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The photosensitizer disulfonated aluminum phthalocyanine reduces uptake and alters trafficking of fluid phase endocytosed drugs in vascular endothelial cells—Impact on efficacy of photochemical internalization

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ABSTRACT

Targeting cancer vasculature is an emerging field in cancer treatment. Photochemical internalization (PCI) is a drug delivery technology based on photochemical lysis of drug-bearing endocytic vesicles originally designed to target cancer cells. Recent investigations have revealed a lower PCI efficacy in vascular endothelial cells (HUVECs) in vitro than in HT1080 fibrosarcoma cells. This manuscript aims to explore the limiting factor for the PCI effect in HUVECs. Cellular uptake of the photosensitizers $AlPcS_{2a}$ and $TPPS_{2a}$, and a model compound for macromolecular drugs taken up by fluid phase endocytosis, $Alexa^{488}$-dextran, was explored by flow cytometry. The uptake of $AlPcS_{2a}$ and $TPPS_{2a}$ was 3.8-fold and 37-fold higher in HUVECs than in HT1080 cells, respectively, while the $Alexa^{488}$-dextran uptake was 50% lower. $AlPcS_{2a}$ (but not $TPPS_{2a}$) was shown to reduce $Alexa^{488}$-dextran uptake in a concentration-dependent manner, resulting in 66% and 33% attenuation of $Alexa^{488}$-dextran uptake at 20 μg/ml $AlPcS_{2a}$ in HUVECs and HT1080 cells respectively. Studies of intracellular localization of $Alexa^{488}$-dextran and $AlPcS_{2a}$ by confocal microscopy in HUVECs uncovered a concentration-dependent $AlPcS_{2a}$-induced inhibition of $Alexa^{488}$-dextran trafficking into $AlPcS_{2a}$-stained and acidic vesicles. The localization of $Alexa^{488}$-dextran to $AlPcS_{2a}$-localizing compartments was reduced by 40% when the $AlPcS_{2a}$ concentration was increased from 5 to 20 μg/ml. The treatment dose of $AlPcS_{2a}$ was found to influence on the efficacy of PCI of saporin, but to a lesser extent than expected considering the data from cellular uptake and intracellular trafficking of $Alexa^{488}$-dextran. The implications of these results for further development of vascular targeting-PCI are discussed.

1. Introduction

Drug delivery to intracellular targets is a major challenge in treatment of cancer. Many potential anticancer drugs are taken up by endocytosis due to lack of efficient transport mechanisms into the cells, and these drugs are to a large extent degraded by lysosomal enzymes before they reach their targets [1]. Photochemical internalization (PCI) is a technology developed to release these drugs into cytosol prior to lysosomal degradation. In PCI, drugs are administrated in combination with amphiphilic photosensitizers (PSs) localized to endolysosomal membranes before being subjected to illumination. Light absorbed by the PSs will in the presence of oxygen form reactive oxygen species, in particular $^1O_2$. The reactive oxygen species rupture the endolysosomal membranes [2] and finally release the endocytosed drug into the cytosol. The PCI drug delivery technology are reported to potentiate the effects of a variety of drugs taken up by endocytosis, such as proteins, DNA, RNA and some chemotherapeutics [3–6], as well as targeted macromolecular therapeutics [7,8]. A major goal in experimental cancer therapy is to develop therapies with better specificity for the target lesions. The tetrapyrrol-based photosensitizers used in PCI and photodynamic therapy (PDT) accumulate preferentially in neoplastic lesions and is pharmacologically active only in the presence of light, usually in the 600–800 nm range, delivered primarily to the target tissue by means of diode lasers equipped with optical fibers [27]. In addition, further specificity may be obtained by utilizing targeted therapeutics activated by PCI. In this manner PCI utilizes three different and independent methods to achieve improved specificity.

