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Photodynamic therapy of human biliary cancer cell line using combination of phosphorus porphyrins and light emitting diode

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ABSTRACT

A series of phosphorus porphyrin complexes ([$(RO)_2$P(tpp)]Cl, tpp = tetraphenylporphyrinato group, R = $-(CH_2CH_2O)_m(CH_2)_nH$; **1a**: $m = 2$, $n = 2$; **1b**: $m = 2$, $n = 4$; **1c**: $m = 2$, $n = 6$; **1d**: $m = 3$, $n = 6$) were used for the photodynamic therapy (PDT) of human biliary cancer cell line (NOZ) when exposed to the irradiation of light emitting diodes (LEDs). A Dulbecco's modified Eagle's medium (DMEM) containing NOZ cells (2000 cell well^{-1}) and **1** (0–100 nM) was introduced into a 96-well microplate and incubated for 24 h to accumulate **1** into the NOZ cells and to multiply the NOZ cells until the cell number reached 10^4 cells well^{-1}. After replacing the DMEM medium containing **1** with a fresh DMEM medium without **1**, the plates were irradiated for 30 min at 610 nm. After incubation was performed for 24 h in dark conditions, the cell viability of the NOZ cells was determined using the MTT assay. The half maximum inhibitory concentrations 50 (IC_{50}) of **1a**–**1d** were found to be in the range of 33.7–58.7 nM for NOZ. These IC_{50} values for the NOZ were one hundredth the IC_{50} value (7.57 μM) for mono-L-aspartyl chlorin e6 (laserphyrin®). Thus, it was found that the PDT activity of **1a**–**1d** was much higher than the mono-L-aspartyl chlorin e6. Similarly, IC_{50} vales of **1a**–**1d** for HeLa cells were found to be 27.8–52.5 nM. This showed that **1a**–**1d** had high photodynamic activity in cancer cells. At the same time, it was speculated that an LED is a useful light source for deactivating the cancer cells because it can excite the sensitizers with peak width in their absorption spectra using the light of the specified wave length with band width of 10–20 nm; LEDs provide a homogeneous light distribution for the target cells.

1. Introduction

Photodynamic treatment has received considerable attention as a methodology leading to the medical applications such as photodynamic therapy (PDT) for tumor cells.[1] Selection of appropriate light sources and photosensitizers are crucial for efficient PDT. Lasers have been used as a PDT light source because lasers can emit intense light at specified narrow-band wave length. As photosensitizers, porphyrin derivatives are attractive singlet-oxygen sensitizers for PDT because of their strong absorption band in the visible-light region.[2–7] In general, free-base porphyrins comprise four Q-bands regions near 515, 550, 590, and 645 nm; these band regions have wider and longer wavelengths in comparison with that of the Q-bands of metalloporphyrins. Therefore, most sensitizers for PDT are based on free-base porphyrins or chlorins (i.e. photofrin® and laserphyrin®).[8]

By contrast, our research group has been interested in the use of phosphorus porphyrins (P-porphyrins) as photosensitizer[9] and light emitting diodes (LEDs) as light source for PDT. Recently LEDs are readily available as a convenient light source. The LED emits a light of a specified wave length with a band width of 10–20 nm and provides a homogeneous light distribution for target cells.[10] Moreover, it is advantageous in P-porphyrins that water-solubilization is easily achieved through the modification of the axial ligands

Abbreviations: A, absorbance; *B*, number of living cell in cell well^{-1}; CLSM, confocal laser scanning microscope; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; HeLa, human cervix adenocarcinoma; IC_{50}, half maximal inhibitory concentration; LD_{50}, lethal dose 50; LED, light emitting diode; MTT, 3 [4,5-Dimethyl-thiazoyl-2-yl]-2,5-diphenyltetrazolium bromide; NOZ, human biliary cancer cell line; PBS, phosphate-buffered saline; PDT, photodynamic therapy; tpp, tetraphenylporphyrinato group; Φ_Δ, overall quantum yield for generation of $^1O_2$.

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and the presence of these axial ligands also prevents the formation of porphyrin-ring aggregates.[11] This aggregation may lower the water solubility of the porphyrins and retard the incorporation of the porphyrins into the target cell. Moreover, it is expected that strong oxidation power due to high-valent metal such as phosphorus (V) can sensitize the inactivation of cells through an electron transfer in addition to an energy transfer mechanism even in low oxygen concentration.[12,13] Among the P-porphyrins, we have focused on di(polyoxaalkyl)tetraphenylporphyrinatophosphorus (V) chloride ([$(RO)_2$P(tpp)]Cl, tpp = tetraphenylporphyrinato group, R = $-(CH_2CH_2O)_m(CH_2)_nH$. **1a**: $m = 2$, $n = 2$; **1b**: $m = 2$, $n = 4$; **1c**: $m = 2$, $n = 6$; **1d**: $m = 3$, $n = 6$, Scheme 1), which can generate $^1O_2$ with high quantum yields.[14] The PDT activity of a series of **1** has been evaluated using *Saccharomyces cerevisiae*.[14] It was found that **1c** operated as a photosensitizer for the photodynamic inactivation of *S. cerevisiae* even at 5 nM. However, it is still unclear whether **1** has any activity in the tumor cells.

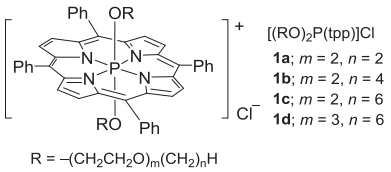
In contrast, Nanashima et al. found that PDT using mono-L-aspartyl chlorin e6 as photosensitizer was effective for medical treatment of the bile duct carcinoma.[15–17] In fact, the PDT using more efficient sensitizers is currently being sought globally for the local treatment of bile duct carcinomas.[18] Therefore, we performed the PDT of a human biliary cancer cell line (NOZ cells) through the combination between **1** and irradiation of LEDs.

