Effect of different chemical bonds in pegylation of zinc protoporphyrin that affects drug release, intracellular uptake, and therapeutic effect in the tumor

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ABSTRACT

Pegylated zinc protoporphyrin (PEG-ZnPP) is a water-soluble inhibitor of heme oxygenase-1. In this study, we prepared two types of PEG-ZnPP conjugates with different chemical bonds between PEG and ZnPP, i.e., ester bonds and ether bonds, where both conjugates also contain amide bonds. Cleavability of these bonds in vitro and in vivo, especially cancer tissue, and upon intracellular uptake, was investigated in parallel with biological activities of the conjugates. Each conjugate showed different cleavability by plasma esterases and tumor proteases, as revealed by HPLC analyses. PEG-ZnPP with ester bond (esPEG-ZnPP) was more sensitive than PEG-ZnPP with ether bond (etPEG-ZnPP) for cleavage of PEG chains. etPEG-ZnPP showed no cleavage of PEG chains and had lower intracellular uptake and antitumor activity than did esPEG-ZnPP. The degradation of esPEG-ZnPP appeared to be facilitated by both serine and cysteine proteases in tumor tissues, whereas it was significantly slower in normal organs except the liver. Depegylated products such as free ZnPP had higher intracellular uptake than did intact PEG-ZnPP. We also studied hydrolytic cleavage by blood plasma of different animal species; mouse plasma showed the fastest cleavage whereas human plasma showed the slowest. These results suggest that ester-linked conjugates manifest more efficient cleavage of PEG, and greater yield of the active principle from the conjugates in tumor tissues than in normal tissues. More efficient intracellular uptake and thus an improved therapeutic effect with ester-linked conjugates are thus anticipated with fair stability, particularly in human blood.

Keywords: PEG-ZnPP, PEG-dilemma, PEG-cleavability, tumor proteases, intracellular uptake, antitumor effect

1. Introduction

Polyethylene glycol (PEG) is essentially a nontoxic biocompatible polymer, and widely utilized in conjugation of pharmaceutical proteins and other drugs so as to mask antigenicity of proteins and improve water solubility of the conjugated drugs as well as prolonging blood circulation time [1–3]. The PEG moiety forms an aqueous layer on the protein or drug surface, which also suppresses protein adsorption and recognition by the reticuloendothelial system, thus conferring stealth characteristics [4,5]. Macromolecular drugs (>40 kDa), after modification with such polymers, also show higher accumulation in tumors than do parental low-molecular-weight drugs, due to the enhanced permeability and retention (EPR) effect [3,6,7]. However, despite tumor selective delivery of pegylated macromolecular antitumor agents, pegylation hampers the therapeutic effect on tumor cells because of decreased intracellular uptake of the conjugates [8]. This problem is now referred as PEG-dilemma [8,9].

To overcome this drawback, many researchers have investigated the possibility of utilizing cleavage of PEG at tumor sites. For this purpose, several mechanisms of cleaving PEG have been reported using enzyme-cleavable peptide-linker for proteases such as matrix metalloproteinases (MMP) and cathepsin B, and esterases, as well as pH-sensitive linkers such as hydrazone-bonded PEG [9–13].

Another PEG-related problem is the formation of antibodies (IgM) against PEG conjugates [14], which might cause undesirable rapid clearance of pegylated drugs from the blood circulation. Various factors such as the size of pegylated drugs, surface density of PEG, and chain length of PEG in eliciting anti-PEG-IgM were investigated previously [15,16].

Previously, we developed pegylated zinc protoporphyrin (PEG-ZnPP) as a water-soluble macromolecular antitumor agent [17]. ZnPP is almost insoluble in water and it inhibits heme oxygenase-1 (HO-1), which is a key enzyme in heme catabolism [18]. HO-1 is also called heat shock protein 32 (HSP-32), and it is also known as a survival factor of tumor cells. On the contrary to HO-2 which is a constitutive isoform of HO, HO-1 is an enzyme that is induced by various stimuli such as ultraviolet radiation, oxidative stress, metalloporphyrins, heavy metals, and nitric oxide [18–22]. HO-1 is also reported to exhibit antioxidative and antiapoptotic effects during different cellular stresses. In addition, high HO-1 expression in various solid tumors is well known [18,20,21]. In many cancer cells, antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase are down-regulated or are

absent [18,23]. Therefore, biological function of HO-1 is thus important for protection of cancer cells against oxidative stress of endogenous reactive oxygen species or that by anticancer agents [21]. It was also reported that ZnPP exhibited antitumor effect by other mechanisms such as oncogene regulation [24,25].

The poor water solubility of ZnPP limits its therapeutic application, while we can successfully improve its water solubility by pegylation: PEG-ZnPP forms micelles and demonstrated high water solubility [17]. Moreover, PEG-ZnPP accumulated in tumors more preferably according to the EPR effect [6,7,26,27]. However, we also found that PEG-ZnPP had lower intracellular uptake than did free ZnPP [28,29], and thus another example of PEG-dilemma in PEG-ZnPP.

In our recent studies, we found that PEG-ZnPP underwent hydrolytic cleavage of PEG chains inside or near cells in vitro [28]. Cells may be able to internalize the depegylated ZnPP derivatives more rapidly. Therefore, freely liberated depegylated ZnPP derivatives may thus show higher cell uptake and hence better therapeutic activity than PEG-ZnPP. This means facilitated depegylation may be important for its biological activity.

In the present study described here, we aimed to clarify the mechanism and kinetics of degradation of PEG-ZnPP in blood circulation and in tumor tissues by utilizing different types of bonds between PEG and ZnPP. To prepare different bonds, we used two types of PEG, namely, PEG containing ester bond, and ether bond, and we refer here ester-bonded PEG-ZnPP (esPEG-ZnPP) and ether-bonded PEG-ZnPP (etPEG-ZnPP). Also, amide bonds are common bond in both esPEG-ZnPP and etPEG-ZnPP.

