Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 338 (2007) 306–309
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Study on liposomalization of zinc-coproporphyrin I as a novel drug in photodynamic therapy

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

[a] *School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan*
[b] *Department of Life Science, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan*
[c] *Photon Medical Research Center, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan*
[d] *Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan*

Received 10 October 2006; received in revised form 9 January 2007; accepted 3 February 2007
Available online 9 February 2007

Abstract

Photodynamic therapy (PDT) with a photosensitizer and laser irradiation has been shown to have potential effects in cancer chemotherapy. However, the commercial drug clinically gave many problems due to the poor solubility of the photosensitizer in water and the photosensitivity as an adverse reaction of PDT. We have examined best condition on the liposomalization of Zn-complexed coproporphyrin I (ZnCPI) as novel photosensitizer.

The difference of pH in buffer significantly changed the ZnCPI entrapped ratio. The entrapped ratio of ZnCPI in PBS(−) buffer was 10.8 ± 0.3%, whereas, these levels in some lactate buffer (below pH 5.0) increased. The change between the molecular form ⇔ ionic form of ZnCPI was occurred due to the change of the pH of buffer, and the amount of ZnCPI in the liposomal membrane changed. The difference of this level was considered to be contributed by the change of zeta potentials. Next, we examined the effect of the different pH of the buffer in liposomal preparation on the ZnCPI distribution in each tissue after each liposome administration. At 2 and 6 h post-injection of ZnCPI liposome (pH 4.6), the ZnCPI concentration in the plasma of Ehrlich ascites carcinoma bearing mice was shown to be higher compared to that in other groups. The ZnCPI concentrations in the tumor after 2 and 6 h of ZnCPI liposome (pH 4.6) treatment were shown to be higher than that in other groups. In conclusion, it is considered that the ZnCPI liposome (pH 4.6) had the effective antitumor activity with laser irradiation without the adverse reactions.

Keywords: Liposome; Photodynamic therapy (PDT); Zinc-coproporphyrin I; Photofrin

1. Introduction

Photodynamic therapy (PDT) with a photosensitizer and laser irradiation has been shown to have some effects on early or superficial tumor (Hirano, 1991; Itou et al., 1991; Kato et al., 1991; Hisazumi, 1987). Furthermore, PDT has been used on some vascular diseases, such as arteriosclerosis or aging macular degeneration (Jenkins et al., 1999; Amemiya et al., 1999; Blumenkranz et al., 2002; Obana et al., 1999). Photofrin as a photosensitizer is a lipophilic agent, and was shown to be slowly metabolized in the body. Importantly, photofrin is retained in the skin and produces some adverse reactions, including skin damage or photosensitivity. The patient, after undergoing PDT, must live in a dark room for 6–8 weeks due to this adverse reaction, causing a decline in the quality of life (Bellnier and Dougherty, 1996). To decrease this adverse reaction or to increase the laser sensitivity in PDT, some novel photosensitizers have been developed (Bonnett, 1995).

We have noted that Zn-complexed coproporphyrin I (ZnCPI, Fig. 1) is water soluble and safe, as a novel photosensitizer. ZnCPI exerts little concentration in the blood of adult humans and was extracted from the meconium in man. As ZnCPI produces active singlet oxygen by laser irradiation, it has been suggested to be an effective tool for PDT (Horiuchi et al., 1991). However, ZnCPI is highly water soluble, disappears rapidly from the blood and is difficult to target to the tumor (Horiuchi et al.,

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

* Corresponding author. Tel.: +81 54 264 5610; fax: +81 54 264 5615.
E-mail address: sadzuka@u-shizuoka-ken.ac.jp (Y. Sadzuka).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.02.003

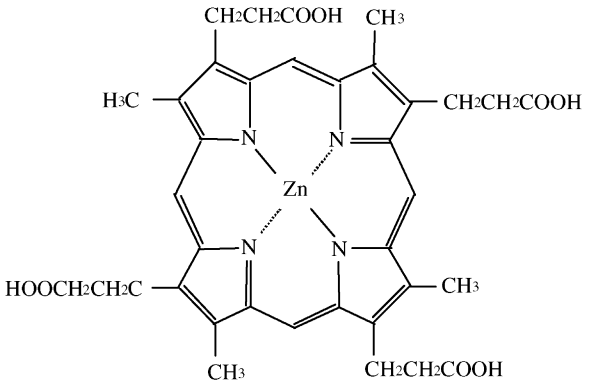

Fig. 1. Structure of Zinc-coproporphyrin I.

