Published in final edited form as:

Nanomedicine (Lond). 2011 April ; 6(3): 475–487. doi:10.2217/nmm.11.8.

Evaluation of bacteriochlorophyll-reconstituted low-density lipoprotein nanoparticles for photodynamic therapy efficacy *in vivo*

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Abstract

Aim—To evaluate the novel nanoparticle reconstituted bacteriochlorin e6 bisoleate low-density lipoprotein (r-Bchl-BOA-LDL) for its efficacy as a photodynamic therapy agent delivery system in xenografts of human hepatoblastoma G2 (HepG2) tumors.

Materials & methods—Bchl-BOA was encapsulated in the nanoparticle low-density lipoprotein (LDL), a native particle whose receptor's overexpression is a cancer signature for a number of neoplasms. Evaluation of r-Bchl-BOA-LDL as a potential photosensitizer was performed using a tumor response and foot response assay.

Results & discussion—When compared with controls, tumor regrowth was significantly delayed at injected murine doses of 2 μ mole/kg r-Bchl-BOA-LDL after illumination at fluences of

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Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

125, 150 or 175 J/cm². Foot response assays showed that although normal tissue toxicity accompanied the higher fluences it was significantly reduced at the lowest fluence tested.

Conclusion—This research demonstrates that r-Bchl-BOA-LDL is an effective photosensitizer and a promising candidate for further investigation.

Keywords

bacteriochlorophyll; low-density lipoprotein; nanoparticle; photodynamic therapy; xenograft hepatoma

Photodynamic therapy (PDT) utilizes a light-activated photosensitizer to induce cytotoxicity in targeted tissue, primarily through the production of the highly reactive singlet oxygen species (¹O₂) [1]. The choice of photosensitizer is an essential determinant of the efficacy of this process. Among the desired criteria for an ideal photosensitizer for solid tumor PDT are good tumor cell selectivity, high photocytotoxicity with minimal dark toxicity, and a strong absorption peak above 630 nm for deep tissue penetration [2,3]. Efforts to optimize such light-activated compounds are ongoing. Photofrin[®] is a first-generation photosensitizer approved worldwide for the treatment of early- and late-stage lung cancer, esophageal cancer and bladder cancer, among other clinical applications [2,4]. However, since its longest wavelength absorption is only 630 nm, tissue penetration is limited [4]. In addition, skin photosensitivity lasts for as long as 12 weeks [3]. Subsequently, second-generation photosensitizers were designed to minimize skin photosensitivity and to extend the absorbance range to 650–850 nm [5]. Levulan[®], approved for the treatment of actinic keratosis, is a second-generation photosensitizer pro-drug whose photosensitizing conversion product, protoporphyrin IX, is associated with reduced skin photosensitivity (1–2 days), but protoporphyrin IX is usually also activated by light at 630 nm [3]. The second-generation photosensitizer Foscan[®], approved for the palliative treatment of head and neck cancer in Europe, is activated at a slighter higher wavelength, 652 nm, which slightly increases the depth of tissue penetration [3,4]. Still deeper penetration is achieved with Visudyne[®], a second-generation photosensitizer activated at 690 nm [5,6]. While initially intended for cancer treatment, it has obtained worldwide approval for treatment of age-related macular degeneration [7].

To enhance tumor cell selectivity, third-generation photosensitizers have been developed by conjugation of second-generation photosensitizers to appropriate carriers [5]. For example, chlorin covalently linked to low-density lipoprotein (LDL; Ce₆: LDL) displayed an approximately fourfold higher uptake in fibrosarcoma and retinoblastoma cells than the isolated dye [8]. Upon illumination at 10 J/cm², Ce₆:LDL and the free dye conferred a 20% and approximately 100% survival of retinoblastoma cells, respectively [8]. Similarly, Vrouenraets *et al.* demonstrate the feasibility of coupling meta-tetrahydroxyphenylchlorin to a monoclonal antibody for tumor targeting [9]. Further optimization efforts have substituted antibody fragments for monoclonal antibodies, whose large size limits conjugate penetration into solid, poorly vascularized tumors [10]. The conjugation of isothiocyanato porphyrin to colorectal tumor-specific scFv (single chain heavy and light chain variable regions) generated a novel photosensitizer that demonstrated *in vitro* selective phototoxic effects on colorectal cancer cells [10].

We have engineered reconstituted bacteriochlorin e6 bisoleate LDL (r-Bchl-BOA-LDL), a novel compound expected to enhance PDT efficacy. Bacteriochlorophyll, a magnesium chelate, is activated at 748 nm in the near-infrared (NIR) region, thus allowing greater tissue penetration than conventional photosensitizers [11]. Since many malignancies overexpress LDL receptors (LDLRs) [12], we incorporated the lipophilic bacteriochlorophyll derivative into the core of LDL to enhance tumor cell delivery [11]. Bis-oleate or bis-stearate moieties

have also been used to incorporate dyes and Gd-chelating groups into the phospholipid monolayer of LDL by intercalation of the lipid chains in the phospholipid and exposure of the hydrophilic dyes (e.g., tricarboxyanines) and Gd chelates to the external aqueous phase [11,13,14]. However, chlorins are neutral and highly hydrophobic. These entities were incorporated into the lipid core by an extraction/reconstitution procedure developed by Krieger *et al.* [15]; hence, these photosensitizers reside at the surface of the lipid core and protrude into the phospholipid layer from the inside of the particle in a manner similar to phthalocyanine PDT agents that we have previously reported [16]. An analogous compound, with only one lipophilic chain, bacteriochlorin e6 cholesteryl oleate (Bchl-CE), was designed in parallel and reconstituted into LDL in a similar manner. However, Bchl-CE demonstrated a reduced LDL payload compared with Bchl-BOA and was subsequently abandoned. Human tumor xenografts of hepatoblastoma G₂ (HepG₂), which overexpress LDLR, were chosen for study. This lipoprotein-based nanoplatform concept for drug delivery is depicted in FIGURE 1. An LDL-based system for delivery of therapeutic and diagnostic agents to tumor cells by LDLR-mediated endocytosis has been previously established [11,17]. In addition, compared with synthetic nanoscale transporters, lipoprotein nanoparticles may minimize immunogenicity problems as they are endogenous carriers found in normal human blood [17]. Here we examine the potential of r-Bchl-BOA-LDL as an effective PDT agent.

