Styrene-maleic acid-copolymer conjugated zinc protoporphyrin as a candidate drug for tumor-targeted therapy and imaging

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Abstract

Previous studies indicated the potential of zinc protoporphyrin (ZnPP) as an antitumor agent targeting to the tumor survival factor heme oxygenase-1, and/or for photodynamic therapy (PDT). In this study, to achieve tumor-targeted delivery, styrene-maleic acid-copolymer conjugated ZnPP (SMA-ZnPP) was synthesized via amide bond, which showed good water solubility, having ZnPP loading of 15%. More importantly, it forms micelles in aqueous solution with a mean particle size of 111.6 nm, whereas it has an apparent Mw of 65 kDa. This micelle formation was not detracted by serum albumin, suggesting it is stable in circulation. Further SMA-ZnPP conjugate will behave as an albumin complex in blood with much larger size (235 kDa) by virtue of the albumin binding property of SMA. Consequently, SMA-ZnPP conjugate exhibited prolonged circulating retention and preferential tumor accumulation by taking advantage of enhanced permeability and retention (EPR) effect. Clear tumor imaging was thus achieved by detecting the fluorescence of ZnPP. In addition, the cytotoxicity and PDT effect of SMA-ZnPP conjugate was confirmed in human cervical cancer HeLa cells. Light irradiation remarkably increased the cytotoxicity (IC50, from 33 to 5 μM). These findings may provide new options and knowledge for developing ZnPP based anticancer theranostic drugs.

Keywords

Antitumor therapy, EPR effect, styrene-maleic acid copolymer, tumor imaging, tumor targeting, zinc protoporphyrin

Introduction

Zinc protoporphyrin (ZnPP) is a porphyrin derivative with versatile functions and characteristics. We first focused on it as a potent inhibitor of heme oxygenase-1 (HO-1) which is an important antioxidative, antiapoptotic molecule that is highly expressed in many tumors and is considered a survival factor of tumor to protect tumor cells against the attack from the host [1,2]. Use of ZnPP to target HO-1 was described by us as a new antitumor strategy, and it was verified effective in many solid tumors as well as leukemia [1,3–8].

Recently, the potential of ZnPP as a photosensitizer in photodynamic therapy (PDT) has been receiving more attentions as many other porphyrin derivatives [9–12]. Namely, after irradiated by a light having an absorption wavelength, it will undergo intersystem crossing with molecular oxygen to generate singlet oxygen (1O2) and other reactive oxygen species (ROS) that kill tumor cells [13]. Meanwhile, ZnPP also exhibit extensive fluorescence, which means that it may be used for tumor imaging and detection if it could be selectively delivered to tumors.

Tumor-targeted delivery is one of the upmost important issues for successful anticancer therapy and it is becoming a hot topic and gold standard for development of anticancer drugs [14]. During the past few decades, a tumor-targeting strategy was developed based on the unique anatomical and pathophysiological characteristics of tumor blood vasculature, which are generally have a defective vascular architecture, with excessive production of vascular mediators that lead to high vascular permeability [14]. Consequently, macromolecules larger than 40–50 kDa could permeate in tumor tissues and further remain there due to the poor lymphatic recovery system in tumor tissues, whereas far less distribution in normal tissues could be seen for such macromolecules. This phenomenon was first reported in 1986 as the enhanced permeability and retention (EPR) effect [15], which is now becoming a basic principle for the development of anticancer nanomedicines, including micelles, polymer conjugates and liposomes [14,16–19].

Based on the EPR effect, in our laboratory, we previously prepared polymer conjugate of ZnPP with poly(ethylene glycol) (PEG-ZnPP), which not only greatly improved the solubility of ZnPP but also showed high tumor selectivity [20]. The tumor-targeted inhibition of HO-1 consequently resulted in the remarkable suppression of tumor growth [4]. Moreover, in combination with an xenon light, further
significantly increased antitumor tumor effect was achieved [9]. Along this line, recently we developed a ZnPP micelle with styrene-maleic acid copolymer (SMA), via the non-covalent bonding between ZnPP and styrene moiety of SMA, which exhibited good micelle forming property in aqueous solutions, as well as remarkable antitumor activity in both in vitro and in vivo studies [5,21]. However, the pharmacokinetics of this SMA-ZnPP micelle showed insignificant increase of tumor accumulation and plasma half-life, probably due to the disruption of micelle and release of free ZnPP in blood stream [5].

