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# Water-soluble metalloporphyrinates with excellent photo-induced anticancer activity resulting from high tumor accumulation

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ABSTRACT

To develop a water-soluble and tumor-targeted photosensitizer for photodynamic therapy (PDT), a porphyrin framework containing the metal ion gallium(III) was combined with platinum(II)-based groups to produce two new pentacationic metalloporphyrinates, Ga-4cisPtTPyP (5,10,15,20-tetrakis{*cis*-diammine-chloro-platinum(II)}(4-pyridyl)-porphyrinato gallium(III) hydroxide tetranitrate) and Ga-4transPtTPyP (5,10,15,20-tetrakis{trans-diammine-chloro-platinum(II)} (4-pyridyl)-porphyrinato gallium(III) hydroxide tetranitrate). Both complexes exhibited high singlet oxygen quantum yields ($\Phi_\Delta$) and remarkable photocytotoxicity with appreciable phototoxic indexes (PIs). In particular, Ga-4cisPtTPyP showed a low $IC_{50}$ value (Colon 26: 0.12 μM; Sarcoma 180: 0.08 μM) under illumination and its PI up to 1000. With outstanding tumor accumulation (tumor/muscle ratio > 9), Ga-4cisPtTPyP almost completely inhibited tumor growth over two weeks in an in vivo PDT assay. These results imply that Ga-4cisPtTPyP could be a promising anticancer agent for use in PDT.



## 1. Introduction

Porphyrins are quite unique molecules that are highly hydrophobic because of the presence of an extended π system. Functionalization with charged peripheral groups or insertion of metals into the core could make them water-soluble [1–6], markedly affecting their biodistribution and in vivo efficacy [7,8]. With an ability to generate singlet oxygen and other reactive oxygen species, metalloporphyrins are thermodynamically and kinetically stable and show great retention or accumulation in tumors as the result of preferential binding to low-density lipoproteins [7,9–11]. Therefore, porphyrin-based photosensitizers are promising and useful agents for cancer treatment in photodynamic therapy (PDT). PDT is a minimally invasive therapy with negligible toxicity, involving three key components: a photosensitizer, a light source, and tissue oxygen. The combination of these three components results in the chemical destruction of any tissues that have either selectively taken up the photosensitizer or have been locally exposed to light [7,12]. High triplet state quantum yields and long triplet lifetimes, which generate higher singlet oxygen quantum yields, are essential for efficient sensitizers. Closed-shell and diamagnetic ions, such as $Zn^{2+}$, $Ga^{3+}$, and $Si^{4+}$, give porphyrin complexes that possess both high triplet yields and long lifetimes [13–20]. For the above reasons, gallium(III) porphyrin complexes have been proven to act as an effective framework for photosensitizers used in PDT [20–24].

Despite their advantages, the notorious insolubility and aggregation of porphyrins in aqueous solutions negatively affect their cellular uptake and restrict in vivo activity and applications. Given these limitations, efforts have been devoted for developing water-soluble cationic porphyrins that can eliminate the formation of aggregates by increasing the electrostatic repulsion among charged functional groups [3]. Meanwhile, some porphyrin and platinum conjugates have been synthesized with the purpose of improving both hydrophilicity and antitumor activity; these complexes exhibited excellent efficacy in sequential PDT treatments [25–31]. Jana et al. prepared a series of Zn(II) porphyrin–Pt(II) C^N^N acetylide conjugates, all of which were effective photosensitizers for singlet oxygen generation [32]. Lau et al. reported a novel Zn(II) phthalocyanine appended with oxaliplatin. The two components worked in a cooperative manner, and the combination showed high photodynamic activity and cellular uptake and induced cell death via apoptosis [27]. Consequently, the system combinating porphyrins and Pt-based complexes generated interesting synergistic treatment effects, achieving a tremendous enhancement in anticancer effects [25,26,29,33–36].

In light of the potential advantages, we have focused on metalloporphyrinates substituted with platinum-based anticancer drugs, aiming to enhance their hydrophilicity, thereby enabling in vivo evaluation. We also anticipated tumor-targeted effects. In this study, a porphyrin framework, platinum(II)-based groups, and a metal ion gallium(III) were combined to produce two new pentacationic porphyrinates Ga-4cisPtTPyP (5,10,15,20-tetrakis{cis-diammine-chloro-platinum(II)} (4-pyridyl)-porphyrinato gallium(III) hydroxide

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tetranitrate) and Ga-4transPtTPyP (5,10,15,20-tetrakis{trans-diammine-chloro-platinum(II)}(4-pyridyl)-porphyrinato gallium(III) hydroxide tetranitrate). Their photophysical and photochemical properties were investigated, and the photodynamic activities of these complexes in vitro and in vivo were evaluated. The complexes were found to manifest highly potent anticancer activity in vitro and in vivo.

