Synthesis, Characterization, and Plasma Lipoprotein Association of a Nucleus-Targeted Boronated Porphyrin

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Abstract: The efficacy of binary cancer therapies such as BNCT and PDT depends critically on the subcellular localization site of the sensitizer. This work presents the synthesis and plasma lipoprotein binding properties of the first reported binary conjugate of a boronated porphyrin with a peptide nuclear localization sequence. The porphyrin−NLS conjugate associates in vitro predominantly with low density lipoproteins. Such association provides a potentially selective entry pathway into malignant cells that overexpress the LDL receptor.

One of the distinctive hallmarks of binary cancer therapies such as boron neutron capture therapy (BNCT) and photodynamic therapy (PDT) is the selectivity they afford. Only cells that take up the sensitizer will be killed when the sensitizer is activated, either by thermal neutrons (BNCT) or by visible light (PDT), because the resulting cytotoxic products remain largely within the cellular target. In both BNCT and particularly in PDT, the subcellular site of the sensitizer is of critical importance.[1] Currently available BNCT agents localize in a variety of cell organelles, but none is known to localize selectively in the most exquisitely sensitive target of all, the cancer cell nucleus. For example, the boronated protoporphyrin BOPP localizes in both the mitochondria and lysosomal compartments of cancer cells, but not in the nucleus.[2,3] Hawthorne et al. have found nuclear uptake of boronated oligomeric phosphates in TC7 cells after either microinjection or permeabilization of the plasma membrane, but these techniques do not lend themselves to reproduction in vivo.[4] None of the currently available photosensitizers, including both clinically approved and preclinical drugs, is known to localize in the nucleus. Creation of a molecule of singlet oxygen in close proximity to the cancer cell DNA would presumably increase dramatically the odds of cell death whether by induction of apoptosis or by necrosis. Thus, it seems that a logical goal of drug design for both binary therapies would be a general method for nuclear sensitizer delivery.

The nuclear targeting of proteins and other macromolecules has been extensively studied and reviewed.[5,6] All passive and active transport in to and out of the nucleus takes place through the nuclear pore complexes in the nuclear membrane, which acts much like a molecular sieve. Molecules smaller than ∼45 kDa can in principle diffuse freely, while larger species require the presence of peptide nuclear localization sequences (NLS) to gain entry into the interior of the nuclear membrane. Heitz and co-workers have shown a 100-fold increase in DNA cleavage in tumor cell culture by a manganese(III) porphyrin−NLS conjugate over the metalloporphyrin itself.[7] This finding suggests that the nuclear localization and drug efficacy of even small molecule constructs can be dramatically increased using NLS targeting. Although there has been widespread study of this strategy for the nuclear delivery of large molecules (e.g. peptides, oligonucleotides, peptide nucleic acids, nanoparticles), relatively little advantage of this process has been taken for therapeutic small molecule or sensitizer delivery. Sobolev and Jans have prepared high molecular weight constructs of bovine serum albumin, insulin, and the photosensitizer chlorin e6.[8,9] Endocytosis and localization of the conjugate into and around the nucleus of human hepatoma cells was observed visually using fluorescence labeling techniques. When these cells were irradiated with light, the photodynamic efficacy brought about by nuclear delivery of the conjugate was enhanced by more than 2000-fold over chlorin e6 alone. Although impressive, there are several drawbacks to the use of such a construct. Aside from their complexity, the most critical problem is lack of target cell selectivity. A simpler conjugate carrier is required with the ability to associate itself with some tumor specific entity. For this latter function, we have selected low-density lipoproteins (LDL) since the LDL receptor (LDLR) is up-regulated in many tumors, including gliomas.[10]

There is substantial experimental evidence that amphiphilic sensitizers on administration become associated with plasma lipoproteins, particularly LDL, and, to a lesser extent, high-density lipoprotein (HDL).[11] The model suggests that the hydrophobic portion of the molecule interacts with the cholesterol ester-rich interior of the LDL particle, while the hydrophilic end remains at the phospholipid surface. This noncovalent association is responsible for the resulting passive intracellular uptake in a "Trojan horse" fashion that is initiated when the apo-B100 protein of LDL binds to the LDLR on the cell surface. Internalization of the complex and transport to the lysosomal compartment introduces the porphyrin to the cytoplasm. BOPP uptake into SF-767 human glioma cells is mainly dependent upon the LDL pathway and is significantly reduced when LDL is removed from the incubation medium, supporting the role of LDL as its transporter.[3] Seven distinct human glioma cell lines show significant upregulation of the LDLR relative to normal fibroblasts.[10] The absence of LDLR expression in normal adult glial cells provides the possibility for utilizing the LDL-LDLR pathway for the selective delivery of sensitizers for BNCT and PDT. In this communication, we describe the synthesis of a new boronated porphyrin, its fusion with a peptide NLS, and evidence that the resulting conjugate associates with human lipoproteins.

