Journal of Innovative Optical Health Sciences
Vol. 8, No. 5 (2015) 1550027 (8 pages)
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DOI: 10.1142/S1793545815500273

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An *in vitro* investigation of photodynamic efficacy of FosPeg® on human colon cancer cells

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Received 18 September 2014
Accepted 15 December 2014
Published 28 January 2015

Photodynamic therapy (PDT) is a novel therapeutic approach for combating various cancers. PDT involves the administration of a photosensitizer which generates singlet oxygen after light activation. FosPeg® is the liposomal formulation of mTHPC. In this *in vitro* study, the photodynamic efficacy of FosPeg® on a human colon cancer cell line (HT29) was investigated via studying the cellular uptake of FosPeg® , FosPeg® PDT mediated photocytotoxicity and the cell death mechanism were triggered. FosPeg® PDT demonstrated its antitumor effect in a drug and light dose-dependent manner in HT-29 cells. Lethal dose (LD_{50}) was achieved with 0.4 μg/mL of drug and 3 J/cm^{-2} of light dose. FosPeg® PDT triggered apoptotic cell death via activating caspase cascade and regulating cell cycle progression. In conclusion, FosPeg®-PDT is an effective measure to combat human colon cancer cells.

Keywords: FosPeg®; HT-29; apoptosis; caspase-3.

1. Introduction

Colorectal cancer is a malignant tumor that develops in the colon or rectum region.[1] Risk factors of colorectal cancer include unhealthy lifestyle, alcohol consumption, red meat intake and lack of physical exercise.[2] According to the statistics of World Health Organization published in 2009, colorectal cancer ranked as the third killing-cancer in the

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world, leading to 639,000 deaths every year. The conventional treatment for colorectal cancer includes surgery, radiotherapy, chemotherapy and targeted therapy.[3] However, the choice of treatment is limited by the stage of cancer and patient's general health status. Patients with advance stage colorectal cancer will only receive palliative treatment.[4] Photodynamic therapy (PDT) could be one of the novel therapeutic approaches for the treatment of colorectal cancer.

PDT has been approved worldwide for various cancers, including bladder cancer, esophagus cancer, lung cancer and cervical cancer.[5,6] PDT destroys cancer cells based on the selective accumulation of photosensitizer (PS) in cancer cells. Upon photoactivation, PS will undergo different types of photochemical reactions to produce reactive oxygen species (ROS, such as singlet oxygen), causing oxidative damage to targeted cells.[7–9]

Meta-tetra (hydroxyphenyl) chlorine (mTHPC) is a second generation PS with hydrophobic nature that has good photocytotoxicity. The absorption peak of mTHPC shifts to the longer wavelength of 652 nm in the red spectrum, which is favorable for a deeper tissue penetration.[10] However, the major drawbacks of mTHPC include poor biodistribution, clearance and selectivity of tumor uptake. These problems are related to the photochemical properties of mTHPC. The hydrophobicity leads to poor solubility of mTHPC in physiologically acceptable media, which complicates its formulation and administration. mTHPC will also accumulate in subcutaneous fat tissues near intravenous administration and prolongs the clearance rate for 4–6 weeks after injection. Some of these problems might be overcome by the liposomal formulation of mTHPC (e.g., Foscan®).[11,12] FosPeg® is a new liposomal formulation of m-THPC produced in recent years with improved biodistribution and increased intracellular accumulation in tumor tissues.[13] In this study, we aim to investigate FosPeg® mediated photocytotoxicity on human colorectal adenocarcinoma cell line HT-29 and reveal the cell death mechanism induced by FosPeg®-PDT.