1991). Furthermore, it was expected that ZnCPI was not easy to use in clinical therapy because of too high sensitivity against pH. It is speculated that liposomalization of ZnCPI is able to solve these problems by the stability of intraliposomal conditions. We have suggested that the photosinseitizer in liposomes was shown to be high generation of singlet oxygen (Sadzuka et al., 2006). In addition, ZnCPI has the ability as a photosinsitizer in physiological condition. We have tried the liposomalization of ZnCPI and examined the blood circulation and tumor accumulation.

In this study, some prepared liposomes were determined as to their particle size and zeta potential on the surface of liposomes. Furthermore, the preparation of liposomes was performed in different pH buffers, as ZnCPI has four pK_a's (pK_a: 4.17, 4.43, 4.77 and 4.87), and examined the entrapment ratio of ZnCPI and the tissue distribution of ZnCPI *in vivo*.

2. Materials and methods

2.1. Materials

ZnCPI was synthesized from coproporphyrin I in our laboratory. L-α-distearoylphosphatidylcholine (DSPC) and L-α-distearoylphosphatidyl-DL-glycerol (DSPG) were kindly donated by Nippon Oil & Fat Co. Ltd. (Tokyo, Japan). The other chemicals used in this study were of the highest purity available.

2.2. Preparation of liposomes

ZnCPI 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 was dissolved in a chloroform/methanol mixture (3:7, v/v), and the mixture was perfectly dispersed 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 phosphate buffered saline (−) (PBS(−)) or 9.0% sucrose in 10 mM lactate buffer 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.

Each liposome suspension was dialyzed against prepared buffer at 4 °C for 16 hr to remove and untrapped ZnCPI. The particle sizes and zeta potentials of the liposomes were measured with an electophoretic light scattering apparatus (ELS 8000; Otsuka Electronics Co. Ltd., Osaka, Japan). The entrapment efficiency of ZnCPI in the liposome, as liposomes encapsulating ZnCPI and adsorbed ZnCPI on liposomal surface, was measured in the following way. The liposomal suspension was were mixed for 30 s with 9.0% sucrose in 10 mM lactate buffer (pH 4.0) and chloroform/isopropanol (1/1, v/v), and then centrifuged at 1200 × *g* for 15 min. The ZnCPI as the molecular form (no the ionic form) in the organic phase was calculated with a fluorescence spectrophotometer (Hitachi F2000; Hitachi Ltd., Tokyo), at an excitation wavelength of 405 nm and an emission wavelength of 580 nm.

2.3. Animals

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 regular chow pellets and water.

2.4. Biodistribution of zinc-coproporphyrin I liposome

Ehrlich ascites carcinoma (Ehrlich) cells (5×10^5 cells/animal) were transplanted onto the backs of CDF_1 mice.

ZnCPI (10 mg/kg, i.v.) was administered at the 14 days after the inoculation. The mice were killed by cervical dislocation at the second or sixth hours, and then the plasma was collected and the solid tumors were immediately removed and weighed. Tissue samples were homogenized in 10 volumes (w/v) of 10 mM phosphate buffer (pH 7.8). To each suspension (1.0 ml) was added NaCl (0.5 g) and it was mixed for 60 s with 4.0 ml of tetrahydrofuran and then centrifuged (1200 × *g*, 15 min). The ZnCPI concentration was determined according to as Section 2.2.

2.5. Statistical analysis

Statistical analysis was carried out using Student's *t*-test and ANOVA.

3. Results

3.1. Physicochemical characteristics of liposomes

The particle size and zeta potentials of some prepared liposomes using various pH in buffers are shown in Table 1. The particle sizes of the liposome in PBS(−) were indicated to be 115.3 ± 1.2 nm, whereas those in lactate buffer increased.

Table 1
Physical properties of liposomal ZnCPI

Buffer	pH	Particle size (nm)	ζ potential (mV)
PBS	7.4	115.3 ± 1.2	−32.2 ± 1.6
Lactate	4.3	167.4 ± 5.6	−17.5 ± 0.7
	4.6	154.4 ± 3.8	−29.2 ± 6.0
	4.82	173.1 ± 5.3	−29.1 ± 7.3
	5.0	172.3 ± 4.0	−35.3 ± 8.4

Each data is expressed as means ± S.D. (n = 3–4).

