# Enhanced gene transfection by photochemical internalization of protomine sulfate/DNA complexes.

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## Abstract

**Introduction:** One of many limitations for cancer gene therapy is the inability of the therapeutic gene to transfect a sufficient number of tumor cells. Photochemical internalization (PCI) is a photodynamic therapy-based approach for improving the delivery of macromolecules and genes into the cell cytosol. The utility of PCI for the delivery of the GFP indicator gene on the same plasmid as a tumor suppressor gene (PTEN) was investigated in monolayers of U251 human glioma cells.

**Materials and Methods**: U251 monolayers were incubated in $AlPcS_{2a}$ for 18 h. The monolayers were incubated with non-viral vectors for either 4 or 18 hrs. In all cases, light treatment was performed with a diode laser at a wavelength of 670 nm. The non-viral transfection agents, branched PEI or protomine sulfate (PS), were used with the plasmid construct (GFP-PTEN).

**Results:** PS was much less toxic to the gliomas cells compared to BPEI but was highly inefficient at gene transfection. PCI resulted in a 5-10 fold increase in GFP protein expression compared to controls.

**Conclusions**: Collectively, the results suggest that $AlPcS_{2a}$-mediated PCI can be used to enhance transfection of tumor suppressor genes in glioma cells.

## Introduction

Glioblastoma multiforme (GBM, WHO grade IV) represents 60% of all malignant brain tumors, and carries a very dismal prognosis [1, 2]. The tumor cells have astrocytic cell features and are highly invasive; therefore, with current surgical techniques the removal of the tumor is impossible. Despite the recent technological advances in surgery and radiotherapy, these procedures have not been able to significantly improve the health of a patient struggling with GBM. Chemotherapy has only contributed marginal patient benefit [3-5]. Improved treatment modalities, such as gene therapy are clearly required.

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One of many limitations for cancer gene therapy is the inability of the therapeutic gene to transfect a sufficient number of tumor cells. Cells are not prone to take up and utilize large and negatively charged macromolecules such as plasmid DNA. This necessitates the use of delivery carriers in order to overcome multiple extracellular and intracellular pathways. Among many barriers in nonviral gene delivery, cytosolic release (endosomal escape) and dissociation of nucleic acids from the carriers once arrived at their intracellular targets are crucial.
Photochemical internalization (PCI) is a technique which utilizes the photochemical properties of photodynamic therapy ( PDT) for the enhanced delivery of macromolecules into the cell cytosol. These macromolecules lack the ability to naturally permeate intracellular barriers such as the plasma and endocytic membranes [6]. Therefore, PCI a physical targeting technique has been used in combination with adenovirus transduction introducing up to 20-fold increase in transgene expression in photochemically treated cells [7]. The concept of PCI is based on using photosensitzers, which will localize in the membranes of the endocytic vesicles. When light is applied, the photosensitzers will react with oxygen and cause the membrane to break apart releasing all of the encapsulated macromolecules so more genes will be released into the cells instead of being degraded by lysosomes. With regard to PCI studies, viral vectors have been used but alternative approaches with non-viral carriers have also been proposed. [8] Despite their low transfection efficiency, non-viral vectors like polyethylenimine (PEI), which form stable ionic complexes with plasmid DNA, are interesting due to their ability to circumvent the immune response occurring against viral proteins, which limits repeated administration [9]. In this case, the photosensitizer and DNA complexes both enter cells by endocytosis, suggesting that they can be combined with PCI for efficient gene transfer.

## Materials and Methods

### Cell Lines and Plasmid construct

The **U-251 MG** tumor cells were grown as monolayers in DMEM medium (Invitrogen Corp., Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS), 50 mM HEPES buffer (pH 7.4), penicillin (100 U ml–1), and streptomycin (100 mgml–1) at 37°C and 5% CO2.
The plasmid construct, PTEN-GFP. (shown below) was used in all the nonviral transfection experiments.