At first, we investigated the in vitro PEG cleavability of these two types of PEG-ZnPP in blood and in tumor tissues by using blood plasma and tumor tissue homogenates, respectively. Furthermore, we used cultured tumor cells to study the intracellular uptake and cytotoxicity of esPEG-ZnPP and etPEG-ZnPP in vitro. Then, we investigated antitumor effect, tissue distribution, and the PEG cleavability of two types of PEG-ZnPP in vivo.

2. Materials and Methods

2.1. Materials

Protoporphyrin IX (PP) and leupeptin were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO). Free ZnPP was obtained from Frontier Scientific, Inc. (Salt Lake City, UT). *N*-Succinimidyl PEG containing ester bond (ME-20CS) with a mean molecular weight of 2322 and *N*-succinimidyl PEG containing ether bond (ME-20AS) with a mean molecular weight of 2280 were obtained from NOF (Tokyo, Japan). Fetal bovine serum was obtained from GIBCO (Grand Island, NY). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo Chemical Laboratories (Kumamoto, Japan). Succinic anhydride, RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM), penicillin G, streptomycin, diisopropylfluorophosphate (DFP), phenylmethylsulfonylfluoride (PMSF), *N*-tosyl-L-phenylalaninechloromethyl ketone (TPCK), iodoacetamide, and *N*-acetylcysteine were purchased from Wako Pure Chemical (Osaka, Japan). All other reagents were reagent grade and used without further purification. Samples of human colorectal tumor tissues (well-differentiated adenocarcinoma) as well as the neighboring normal colon tissues were supplied from National Hospital Organization Kumamoto Medical Center (Kumamoto, Japan). Use of the human tumor tissues was approved by the ethical committee of National Hospital Organization Kumamoto Medical Center.

2.2. Synthesis of two types of PEG-ZnPP

Two different types of PEG were conjugated to PP as described previously with some modification [17]. Briefly, before pegylation, PP (100 mg in 20 mL of tetrahydrofuran) was first conjugated with ethylenediamine (2.4 mL) via formation of an amide bond. The crude *bis*-diaminoethyl protoporphyrin (PPED) obtained was adsorbed to activated alumina and was washed five times with chloroform containing 0.2% ethylenediamine. PPED was then eluted from activated alumina with chloroform containing 5% ethylenediamine. To prepare esPEG-ZnPP (Figure 1A), PEG containing ester bond (ME-20CS, NOF) (54 mg) was reacted with the PPED (5 mg in 20 mL of chloroform) at room temperature for 4 h. The reaction mixture was applied to an activated alumina column (25 × 50 mm) equilibrated and eluted with chloroform to remove unreacted PPED. A 100 molar excess of zinc acetate powder was then added to the conjugate in chloroform with stirring at room temperature for 2 h, thereby

chelating the zinc. Chloroform was removed by evaporation in vacuo at 40 °C, which yielded esPEG-ZnPP as a dark reddish powder. esPEG-ZnPP was further purified by using the Labscale Tangential Flow Filtration System (Millipore, Bedford, MA) with a 10-kDa cutoff membrane filter under pressure, followed by lyophilization. The ZnPP content was 10% (wt/wt).

etPEG-ZnPP (Figure 1B) was prepared by using a procedure similar to that for esPEG-ZnPP except with using PEG containing ether bond (ME-20AS, NOF) for pegylation. Both esPEG-ZnPP and etPEG-ZnPP contain two amides derived from ethylenediamine (Figure 1A, B).

2.3. Synthesis of bis-diaminoethyl zinc protoporphyrin (ZnPPED) and succinyl ZnPPEDs (mono- and bis-succinyl ZnPPED)

ZnPPED (Figure 1D) was synthesized as a putative degradation product of PEG-ZnPP after depegylation by hydrolysis of the amide bonds adjacent to the PEG chains. PPED was dissolved in chloroform after which 10 molar excess zinc acetate was added with stirring at room temperature for 2 h so as to chelate zinc. After the reaction, chloroform was removed by evaporation. ZnPPED thus obtained was washed with distilled water five times, followed by lyophilization, yielding ZnPPED powder.

In addition to free ZnPP (Figure 1C) and ZnPPED (Figure 1D), succinyl ZnPPEDs (mono- and bis-succinyl ZnPPED) (Figure 1E, F) were candidate products for depegylation. Succinyl ZnPPEDs were synthesized by using a 10 molar excess of succinic anhydride in tetrahydrofuran under stirring at room temperature for 5 h reacting with ZnPPED.

2.4. High performance liquid chromatography (HPLC)

HPLC was performed to separate cleavage products of PEG-ZnPP by using LC-2000Plus series HPLC system (JASCO, Tokyo, Japan) equipped with PU-2080 pump, UV-2075 UV/Visible detector (Integrator output scale was 1 V/1.0 AU), and 807-IT integrator (The settings were: ATTENUATION: 32 mV F. S.; CHART SPEED: 5 mm/min; MIN WIDTH: 0.10 min; MIN HEIGHT: 100 μ V; MIN AREA: 100 μ V; DRIFT; 0 μ V/min; DOUBLE WIDTH TIME; 0 min; NOISE LEVEL: 10 μ V). The column was Asahipak GF-310 HQ column (7.5 $\times$ 300 mm) (Showa Denko, Tokyo, Japan), and the prefilter was Asahipak GF-1G 7B column (7.5 $\times$ 50 mm) (Showa Denko, Tokyo, Japan). The mobile phase

consisted of 70% methanol, 30% dimethyl sulfoxide (DMSO), and 0.001% trifluoroacetic acid at a flow rate of 0.8 mL/min; eluate was monitored at 415 nm for ZnPP. The injection volume was 20 μ L. PEG-ZnPP and its degradation products were quantified by their peak areas, and in most studies they were described as percent ratio to the total peak areas.

2.5. MALDI-TOF MS

The molecular weights of PEG-ZnPP and cleavage products of PEG-ZnPP were analyzed by using MALDI-TOF MS (Autoflex II TOF/TOF, Bruker Daltonics, Bremen, Germany).

