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Efficient anti-tumor effect of photodynamic treatment with polymeric nanoparticles composed of polyethylene glycol and polylactic acid block copolymer encapsulating hydrophobic porphyrin derivative

Ken-ichi Ogawara [a], Taro Shiraishi [a], Tomoya Araki [a], Taka-ichi Watanabe [b], Tsutomu Ono [b], Kazutaka Higaki [a,*]

[a] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University, Japan
[b] Graduate School of Natural Science and Technology, Okayama University, Japan

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ABSTRACT

To develop potent and safer formulation of photosensitizer for cancer photodynamic therapy (PDT), we tried to formulate hydrophobic porphyrin derivative, photoprotoporphyrin IX dimethyl ester (PppIX-DME), into polymeric nanoparticles composed of polyethylene glycol and polylactic acid block copolymer (PN-Por). The mean particle size of PN-Por prepared was around 80 nm and the zeta potential was determined to be weakly negative. *In vitro* phototoxicity study for PN-Por clearly indicated the significant phototoxicity of PN-Por for three types of tumor cells tested (Colon-26 carcinoma (C26), B16BL6 melanoma and Lewis lung cancer cells) in the PppIX-DME concentration-dependent fashion. Furthermore, it was suggested that the release of PppIX-DME from PN-Por would gradually occur to provide the sustained release of PppIX-DME. *In vivo* pharmacokinetics of PN-Por after intravenous administration was evaluated in C26 tumor-bearing mice, and PN-Por exhibited low affinity to the liver and spleen and was therefore retained in the blood circulation for a long time, leading to the efficient tumor disposition of PN-Por. Furthermore, significant and highly effective anti-tumor effect was confirmed in C26 tumor-bearing mice with the local light irradiation onto C26 tumor tissues after PN-Por injection. These findings indicate the potency of PN-Por for the development of more efficient PDT-based cancer treatments.

1. Introduction

Photodynamic therapy (PDT) is a unique cancer treatment modality based on the dye-sensitized photo-oxidation of biological substances in the target tissue (Agostinis et al., 2011; Kübler, 2005). Basically, PDT-based cancer treatment consists of the following two major steps: (i) topical or systemic administration of photosensitizers (PS) and (ii) irradiation of non-thermal light in the visible range (635–760 nm) to tumor tissues. The light irradiation to the tumor leads to the excited singlet state of PS within tumor tissues from ground singlet state. A fraction of the excited singlet state PS is transformed via intersystem crossing to the relatively long-lived excited triplet state. The interaction of the excited triplet PS and molecular oxygen results in the formation of singlet oxygen ($^1O_2$), which is the primary phototoxic species generated upon the light irradiation. The damage induced by the singlet oxygen results first in injury to cellular function and structure, and ultimately in the death of cancer cells and regression of tumor growth (O'Connor et al., 2009). Since these reactive oxygen species are only generated when all of the three factors, PS, light with specific range of wavelength, and oxygen molecule, co-exist, the tissue selectivity of this treatment toward tumor tissues can be expected (Buytaert et al., 2007; Robertson et al., 2009). In addition, unlike other conventional cancer chemotherapy, drug resistance is expected to be hardly acquired and therefore repeated treatments would be possible (Dolmans et al., 2003).

However, PDT has several disadvantages that have to be improved. Firstly, most of PS are lipophilic and essentially poorly water-soluble under physiological conditions and are easily aggregated after administration, which not only complicates the formulation of PS, but also dramatically reduces the photodynamic activity of PS against tumor (de Visscher et al., 2011). Secondly, even if PS is solubilized in the aqueous vehicle and is given as solution, it is known that PS nonspecifically distributes throughout the body and induces various side effects in the skin and eyes that are exposed to daylight. Skin photosensitivity reactions are characterized by erythema, edema, blistering, hyperpigmentation and sunburn (Wolf et al., 1993), and these phototoxicities to skin significantly reduce the quality of life of patients who receive PDT. To overcome these problems that associate with poor solubility and nonspecific *in vivo* disposition characteristics of PS, a lot of attention has been paid to the development of a safer dosage form for PS with higher solubilizing capability and better tumor targeting properties while reducing non-specific disposition to other tissues/organs. To date, although various nanoparticulate PS formulations such as liposome (Bovis et al., 2012; García-Díaz et al., 2012), emulsion (Garbo et al., 1998) or others (Konan et al., 2003; Sibata et al., 2004) have been

* Corresponding author at: Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University, 1-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan.
E-mail address: higaki@pharm.okayama-u.ac.jp (K. Higaki).

