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Authors
Sun, Chung-Ho
Berg, Kristian
Hirschberg, Henry

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Photochemical Internalization enhanced non-viral suicide gene therapy

Sun Chung-Ho[1], **Kristian Berg**[2], and **Henry Hirschberg**[1,#]

[1]Beckman Laser Institute and Medical Clinic, University of California, Irvine USA

[2]Dept. of Radiation Biology, The Norwegian Radium Hospital, Oslo University Hospital, Oslo Norway

Abstract

Non-viral gene transfection overcomes some of the disadvantages of viral vectors, such as undesired immune responses, safety concerns, issues relating to bulk production, payload capacity and quality control, but generally have low transfection efficiency. Here we describe the effects of a modified form of photodynamic therapy (PDT), i.e. photochemical internalization (PCI) to: 1) greatly increase nonviral cytosine deaminase gene (CD) transfection into tumor cells which significantly increases the conversion of 5- fluorocytosine (5-FC) to 5-fluorouracil (5-FU) and 2) enhance the toxic efficacy of the locally produced 5-FU to induce cell death on both transfected and non-transfected bystander cells.

Keywords

Non-viral gene transfection; cytosine deaminase gene; suicide gene therapy; photodynamic therapy; photochemical internalization; endosomal escape

1 Introduction.

One important limitation for suicide gene therapy, in particular when nonviral gene carriers are employed, is the inability of the gene to transfect a sufficient number of tumor cells. This in turn sets a limit to the amount of on-site conversion of prodrug to active drug. The well established suicide gene therapy modality, utilizing the transfection of the cytosine deaminase (CD) gene, which codes for an enzyme that converts the antifungal agent 5-fluorocytosine (5-FC) into its antimetabolite 5-fluorouracil (5-FU) was used in the development of this protocol [6–8]. Additionally the bystander effect, where active drug is exported from the transfected cancer cells into the tumor microenvironment, plays an important role by inhibiting growth of adjacent non-transfected tumor cells. Methods to enhance the initial gene transfection efficiency and the efficacy of the locally produced and consequently exported drug would therefore offer a distinct advantage. A modified form of photodynamic therapy (PDT), photochemical internalization (PCI) has been shown to greatly enhance gene insertion efficiency for both viral and nonviral gene transfection

[#]corresponding author hhirschb@uci.edu.

protocols as well as significantly increasing the efficacy of a variety of anti- cancer agents [1–5]. DNA polyplexes or drugs that are internalized into cells via endocytosis end up inside and trapped in the intracellular endosomes and lysosomes. The concept of PCI is based on using photosensitizers which localize to endosomes and lysosomes. When light is applied, the photosensitizer will react with oxygen causing endo-lysosome membrane rupture releasing the trapped macromolecules into the cell cytosol, avoiding lysosomal degradation. The released gene or drug can therefore exert its full biological activity, in contrast to being degraded by lysosomal hydrolases.

Here we describe an *in vitro* 3 dimensional multi-cell glioma spheroid tumor model [9] to demonstrate that PCI could: 1) Greatly increase the subpopulation of CD gene transfection cells within the spheroid resulting in increased amounts of 5-FU converted from 5-FC and 2) significantly increase the toxic efficacy of the locally produced and exported 5-FU both on the CD transfected as well as the non transfected bystander cells. Due to the rapid attenuation of light in tissue this enhanced gene transfection efficiency and increased drug efficacy would be limited to the illuminated targeted tumor site, thus reducing damage to off target normal tissue. Thus, the light based therapy PCI, can both increase gene transfection efficiency as well as the efficacy of the compounds converted by the suicide gene induced enzyme, in a targeted site and time specific manner.

2 Materials

2.1 CD gene plasmid

1. Plasmid pPH36, Addgene (Cambridge MA) cat #35101. The plasmid is received as bacteria E. Coli (high copy number DH5alpha strain).
2. LB agar ampicillin-100, SigmaAldrich Cat# L5667, Prepoured LB agar plates with 100ug/ml ampicillin. Store at 2 to 8°C.
3. LB Broth, Mediatech Cellgro from Fisher Scientific Cat#MT-46–050-CM.
4. Ampicillin, EMD Millipore Calbiochem from Fisher Scientific Cat# 17–125-520ML, 20ml at 10mg/ml H_2O, sodium salt, sterile and tissue culture grade.
5. Inoculation loop 1 ul, STARTEDT Cat# 86.1567.050, white, 48 sterile pieces packed in the bag. SARSTEDT (Germany) with sale office at Sparks NV USA.
6. Plain Polycarbonate Fisherbrand Shaker Flasks with vented polypropylene closure, the vent is with 0.22 um PTFE pore. Both flasks and closures are autoclavable.
7. Thick wall Polypropylene 50ml tube for Beckman JA-20 rotor, Beckman Coulter Cat# 357005.

