Photodynamic therapy excited by Cerenkov radiation from Cesium-137 irradiator: *In vitro studies*

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ABSTRACT

Photodynamic therapy (PDT) is a noninvasive cancer therapy method that has been clinically approved for many years. Due to strong optical scattering and absorption of tissues, optical photons can only penetrate tissues several millimeters which limits the applications of PDT to superficial lesions. To overcome the limitation of penetration depth, here we applied Cerenkov radiation, as generated by the high-energy gamma rays from radionuclide Cesium-137, to directly activate the porphyrin-based photosensitizer MPPa (Pyropheophorbide-a methyl ester) without any additional energy mediators. Experiments were conducted with A549 human lung carcinoma cells lines. Moreover, to reduce the effects of possible plastic scintillation on PDT, we used black cell culture plates in these studies. We have also shown that the effects of the scintillations on PDT could be minimized. In our studies, we have excluded the effects of radiotherapy and the drug toxicity. Our results indicated that the Cerenkov radiation generated from high energy gamma rays can be used to activate the photosensitizer MPPa in PDT, which could potentially overcome the penetration limitations of optical photon based PDT, making the PDT a feasible and complementary cancer therapy for deep lesions.

Key words: Photodynamic therapy, Cerenkov radiation, Cesium-137 irradiator

INTRODUCTION

Cancer is the second leading cause of death in the United States, exceeded only by heart diseases. One of every four deaths in the United States is due to cancer. [1,2] As such, more efforts have been made to improve cancer diagnosis and therapy methods in recent decades. Specifically, chemotherapy and radiotherapy are the primary therapeutic approaches that have been used to treat cancers. However, their drawbacks and side-effects are well-known, including but not limited to extreme pain and discomfort reflected on patients, especially for senior patients. [3,4]

Founded by R. L. Lipson and S. Schwart from Mayo Clinic in 1960s, Photodynamic Therapy (PDT) has become a cancer treatment approach by illuminating dye agents, called photosensitizers, to kill cancer cells. [5,6] The basic principle of PDT is that the excited photosensitizer reacts with oxygen to generate reactive oxygen species (ROS) which are cytotoxic. [7,8] Compared to other therapeutic methods, The photosensitizer is nontoxic until it is excited and the excitation can be selectively delivered to cancerous targets only. Therefore, the side-effects of PDT to normal tissues can be fully controlled and minimized. [9]

A problem with conventional PDT is that the absorption and strong scattering of optical photons from tissues make it difficult to deliver optical photons to deep targets with a typical photon penetration depth of several millimeters, [10,11] which limits the applications of PDT to superficial lesions such as skin cancers or lesions reachable by a light guide. [12,13] Studies have shown that PDT has been applied to treat superficial lesions such as neck cancer, early stage oral cancers, and nasopharyngeal carcinoma. [9,10] However, to date, there are no reports of applications for deep cancers. To overcome these limitations, high energy photons with high penetration power were introduced to deliver energy for PDT treatment. [13]

Cerenkov radiation is produced when a charged particle travels in a medium with a velocity faster than the speed of light in that medium. [14] Inside tissues, β particles can generate Cerenkov radiation when their energy is larger than $250\,\mathrm{keV}$. [15,16] High energy x-rays or γ-rays can induce highly energetic secondary electrons that can result in Cerenkov radiation emission. [17] The high-energy radiation can penetrate deep tissues and deliver the energy to tumors deep inside of the body. Thus, there are substantial advantages for Cherenkov radiation induced PDT. Recent studies have reported the application of Cerenkov radiation activated PDT using energy mediator titanium dioxide. [18] However, the efficiency of the treatment could be limited due to administration process. We hypothesized that high energy γ-rays can result in sufficient Cerenkov radiation as the light source for deep tumor target PDT without nanoparticles as energy mediators. Considering that there are high energy x-rays in radiotherapy and that there are many photosensitizers for clinical applications, we believe the hypothesized approach can be a good complementary cancer therapy for enhancing the efficacy of radiotherapy.