It has been known that polymeric micelles with covalent interactions showed better pharmacokinetics and higher tumor accumulation than non-covalent micellar drugs [22,23]. The critical issue regarding this difference is the stability of micelles. Usually, the stability of drug-encapsulating micelles by non-covalent bond is not adequate, resulting in the bursting of micelles and the release of parent low molecular weight drugs during circulation [24,25]. Thus, use of polymer-conjugated drugs via covalent bonds may be better options to achieve high plasma stability as well as high tumor-targeted delivery based on the EPR effect.

In this context, we developed a SMA-ZnPP conjugate with covalent bonding, and we report here the preparation, characterization and therapeutic effect of the SMA-ZnPP conjugate, by emphasizing its micelle-forming property as well as in vivo pharmacokinetics and tumor-targeting profiles.

Materials and methods

Materials

Protoporphyrin IX (PP) and SMA anhydride (Mr 7000) were purchased form Sigma-Aldrich Japan (Tokyo, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and water soluble carbodiimide (WSC) were purchased from Dojindo Chemical Laboratories (Kumamoto, Japan). All other chemicals and reagents were from Wako Pure Chemical (Osaka, Japan), and were used without purification.

Synthesis of SMA-ZnPP conjugate

The scheme for synthesizing SMA-ZnPP conjugate is shown in Figure 1. Amino groups were first introduced into PP to obtain bis(ethylenediamino)-protoporphyrin (PPED) as reported previously [20]. Then chelation of zinc into PPED was carried out as described in the previous reported protocol with some modifications [21]. Briefly, 10 mol excess of zinc acetate was added to PPED in dimethyl sulfoxide (DMSO) and stirred at 60°C for 2 h. The reaction mixture was then cooled to room temperature and cooled deionized water was added 10 folds to the original volume of DMSO. The resulted bis(ethylenediamino)-zinc protoporphyrin (ZnPPED) as precipitate was purified by centrifugation to remove excess zinc acetate that was in the supernatant. The chelation of zinc into protoporphyrin ring was confirmed by UV spectroscopy; After Zn insertion, completed shift of λ_max peak from 406 to 422 nm was observed, suggesting no vacant protoporphyrin after the reaction.

Amino groups of ZnPPED were used to react with the maleic anhydride residue of SMA to form amide bond. Briefly, 100 mg SMA anhydride and 20 mg of ZnPPED were dissolved in 10 mL N,N-dimethylformamide (DMF), into which 10-time mole excess (versus ZnPPED) of triethylamine and WSC were added. The reaction was carried out under stirring for 24 h at 60°C in the dark. After the reaction, SMA-ZnPP conjugates were precipitated and washed by diethyl ether, which was then dissolved in 40 mL of 0.2 M Na2CO3, stirring for further 24 h at 60°C to hydrolyze remaining maleic anhydride residues. Finally, after dialysis against distilled water, the powder of SMA-ZnPP conjugate was obtained by lyophilization.

High performance liquid chromatography

High performance liquid chromatography (HPLC) was carried out using the LC-2000Plus series HPLC system (JASCO, Tokyo, Japan) equipped with a UV-2075 UV/visible detector. Multimode size exclusion chromatography was carried out using an Asahipak GF-310 HQ column (7.5 x 9 x 300 mm; Showa Denko, Tokyo, Japan), and WSC was used as mobile phase at a flow rate of 0.5 mL/min; eluate was monitored at 415 nm of ZnPP.

Dynamic light scattering and zeta potential

SMA-ZnPP conjugate was dissolved in PBS (pH 7.4) at 2 mg/mL and filtered through a 0.45-nm filter. Particle size and surface charge (zeta potential) were measured by dynamic light scattering (ELS-Z2; Photol Otsuka Electronics, Osaka, Japan), using cumulant method and histogram method.

Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectra were recorded on FT/IR-4200 spectrometer (JASCO Corporation, Tokyo, Japan) using KBr discs.

Fluorescence spectroscopy

Fluorescence spectrum was recorded on F-4500 spectrometer (Hitachi, Tokyo, Japan). The sample solution was excited at 420 nm (corresponding to ZnPP) and emission from 550 to 700 nm was recorded.

Sephacryl S-200HR size exclusion chromatography

Size exclusion chromatography (SEC) with Sephacryl S-200HR (GE Healthcare, Tokyo, Japan) was carried out with 0.01 M phosphate buffered 0.15 M saline (PBS, pH 7.4; column, φ = 15 mm, L = 700 mm).

Cell culture and in vitro cytotoxicity assay

Human cervical cancer HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Wako) with 10% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan), at 37°C under atmosphere of 5% CO₂/95% air. For cytotoxicity assay, cells (3000 cells/well) were plated in 96-well plates (Corning, Corning, NY). After overnight incubation, different concentrations of SMA-ZnPP conjugate were added. After further 48 h of culture, MTT assay was
carried out to quantify the viable cells. In some experiments, light irradiation using a fluorescent blue light having peak emission at 420 nm (TL-D; Philips, Eindhoven, Netherland) with 1.0 J/cm² per 15 min was performed at 24 h after addition of SMA-ZnPP.

Tissue distribution of SMA-ZnPP conjugate after intravenous (i.v.) injection

Male ddY mice, 6 weeks old, weighing 20–25 g, were obtained from Kyudo Inc. (Saga, Japan) and were used for this study. All animals were maintained under standard conditions and were fed water and murine chow ad libitum. All animal experiments were carried out according to the Guidelines of the Laboratory Protocol of Animal Handling, Sojo University.

Mouse sarcoma S-180 cells (2 × 10⁶ cells) were implanted subcutaneously (s.c.) in the dorsal skin of ddY mice. When diameters of the tumors reached approximately 10 mm, 15 mg/kg (ZnPP equivalent) of SMA-ZnPP conjugate dissolved in saline was injected i.v. into the tail vein. At 24 h after i.v. injection, mice were sacrificed and perfused with saline followed by removal of each organ. Tissues were dissected, weighed and DMSO (1 mL per 100 mg of tissue) was added, and samples were homogenized and centrifuged (12 000 × g, 25°C, 10 min) to precipitate insoluble tissue debris, SMA-ZnPP in the supernatant was quantified by fluorescence intensity (excitation at 422 nm, emission at 590 nm).

In this study, tumors of about 1 cm in diameter was used because it was known that usually experimentally induced tumors smaller than 1 cm diameter are suitable for EPR-based drug delivery, but tumors larger than 3 cm in diameter may show heterogeneity of EPR effect with some necrotic/avascular regions [14].

In vivo fluorescence imaging

Tumor-bearing mice as described above were injected with 15 mg/kg of SMA-ZnPP (ZnPP equivalent) i.v. At indicated time after injection, the hair around tumor was shaved and, under isoflurane gas anesthesia the mice were subjected to in vivo fluorescence imaging using IVIS XR (Caliper Life Science, Hopkinton, MA) (excitation at 430 ±15 nm and emission at 695–770 nm).

HO inhibitory activity of SMA-ZnPP

Rat splenic microsomal fraction liver cytosolic fractions were prepared for measurement of splenic HO activity according to the previous literature [20]. The HO reaction mixture was composed of the splenic microsomal fraction (1.0 mg of protein), the liver cytosolic fraction (3.0 mg of protein) and 333 µM nicotinamide adenine dinucleotide phosphate (NADPH), without or with inhibitors at a given concentration, in 1.0 mL of 90 mM potassium phosphate buffer (pH 7.4). After 30 min incubation, the reaction was initiated by adding
than hydrolyzed SMA that shows approximately \( C_0/C_1 \)