For analysis of PEG-ZnPP, 10 μ L of PEG-ZnPP (100 μ g/mL) in THF was mixed with 10 μ L of dithranol (10 mg/mL, Bruker Daltonics, Bremen, Germany) dissolved in THF. Then, 1 μ L of the mixture was spotted on a target plate (MTP 384 target plate ground steel T F, Bruker Daltonics, Bremen, Germany) and allowed to dry at room temperature. Mass spectra were measured with flexControl (Bruker Daltonics, Bremen, Germany). The settings were: method: reflector positive mode; shots: 200; frequency: 100; laser power: 10-30%. And the mass spectra were analyzed with flexAnalysis (Bruker Daltonics, Bremen, Germany).

The cleavage products of PEG-ZnPP were isolated by HPLC respectively as described above. About 5 mL of each peak fraction was collected and methanol in mobile phase was removed by evaporation in vacuo at 40 $^{\circ}$ C. And 10 mL of distilled water was added, and lyophilization. The obtained powder was dissolved in 20 μ L of THF and mixed with 20 μ L of dithranol (10 mg/mL) dissolved in THF. Then, 1 μ L of the mixture was spotted on a target plate (MTP 384 target plate ground steel T F) and allowed to dry at room temperature. Mass spectra were measured with flexControl. The settings were: method: reflector positive mode; shots: 200; frequency: 100; laser power: 10-30%. And the mass spectra were analyzed with flexAnalysis.

2.6. Stability/PEG cleavability of PEG-ZnPPs in vitro: Treatment of PEG-ZnPPs with blood plasma, tumor homogenates, and normal tissue homogenates

To investigate the stability of two PEG-ZnPPs in blood, es- and et- PEG-ZnPP (1 mg/mL) was incubated at 37 $^{\circ}$ C in PBS (0.01 M phosphate-buffered 0.15 M saline, pH 7.4) containing 10% blood plasma of different animal species (human, rabbit, mouse, or rat). In some experiments, esPEG-ZnPP was further incubated in the presence of a serine-type

esterase/protease inhibitor, DFP (1 mM) which was diluted by PBS (pH 7.4). At the indicated times, 10 μ L of each incubation mixture was taken to add to 1 mL of the mobile phase of the HPLC. Then it was centrifuged to remove insoluble materials, and the supernatant was subjected to HPLC.

To investigate the PEG cleavability of two PEG-ZnPPs in tumor tissue, es- and et-PEG-ZnPP (1 mg/mL) was incubated at 37 °C in 50 mM phosphate buffer (pH 6.2) containing supernatant of mouse sarcoma 180 (S-180) or human colorectal tumor (well-differentiated adenocarcinoma) homogenates (final conc.: 2 or 5 mg/mL protein). With supernatant of S-180 tumor homogenates at 5 mg/mL protein, esPEG-ZnPP was incubated in the absence or presence of protease inhibitors, TPCK (1 mM), PMSF (1 mM), leupeptin (10 μ g/mL), and iodoacetamide (1 mM). TPCK and PMSF were dissolved in ethanol and DMSO respectively, and final concentration of ethanol and DMSO in incubation mixture was 0.1% (v/v). Leupeptin and iodoacetamide were dissolved in 50 mM phosphate buffer (pH 6.2). With supernatant of human colorectal tumor at 5 mg/mL protein, esPEG-ZnPP was incubated in the absence or presence of *N*-acetylcysteine (100 μ M or 1 mM). *N*-acetylcysteine was dissolved in 50 mM phosphate buffer (pH 6.2). At the indicated times, HPLC analysis was performed as described above.

To investigate the PEG cleavability of esPEG-ZnPP in normal tissues, esPEG-ZnPP (1 mg/mL) was incubated at 37 °C in PBS (pH 7.4) containing supernatant of mouse normal tissues (spleen, kidney, liver) homogenates (final conc.: 2 mg/mL protein) or human normal colon tissue homogenates (final conc.: 5 mg/mL protein). At the indicated times, HPLC analysis was performed as described above.

2.7. Cell culture

Human chronic myelogenous leukemia cells (K562) were cultured as usual in RPMI 1640 medium with inactivated 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μ g/mL streptomycin at 37 °C in 5% CO₂/95% air.

Human esophageal squamous carcinoma cells (KYSE150), supplied by Dr. Hiroshi Kumimoto, Aichi Cancer Center Research Institute, and human colon cancer cells (HT-29) were cultured similarly to K562 cells but in DMEM.

2.8. Quantification of ZnPP derivatives in cells

K562 cells (5×10^5 cells/well) were placed in a 12-well plate (22.1 mm in well diameter). After overnight incubation, cells were treated with esPEG-ZnPP, etPEG-ZnPP, free ZnPP, or ZnPPED at 20 μ M each (ZnPP equivalent) for the indicated times. The esPEG-ZnPP and etPEG-ZnPP were dissolved in PBS (pH 7.4); free ZnPP and ZnPPED were dissolved in DMSO. After incubation for indicated times, cells were collected by centrifugation and washed three times with PBS at room temperature. Amounts of the intracellular ZnPP derivatives were quantified by fluorescence intensity by fluorescence-activated cell sorter (FACS) analysis using FACSCalibur (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). For analysis of the desired cell peaks, 1×10^4 cells were collected, and the mean fluorescence intensity of each sample was recorded and analyzed.

Intracellular uptake of ZnPP derivatives was also analyzed using HT-29 cells. HT-29 cells (5×10^5 cells/well) were placed in a 12-well plate. After overnight incubation, cells were treated with ZnPP derivatives at 20 μ M each (ZnPP equivalent) for the indicated times. After incubation for the indicated times, cells were washed with PBS twice, trypsinized, and were resuspended in methanol. Then, cells were sonicated (30 W, 30 s, Hielscher, Teltow, Germany) at ice chilled condition, followed by centrifugation to collect supernatant containing ZnPP derivatives, and fluorescence intensity (excitation at 420 nm, emission at 590 nm) of the methanol extract was measured using fluorescence spectrophotometer (FP-6600; JASCO, Tokyo, Japan).