2. Materials and methods

2.1. *Materials*

HT-29 human colorectal adenocarcinoma cell line was obtained from ATCC. FosPeg® was kindly provided by Biolitec AG (Jena, Germany). FosPeg® stock solution (10 μg/mL) was prepared in miliQ water. Phosphate buffered saline (PBS), RPMI-1640 medium and fetal bovine serum (FBS) was obtained from Gibco. Penicillin-Streptomycin-Neomycin (PSN) antibiotic mixture was purchased from Invitrogen. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma Chemical Co. (Sigma-Aldrich, St. Louis, MO, USA). Phycoerythrin (PE)-conjugated anti-active caspase-3 antibody was purchased from BD (BD PharMingen, USA). Annexin V-FITC/7-AAD kit was purchased from Beckman Coulter for cell cycle analysis.

2.2. *Cell culture*

HT-29 cells were grown in RPMI-1640 supplemented with 10% FBS (Gibco BRL, Carlsbad, CA, USA) and 1% PSN antibiotic at 37°C in a humidified 5% CO_2 incubator.

2.3. *Drug uptake assay*

Sub-confluent HT-29 cells were incubated with FosPeg® (0.6 μg/mL) in the dark for 2, 4, and 6 h. Cells were then washed and re-suspended in PBS. Cellular uptake of FosPeg® was determined by flow cytometry (Cytomics FC500, Beckman Coulter) equipped with a 15 mW argon ion laser providing excitation light at 488 nm. Cell suspensions were excited and cellular mTHPC fluorescence signals were detected by a photomultiplier tube with a 610 nm long-pass filter. A minimum of 10,000 cells per sample was analyzed in three independent experiments. The uptake of FosPeg® in terms of mTHPC fluorescence intensity at single-cell level was quantitated.[14]

2.4. *Viability assay*

HT-29 cells (3×10^4/well) were incubated in RPMI-1640 medium in a 96-well flat-bottomed microplate overnight without FBS. FosPeg® (0–1.2 μg/mL) was added to each well and incubated for 4 h before light illumination. Cells were irradiated with 0-3 J/cm^2 of light emitting diode (LED) light as described in our previous publication.[14] Afterward, the drug was removed and replenished with cRPMI. HT-29 cells were further incubated for 20 h in the dark. Then, 10 μL of 5 mg/mL MTT reagent was added and mixed, and then re-incubated for 4 h for the formation of formazan crystal. After that, the

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microplate was centrifuged at $300 \times g$ for 10 min. The medium was removed and the purple formazan in cells was solubilized in dimethyl sulfoxide (DMSO). The color intensity of each well was recorded on a microplate spectrophotometer (Bio-Rad Laboratories).

2.5. *Apoptosis assay*

The staining procedures were modified according to the manufacturer's instructions. In brief, FosPeg® treated and untreated cells (1×10^6) were harvested and washed in ice-cold PBS. Pellet was then resuspended and stained in the staining solution containing 100 μL binding buffer, 10 μL annexin V-FITC dye and 20 μL 7-AAD viability dye. Staining mixture was kept in the dark and on ice for 15 min. After staining, 400 μL of binding buffer was added and the samples were analyzed immediately by the flow cytometry in FL1 and FL3 channels. For active caspase-3 assay, pellet was resuspended in the BD Cytofix/Cytoperm™ solution. The cells were incubated on ice bath for 20 min and then washed in BD Perm/Wash™ buffer twice. Cell pellets were then stained by adding 50μL BD Perm/Wash™ buffer and 2μL anti-caspase-3 antibody at room temperature for 30 min. After staining, cells were washed in 1 mL BD Perm/Wash™ buffer once then resuspended in 500 μL BD Perm/Wash™ buffer for analysis by flow cytometry immediately using FL2 channel.

2.6. *Cell cycle analysis*

Cell cycle phase distribution was analyzed by flow cytometry with PI staining. HT-29 cells (10^6 cells/dish) were cultured and synchronized in RPMI-1640 without FBS for 24 h. At 24 and 48 h post-FosPeg®-PDT, treated cells were harvested, washed and suspended in PBS. Cells were fixed with ice cold 80% ethanol overnight (at $-20°$C). Fixed cells were washed twice with PBS, then re-suspended in PBS containing PI staining solution (10 μg/mL PI, 10 mg/mL RNase A and triton X-100) followed by incubation at 37°C for 30 min. For each sample, 10,000 events in triplicates were counted by Cytomics FC500 (Beckman Coulter) and analyzed by FlowJo Software (Version 5.7.2) to estimate the proportion of the cell cycle phases in the form of the cell number against DNA content histograms. The debris and doublets were gated out and excluded by the software.