## 2. Experimental

### 2.1. Materials and methods

All the reagents used were of analytical or chemically pure grade. The complex of 4cisPtTPyP (5,10,15,20-tetrakis{*cis*-diammine-chloro-platinum(II)}(4-pyridyl)-21*H*, 23*H*-porphyrin nitrate) was synthesized referring to the reported methods [26,27]. Ultraviolet (UV) spectra were recorded on a UV-3150 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). C, H, and N analysis was carried out on a LECO CHN-900 instrument (LECO Corporation, St. Joseph, Michigan, USA). Fluorescence spectra were measured using a Jasco FP 6500 spectrofluorometer (Jasco, Tokyo, Japan). $^1$H NMR spectra were obtained on a JEOL JNM-ECS400 spectrometer (JEOL Ltd., Tokyo, Japan) using tetramethylsilane as an internal reference. The absorbance was measured at 570 nm with a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Irradiation was performed using a 50-W LED light source containing 45 red and 5 blue LEDs. Colon 26, a colorectal adenocarcinoma cell line derived from BALB/c mice, was used (TKG 0518, Cell Resource Center for Biomedical Research, Tohoku University, Japan). Sarcoma 180 (CCRF S180II) was obtained from Japanese Cancer Research Resource Bank (Tokyo, Japan). LS180 cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Female BALB/c mice (5 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). The biodistribution of Ga-4cisPtTPyP in blood and tissue was determined by measuring the Pt and Ga concentrations using inductively coupled plasma–atomic emission spectrometry (ICP–AES, ICAP-6300, Thermo Fisher Scientific, Waltham, MA, USA). The partition coefficients (log*P* values) of the complexes were measured in an *n*-octanol/water system; the combined phases were shaken by vortex for 10 min, centrifuged for 20 min at 10,000*g*, and each phase was analyzed using UV/Vis spectrophotometry [33,37].

### 2.2. Synthesis of Ga-4cisPtTPyP and Ga-4transPtTPyP

5,10,15,20-Tetra(4-pyridyl)-21*H*,23*H*-porphine ($H_2$TPyP) and the gallium(III) complex GaTPyP (5,10,15,20-tetra(4-pyridyl)-porphyrinato gallium(III) acetate) were obtained according to the methods reported previously [2,38–40]. To synthesize Ga-4cisPtTPyP, the following method was used. Cisplatin (0.129 mmol, 39 mg) and silver nitrate (0.129 mmol, 22 mg) were dissolved in 2 mL of *N*,*N*-dimethylformamide (DMF) and stirred at room temperature for 24 h. The resultant turbid solution was filtered to remove white silver chloride. The light yellow-colored filtrate was added to a solution of GaTPyP (0.0325 mmol, 24 mg) in DMF (2 mL). After stirring at 50 °C for 72 h, the mixture was cooled to ambient temperature and the product was precipitated by the addition of diethyl ether. The solid was filtered and washed with water, methanol, chloroform, and diethyl ether. The solid was dried under vacuum to afford Ga-4cisPtTPyP in the form of a purple powder (43 mg, 64%). $^1$H NMR (400 MHz; DMSO-$d_6$): $\delta$ 9.23 (*o*-pyridyl and $\beta$-pyrrole, m, 16H), 8.45 (*m*-pyridyl, d, 8H, $J$ = 6.4 Hz), 5.08 ($NH_3$, s, 12H), 4.56 ($NH_3$, s, 12H), 7.95 (CH, DMF), 2.90 ($CH_3$, DMF), 2.74 ($CH_3$, DMF), 3.16 ($CH_3$, MeOH); Elemental analysis calcd. for $C_{40}H_{49}Cl_4N_{20}O_{13}Pt_4Ga\cdot DMF\cdot 5CH_3OH\cdot 2H_2O$: C 25.29, H 3.47, N 12.91; obsd. C 25.36, H 3.10, N 12.59.