2.9. Confocal fluorescence microscopic analysis of ZnPP derivatives in cells for intracellular uptake

To visualize ZnPP derivatives in the cells, confocal microscopy was performed with Nikon Eclipse TE2000-E Confocal Microscope (Nikon, Tokyo, Japan). KYSE150 cells were cultured in DMEM without phenol red with a round glass bottom petri dish ($\phi = 35$ mm, Matsunami Glass, Osaka, Japan). Cells were treated with at 30 μ M each of esPEG-ZnPP, etPEG-ZnPP, free ZnPP, or ZnPPED for the indicated times at 37 °C. After incubation, analysis of ZnPP derivatives in cells carried out at 488 nm, and fluorescence was detected at 565–615 nm.

2.10. Cytotoxicity assay

In vitro cytotoxicity of ZnPP derivatives was investigated by using the MTT assay.

K562 cells and HT-29 cells were plated in 96-well culture plates (3000 cells/well). After overnight incubation, esPEG-ZnPP, etPEG-ZnPP, free ZnPP, or ZnPPED was added. The MTT assay was performed to quantify the viable cells with absorbance at 570 nm after 72 h of culture, by a microplate reader (Infinite M200 PRO; Tecan Japan Co., Ltd., Kanagawa, Japan).

2.11. Antitumor effect of es- and et- PEG-ZnPP in vivo

Routine care and maintenance of animals were carried out in accordance with the institutional guidelines of the Institutional Animal Care and Use Committee of Sojo University. In vivo antitumor activity of two types of PEG-ZnPP was examined with S-180 tumor bearing male ddY mice. Male ddY mice, 6 weeks of age, were purchased from Kyudo Co., Ltd (Saga, Japan). S-180 cells (2×10^6 cells) were implanted s.c. in the dorsal skin of ddY mice. When tumor reached to diameter of about 3-5 mm, 15 mg/kg of ZnPP equivalent drugs in saline was administered intravenously (i.v.) via the tail vein. Tumor volume (mm^3) was calculated as $(W^2 \times L)/2$ by measuring the length (L) and width (W) of the tumor on the dorsal skin.

2.12. Tissue distribution and cleavability of es- and et- PEG-ZnPP in vivo

Tissue distribution and the PEG cleavability of es- and et- PEG-ZnPP in vivo were examined by using mouse S-180 tumor bearing male ddY mice. S-180 cells were similarly used as above. When diameter of tumor reached to about 10 mm, 40 mg/kg of ZnPP equivalent drugs in saline was injected i.v. via the tail vein. At the indicated time after i.v. injection, mice were killed and perfused with physiological saline to remove residual blood, and organs were dissected. The mobile phase of HPLC (500 μL per 100 mg of tissue) was added to the tissue samples, then the samples were homogenized at ice chilled condition and centrifuged to remove insoluble tissue debris. Then, the supernatant was subjected to HPLC as described above.

3. Results and Discussion

3.1. Analyses of the cleavage products of PEG-ZnPPs after treatment with blood plasma or supernatant of tumor tissue homogenates

Figure 2A-i showed the chromatogram of PEG-ZnPP which showed had a main single peak at 7.5 min. When it was treated with 10% blood plasma or with supernatant of tumor homogenates (final conc.: 2 or 5 mg/mL protein), several peaks were detected, where peak retention time varied from 7.5 min (intact PEG-ZnPP, Figure 2A-i) to 8.3, 11.2, 11.8, and 12.8 min (cleavage products) as shown in Figure 2A-ii (a sample of esPEG-ZnPP treated with 10% mouse blood plasma for 6 h). The precision, repeatability of this HPLC method was tested by using PEG-ZnPP incubated in supernatant of S-180 tumor homogenates (final conc.: 5 mg/mL protein) for 72 h; the relative standard deviations (%RSD) were less than 0.1% for retention time of each peak (Table S1) and less than 3.7% for peak area of each peak (Table S2). Total of peak area of all peaks showed no significant change between 0 h and 72 h after the incubation (Table S3). These results may at least partly support the repeatability and reliability of this HPLC assay. In addition, the lower limit of detection for PEG-ZnPP in this analysis was 1 ng or 0.1 ng of ZnPP.

The intact PEG-ZnPP (peak 1) has a mean molecular weight of about 5000, which was also supported by MALDI-TOF MS (Figure S2A). The peak 2 had a mean molecular weight of about 3000 (Figure S2B), and it was detected only with esPEG-ZnPP (see Figure 3B, D). From these results, peak 2 was considered as mono esPEG-ZnPP, with one PEG chain detached at the ester bond (see Figure S1). Though MALDI-TOF MS could not accurately determine the molecular weight of the peaks 3-5, products with molecular weight of more than 1000 were not detected (Figure S2C-E), suggesting they are further depegylated products. When we compared these peaks with free ZnPP (12.8 min), ZnPPED (6.5 min), and succinyl ZnPPEDs (11.2 and 11.8 min) (Figure 2B), we found that peak 5 was agreement with the peak of free ZnPP; peaks 3 and 4 were accorded with the peaks of succinyl ZnPPEDs. These findings support that peaks 3-5 may be completely depegylated products. In addition, there was no peak corresponding to ZnPPED which was also completely depegylated product in the cleaved products as seen by HPLC (Figure 2A-ii, B).

