Toxicology Letters 182 (2008) 110–114

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Phototoxicity of coproporphyrin as a novel photodynamic therapy was enhanced by liposomalization

Yasuyuki Sadzuka[a,b,*], Fumiaki Iwasaki[b], Ikumi Sugiyama[a], Kentaro Horiuchi[c], Toru Hirano[d], Hidechika Ozawa[e], Naohiro Kanayama[e], Naoto Oku[b]

[a] School of Pharmacy, Iwate Medical University, 2-1-1 Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan
[b] School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
[c] Department of Life Science, Hamamatsu University School of Medicine 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan
[d] Photon Medical Research Center, Hamamatsu University School of Medicine 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan
[e] Department of Obstetrics and Gynecology, School of Medicine, Hamamatsu University School of Medicine 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan

ARTICLE INFO

Article history:
Received 12 June 2008
Received in revised form 2 September 2008
Accepted 2 September 2008
Available online 12 September 2008

Keywords:
Liposome
Photodynamic therapy
Coproporphyrin
Singlet oxygen
Cytotoxicity

ABSTRACT

This study attempted the liposomalization of coproporphyrin I (CPI) with hydrophobic properties. Liposomalization of CPI was not successful at any pH when using lactate buffer. In contrast, when using 9% sucrose/10 mM phosphate buffer (pH 7.8), CPI liposomes (Lipo-CPI) and polyethyleneglycol (PEG) modified liposomes (PEG-CPI) were prepared with a high entrapment ratio of CPI and small particle size. Plasma CPI concentration at 6 h after PEG-CPI injection were 6.5-fold greater than that after the injection of Lipo-CPI. In tumors, the CPI concentration was higher after PEG-CPI injection than after Lipo-CPI or CPI solution. Therefore, PEG-CPI was likely to increase blood circulation and achieve greater accumulation of CPI in the tumor. When loaded into tumor cells, photosensitizers generate singlet oxygen during laser irradiation, resulting in the induction of necrosis in the cells. The order of magnitude of CPI tumor cells uptake was PEG-CPI > Lipo-CPI > CPI solution. Thus, the PEG modification of CPI liposomes improved its tumor cell uptake. Furthermore, it is likely that the order of the ability to produce singlet oxygen was PEG-CPI ≒ Lipo-CPI > CPI solution. The cytotoxicity of PEG-CPI was significantly greater than the other formulations, suggesting that the cytotoxicity reflected the CPI concentration in tumor cells. In conclusion, PEG-CPI was confirmed to show effective tissue distribution, elevated CPI concentration in the tumor cells, to produce singlet oxygen, and cytotoxicity by PDT.

1. Introduction

Recently, photodynamic therapy (PDT) has started to play an important role in cancer chemotherapy as a less invasive treatment for superficial tumors. Porphyrin derivatives have been developed as photosensitizers, and photofrin was shown to have a superior effect in clinical therapy (Dougherty et al., 1998; McCaughan, 1999). However, photofrin is retained in the skin and produces some adverse reactions, including skin damage or photosensitivity (Bellnier and Dougherty, 1996). To solve this problem, photofrin was incorporated into liposomes with a resultant increase in photofrin-induced antitumor activity (Sadzuka et al., 2006). Furthermore, water soluble Zn coproporphyrin I (ZnCPI) was developed as a novel photosensitizer (Horiuchi et al., 1991). In the previous paper, the liposomalization of ZnCPI was attempted and a pharmaceutically useful liposome containing ZnCPI was developed (Sadzuka et al., 2007). However, it is not necessary to increase the water solubility of drugs entrapped in liposomes. The entrapment ratio was increased by the altered molecular form of ZnCPI (Sadzuka et al., 2007). In this paper, the tissue distribution (*in vivo*) and tumor cell uptake (*in vitro*) of ZnCPI-containing liposomes were examined. Coproporphyrin I (CPI), which is not suitable for injection due to its hydrophobic properties, was also examined. CPI-containing liposomes (Lipo-CPI) and polyethyleneglycol (PEG) modified Lipo-CPI (PEG-CPI) were prepared, and their efficacies were examined.

Photosensitizers loaded into tumor cells generate singlet oxygen during laser irradiation, resulting in induction of necrosis in tumor cells (Bockstahler et al., 1979). Therefore, the efficacy of PDT therapy is expected to correlate with the level of singlet oxygen generated from photosensitizers during laser irradiation (Krasnovsky, 1979). Namely, the monitoring of singlet oxygen formation is

Abbreviations: CPI, coproporphyrin I; DSPC, L-α-distearoylphosphatidylcholine; DSPG, L-α-distearoylphosphatidylcholine-DL-glycerol; PBS, phosphate-buffered saline; PDT, photodynamic therapy; ZnCPI, zinc coproporphyrin I.