Materials & methods

Materials

Reagent grade chemicals were purchased from Aldrich (Milwaukee, Wisconsin). The Lowry protein assay kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemical syntheses reactions were conducted under N₂ or Ar and were monitored by precoated (0.20 mm) silica TLC plastic sheet (20 × 20 cm) strips (POLYGRAM SIL N-HR) and/or UV-visible spectroscopy. Silica gel 60 (70–230 mesh, Merck) was utilized for column chromatography. UV-visible and fluorescence spectra were recorded on a Varian (Cary-50 Bio) spectrophotometer and a FluoroMax[®]-4 (HORIBA Scientific, Kyoto, Japan) spectrofluorometer, respectively, for chemical syntheses or on a Perkin-Elmer Lambda 2 spectrophotometer and a LS50B spectrofluorometer, respectively, for reconstitution characterization. Bacteriopheophorbide a (Bchl-acid) was obtained from *Rhodospirillum rubrum* (Frontier Sciences, Utah). Proton NMR spectra were recorded on a Bruker AMX 400MHz NMR spectrometer at 303°K. Proton chemical shifts (δ) are specified in parts per million (ppm) relative to CDCl₃ (7.26 ppm) or TMS (0.00 ppm). MS and purity data were obtained from the Waters Analytical high-performance liquid chromatography (HPLC) mass spectroscopy facility. Analytical HPLC was also used to assess the purity of compounds. A Waters (Milford, MA, USA) system including a Waters 600 Controller, a Delta 600 pump and a 996 Photodiode Array Detector was used. A reverse phase, Symmetry C18, 5 μ m, 4.6 × 150 mm column (Waters; made in Ireland) was utilized under an isocratic setting of MeCN/H₂O for all compounds. The solvent flow rate was kept constant at 1.00 ml/min and the detector was set at 700 nm for all compounds. All final products were at least 95% pure.

Cell line

Human hepatoblastoma G₂ (HepG₂; LDLR⁺) cells obtained from Theo van Berkel's laboratory (University of Leiden, The Netherlands) were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES with 100 U/ml penicillin G sodium and 100 μ g/ml streptomycin sulfate at 37°C and 5% CO₂ in a humidified incubator.

Bchl-BOA & Bchl-CE synthesis

The total synthesis of bacteriochlorophyll analogs including Bchl-CE and Bchl-BOA is depicted in FIGURE 2. Part of the synthesis scheme including some compound characterizations has been reported in a previous communication [18].

Synthesis of Bchl-CE

Synthesis of bacteriopheophorbide a (Bchl-acid)—Bacteriopheophorbide a (Bchl-acid, see FIGURE 2A) was synthesized from Bchl extracted from *R. sphaeroides* in an inert atmosphere using 1-propanol following literature methods [19].

Synthesis of bacteriopheophorbide a methyl ester (Bchl-Me-ester)—Bchl-acid (1.0 g, 1.64 mmol) was dissolved in 500 ml dichloromethane and then treated with excess diazomethane. The completion of reaction was monitored and excess diazomethane was destroyed by addition of glacial acetic acid. The solvent was evaporated and the crude residue was purified by silica gel chromatography with 5% acetone dichloromethane as eluent to obtain Bchl-Me-ester (see FIGURE 2B; 870 mg, 1.39 mmol) in 85% yield.

Bchl-Me-ester: Mass calculated for $C_{36}H_{40}N_4O_6$: 624.29. Found by ESI-MS: 625.22 ($M+1$)⁺. ¹H NMR ($CDCl_3$, δ ppm): 8.99, 8.50 and 8.43 (each, s, 3H for 10, 5, 20-H), 6.10 (s, 1H, 15¹-CH), 4.33 (m, 2H), 4.05 (m, 2H), 3.87 (s, 3H, 17³-CO₂CH₃), 3.63 (s, 3H, 15²-CO₂CH₃), 3.50 (s, 3H, 12-CH₃), 3.46 (s, 3H, 2-CH₃), 3.17 (s, 3H, 3-COCH₃), 2.54 (m, 2H), 2.25–2.09 (m, 4H), 1.82 (d, 3H, J = 8.0 Hz), 1.79 (d, 3H, J = 8.0 Hz), 1.14 (t, 3H, J = 7.6 Hz, 8²-CH₃), 0.49 and 0.10 (each, s, 2H, 2 × NH).

Synthesis of bacteriochlorin e6 N-Boc-aminopropylamide (Bchl-BOC)

Bacteriopheophorbide a methyl ester (625 mg, 1 mmol) was dissolved in chloroform (50 ml) and *N*-Boc-1,3-diaminopropane (380 mg, 2.18 mmol) was dissolved in 15 ml benzene. The mixture was refluxed at 78°C under Ar for 48 h. After removing solvent, the crude residue was purified by silica gel column chromatography with 10% acetone in dichloromethane. The desired product was obtained in 70% yield (561 mg, 0.70 mmol; see FIGURE 2C).