2.7. *Statistical analysis*

Data were analyzed by GraphPad 4.0, and presented as mean ± SEM. Comparison of results from MTT cell proliferation assay and cell cycle analysis was evaluated by one way ANOVA followed by Dunnett post-tests. The p-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. *Intracellular accumulation of FosPeg®*

The intracellular accumulation of FosPeg® in HT-29 cells at different time points were determined by flow cytometry. Figure 1 summarized the kinetics of FosPeg® accumulation and showed a raise for up to

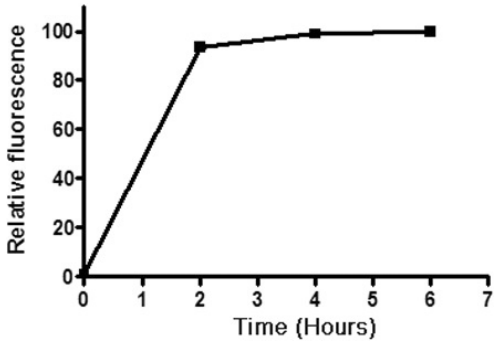

Fig. 1. Uptake of FosPeg® in HT-29 cells. The fluorescence intensity represents the relative cellular content of FosPeg® in HT-29 cells after incubating with FosPeg® (0.6 μg/mL) for 0–6 h. Results were obtained from three independent experiments.

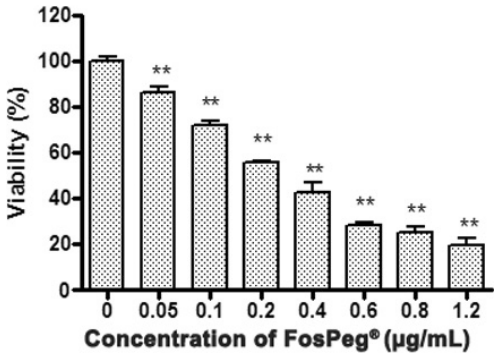

Fig. 2. Photocytotoxicity of FosPeg® PDT in HT-29 cells. Viability of HT-29 cells was presented as percentage of control and measured at 24 h post-FosPeg® PDT treatment. LD_{50} and LD_{70} were obtained at 0.3 μg/mL and 0.6 μg/mL under the light dose of 3 J/cm^2, respectively. The values of percentages represented the means of three independent experiments and the bar indicated the SEM ($n = 3$, **: $p < 0.05$).

6 h of incubation. This test suggested that 4 h of incubation was necessary for FosPeg® to be accumulated in HT-29 cells prior to light irradiation.

3.2. *Photocytotoxicity of FosPeg®*

MTT assay was used to evaluate the phototoxicity of FosPeg®-PDT on HT-29 cells. Results showed that FosPeg®-PDT induced a significant ($p < 0.05$) cell death in a dose-dependent manner (Fig. 2). Within the range of FosPeg® concentrations of 0.05 μg/mL to 1.2 μg/mL, the FosPeg®-PDT cytotoxic effect on HT-29 cells increased progressively at the light dose of 3 J/cm^2. The effect of drug alone (dark toxicity) and light irradiation alone were negligible (data not shown). The lethal doses of 50%

