bonsted A, Engesaeter BO, Angell-Petersen E, Warloe T, Frandsen N, Hogset A. Porphyrin-related photosensitizers for cancer imaging and therapeutic applications. *J Microsc* 2005;218:133–147. [PubMed: 15857375]
2. Licha K. Contrast Agents for Optical Imaging. *Top Curr Chem* 2002;222:1–29.
3. Oleinick NL, Evans HH. The photobiology of photodynamic therapy: cellular targets and mechanisms. *Radiat Res* 1998;150:S146–156. [PubMed: 9806617]
4. Bogaards A, Varma A, Collens SP, Lin A, Giles A, Yang VX, Bilbao JM, Lilge LD, Muller PJ, Wilson BC. Increased brain tumor resection using fluorescence image guidance in a preclinical model. *Lasers Surg Med* 2004;35:181–190. [PubMed: 15389738]
5. Coleman CN. Linking radiation oncology and imaging through molecular biology (or now that therapy and diagnosis have separated, it's time to get together again!). *Radiology* 2003;228:29–35. [PubMed: 12832573]
6. Huang X, El-Sayed IH, Qian W, El-Sayed MA. Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods. *J Am Chem Soc* 2006;128:2115–2120. [PubMed: 16464114]
7. Dougherty TJ. Photosensitizers: therapy and detection of malignant tumors. *Photochem Photobiol* 1987;45:879–889. [PubMed: 2957705]
8. Gurfinkel M, Thompson AB, Ralston W, Troy TL, Moore AL, Moore TA, Gust JD, Tatman D, Reynolds JS, Muggenburg B, Nikula K, Pandey R, Mayer RH, Hawrysz DJ, Sevcik-Muraca EM. Pharmacokinetics of ICG and HPPH-car for the detection of normal and tumor tissue using fluorescence, near-infrared reflectance imaging: a case study. *Photochem Photobiol* 2000;72:94–102. [PubMed: 10911733]
9. Moon WK, Lin Y, O'Loughlin T, Tang Y, Kim DE, Weissleder R, Tung CH. Enhanced tumor detection using a folate receptor-targeted near-infrared fluorochrome conjugate. *Bioconjug Chem* 2003;14:539–545. [PubMed: 12757377]
10. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbek M, Moan J, Peng Q. Photodynamic therapy. *J Natl Cancer Inst* 1998;90:889–905. [PubMed: 9637138]
11. Niedere MJ, Secord AJ, Patterson MS, Wilson BC. In vitro tests of the validity of singlet oxygen luminescence measurements as a dose metric in photodynamic therapy. *Cancer Res* 2003;63:7986–7994. [PubMed: 14633731]
12. Jori G. Tumour photosensitizers: approaches to enhance the selectivity and efficiency of photodynamic therapy. *J Photochem Photobiol, B* 1996;36:87–93. [PubMed: 9002244]
13. Chen J, Stefflova K, Niedere M, Wilson B, Chance B, Glickson JD, Zheng G. Protease-triggered photosensitizing beacon based on singlet oxygen quenching and activation. *J Am Chem Soc* 2004;126:11450–11451. [PubMed: 15366886]
14. Sun X, Leung WN. Photodynamic therapy with pyropheophorbide-a methyl ester in human lung carcinoma cancer cell: efficacy, localization and apoptosis. *Photochem Photobiol* 2002;75:644–651. [PubMed: 12081327]
15. Bellnier DA, Greco WR, Loewen GM, Nava H, Oseroff AR, Pandey RK, Tsuchida T, Dougherty TJ. Population pharmacokinetics of the photodynamic therapy agent 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a in cancer patients. *Cancer Res* 2003;63:1806–1813. [PubMed: 12702566]
16. Reddy JA, Allagadda VM, Leamon CP. Targeting therapeutic and imaging agents to folate receptor positive tumors. *Curr Pharm Biotechnol* 2005;6:131–150. [PubMed: 15853692]