Bchl-BOC: Mass calculated for $C_{39}H_{58}N_6O_6$: 798.43. Found by ESI-MS: 799.43 ($M+1$)⁺. ¹H NMR ($CDCl_3$, δ ppm): 9.33, 8.71 and 8.58 (each, s, 3H for 5, 10, 20-H), 6.96 (brs, 1H, NH), 5.41 (d, 1H, 15¹-CH₂), 5.12 (d, 2H, 15¹-CH₂ and NHBoc), 4.35 (m, 1H), 4.26 (m, 1H), 4.17 (m, 2H, 7 and 8-H), 3.78 (m, 1H), 3.76 (s, 3H, 17³-CO₂CH₃), 3.64 (s, 3H, 12-CH₃), 3.59 (s, 3H, 2-CH₃), 3.36 (m, 5H), 3.19 (s, 3H, 3-COCH₃), 2.38–2.06 (m, 5H), 1.87 (d, 5H), 1.75 (m, 2H), 1.44 (d, 3H, J = 8.0 Hz, 18-CH₃), 1.41 (s, 9H, N-CO₂-Boc), 1.09 (t, 3H, 8²-CH₃).

Synthesis of bacteriochlorin e6 isothiocyanatopropylamide (Bchl-NCS)—Bchl-BOC (400 mg, 0.5 mmol) was dissolved in 6 ml trifluoroacetic acid (TFA). The mixture was stirred at room temperature (rt) under Ar for 1 h. TFA was removed by vacuum and the crude residue was diluted with 40 ml dichloromethane, washed once with 30 ml NaHCO₃ and twice with 30 ml water. The organic layer was dried over anhydrous Na₂SO₄. After removing solvent, Bchl-NH₂ was obtained (300 mg, 0.43 mmol) at an 86% yield. Mass calculated for $C_{39}H_{50}N_6O_6$: 698.38. Found by ESI-MS: 698.26. Bchl-NH₂ was then used directly for the next reaction step without further purification. The Bchl-NH₂ (300 mg, 0.43 mmol) and 1,1'-thiocarbonyldiimidazole (71.3 mg) were dissolved in 20 ml dichloromethane. The reaction mixture was subsequently refluxed at 40°C under Ar for 3 h and the solvent was removed. The residue was purified by silica gel chromatography with 2% MeOH-CH₂Cl₂ and 95 mg of Bchl-NCS was collected (yield 75%; see FIGURE 2D).

Bchl-NCS: Mass calculated for $C_{39}H_{48}N_6O_6S$: 740.34. Found by ESI-MS: 763.21 ($M + Na$)⁺. ¹H NMR ($CDCl_3$, δ ppm): 9.32, 8.71 and 8.59 (each, s, 3H for 5, 10, 20-H), 6.67 (brs, 1H, NH), 5.31 (d, 1H, 15^1-CH_2), 5.10 (d, 1H, 15^1-CH_2), 4.34 (m, 1H), 4.26 (m, 1H), 4.17 (m, 2H, 7 and 8-H), 3.79 (m, 1H), 3.74 (s, 3H, $17^3-CO_2CH_3$), 3.64 (s, 3H, $12-CH_3$), 3.61 (s, 5H), 3.32 (s, 3H, $15^2-CO_2CH_3$), 3.17 (s, 3H, 3-COCH₃), 2.59–2.03 (m, 7H), 1.86 (d, 3H, $J = 8.0$ Hz, 7-CH₃), 1.72 (m, 2H), 1.62 (d, 3H, $J = 8.0$ Hz, 18-CH₃), 1.08 (t, 3H, $J = 7.6$ Hz, 8^2-CH_3).

Synthesis of bacteriochlorin e6 cholesteryl oleate (Bchl-CE)—Bchl-NCS (20 mg, 0.027 mmol) was dissolved in 3 ml dichloromethane followed by addition of a solution containing (22 mg, 0.397 mmol) 5-androsten-17 β -amino-3 β -yl oleate [20] and 30 μ l diisopropylethylamine (DIPEA) in 2 ml dichloromethane. The reaction mixture was stirred at rt under Ar for 24 h. After removing solvent, the crude residue was purified by silica gel chromatography and eluted with 3% methanol in dichloromethane. The desired conjugate Bchl-CE was obtained in 86% yield (30 mg; see FIGURE 2E).

Bchl-CE: Mass calculated for $C_{77}H_{111}N_7O_8S$: 1294.81. Found by ESI-MS: 1294.72 (M^+) and 1318.47 ($M + Na$)⁺. ¹H NMR ($CDCl_3$, δ ppm): 9.25, 8.76 and 8.63 (each, s, 3H for 5, 10, 20-H), 5.35–5.12 (m, 5H, 15^1-CH_2 , vinyl-H from oleoyl and NH), 4.27–4.15 (m, 7H), 3.95–3.75 (m, 3H), 3.74 (m, 5H, $17^3-CO_2CH_3$ and CH_2), 3.59 (s, 3H, $12-CH_3$), 3.55 (s, 3H, 2-CH₃), 3.34 (s, 3H, $13^2-CO_2CH_3$), 3.30 (t, 1H), 3.15 (s, 3H, 3-COCH₃), 2.65 (m, 1H), 2.45 (m, 1H), 2.25–2.00 (m, 16H), 1.83 (d, 3H, $J = 7.2$ Hz, 7-CH₃), 1.79–1.56 (m, 10H, 18-CH₃ and CH_2), 1.45–1.10 (m, 30H, CH_2), 1.07–0.08 (m, 12H, $J = 7.6$ Hz, 8^2-CH_3), –1.13 and –1.18 (each, s, 2H, 2 $\times$ NH).

Synthesis of Bchl-BOA

Bchl-acid, the starting compound in FIGURE 2, was synthesized from Bchl extracted from *R. sphaeroides* in an inert atmosphere using 1-propanol following literature methods [19].