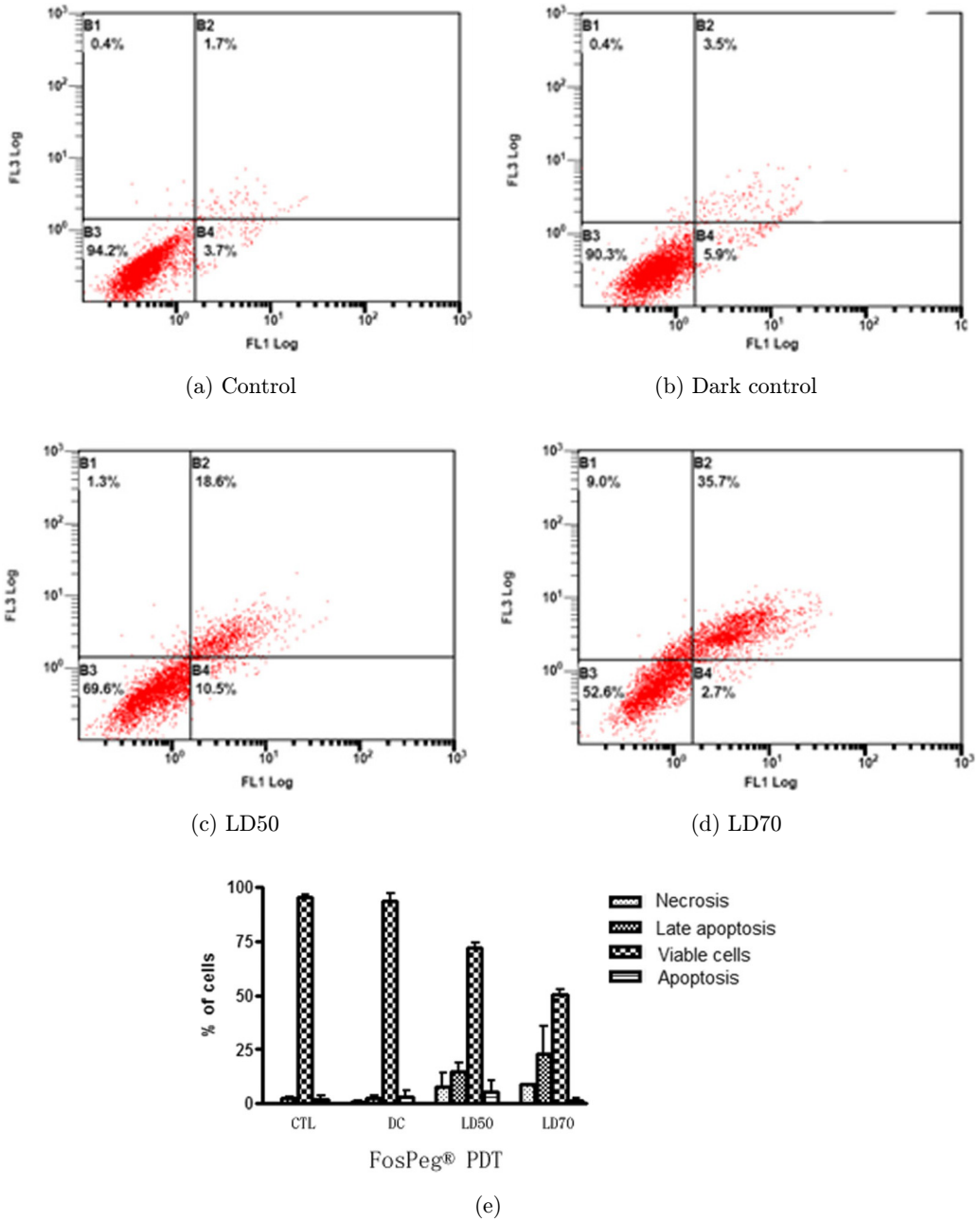

Fig. 3. Flow cytometry assay of apoptosis. (a)–(d): Dot plot graphs of Annexin V/7AAD apoptotic assay and (e) the percentage of cell distribution in different quadrants. HT-29 cells appeared to have two cell populations and shifted from B3 region to all other regions after FosPeg® PDT, suggesting that these cells were necrotic (B2 region) and some were apoptotic (B4 region) at the same time (CTL: Control cells; DC: Dark control).