The micelle was fluoresced from SMA-ZnPP conjugate in PBS as compared to aqueous solution was further supported by the quenching of (Figure 2A). The micelle structure of SMA-ZnPP conjugate in particles with a mean hydrodynamic diameter of 111.6 nm that in aqueous solution SMA-ZnPP conjugate appears as hydrophilic SMA chain as tail would form an outer surface head group can form a hydrophobic inner core, while the interaction as reported earlier [21]. Namely, ZnPP containing solution, similar to the SMA-ZnPP micelle with non-covalent ZnPP conjugate would form micellar structures in aqueous As ZnPP is highly hydrophobic, we anticipated that SMA-ZnPP conjugate after i.v. administration

Micelle formation of SMA-ZnPP conjugate in aqueous solution

As ZnPP is highly hydrophobic, we anticipated that SMA-ZnPP conjugate would form micellar structures in aqueous solution, similar to the SMA-ZnPP micelle with non-covalent interaction as reported earlier [21]. Namely, ZnPP containing head group can form a hydrophobic inner core, while the hydrophilic SMA chain as tail would form an outer surface layer facing toward water. The micelle formation was first demonstrated by dynamic light scattering analyses showing that in aqueous solution SMA-ZnPP conjugate appears as particles with a mean hydrodynamic diameter of 111.6 nm (Figure 2A). The micelle structure of SMA-ZnPP conjugate in aqueous solution was further supported by the quenching of fluorescence from SMA-ZnPP conjugate in PBS as compared to that dissolved in DMSO (Figure 2B). The zeta potential of the micelle was \(-32.38\) mV in PBS, with less negative charge than hydrolyzed SMA that shows approximately \(-50\) mV (data not shown), suggesting the masking of carboxyl groups of hydrolyzed SMA.

The apparent molecular weight of SMA-ZnPP conjugate in PBS, as determined by SEC using Sephacryl S-200HR, was supposed to be 65 kDa (Figure 2C and D). There is a gap of molecular mass obtained by SEC compared to that measured by DLS (111.6 nm), e.g. bovine serum albumin (BSA) of 67 kDa shows 6–8 nm by DLS. This gap may be due to the interaction of SMA-ZnPP conjugate with gel.

However, using the same SEC system, we further examined the effect of BSA on the behavior of SMA-ZnPP conjugate, because albumin binding is known as a noteworthy property of SMA [26]. As shown in Figure 3(C), SMA-ZnPP conjugate exhibited larger molecular weight in the presence of BSA in a dose-dependent manner, mostly forming a complex of about 235 kDa at the BSA concentration of 10 mg/mL (Figure 2C and D), whereas further increase of BSA did not induce further increase of apparent molecular size perhaps due to saturation (data not shown).

Factors affecting the micelle formation of SMA-ZnPP conjugate

We further investigated the possible factors showing influence on the micelle formation, by measuring the fluorescence emitting from SMA-ZnPP conjugate under different conditions. As shown in Figure 3(A), BSA enhanced the quenching of fluorescence in SMA-ZnPP conjugate dose-dependently, indicating that the conjugate may interact with albumin without disturbing the micellar structure, namely, SMA-ZnPP conjugates bind to albumin as polymeric micelles which may stabilize the micelle formation.

Fluorescence intensity of SMA-ZnPP conjugate was increased in the presence of detergent Tween 20 (Figure 3B), suggesting the involvement of hydrophobic interaction in the micelle structure. In contrast, fluorescence was rather suppressed by urea (Figure 3C), which suggested \( \pi-\pi \) interaction of tetrapyrol ring seems increased, or indicated the tightening of micelle structure. More important, the fluorescence intensity of SMA-ZnPP conjugate clearly increased after addition of lecithin that is a major component of cell membrane, in a dose-dependent manner (Figure 3D).