2. Experimental

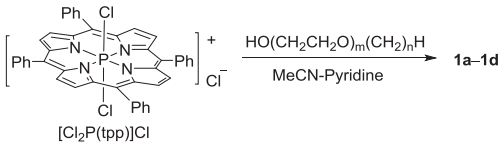
2.1. Materials

Based on previously reported methods,[14] the preparation of **1** was performed as follows. The preparation of **1b** was a typical procedure. The **1b** was prepared by heating the MeCN (20 mL) solution of dichloro(tetraphenylporphyrinato)phosphorus chloride ([Cl_2P(tpp)]Cl, 75 mg) with 3,6-dioxa-1-decanol (10 mL) in the presence of small amounts of pyridine (0.75 mL) at reflux temperature for about 24 h until the Soret band shifted from 436 nm to 423 nm (Scheme 2). The general follow-up procedure was as follows: After evaporation, the residue was dissolved into $CHCl_3$ (20 mL). The $CHCl_3$ solution was washed with water (20 mL) thrice, and the washed $CHCl_3$ solution was subjected to precipitation with hexane (200 mL) to remove the residual alkanol and other impurities. Moreover, the resulting precipitate was purified by column chromatography on SiO_2 using $CHCl_3$–MeOH (100:1–30:1 v/v). The structure of **1a**–**1d** was confirmed by NMR and MS spectra.[14]

Mono-L-aspartyl chlorin e6[19] (molecular weight 799.7 g mol^{-1}) was supplied from Meiji Seika Pharma Co. Ltd (Tokyo, Japan) as laserphyrin®. DMEM (Dulbecco's modified Eagle's medium) was prepared by mixing 10% fetal bovine serum (Sigma, #172012), glutamine (0.6 mg mL^{-1}), penicillin (100 units mL^{-1}) and streptomycin (100 mg mL^{-1}) in water. Aqueous stock solutions of **1a**–**1d** (100 μM) were prepared by dissolving **1** in DMSO (dimethyl sulfoxide)–water (1: 9 v/v, 20 mL) and adjusting the given concentration using the molar absorption coefficients. These stock solutions were sterilized by filtration through a syringe filter (Millex-LG, pore size = 0.20 μm, Millipore). DMEM mediums containing **1**



Scheme 1.



Scheme 2.

(0–400 μM) or mono-L-aspartyl chlorin e6 (0–10 μM) were prepared from the stock solutions before use.

2.2. Apparatus

PDT experiments were performed on a 96-well microplate (128 × 86 mm) with a cover. In the case of the PDT using **1**, irradiation was performed from the bottom of the well plates for 30 min using LEDs (λ = 610 nm, half with = 15 nm, light intensity = 4.09 mW cm^{-2}; L610-04, Ushi Opto Semiconductors, Kyoto, Japan), as illustrated in Fig. 1. An LED light source was fabricated using 100 plug-in LED arrays on a LED substrate (SPL-100-LC, Revox, Kanagawa, Japan). Heat emitted from LEDs was moderated using a heat-adsorbing filter filled with water (100 mm ϕ, 20 mm thickness); the heat-adsorbing filter was located between the microplate and the LED light source. In the case of PDT using mono-L-aspartyl chlorin e6, irradiation was performed at 660 nm using 100 LEDs (λ = 660 nm, half width = 15 nm, light intensity = 8.96 mW cm^{-2}; L660N-04, Ushi Opto Semiconductors, Kyoto, Japan). It should be noted that the LEDs can provide uniform light flow for 96-well plates.

Fluorescence microscopy images were obtained with an Olympus FV-300 confocal laser scanning microscope (CLSM) equipped with a 60 × water immersion objective lens (numerical aperture = 1.2). The wavelength of excitation laser/emission filter was 543 nm/565 nm for **1**, respectively.

2.3. Cancer cell culture

The NOZ cells (human biliary cancer cell line, JCRB1033: Japanese Collection of Research Bioresources, Tokyo, Japan) and HeLa cells (human cervix adenocarcinoma, JCRB9004) were cultured in DMEM at 37 °C in a humidified atmosphere with 5% CO_2 in air.

2.4. Cell viability assay

Cell numbers of NOZ and HeLa cells were determined by an MTT assay using 3-[4,5-dimethyl-thiazoyl-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) as follows.[20] At first, the PDT-treated samples were incubated under dark conditions for 24 h; by the comparison of the number of cells of the PDT-treated NOZ incubated for 24 h and 48 h, the optimal incubation time was found to be 24 h. Then, an MTT solution (10 μL), which was prepared by adding MTT (5 mg) in phosphate-buffered saline (PBS, 1 mL), was added to the cells in each well and then incubated for 2 h. DMSO (100 μL) was added to each well, and the MTT-formazan was completely solubilized by vortexing the plate. The absorbance (A) of each cell was recorded at 562 nm using an Immuno reader (Nihon Inter Med, NJ-2000, Tokyo, Japan). The A was converted to the number of living cells (B in cell $well^{-1}$) using the following calibration line: $B = 10^4 \times A/0.672$.

3. Results and discussion

3.1. Water-soluble P-porphyrins (*1*)

Based on a previously reported method,[14] water-soluble [$(RO)_2$P(tpp)]Cl (**1**, R = $-(CH_2CH_2O)_m(CH_2)_nH$) was easily prepared by the