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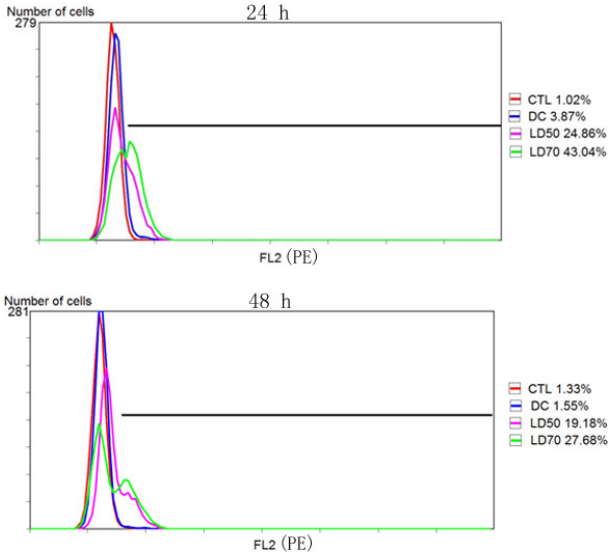

Fig. 4. Caspase-3 activation. Top: 24 h after PDT; bottom: 48 h after PDT. Results indicated that FosPeg® PDT activated caspase-3 protein expression. (CTL: control cells; DC: dark control with FosPeg® only; LD50: cells treated with 0.3 μg/mL of FosPeg® and 3 J/cm^2 of light; LD70: cells treated with 0.6 μg/mL of FosPeg® and 3 J/cm^2 of light).

(LD_{50}) and 70% (LD_{70}) were achieved at 0.3 μg/mL and 0.6 μg/mL, respectively. From the cytotoxicity data, LD_{50} and LD_{70} at 3 J/cm^2 were selected for subsequent experiments.

3.3. *Induction of apoptosis*

The induction of apoptosis was measured by Annexin V apoptosis assay at 24 h post-FosPeg® PDT treatment. As shown in Figs. 3(a)–3(d), cells stained by Annexin V only represented early apoptotic cells (B4 region). Cells stained by both Annexin V and 7AAD indicated either late apoptotic cells or necrotic cells (B2 region). Cells stained by 7AAD indicated late necrotic cells (B1 region). Figure 3(e) summarized the percentage of cells undergoing necrosis and apoptosis under LD_{50} and LD_{70}. Cell morphological changes were observed in HT-29 cells after FosPeg® PDT treatment. Results showed a gradual decrease of viable cells with increased apoptotic and/or necrotic cells as drug dose increased.

Figure 4 shows that an increase in fluorescence intensity was obtained in FosPeg® PDT treated cells when compared to control cells. The increase in fluorescence intensity indicated that FosPeg® PDT activated caspase-3 protein in HT-29 cells. At 24 h post-PDT treatment, 25% and 43% of cells demonstrated caspase-3 activation at LD_{50} and LD_{70}, respectively. While at 48 h post-PDT treatment, 20% and 27% of cells demonstrated caspase-3 activation at LD_{50} and LD_{70}, respectively.

3.4. *Alteration of cell cycle phase distribution and DNA content*

The FosPeg®-PDT effect on the cell cycle phase distribution was determined by flow cytometric analysis. Table 1 shows that FosPeg® PDT triggered time-dependent DNA content changes in HT-29 cells. Representative data are shown in Fig. 5. The sub-G1, G1/G0, S and G2/M phases were labeled as (C) (D), (E) and (F) in the histograms,

Table 1. Dose-and Time-effect of FosPeg® PDT on Cell Cycle Distribution in HT-29.

	CTL	DC	LD50	LD70
			24 h	
Sub-G1	2.90 ± 3.11	12.03 ± 12.43	21.21 ± 6.51*	13.51 ± 12.69
G1/G0	42.19 ± 16.74	50.74 ± 3.98	39.15 ± 5.61	34.44 ± 4.92
S	49.11 ± 23.95	32.78 ± 2.85	33.54 ± 4.43	46.55 ± 12.78
G2M	4.55 ± 3.00	3.63 ± 4.61	4.62 ± 5.92	3.76 ± 3.05
			48 h	
Sub-G1	1.11 ± 0.11	0.80 ± 0.58	35.29 ± 8.82****	52.25 ± 8.24****
G1/G0	45.04 ± 0.13	44.18 ± 2.57	33.87 ± 6.22***	26.43 ± 5.40****
S	34.64 ± 0.92	34.17 ± 0.30	22.44 ± 4.96****	17.23 ± 5.06****
G2M	19.06 ± 1.09	20.54 ± 2.48	9.35 ± 0.29**	5.31 ± 0.04****