2.2 CD gene DNA isolation

1. QIAGEN Plasmid Midi Kit Cat# 12143, This kit can be stored at room temperature (15–25°C) for up to 2 years.

2. Buffer P1 (suspension buffer): 50mM Tris-HCl, pH:8.0, 10 mM EDTA (inactivate DNase activity by chelating out Ca^{++} and Mg^{++} which are essential cofactors), 100 ug/ml RNase A, resuspend before each use when LyseBlue is added.
3. Buffer P2 (lysis buffer): 200 mM sodium hydroxide (alkaline mixture to rupture the cells, break down the cell wall and denature dsDNA to ssDNA), 1% SDS w/v (breaks apart the lipid membranes and solubilizes cellular proteins, keep the bottle closed when not in use to avoid acidification of the buffer by CO_2 in the air).
4. Buffer P3 (neutralization buffer): Neutralize to allow DNA strands to renature which is easy for plasmid DNA: (acetic acid), 3.0 M potassium acetate pH:5 (precipitate SDS out along with cellular debris and genomic DNA, e coli chromosomal DNA while plasmid DNA remains in solution).
5. Buffer QBT (equilibrium buffer): isopropanol 15% (v/v), 0.15% Triton X 100 (v/v), 750 mM NaCl, 10 mM MOPS, pH: 7.0.
6. Buffer QC (wash buffer): isopropanol 15% (v/v), 1.0 M NaCl, 50 mM MOPS, pH:7.0.
7. Buffer QF (elution buffer): isopropanol 15% (v/v), 1.0M NaCl and 50 mM Tris-HCl, pH:8.5.
8. RNase A
9. LyseBlue
10. QIAGEN-tip 100: anion exchange resin, plasmid DNA binds to the resin under appropriate low salt and pH condition, RNA, proteins and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer followed by isopropanol precipitation to concentrate and desalt the plasmid DNA.

2.3 CD gene DNA/gene carrier polyplex

1. DI water (Nuclease-free water (Fisher Scientific, Pittsburgh, PA).
2. Protamine sulfate (PS, 5.1 kDa), Fisher Scientific (Pittsburgh, PA).
3. Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA)

2.4 Tumor spheroids

1. *Cell lines*: Rat F98 glioma cell line obtained from the American Type Culture Collection (Manassas, VA, USA).
2. Ultra-low attachment surface 96-well round-bottomed plates (Corning In., NY).
3. Advanced DMEM medium (Invitrogen Corp., Carlsbad CA., Cat. # 35101)
4. TrypLE Express
5. Tissue Culture Flask T25

6. Serological Pipettes 5ml
7. P10
8. Disposable plastic hemocytometer C-chip

2.5 Laser light treatment

1. Photosensitizer $AlPcS_{2a}$ (Frontier Scientific, Inc., Logan, UT)
2. Laser fiber Frontal light distributor Model FD1 (Med*light* Ecublens Switzerland)
3. 670 nm Diode laser; Intense-HPD 7404 (Intense HPD, New Jersey, USA).