17. Lu Y, Low PS. Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv Drug Deliver Rev* 2002;54:675–693.
18. Sabharanjak S, Mayor S. Folate receptor endocytosis and trafficking. *Adv Drug Deliver Rev* 2004;56:1099–1109.
19. Paulos CM, Turk MJ, Breur GJ, Low PS. Folate receptor-mediated targeting of therapeutic and imaging agents to activated macrophages in rheumatoid arthritis. *Adv Drug Deliver Rev* 2004;56:1205–1217.
20. Hilgenbrink AR, Low PS. Folate receptor-mediated drug targeting: from therapeutics to diagnostics. *J Pharm Sci* 2005;94:2135–2146. [PubMed: 16136558]
21. Sibrian-Vazquez M, Jensen TJ, Hammer RP, Vicente MG. Peptide-mediated cell transport of water soluble porphyrin conjugates. *J Med Chem* 2006;49:1364–1372. [PubMed: 16480271]
22. Rosenkranz AA, Jans DA, Sobolev AS. Targeted intracellular delivery of photosensitizers to enhance photodynamic efficiency. *Immunol Cell Biol* 2000;78:452–464. [PubMed: 10947873]
23. Dozzo P, Koo MS, Berger S, Forte TM, Kahl SB. Synthesis, characterization, and plasma lipoprotein association of a nucleus-targeted boronated porphyrin. *J Med Chem* 2005;48:357–359. [PubMed: 15658849]
24. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70. [PubMed: 10647931]
25. Sharman WM, van Lier JE, Allen CM. Targeted photodynamic therapy via receptor mediated delivery systems. *Adv Drug Deliver Rev* 2004;56:53–76.
26. Konan YN, Gurny R, Allemann E. State of the art in the delivery of photosensitizers for photodynamic therapy. *J Photochem Photobiol, B* 2002;66:89–106. [PubMed: 11897509]
27. Hamblin MR, Miller JL, Ortel B. Scavenger-receptor targeted photodynamic therapy. *Photochem Photobiol* 2000;72:533–540. [PubMed: 11045726]
28. Derycke AS, Kamuhabwa A, Gijssens A, Roskams T, De Vos D, Kasran A, Huwyler J, Missiaen L, de Witte PA. Transferrin-conjugated liposome targeting of photosensitizer ALPcS4 to rat bladder carcinoma cells. *J Natl Cancer Inst* 2004;96:1620–1630. [PubMed: 15523091]
29. Zheng G, Chen J, Li H, Glickson JD. Rerouting lipoprotein nanoparticles to selected alternate receptors for the targeted delivery of cancer diagnostic and therapeutic agents. *Proc Natl Acad Sci U S A* 2005;102:17757–17762. [PubMed: 16306263]
30. Morgan J, Gray AG, Huehns ER. Specific targeting and toxicity of sulphonated aluminium phthalocyanine photosensitised liposomes directed to cells by monoclonal antibody in vitro. *Brit J Cancer* 1989;59:366–370. [PubMed: 2930700]
31. Chen X, Hui L, Foster DA, Drain CM. Efficient synthesis and photodynamic activity of porphyrin-saccharide conjugates: targeting and incapacitating cancer cells. *Biochemistry* 2004;43:10918–10929. [PubMed: 15323552]
32. Farokhzad OC, Karp JM, Langer R. Nanoparticle-aptamer bioconjugates for cancer targeting. *Expert Opin Drug Deliver* 2006;3:311–324.
33. Ichikawa K, Hikita T, Maeda N, Yonezawa S, Takeuchi Y, Asai T, Namba Y, Oku N. Antiangiogenic photodynamic therapy (PDT) by using long-circulating liposomes modified with peptide specific to angiogenic vessels. *Biochim Biophys Acta* 2005;1669:69–74. [PubMed: 15843001]
34. Renno RZ, Terada Y, Haddadin MJ, Michaud NA, Gragoudas ES, Miller JW. Selective photodynamic therapy by targeted verteporfin delivery to experimental choroidal neovascularization mediated by a homing peptide to vascular endothelial growth factor receptor-2. *Arch Ophthalmol* 2004;122:1002–1011.
35. Bisland SK, Singh D, Gariepy J. Potentiation of chlorin e6 photodynamic activity in vitro with peptide-based intracellular vehicles. *Bioconjug Chem* 1999;10:982–992. [PubMed: 10563767]
36. Kukowska-Latallo JF, Candido KA, Cao Z, Nigavekar SS, Majoros IJ, Thomas TP, Balogh LP, Khan MK, Baker JR Jr. Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. *Cancer Res* 2005;65:5317–5324. [PubMed: 15958579]
37. Schneider R, Schmitt F, Frochot C, Fort Y, Lourette N, Guillemin F, Muller JF, Barberi-Heyob M. Design, synthesis, and biological evaluation of folic acid targeted tetraphenylporphyrin as novel photosensitizers for selective photodynamic therapy. *Bioorg Med Chem* 2005;13:2799–2808. [PubMed: 15781391]

38. Schottelius M, Rau F, Reubi JC, Schwaiger M, Wester HJ. Modulation of pharmacokinetics of radioiodinated sugar-conjugated somatostatin analogues by variation of peptide net charge and carbohydrate chemistry. *Bioconj Chem* 2005;16:429–437. [PubMed: 15769098]
39. Zheng G, Chen Y, Intes X, Chance B, Glickson JD. Contrast-enhanced near-infrared (NIR) optical imaging for subsurface cancer detection. *J Porphyrins Phthalocyanines* 2004;8:1106–1117.
40. Li H, Marotta DE, Kim S, Busch TM, Wileyto EP, Zheng G. High payload delivery of optical imaging and photodynamic therapy agents to tumors using phthalocyanine-reconstituted low-density lipoprotein nanoparticles. *J Biomed Opt* 2005;10:41203. [PubMed: 16178627]

Figure 1. Synthesis (A), MALDI-ToF (B, mass calculated: 1800.76, found: 1799.79) and HPLC (C, absorbance at 410nm) of Pyro-GDEVDSGK-Folate.