Synthesis of bacteriochlorin e6 Di-N-Boc-aminopropylamide (Bchl-2 BOC)

Bchl-acid (330 mg, 0.54 mmol) was first dissolved in 20 ml dichloromethane to which 160 mg of 4-*N,N*-dimethylaminopyridine (DMAP, 1.31 mmol) and 400 mg of *N*-Boc-1,3-diaminopropane (2.30 mmol) were added. This mixture was stirred under Ar for 30 min at rt, after which 260 mg of dicyclohexylcarbodiimide (DCC, 1.26 mmol) was added and the mixture was stirred for 40 h. The solvent was removed under vacuum. The resultant crude product was purified by silica gel column chromatography with 8% acetone in dichloromethane and then with 4% methanol in dichloromethane. The desired product, Bchl-2 BOC (368.3 mg, 0.39 mmol; see FIGURE 2F), was obtained in 72.2% yield.

Bchl-2 BOC: Mass calculated for $C_{51}H_{72}N_8O_9$: 941.17. Found by ESI-MS: 941.8 (M^+). ¹H NMR ($CDCl_3$, δ ppm): 9.31, 8.65 and 8.56 (each, s, 3H for 5, 10, 20-H), 7.30, 6.42, 6.07 and 5.98 (each, brs, 4H for 4 $\times$ NH), 5.21 (s, 2H, 15^1-CH_2), 4.32 (br, 1H, 18-H), 4.25 (brs, 1H, 7-H), 4.23 (d, 1H, 17-H), 4.14 (brs, 1H, 8-H), 3.72 (s, 3H, $15^2-CO_2CH_3$), 3.69 (m, 2H, 13^3-NCH_2), 3.39 (s, 3H, $12-CH_3$), 3.38 (m, 2H, 17^5-NCH_2), 3.32 (s, 3H, 2-CH₃), 3.17 (s, 3H, 3-COCH₃), 2.84–2.59 (m, 4H, for 13^5-CH_2N , 17^7-CH_2N), 2.36 (m, 1H, 17^1-H), 2.22 (m, 1H, 17^1-H), 2.04 (m, 2H, 17^2-CH_2), 1.94 (m, 2H, 8^1-CH_2), 1.82 (d, 3H, 18-CH₃), 1.63 (m, 5H, for 7-CH₃, 13^4-CH_2), 1.40–1.23 (brs, 20H, for 17^6-CH_2 , $13^9-C(CH_3)_3$, $17^{11}-C(CH_3)_3$), 1.08 (t, 3H, 8^2-CH_3), –1.21 and –1.25 (each, s, 2H, for 2 $\times$ NH).

Synthesis of bacteriochlorin e6 bisoleate (Bchl-BOA)—Bchl-2 BOC (40 mg, 0.043 mmol) was dissolved in 1.5 ml TFA. The solution was stirred first in an ice-water bath under Ar for 1 h and then at rt for 1 h. TFA was removed under vacuum. The crude product

bacteriochlorin e6 diaminopropylamide (Bchl-2 NH₂) required no further purification and was used directly for the next reaction. Bchl-2 NH₂ was dissolved in 5 ml dichloromethane to which 0.15 ml DIPEA was added. The mixture was then stirred under Ar at rt for 0.5 h. Oleoyl chloride (0.15 ml) was added and the mixture was stirred for an additional 4 h at rt. The solution was subsequently poured into 50 ml ice water and washed with ice water three times (3 × 20 ml). The organic layer was dried over anhydrous Na₂SO₄ for 1 h. After the solvent was removed under vacuum, the crude product was purified by silica gel column chromatography with 5% methanol in dichloromethane. Bchl-BOA (30 mg, 0.024 mmol; see FIGURE 2G) was obtained in 63% yield in two steps.

Bchl-2 NH₂: Mass calculated for C₄₁H₅₆N₈O₅: 740.93. Found by ESI-MS: 741.5(M⁺).

Bchl-BOA: UV-Vis in CHCl₃ λ_{max}: 357, 386, 522 and 752 nm. Mass calculated for C₇₇H₁₂₀N₈O₇: 1269.83. Found by ESI-MS: 1270.2 (M⁺). ¹H NMR (CDCl₃, δ ppm): 9.31, 8.67 and 8.57 (each s, 3H for 5-H, 10-H and 20-H), 7.16, 6.36, 5.98 and 5.82 (each brs, 4H for 4 × NH), 5.31–5.15 (m, 6H, for 2 × CH = CH in oleoyl and 15¹-CH₂), 4.32 (brs, 1H, 18-H), 4.25 (brs, 1H, 7-H), 4.22 (brs, 1H, 17-H), 4.13 (brs, 1H, 8-H), 3.68 (s, 3H, 15-CO₂CH₃), 3.66 (m, 2H, 13³-NCH₂), 3.55 (s, 3H, 12-CH₃), 3.47 (m, 2H, 17⁵-NCH₂), 3.30 (s, 3H, 2-CH₃), 3.15 (s, 3H, 3-COCH₃), 2.91–2.83 (m, 4H, for 13⁵-CH₂N, 17⁷-CH₂N), 2.37 (m, 1H, 17¹-H), 2.08–1.94 (m, 25H, for 17¹-H, 13¹⁴-CH₂-CH=, 17¹⁶-CH₂-CH=, 13¹⁷-CH=CH₂, 13¹⁹-CH=CH₂, 13⁸-COCH₂, 17¹⁰ COCH₂, 17²-CH₂, 13¹²-CH₂, 13¹³-CH₂, 17¹⁴-CH₂, 17¹⁵-CH₂ and 8¹-CH₂), 1.82 (d, 3H, 18-CH₃), 1.61 (d, 3H, 7-CH₃), 1.51 (m, 2H, 13⁴-CH₂), 1.43 (m, 2H, 17⁶-CH₂), 1.23–1.17 (brs, 34H, other H in oleoyl), 1.08 (t, 3H, 8²-CH₃), 0.85 (m, 6H, 13²⁴-CH₃, 17²⁶-CH₃), −1.24 and −1.27 (each s, 1H, 2 × NH).