The boronated porphyrin portion of the synthesis is shown in Scheme 1. Catalytic hydroxylation of the two

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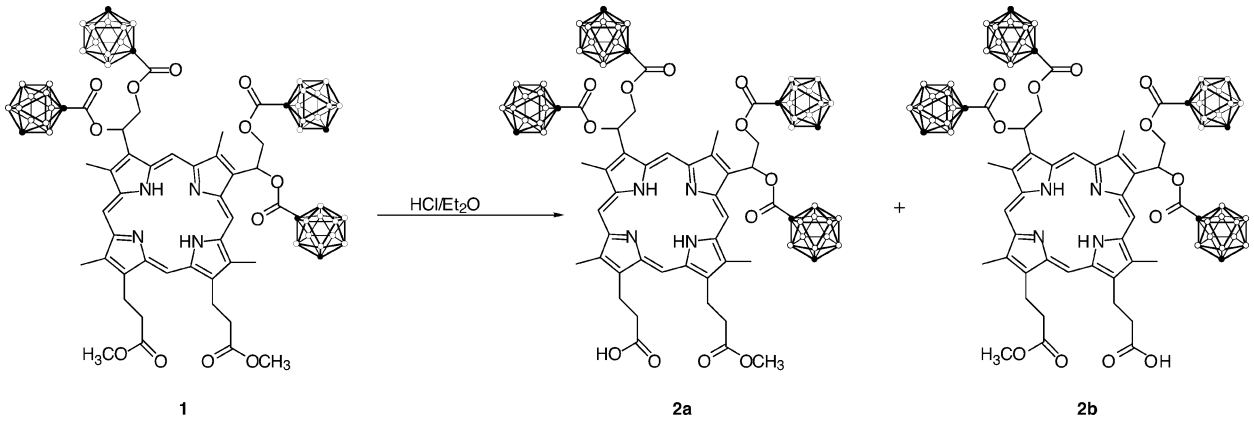
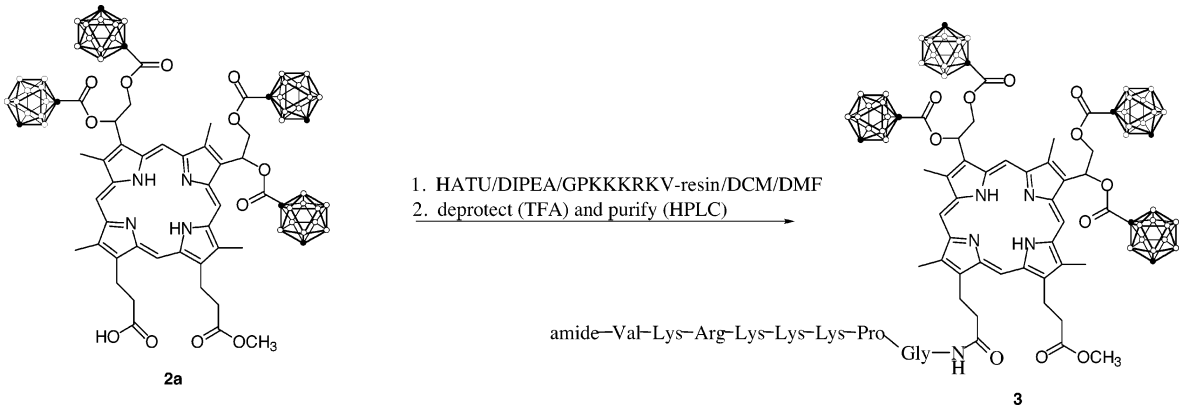
Scheme 1. Synthesis of Isomeric *m*-BOPP Monoacids