3.2. Stability of es- and et- PEG-ZnPP after incubation with blood plasma

Pegylated antitumor drugs must be stable and retain their macromolecular integrity during blood circulation. As a result, they can achieve high accumulation in tumor due to the EPR effect [6,30]. In this study, we investigated the stability of both es- and et- PEG-ZnPP under incubation with 10% blood plasma from various animal species. Because peak 1 and 2, which were pegylated ZnPP derivatives, were important for macromolecular integrity/micelle formation during blood circulation, changes of peak 1 and 2 were used to evaluate stability of es- and et- PEG-ZnPP in circulation. Figure 3A shows the comparison of peak 1 (intact PEG-ZnPP) degradation of esPEG-ZnPP and etPEG-ZnPP. Peak 1 of etPEG-ZnPP showed no apparent change for 48 h and etPEG-ZnPP remained uncleaved, whereas esPEG-ZnPP was degraded in a time-dependent manner, with the decrease being faster in mouse and rat plasma than in human and rabbit plasma. Similar results were noted for peak 2 (Figure 3B): at 24 h after incubation, the degradation of peak 2 in human plasma was about 1/8 of that of mouse and rat plasma.

In our previous study, we found that both esPEG-ZnPP and etPEG-ZnPP had a longer blood circulation time, resulting in higher accumulation in tumors because of the EPR effect, than that seen for free ZnPP in a mouse model [26,27]. These facts together with our present findings indicated that although esPEG-ZnPP was relatively cleavable in blood circulation than etPEG-ZnPP as described above, the pharmacokinetic properties of this PEG-ZnPP conjugate were sufficient for achieving EPR effect, e.g., more than 60-80% of esPEG-ZnPP remain pegylation (peak 1 and 2) after 4-6 h incubation in blood plasma (Figure 3). A more important and interesting finding is the significant species differences in cleavability of the PEG: degradation of peaks 1 and 2 in human plasma was much slower than that in mouse and rat plasma. These results suggested that esPEG-ZnPP was more stable in human plasma than in other animal plasma, and thus would exhibit a superior accumulation in tumors in patients in view of the EPR effect.

3.3. Effect of DFP on stability of esPEG-ZnPP

From the data in Figure 3A and B, we have observed a significant difference in stability of esPEG-ZnPP in plasma from different animal species. We then investigated the causes of cleavage of PEG from esPEG-ZnPP and species differences in plasma. Species differences in activities of hydrolytic enzymes such as esterase in blood plasma have been reported [31]. We thus examined the stability of esPEG-ZnPP in 10% human or mouse plasma

in the presence of DFP (1 mM), a serine-type esterase/protease inhibitor. Figure 3C shows that DFP suppressed the peak 1 degradation in mouse plasma but not much in human plasma. The results observed for changes in peak 2 was more notable for mouse plasma but not much in human plasma (Figure 3D). DFP had less effect in human plasma compared with that in mouse plasma. These findings suggest that esterases such as carboxylesterase, which is abundant in mouse blood plasma [31], are involved in depegylation of esPEG-ZnPP in blood plasma and may lead to the species differences in stability. In addition, because DFP is also a serine protease inhibitor, these findings suggest that serine proteases may also involve in cleavage between PEG and ZnPP in plasma, which warrants additional investigation.

3.4. Cleavability of the PEG from es- and et- PEG-ZnPP after incubation with supernatant of mouse tumor homogenates

Stability of the pegylated drugs during blood circulation is a prerequisite for their delivery to tumor sites or lesions, after which depegylation is needed for efficient intracellular uptake and therapeutic effect. Namely, it is important that completely depegylated products are cleaved from PEG-ZnPP in tumor tissue. Therefore, yield of peak 3, 4 and 5 which were presumed to be completely depegylated products, were then evaluated.

To investigate the depegylation from esPEG-ZnPP and etPEG-ZnPP at the tumor site, we incubated esPEG-ZnPP and etPEG-ZnPP in 50 mM phosphate buffer (pH 6.2) with supernatant of mouse S-180 tumor homogenates (final conc.: 2 or 5 mg/mL protein). After incubation of esPEG-ZnPP without supernatant of tumor homogenates, peaks 3 and 4 were not generated up to 72 h (Figure 4A, B), and only about 5% of peak 5 was generated after 72 h incubation (Figure 4C). In contrast, the cleavage of esPEG-ZnPP increased after incubation with supernatant of tumor homogenates in a dose- and time-dependent manner (Figure 4A, B, C). However, no generation of these products occurred in etPEG-ZnPP after incubation with supernatant of tumor homogenates (final conc.: 5 mg/mL protein) (Figure 4A, B, C). These results suggest that PEG was not removed from etPEG-ZnPP by the supernatant of tumor homogenates.

3.5. Effect of protease inhibitors on cleavability of the PEG from esPEG-ZnPP

To investigate the factors involved in cleavage of the PEG from esPEG-ZnPP at a tumor site, we focused on serine and cysteine proteases, which are highly expressed in tumors

and facilitate tumor invasion and metastasis [32]. We investigated the effect of protease inhibitors on cleavability of the PEG during incubation with supernatant of mouse S-180 tumor homogenates (final conc.: 5 mg/mL protein). We used TPCK (1 mM), PMSF (1 mM), leupeptin (10 µg/mL), and iodoacetamide (1 mM) in this study. As Figure 5A illustrates, PMSF almost completely suppressed the generation of peak 3 through 72 h. Other inhibitors also suppressed the generation of peak 3, and the inhibitory effect was found in the order of TPCK > iodoacetamide > leupeptin, suggesting both serine and cysteine proteases appear to be involved in the depegylation from esPEG-ZnPP at the tumor tissue. Similar results were observed for peak 4, but the protease inhibitors in general had a reduced effect (Figure 5B). However, all protease inhibitors promoted the generation of peak 5 at 72 h (Figure 5C), which suggests that other proteases may also participate in the cleavage of PEG-ZnPP. Serine and cysteine proteases, which were involved in the generation of peaks 3 and 4, may be suppressed by TPCK, PMSF, leupeptin, or iodoacetamide, which may result in the accumulation of peak 5.