Note: The cell percentages of different cell cycle phases for different concentrations of FosPeg® were expressed as mean ± SD. Significant difference between treatment and control groups were analyzed by two-way ANOVA and followed by Bonferroni's post-tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

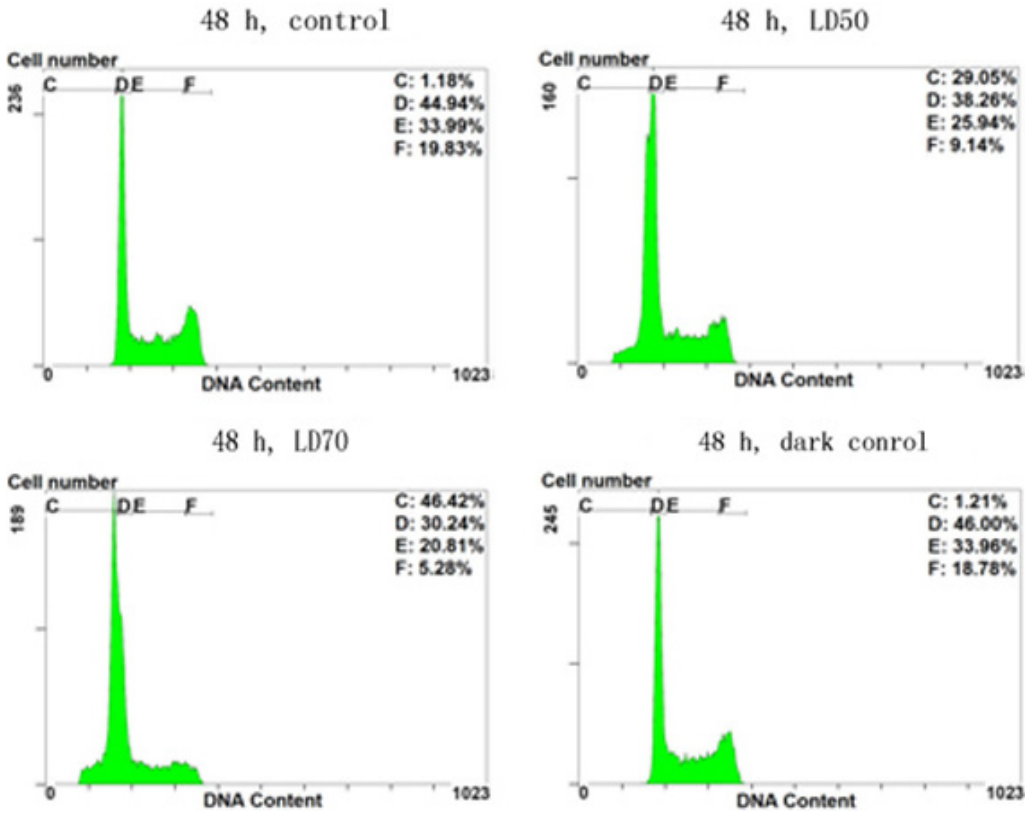

Fig. 5. Flow cytometric analysis of FosPeg® PDT on cell cycle distribution in HT-29 cells. Results show that FosPeg® PDT modulated cell cycle distribution at 48 h post PDT. Phase indication: C = sub-G1 phase, D = G1/G0 phase, E = S phase, F = G2/M phase. Data were obtained from three-independent experiments.

respectively. Cell cycle and DNA content analysis showed that an increase in sub-G1 portion of HT-29 cells was obtained in a time-dependent manner. The increased sub-G1 peak suggested that FosPeg® PDT induced apoptotic cell death in HT-29 cells when comparing the treated cells with the control and the dark control groups. At LD_{50}, a significant increase in the percentage of sub-G1 phase cells was obtained at 24 h and 48 h post-FosPeg® PDT, with 21.2% and 35.3% of HT-29 cells undergoing apoptosis ($p < 0.05$).