LDL reconstitution & characterization

Low-density lipoprotein was isolated from the plasma of healthy donors by sequential ultracentrifugation as described previously [21]. Bchl-BOA was reconstituted into LDL similarly to the method described by Krieger [15]. Specifically, 1.9 mg of dialyzed LDL was lyophilized with 25 mg starch and subsequently extracted three times with 5 ml of heptane at −10°C. Following the last heptane extraction, 0.6 mg of Bchl-BOA dissolved in 200 μl of benzene and 5.4 μl Triolein (Sigma) were added to the LDL. The sample was stored at 4°C for 90 min, after which benzene and residual heptane were removed under N₂ flow in an ice salt bath. The r-Bchl-BOA-LDL was dissolved in 1 ml of sterile 10 mM Tricine (pH: 8.2–8.4) at 4°C for approximately 24 h. Starch was removed from the solution by one low-speed centrifugation (2000 rpm for 10 min at 4°C) and two subsequent high-speed centrifugations (10,000 rpm for 10 min at 4°C). Reconstituted Bchl-BOA-LDL (r-Bchl-BOA-LDL) was stored under N₂ at 4°C until use. Reconstituted Bchl-CE-LDL (r-Bchl-CE-LDL) was prepared similarly. The Lowry assay was performed at OD_{500nm} to determine protein concentrations (Perkin-Elmer Lambda 2 spectrophotometer) [22]. To determine Bchl-BOA/CE concentration, the sample was added to a sodium chloride solution (500 μl total volume) and extracted with 1 ml of a chloroform/methanol mixture (2:1). The absorption spectra of Bchl-BOA/CE were subsequently measured (LS50B spectrofluorometer) and the concentrations were determined using a modification of Beer–Lambert's Law ($c = (A/\epsilon l) \times D$) where c is concentration of the probe, A is the OD_{754nm} value, l is the path length, D is the dilution fold and ϵ is the extinction coefficient (97,800 for Bchl-BOA/CE in methanol). Probe/protein molar ratios were calculated using the molecular mass of the solitary ApoB-100 protein (514 kDa) per LDL particle. Electron microscopy was utilized to measure native LDL, r-Bchl-BOA-LDL and r-Bchl-CE-LDL particle dimensions.

Murine model & PDT

The 6-week-old female athymic nude mice were purchased from NCI (Frederick, MD, USA). Animal studies were conducted in accordance with the guidelines established by the University of Pennsylvania (PA, USA). Cultured HepG₂ cells were trypsinized and resuspended in Hank's Balanced Salt Solution (HBSS) at 10⁸ cells/ml. Each mouse was inoculated on the left flank with 10⁷ HepG₂ cells. Experiments were initiated once tumors attained diameters of between 3.0 and 5.0 mm. PDT was performed using a custom-built 750 nm diode laser (laboratory of Britton Chance, University of Pennsylvania). At 3 h after intravenous administration of 2 µmole/kg of drug (via tail vein) 750nm light was delivered to a 1 cm diameter circular region of the tumor through a microlens-tipped fiber (CardioFocus, MA, USA). PDT was performed at light doses of 125–175 J/cm² in combination with a drug dose of 2 µmole/kg, based on the results of a pilot study that evaluated a broader range of light (75–200 J/cm²) and drug (0.5–2 µmole/kg) doses. All treatments were at a fluence rate of 75 mW/cm². Controls included mice receiving light alone (175 J/cm²), drug alone (2 µmole/kg) and no treatment. Prior to illumination mice were anesthetized with 100 µl of ketamine (10 mg/ml)/acepromazine (1 mg/ml).

Tumor response assay

The study end point was tumor volume doubling in comparison with its volume immediately before PDT. Tumor volume was measured in two orthogonal directions and calculated as $(\text{length} \times \text{width}^2 \times 3.14)/6$. Animals were followed for 60 days after PDT to determine the tumor volume doubling time. Any animals that did not experience tumor volume doubling by day 60 after PDT were censored in data analysis. For each group $3 \leq N \leq 8$.

Redox ratio comparison

A drug control mouse bearing a HepG₂ tumor on its left flank was injected via tail vein with 2 µmole/kg r-Bchl-BOA-LDL and sacrificed at 3 h. An untreated control mouse with a corresponding HepG₂ tumor was sacrificed in parallel. Sacrificed mice were immersed in precooled isopentane (−160°C) for 5 min. Subsequently, they were transferred to liquid N₂ (−196°C). Tumor, liver, muscle and kidney tissues were surgically excised. Tissues were embedded in a frozen ethanol/glycerol/water mixture and mounted prior to scanning. Samples were imaged by surface reflectance spectrophotometry with a raster scanner incorporated in a 3D Cryo-Imager (128 × 128 steps covering 1.024 × 1.024 cm²) while the light guide remained at a fixed distance from the surface (70 µm); the surface was then milled away to a depth of 100 µm and the imaging process was repeated until the entire sample had been imaged [23,24]. The fluorescent signals of flavoprotein (Fp; filters: Ex: 440DF20; Em: 525DF50), NADH (filters: Ex: 350HT25; Em: 455DF70) and r-Bchl-BOA-LDL (filters: Ex: 740DF25; Em: 780EFLP) were imaged for each depth, digitized and recorded on a PC. The redox ratio of NADH/(FP + NADH) was calculated using MATLAB.

Foot response assay

Mice without tumors were subjected to PDT on the left hind paw at the drug and light doses under investigation (2 µmole/kg drug and 125, 150 or 175 J/cm² light exposure delivered at 75 mW/cm²). Photosensitizer-free controls at the highest light dose (175 J/cm²) were included. Subsequently, mice were monitored for 1 month and morphology was scored based upon the established criteria displayed in TABLE 1 [1].

