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Evaluation of one- and two-photon activated photodynamic therapy with pyropheophorbide-a methyl ester in human cervical, lung and ovarian cancer cells

Ting Luo [a], Brian C. Wilson [b], Qing-Bin Lu [a,c,*]

[a] Department of Physics and Astronomy, University of Waterloo, 200 University Avenue West, Waterloo, ON N2L 3G1, Canada
[b] Department of Medical Biophysics, University of Toronto, and Ontario Cancer Institute, 610 University Avenue, Toronto, ON M5G 2M9, Canada
[c] Departments of Biology and Chemistry, University of Waterloo, 200 University Avenue West, Waterloo, ON N2L 3G1, Canada

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ABSTRACT

Two-photon activated photodynamic therapy (2-γ PDT) has the potential of treating deeper tumors and/or improving tumor targeting. Here, we evaluated the one- and two-photon activated PDT efficacy of pyropheophorbide-a methyl ester (MPPa), a second-generation photosensitizer derived from chlorophyll *a*. We show that MPPa, when activated by femtosecond (fs) laser pulses at 674 nm, has high one-photon (1-γ) PDT efficacy against cisplatin-sensitive human cervical (HeLa) and cisplatin-resistant human lung (A549) and ovarian (NIH:OVCAR-3) cancer cells. At a low light dose of 0.06 J cm^{-2}, the IC_{50} (the MPPa concentration required to kill 50% of the cells) was determined to be 5.3 ± 0.3, 3.4 ± 0.3 and 3.6 ± 0.4 μM for HeLa, A549 and NIH:OVCAR-3 cells, respectively. More significantly, we also show that MPPa can be effectively activated by an 800 nm, 120 fs laser through 2-γ excitation; at a light dose causing no measurable photocytotoxicity in the absence of photosensitizer, the corresponding IC_{50} values were measured to be 4.1 ± 0.3, 9.6 ± 1.0 and 1.6 ± 0.3 μM, respectively. These results indicate that MPPa is a potent photosensitizer for both 1- and 2-γ activated PDT with potential applications for difficult-to-treat tumors by conventional therapies.

1. Introduction

Photodynamic therapy (PDT) has emerged as a novel clinical approach that involves the administration of a photoactivatable compound (photosensitizer) and subsequent exposure of the target diseased tissue to light (including laser sources) for the treatment of various tumors and other non-malignant conditions [1–4]. PDT has potential advantages over surgery and other therapies of being minimally invasive, local targeting, and having few systemic side effects. PDT has been successfully applied for the treatment of various types of cancer, including esophageal, lung and skin cancers [1–4]. To date, however, only a few photosensitizers have been approved for clinical use and mostly activated at wavelengths (λ) in the range of 630–690 nm, at which the light tissue penetration and resulting effective depth of treatment are limited [5].

The penetration depth of light into tissue depends strongly on the wavelength. In particular, there is an "optical window" between 700 and 950 nm, where the absorbance of light by biological molecules is relatively low [5]. Below about 700 nm endogenous chromophores, particularly hemoglobin/oxyhemoglobin and melanin, are highly absorbing, while above about 900 nm absorption by water becomes dominant [5,6]. The spectral mismatch between the photosensitizer absorption spectrum and the optimum wavelength for tissue penetration has hindered the application of PDT to the treatment of larger or thicker solid tumor masses. Hence, one approach to improve the clinical effectiveness of PDT is to develop new photosensitizers that can be activated at near-infrared (NIR) wavelengths [7,8].

Two-photon excitation, originally predicted by the Nobel Laureate Maria Goeppert-Mayer in the 1930s [9], is a non-linear optical process in which a molecule is promoted to an excited state by simultaneous absorption of two photons. This concept has been successfully applied in confocal fluorescence microcopy for more than two decades [10–12]. Two-photon activated photodynamic therapy, denoted here as 2-γ PDT, has also been explored since 1990 [13–21] and has several potential advantages over conventional one-photon (1-γ) PDT using (quasi) continuous-wave (CW) illumination. First, there is experimental evidence *in vivo* that 2-γ PDT can treat to greater depth in tissue [19,21] by using NIR light,

* Corresponding author at: Department of Physics and Astronomy, University of Waterloo, 200 University Avenue West, Waterloo, ON N2L 3G1, Canada. Tel.: +1 519 888 4567.
E-mail address: qblu@uwaterloo.ca (Q.-B. Lu).