3 Methods

3.1 CD gene plasmid generation

1. Take out one LB agar plate with 100ug/ml ampicillin from refrigerator and label the bottom of the plate with pPH36, ampicillin 100, date and your initial. Bring the labeled agar plate, pPH36 stab and a pack of sterile 1 ul inoculation loop to the clean bench. Insert and touch the punctured area of the stab culture with the sterile loop and hold the loop at an angle to make several broad zigzag strokes to spread the bacteria over about a one third section of the surface of the plate to generate streak #1. Take a new sterile loop and drag through streak #1 at an slight angle and spread the bacteria over a second section of the plate in the broad zigzag stroke to generate streak #2. Take a third new sterile loop and drag through streak #2 at an angle and spread the bacteria over the third and last section of the plate in the broad zig-zag stroke to generate streak #3.
2. Place the inoculated plate in a 37°C incubator overnight (12 to 18 hours). Next morning, single colonies looking like white dots should be visible on the solid agar plate. Each single colony is composed of millions of genetically identical bacteria grown from a single bacterium. Store the plate in the refrigerator until ready for expansion in liquid bacteria culture.
3. Add 20ml LB broth into a sterile 125ml shaker flask with vented closure. Label the flask the sam as the plate with new date. Add total 200 ul of ampicillin (EMD Millipore Calbiochem Fisher Scientific Cat# 17–125-520ML). Use a sterile loop to pick a single clone from the inoculated agar plate. Dip and swirl the tip of the loop into the 125 ml shaker flask containing the 20ml LB broth and ampicillin. Cap the flask with the vented closure and hook up the flask onto a shaking incubator. Incubate the liquid bacteria culture overnight at 37°C.
4. After incubation, the LB broth should look cloudy. For incubation to obtain bacteria culture at log phase of growth, check the density of the culture at OD600.
5. Further expanding the culture by preparing 20 of 125 ml shaker flasks with 40 ml LB broth supplemented with 400ul ampicillin each. Transfer 1ml of the initial liquid culture to each of the new sterile shaker flasks and incubate overnight at

37°C in a shaking incubator. Transfer the bacteria culture in log phase from each shaker flask to a thick wall polypropylene 50 ml tube (Beckman Coulter cat# 357005). Centrifuge at 6000 x g (7000rpm for Beckman J2–21 centrifuge with JA-20 rotor) for 15 minutes at 4°C, discard the supernatant and store the bacteria pellets at −20°C until ready to isolate the plasmid DNA.

3.2 Isolation of CD gene plasmid DNA (see note 1)

1. Thaw out the bacteria pellets from −20°C freezer at room temperature for 10 minutes. Re-centrifuge at 7000 rpm for 10 minutes. Remove residue LB broth by inverting the tubes on a paper towel covered with alcohol wipe. This step is not necessary if thawed pellet is relatively dry. Re-suspend the bacteria pellet in each 50 ml tube in 4 ml Buffer P1. Add 4 ml Buffer P2, mix thoroughly by vigorously inverting 4 to 6 times and incubate at room temperature (15 to 25°C) for 5 minutes. If using LyyseBlue reagent, the solution will turn blue. Maximum number of tubes to use each time is 8 when using Beckman J2–21 with JA-20 rotor. Add 4 ml pre-chilled Buffer P3, mix thoroughly by vigorously inverting 4 to 6 times. Incubate on ice for 15 minutes. If using LyseBlue reagent, mix the solution until it is colorless.
2. Equilibrate a QIAGEN-tip 100 by applying 4 ml Buffer QBT, and allow column to empty by gravity flow into sterile 50 ml conical tube. Use the blue column holder. Setup the same number of column with the number of bacteria pellet containing tubes. Centrifuge all the 50 ml tubes in Beckman J2–21 centrifuge with JA-20 rotor at 20,000g (13,000 rpm) for 30 minutes at 4°C. Re-centrifuge at the same speed for 15 minutes if the supernatant is not clear. Pellets contain genomic DNA, proteins, cell debris and potassium salt of SDS is discarded. Apply by dumping directly the supernatant from each tube into each QIAGEN-tip 100 column. Allow the supernatant to enter the resin by gravity flow.
3. Wash the QIAGEN-tip 100 with 2 × 10 ml Buffer QC. Allow Buffer QC to move through the QIAGEN-tip by gravity flow. Elute plasmid DNA with 5 ml Buffer QF just removed from 65°C water bath from the QIAGEN-tip 100 column the into a sterile Beckman 50 ml tube. For constructs larger than 45 kb like ours, pre-warming the elution buffer to 65°C may help increase the yield.
4. Precipitate plasmid DNA by adding 3.5 ml room-temperature isopropanol (room temperature to minimize salt precipitation) and mix. Solution should look a little bit hazy. Centrifuge again in Beckman J2–21 centrifuge with JA-20 rotor at minimum 15,000 x g for 30 minutes at 4°C. Carefully decant the supernatant. Keep the precipitates.
5. Wash the plasmid DNA pellet with 2 ml room- temperature 70% ethanol (prepared with7 ml 100% ethanol and 3 ml autoclaved double deionized water