In vivo fluorescence imaging and tissue distribution of SMA-ZnPP conjugate after i.v. administration

Due to the micelle structure of SMA-ZnPP conjugate, we anticipated the tumor-selective accumulation of SMA-ZnPP due to the EPR effect. This was clearly demonstrated by fluorescence imaging using the in vivo fluorescence imaging system (IVIS Lumina-XR; Figure 4A). Preferential fluorescence of tumor tissue was observed at 2 h after i.v. administration of SMA-ZnPP conjugate indicating the accumulation of SMA-ZnPP conjugate in tumor (Figure 4A). And, the fluorescence in tumor increased with time; apparently stronger fluorescence was found at 6 h than that at 2 h after i.v. infusion, and the fluorescence of tumor remained strong intensity till 24 h whereas very little fluorescence could be seen in the surrounding normal skin (Figure 4A). These findings suggest that SMA-ZnPP conjugate can preferentially accumulate in tumor tissue and it may be used for sensitive visualization or imaging of tumor.

We then examined the tissue distribution of SMA-ZnPP conjugate after 24 h i.v. injection. As shown in Figure 4B, high accumulation of SMA-ZnPP conjugate was observed in the liver and the spleen, both of which have an active reticuloendothelial system (RES) [27], which is similar to our previous reports for pegylated ZnPP [4], suggesting that...
SMA-ZnPP conjugate after systemic administration is trapped by the RES. Besides, the liver and spleen, tumor tissue showed a significantly higher accumulation of SMA-ZnPP conjugate than that in other normal tissues (Figure 4B), which further supported the preferential accumulation of SMA-ZnPP conjugate in tumor.

Cytotoxicity and HO-1 inhibitory activity of SMA-ZnPP conjugate

In cultured HeLa cell, SMA-ZnPP conjugate showed a dose-dependent cytotoxicity with a 50% inhibitory concentration (IC50) of about 33 μg/mL of ZnPP equivalent, which is about 1/6 the effect of free ZnPP (IC50 of about 5 μg/mL; Figure 5). However, with light irradiation, SMA-ZnPP conjugate exhibited markedly increased cytotoxicity; 1.0 J/cm² of blue fluorescent light with an irradiation peak at 420 nm resulted in a three-time increase of cytotoxicity (IC50 of about 12 μg/mL; Figure 5).

We considered the cytotoxicity of SMA-ZnPP conjugate alone (without light irradiation) is mostly due to its HO-1 inhibitory activity. As expected, SMA-ZnPP conjugate showed comparable HO-1 inhibitory activity to free ZnPP; HO-1 activity decreased to about 30% by 1 μM of SMA-ZnPP conjugate (ZnPP equivalent) (Supplementary Figure S1).

Discussion

We report here the chemical conjugation of ZnPP with a highly biocompatible SMA-copolymer to produce SMA-ZnPP conjugate via amide bond (Figure 1A). This is the pendant-type conjugation that is similar to our recently developed polymeric ZnPP using N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer [10], which resulted in improved loading of ZnPP (i.e. 15%) compared to pegylated ZnPP, the first polymer conjugate of ZnPP previously synthesized in our group that has one ZnPP in the terminal end of PEG chain [4]. Improved ZnPP loading will be a preferable character of this polymeric ZnPP, and it did not affect water solubility of SMA-ZnPP conjugate that is one of the major drawbacks of ZnPP, i.e. SMA-ZnPP conjugate could be dissolved in PBS or saline at 20 mg/mL or more.

Due to the highly hydrophobic property of ZnPP, SMA-ZnPP conjugate was considered to behave as micelle in aqueous solutions, with the hydrophobic inner core of ZnPP and hydrophilic polyanionic chain of SMA as outer surface. As expected, dynamic light scattering showed that SMA-ZnPP conjugate in PBS exhibited a single peak distribution having an apparent hydrodynamic diameter of 111.6 nm (Figure 2A), and Sephacryl S-200HR chromatography of SMA-ZnPP conjugate exhibited a single peak of apparent
molecular weight of 65 kDa (Figure 2C). As there is a gap of molecular size between these two methods, we considered that SMA-ZnPP micelles exist as aggregated form in physiological solutions, whereas interactions between SMA-ZnPP conjugate and gel may result in the disaggregation and/or elution delay of SMA-ZnPP conjugate.

