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Targeting gastrin-releasing peptide receptors of prostate cancer cells for photodynamic therapy with a phthalocyanine–bombesin conjugate

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Abstract—Sulfonated aluminum phthalocyanines (AlPcS) are potent photosensitizers for the photodynamic therapy (PDT) of cancer. In this study we evaluate the possibility to improve the efficacy of AlPcS-PDT for prostate cancer by targeting tetrasulfonated aluminum phthalocyanines ($AlPcS_4$) to the gastrin-releasing peptide receptor (GRPR) through coupling to bombesin. A mono-carbohexyl derivative of $AlPcS_4$ is attached to 8-Aoc-bombesin(7–14)NH_2 via an amide bridge to yield a bombesin–$AlPcS_4$ conjugate linked by a C-14 spacer chain. The conjugate is characterized by mass spectroscopy and shown to bind to the GRPR with a relative binding affinity (RBA) of 2.3, taking bombesin (RBA = 100) as unity. The in vitro photodynamic efficacy of the conjugate against PC-3 human prostate cancer cells is improved by a factor 2.5 over the non-conjugated mono-carbohexyl derivative of $AlPcS_4$.
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Photodynamic therapy (PDT) of cancer involves systemic administration of a photosensitizer followed by red-light activation of the diseased tissue. The activated photosensitizer interacts with ground state molecular oxygen to yield activated oxygen species (ROS) including singlet oxygen and different radical species. The resulting oxidative stress initiates a cascade of biochemical reactions resulting in cell death either by direct cell kill or by destruction of the micro vasculature of the tumor.[1] Selective retention of the photosensitizer in the tumor tissue combined with local illumination thus induces tumor regression without affecting the surrounding healthy tissues. Problems associated with photosensitizers presently approved for clinical use, such as Photofrin®, include lack of chemical homogeneity, non-optimal light absorbing properties, prolonged retention in non-target tissues such as the skin and low selectivity for tumor tissues.[2]

Targeting photosensitizers as receptor mediated delivery systems has received increased attention as a means to improve the outcome of PDT.[3,4]

Sulfonated aluminum phthalocyanines (AlPcS) are water soluble, second generation photosensitizers that exhibit a strong absorption band between 660 and 700 nm with a peak at 680 nm, where tissue penetration is optimal. A mixture of mono- through tetrasulfonated aluminum phthalocyanines has been used for over a decade for clinical PDT of cancer in Russia.[5] The negatively charged peripheral sulfonate groups assure water solubility, while the chelated central aluminum ion provides the complex with long-lived excited triplet states and good capacity to generate reactive singlet oxygen. We have previously shown that the tetrasulfonated analog, that is, $AlPcS_4$, can be modified with a free C6-carboxyl sulfonamide side-chain ($AlPcS_4A_1$), allowing facile amide-coupling to free amine groups of proteins and peptides.[6,7]

Bombesin, first discovered in skin of the frog Bombina bombina, and its human counterpart, gastrin-releasing

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peptide (GRP), are neurohormones with a wide range of biological effects such as stimulation of hormone secretion, memory retention, body temperature, cardiac output, blood pressure, control of food intake and changes in the dorsal vagal complex neuron.[8,9] Bombesin also has been shown to affect tumor growth, cell proliferation and inflammation.[10] In the case of prostate cancer, the receptor subtype GRPR is expressed early in tumor development and is correlated with tumor aggressiveness.[11] Therefore, GRPR is an obvious target for the development of novel agents for treatment and diagnosis of prostate cancer.[12] In this study we evaluate the potential to improve the efficacy of phthalocyanine-based PDT of prostate cancer by targeting the photosensitizer to GRPR using 8-Aoc-bombesin(7–14)NH_2, a potent bombesin analog previously developed by Hoffman.[13]

The preparation of the bombesin–$AlPcS_4$ conjugate (Scheme 1) involves the following steps.[14] A solution of 8-Aoc-bombesin(7–14)NH_2 (1.8 mg, 1 μmol) in 1 mL of sodium carbonate (0.2 M, pH 8.5) was added to the lyophilized, carbodiimide activated aluminum mono-(6-carboxypentylamino-sulfonyl)-trisulfophthalocyanine ($AlPcS_4A_1$) (5 μmol, 5 equiv) and stirred 1 h at 25 °C while the pH was maintained at 8.5. The reaction mixture was kept in the dark at 4 °C overnight. The bombesin–$AlPcS_4$ conjugate was purified by HPLC on a spherisorb ODS-2 column and characterized by MALDI-MS: $C_{89}H_{107}O_{23}N_{23}S_5Al$, found (M–$H_2O$): 2035.09; calcd.: 2034.62.