In this study, we used a Cesium-137 irradiator as the high-energy γ-rays sources to excite a photosensitizer, MPPa (Pyropheophorbide-a methyl ester), in cancer cells inside cell plates to establish the feasibility of the proposed approach.

METHOD

Photosensitizer and cell line

The photosensitizer, MPPa ($C_{34}H_{36}N_4O_3$, molecular mass 548.7 gram per mole, 95% purity, Sigma-Aldrich Co. LLC.), was used in this study (Fig. 1). MPPa was first dissolved in acetone (1 mM) and then filtered by 0.2 μm polytetrafluoroethylene syringe filter (Alltech Association Inc., Deerfield, IL). The filtered MPPa was then stored in a dark refrigerator at -20°C.
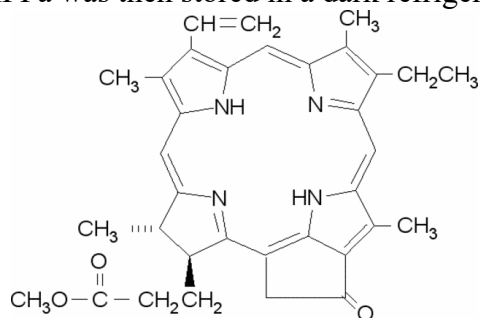

Figure 1. Chemical structure of photosensitizer, MPPa

All the following *in vitro* photodynamic therapy experiments were performed on A549 human lung carcinoma cells (Sigma-Aldrich Co. LLC). The cancer cells were cultured using fresh Ham's F12 nutrient mixture medium (L0136, Biowest) supplemented with 100U/mL penicillin and 10% (v/v) Fetal Bovine Serum (FBS, GIBCO) in 5% CO_2.

Cesium (Cs)-137 irradiator excited PDT

A 2008 manufactured J.L. Shepherd and Associates Mark I-68A 4000Ci Cs-137 irradiator is located at UC Merced Department of Animal Research Service facility as shown in Fig. 2A. The Cs-137 source emits γ-rays with an energy peak of 662 keV. Fig. 2B indicates the three irradiation positions (1, 2, and 3). From the irradiation position and exposure time of samples, we can calculate the radiation dose.

Laser excited PDT

In this study, to validated the efficacy of the photosensitizer, MPPa, we used a pigtailed diode laser (BWF-OEM-650, B&W Tek, 650 nm) with a laser power of 150 mW. As shown in Fig. 2C the laser beam was expanded to cover the major part of a cell culture plate with a measured photon density of 4.6±0.05 mW/cm².

A549 cells (2.5×10^3 cells per well) were seeded in the wells of each plate. The pre-treated drug solution (1 mM/ ml) was firstly 1:100 diluted. The mixture was then 100%, 50%, 25% and 10% v/v added to cell suspension and the different concentrations of photosensitizer assessment now reads at 10, 5, 2.5, 1 and 0 μM. After rinsing with PBS and fresh F12 medium, the experimental group of plates were irradiated for 7, 15 and 30 minutes respectively. The control group of plates was incubated in dark conditions.

Cs-137 irradiator dose calibration

A dose rate for each position was first established by measuring the accumulated dose and dividing by the irradiator exposure time then creating a simple linear function to fit our data. The exposure

times were ensured to be consistent since the built-in irradiator timer was used (where 1.00 corresponds to 60 secs exposure time). Next, Gafchromic EBT3 films were calibrated with a procedure where the net optical density (NOD) was determined for different doses (determined using the dose rate function).[21] For all films, scanning was performed 3 times each to average the pixel values and reduce noise effects. All film analysis was performed in MATLAB (R2016b, MathWorks) with our own in-house algorithm. Once NOD was determined, the data was plotted as a dose (Gray unit [Gy]) versus NOD plot and the data was fit with a two-term exponential function of the following form:

$$f(x) = a \times \exp(b \times x) + c \times \exp(d \times x)$$

Similar equations were generated for Positions 1 and 2 and then from the equations, the isodose curves can be generated.