We also investigated the cleavability of the PEG from esPEG-ZnPP by using homogenates of other tissues from male ddY mouse: the spleen, the kidney, and the liver. Figure 6 shows the generation of cleavage products, as a total of peaks 3-5 combined, after incubation with supernatants of homogenates of these normal tissues at pH 7.4 and with supernatant of tumor homogenates at pH 6.2 (final conc.: 2 mg/mL protein). Supernatants of normal tissues also generated cleavage products, with the yield being in the order of liver > tumor > kidney and spleen. This result may reflect high hydrolytic enzymes in the liver. This indicates if esPEG-ZnPP is delivered to tumor it would generate more depegylated ZnPP derivatives than other normal tissue, except liver. This is preferable point. ZnPP is not toxic to the liver [26], thus results are favorable for more tumor selective toxicity.

3.6. Cleavability of the PEG from esPEG-ZnPP after incubation with supernatant of human tumor homogenates

We investigated similar cleavability of PEG chains from esPEG-ZnPP by using homogenates of human colorectal tumor (well-differentiated adenocarcinoma), as well as mouse S-180 tumor. As shown in Figure 7A, cleavage products (total of peaks 3-5 combined) were generated after incubation with supernatant of human colorectal tumor homogenates (final conc.: 5 mg/mL protein) at pH 6.2.

From the data in Figure 5, we found that the depegylation of esPEG-ZnPP was facilitated by both serine and cysteine proteases in tumor tissues. Cysteine proteases are activated by more reducing condition and agents such as 2-mercaptoethanol and dithiothreitol by preserving active thiol residue. For this purpose, *N*-acetylcysteine was used. *N*-acetylcysteine did facilitated generation of cleaved products (total of peaks 3-5 combined) (Figure 7B). This result suggests that esPEG-ZnPP may be more effectively cleaved in vivo setting of tumor, where proteases such as cysteine proteases, e.g. cathepsin B, were known up-regulated [32,33].

Moreover, we compared cleavability of PEG from esPEG-ZnPP after incubation with supernatants of human colorectal tumor at pH 6.4, and that of normal large intestine at pH 7.4. After 24 h of incubation, yield of cleavage products (total of peaks 3-5 combined) after incubation with supernatant of homogenate of human normal large intestine was lower than that with supernatant of corresponding colorectal tumor (Figure 7C). This result indicates that more preferential cleavage of PEG may proceed in tumor tissues than in normal tissues.

3.7. Intracellular uptake and cytotoxicity of PEG-ZnPPs and the cleavage products in vitro, and antitumor effect of PEG-ZnPPs in vivo

Intracellular uptake of pegylated drugs was reported that it was improved after depegylation [8]. We examined this by using K562 cells and HT-29 cells whether depegylated products would show higher intracellular uptake than intact PEG-ZnPPs. For this study, free ZnPP (Figure 1C) and ZnPPED (Figure 1D) were used as references. Figure 8A shows that the depegylated products were incorporated into cells much faster than esPEG-ZnPP and etPEG-ZnPP in K562 cells. Similar results were observed in HT-29 cells (Figure 8B). Confocal microscopic analysis with KYSE150 cells after 2.5 h of incubation confirmed these findings, as seen in Figure 8C. More important finding, esPEG-ZnPP and etPEG-ZnPP were taken up by cells in a time-dependent manner, and esPEG-ZnPP at 8 h in K562 cells, at 16 h and 24 h in HT-29 cells after incubation showed a significantly higher intracellular uptake compared with etPEG-ZnPP (Figure 8A, B).

These findings strongly support the concept of PEG-dilemma, i.e., pegylated drugs generally show slower intracellular uptake than parental non-pegylated drugs. More important, the high uptake of the depegylated products (free ZnPP and ZnPPED) and higher uptake of esPEG-ZnPP than that of etPEG-ZnPP suggested clear advantage of esPEG-ZnPP over

etPEG-ZnPP, because of its high cleavability in vivo at the tumor site.

To elaborate further, we performed an in vitro MTT assay with K562 cells to compare the cytotoxicity of esPEG-ZnPP, etPEG-ZnPP, free ZnPP, and ZnPPED (Figure 9A, Table 1). As expected, free ZnPP (IC_{50} of 0.97 μ M) and ZnPPED (IC_{50} of 0.85 μ M) demonstrated much higher cytotoxicity than esPEG-ZnPP and etPEG-ZnPP. And esPEG-ZnPP showed relatively potent cytotoxicity compared with etPEG-ZnPP (IC_{50} 2.88 μ M vs 39.1 μ M, respectively). Similar results were observed in HT-29 cells (Figure 9B, Table 2). IC_{50} of free ZnPP, ZnPPED, esPEG-ZnPP, and etPEG-ZnPP were 4.16 μ M, 2.82 μ M, 27.7 μ M, and >100 μ M, respectively. These findings explain the difference between the two PEG-ZnPPs in which cleavability reflects more responsible for biological activity. Utilization of PEG via ester linkages may thus be beneficial for development of various PEG-conjugates, and it is not as labile as envisaged. Further, a great difference between different origin of blood plasma was observed (see Figure 3).

Then, in vivo antitumor effect of es- and et- PEG-ZnPP against S-180 tumor was investigated. As shown in Figure 9C, esPEG-ZnPP exhibited significant higher antitumor effect than etPEG-ZnPP, which is accorded with in vitro higher cleavability, intracellular uptake, and cytotoxicity of esPEG-ZnPP than etPEG-ZnPP.

3.8. Tissue distribution and PEG cleavability of es- and et- PEG-ZnPP in vivo

Figure 10A shows tissue distribution (described as total of peaks 1-5) of es- and et- PEG-ZnPP at 24 h after i.v. injection in S-180 tumor bearing male ddY mice. At 24 h after i.v. injection, both types of PEG-ZnPP showed almost similar tumor accumulation. However, most importantly, significant difference was observed in depegylation. Figure 10B shows the amount of each peak of esPEG-ZnPP in each tissue at 24 h after i.v. injection, where depegylated products (total of peaks 3-5, black bar) were observed in tumor. Meanwhile, etPEG-ZnPP showed only peak 1 (intact etPEG-ZnPP, white bar) largely in each tissue at 24 h (Figure 10C). For esPEG-ZnPP, the depegylated products (total of peaks 3-5 combined) in the liver was highest, due to unique affinity of protoporphyrin, followed by that in tumor (Figure 10B). Despite of high liver accumulation, no toxicity of esPEG-ZnPP for liver function was found in our previous study [26].