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although a rigorous quantitative understanding of this has not been reported. Second, the reduced interaction of the NIR light with the tissue leads to higher differential excitation of the photosensitizer and so, in principle, minimizes the side effects and allows higher light doses to be administered. Third, due to the non-linear nature of 2-γ excitation, the photosensitizer activation is confined to the region of highest light intensity (the basis of 2-γ confocal fluorescence microscopy), which further improves 3D control of the spatial localization of treatment. This has been demonstrated in the use of 2-γ PDT to target single blood vessels in preclinical models [18].

However, the utility of 2-γ PDT has been limited by the low 2-γ cross-section of currently used photosensitizers. For example, at a peak irradiance of 1.75×10^{11} W cm^{-2}, the light doses required to kill 50% of cells (LD_{50}) of two clinically-approved photosensitizers, Photofrin® with a 2-γ absorption cross section $\sigma = 10$ in Goeppert-Mayer (GM) units (1 GM = 10^{-50} cm^4 s photon^{-1}) at 800 nm and Visudyne® with $\sigma = 51$ GM at 900 nm, were found to be 11,300 J cm^{-2} and 1700 J cm^{-2}, respectively [17], which are about 2 orders of magnitude higher than those required for 1-γ PDT of the same compounds. Several novel photosensitizers designed explicitly to have much higher σ values (up to about 17,000 GM) have been synthesized and their photodynamic properties have been explored [8,18–21], with promising initial results. For example, *in vivo* experiments by Collins et al. [18] demonstrated single blood-vessel closure by 2-γ PDT using a conjugated porphyrin dimer activated by a 300 femtosecond (fs) laser beam at 920 nm, while Spangler et al. [21] showed effective treatment up to about 2 cm depth in tumor tissue *in vivo* using a new 2-γ PDT triad. These positive initial findings have inspired further investigation of 2-γ PDT, including the work reported here.

Pyropheophorbide-a methyl ester (MPPa, also known as PPME), a semi-synthetic molecule derived from chlorophyll *a*, is one of a number of emerging second-generation PDT agents [22–30]. Compared with the FDA-approved photosensitizer Photofrin®, MPPa has a much stronger absorption in the red part of the visible spectrum: its molar extinction coefficient $\varepsilon = 47{,}500$ M^{-1} cm^{-1} at $\lambda = 667$ nm [23] is about 15 times that of Photofrin® with $\varepsilon = 3200$ M^{-1} cm^{-1} at $\lambda = 630$ nm [14]. The antitumor efficacy of 1-γ PDT using MPPa has been studied in a number of cancer cell lines *in vitro*, including colon (HCT-116), lung (NCI-h446), nasopharyngeal (HONE-1), prostate (PC-3M), ovarian (COC1/DDP) and breast (MDA-MB-231, MDA-MB-435, and MCF-7) [22–30]. Of particular relevance here, MPPa has a very strong absorption peak around 400 nm, nearly double that at 667 nm, as shown in Fig. S1B. However, no previous studies have examined the effectiveness of PDT with MPPa via 2-γ excitation at 800 nm. Here, we demonstrate that 1-γ PDT with MPPa is highly cytotoxic against cisplatin-sensitive human cervical (HeLa) and cisplatin-resistant lung (A549) and ovarian (NIH:OVCAR-3) cancer cells *in vitro*. For experimental convenience, these studies were performed using 674 nm, ~120 fs laser pulses. Moreover, we also showed that the 2-γ PDT cytotoxicity of MPPa could be effective at the optimal tissue-penetrating NIR wavelength of 800 nm, suggesting a novel strategy via 1-γ and 2-γ PDT for effective treatment of cervical, lung and ovarian cancers that are currently difficult to cure by conventional methods [31–34].