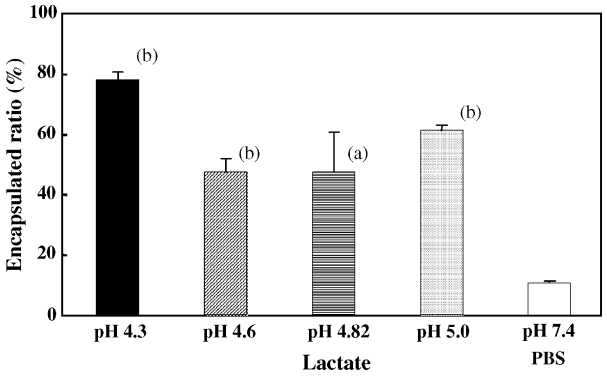

Fig. 2. Encapsulation efficiency of ZnCPI in liposomes. Each column represents the mean ± S.D. of 3–4 samples. Significant differences from the level of the PBS(−) group are indicated by (a) $P < 0.05$ and (b) $P < 0.001$.

3.2. Encapsulation ratio of ZnCPI in liposomes

The entrapment ratio of ZnCPI into liposome (PBS(−)) was 10.8 ± 0.3% whereas those in liposomes (lactate buffer) increased, and those in liposomes (pH 4.3, 4.6, 4.82 and 5.0) were 78.0 ± 2.5, 47.2 ± 4.5, 47.1 ± 13.7 and 61.4 ± 1.4%, respectively (Fig. 2). However, liposomes were not formed in the lactate buffer (pH 4.0). From these results, the entrapment ratio of ZnCPI into liposomes using lactate buffer (pH 4.3–5.0) was clarified to increase, compared with that using PBS(−). Furthermore, in the judge from Table 1 and Fig. 2, the entrapment ratio of ZnCPI was confirmed to be reversed in connection with the absolute values of the zeta potentials.

3.3. Effect of liposomalization on the ZnCPI concentrations in tissues

The ZnCPI concentration in the plasma reached a maximum level (2.03 ± 0.31 μg/ml plasma) after 2 h of ZnCPI liposome (pH 4.6) administration, showing a significant high level ($P < 0.01$) compared with that in ZnCPI solution and other liposomes. In contrast, these levels in the ZnCPI liposome (pH 4.3 or pH 5.0) groups were similar to that in the ZnCPI solution group. At 6 h after treatment, it was shown that the ZnCPI concentration in the plasma in the ZnCPI liposome (pH 4.6) group had a tendency to increase compared to that in the other groups (Fig. 3(A)).

In the tumor, the ZnCPI concentration at 2 h after ZnCPI liposome (pH 4.6) administration was 1.68 ± 0.19 μg/g tumor and significantly increased compared to that in the other groups ($P < 0.01$). Furthermore, the ZnCPI concentrations at 6 h injection in the tumor in the ZnCPI liposome (pH 4.6 or 5.0) groups were higher than those in other groups (Fig. 3(B)).

4. Discussion

PDT using a photosensitizer has been applied in the early stage of lung, gastric, esophagus and uterine cervix tumors (Hirano, 1991; Itou et al., 1991; Kato et al., 1991; Hisazumi, 1987). However, there are many problems due to the poor solubility of the photosensitizer in water and photosensitivity as an adverse reaction of PDT, although many compounds have been examined as photosensitizer candidates. We have noted ZnCPI (Fig. 1) as an novel photosensitizer. For the purpose of the investment of clinical utility, we have examined best condition for the liposomalization of ZnCPI, judging from size distribution, zeta potential, entrapment ratio and tissue distribution.