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examined, and all of these formulations achieved certain improvements in PS solubility, the outcome especially from *in vivo* studies is still limited and unsatisfactory. Among nanoparticulate drug carriers available, polymeric nanoparticles composed of an amphiphilic diblock copolymer are one of promising drug carriers (Ayen et al., 2011; Yang et al., 2011). In terms of structural and functional properties, polymeric nanoparticles have a hydrophobic core where hydrophobic drugs can be easily incorporated, and their hydrophilic outer shell is associated with an aqueous layer, which protects from the interaction with plasma proteins such as opsonins enhancing phagocytosis by macrophages in the liver and spleen, and which therefore provides a prolonged blood circulation time. It is well known that nanoparticles with certain physicochemical properties preferentially accumulate in many types of solid tumors due to the unique pathophysiological characteristics of tumors, so-called "enhanced permeability and retention effect (EPR effect)" (Maeda et al., 2000; Matsumura and Maeda, 1986). On the other hand, since nanoparticles usually cannot permeate the small vessels, the tissue distribution should be highly limited. Thus, polymeric nanoparticles could be a suitable vehicle for hydrophobic compounds including PS to achieve their efficient tumor delivery with lower side effects.

In the present study, therefore, we used a hydrophobic porphyrin derivative, photoprotoporphyrin IX dimethyl ester (PppIX-DME), as PS, and incorporated it into polymeric nanoparticles (PN) composed of poly(ethylene glycol)-block-polylactic acid (PEG–PLA) (PN-Por). The stable incorporation of hydrophobic drugs within PN is dependent on the physicochemical properties of the copolymer used. In general, block copolymers with a similar length of hydrophobic and hydrophilic segments with molecular weights ranging from 5000 to 15,000 are used for preparing polymeric micelles (Lee et al., 2007; Liu et al., 2007). However, due to the unstable incorporation of drugs in these conventional polymeric micelles in the systemic circulation, the chemical conjugation of doxorubicin to a diblock copolymer (Nakanishi et al., 2001) or chemical introduction of a hydrophobic group to paclitaxel (Negishi et al., 2006) was necessary to more stably incorporate these drugs into, and to reduce their excess release from, polymeric micelles in the systemic circulation. In the present study, to achieve stable incorporation of PppIX-DME in the blood circulation, a PEG–PLA polymer (Mw: 42,000) composed of long PLA chain (37,000) was applied (Araki et al., 2012). First, the *in vitro* phototoxicity of PN-Por was assessed for various types of cancer cells and the release property of PppIX-DME from PN-Por prepared was also evaluated. Moreover, *in vivo* disposition characteristics of PN-Por labeled with ^{3}H-cholesteryl hexadecylether (^{3}H-CHE) and *in vivo* antitumor effect of PN-Por with the local light irradiation to tumor tissue was assessed after its intravenous injection into Colon-26 carcinoma (C26) solid tumor-bearing mice.

2. Materials and methods

2.1. Materials

Phosphate buffered saline (PBS), RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT), fetal bovine serum (FBS) and penicillin–streptomycin solution were purchased from Sigma (St. Louis, MO, USA). Gentamicin was purchased from Tokyo Chemical Industry (Tokyo, Japan). [^{3}H] Cholesteryl hexadecylether (^{3}H-CHE) was obtained from PerkinElmer Inc. (Waltham, MA, USA). Photoprotoporphyrin IX dimethyl ester (PppIX-DME), a hydrophobic porphyrin derivative, was kindly gifted from Porphyrin Lab. (Okayama, Japan). All other chemicals were of the finest grade available.