* Corresponding author. Tel.: +81 19 651 5111; fax: +81 19 698 1832.
E-mail address: ysadzuka@iwate-med.ac.jp (Y. Sadzuka).

0378-4274/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.toxlet.2008.09.002

very important in the development and assessment of photosensitizers and therapeutic protocols. When singlet oxygen decays to the triplet state, 1270 nm light is released. This emission was detected using a photon-counting method with a high-sensitivity singlet channel detector, photomultiplier tube, or a multichannel detector.

2. Materials and methods

2.1. Materials

ZnCPI was synthesized from coproporphyrin I in our laboratory. CPI was purchased from Aldrich (USA). L-α-distearoylphosphatidylcholine (DSPC) and L-α-distearoylphosphatidyl-DL-glycerol (DSPG) were kindly donated by NOF Co., Ltd (Tokyo, Japan). Monomethoxypolyethyleneglycol-2,3-distearoylglycerol (PEG-DSG) was kindly provided by NOF Co., Ltd. Eagle's MEM medium was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). The other chemicals used in this study were of the highest purity available.

2.2. Preparation of liposomes

ZnCPI- or CPI-containing liposomes were prepared according to a modification of the method of Bangham (Bangham et al., 1965). DSPC/cholesterol/DSPG (100:100:60 μmol) and ZnCPI or CPI were dissolved in a chloroform/methanol mixture (3:7, v/v), and the mixture was dispersed perfectly by sonication. The chloroform and methanol were then evaporated to dryness under a stream of nitrogen gas. The thin lipid film was evacuated in a desiccator and then hydrated with 10 ml of 9.0% sucrose in 10 mM lactate buffer (pH 4.6) for ZnCPI film or phosphate buffered saline (-) (PBS (-)) for CPI film in a water bath at 75 °C for 10 min. The suspension was sonicated for 20 min at 75 °C after nitrogen gas bubbling. The liposome suspension was extruded through two stacked polycarbonate membrane filters with 0.2 μm pores, and then passed five times through polycarbonate membrane filters with 0.1 μm pores to obtain liposomes that were homogeneous in size. On the other hand, PEG-modification was prepared by adding 2.0 ml of PBS (−) or lactate buffer containing 15 μmol PEG-DSG, and then sonicating for 5 min.

Each liposome suspension was dialyzed against the preparation buffer at 4 °C for 16 h to remove unencapsulated drugs. The particle sizes and zeta potentials of the liposomes were measured with an electrophoretic light scattering apparatus (ELS 8000; Otsuka Electronics, Co., Ltd., Osaka, Japan). The entrapment efficiency of ZnCPI or CPI into the liposomes was measured as the encapsulated drug and adsorbed drug on the liposomal surface. The liposomal suspension was mixed for 30 s with PBS (−) or 9.0% sucrose in 10 mM lactate buffer (pH 4.6) or chloroform/isopropanol (1/1, v/v), and then centrifuged at 1200 *g* for 15 min. ZnCPI or CPI as their molecular forms (not ionic forms) in the organic phase was calculated using a fluorescence spectrophotometer (Hitachi F2000; Hitachi Ltd., Tokyo) at an excitation wavelength of 405 nm and an emission wavelength of 580 nm.

2.3. Biodistribution of ZnCPI and CPI liposomes

Male CDF_1 mice (5 weeks old and weighing 20–25 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The animals were housed in a room maintained at 25 ± 1 °C and 55 ± 5% relative humidity and were given free access to standard chow pellets and water.

Ehrlich ascites carcinoma (Ehrlich) cells (5.0×10^5 cells/animal) were transplanted onto the backs of CDF_1 mice. ZnCPI or CPI liposomes (10 mg/kg, i.v.) were administered at 14 days after inoculation. The mice were sacrificed by cervical dislocation after 2 or 6 h, and then plasma was collected and solid tumors were removed immediately and weighed. Tissue samples were homogenized in 10 vol. (w/v) of 10 mM phosphate buffer (pH 7.8). NaCl (0.5 g) was added to each suspension (1.0 ml), mixed for 60 s with 4.0 ml of tetrahydrofuran, and then centrifuged (1200 *g*, 15 min). The ZnCPI or CPI concentrations were determined as in Section 2.2.

2.4. Uptake of ZnCPI and CPI into tumor cells

Ehrlich ascites carcinoma cells (1.0×10^6 cells/animal) were transplanted intraperitonally into male ddY mice. Ascites fluid was collected on the 7th day after transplantation. The Ehrlich cells were washed twice and then resuspended (5×10^6 cells/ml) in Eagle's MEM medium containing 10% FBS.