In this study, the preparation of the ZnCPI liposome was performed according to the Bangham method as a common preparation method. It was expected that the liposome preparations in the different pH of buffers would change the entrapment ratio of ZnCPI, as ZnCPI has four pK_a's (4.17, 4.43, 4.77 and 4.87). The entrapment ratio of ZnCPI in PBS(−) buffer was 10.8 ± 0.3, whereas these levels in certain lactate buffers (below pH 5.0) increased. Particularly, this level in lactate buffer (pH

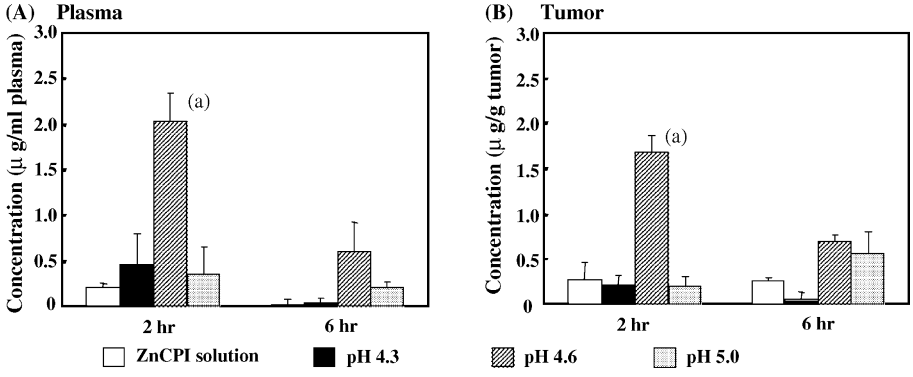

Fig. 3. Biodistribution of ZnCPI in tumor bearing mice after liposomal ZnCPI administration in the plasma (A) and tumor (B). Ehrlich ascites carcinoma bearing mice were injected with each liposome (ZnCPI dose: 10 mg/kg). Data presented as the mean ± S.D. (n = 3). A significant difference from the level of the ZnCPI solution, pH 4.3 and pH 5.0 group is indicated by (a) $P < 0.01$.

4.3) was shown to be 78.0 ± 2.5% as the maximum level. Thus, the difference in the pH of the buffer significantly changed the ZnCPI entrapment ratio (Fig. 2).

In general, when a drug is entrapped into a liposome using the Bangham method, the entrapment ratio of hydrophilic drugs is expected to be at a low level, and to be entrapped according to volume ratio between the intraliposomal volume/extraliposomal volume. In the case of using PBS(−), the entrapment ratio of ZnCPI is considered to be at a low level, as ZnCPI was entrapped into the water phase of the liposome by high ionic form, from the judgement of pK_a in ZnCPI. On the other hand, the increase of the ZnCPI entrapment ratio into liposomes (lactate buffer) was expected to be contributed by the high lipophilicity, and then the solubility of ZnCPI into the liposomal membrane. This condition was considered to be suitable for liposomal preparation. However, in the preparation with lactate buffer (pH 4.0), a lipid film was not formed and no liposomal suspension was prepared. It was speculated that this phenomenon was due to the formation of the molecular aggregation of ZnCPI before lipid film formation.

As for the zeta potential of these liposomes, except that in lactate buffer (pH 5.0), the difference of the entrapment ratio of ZnCPI was considered to be caused by the change of the zeta potentials. From these results, the difference of the existing site of ZnCPI into the liposome with the pH of the buffer was discussed. Namely, the change of molecular form ⇔ ionic form of ZnCPI occurred due to the change of the pH of the buffer, and the amount of ZnCPI into liposomal membrane changed. The difference of this level was considered to be caused by the change of the zeta potentials. Thus, the ZnCPI liposome using by lactate buffer (pH 4.3) was useful for the pharmaceutical sciences.

Next, we have examined the effect of different pH of buffers in liposomal preparation on the ZnCPI distribution in each tissue after each liposome administration. In this study, we examined ZnCPI distribution, not liposome distribution, as ZnCPI has antitumor activity. At 2 and 6 h post-injection of the ZnCPI liposome (pH 4.6), the ZnCPI concentration in the plasma of Ehrlich ascites carcinoma bearing mice was shown to be at a high level compared to that in other groups. In contrast, these levels in the liposome (pH 4.3 and 5.0) groups were similar to that in ZnCPI solution group (Fig. 3(A)). In the preparation of ZnCPI liposomes using lactate buffer (pH 4.3), ZnCPI was lipophilic in this condition and was expected to be present in the liposomal membranes. Thus, it is expected that this entrapment site in the liposome induced a high ZnCPI level in the plasma. However, the stability of the ZnCPI liposome (pH 4.3) was not maintained in the plasma. Although this liposome was superior regarding the entrapment ratio in the pharmaceutical properties, it is expected that the drug aggregation was strong due to the high lipophilic properties of ZnCPI at this pH, and the stability of the ZnCPI liposome in the plasma was expected to be lower. In the preparation of the ZnCPI liposome (pH 5.0), ZnCPI had hydrophilic properties and ZnCPI was speculated to exist in intraliposomal space and to be adsorbed onto the extraliposomal surface. As the adsorbed ZnCPI on the liposomal surface was desorbed in the blood circulation of this liposome, the ZnCPI concentration in the plasma on this liposome group was considered to be low. In vitro experiment, the released level of ZnCPI from ZnCPI liposome in the buffer (pH 5.0) was higher than that in other group (data not shown). In contrast, pH 4.6 in liposomal condition is the medium value between four pK_a's of ZnCPI. It is expected that good balance of lipophilicity—hydrophilic as drug carrier was keep in this pH. Namely, ZnCPI liposome (pH 4.6) was shown to be useful in the high entrapment ratio of ZnCPI and the effective blood circulation of ZnCPI.