[1]Preparation for Isolation of CD gene plasmid DNA: Place Buffer QF from QIAGEN Plasmid Midi Kit (QIAGEN Cat# 12143) in a 65^0C waterbath and make sure the bottle is secured to stay upright. Add RNase A solution to Buffer P1, mix and store at 2–8°C. Label as RNaseA added with the date. The combined buffer is good for 6 months. Add Lyse Blue reagent to Buffer P1 with RNase A at a ratio of 1:1000. Check buffer P2 for SDS precipitation and pre-chill Buffer P3 at 4°C.

adjusted to pH:8.0). This step is to remove precipitated salt and replace isopropanol with the more volatile ethanol) and centrifuge at minimum 15,000 x g for 10 minutes. Carefully decant supernatants. Air-dry plasmid DNA pellet for 5–10 minutes and re-dissolve DNA in a suitable volume of autoclave ddH^2O at pH: 8.0. We use 150 ul for each tube. DNA dissolves better in alkaline condition. Over drying will make DNA difficult to redissolve. Be careful not shearing DNA by over pipetting to promote resuspension.

6. Save 2ul of plasmid DNA sample from each tube and use Nanodrop 1000 to check the plasmid DNA concentration and quality based on the absorbance at 280 nm, 260 nm, 230 nm and the ratio of 260nm/280 nm and the ratio of 260nm/230 nm. The unit uses 340nm for baseline normalization and 50 ng-cm/μl as a constant for dsDNA. The read out unit of the plasmid DNA concentration is ng/ul. The result is acceptable when the ratio of 260/280 is in the range of 1.8 to 2.0 and the ratio of 260/230 is in the range of 2.0 to 2.2 (see note 2)

3.3 Polyplex preparation.

Protamine sulfate (PrS)/DNA polyplexes formed as follows (see note 3).

1. Disperse 20μg of pDNA in 500μL of DI water.
2. Add drop-wise pDNA in water to protamine sulfate solution while vortexing.
3. Leave the resulting polyplex undisturbed for 30min to allow polyplex formation. Refrigerate prior to use for efficient complexation. N/P ratio of the PrS/DNA polyplexes; 10:1 (molecular ratio of amines of polyamine to phosphates of DNA)
4. Characterize the size and surface charge of the prepared polyplex by dynamic light scattering and zeta-potential analysis (Malvern Zetasizer Nano ZS).

3.4 Cell preparation

1. Grow the tumor cells (F98) as adherent monolayers in T25 culture flask in Advanced MEM medium (Gibco) supplemented with GlutaMax (Gibco), 2% heat–inactivated fetal Bovine serum (FBS) and GS/AB (Gibco) in humidified CO2 incubator at 37C and 7.5% CO2 (see note 4). When the cells have reached sub-confluence at late log growth phase, remove the entire growth medium from the T25 flask along with any floating F98 cells.
2. Add 2ml TrypLE Express with flask standing with cap on top and tilted at a slight angle to avoid direct contact of TrypLE Express with the cell monolayer. Place the flask under the inverted microscope with the cell side down to allow

[2]Aromatic amino acids side chains within the proteins are responsible for the absorbance at 280 nm. The aromatic base moieties within the structure of nucleic acids are responsible for the peak absorbance of purines and pyrimidines at 260 nm. Many organic compounds have strong absorbance at around 225 nm along with peptide bonds in proteins absorb light between 200 and 230 nm. The 260/280 ratio is used to assess the purity of DNA and RNA with a ratio of ~1.8 for DNA and a ration of ~2.0 for RNA. A lower ratio indicates the sample is contaminated with proteins. The ratio of 260/230 is used as a secondary measure of nucleic acid purity with expected 260/230 ratio in the range of 2.0 to 2.2.

[3]The gene carrier PrS Protamine Sulfate was used here due to its low toxicity. Alternate carriers, such as branched polyethylenimine (bPEI), MW 25,000, (Sigma, St. Louis, MO), can also be used.

[4]The F98 cells have a short doubling cycle and require splitting often. One day after confluence, they are no longer useable.

direct contact of TrypLE Express with the cell monolayer under the microscope. Observed the cells and turn the flask with cap on top as soon as some of the cells start to detach from each other. Keep the TrypLE Express from direct contact with the cell monolayer and remove all the TrypLE Express with a serological pipette.