2.2. Cells

C26, B16BL6 Melanoma (B16) and Lewis Lung Cancer (LLC) were provided from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). C26 or B16 was cultured in RPMI-1640, and LLC was cultured in DMEM, both supplemented with 10% heat-inactivated FBS, 2 mM $_{L}$-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 20 μg/mL gentamicin at 37 °C under 5% CO_2/95% air.

2.3. Synthesis of PEG–PLA

PEG–PLA was synthesized by ring-opening polymerization of $_{D,L}$-lactic acid (Purac, Netherland) using MeOPEG (molecular weight (Mw) = 5000, NOF Inc., Tokyo) as an initiator in the presence of tin(II) 2-ethylhexanoate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a catalyst as previously reported (Muranaka et al., 2010). In this study, we used two different PEG–PLA block copolymers with different PLA segment lengths (Araki et al., 2012). One was a water-soluble PEG–PLA (Mw: 6000 (PEG 5000; PLA 1000), hydrophile–lipophile balance (HLB) = 15.65) and the other was oil-soluble PEG–PLA (Mw: 42,000 (PEG 5000; PLA 37000), HLB = 2.65).

2.4. Preparation of PN-Por

PN-Por was prepared by the emulsion solvent diffusion method as reported previously (Araki et al., 2012). In brief, PppIX-DME and oil-soluble PEG–PLA dissolved in ethyl acetate was mixed with purified water containing water-soluble PEG–PLA. To prepare ^{3}H-CHE radiolabeled PN-Por, a tracer amount of ^{3}H-CHE was further added to ethyl acetate. Oil/water (O/W) emulsion was prepared by a probe-type sonicator (50 W, Ohtake Works, Tokyo) and the obtained emulsion was then diluted with purified water. To remove free PppIX-DME which was not properly encapsulated into PN, PN-Por suspension was ultracentrifuged at 110,000 × *g* for 2 h and washed with water two times. Recovery rate of PppIX-DME was determined as the ratio of the amount of PppIX-DME recovered to the initial amount by spectroscopic analysis (λ = 420 nm) to be approximately 55%.

2.5. Size distribution and zeta potential of PN-Por

Particle size and zeta potential of PN-Por were assessed by measuring their dynamic light scattering and electrophoretic mobility, respectively (Zetasizer Nano, Malvern Instruments Ltd., Worcestershire, UK) after the adequate dilution (approximately one in hundred) of the final preparation of PN-Por with PBS (pH 7.4). As for the stability of PN, our preliminary study revealed that the freeze-dried PN preserved for more than one year provided PN with the size quite similar to that of PN freshly prepared. In addition, the particle size of PN-Por in the presence of serum (5%) (90.2 ± 3.1 nm) did not change for at least one week (91.1 ± 2.6 nm).

2.6. In vitro cytotoxicity of PN-Por

Phototoxicity of PN-Por was indirectly evaluated by MTT assay. Briefly, C26, B16 or LLC was seeded (5000 cells/well) in a 96-well plate (Asahi Techno Glass, Chiba, Japan). After the plate was incubated at 37 °C for 24 h in a humidified 5% CO_2 atmosphere, the medium with various concentrations of PppIX-DME (0.001, 0.005, 0.01, 0.05 and 0.1 μM) was added as PN-Por to each well in the plate. After 12 h incubation, each well was irradiated by halogen laser light (150 W, MORITEX, Saitama, Japan) for 15 s using light guide (MSG4-500R, MORITEX) with the filter to cut off the wavelength below 600 nm. Following the further incubation for 24 h after the light irradiation, each well was washed and rinsed with medium. MTT solution (0.5 mg/mL) was then added to each well and the cultures were further incubated for 4 h. The formazan crystals were dissolved in 0.04 M HCl-isopropanol and subjected to sonication in a bath-type sonicator (ASONE Corporation, Osaka, Japan) for 15 min. Each plate was set into a microplate reader and the absorbance values were measured at 570 nm (test wavelength) and 750 nm (reference