These findings suggest that esPEG-ZnPP will accumulate in tumor tissue by EPR

effect, and it will remain there for long time, e.g., several days [6], during which time the PEG chains will undergo hydrolytic removal by various proteases in the tumor tissue. The liberated active components will then be internalized quickly by tumor cells as demonstrated in Figure 8A, B, and C, and thus antitumor effect will become apparent. This finding may explain the higher in vivo antitumor activity of esPEG-ZnPP than that of etPEG-ZnPP (Figure 9C).

3.9. Mechanism of cleavage of PEG from esPEG-ZnPP

Figure S1 summarizes the conceptual mechanism of cleavage of PEG from esPEG-ZnPP. In this study, we also found that peaks 3-5 were generated together with a decrease in peak 2, which suggested that peaks 3-5 were generated via peak 2 (mono PEG-ZnPP), and not directly from peak 1 (intact PEG-ZnPP). Generation of peak 2 (mono PEG-ZnPP) is probably critical for further degradation. Additional degradation of mono PEG-ZnPP in the tumor tissue may proceed involving various proteases. Amide bonds exist in both es- and et- PEG-ZnPP (Figure 1A, B), appeared not readily cleavable by hydrolytic enzymes, perhaps because of steric hindrance. The putative structures such as the succinyl ZnPPEDs, ZnPPED, and free ZnPP shown in Figure S1 may correspond to the products indicated by peaks 3-5.

Depegylated products of esPEG-ZnPP will be generated by cleavage of ester bonds and amide bonds between PEG and ZnPP. Although amide bond is more stable than ester bond generally, it is also known that amide-linkage in amide prodrugs of amantadine is hydrolyzed by rat liver homogenate [34]. Once PEG is detached, amide bonds derived from ethylenediamine may be gradually cleaved.

Different numbers of methylene group adjacent to ester carbonyl was recently reported to have an impact on the cleavability of ester bonds in vitro [35]. Therefore, use of different carboxylic ester spacers in PEG-ZnPP may control depegylation rate of the PEG conjugate during blood circulation and in the tumor tissue.

4. Conclusions

We described herein depegylation of PEG-conjugated ZnPP in view of chemical bond between PEG and the prosthetic group. PEG was cleaved effectively from the bonds in the following order: ester > ether/amide. For the proteolytic cleavage activity in vivo, cysteine proteases and serine proteases (esterases) in solid tumor tissues play more active roles than

those of normal organs except the liver. This finding indicates more preferential cleavage of the conjugate-linker to PEG in tumor tissues than in normal tissues would occur. Moreover, the cleavage products showed higher intracellular uptake than the original PEG-ZnPP conjugates, which also explains higher in vitro cytotoxicity and in vivo antitumor effect of esPEG-ZnPP compared with non-cleavable etPEG-ZnPP. Thus, esPEG-ZnPP has beneficial facts favoring more tumor selectivity than normal tissues. Namely, the EPR effect driven tumor accumulation, tumor proteases dependent yield of active ZnPP moieties, and upregulated uptake of active ZnPP moieties into the tumor cells. The present study thus suggests a possible method to overcome PEG-dilemma, by using ester linkage between PEG and low-molecular-weight drugs for the antitumor drug development.

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Figure Legend

Figure 1. Chemical structures of (A) esPEG-ZnPP, (B) etPEG-ZnPP, (C) free ZnPP, (D) ZnPPED, (E) *mono*-succinyl ZnPPED, and (F) *bis*-succinyl ZnPPED.

Figure 2. Analyses of PEG-ZnPP by HPLC after treatment with blood plasma or with supernatant of tumor homogenates. (A) The peak of intact PEG-ZnPP, the original sample (A-i), and peaks of the cleavage products after incubation of esPEG-ZnPP with 10% mouse blood plasma for 6 h (A-ii). Similar results were obtained for cleavage products of esPEG-ZnPP incubated with supernatant of mouse and human tumor homogenates. (B) Peaks of standards: free ZnPP, ZnPPED, and succinyl ZnPPEDs (*mono*- and *bis*-succinyl ZnPPED, synthesized), all at about 10 ng/20 μ L, measured by using HPLC. Note that Figure A-i and A-ii are plotted at same y-axis, but Figure B is at different scale.

Figure 3. Stability of es- and et- PEG-ZnPP in 10% blood plasma from different species and effect of DFP (1 mM) added to 10% human or mouse blood plasma at pH 7.4, 37 °C, analyzed by using HPLC. (A, B) Changes in peak 1 for esPEG-ZnPP and etPEG-ZnPP (A), changes in peak 2 for esPEG-ZnPP (B), after incubation in 10% blood plasma of human, rabbit, mouse, or rat blood plasma. (C, D) Changes in peak 1 (C), peak 2 (D) for esPEG-ZnPP after incubation in 10% human or mouse blood plasma with (solid) or without (open) 1 mM DFP. Symbols in (B) and (D) are same as those in (A) and (C), respectively. Values are means $\pm$ S.E. ($n = 3$). H, Human; Rab, Rabbit; M, Mouse; R, Rat.

Figure 4. Cleavability of PEG chains from esPEG-ZnPP and etPEG-ZnPP after incubation with supernatant of mouse S-180 tumor homogenates (final conc.: 2 or 5 mg/mL protein) at pH 6.2, 37 °C, analyzed by HPLC. Yield of peak 3 (A), peak 4 (B), and peak 5 (C) after incubation of esPEG-ZnPP or etPEG-ZnPP with supernatant of tumor homogenate. Notations of bars in (A) apply to (B) and (C) as well. Values are means $\pm$ S.E. ($n = 3$).

