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Production of singlet oxygen on irradiation of a photodynamic therapy agent, zinc-coproporphyrin III, with low host toxicity

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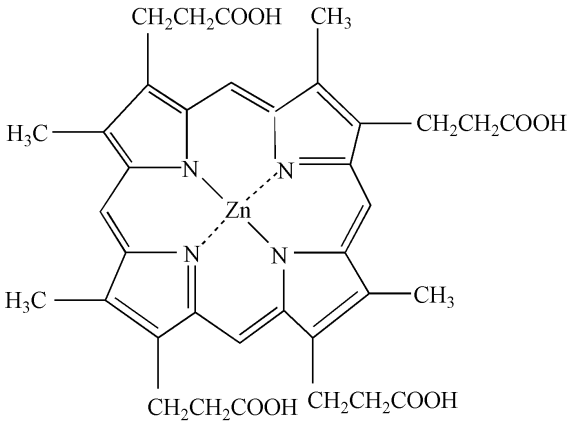
Abstract

Zinc-coproporphyrin III (Zincphyrin) acts efficiently as a photodynamic therapy (PDT) agent in mice, while it shows no tumor cell-killing activity *in vitro* and has a high LD_{50} (low toxicity) in mice. It appears to have advantages over other porphyrins as a practical PDT reagent. In order to examine the action mechanism of Zincphyrin in PDT, we evaluated the photochemical characteristics of Zincphyrin by measurement of the near-infrared emission at 1268 nm, which provides direct evidence for formation of $^1O_2$. Intense emission was observed in the presence of Zincphyrin, and was completely inhibited by NaN_3, a $^1O_2$ scavenger. Based on a quenching study, the rate constant of the reaction of $^1O_2$ with NaN_3 was determined to be 1.5–3.5 M^{-1} s^{-1}, which is close to the reported value (3.8×10^8 M^{-1} s^{-1}). The intensity of the $^1O_2$-specific emission was proportional to both the laser power and the concentration of Zincphyrin. The fluorescence quantum yield of Zincphyrin was 0.004 in phosphate buffer (100 mM, pH 7.4), which indicates that the excited state decays via other pathway(s) faster than through the fluorescence emission pathway. The lifetime of the triplet state of Zincphyrin (210 μs) was relatively long compared to that of other porphyrins, such as hematoporphyrin (Hp) (40 μs), coproporphyrin I (50 μs), or coproporphyrin III (36 μs). These results demonstrate the photodynamic generation of $^1O_2$ by Zincphyrin.

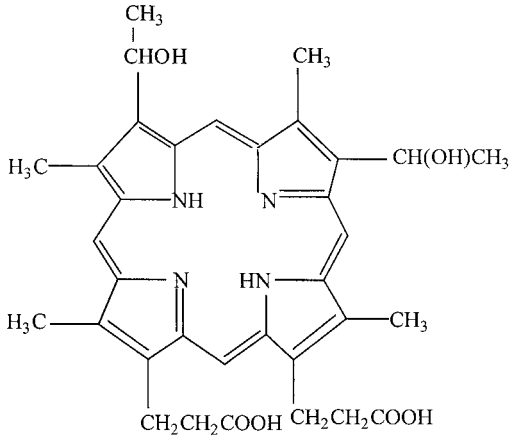
Introduction

Zinc-coproporphyrin III, named Zincphyrin (Scheme 1), is a photodynamic therapy (PDT) agent that is as effective as hematoporphyrin (Hp) against B-16 melanoma- or sarcoma-180-implanted mice, while having much less toxicity than Hp in terms of LD_{50}. In addition, irradiation of Zincphyrin shows no cytocidal activity against tumor cells such as L5178Y or sarcoma-180 *in vitro* (Toriya *et al.* 2001). PDT is a cancer therapy that is based on the administration of a photosensitizer, which is preferentially accumulated in tumor cells and then excited by irradiation with visible light, the resultant generation of singlet oxygen ($^1O_2$) leads to oxidation of many cellular constituents, killing the tumor cells (Pervaiz 2001). However, these porphyrins also accumulate in normal cells to some extent, resulting in serious side effects.

