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Research paper

# 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 fain stability, particularly in human blood.



## 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

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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 (Fig. 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 (Fig. 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 (Fig. 1A and B).

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

ZnPPED (Fig. 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 (Fig. 1C) and ZnPPED (Fig. 1D), succinyl ZnPPEDs (*mono*- and *bis*-succinyl ZnPPED) (Fig. 1E and 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