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FosPeg® PDT alters the EBV miRNAs and LMP1 protein expression in EBV positive nasopharyngeal carcinoma cells

Ricky W.K. Wu [a], Ellie S.M. Chu [b], Zheng Huang [c], C.S. Xu [d], C.W. IP [a], Christine M.N. Yow [a,*]

[a] Medical Laboratory Science Section, Department of Health Technology & Informatics, Hong Kong Polytechnic University, Hong Kong Special Administrative Region
[b] Department of Medical Science, Tung Wah College, Hong Kong Special Administrative Region
[c] Cancer Center, University of Colorado Denver, USA
[d] School of Chinese Medicine, The Chinese University of Hong Kong, Hong Kong Special Administrative Region

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is one of the top ten cancers highly prevalent in Hong Kong and South China. Epstein-Barr virus (EBV) infection contributes to the tumorigenesis of NPC through the expression of different viral proteins. Among these, Latent Membrane Protein 1(LMP1) is the major oncoprotein expressed by EBV. Foscan® (Biolitec AG), m-tetrahydroxyphenylchlorin (mTHPC)-based photosensitizing drug, has been used in the photodynamic therapy (PDT) for head and neck cancers. FosPeg® (Biolitec AG) is a new formulation of mTHPC contained in PEGylated liposomes with optimized distribution properties. In this *in vitro* study, the potential of FosPeg®-PDT on human EBV positive NPC cell (c666-1) and EBV negative cells (HK1 and CNE2) were investigated. Effects of FosPeg®-PDT on the expression of EBV BART miRNAs (EBV miRNA BART 1-5p, BART 16, and BART 17-5p), LMP1 mRNA and proteins on c666-1 cells were also elucidated.

The killing efficacy of FosPeg®-PDT on NPC cells were determined by MTT assay after LED activation. Effects of FosPeg®-PDT on the expression of LMP1 mRNA and protein were examined by real time PCR and western blot analysis.

FosPeg®-PDT demonstrated its antitumor effect on c666-1 cells in a drug and light dose dependent manner. LD_{30}, LD_{50} and LD_{70} were achieved by applying LED activation (3 J/cm^2) at 4 h post incubated cells with 0.05 μg/ml, 0.07 μg/ml and 0.3 μg/ml FosPeg®, respectively. Up-regulation of both LMP1 mRNA and protein were observed after FosPeg®-PDT in a dose dependent manner.

FosPeg®-PDT exerted antitumor effect on c666-1 cells through up-regulation of LMP1 protein. Understanding the mechanism of FosPeg®-PDT may help to develop better strategies for the treatment of NPC.

1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the top ten cancers highly prevalent in Southern China, especially in Hong Kong and Guangdong [1,2]. NPC encompasses any squamous cell carcinoma arising in the epithelial lining of the nasopharynx, a tubular space situated at the base of the skull. Different from other head and neck cancers, NPC is strongly associated with Epstein-Barr virus (EBV). EBV is a herpes virus that infects over 90% of adult population worldwide [3]. It is known as the most potent transforming agent for human cells and is associated with numbers of malignancies, include the Burkitt's lymphoma, nasopharyngeal carcinoma, T cell lymphomas, lung carcinoma and gastric carcinoma [4,5]. The tumorigenic potential of EBV is mainly related to a unique set of latent gene products, include the latent membrane proteins (LMP1, LMP2A, LMP2B) and EBV-determined nuclear antigens (EBNA1 and EBNA2) [3]. Among these, LMP1 is the principal oncogene involved in the process of EBV-associated oncogenesis of NPC [6,7]. LMP1 abnormally activates a number of signaling pathways in NPC cells, including the nuclear factor kappa B (NF-kB) and mitogen-activated protein kinases (MAPK) pathways, and results in inhibiting apoptosis; inducing cell immortality; promoting cell proliferation; and influencing the cell invasion and metastasis [6,8]. Interestingly, several studies revealed that transforming potential of LMP1 was dose-dependent [9].

EBV was the first human virus reported to encode micro-RNAs (miRNAs). miRNA is a class of 20–25-nucleotide non-coding RNA which could bind with their target mRNA, results as inhibition of gene expression [10]. EBV encoded miRNAs were found mainly in two clusters, which were known as the BamHI-A rightward transcripts (BARTs) cluster 1 and 2. Recently, researchers have been

* Corresponding author. Address: Room 930, Block Y, The Hong Kong Polytechnic University, Hung Hom, Hong Kong Special Administrative Region. Tel.: +852 3400 8575; fax: +852 2362 4365.
E-mail address: htcyow@polyu.edu.hk (C.M.N. Yow).

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identified at least three BARTs cluster 1 miRNAs (*ebv-miR-BART1-5p*, *16* and *17-5p*) targeting the LMP1 gene and down regulating the LMP1 protein expression [6,11,12].

The conventional treatments for NPC are radiotherapy and chemotherapy [13,14]. However, different complications were resulted after receiving radiotherapy and chemotherapy and were mainly caused by radiation toxicities, distant recurrence and development of multi-drug resistance phenotypes [15,16]. Alternative treatment is advisable to NPC as it is often inoperable because of its complex anatomical location [17,18]. The development of improved therapeutic strategies, photodynamic therapy (PDT), shed light on the development of NPC treatment [19–21]. As a potential powerful treatment for cancer, PDT could be applied solitary or in combination with chemotherapy, radiotherapy, or surgery.