Ga-4transPtTPyP was synthesized from transplatin using the same method (38 mg, 57%). $^1$H NMR (400 MHz; DMSO-$d_6$): $\delta$ 9.24 (*o*-pyridyl and $\beta$-pyrrole, m, 16H), 8.46 (*m*-pyridyl, d, 8H, $J$ = 6.4 Hz), 5.02 ($NH_3$, s, 12H), 4.52 ($NH_3$, s, 12H), 7.95 (CH, DMF), 2.88 ($CH_3$, DMF), 2.72 ($CH_3$, DMF), 3.16 ($CH_3$, MeOH); Elemental analysis calcd. for $C_{40}H_{49}Cl_4N_{20}O_{13}Pt_4Ga\cdot DMF\cdot 4CH_3OH\cdot H_2O$: C 25.31, H 3.31, N 13.20; obsd. C 25.63, H 3.27, N 13.29.

### 2.3. Singlet oxygen quantum yields

Singlet oxygen quantum yields ($\Phi_\Delta$) were determined by photooxidation of 9,10-dimethylanthracene (DMA) and 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) via steady-state photolysis. Tetraphenylporphyrin (TPP) in dimethylsulfoxide (DMSO) ( $\Phi_\Delta^R$ = 0.52) and Rose Bengal (RB) in aqueous solution were used as references [13,41–44]. DMA solution ($1.45\times10^{-4}$ M in DMSO) or ABDA solution ($2\times10^{-4}$ M in aqueous solution) was mixed with the photosensitizer in a 1-cm quartz cuvette. The initial absorbance reading of the reaction mixture was taken at 401 nm or 380 nm, and the cuvette was irradiated by a 50-W LED light. The kinetics of DMA and ABDA photooxidation were measured. The observed rate constants were obtained by a linear least-squares fit of the semilogarithmic plot of $\ln A_0/A$ versus time. The singlet oxygen generated by a photosensitizer was calculated using the following equation:

$$\Phi_\Delta^S = \Phi_\Delta^R \frac{K^S I^R}{K^R I^S} \quad (1)$$

where $K^R$ and $K^S$ are the slopes of the kinetic plot of DMA and ABDA disappearance photosensitized by the sample and standard, respectively, and $I^R$ and $I^S$ are the total light intensities absorbed by the sample and standard, respectively. All measurements were performed in triplicate.

### 2.4. Photobiological assays

Colon 26 (mouse colon adenocarcinoma), Sarcoma 180 (mouse sarcoma), and LS 180 (human colon carcinoma) cell lines were plated in 96-well plates at a density of $5\times10^3$ cells/well. The cells were incubated with different concentrations of Ga-4cisPtTPyP, Ga-4transPtTPyP, 4cisPtTPyP, GaTPyP, a mixture of GaTPyP and four equivalents of cisplatin, and cisplatin for 24 h or 48 h (DMSO = 0.4%). After this period, the complexes were removed by washing with phosphate-buffered saline (PBS) and the medium was replaced. The cells were immediately irradiated by a 50-W LED light or left without irradiation (in the case of dark control cells) for 30 min. The plates were kept in a $CO_2$ incubator for 48 h for further cultivation. Cell viability was estimated by the standard MTT assay with a microplate reader at an absorbance of 570 nm [45,46]. All experiments were independently replicated thrice.

### 2.5. Cellular uptake

To compare the uptake ability of Ga-4cisPtTPyP and 4cisPtTPyP, Colon 26 cells were treated with 10 μM Ga-4cisPtTPyP or 4cisPtTPyP at 37 °C for 2 h. After incubation, cells were washed twice with ice-cold PBS and resolved by 1 mL of 1 M NaOH. After adding 3 mL 5% $HNO_3$, the samples were analyzed using ICP–AES. The Pt content of each sample was normalized for the protein level, which was determined using a Protein Assay Bicinchoninate Kit (Nacalai Tesque) [47].

### 2.6. Subcellular localization

To measure the cellular uptake of the promising agent Ga-4cisPtTPyP, Colon 26 cells were treated with 20 μM Ga-4cisPtTPyP at 37 °C for 2 h and 4 h. Then the medium was removed, the cells were washed with PBS solution, harvested, and centrifuged. The pellet was suspended in a sucrose solution and incubated with buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.2 mM EDTA, 1.0 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) on ice for 15 min. After adding 5% NP-40, the suspension was centrifuged and the supernatant was collected to get the cytosol. The pellet was re-dissolved in a sucrose