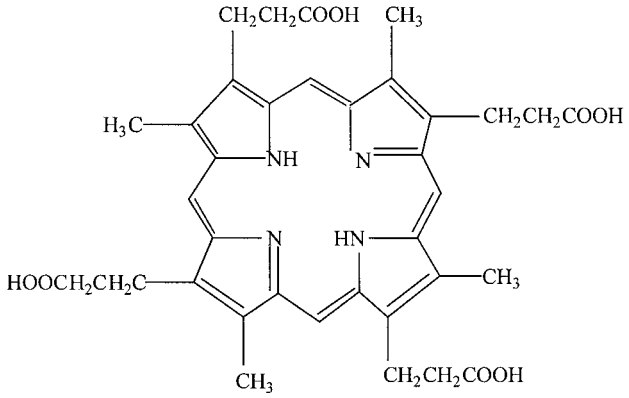
The findings that Zincphyrin has no tumor cell-killing activity *in vitro* and low host toxicity *in vivo* led us to examine the action mechanism of Zincphyrin in PDT. It is important to know whether Zincphyrin on irradiation can produce $^1O_2$ or not, because there are two major mechanisms of the photodynamic action of photosensitizers in biological systems, i.e., sensitizers



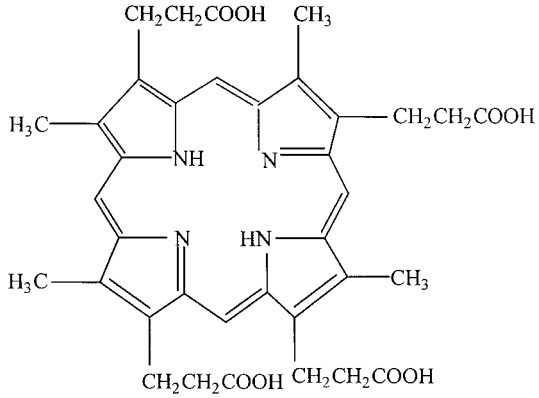
Zincphyrin



hematoporphyrin IX



coproporphyrin I



coproporphyrin III

Scheme 1. Structures of Zincphyrin, hematoporphyrin, coproporphyrin I and coproporphyrin III.

excited to the triplet state react directly with biological substrates (Type I reaction), and the photo-generated triplet state of sensitizers reacts with oxygen via an energy transfer process to produce $^1O_2$ (Type II reaction) (Sibata *et al.* 2001).

Kobayashi *et al.* proposed the photodynamic DNA strand-breaking activity by reactive oxygen species (ROS) as the possible mechanism in the case of Zincphyrin, though there is no direct evidence of the formation of ROS upon irradiation of Zincphyrin (Kobayashi *et al.* 1994). In Type II reaction, the lifetime of the excited triplet state of porphyrin and the amount of $^1O_2$ production are essential factors for PDT activity.

In this study we measured the near-infrared emission at 1268 nm, which provides direct evidence for the formation of $^1O_2$, and the lifetime of the excited triplet state in order to evaluate the photochemical properties of Zincphyrin in relation to PDT action.

Materials and methods

Chemicals

Zincphyrin was prepared by the methods described in our previous paper (Toriya *et al.* 1993). Hematoporphyrin IX (Hp), coproporphyrin I and coproporphyrin III (Scheme 1) were obtained from Frontier Scientific, UT, U.S.A.

Measurement of $^1O_2$ near-infrared emission of Zincphyrin

The $^1O_2$ near-infrared emission of Zincphyrin was analyzed using the method reported by Nagano (Nagano *et al.* 1994; Umezawa *et al.* 1997). The experimental setup consisted of an Ar laser (Innova 70-4; Coherent Inc., U.S.A.) with a liquid nitrogen-cooled near-infrared Ge-detector (model 403HS; Applied Detector Co., U.S.A.) connected to the exit slit of a monochromator (Model CT10; JASCO, Japan) having a blaze wavelength at 1250 nm to minimize the grating loss. An IR-80 cutoff filter with 0% transmittance at less than 750 nm and 35% transmittance at 800 nm was placed at the entrance slit of the monochromator. A collecting lens focused the monochromator output the detector crystal. The Ar laser output was chopped at 800 Hz by an acousto-optic modulator (A-160; Hoya, Japan) driven by a driver (110-DS; Hoya). The laser light contained three wavelengths in the UVA range (334.0, 351.1, and 363.8 nm). The signal output from the Ge-detector was fed to a Model 124A lock-in amplifier via a Model 116 preamplifier (both from E.G. & G. Princeton Applied Research, U.S.A.) and synchronized with the internal reference signal of the lock-in amplifier. The signal output from the lock-in amplifier was fed to an XY recorder, and the emission spectrum was recorded by scanning the grating with a motor. In order to minimize photobleaching of the drugs, the solution was circulated by a peristaltic pump through a quartz flow cell (3 × 3 mm).