Competition assays for GRPR binding in PC-3 human prostate tumor cells against $^{125}I[Tyr^4]$-bombesin were performed as described in the literature.[15] Bombesin and 8-Aoc-bombesin(7–14)NH_2 show similar strong binding affinity for the GRPR with EC_{50} of 2.58×10^{-10} M and 3.73×10^{-10} M, respectively. These values correspond to dissociation coefficients (K_d) of 2.17×10^{-10} and 3.33×10^{-10}, respectively, and are in good agreement with reported literature values (1.10×10^{-10} and 4.90×10^{-10}, respectively).[16,17] In the same competition assay, binding affinity of the bombesin–$AlPcS_4$ conjugate also shows a characteristic sigmoid curve clearly indicating competition with $^{125}I[Tyr^4]$-bombesin, confirming retention of binding affinity for GRPR (Fig. 1). However, the lower EC_{50} of 2.94×10^{-8} M (K_d 2.9×10^{-8}) indicates loss of specificity, which is reflected in a lower relative binding affinity (RBA = 2.3), taking bombesin as unity (RBA = 100).

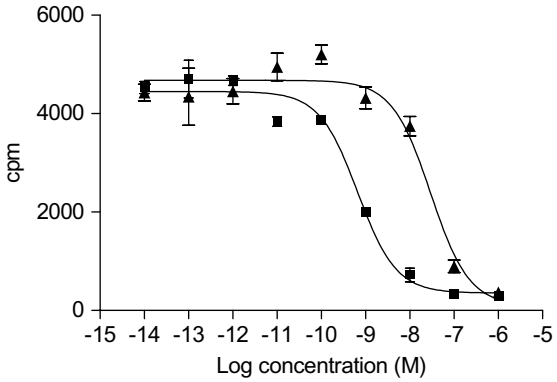
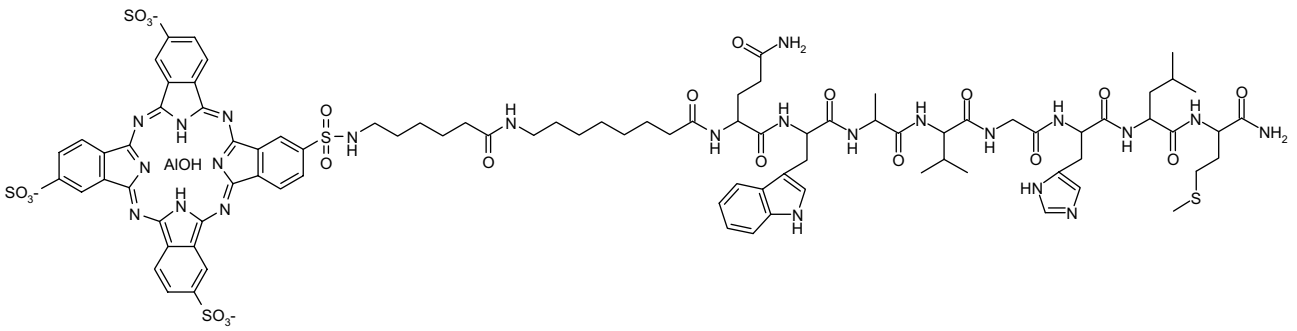


Figure 1. Competitive binding assay of the $AlPcS_4$–bombesin conjugate (▲) and bombesin (■) versus ^{125}I-Tyr4-bombesin in PC-3 cells.

Cellular phototoxicity was evaluated in PC-3 human prostate tumor cells.[18] Cell metabolic activity curves after incubation with free and conjugated photosensitizer in concentrations ranging from 1 to 20 μM as a function of red-light fluences are presented in Figure 2. In all cases, conjugated bombesin–$AlPcS_4$ exhibits a slightly higher phototoxicity than the free $AlPcS_4A_1$ and a substantial higher phototoxicity when compared to the non-substituted $AlPcS_4$. These differences in efficacy are more apparent when the LD_{50} and LC_{50} values, derived from the cell survival curves, are plotted against photosensitizer concentration and light dose, respectively (Fig. 3). The latter reveal a significant two- to threefold increase in photodynamic efficacy of conjugated over free $AlPcS_4A_1$ or $AlPcS_4$ at lower drug and/or light doses. At 3 J/cm^2 the conjugate requires 2.5 and >5 times less photosensitizer to inflict 50% cell inactivation as compared to the non-conjugated $AlPcS_4A_1$ and free $AlPcS_4$, respectively. It should be noted that mono-carbohexyl substituted $AlPcS_4A_1$ already exhibits significantly higher phototoxicity than the non-substituted $AlPcS_4$ reflecting an increase in amphiphilic nature of the latter. Differences in the aggregation state of $AlPcS_4$ in the three photosensitizer preparations do not appear to play a role in the observed differences in phototoxicity.