Cell viability assay

Photocolorimetric determination of cytotoxicity was assessed using CCK-8 dye (Dojindo Laboratories, Tokyo, Japan) to evaluate cell viability.[19,20] After PDT experiments, cells were incubated in dark for 24 hours and then were washed with PBS buffer. 10% (v/v) CCK-8 assay were added to each well. Then we incubated cells for another 4 hours at 37°C. Finally, the Microplate Reader was used to record optical density (OD) of each well. Using the equation: $(OD_{experimental} - OD_{blank})/(OD_{control} - OD_{blank})$, we can calculate the cell viability in each well. As shown in Fig. 2D, we used 24 wells in 6 columns and 4 rows. The same numbers of cells were cultured in each well in the left 5 columns. Then photosensitizers with concentrations of 10 μM, 5 μM, 2.5 μM, 1.0 μM, nd 0 μM were added to each column from left to right. The rightmost column, only filled with F-12 medium without cells, was used as background reference (OD_{blank}). For each column, we averaged the measurements from the Microplate Reader (Model number, company) from four wells with a standard deviation.

Eight 96-well plates were divided into four experimental and one control groups, respectively. A549 cells (2.5×10^3 cells per well) were seeded on the plate wells. Then cells in all plates were administrated to different concentrations of photosensitizer (0, 1, 2.5, 5, 10 μM). After rinsing with PBS and fresh F12 medium, the four experimental plates were irradiated by the Cs-137 irradiator for 30, 15, 7 and 3 minutes, respectively. Accordingly, the corresponding plates in the control group were placed inside the irradiator for the same times with the radiation source off. Thus, the no radiation group indicates the effects from background light. Cs-137 irradiator delivers γ-rays with an energy peak of 662 keV which is far beyond the threshold of Cerenkov radiation. When using transparent cell plates, the photons could possibly result from plastic scintillation. However, the photons from plastic scintillation inside the black colored plates were absorbed by the black plates thus the plastic scintillation effects on PDT were removed.
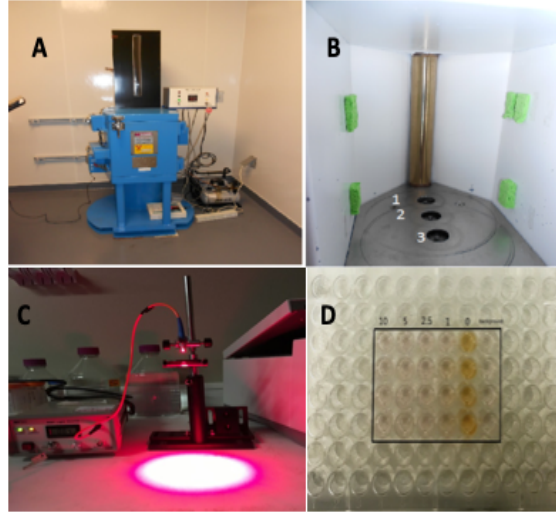
Figure 2. A: The Cs-137 irradiator in UC Merced. B: Inside of Cs-137 irradiator chamber, where the numbers indicate the irradiation positions for dose estimation. C: The laser beam of a pigtailed diode laser was expanded and was used for the laser excited PDT. D: The photo of a typical 96-well plate after adding CCK-8 assay and incubated for another 4 hours.