Fluorometric analysis

UV-Visible spectra were obtained on a Shimadzu UV-1600. Fluorescence spectroscopic studies were performed on a Hitachi F4500. The slit width was 2.5 nm for both excitation and emission. The photon multiplier voltage was 950 V. Relative quantum efficiencies of fluorescence of Zincphyrin were obtained by comparing the area under the corrected spectrum excited at 492 nm in the phosphate buffer (100 mM, pH 7.4)

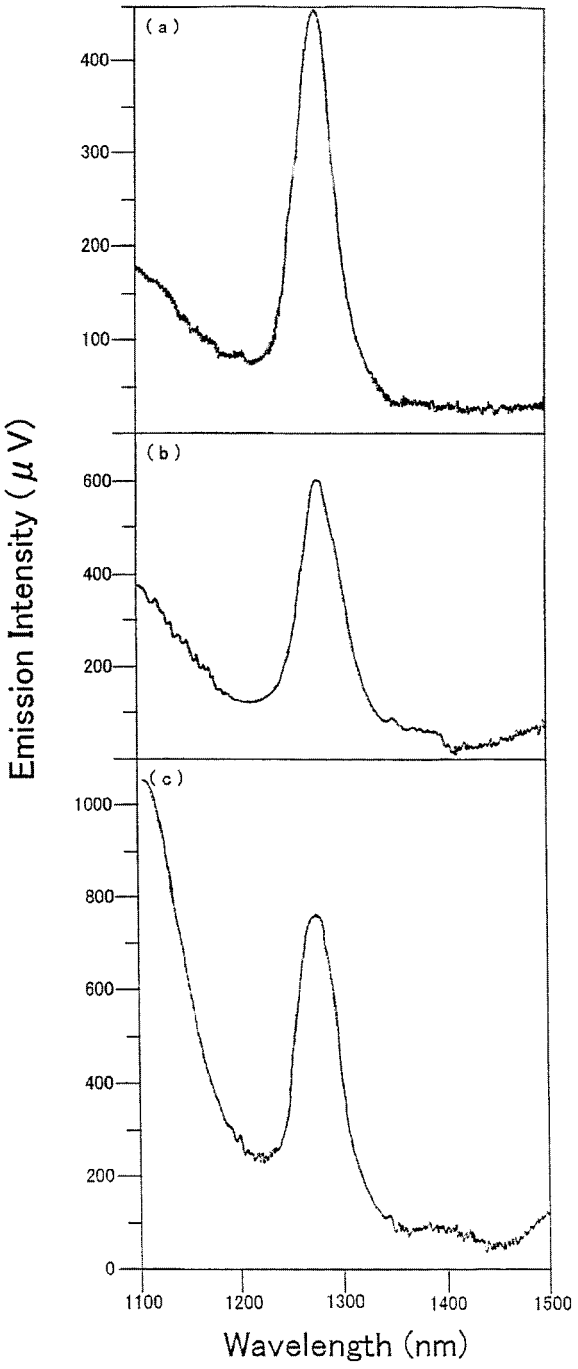


Fig. 1. Emission spectrum at near-infrared wavelength in the presence of porphyrins in methanol. The measurements were carried out at 100 μM porphyrin (a: Zincphyrin; b: Hp; c: coporoporphyrin III), with Ar laser light excitation at 514.5 nm (200 mW output power).