Scheme 1. Structure of the bombesin–$AlPcS_4$ conjugate.

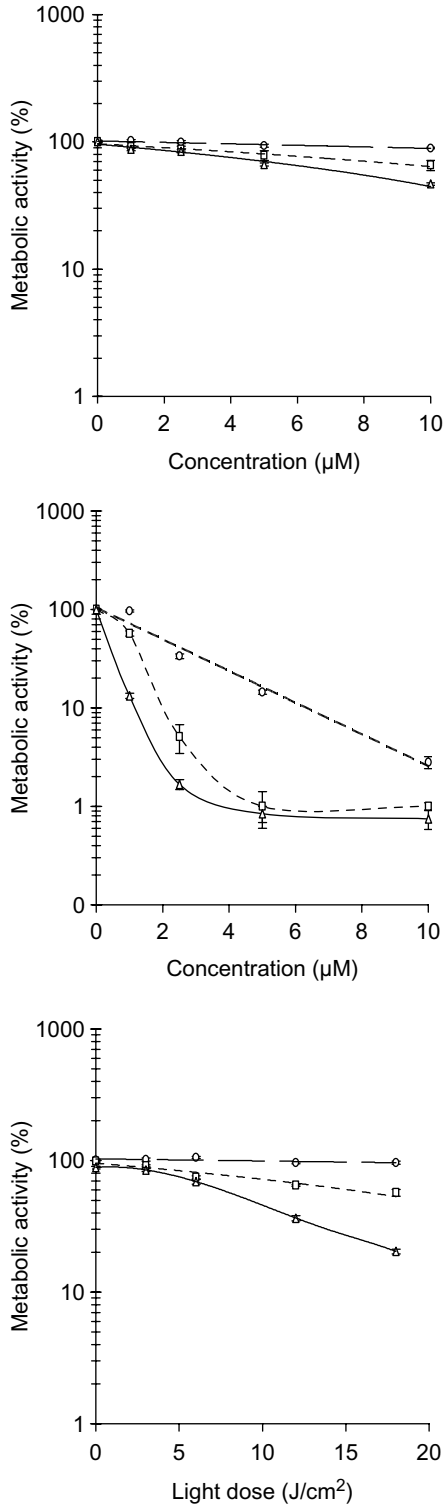


Figure 2. PC-3 cell inactivation after 24 h incubation with 1–20 μM of $AlPcS_4$ (○), $AlPcS_4A_1$ (□) or bombesin–$AlPcS_4$ conjugate (Δ) following exposure to 3 J/cm^2 (top) or 18 J/cm^2 (middle) of red light, and after 24 h incubation with 1 μM of $AlPcS_4$ (○), $AlPcS_4A_1$ (□) or bombesin–$AlPcS_4$ conjugate (Δ) following exposure to 3–18 J/cm^2 graded doses of red light (bottom).

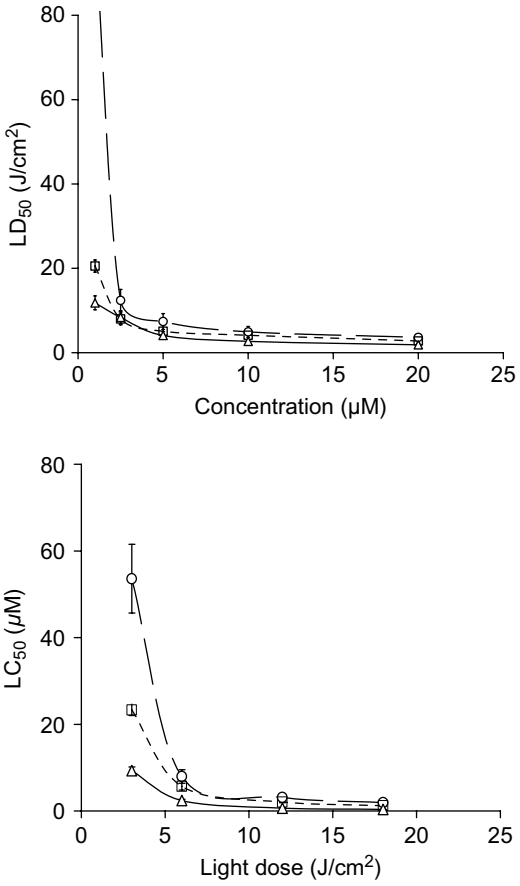


Figure 3. Light dose (top: LD_{50} in J/cm^2) and drug dose (lower: LC_{50} in μM) required to induce 50% PC-3 cell inactivation after 24 h incubation with $AlPcS_4$ (○), $AlPcS_4A_1$ (□) or bombesin–$AlPcS_4$ conjugate (Δ).