2. Materials and methods

2.1. Chemicals and cell lines

Pyropheophorbide-a methyl ester (MPPa; $C_{34}H_{36}N_4O_3$; MW = 548.7 g/mol; 95% of purity) and Zn(II) phthalocyanine tetrasulfonic acid ($ZnPcS_4$; $C_{32}H_{16}N_8O_{12}S_4Zn$; MW = 898.19 g/mol) purchased from Frontier Scientific Inc. (Logan, UT, USA) were used without any further purification. A stock solution of 2.5 mM MPPa was made in acetone and then stored in the dark at −20 °C. A stock solution of 5 mM $ZnPcS_4$ was made in ultrapure water with a resistivity of >18 MΩ/cm obtained from a Barnstead Nanopure (Thermo Scientific, Dubuque, IA, USA) water system and stored in the dark at 4 °C. Penicillin–streptomycin antibiotics (PS), minimum essential medium Eagle (MEM), nutrient mixture F12 Ham Kaighn's modification (F12K), RPMI-1640 Medium, and trypsin–EDTA (0.5 g/L porcine trypsin and 0.2 g/L EDTA 4Na in Hank's Balanced Salt Solution with phenol red) were purchased from Sigma–Aldrich (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) and fetal bovine serum (FBS) from Hyclone Laboratories (Logan, UT, USA). The HeLa (ATCC#: CCL-2™), A549 (ATCC#: CCL-185™) and NIH:OVCAR-3 (ATCC#: HTB-161™) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HeLa cells were cultivated in MEM supplemented with 10% FBS, 100 units/mL penicillin G and 100 μg/mL streptomycin. A549 cells were cultivated in F12K supplemented with 10% FBS, 100 units/mL penicillin G and 100 μg/mL streptomycin. The complete growth medium for NIH:OVCAR-3 cells was RPMI-1640 medium supplemented with 20% FBS, 100 units/mL penicillin G, 100 μg/mL streptomycin and 0.01 mg/mL bovine insulin. The cell culture was kept at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Subcellular localization

Exponentially growing HeLa, A549 and NIH:OVCAR-3 cells were seeded in 96-well microplates at densities of 3×10^3, 3×10^3 and 4×10^3 cells/well, respectively. After overnight incubation, the cells were incubated with 5 μM MPPa for 24 h in the dark. For the last 30 min of incubation, Hoechst 33342 was added for nuclear staining at a final concentration of 1 μg/mL. After incubation, the cells were washed twice with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde so that the imaging could be done at any time thereafter and to avoid any additional photodynamic damage during imaging. The images were taken on a Nikon Eclipse TS100/TS100-F microscope with filter sets of Ex/Em of BP510-560/LP590 nm and BP330-380/LP420 nm for MPPa and Hoechst 33342, respectively.

2.3. One-photon PDT treatment

Exponentially growing HeLa, A549 and NIH:OVCAR-3 cells were seeded in 96-well microplates at densities of 5×10^3, 5×10^3 and 7×10^3 cells/well, respectively. After overnight incubation, the cells were incubated with various concentrations of MPPa for 18 h in the dark. There was little difference between 18 and 24 h incubation. For instance, Tian et al. [26] reported that cytotoxicity induced by 1-photon excitation of MPPa reached a plateau at a drug incubation time $\geqslant$12 h in PC-3M cells. Cells were then washed twice with PBS and fresh complete culture medium was added before irradiation. The irradiation was done using a 674 nm laser with a pulse duration of 120 fs and a pulse repetition rate of 500 Hz. The average irradiance and corresponding peak irradiance were 1 mW cm^{-2} and 1.57×10^7 W cm^{-2}, respectively. The cells were exposed for different times to vary the total light dose (J cm^{-2}). The irradiated cells were then kept in the incubator for 48 h and the cell viability was evaluated by the MTT assay, a widely used colorimetric technique that measures cell viability (see below).

Apoptosis was assessed by Hoechst 33342 staining, for which HeLa cells were seeded in 96-well microplates at a density of 3×10^3 cells/well. After overnight incubation, the cells were incubated with 10 μM MPPa, light-irradiated at 0.04 J cm^{-2} and incubated for 24 h. For the final 30 min of incubation, Hoechst 33342