3. Place the flask in the 37C and 7.5% CO2 incubator for 10 minutes. Check under the inverted microscope to make sure all the cells have rounded up. Tap the side of the flasks gently to dislodge all the cells before adding 2 ml fresh medium to wash down the cells. Transfer the cell suspension to a 15 ml. Pipette the cells up and down gently several times to generate single cell suspension.

4. Count the cell number either manually (C-chip or Neubauer improved hemocytometer) or with a Coulter counter. Re-suspend the cells in culture medium at 25×10^4/ml

3.5 PCI phase 1: Initial PCI mediated CD gene transfection.

1. Aliquot 2.5 $\times10^3$ F98 cells per well in all wells. Spin the plate 1000 rpm for 30 minutes. Incubate the plate for 48hrs (37^0C, 7.5% CO_2) (see note 5).

2. Measure the diameters of each spheroid using an inverted microscope with a calibrated eyepiece micrometer, and record the measurements (see note 6).

3. Aliquot 0.5μg/ml of the photosensitizer $AlPcS_{2a}$ and the PrS/CD DNA polyplexes into the wells that will be receiving light treatment. A typical plate layout is shown in fig. 2. Loosely wrap the plate in aluminum foil (to shield it from ambient light). Incubated for 18 hours (see note 7).

4. Replace half of the medium in each well (100μl) with fresh medium. Repeat 3 times with 10 minutes between cycles (see notes 7&8). Incubate the plates for 4 hours 37^0C, 7.5% CO_2 following the washing procedure. The incubator must have port for laser fiber insertion (See figure 3 for set up details).

5. Initiate light treatment, covering the entire plate (12cm beam) for 8 minutes exposures at an irradiance of 2 mW/cm^2, administered from λ = 670 nm light from a diode laser (see notes 9&10).

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[5]Other cell lines that easily form spheroids and can be used in place of the rat F98 cell line are human; glioma U87, squamous cell carcinoma FaDu, and breast tumor MCF-7 (all obtained from the American Type Culture Collection Manassas, VA, USA). If other cell lines are too be used a light dose titration should be done (PDT control) PCI is most efficient when PDT (ie no drug) cell or spheroid viability is 70–80%.

[6]Spheroids usually form 48 hours following centrifugation but occasionally an extra day of incubation is required.

[7]All handling of the plates after the addition of the photosensitizer must be done in subdued external room and bench lighting. A dim blue light source can be used to facilitate work on the plate.

[8]Careful washing of the spheroids is required to remove excess photosensitizer while avoiding the accidental removal during the washing process.

[9]All light treatment should be done at 37^0C in an incubator that will allow accesses for the laser fiber.

[10]The laser power required given in mW is: fluence rate x the area of the laser beam to cover the plate. In the protocol described here with a 12cm diameter beam gives: 2mWx113=226mW

3.6 PCI Phase 2: Efficacy enhancement of converted 5-FU from 5-FC (Fig 1)

1. Following light treatment, incubate the plate for 18hrs. Each well should contain 200μl. Replace 100μl in each well with 100μl of medium containing 50μM 5-FC (see note 7). Incubate the plate for 4 hours.
2. Place a rectangle of aluminum foil under the micro plate shielding columns 1–8 from the beam (only columns 9–12 should receive irradiance). Initiate light treatment with the laser beam only illuminating columns 9–12, for 8 minutes exposures at an irradiance of 2 mW/cm^2, $\lambda = 670$ nm.
3. Incubate the plate for 3 days (wrapped in foil). Do not expose to light during this interval (see note 11)
4. 4. Measure the diameters of each spheroid using an inverted microscope with a calibrated eyepiece micrometer, and record the measurements. Repeat the measurement every 3–5 days Replace half of the culture medium in the wells every third day with fresh medium taking care not to accidently remove the spheroid. Terminate the plates 14 days after initiation.
5. Calculate the volume of the spheroids assuming a perfect sphere. $(4/3\pi r^3)$

Figure 4 illustrates typical results following both phase 1 and 2 of PCI treatment with calculated volumes.

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[11]Do not expose the plates to external light either ambient or by observation through the inverted microscope until 3 days following light treatment.