As estimated from the ratios of the absorbance maxima at 635 nm (aggregate) and 680 nm (monomer),[6] both the $AlPcS_4$ and $AlPcS_4A_1$ are largely monomeric in cell medium (74% and 68%, respectively), while for the bombesin conjugate the $AlPcS_4$ appears >90% aggregated. Thus the significantly higher phototoxicity of the latter below 2 μM suggests monomerization of the photosensitizer conjugate at the cellular level permitting internalization by GRPR. At higher drug and/or light doses the differences between the activities of the three photosensitizer preparations are less pronounced due to the higher level of phototoxicity under these extreme conditions.

In conclusion, linking $AlPcS_4$ to bombesin via a C-14 spacer chain yields a conjugate that binds to GRPR with a relative binding affinity of 2.3 and that provides a modest gain in photodynamic potency against GRPR-rich prostate cancer cells. Further studies on the effect of the length of the spacer chain and polarity of the photosensitizer moiety on the GRPR binding affinity and cell penetrating capacity will be required in order to develop a bombesin–phthalocyanine conjugate with optimal properties for PDT of prostate cancer.

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14. The HPLC system was equipped with a UV–vis detector set at 215 nm and 670 nm. The conjugate was eluted with a linear gradient from solvent system A/B (95:5) to A/B (3:7), over a period of 40 min at a flow rate of 2 ml/min (A = 0.1% TFA in H_2O and B = 0.05% TFA in CH_3CN). The MALDI-MS spectra were acquired in reflector mode using a Tof Spec 2E mass spectrometer (Micromass, Waters, Canada) with operating and pulse voltages set to 20 kV and 2175 V, respectively. The sample was dissolved in water and diluted 100× in a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, prior to application of 1 μL onto the sample probe. One point calibration was carried out with [Glu^1]-fibrinopeptide B (Oakville, ON, Canada).
15. For competition assays[13] cells were grown in Petri dishes to a confluence of about 70% and incubated at 37 °C for 40 min with 4.9 mL of reaction medium (RPMI with 0.2% BSA, 1% penicillin/streptomycin, 2% Hepes), 50 μL of a dilution of 20,000 cpm of ^{125}I-bombesin (4.1×10^{-11}mol) (Perkin-Elmer) and 50 μL of competitor solution (i.e. bombesin–$AlPcS_4$ conjugate, 8-Aoc-bombesin(7–14)NH_2 or bombesin) at increasing concentrations from 1×10^{-6} to 1×10^{-13} mol. After incubation, cells were incubated with 1 mL of 0.05% (w/v) trypsin and counted. A competition curve of the cpm bound as a function of the concentration of cold competitor was made with GraphPad Prism computer-fitting program (GraphPad software, Inc.), providing EC_{50} values, dissociation coefficients (K_d) and relative binding affinities (RBA: bombesin concentration required for 50% competition, divided by the competitor's concentration required for 50% competition, 100×).
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18. Cell phototoxicity was estimated using a colorimetric MTT assay as described previously.[6] Briefly, 1×10^5 PC-3 cells per well in Ham's F12 growth medium were inoculated in 96 multiwell plates and incubated overnight at 37 °C in the presence of 5% CO_2. The cells were rinsed with PBS and incubated with drug at concentrations ranging between 1.0 and 20 μM of $AlPcS_4$ (free or conjugated) in Ham's F12 1% FBS for 24 h at 37 °C and 5% CO_2. After incubation, the cells were rinsed with PBS, refed with Ham' F12 medium and exposed to red light.[6] The cells were incubated at 37 °C and 5% CO_2 overnight before assessing cell viability. MTT in Ham's F12 growth medium was added to each well. After 4 h, sodium dodecyl sulfate (SDS) was added. Plates were incubated overnight at 37 °C and the absorbance was read the next day at 570 nm with a microplate reader (BIO-TEK Instruments Inc., Winooski, VT). Average absorbance of blank wells is subtracted from the readings. The average absorbance of control cells incubated with Ham's F12 1% FBS, was taken as 100% cell survival. The light dose required to inactivate 50% of cells (LD_{50}) at a given drug dose and the photosensitizer concentration required to kill 50% of cells (LC_{50}) at a given light dose was extrapolated from survival curves.