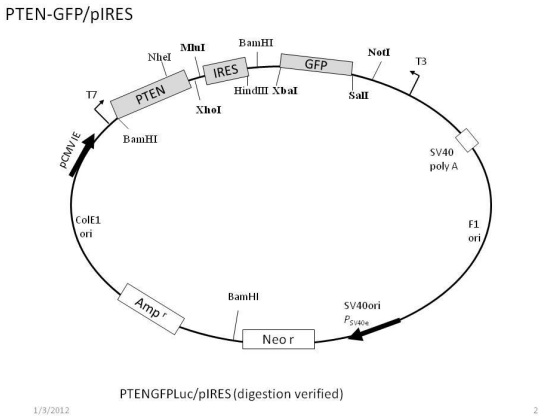


Figure 1 Plasmid PTEN/GFP DNA construct.

***Non-viral transfection of glioma cells***

U251 cells were cultured in 35mm imaginging dishes at 200,000 cells per well and allowed to grow overnight. 1 ug/ml $AlPcS_{2a}$ and the BPEI or PS/DNA polyplexes were then added to the cell culture for an additional 18h, followed by a triple wash. The cells were incubated for 4 hours in fresh medium. Light treatment at fluence levels of 0, 0.75 J/cm$^2$ @ 5 mW/cm$^2$ was administered.

Controls consisted of cultures that contained BPEI or PS/DNA plasmids but were not exposed to light (dark controls). Following transfection the cells were incubated for an additional 24 hrs and then imaged in a two photon microscope.

## Results

Since PCI is optimal with a light fluence level that allows 70-80 % survival, we performed $AlPcS_{2a}$ mediated PDT at increasing light doses. Live /dead assay of U251 following PDT is shown in fig. 2 for 0 and 1.5J respectively. Fluence levels of 1.5J/cm$^2$ proved highly toxic killing more than 80% of the cells. Fluence level of 0.5-0.75J seemed optimal.

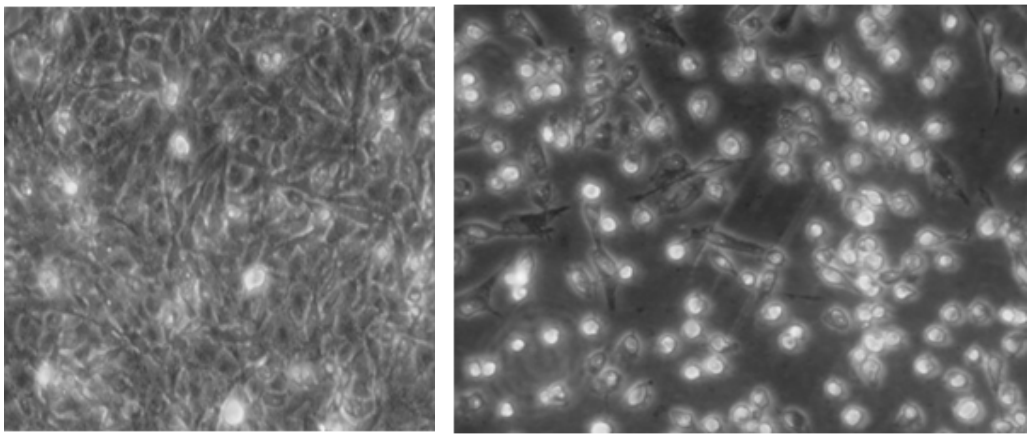

Figure 2 Live dead assay of U251 cell monolayers following $AlPcS_{2a}$ PDT at 0J (left panel) and 1.5J/cm$^2$ (right panel).

***Effects of PCI on GFP transfection with bPEI/DNA polyplexes***

In order to optimize the various parameters for effective PCI mediated gene transfection, experiments were carried out on U251 monolayers employing a DNA plasmid for GFP. Branched PEI of various PEI/DNA (N/P) ratios and concentrations was investigated as the delivery carrier and a N/P ratio of 5:1 proved optimal. PCI could improve transfection efficiency from 10% to 27% in this model, (fig 3) comparable to virus vectors. As can be seen from the figure transfection efficiency following 18hr incubation of the bPEI/DNA polyplexes was significantly increased compared to 4 hour incubation.