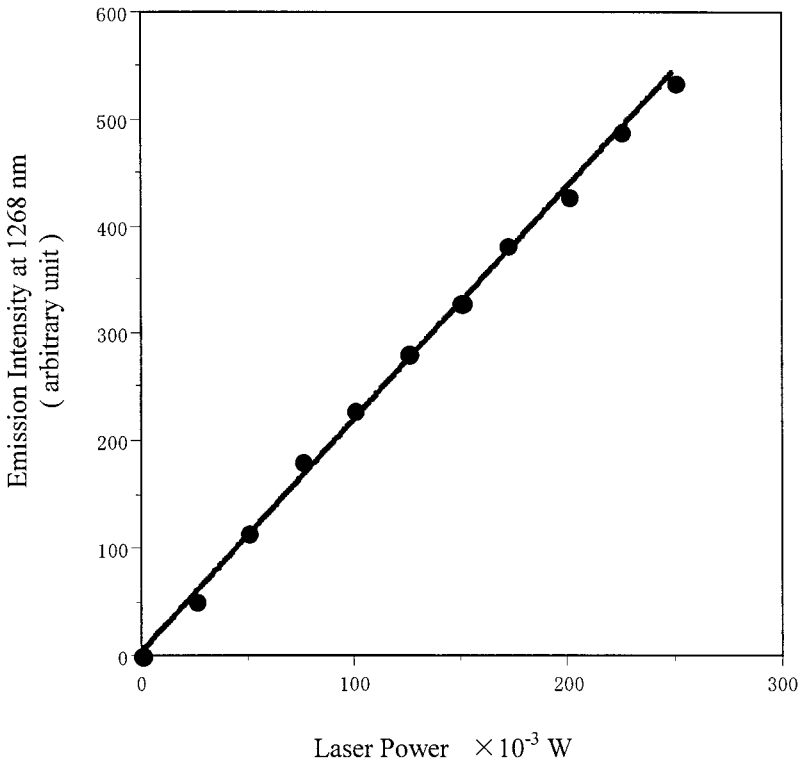


Fig. 2. Correlation between emission intensity at 1268 nm and laser power. The measurements were done under the same conditions as in the legend to Figure 1 except for output power.

with that of a solution of fluorescein in 0.1 M NaOH, which has a quantum efficiency of 0.85 according to the literature (Parker & Rees 1960).

Measurement of lifetime of photoexcited triplet state of Zincphyrin

The lifetime of the photoexcited triplet state of Zincphyrin was measured by conventional laser flash photolysis (Amao *et al.* 1998; Furuto *et al.* 1998). These experiments were carried out by using an Nd:YAG laser (Model DCR-3 from Quanta Ray Inc.) as the excitation source. An excitation wavelength of 532 nm, a pulse duration of 10 ns and a repetition rate of 10 Hz were used for the excitation. Light that passed through the sample cell was collimated into the entrance slit of a monochromator (Model 1410 from Applied Photophysics Co., Ltd.). The output signal from a photomultiplier (Model 1445 from Applied Photophysics Co., Ltd.) attached to the slit of the monochromator was displayed on a digitizing oscilloscope (Model 11401 from SONY-Tektronix) and averaged over 64–128 flashes. Sample solutions were adjusted to possess an absorbance at 532 nm of 0.20 and were deaerated by repeated freeze-pump-thaw cycles (four times) to remove dissolved oxygen.

Results

$^1O_2$ formation of Zincphyrin

The near-infrared emission was measured at 1100–1500 nm using laser-excited solutions of 100 μM Zincphyrin in methanol. The spectra made it clear that Zincphyrin can act as a photosensitizer for production of $^1O_2$. There are some differences of emission intensity between the porphyrins, Zincphyrin, Hp and coproporphyrin III, as shown in Figure 1. Generally, metal ions such as Fe^{2+} and Cu^{2+} quench the emission at 1268 nm due to the photosensitizer, but zinc ion did not inhibit the photosensitizing activity.

Figure 2 shows the relationship between the laser power and the emission intensity at 1268 nm in Zincphyrin solution. The emission intensity due to $^1O_2$ from laser-excited Zincphyrin increased with increase of the laser power. Figure 3 shows the dependence of the emission intensity at 1268 nm on the concentration