The micelle formation of SMA-ZnPP conjugate was also evidenced by fluorescence quenching. Namely, fluorescence intensity of SMA-ZnPP conjugate in distilled water was much lower than that in DMSO (Figure 2B), in which the aggregation of hydrophobic ZnPP core will be disintegrated.

When we further examined the micelle structure of SMA-ZnPP conjugate, we found the micelles were dissociated by detergents such as Tween 20 and sodium dodecyl sulfate (data not shown), but the micelle structure was not disrupted by urea (Figure 3B and C). These findings suggested that in aqueous solutions ZnPP is clustered in the core of SMA-ZnPP conjugate probably by hydrophobic interaction but not by hydrogen bonding. Compared to our previously reported SMA encapsulating non-covalent type ZnPP micelle which showed almost completed quenching of fluorescence in distilled water and the fluorescence quenching was restored by urea [21], the findings in this study suggested that the micelle structure of SMA-ZnPP covalent conjugate is different from that of non-covalent ZnPP micelle encapsulated by SMA, in which the covalent chemical bond may play an essential role. Further investigations are warranted to clarify this issue.

It is noteworthy that in the presence of BSA, the micelle structure of SMA-ZnPP conjugate was not dissociated, but rather reinforced the fluorescence quenching (Figure 3A), suggesting the increased stability of micelles in circulation. Circulation stability is one of the upmost important issues for micellar type drugs; unstable micelles especially those with non-covalent bonds, usually experience rapid disrupting/drug release during circulating which resulted in the short plasma half-life and the consequent less EPR-based tumor accumulation [5,22–24,28,29]. The micelle stability of SMA-ZnPP conjugate thus resulted in its prolonged circulation time, i.e. relative high concentration of SMA-ZnPP conjugate remained in plasma that was higher than those of most normal tissues at 24 h after i.v. injection (Figure 5). In contrast, ZnPP micelles non-covalently encapsulated by SMA showed much shorter plasma half-life, more than 50% of the micelles were removed from circulation during 10 min after i.v. injection [5]. Consequently, high tumor accumulation of SMA-ZnPP conjugate was achieved by taking advantage of the EPR effect.

In addition, of greater interest, we found dissociation of the micelles by cell-membrane components or lecithin as evidenced by the regeneration of fluorescence (Figure 3D), indicating micellar disruption during entry into the cells. Namely, when the micelles were internalized into the cells by endocytosis, the endocytic vesicular lecithin component will facilitate intracellular disruption of the micelles, which
will thus led to more effective antitumor and PDT effect as reported previously [10,30,31].

Moreover, similar to many other SMA conjugates and micelles [26,32–34], SMA-ZnPP conjugate showed larger molecular weight in the presence of BSA by SEC, which may be partly due to the blocking of SMA-ZnPP conjugate/gel interaction by BSA, and more important these results indicate the albumin binding property of SMA-ZnPP conjugate. The albumin binding is an important capacity of SMA which not only ensures prolonged blood circulation, but also confers higher biocompatibility of SMA modified drugs [32,35]. As albumin is one of the major components in blood, it is expected that most SMA-ZnPP conjugate exists as a complex with albumin \textit{in vivo}, consequently leading to prolonged circulation time and high tumor accumulation by the EPR effect which made \textit{in vivo} imaging possible for detection of tumors, as shown in Figure 4.

However, pronounced accumulation of SMA-ZnPP conjugate in liver and spleen was also observed (Figure 4B), which is a similar issue observed for many other polymeric drugs and liposomal drugs probably by the RES trapping [4,27,36]. In addition, because spleen is the major organ to degrade heme and other porphyrin derivatives, high affinity/accumulation of ZnPP and polymeric ZnPP was usually observed [4,5,10].