Statistics

Data were analyzed using Stata software (Stata Corporation, College Station, TX, USA). We used Cox regression to analyze the time-to-doubling outcomes. Observations were censored at 60 days. The Wald Chi-square was used for comparison of treatment effects. Controls

were tested against each other as were the various PDT doses and were combined if they were found not to vary significantly. We used the two-sample rank-sum test to analyze differences in the foot-toxicity assay because mice in one treatment group were all censored after reaching the maximum allowable response.

Results

Bchl-BOA & Bchl-CE synthesis

Bchl-CE was synthesized via a multistep procedure: Bchl was extracted from *R. sphaeroides* and was hydrolyzed in dilute HCl to generate the key starting material, Bchl-acid [19]. Esterification of Bchl-acid using diazomethane yielded bacteriopheophorbide a methyl ester in high yield. *N*-Boc-1,3-diaminopropane reacted with the 13-carbonyl of the β -keto ester, resulting in ring-opening to form an amide bond producing Bchl-BOC in 70% yield. After deprotection of the BOC group in TFA, the conversion of amino to isothiocyanato groups was performed with 1.1'-thiocarbonyldiimidazole in dichloromethane under reflux to produce Bchl-NCS. The overall yield for deprotection and transformation was approximately 65%. Bchl-CE was obtained in good yield by the reaction of Bchl-NCS with 5-androsten-17 β -amino-3 β -yl oleate [20] in dichloromethane in the presence of DIPEA. Bchl-BOA was synthesized as follows: Bchl-acid was reacted with *N*-Boc-1,3-diaminopropane in the presence of DMAP and DCC to produce Bchl-2 BOC in 72% yield. Deprotection of the BOC group generated Bchl-2 NH₂. Bchl-BOA was synthesized through the reaction of Bchl-2 NH₂ with oleoyl chloride in the presence of DIPEA in dichloromethane. The purity of the product was 96% by analytical HPLC and the overall yield for deprotection and acylation from Bchl-2 BOC was 63%.

LDL reconstitution & characterization

Based upon Lowry assay [22] measurements, protein concentrations for r-Bchl-BOA-LDL varied between approximately 0.65 and 0.85 mg/ml. The Bchl-BOA concentration of r-Bchl-BOA-LDL was generally approximately 70 μ M. An administered drug dose of 2 μ mole/kg refers to the amount of Bchl-BOA/kg. The probe/LDL ratio was 48:1 based upon the molecular mass of the ApoB protein (514 kDa), with one equivalent of protein per LDL particle. Reconstituted Bchl-CE-LDL exhibited a considerably lower probe/LDL ratio (~20:1). The average native LDL, r-Bchl-BOA-LDL and r-Bchl-CE-LDL particle sizes were 20 ± 2.7 nm, 30.9 ± 5.6 nm and 26.2 ± 4.8 nm, respectively. Based upon similar size measurements for the reconstituted particles and r-Bchl-BOA-LDL's higher probe/LDL ratio, r-Bchl-CE-LDL was excluded from further consideration.

Tumor response assay

In tumor response studies, we compared tumors treated with PDT at 125, 150 or 175 J/cm² and 2 μ mole/kg r-Bchl-BOA-LDL against untreated controls, controls treated with drug only (2 μ mole/kg) and controls only irradiated with light (175 J/cm²). All of these PDT treatments (drug + light) were effective in controlling tumor regrowth compared with the control groups ($p < 0.0001$). By day 12, there were no control animals remaining whose tumor volume had not doubled. Conversely, 100, 66.7 and 80% of mice treated with PDT at 125, 150 or 175 J/cm², respectively, demonstrated no doubling of tumor volume by day 60. Differences observed among PDT treatment groups were not statistically significant. Thus, these doses were highly effective and pilot studies found a further increase in light dose to 200 J/cm² to be accompanied by elevated toxicity. A nude mouse with a tumor xenograft is displayed in FIGURE 3 pre- and post-PDT treatment. Survival curves (time to doubling) for PDT treatments and control groups are depicted in FIGURE 4.

Redox ratio comparison

We utilized redox ratio imaging of mitochondrial function to investigate the dark toxicity of r-Bchl-BOA-LDL. Coenzymes NADH (reduced nicotinamide adenine dinucleotide) and FADH₂ (reduced flavin adenine dinucleotide) contribute electrons to generate a proton gradient across the mitochondrial inner membrane, resulting in ATP synthesis [24]. Consequently, since the relative intrinsic fluorescence levels of NADH and oxidized Fp are indicators of mitochondria metabolic activity, altered redox ratios derived from NADH and Fp fluorescence signals can signify tissue damage [25]. Specifically, the mitochondrial reduction ratio, NADH/(Fp + NADH), which reflects the concentration of reducing equivalents in the mitochondria, was calculated. This approach exerts less stringent demands upon the instrumentation and reduces extraneous pigment interference when compared with the measurement of absolute values [26]. FIGURE 5A–5H depicts mitochondrial redox ratio images in specified tissue samples for the untreated and r-Bchl-BOA-LDL drug-only control mice, along with the corresponding histograms.

Redox ratios generated for the specified tissue types investigated in HepG₂ tumor-bearing mice indicate that there are no substantial metabolic differences between untreated and drug-only (2 μ mole/kg r-Bchl-BOA-LDL) controls at 3 h postinjection (TABLE 2). This absence of effect observed between the controls is further validated by the demonstration that PDT-induced damage is readily detected by redox ratio [25]. Zhang *et al.* have shown that pyropheophorbide-2-deoxyglucosamide-induced PDT elevated singlet oxygen production and generated a highly oxidized state of the mitochondria as reflected in the high Fp redox ratios [25]. This suggests that r-Bchl-BOA-LDL alone is not acutely toxic and that the tumoricidal effect observed only occurs when r-Bchl-BOA-LDL is combined with the appropriate light dose.