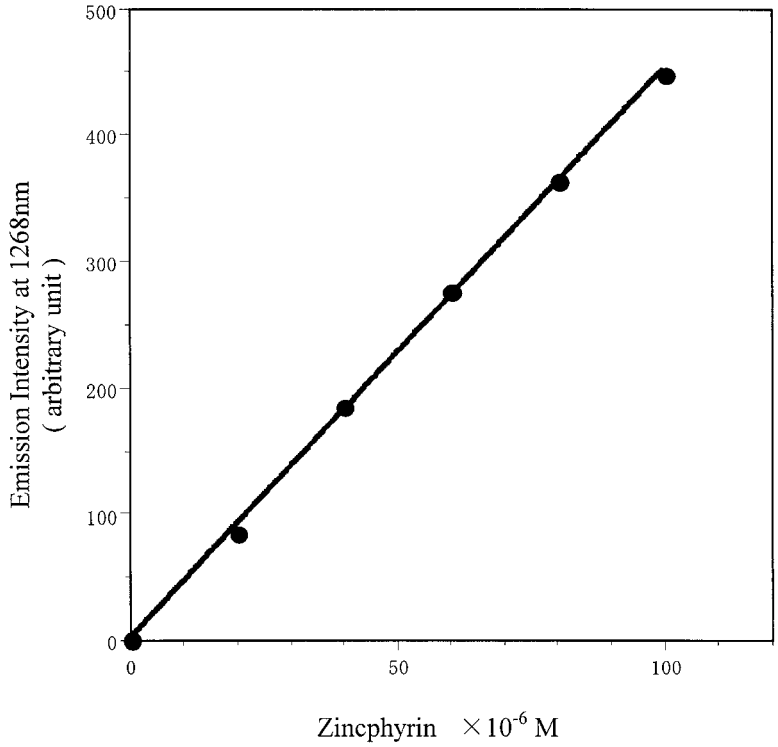


Fig. 3. Correlation between emission intensity at 1268 nm and concentration of Zincphyrin. The measurements were done under the same conditions as in the legend to Figure 1 except for the concentration of Zincphyrin (20–100 μM).

of Zincphyrin. The relation was linear from 2×10^{-5} to 1×10^{-4} M. The emission at 1268 nm was inhibited by the addition of a $^1O_2$ quencher, NaN_3 (Figure 4). A quenching study of the $^1O_2$ emission at 1268 nm gave a value for the rate constant of the reaction of $^1O_2$ with NaN_3 of 1.5–3.5 M^{-1} s^{-1}, which is close to the reported value (3.8×10^8 M^{-1} s^{-1}).

The fluorescence quantum yield of Zincphyrin was 0.004 in phosphate buffer. The low quantum yield indicates the existence of fast decay pathway(s) other than the fluorescence-emitting pathway. The intersystem crossing (ISC) pathway could be involved in the production of $^1O_2$ via a meta-stable excited triplet state of Zincphyrin.

The results confirm that Zincphyrin has the activity to produce $^1O_2$ on irradiation.

Lifetime of photoexcited triplet state of Zincphyrin

The lifetime of the photoexcited triplet state of Zincphyrin (210 μs) was longer than that of Hp (40 μs), coproporphyrin I (50 μs), or coproporphyrin III (36 μs).

Discussion

The photochemical properties of Zincphyrin were examined in order to understand the difference of PDT activity between *in vitro* (cytocidal activity on tumor cells) and *in vivo* (tumor cell-killing activity using mice).

Takemura *et al.* compared the lifetime of Fe- or Cu-chelating porphyrin with that of the corresponding free base, and showed the triplet lifetime of the photosensitizer to be critically important for PDT (Takemura *et al.* 1989; Ando *et al.* 1993). Ando *et al.* reported that the PDT activity of photosensitizers correlates well with the triplet lifetime, using HGC-27 cells (Ando *et al.* 1990). Fukuzumi et al. also examined the photochemical properties of zinc chlorin-C60 dyad as compared to the corresponding free-base chlorine-C60, free-base porphyrin-C60 and zinc porphyrin-C60 dyads (Fukuzumi *et al.* 2001). Scott et al. reported RBC hemolysis by protoporphyrin IX (Pp) and some metal-chelated Pp under irradiated or non-irradiated conditions. Hp and Pp showed remarkable PDT effects, whereas zinc-Pp was much less effective, and they concluded that zinc reduced the