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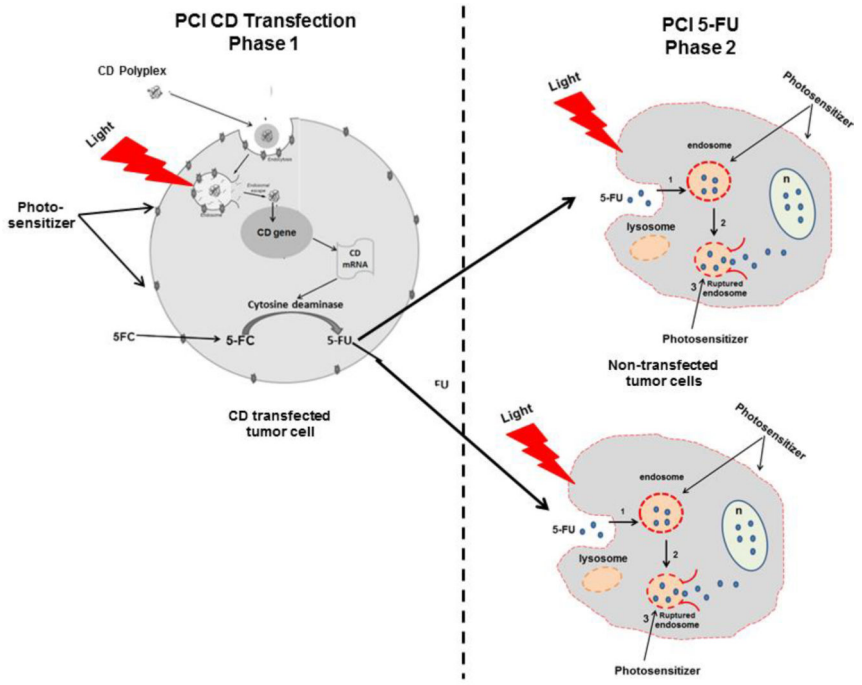

Figure 1. Basic concept of PCI enhanced gene transfection followed by PCI enhanced drug efficacy.

Phase 1: 1), cell exposed to photosensitizer 2). CD gene and carrier polyplex taken into cells via endocytosis 3). transported to endosome; released from entrapment by light treatment, 40 Induction of CD enzyme, converts 5-FC 5-FU 5) 5-FU exported to bystander non-transfected tumor cell;

Phase 2. Second light treatment synergistically enhances the effect of the toxic metabolites of 5-FU, leading to enhanced transfected and non-transfected tumor cell death.

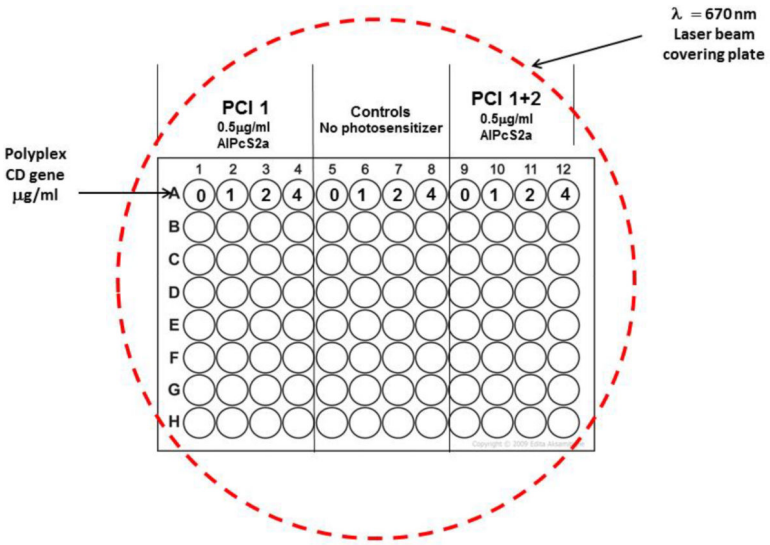

Figure 2. Micro plate layout tumor for gene DNA titration.

Spheroids formed in the wells. CD gene polyplexes at increasing concentrations and photosensitizer added as shown. First light treatment covers the entire plate (PCI phase 1). 5-FC added to all wells. Second light treatment administered only to columns 9–12.