Photodynamic therapy (PDT) is an evolving cancer treatment regimen with approval for use in USA, EU, Canada, Russia and Japan. PDT uses a combination of photosensitizing agents (PSs), visible light and molecular oxygen to selectively destroy the biological targets in tumor cells. None of these is individually toxic, but together they initiate photo-destruction to biological targets. Effectiveness of PDT depends on the tumor localizing photosensitizer, which absorbs light to produce reactive oxygen species (ROS) [22–24]. Advanced development of light source could also enhance the PDT efficiency. The clinical efficacy of PDT is depend on complex dosimetry, including total light dose, light exposure time, and light delivery mode. Light-emitting diodes (LEDs) is one of the alternative light sources with several advantages, including relatively narrow spectral bandwidths, high fluence rates, small and cost-effective, simple to install, and a longer operational life [22,24]. A novel light source with light emitting diode (LED) has been setup by our group showing promising results in activation of the nano-photosensitizer hypocrellin in nasopharyngeal carcinoma cells [25].

FosPeg® (Biolitec AG) is the derivative of meta-tetra (hydroxyphenyl) chlorine (mTHPC) contained in PEGylated liposomes. mTHPC is a chlorine-like 2nd generation photosensitizer which has been shown to be highly effective in treating skin, prostate and pancreatic cancer [26,27]. However, the major drawback of mTHPC is related to its photochemical properties. The hydrophobicity of mTHPC leads to poor solubility in physiologically acceptable media and complicates its formulation, administration and bio-distribution. Thus the liposomal formulations using PEGylated liposomes as the nanocarriers to encapsulate mTHPC have been developed. The aim of using liposomes with long-circulating poly-ethylene glycol (PEG) as an improved delivery system is to enhance its therapeutic effects by solubilizing the photosensitizer at suitable concentration, increasing drug uptake as well as tumor eradication [28,29].

In this study, we aim to investigate the photodynamic efficacy of FosPeg® – a new liposomal formulation of mTHPC in EBV positive and EBV negative NPC cell lines and to reveal the effect of FosPeg® PDT on the expression of LMP1 at both molecular and protein levels.

2. Materials and methods

2.1. Photosensitizer, chemicals and antibodies

FosPeg® (1.5 mg mTHPC/mL) was kindly provided by Biolitec AG (Jena, Germany). FosPeg® stock solution (10 μg/mL) was prepared in miliQ water. 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). Primary mouse anti-LMP1 antibody (CS1-4) was purchased from Dako (Glastrup, Denmark). Secondary anti-mouse antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.2. Cell lines and cell culture

EBV-positive NPC cell line c666-1 (an undifferentiated NPC cell line) and EBV-negative NPC cell line HK1 (a highly differentiated NPC cell line) were kindly provided by Chinese University of Hong Kong [30,31]. EBV-negative NPC cell line CNE2 (a poor differentiated NPC cell line) was purchased from Shanghai Biosis Biotechnology Co., Ltd. (Shanghai, China) [25]. c666-1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA, USA), 1% glutamax and 1% antibiotics PSN (50 IU/ml penicillin, 50 mg/ml streptomycin and 100 mg/ml neomycin) (Gibco BRL, Carlsbad, CA, USA). HK1 and CNE2 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA, USA) and 1% antibiotics PSN (50 IU/ml penicillin, 50 mg/ml streptomycin and 100 mg/ml neomycin). Cells were grown at 37 °C in a humidified 5% CO_2 incubator.

2.3. Examination of intracellular drug uptake and distribution

Sub-confluent NPC cells were incubated with FosPeg® (0.1 μg/mL) in dark for 1, 2, 4, 8 and 24 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 the fluorescence signal of drug uptake by the cells was 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 acquired [19].

To determine the intracellular localization of FosPeg®, cells were incubated with FosPeg® (1 μg/mL) for 4 h in darkness followed by co-incubation with 100 nM MitoTracker Green (Molecular Probe, Invitrogen, Carlsbad, CA, USA) and LysoTracker Green (Molecular Probe, Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C with 5% CO_2. The MitoTracker Green and LysoTracker Green were two fluorescent probes used specifically for the identification of sub-cellular organelles of the mitochondria and lysosomes, respectively. Prior to the visualization, the excess probes were washed off and the image analysis was accomplished with a fluorescence microscope (EZ-C1, Nikon). The fluorescent images were captured with a magnification of 400×. All parameters were kept constant to ensure reliable comparison throughout the experiment. The imaging measurements were repeated at least six times and several hundred cells were observed.

2.4. Light Irradiation

NPC cells (3×10^4 cells/well) were seeded in 96-well tissue culture plates and co-incubated with various concentrations of FosPeg® (0.03–0.3 μg/mL) for 4 h. Cells then were irradiated with 0–3 J/cm^2 of light from light emitting diode (LED) with the wavelength of 630 nm and the highest output of 130 mW/cm^2 distributing uniformly over an area of 78.5 cm^2 as described previously [25].

2.5. MTT assay

3×10^4 cells/well were seeded in 96 well plates for 24 h and incubated with a range of 0.03–0.3 μg/mL FosPeg® for 4 h. Light irradiated cells were washed and further incubated for 24 h. MTT (5 mg/mL) was added to each well and incubated for 3 h. Viable cells took up MTT and reduced it to form dark blue water insoluble