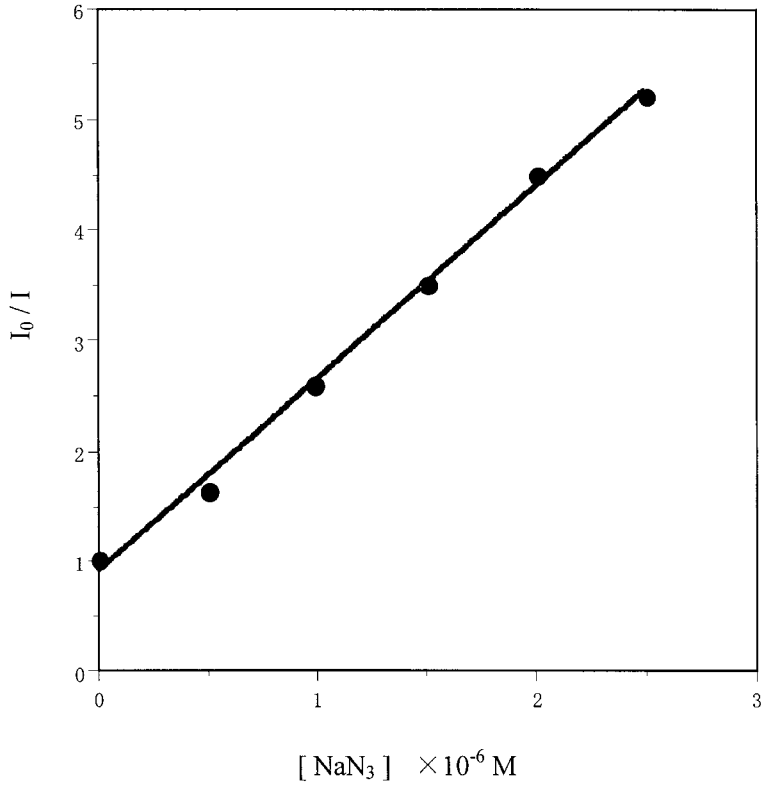


Fig. 4. Inhibitiory effect of NaN_3 on emission intensity at 1268 nm based on Zincphyrin. $^1O_2$ was generated in Zincphyrin methanol solution excited with Ar laser light at 514.5 nm (200 mW output power) and inhibitiory activity was monitored by measuring the emission intensity at 1268 nm after addition of NaN_3 at various concentrations.

phototoxic activity of Pp (Scott *et al.* 1990). On the other hand, zinc-Pp was reported to have a higher phototoxicity than Pp *in vitro* using HeLa cells (Koide *et al.* 2002). These differences may be due to the differences in the tumor cells used.

However, our finding is that the lifetime of the triplet state of Zincphyrin was 5–6 times longer than those of Hp, coproporphyrin I and coproporphyrin III, but the emission intensity of Zincphyrin at 1268 nm was almost the same as those of the other three porphyrins. These data were obtained in a cell-free system, independent of tumor cells. Takemura *et al.* also reported that zinc-chelating porphyrins have a long triplet lifetime compared to the corresponding free base and some metal porphyrins. In conclusion, the amounts of $^1O_2$ production do not correlate linearly with the lifetime of the triplet state of a photosensitizer, though porphyrins with extremely short lifetime cannot be effective photosensitizers.

Zincphyrin showed PDT effects in B-16 melanoma or sarcoma-180 implanted mice, though the efficiency of PDT with Zincphyrin in a cellular system (*in vitro*) was much lower than with Hp, as we showed in our previous paper (Toriya *et al.* 2001). These results indicate that the order of $^1O_2$-forming ability is not correlated with the order of *in vitro* photo-cytocidal activity, and the PDT effect does not simply depend on the amount of $^1O_2$ production and the triplet lifetime of the photosensitizer. The tumor vasculature, apart from membranous intracellular organelles such as mitochondria, lysosomes, and nuclei, may be a potential target for $^1O_2$ generated on photoactivation of PDT agents (Krammer 2001). Zincphyrin may merely cause vascular impairment that restricts blood supply to the region around tumor cells, while other porphyrins do direct damage to tumor cells. Thus, anti-angiogenesis activity of Zincphyrin based on the damage of the neovascularization may indirectly cause to kill tumor cells. The cell membrane permeability and/or the location of Zincphyrin could also contribute to the activity. The photochemical properties of Zincphyrin in this study thus provide a clue to the PDT mechanism of Zincphyrin, and further studies are in progress.

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