Studies performed by our group and others have documented the cytotoxic efficacy of PCI on xenotransplanted cancers [9]. When compared to photochemical treatment alone (i.e. PDT), PCI has

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been shown to induce a stronger cancer growth delay and increased overall survival in several animal models [10–12]. Additionally, a study of contrast-enhanced MRI of HT1080 fibrosarcoma xenografts treated by PDT and PCI revealed reduced tumor perfusion [13], indicating a vascular disrupting effect of these treatment modalities. We have recently discovered that the uptake of the PSs $AlPcS_{2a}$ (aluminum phthalocyanine with two sulfonate groups on adjacent phenyl rings) and $TPPS_{2a}$ (meso-tetraphenylporphin with two sulfonate groups on adjacent phenyl rings) is 10–20-fold higher in endothelial cells (Human Umbilical Vein Endothelial Cells (HUVECs)) than in the HT1080 cells (Vikdal et al. accepted). This observation makes endothelial cells an attractive target for PCI-based drug delivery. However, it was found that PCI, in particular with $AlPcS_{2a}$ as PS, activating the hydrophilic non-targeted protein toxin saporin exerted a lower effect on HUVECs than HT1080 cells. The PCI efficacy was also approximately 20-fold lower when utilizing $AlPcS_{2a}$ as PS instead of $TPPS_{2a}$. These results indicate that the rate of endocytosis or intracellular routing of at least non-targeted macromolecules may attenuate the PCI efficacy in endothelial cells.

The present study was initiated to measure cell line differences in macromolecular drug uptake and the impact of $AlPcS_{2a}$ on endocytosis in endothelial cells. The biologically stable $Alexa^{488}$ labeled dextran (hereby abbreviated only as dextran) particles was utilized as a fluid phase endocytosis marker for non-targeted macromolecular therapeutics. Studies by confocal microscopy were performed to uncover the impact of $AlPcS_{2a}$ on its colocalization to the macromolecular dextran in endothelial cells. Finally, the impact of these findings in HUVECs were evaluated by estimations of the relative PCI-efficacy in cytotoxicity assays of PDT and PCI at high (20 μg/ml) and low (5 μg/ml) concentrations of $AlPcS_{2a}$. This report is the first to document that a PS can influence on endocytic activities and PCI efficacy.

2. Materials and methods

2.1. Cell lines and culturing

The HT1080 fibrosarcoma cell line was purchased from the American Type Culture Collection (ATCC, **CCL-121** Manassas, VA) and grown in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% (v/v) fetal calf serum (GIBCO BRL, Paisley, U.K.), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma–Aldrich, St. Louis, MO). The Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from ATCC (CRL-1730) and cultured in growth media specialized for endothelial cells (EGM®-2 BulletKit, #CC-3162, Lonza, Switzerland). Both cell lines were subcultured by trypsinization twice a week and experiments were performed during the passages 5–20 for HT1080 cells and 3–15 for HUVECs. The cells were grown and incubated in 75 cm^2 flasks (Nalge Nunc Int., Naperville, IL, USA) at 37 °C in a humidified tissue culture incubator with 5% CO_2.

2.2. Chemicals

$AlPcS_{2a}$ was purchased from Frontier Scientific (Logan, UT). $AlPcS_{2a}$ was first dissolved in 0.1 M NaOH and thereafter diluted in phosphate-buffered saline to a stock solution of 1.23 mg/ml. The photosensitizer was protected from light and stored at −20 °C until use. $TPPS_{2a}$ (LumiTrans®), was a generous gift from PCI Biotech AS (Lysaker, Norway). A stock solution of 0.35 mg/ml $TPPS_{2a}$ in DMSO (dimethyl sulfoxide, Merck KGaA, Darmstadt, Germany) was kept at −20 °C. All procedures in presence of PSs were carried out under subdued light. Saporin (Sigma–Aldrich) was dissolved in phosphate-buffered saline to a stock concentration of 1 mg/ml, aliquoted and stored at −20 °C. LysoTracker® Blue DND-22 (Invitrogen, Carlsbad, CA) and $Alexa^{488}$-dextran (hereafter abbreviated dextran, MW 10 000, No. D22910, Molecular Probes, Invitrogen, CA, USA) were kept in aliquots at −20 °C as recommended by the producer.