Foot response assay

The phototoxicity of r-Bchl-BOA-LDL to normal tissues was investigated using the foot response assay [1] modified for our treatment conditions. This assay was performed to determine whether differences exist in normal tissue toxicity despite similar tumor response findings. Focus was placed on the lowest fluences because of the efficacy of 125 J/cm² in producing tumor response. Mice were entered into PDT-treatment groups as shown in FIGURE 6. Morphology of the treated left hind paw was rated over approximately 30 days according to the established protocol [1]. PDT at the lowest fluence of 125 J/cm² produced a morphological change with a corresponding average maximum value ($\pm$ SE) of 1.47 ± 0.27 . This was a significantly ($p < 0.05$) less severe response than that resulting from treatment at 150 J/cm² (morphological damage rating of 2.5). Importantly, all of the mice treated at 150 J/cm² developed severe normal tissue damage (a score of 2.5) that required euthanasia, whereas none of the animals treated with 125 J/cm² developed a score above 2. Two animals were also treated at the highest fluence of 175 J/cm² with one demonstrating an unexpected death at several weeks after PDT (foot response score of 0.75 at the time of death) and the second demonstrating severe tissue damage that required euthanasia. Animals treated as light-only controls exhibited no evidence of foot damage over the time course of this study.

Discussion

Owing to the need to improve targeting in PDT, efforts to develop effective third-generation photosensitizers that are more specific to the tissue of interest are underway. Antibodies, LDL and synthetic nanodevices are currently under investigation as putative drug targeting approaches [16,17,27]. Although each modality of drug delivery shows promise, each has limitations. For instance, antibody distortion induced upon photosensitizer binding may prohibit requisite binding of vehicle to the antigen [28]. In addition, synthetic nanodevices

often induce immunogenicity [17]. Conversely, reconstituted LDL and native LDL demonstrate similar capacities to bind LDLR and become internalized [16]. Furthermore, since lipoproteins are endogenous carriers in the blood, they are not expected to be detected by the human immune system and avoid reticuloendothelial system absorption [17]. However, lipoprotein isolation from human blood raises concerns about blood borne pathogen introduction and development of diseases such as hepatitis or AIDS [11]. Commercialized sources of pathogen-free blood proteins could help alleviate such concerns [11]. Alternatively, one could use high-density lipoprotein, which can be prepared in recombinant form. Based upon the advantages of LDL over competing drug-delivery vehicles such as antibodies and synthetic nanodevices, we have chosen to incorporate Bchl-BOA into LDL and investigate its PDT efficacy using a murine model. In addition, the Bchl fluorophore in Bchl-BOA has strong NIR absorption at 748 nm, helping ensure that the photosensitizer, rather than endogenous chromophores, is the primary absorber [29]. For our studies, we elected to use the more lipophilic Bchl-BOA compound rather than the Bchl-CE candidate, which demonstrated a reduced LDL payload compared with Bchl-BOA (20:1 vs 48:1). Preliminary biodistribution analysis in the murine model has demonstrated that r-Bchl-BOA-LDL is preferentially internalized by HepG₂ tumor cells overexpressing LDLR. At 3 h postintravenous administration of 1 μ mole/kg r-Bchl-BOA-LDL, fluorescence measurements in harvested tissue indicated that the probe concentration tumor to muscle ratio was 30:1.

Our research demonstrates that across a range of fluences, our photosensitizer, r-Bchl-BOA-LDL, significantly delayed tumor regrowth compared with averaged controls (untreated, 175 J/cm² only and 2 μ mole/kg r-Bchl-BOA-LDL alone). Among the three PDT treatments that were evaluated (2 μ mole/kg r-Bchl-BOA-LDL and 125, 150 or 175 J/cm²), tumor regrowth delay was not significantly different. Although the drug is targeted, we speculate that residual drug in the plasma may exert vascular effects, thus accounting for the morphological differences observed over the range of fluences (125–175 J/cm²) in our foot response assay. Our drug–light interval was based upon preliminary pharmacokinetic studies that demonstrated substantial tumor accumulation of Bchl-BOA-LDL at 3 h postinjection with a return to baseline levels 24 h after injection. However, blood clearance studies indicate that circulating probe remained at approximately 35% of maximum levels 2–6 h postinjection. Additional pharmacokinetic analysis is required to determine the optimal drug–light interval whereby selective tumor toxicity is maximized while damage to normal tissue is minimized. However, these results establish that r-Bchl-BOA-LDL is effective as a photosensitizer at reasonable drug and light doses and thus deserves further consideration as a third-generation photosensitizer.

Conclusion & future perspective

Tailored personalized therapy is revolutionizing clinical nanomedicine. Nanocarrier delivery systems have been designed to target compounds to cancer cells [8, 9]. Specifically, LDL offers great promise as an effective nanocarrier in the treatment of cancers because LDLR expression is elevated in numerous malignancies, including colon, adrenal, prostate and breast cancers [30]. Also, LDL can be retargeted to folate and other receptors that are more specific to certain forms of human cancer [17]. In addition, LDL is biocompatible, biodegradable and nonimmunogenic [30]. Bacteriochlorin e6 bisoleate was incorporated into LDL to generate the effective photosensitizer r-Bchl-BOA-LDL. Although the nanoparticle has an established payload of approximately 50:1, further investigation is warranted to elucidate immunogenicity issues as well as pharmacokinetics, including drug stability and biodistribution. Specifically, drug leakage, previously observed in lipid-based carriers, should be examined, although it is speculated that the bisoleate moiety in r-Bchl-BOA-LDL confers lipid-anchoring [30]. In addition, the liver, adrenal and reproductive organs express

elevated LDLR, although such expression in these tissues may be modulated by steroids or dietary fats [30]. Identifying the optimal drug–light interval whereby selective tumor toxicity is maximized and normal tissue damage is minimized is the requisite next step in determining r-Bchl-BOA-LDL's capacity as a broad spectrum PDT agent. We speculate that the advent of such targeted nanoparticles will radically transform the exciting, new frontier of personalized medicine.