Figure 4. \textit{In vivo} tumor imaging (A) and body distribution of SMA-ZnPP conjugate (B) after i.v. injection in tumor-bearing mice. ddY mice bearing mouse sarcoma S180 was used in this study. SMA-ZnPP conjugate (15 mg/kg ZnPP equivalent) was i.v. injected when tumor grew to size of about 10 mm in diameter, tumor imaging was carried out at different time (e.g. 2, 6 and 24 h) after SMA-ZnPP administration by IVIS \textit{in vivo} imaging system. At 24 h after injection of SMA-ZnPP, the mice were sacrificed and each tissue including tumor tissue was collected, and the amount of SMA-ZnPP in each tissue was quantified by detecting the fluorescence of ZnPP. In A, circles indicate the tumors. In B, inset shows the results without liver and spleen. See text for details. Data are mean ± SE.

Figure 5. \textit{In vitro} cytotoxicity and PDT effect of SMA-ZnPP conjugate in human cervical cancer HeLa cells. HeLa cells (3000/well) were seeded in 96-well plate, after overnight pre-incubation, different concentrations of SMA-ZnPP conjugate were added, and after further 48 incubation, MTT assay was carried out to determine the viability of cells. In a separate study, light from a fluorescence light source with maximum emitting wavelength of 420 nm (1 J/cm²) was subjected to cells at 24 h after SMA-ZnPP conjugate treatment. Data are mean ± SE. See text for details.
Recent studies suggested that it may be possible to suppress the liver RES trapping by blocking scavenge receptor using such as rifampicin [37], whereas needs further investigations.

It is also important to mention that, ZnPP, not like many other antitumor drugs, exhibits less direct cytotoxicity towards cells, whose antitumor effect is mostly due to the HO-1 inhibition or PDT after light irradiation [1,4,9,10]. We previously reported that ZnPP showed negligible toxicity to normal hepatocytes but much stronger cytotoxicity was found for liver cancer cells [5]. Thus, no or very little toxicity would be induced to normal liver and spleen, similar to the toxicity profiles of pegylated ZnPP as reported previously [4]. Moreover, polymer-conjugated drugs usually exhibit lower in vitro cytotoxicity compared to free drugs [10,22,29], which was also described in this study. Namely, SMA-ZnPP conjugate showed an about six-time higher IC50 than free ZnPP (Figure 5), probably due to the lower intracellular uptake of polymer drugs [10,29,30,38]. The low cytotoxicity of SMA-ZnPP conjugate will further contribute to less damage/side effects toward normal tissues. Accumulation of SMA-ZnPP conjugate in tumor, however, could ensure sufficient drug concentrations to inhibit HO-1 in tumor cells thus exhibiting antitumor effect, and further fulfill more potent PDT effect upon light irradiation as indicated in Figure 5.

Compared to other polymer-modified ZnPP developed in our laboratory, e.g. pegylated ZnPP [4], HPMA-ZnPP [10] and SMA encapsulating ZnPP micelle [21], SMA-ZnPP conjugate showed higher drug loading than pegylated ZnPP and improved tumor accumulation/EPR effect than non-covalent micelles, also this conjugate exhibited comparable antitumor effect not mentioning its PDT potential (Figure 5) which may be an advantage compared to HPMA-ZnPP that is applicable only for PDT [10].

In addition, immune stimulatory properties was proven to be an important property of SMA, such as activation of macrophage, T-cells, NK cells and induction of interferons in animal models [39–42]. These features of SMA-ZnPP conjugate may provide new options and knowledge for ZnPP-based theranostic nanomedicine toward cancer. Future studies will focus on the in vivo behaviors especially therapeutic effect including PDT effect of SMA-ZnPP conjugate.

**Conclusions**

We were successful in synthesizing a SMA conjugated ZnPP, which exhibited good water-solubility, relatively high ZnPP loading and more importantly micelle formation in aqueous solutions. In combination with the albumin binding property of SMA, SMA-ZnPP conjugate behaves as stable macromolecular micelles in circulation, consequently resulting in preferable tumor-targeted accumulation by taking advantage of the EPR effect. This tumor accumulation subsequently led to clear tumor imaging effect upon light irradiation based on the properties of ZnPP as a photosensitizer and fluorescing agent. Thus we expected the potential of SMA-ZnPP conjugate as a candidate of theranostic nanomedicine for cancer.

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**Declaration of interest**

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Supplementary material available online
Supplementary Figure S1