Scheme 2. Synthesis of *m*-BOPP−NLS Conjugate



vinyl groups of protoporphyrin IX dimethyl ester proceeded in high yield to give the bis-glycol (not shown).[12] Acylation of this porphyrin tetraalcohol with *m*-carborane carbonyl chloride in dry methylene chloride in the presence of 4-(dimethylamino)pyridine (DMAP) efficiently provided the *m*-carborane analogue **1** of BOPP dimethyl ester. *m*-Carborane was chosen in preference to the ortho isomer due to its greater lipophilicity and superior resistance of its α-carbonyl derivatives to base-assisted cage-opening reactions.[13] The resultant tetracarboranyl porphyrin showed a typical etio-type porphyrin visible absorption spectrum with a Soret band at 404 nm and Q-band peaks at 500, 534, 570, and 624 nm in CH_2Cl_2. The molecular ion envelope in the mass spectrum of **1** (centered at *m/e* = 1340.6 for MH^+) confirmed the presence of the boron cages and matched the shape calculated for a compound with the formula $C_{48}H_{82}N_4O_{12}B_{40}$. This dimethyl ester, which we call *m*-BOPP to distinguish it from BOPP, was selectively deprotected to monoacid monoester isomers **2a** and **2b** by careful treatment with 25% hydrochloric acid in ether (Scheme 1). Isomeric monoacids **2a** and **2b** are readily separated from the unreacted diester and the completely hydrolyzed diacid, but can be separated from each other only with difficulty. This was done to provide analytical samples of each isomer, but since the association of the porphyrin−NLS conjugate with LDL was assumed to be nonstereoselective, the isomeric porphyrin mixture of **2a** and **2b** was used for linkage to the peptide NLS sequence. Porphyrin isomers **2a** and **2b** have identical UV−visible spectra which are also identical to diester **1**, as expected. Their mass spectra are also very similar to each other and show a molecular ion MH^+ cluster centered at *m/e* = 1326.9 corresponding to the expected formula $C_{47}H_{80}N_4O_{12}B_{40}$.

Several peptide sequences have nuclear localizing properties. The SV40 large T antigen NLS was selected for its brevity and high content of charged (basic) amino acids (PKKKRKV) to enhance aqueous solubility. A glycine residue was added at the amino terminus to act as a spacer between the porphyrin and peptide. The protected sequence was prepared on a Gilson synthesizer using FMOC chemistry and Rink amide resin. The boronated porphyrin was coupled to the resin-bound peptide off-column using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′*N*′-tetramethyluronium hexafluorophosphate (HATU) and diisopropylamine as shown in Scheme 2. Coupling efficiency was monitored by ninhydrin Kaiser test. Cleavage from the resin with trifluoroacetic acid (TFA) followed by reverse phase HPLC purification gave the desired porphyrin−NLS conjugate **3**. The deconvoluted ESI mass spectrum of **3** showed a parent ion peak at 2246.6 compared to the predicted mass of 2246.63 and corresponding to the expected formula of $C_{89}H_{161}B_{40}N_{20}O_{19}$. Also observed were peaks at 1123.8, 749.9, and 562.9 corresponding to masses of $(m + 2)/2$, $(m + 3)/3$, and $(m + 4)/4$, respectively. As expected, the visible spectrum of the *m*-BOPP−NLS conjugate was nearly identical with that of the precursor porphyrins **1** and **2** with a Soret band at 402 nm and Q-bands at 504, 536, 572, and 624 nm in CH_2Cl_2/CH_3OH solution. This compound has pronounced amphiphilic character with substantial solubility in both water and ether. To our knowledge, it represents the first reported example of a porphyrin−NLS conjugate in which the peptide NLS is directly bound to the porphyrin.

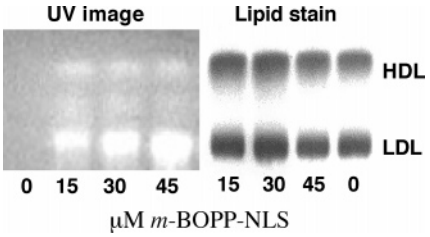


Figure 1. Agarose gel electrophoresis using Paragon Lipogels indicates that the *m*-BOPP−NLS conjugate associates with LDL. Human plasma was incubated with increasing amounts of conjugate and applied to the gel. After electrophoresis, the gel was cut in half and one-half stained with Sudan black lipid stain to visualize the lipoprotein components (right) and the other half examined under UV to visualize the distribution of the *m*-BOPP−NLS (left).

Central to our working hypothesis is the noncovalent association between the porphyrin−NLS conjugate and LDL. To test this, aqueous solutions of *m*-BOPP−NLS conjugate **3** were incubated with normal human plasma for 30 min at 37 °C in the dark. For identification of the lipoproteins, the samples were then applied to Paragon Lipogels for electrophoresis (Figure 1). One-half of the gel was used for UV examination of the electrophoretically separated lipoproteins (left) and the other half was lipid stained to identify the positions of LDL and HDL (right). The *m*-BOPP−NLS is mostly associated with the LDL with only a weak signal associated with HDL. Densitometric scans of the fluorescent bands indicate that at 30 μM and 45 μM *m*-BOPP−NLS there was a 1.4-fold and 2.1-fold increase in UV intensity of LDL, respectively, over 15 μM compound, suggesting a dose-dependent increase in fluorescence. Importantly, as seen in the lipid-stained profiles, association of the porphyrin−NLS conjugate with these lipoproteins does not alter their electrophoretic mobility as would be expected if the association appreciably changes the surface charge characteristics of the lipoproteins.