4. Discussion

PDT uses a combination of PS, visible light and molecular oxygen to selectively destroy the biological targets in cancer cells. It is an evolving cancer treatment regimen which has been approved for clinical application in many countries.[15] However, PDT efficacy can be affected by PS uptake and localization in cancer cells.[16] FosPeg® is a new liposomal formulation of mTHPC. In general, liposomal formulation can facilitate the cellular uptake of PS.

Studies demonstrate that FosPeg® PDT is effective for killing nasopharyngeal carcinoma cells and hepatocellular carcinoma cells.[14,17] Yet its ability to eradicate colon cells is still unknown. Therefore, the possibility of FosPeg® PDT to be used in treating colorectal cancer is worthy of being investigated. This *in vitro* study was aimed to investigate the FosPeg® mediated photocytotoxicity and mode of cell death by using HT-29 cells as a model.

Our data suggested that FosPeg® PDT was effective in destroying HT-29 cells. According to the MTT assay results (see Fig. 2), it inhibited HT-29 cells growth in a dose-dependent fashion and the lethal doses of 50% and 70% were achieved as low as 0.3 μg/mL and 0.6 μg/mL FosPeg® under 3 J/cm^{-2} of light activation, respectively.

The mode of cell death induced by FosPeg® PDT was illustrated via Annexin V Apoptosis Assay. Annexin V/7AAD co-staining differentiates apoptotic and necrotic cells by measuring the morphological changes and the expression of apoptotic-specific

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antigen that increased affinity to PI in necrotic cells. Results of Annexin V/7AAD staining (see Fig. 3) showed that FosPeg® PDT induced apoptotic cell death with a decrease in viable cells in both LD_{50} and LD_{70} after 24 h incubation. When comparing the cell distribution at LD_{50} and LD_{70}, LD_{70} had less apoptotic cells but a greater increase in necrotic cells (see Fig. 3(e)). This suggested that at a higher lethal dose, necrosis was more favorable than apoptosis. Results were in line with other studies. Our group reported previously that FosPeg® induced apoptotic cell death in nasopharyngeal carcinoma cells.[14] Sherifa, *et al.*, also reported that FosPeg® PDT induced apoptotic cell death in hepatocellular carcinoma cells.[17]

The mode of cell death induced by FosPeg® PDT was further revealed by Active Caspase-3 Apoptosis Assay. Results showed that active caspase-3 expression in FosPeg® PDT treated HT-29 cells were also in a dose-dependent manner (see Fig. 4). There were 25% and 43% of cells undergoing apoptosis at LD_{50} and LD_{70} at 20 h post-PDT treatment. Active caspase-3 enzymes were demonstrated in FosPeg® PDT treated cells, indicating that FosPeg® PDT induced apoptotic cell death via activation of caspase-3 cascade. Our study also demonstrated that FosPeg® PDT triggered cell cycle arrest at sub-G1 phase of HT-29 cells (see Fig. 5 and Table 1). The cell cycle analysis showed that sub-G1 peak appeared in all the treated groups but not the control groups. It suggested that FosPeg® PDT induced apoptosis in HT-29 cells. The sub-G1 peak has also shown to be dose dependent.

In summary, our findings confirmed that FosPeg® PDT was effective in inducing apoptotic death of human HT-29 cells, which was likely via the activation of caspase-3. Understanding the mechanism of FosPeg® PDT may shed light on the usefulness of PDT for treating colorectal cancer.

Acknowledgments

We thank Biolitec for generously providing the photosensitizer FosPeg®. This study was supported by The Hong Kong Polytechnic University Central Research Grant (G-YJ67 and G-U737).

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