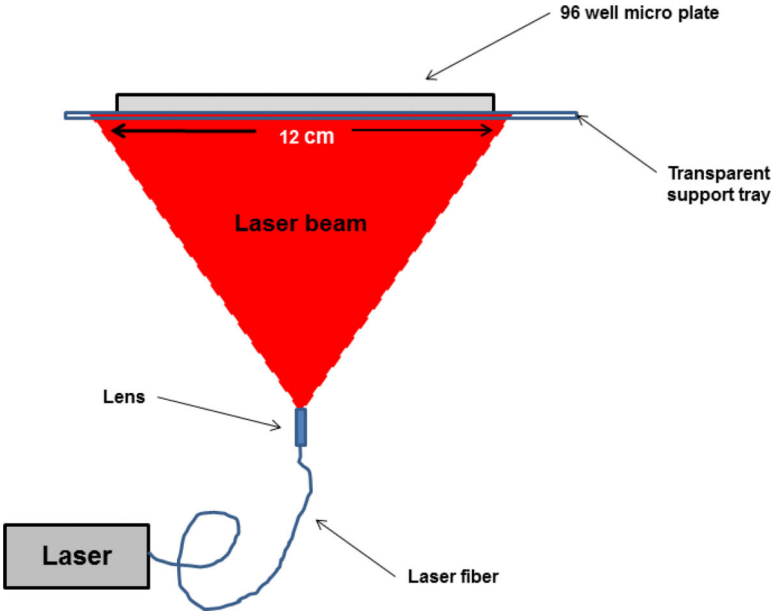

Fig 3. Light treatment.

Irradiance exposure time intervals generate various fluence levels at a constant irradiance of 2 mW/cm^2 administered with λ = 670 nm light from a diode laser (Intense HPD model 7404 New Jersey USA). Laser light is coupled into a 200 μm dia. optical fiber containing a micro-lens at the output end (Frontal light distributor Med lightSA, Switzerland), The lens will form conical light beam. The distance from the micro-lens to the microplate is adjusted to form a 12cm diameter beam pattern at the bottom of the plate. Irradiation is performed through the plate bottom with the micro plate supported on a transparent plastic plate.

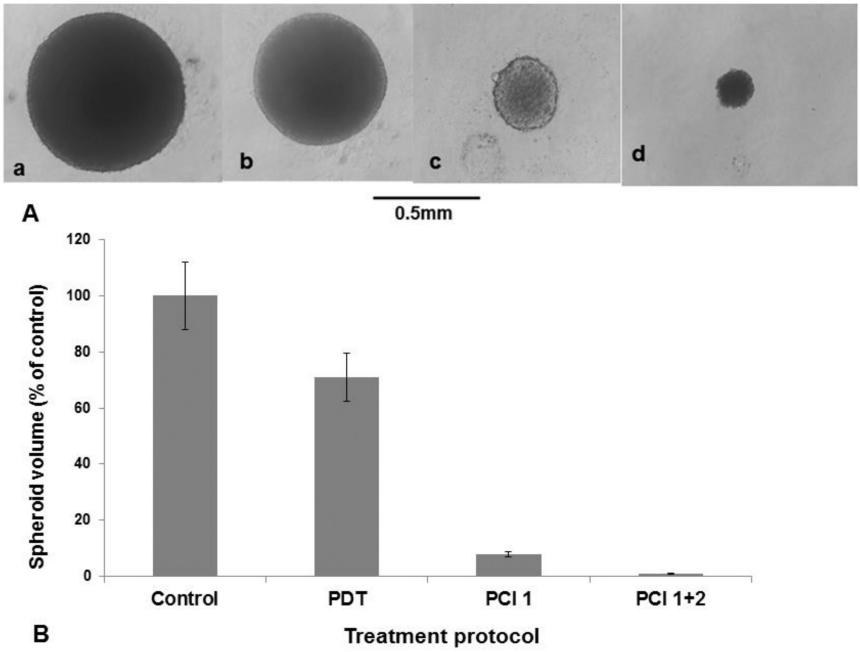

Fig. 4. Effects of PCI mediated suicide CD gene therapy on spheroid growth.

A; Light micrograph of F98 tumor spheroids 14 days post treatment. (a) Non treated control, (B) PDT control (column 9, fig 2), (c) PCI phase 1 CD transfection, 2ug/ml DNA +5-FC (column 3 fig. 2), (d) PCI phase 1+2 CD transfection, 2ug/ml DNA + second light treatment PCI drug enhancement (column 11 fig. 2) **B;** Calculated spheroid volumes from the measured diameters (shown in **A**) expressed as a % of non-treated controls 14 days post treatment.