2.3. Flow cytometry analyses

HT1080 cells and HUVECs were seeded out at 8000 and 25 000 cells/cm^2, respectively, in 12-well plates and left to attach overnight. The cells were incubated with either one of the PSs (20 μg/ml $AlPcS_{2a}$ or 0.5 μg/ml $TPPS_{2a}$), dextran alone (10 μM) or the two in combination for 18 h followed by a 4 h incubation in drug-free medium. Additionally, co-incubations of 10 μM dextran at increasing concentrations of $AlPcS_{2a}$ (5–20 μg/ml) were performed in both cell lines. Cells were harvested by trypsinization and washed in phosphate-buffered saline containing 1% (v/v) fetal calf serum (flow buffer). Samples were diluted in flow buffer prior to flow cytometric analyzes. Analyses were performed by the BD™ LSR II SORP flow cytometer (San Jose, CA, USA). Detection of dextran was performed by excitation at 488 nm, using band pass emission filter set at 525/50. $AlPcS_{2a}$ was recorded at 640 nm excitation and a LP710 emission filter, and $TPPS_{2a}$ was excited at 405 nm with the BP emission filter set at 660/20. Software used for flow cytometric analyses was the BD FACS Diva™ and Flow-Jo 7.6 (Tree Star Inc., OR, USA).

2.4. Confocal microscopy studies

HUVECs were seeded and incubated on poly-D-lysine coated glass-bottom dishes for confocal microscopy (35 mm, MatTek Corp. Ashland, MA). The cells were incubated with dextran and/or $AlPcS_{2a}$ for 18 h, washed twice and incubated in drug-free media (chase) for 4 h in cell culture medium containing fetal calf serum. One μM LysoTracker Blue was added the last hour of the chase period for staining of acidic compartments. Imaging was performed in the presence of one, two or three of the chromophores. Laser settings were fixed throughout the assay, and each cell area was strictly exposed to light only once using the LSM 710 confocal microscope (Carl Zeiss, Germany). A total of 110 micrographs with 5 μg/ml $AlPcS_{2a}$ and 203 micrographs with 20 μg/ml $AlPcS_{2a}$ were analyzed. To avoid possible cross-talking between the channels, the images were obtained using sequential scanning for each channel. Only the images with a signal within the dynamic range (with no saturation) were used for the analysis. Background correction parameters were kept equal for all pictures.

In order to quantify colocalization, a variety of correlation coefficients may be utilized. In confocal imaging quantification is based on pixels analyses [14]. The Pearson's correlation coefficient was utilized to explore the linearity of intensity covariance between two chromophores. The Pearson's correlation coefficient (PCC) estimates the ratio of covariance between two chromophores within the colocalized volume, expressed by the formula:

$$\mathrm{PCC} = \frac{\Sigma(A_i - A_{avg.})(B_i - B_{avg.})}{\sqrt{\Sigma(A_i - A_{avg.})^2 (B_i - B_{avg.})^2}}$$

where A_i and B_i describes the fluorescence intensity from chromophore A and B in each pixel, and $A_{avg.}$ and $B_{avg.}$ are the average intensity values of chromophore A and B.

Presentation of colocalization of one chromophore compared to another is performed by the Manders' thresholded coefficient (MTC). The coefficient designates the proportion of overlap of each chromophore with the other, and formulated by:

$$\mathrm{MTC}_{A_i} = \frac{\Sigma A_{i,coloc}}{\Sigma A_i}; \qquad \mathrm{MTC}_{B_i} = \frac{\Sigma B_{i,coloc}}{\Sigma B_i}$$