Figure 2. Accumulation of Pyro-GDEVDGSGK-Folate (**PPF**) in KB (FR+) vs. HT 1080 (FR-) cells. A) KB cells alone, B) KB cells incubated with 50 μM **PPF** for 5 hours, C) HT 1080 cells alone, D) HT 1080 cells incubated with 50 μM **PPF** for 5 hours. **PPF** accumulates preferentially in KB cells. The scale bar represents 37.5 μm.

Figure 3.

Monitoring the accumulation of **PPF** (Pyro-GDEVDSGK-Folate) in CHO (FR-), HT 1080 (FR-) and KB (FR+) cells using flow cytometry and inhibition of this uptake with free folic acid. CHO cells (A) and HT 1080 cells (B) incubated with 5 μ M **PPF** (blue) or 5 μ M **PPF** and 5mM Folic acid (orange/red) show very similar uptake of **PPF** (695nm fluorescence) with or without inhibition (PPF+FA vs. PPF). Only KB cells (C) show significant (70%) reduction in **PPF**'s uptake when incubated with 5 μ M **PPF** and 5mM Folic acid (PPF-blue vs. PPF+FA-orange), suggesting that folate-receptor mediated endocytosis is the main delivery pathway for KB, but not for CHO and HT 1080 cells.

Figure 4.

Viability of KB cells (FR+, cancer cells), HT 1080 cells (FR-, cancer cells) and CHO cells (FR-, normal cells) incubated with 0.5, 5, and 15 μM PPF and treated with light (5 or 10 J/cm²) or kept in the dark (0 J/cm²). KB cells have viability reduced below 10% when incubated with the lowest drug dose (0.5 μM) and treated with 5 J/cm². CHO cells reach this level of cell death for the same concentration (5 μM) only when treated with the highest light dose (10 J/cm²) and in case of HT 1080, even the highest drug dose (15 μM) and light dose (10 J/cm²) did not reduce their viability below 10%.

Figure 5.

Monitoring fluorescence signal distribution after intravenous injection of three different doses of **PPF** to double tumor bearing mice that have a KB tumor (FR+) on the right side and an HT 1080 tumor (FR-) on the left side, using a Xenogen imager. A) Prescan, B) 5 minutes after injection of 50nmol (left mouse), 100nmol (middle mouse) and 150nmol (right mouse) of **PPF**, C) 6 hours after injection, and D) 24 hours after injection. At this point it is clear that **PPF** preferentially accumulates in the KB tumor.

Figure 6.

Structure of **PPF** (both peptide and Folate modulation), **PP** (lacking the Folate), and **PKF** (lacking the peptide module).

Figure 7.

In vivo and *ex vivo* distribution of *iv* injected Pyro-GDEVDSGK-Folate (**PPF**, targeting FR + KB tumor) and Pyro-GDEVDSGK (**PP**, no targeting). Xenogen images of double tumor mice (HT 1080 tumor left, KB tumor right) injected with **PPF** (left) or **PP** (right) at 6.5 hours after injection (upper left corner) shows higher accumulation of **PPF** in the KB tumor. This was confirmed by images of the dissected organs (lower left corner): 1A-KB tumor, 1B-HT 1080 tumor, 2A-muscle, 2B-heart, 3A-adrenal, 3B-kidney, 4A-spleen, 4B-liver (left-**PPF** and right-**PP**). Fluorescence from homogenized organs (right; **PPF**-orange, **PP**-green), corrected for weight and expressed as a ratio of the signal of muscle (=1) show a significantly higher accumulation of **PPF** in the KB tumor than in the HT 1080 tumor but a similar distribution of non-targeting **PP** in both tumors. The asterisk identifies those organs of the **PPF** mouse whose relative fluorescence is significantly ($P < 0.05$, $n=3$) different from KB tumor showing that KB tumor has the highest accumulation of **PPF**, comparable only with kidney, while HT 1080 tumor has an average signal that is 2.5 times lower.

Figure 8.

Double tumor mice (HT 1080 tumor left, KB tumor right) injected with Pyro-GDEVDSGK-Folate (**PPF**, row 1), Pyro-GDEVDSGK (**PP**, row 2), or Pyro-K-Folate (**PKF**, row 3) and imaged before the injection (“Prescan”, A) and 5 minutes (B), 1.5 hours (C) and 6.5 hours (D) after the drug injection. It is clear that 6.5 hours after the injection **PPF** accumulates mostly in the KB tumor (1D) and **PP** equally in both tumors (2D), demonstrating the folate-introduced cancer specificity. This is in contrast to **PKF** (3D) that has minimal tumor accumulation and is retained in organs responsible for drug clearance, clearly identifying the peptide as the pharmacomodulating component.

Scheme 1.

Pyro-GDEVDGSGK-Folate comprises three principal components: 1) Fluorescent photosensitizer Pyropheophorbide *a* is an imaging and therapeutic agent, 2) Peptide sequence is a stable and pharmacomodulating linker that can be exchanged with any organelle-targeting sequence, and 3) Folate, serving as a cancer-specific delivery vehicle.