RESULTS

Cs-137 irradiator dose calibration results and delivered dose

The resulting isodose curves for each drive shaft position are shown below in Fig.3A. The well-plate for each trial was placed at position 3 of the irradiator floor and was stationary during irradiation. Using the generated isodose curve for position 3, the dose rate at the corresponding position is approximately 3.90 Gy/min. With the NOD versus dose function, we created isodose curves for each of the three irradiator drive shaft positions (Fig. 2B). Exposure times for positions 1, 2, and 3 were set to 75s, 90s, and 120s respectively. The Dose (Gy) versus NOD plot for position 3 where a two-term exponential function is fit to our data points is shown in Fig 3B. The resulting exponential function for this position is given by the following ($R^2 = 0.9999$, RMSE = 0.03339)

$$: \text{Dose (Gy)} = 1.594 * \exp(2.901 * \text{NOD}) - 1.619 * \exp((-0.9637) * \text{NOD})$$
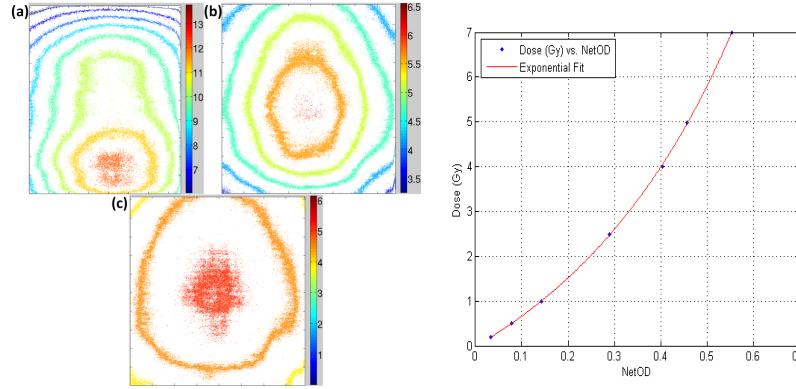
Figure 3 A. Isodose curves for each irradiator drive shaft position. (a) Position 1, (b) Position 2, and (c) Position 3. The color bars are given in units of Gray units [Gy]. B: Plot of NetOD versus Dose (Gy) at Position 3. A two-term exponential function was fit to the data points.

After the irradiator dose calibration, we can calculate the irradiation dose for each positions in the irradiation chamber. For the position 3, the corresponding radiation dose with exposure times of 3, 7, 15, and 30 minutes were calculated to be 5.85, 13.65, 29.25 and 58.5 Gy, respectively, as shown in Table 1.

Table 1. Dose Delivered from Cs-137 exposure.

Exposure Time (mins)	Dose Delivered (Gy)
30	58.5
15	29.25
7	13.65
3	5.85

Cerenkov radiation excited PDT *in vitro*

Figure 4A shows the cell viability in black cell culture plates with irradiation by the Cs-137 irradiator for 30 min and without irradiation. From left to right, each column of the black cell culture plates was treated with different concentrations of the photosensitizer. Compared with the results without irradiation (black triangles), the cell tends to lose their viability with higher MPPa concentration which indicates for Cerenkov radiation induced PDT, the photosensitizer concentration is the main factor. Fig. 4B shows the stained cancer cell organelles. The imaging of top row represented endoplasmic reticulum (ER) and bottom row was another subcellular fraction: Mitochondria. Left column in red is MPPa image; middle column in green was the organelle image; right column was the merged of two. The cancer cell imaging results of the stained cell organelles, which indicates that MPPa targets both ER and mitochondria as indicated by yellow color on the right.
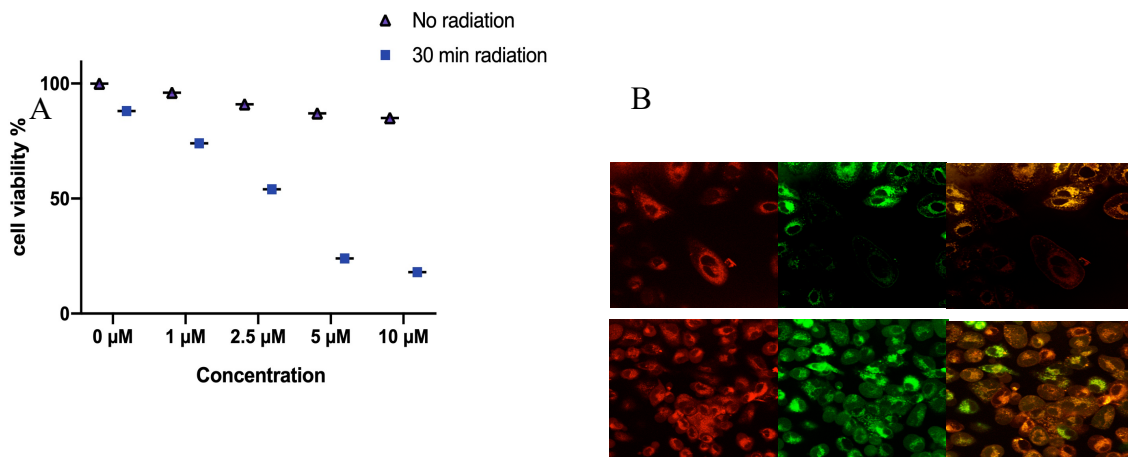
Figure 4. A: The Cs-137 irradiator excited PDT using different concentration of photosensitizer. B: Stained images showed Organelle staining and imaging for ER (top row) and Mitochondria (bottom row). Left column is MPPa image; middle column is the organelle image; right column is merged.