Executive summary

- Bacteriochlorin e6 bisoleate was encapsulated in low-density lipoprotein (LDL). A payload of approximately 50:1 was achieved.
- A murine model expressing the human hepatoblastoma G₂ (HepG₂) xenograft was chosen to evaluate the photodynamic therapeutic potential of r-Bchl-BOA-LDL.
- Photodynamic therapy (PDT) at 2 μ mole/kg r-Bchl-BOA-LDL and 125, 150 or 175 J/cm² significantly delayed tumor regrowth compared with controls.
- No dark toxicity was evident under redox ratio imaging while the foot response assay demonstrated that phototoxicity was significantly reduced at the lowest fluence tested.
- Reconstituted Bchl-BOA-LDL is an effective photosensitizer whose capacity as a broad spectrum PDT agent warrants further investigation.

Acknowledgments

We are grateful to Tuoxiu Bleu Zhong for her invaluable redox scan assistance. Additionally, we extend our sincere thanks to Lin Li for manuscript assistance. Christopher Marotta is appreciated for his continued support.

This research was supported by NIH grants N01C037119 (Gang Zheng) and R01CA085831 (Theresa Busch).

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Figure 1. The lipoprotein-based nanoplatform concept for targeted drug delivery
Reconstituted low-density lipoprotein core labeled with Bchl-BOA is depicted along with the solitary ApoB-100 protein and phospholipid monolayer.

Figure 2. Synthesis of bacteriochlorophyll analogs suitable for low-density lipoprotein reconstitution

(A) Bchl-acid. (B) Bchl-Meester. (C) Bchl-BOC. (D) Bchl-NCS. (E) Bchl-CE. (F) Bchl-2 BOC. (G) Bchl-BOA. (i) CH_2N_2 , dichloromethane, room temperature (rt) 1 h, 85%. (ii) *N*-Boc-1,3-diaminopropane, CHCl_3 , reflux/Ar, 48 h, 70%. (iii) Trifluoroacetic acid (TFA), rt/Ar, 1 h, 86%. (iv) 1,1'-thiocarbonyldiimidazole, dichloromethane, reflux/Ar, 4 h, 75%. (v) 5-androsten-17 β -amino-3 β -yl oleate, dichloromethane, diisopropylethylamine, rt/Ar, 24 h, 86%. (vi) *N*-Boc-1,3-diaminopropane, 4-*N,N*-dimethylaminopyridine, dicyclohexylcarbodiimide, dichloromethane/Ar, 40 h, 72%. (vii) TFA, rt/Ar, 1 h. (viii) oleoyl chloride, dichloromethane, rt/Ar, 1 h, 63% (vii, viii).

Figure 3. HepG₂-tumored mice pre- and post-photodynamic therapy
(A) Nude mouse bearing a tumor on its left flank. (B) 24 h following photodynamic therapy (PDT; 150 J/cm², 2 μ mole/kg Bchl-BOA-LDL), characteristic burn/edema was visible over tumor while (C) scab formation was observed 9 days post-PDT. (D) Complete tumor ablation (arrow) was seen at 60 days post-PDT.

Figure 4. Survival curves for photodynamic therapy-treated and control groups

PDT conditions evaluated are 125, 150 or 175 J/cm² and 2 μmole/kg r-Bchl-BOA-LDL. Survival for untreated, light only (175 J/cm²) and drug alone (2 μmole/kg r-Bchl-BOA-LDL) controls is also displayed. PDT treatments were significantly more effective in controlling tumor regrowth compared with control groups.

*p < 0.0001. For all groups, 3 ≤ N ≤ 8.

PDT: Photodynamic therapy.

Figure 5. Redox ratios for drug control and untreated mice

Redox ratios of HepG₂ tumor tissue for (A) drug control and (B) untreated mice. Redox ratios of liver tissue for (C) drug control and (D) untreated mice. Redox ratios of muscle tissue for (E) drug control and (F) untreated mice. Redox ratios of kidney tissue for (G) drug control and (H) untreated mice.

Fp: Flavoprotein.

Figure 6. Foot response assay

Average maximum morphology scores are shown for photodynamic therapy (PDT) at 125 and 150 J/cm² (n = 3). PDT at 125 J/cm² displayed a significantly lower average maximum morphology score compared with PDT at 175 J/cm². *p < 0.05. The light control (175 J/cm²) demonstrated no morphological change.

Table 1

Foot response rating system.

Numerical rating	Appearance
0	No apparent difference from normal
0.1	Very slight edema and/or erythema
0.3	Slight edema and/or erythema
0.5	Moderate edema and/or erythema
0.75	Large edema and/or erythema
1.0	Large edema and/or erythema, with exudate
1.2	Erythema with slight scaly or crusty appearance
1.5	Erythema with definite scaly or crusty appearance
1.65	Slight damage to toes
1.75	Definite damage and/or slight fusion of toes
2.0	Most toes fused but general shape unchanged
2.5 [†]	Foot almost shapeless, with only stubs of toes present

[†] Mice were euthanized at a score of 2.5.

Reproduced with permission from [1].

Table 2

Redox ratio comparison across tissue types in the absence and presence of r-Bchl-BOA-LDL.

r-Bchl-BOA-LDL	Tumor	Liver	Muscle	Kidney
+	0.58	0.74	0.63	0.55
–	0.6	0.77	0.61	0.63

NADH/(flavoprotein + NADH) redox ratios, indicating the reduced state of the mitochondria, are shown for tumor, liver, muscle and kidney in the presence (+) and absence (–) of r-Bchl-BOA-LDL.

LDL: Low-density lipoprotein.