Cell suspensions containing each liposome (ZnCPI or CPI concentration, 10 μg/ml) were incubated at 37 °C for 90 min. To determine the time course of the intracellular drug concentration, aliquots of the cell suspension were removed at intervals. Each aliquot was cooled on ice and then centrifuged at 150 *g* for 3 min. The cells were washed and resuspended in 1.0 ml of ice-cold saline, mixed for 30 s with 3.0 ml tetrahydrofuran and 0.5 g NaCl, and then centrifuged at 1200 *g* for 15 min. The concentration of CPI or ZnCPI in the organic phase was determined as described above.

2.5. PDT in vitro

Ehrlich cells (1.0×10^5 cells/ml) were suspended in Eagle's MEM medium containing 10% FBS in a 35 mm cell culture dish. This cell suspension was incubated with CPI liposomes (CPI concentration, 10 μg/ml) for 60 min at 37 °C. Each cell suspension was exposed to laser light at 580 nm with a fluence of 2 J/cm^2 (0.1 W, 192 s) and then incubated for 24 h at 37 °C. Cell survival was determined by a 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-8) assay using a commercially available kit (TetraColor ONE cell proliferation assay system; Seikagaku Co., Tokyo, Japan). Each cell suspension was transferred to a microtube and centrifuged at 300 *g* for 5 min. The cells were washed and resuspended in RPMI-1640 medium. TetraColor ONE (50 μl) was added to each cell suspension and incubated for 3 h at 37 °C. Each cell suspension was centrifuged at 300 *g* for 5 min, and the absorbance of the supernatant at 492 nm and 630 nm was measured using a microplate reader. The absorbance (A492–A630) was correlated to the number of living cells.

2.6. Measurement of singlet oxygen

Each liposome was dissolved in dichloroethane/ethanol (9/1, v/v). Each sample was diluted with PBS (-) or dichloroethane/ethanol (9/1, v/v) in order to achieve an absorption of 0.5 at 630 nm.

A quartz cuvette filled with each sample was irradiated with laser light (580 nm, 20 mW) generated by an excimer dye laser. Reflected or scattered light from the cuvette was guided to the detection system, which contained a spectroscope, a single channel detector PMT (Hamamatsu Photonics, R5509-42), or a multichannel detector (Hamamatsu Photonics, NIR-PII) and a photon-counter. To separate the 1270 nm emission from photosensitizer fluorescence, detectors were gated with a delay time from the onset of laser pulse irradiation and a gate time width synchronized to the laser pulses. The energy generated at 1260–1280 nm was detected by the system.

2.7. Statistical analysis

Statistical analyses were performed using ANOVA and Dunnett's test.

3. Results

3.1. Tissue distribution, tumor cell uptake, and generation of singlet oxygen after ZnCPI-containing liposome administration

At 2 and 6 h after the administration of ZnCPI solution, ZnCPI-containing liposomes (Lipo-ZnCPI), or PEG modified Lipo-ZnCPI (PEG-ZnCPI), the ZnCPI concentration in the plasma of Ehrlich ascites carcinoma-bearing mice in each liposome group was shown to be higher than that of the ZnCPI solution group. In the PEG-ZnCPI group, this increase reached 1.5-fold ($P < 0.05$). In the tumor at 2 h after injection of PEG-ZnCPI or Lipo-ZnCPI, ZnCPI concentrations were significantly higher than that in the ZnCPI solution group. In contrast, the ZnCPI concentration in the liver reticuloendothelial system (RES) was not changed by the PEG modification of liposomal membranes (data not shown).

On examination of ZnCPI uptake into Ehrlich ascites carcinoma cells *in vitro*, the ZnCPI concentration in the PEG-ZnCPI group was significantly higher ($P < 0.01$) than the ZnCPI solution, whereas this level was lower compared to that in Lipo-ZnCPI group (Fig. 1).

To clarify the effect of the liposomalization and PEG modification on the generation of singlet oxygen from ZnCPI by laser irradiation, the accumulated photon counts between 1240 and 1300 nm were determined for each group. The order of generation of singlet oxygen was ZnCPI solution (22863) > PEG-ZnCPI (18894) > Lipo-ZnCPI (3266).

3.2. Entrapment ratio of CPI, particle size, and zeta potential of CPI liposomes

It was attempted to prepare CPI liposomes in PBS (-), various buffers with different pH, and lactate buffer. However, liposomes could not form in lactate buffer, and liposomes with a low CPI entrapment ratio were prepared in PBS (-). In contrast, CPI liposomes prepared in 9.0% sucrose/10 mM phosphate buffer (pH 7.8)