Figure 5. Effect of protease inhibitors on depegylation from esPEG-ZnPP incubated with supernatant of mouse S-180 tumor homogenates (final conc.: 5 mg/mL protein) at pH 6.2, 37 °C, analyzed by HPLC. Yield of peak 3 (A), peak 4 (B), and peak 5 (C) after incubation of esPEG-ZnPP with supernatant of tumor homogenate in the presence or absence of the

indicated protease inhibitors. Notations of bars in (A) apply to (B) and (C) as well. Values are means $\pm$ S.E. ($n = 3$). $*p < 0.05$, a significant difference from tumor homogenate (T), according to Student's t test.

Figure 6. Cleavability of the PEG chains from esPEG-ZnPP after incubation with supernatants of homogenates of different mouse tissues, analyzed by HPLC. Yield of cleavage products (total of peaks 3-5 combined) after incubation of esPEG-ZnPP with supernatants of tissue homogenates of mouse spleen, kidney, and liver (final conc.: 2 mg/mL protein) at pH 7.4 or with supernatant of mouse tumor homogenates at pH 6.2, 37 °C. Values are means $\pm$ S.E. ($n = 3$). $*p < 0.05$, a significant difference from no supernatant, according to Student's t test.

Figure 7. Cleavability of PEG chains from esPEG-ZnPP after incubation with supernatants of human colorectal tumor homogenates at pH 6.2 or human normal large intestine homogenates (final conc.: 5 mg/mL protein) at pH 7.4, 37 °C, analyzed by HPLC. (A) Yield of cleavage products (total of peaks 3-5 combined) after incubation of esPEG-ZnPP with supernatants of tumor homogenates. (B) Yield of cleavage products (total of peaks 3-5 combined) similar to (A) in the presence or absence of activator of thiol proteases, *N*-acetylcysteine. (C) Similar to (A), tumor tissue vs normal large intestine at 24 h after incubation. Values are means $\pm$ S.E. ($n = 3-5$). $*p < 0.05$, a significant difference according to Student's t test.

Figure 8. Intracellular uptake of esPEG-ZnPP, etPEG-ZnPP, free ZnPP, and ZnPPED. (A, B) Intracellular uptake in K562 cells (A) measured by FACS analyses and in HT-29 cells (B) quantified by fluorescence intensity. The insets show comparison of intracellular uptake of es- and et- PEG-ZnPP after 8 h in K562 cells (A), after 16 h and 24 h in HT-29 cells (B). Values are means $\pm$ S.E. ($n = 3$). $*p < 0.05$, a significant difference according to Student's t test. (C) Confocal fluorescence microscopic analysis of KYSE150 cells treated with esPEG-ZnPP, etPEG-ZnPP, free ZnPP, or ZnPPED after 2.5 h incubation.

Figure 9. Cytotoxicity of esPEG-ZnPP, etPEG-ZnPP, free ZnPP, and ZnPPED, and in vivo antitumor effect of esPEG-ZnPP and etPEG-ZnPP. (A, B) Cytotoxicity of esPEG-ZnPP, etPEG-ZnPP, free ZnPP, and ZnPPED to K562 cells (A) and HT-29 cells (B) after 72 h

treatment, analyzed by MTT assay. Values are means $\pm$ S.E. (C) Antitumor effect of esPEG-ZnPP and etPEG-ZnPP at 15 mg/kg of ZnPP equivalent in S-180 tumor bearing male ddY mice. Vertical arrows indicate injection time of drugs. Values are means $\pm$ S.E. ($n = 5$). $*p < 0.05$, a significant difference from etPEG-ZnPP, according to Student's t test.

Figure 10. Tissue distribution and PEG cleavability of esPEG-ZnPP and etPEG-ZnPP in S-180 tumor bearing male ddY mice after administration at 40 mg/kg of ZnPP equivalent, measured by HPLC. (A) Tissue distribution (total of peaks 1-5 combined) of esPEG-ZnPP and etPEG-ZnPP at 24 h after administration. The inset shows amount of each PEG-ZnPP in tumor tissue with magnified scale. (B, C) The amount of each peak in each tissue at 24 h after administration of esPEG-ZnPP (B) and etPEG-ZnPP (C), using results in (A). The insets show amount of each peak in tumor tissue with magnified scale, respectively. Values are means $\pm$ S.E. ($n = 3$). Amount of peak is described as the area of the peak.

Table 1. IC₅₀, IC₃₀, and IC₁₀ of esPEG-ZnPP, etPEG-ZnPP, free ZnPP, and ZnPPED against K562 cells.

Drugs	IC ₅₀ (μM)	IC ₃₀ (μM)	IC ₁₀ (μM)
esPEG-ZnPP	2.88	1.76	0.61
etPEG-ZnPP	39.1	2.71	0.41
Free ZnPP	0.97	0.65	0.32
ZnPPED	0.85	0.56	0.24

Data are calculated from the results in Figure 8D

Table 2. IC₅₀, IC₃₀, and IC₁₀ of esPEG-ZnPP, etPEG-ZnPP, free ZnPP, and ZnPPED against HT-29 cells.

Drugs	IC ₅₀ (μM)	IC ₃₀ (μM)	IC ₁₀ (μM)
esPEG-ZnPP	27.7	2.77	1.03
etPEG-ZnPP	>100	73.4	2.61
Free ZnPP	4.16	1.64	0.14
ZnPPED	2.82	0.65	0.15

Data are calculated from the results in Figure 8E

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Ester bonded PEG-ZnPP (esPEG-ZnPP)

Micelle of esPEG-ZnPP

Aqueous layer
formed by PEG

Ether bonded PEG-ZnPP (etPEG-ZnPP)

Micelle of etPEG-ZnPP

Tumor accumulation
based on the EPR effect Depegylation
by proteases

Tumor cell

Nucleus

Cell uptake ↑ Antitumor effect ↑

No depegylation

Tumor cell

Nucleus

Uptake suppression
by aqueous layer