The cell viability after all plates were irradiated by either the Cs-137 irradiator or laser with different irradiation times using 2.5 µM of MPPa are shown in Fig. 5 where the laser treated group was on the left column. We can see that the cell viability is above 85% with the maximum 30 min radiation time. The right column indicates the cell viability when the cells were treated with the high-energy photons. These columns plot the results of Cerenkov radiation excited PDT with different radiation times thus different radiation doses. For the group irradiated for 30 min, we see that the cell viability is 57% as indicated by the rightmost column. The results showed that the efficiency of Cerenkov radiation activated PDT group was higher than optical photon activated PDT group. It confirmed that the Cerenkov radiation induced PDT can achieve excellent therapeutic efficacy.
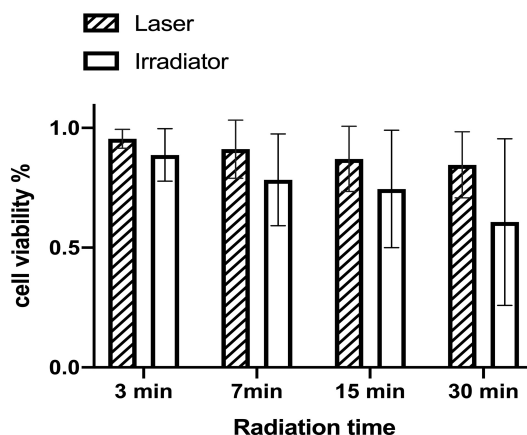
Figure 5. Cell viability (%) of A549 cells after treated by 2.5 µM MPPa photosensitizer and irradiated by the Cs-137 irradiator and laser beam. $p < 0.05$.

CONCLUSION

In our studies, we found it was possible that the second-generation photosensitizer, Pyropheophorbide-a methyl ester (MPPa), can be directly excited by high energy γ-rays through Cerenkov radiation without being bundled to any nanoparticles. Our preliminary finding suggests that high energy excited PDT can expand the applications of PDT to the deep tumors inside of human body due to the greater penetration power of the high-energy photons. No nanoparticles are needed in our approach, which will make the proposed approach more feasible for future clinical applications because there are no nanoparticle toxicity issues. To validate this significant finding, in this work, we used a Cs-137 irradiator as PDT excited light sources to excite MPPa administrated to cultured lung cancer cells. Our results have indicated that the high energy excited PDT could treat deep cancer alone, or can be used as a complementary treatment option with the radiotherapy, in which the high-energy photons excite the photosensitizer to reduce the radiotherapy time for less side-effects from radiation.

ACKNOWLEDGEMENTS

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