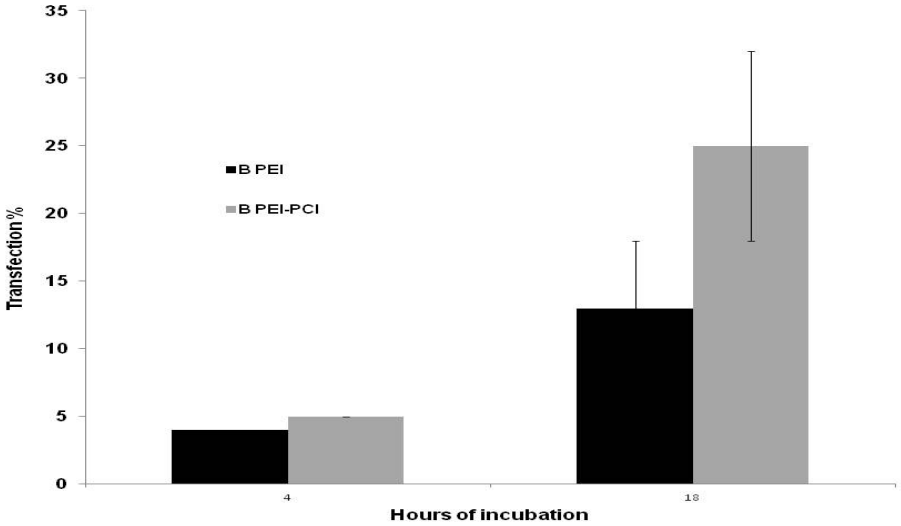


Fig 3 Effects of PCI on GFP transfection with bPEI/DNA polyplexes. DNA complexed with bPEI ,N/P ratio 5:1, DNA concentration 1μg/ml, PDT 0.75J/cm$^2$

***Effects of PCI on protomine-sulfate/DNA polyplexes***

In the initial phase of this project a PEG-conjugated acid-transforming micellar nonviral vector was to be used as a delivery carrier. We have now developed an improved technique utilizing a DNA - protamine sulfate (PS) core that is then packaged in a polymeric nanomaterial that is responsive to changes in pH. The non packaged polyplexes enter the cell by endocytosis and are sequestered outside the nucleolus in endosomes, resulting in very low transfection rates (fig 4). The addition of PCI treatment demonstrated a tenfold increase in transfection rate at optimum PS/DNA concentrations. These results clearly demonstrate the ability of the PCI technique to greatly enhance endosomal escape.