In conclusion, we have prepared and characterized a simple construct composed of 1 mol each of a binary sensitizer and a peptide nuclear localization sequence. This conjugate associates noncovalently with lipoproteins, which retain their electrophoretic mobilities. In vitro experiments in human glioma cells are currently underway to determine if the porphyrin−NLS conjugate localizes intracellularly by an LDL receptor-dependent mechanism and to determine the precise subcellular location of the porphyrin fluorescent signal. This simple sensitizer−NLS complex may prove to be a template for a general method of selectively targeting sensitizers to the malignant cell nucleus.

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Supporting Information Available: Materials, instrumentation used, synthetic procedures, and further spectral data for all compounds (^{1}H NMR, ^{13}C NMR, UV−vis, MS-ESI, and MS-MALDI). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Moan, J.; Berg, K. The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. *Photochem. Photobiol.* **1991**, *53*, 549−553.
(2) Hill, J. S.; Kahl, S. B.; Kaye, A. H.; Stylli, S. S.; Koo, M.-S.; Gonzales, M. F.; Vardaxis, N. J.; Johnson, C. I. Selective tumor uptake of a boronated porphyrin in an animal model of cerebral glioma. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 1785−1789.
(3) Callahan, D. E.; Forte, T. M.; Afzal, S. M. J.; Deen, D. F.; Kahl, S. B.; Bjornstad, K. A.; Bauer, W. F.; Blakely, E. A. Boronated protoporphyrin (BOPP): localization in lysosomes of the human glioma cell line SF-767 with uptake modulated by lipoprotein levels. *Int. J. Radiation Oncol. Biol. Phys.* **1999**, *45*, 761−771.
(4) Nakanishi, A.; Guan, L.; Kane, R. R.; Kasamatsu, H.; Hawthorne, M. F. Toward a cancer therapy with boron-rich phosphate diesters that target the nucleus. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 238−241.
(5) Rosenkranz, A. A.; Jans, D. A.; Sobolev, A. S. Targeted intracellular delivery of photosensitizers to enhance photodynamic efficiency. *Immunol. Cell Biol.* **2000**, *78*, 452−464.
(6) Sobolev, A. S.; Jans, D. A.; Rosenkranz, A. A. Targeted intracellular delivery of photosensitizers. *Prog. Biophys. Mol. Biol.* **2000**, *73*, 51−90.
(7) Chaloin, L.; Bigey, P.; Loup, C.; Marin, M.; Galeotti, N.; Piechaczyk, M.; Heitz, F.; Meunier, B. Improvement of porphyrin cellular delivery and activity by conjugation to a carrier peptide. *Bioconjugate Chem.* **2001**, *12*, 691−700.
(8) Akhlynina, T. V.; Rosenkranz, A. A.; Jans, D. A.; Sobolev, A. S. Insulin-mediated intracellular targeting enhances the photodynamic activity of chlorin-e6. *Cancer Res.* **1995**, *55*, 1014−1019.
(9) Akhlynina, T. V.; Jans, D. A.; Statsyuk, N. V.; Balashova, I. Y.; Toth, G.; Pavo, I.; Rosenkranz, A. A.; Naroditsky, B. S.; Sobolev, A. S. Adenoviruses synergize with nuclear localization signals to enhance nuclear delivery and photodynamic action of internalizabale conjugates containing chlorin e6. *Int. J. Cancer* **1999**, *81*, 734−740.
(10) Maletinska, L.; Blakely, E. A.; Bjornstad, K. A.; Deen, D. F.; Knoff, L. J.; Forte, T. M. Human glioblastoma cell lines: levels of low-density lipoprotein and low-density lipoprotein-related protein. *Cancer Res.* **2000**, *60*, 2300−2303.
(11) Boyle, R. W.; Dolphin, D. Structure and biodistribution relationships of photosensitizers. *Photochem. Photobiol.* **1996**, *64*, 469−485.
(12) Kahl, S. B.; Schaeck, J. J.; Koo, M.-S. Improved methods for the synthesis of porphyrin alcohols and aldehydes from protoporphyrin IX dimethyl ester and their further modification. *J. Org. Chem.* **1997**, *62*, 1875−1880.
(13) Schaeck, J. J.; Kahl, S. B. Rapid cage degradation of 1-formyl- and 1-alkyloxycarbonyl-substituted 1,2-dicarba-*closo*-dodecaboranes by water or methanol in polar organic solvents. *Inorg. Chem.* **1999**, *38*, 204−206.

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