The ZnCPI concentrations in the tumor after 2 and 6 h of ZnCPI liposome (pH 4.6) treatment were shown to be higher than those in other groups (Fig. 3(B)). Namely, it was considered that the ZnCPI liposome (pH 4.6) had the effective antitumor activity by laser irradiation.

In conclusion, in the case of the liposomal preparation of ZnCPI, the ZnCPI entrapment ratio into the liposome using lactate buffer was higher than that with PBS(−). Particularly, the maximum level of the entrapment ratio was shown in the ZnCPI liposome (pH 4.3). However, this liposome (pH 4.3) did not have the properties of blood circulation or tumor accumulation. The ZnCPI (pH 4.6) had effective properties of a high entrapment ratio into the liposome, blood circulation and tumor accumulation. Thus, judging from ZnCPI distribution and physical properties, the ZnCPI liposome (pH 4.6) was expected to be useful for PDT. The development of this liposome is expected to contribute to PDT and cancer chemotherapy.

References

Amemiya, T., Nakajima, T., Katoh, T., 1999. Photodynamic therapy of atherosclerosis using YAG–OPO laser and Porfilmer sodium and comparison with using argon-dye laser. Jpn. Circ. J. 63, 228–295.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ion across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252.

Bellnier, D.A., Dougherty, T.J.A., 1996. Preliminary pharmacokinetic study of intravenous photofrin in patients. J. Clin. Laser Med. Surg. 14, 311–314.

Blumenkranz, M.S., Bressler, N.M., Bressler, S.B., 2002. Verteporfin therapy for subfoveal choroidal neovascularization in age-related macular degeneration. Arch. Ophthalmol. 120, 1307–1314.

Bonnett, R., 1995. Photosensitizers of the porphyrin and phthalocyanine series for photodynamic therapy. Chem. Soc. Rev. 24, 19–33.

Hirano, T., 1991. The present condition of the laser for PDT. J. Jpn. Soc. Laser Surg. Med. 21, 129–135.

Hisazumi, H., 1987. The photodynamic therapy in a urology department domain. J. Jpn. Soc. Laser Surg. Med. 8, 11–14.

Horiuchi, K., Adachi, K., Fujise, Y., Naruse, H., Sumimoto, K., Kanayama, N., Terao, T., 1991. Isolation and characterization of zinc-coproporphyrin I: a major fluorescent component in meconium. Clin. Chem. 37, 1173–1177.

Itou, K., Kano, T., Kobayashi, S., 1991. The photodynamic therapy over endoscope-early stomach cancer. Biotherapy 5, 546–551.

Jenkins, M.P., Buonaccorsi, G.A., Raphael, M., 1999. Clinical study of adjuvant photodynamic therapy to reduce restenosis following femoral angioplasty. Br. J. Surg. 86, 1258–1263.

Kato, H., Takahasi, H., Konaka, T., Ishii, M., Miura, T., 1991. The photodynamic therapy of lung cancer. Biotherapy 5, 536–545.

Obana, A., Gohto, Y., Kaneda, K., 1999. Selective occlusion of choroidal neovascularization by photodynamic therapy with a water-soluble photosensitizer, ATX-S10. Lasers Surg. Med. 24, 209–222.

Sadzuka, Y., Tokutomi, K., Iwasaki, F., Hirano, T., Konno, H., Oku, N., Sonobe, T., 2006. The phototoxicity of photofrin was enhanced by PEGylated liposome in vitro. Cancer Lett. 241, 42–48.