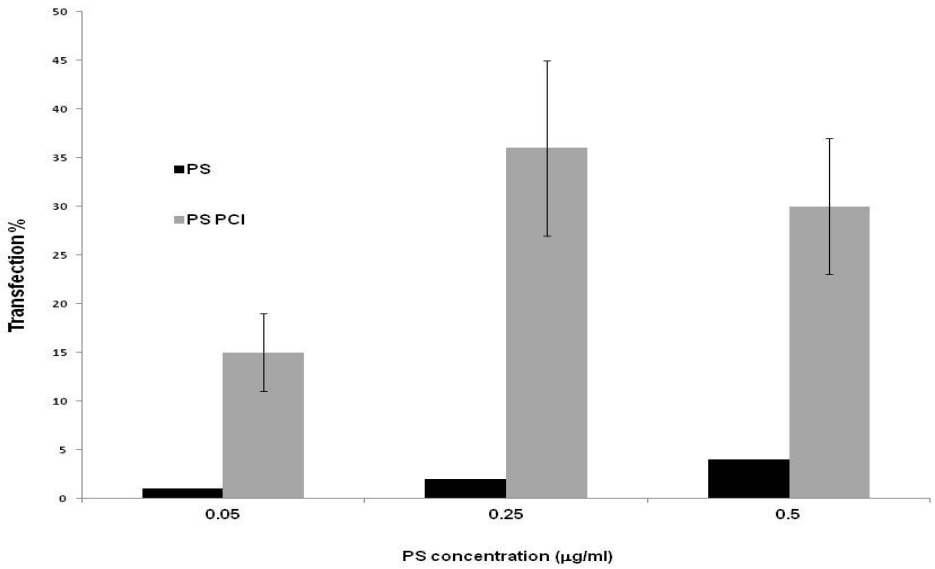


Fig.4 Effects of PCI on GFP gene transfection with protomine-sulfate/DNA polyplexes. DNA complexed with protomine sulfate , DNA concentration 1µg/ml, PDT 0.75J/cm$^2$

## Discussion

The emerging cancer stem cell model suggests that tumors are organized in a hierarchy with a subpopulation of cancer cells responsible for tumor maintenance and progression. The brain tumor stem cell is considered to be responsible for the initiation, propagation, and recurrence of gliomas [10]. The mechanisms leading to GBM development are not well understood but animal studies support the hypothesis that inactivation or mutations of tumor suppressor genes in neural stem cells (NSC), transforming them to tumor stem cells (TSC), is required and sufficient to induce glial cancers [11]. NSC niches in the brain may harbor and act as reservoirs for TSCs from where they initiate and repopulate tumors suggesting new targets for directed therapy. High

doses of ionizing radiation to these stem cell niches have improved patient survival [12] but even low dose radiation therapy can elicit cognitive dysfunction that are associated with the depletion of neural stem cells [13], greatly decreasing the quality of life for patients .

The ability to insert functioning suppressor genes into TSCs and glioma cells would therefore be of considerable interest as a potential treatment modality.

PTEN: is one of the most commonly lost tumor suppressors in human cancer. Mutations and deletions of PTEN occur that inactivate its enzymatic activity leading to increased cell proliferation and reduced cell death. Frequent genetic inactivation of PTEN occurs in glioblastoma, endometrial cancer, and prostate cancer; and reduced expression is found in many other tumor types such as lung and breast cancer.

The results reported here and in a previous paper [14] demonstrate the ability of PCI to increase transfection rates in gliomas cells. Although bPEI is an effective gene carrier it is highly toxic and is not well suited for in vivo applications. PCI of bPEI showed only a modest increase compared to dark controls (fig 3). I contrast PCI of PS/DNA polyplexes demonstrated a tenfold increase in transfection rate at optimum PS/DNA concentrations. Protamine is a cationic small protein with high arginine content. Since Protamine is a nuclear protein that helps DNA packaging in sperm cells it is also used as transfection accelerator in gene delivery [15]. In agreement with the results reported here protamine/DNA polyplexes have been reported to have relatively low transfection efficiency [16]. Some possible reasons of this finding may lie in the strong hydrophilicity of Protamine, which makes it difficult to cross cellular membranes, inhibiting escape through endosomal membranes. We have previously shown that PS polyplexes enter cells in similar amounts compared to PEI [17]. The difference in transfection efficiency between these two gene carriers is most probably due to increased endosomal escape by PEI/DNA polyplexes. Since PCI greatly enhances endosomal escape, the dramatic effects of PCI shown in fig.4 for PS/DNA polyplexes support this interpretation.

In order to increase the bioavailability of PS/DNA polyplexes they can be shelled with an acid degradable polyketal (PK) layer, which protected the gene-carrying core during circulation and cellular internalization[17]. The resulting polyamine/PK core-shell nanoparticles have a gene-carrying polyamine/ DNA polyplex core shielded by PK shell, mimicking a viral vector consisting of a gene-carrying capsid core and an outer envelope. In the mildly acidic endosome, the outer PK shell hydrolyzes and releases the polyamine/DNA core. We are presently studying the effects of PCI on glioma cell transfection employing these nanoparticles.

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