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# Photodynamic Cell Killing Effects and Acute Skin Photosensitivity of Aluminum-chloro-tetrasulfonated Phthalocyanine and Hematoporphyrin Derivative

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**Aluminum-chloro-tetrasulfonated phthalocyanine (PC) showing an absorption peak at 678 nm was compared to hematoporphyrin derivative (HpD), a photosensitizer commonly used in the photodynamic therapy (PDT) of cancers. *In vitro* studies: KK-47 cells were exposed to long-wavelength ultraviolet (UVA) or red light (>600 nm, >640 nm and >660 nm) after drug sensitization. With UVA irradiation, a higher photodynamic cell killing effect was observed in the cells treated with HpD than with PC. However, with red light irradiation (both >640 nm and >660 nm) PC resulted in greater cell damage. PC was less toxic to KK-47 cells in the dark. *In vivo* studies: Using a gold vapor laser (GVL: 627.8 nm, 200 mW/cm$^2$, 200 J/cm$^2$), the photodynamic tumor response was determined in C3H/He mice bearing transplantable squamous cell carcinoma. No significant difference was observed in the tumor volume between the PC and HpD groups, except that the PC group (10.0 mg/kg body weight) showed a significantly higher remission rate (3/6) than the control group (0/10, $P<0.05$). Skin photosensitivity test: Skin photosensitivity was estimated by measuring changes in back skin thickness due to photosensitization. With UVA irradiation, a stronger skin reaction was observed in the HpD group, while with visible light irradiation there was no significant difference between the HpD and PC groups. Based on the superior cell killing effect with red light, reduced toxicity to the cells in the dark and mild skin reaction with UVA, PC may be a more promising photosensitizer for PDT.**

Key words: Photodynamic therapy — Aluminum-chloro-tetrasulfonated phthalocyanine — Hematoporphyrin derivative — Gold vapor laser — Acute skin photosensitivity

The clinical application of photodynamic therapy (PDT)[1–4] to carcinoma of the urinary bladder, especially carcinoma *in situ*, has been attempted at some institutions with encouraging results.[5–8] The photosensitizer preparation most commonly used is a complex mixture of porphyrins termed hematoporphyrin derivative (HpD) and Photofrin II™ [4] which contains the presumed active component dihematoporphyrin ether, ester or ethers and esters. HpD, however, poorly absorbs light at 630 nm, which is the commonly used laser irradiation wavelength. Furthermore, a major adverse effect of HpD-PDT is skin photosensitivity after drug sensitization.[9–11] This skin reaction is potentially so severe that patients must be warned to avoid exposure to direct sunlight for several weeks. This photosensitivity is known to be evoked by light in the long-wavelength ultraviolet (UVA) range.[12] These facts have resulted in a considerable interest in identifying new photosensitizers for PDT. Recently it has been suggested that phthalocyanines (PC) may be promising photosensitizers having the advantage of good absorption in the red light region with a peak at 670 to 680 nm.[13–16] Uptake of PC by tumors has also been reported to be good.[17, 18]

Abbreviations used in this paper: PDT, photodynamic therapy; PC, phthalocyanine; HpD, hematoporphyrin derivative; UVA, long-wavelength ultraviolet; GVL, gold vapor laser

Considering these attractive characteristics of this dye, *in vitro* experiments were designed to compare the photodynamic cell killing effect of PC with that of HpD employing UVA or red light (>600 nm, >640 nm and >660 nm) irradiation of an established human bladder cancer cell line (KK-47). As an *in vivo* study, the photodynamic effect was evaluated in PC- and HpD-injected groups of C3H/He mice bearing transplanted squamous cell carcinoma and irradiated at 627.8 nm with a gold vapor laser (GVL). This laser is reported to have some advantages[19] over an argon dye laser, which has been widely used in the clinical field. Further, skin photosensitivity to UVA and visible light was assessed by using a mouse back skin model following sensitization with each of the photosensitizers.

## MATERIALS AND METHODS

**Mice** Seven- to ten-week-old male C3H/He mice were obtained from Sankyo Laboratories, Toyama. They were housed 3 mice per cage and fed autoclaved lab chow and water *ad libitum*.

**Tumor systems** Two tumor systems were used in the present study. KK-47 human bladder cancer cell line[20] was maintained as an adherent monolayer culture in RPMI 1640 supplemented with 10% fetal calf serum, 1% glutamine, penicillin and streptomycin. The cells were

incubated in a 5% $CO_2$ atmosphere at 37°C. A block of squamous cell carcinoma tissue which arose spontaneously in C3H/He mice,[21] 4×4×4 mm in size, was implanted subcutaneously into the dorsal region of each mouse. This tumor system was kindly provided by Professor K. Ueda (Department of Dermatology, Fukui Medical School, Fukui).

**Photosensitizers** Hematoporphyrin derivative (HpD: Photofrin I™) was purchased from Oncology Research and Development Inc., Cheektowaga, NY. HpD was stored in the dark at −20°C until immediately before use. Aluminum-chloro-tetrasulfonated phthalocyanine (Fig. 1) was kindly provided by Sumitomo Pharmaceuticals Co., Ltd., Osaka. The absorption spectrum of PC shows that the main absorption is in the UV and red light regions with a peak at 678 nm in physiological saline. HpD, however, mainly absorbs light in the UV region (Fig. 2).

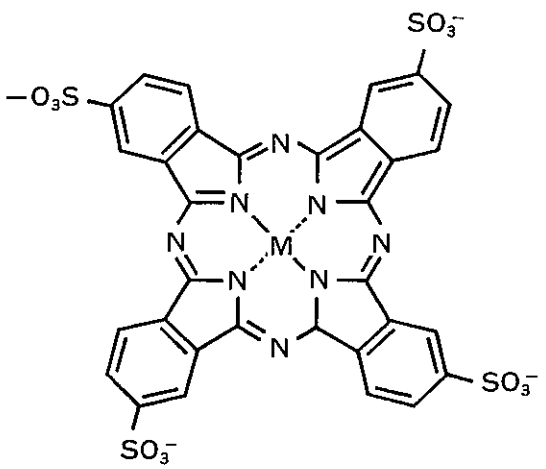


Fig. 1. Structural diagram of aluminum-chloro-tetrasulfonated phthalocyanine (PC). M: AlCl.

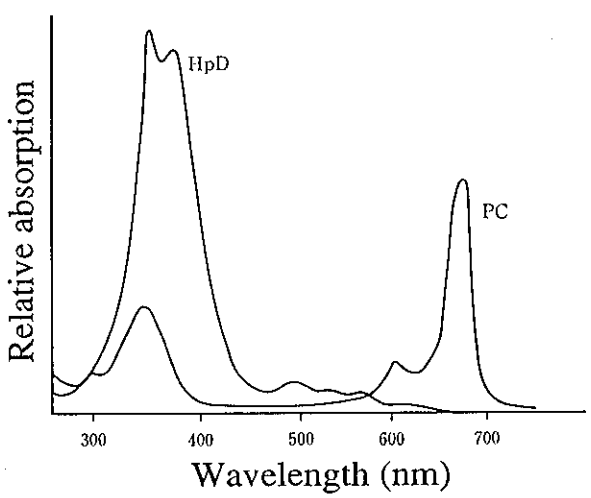


Fig. 2. Absorption spectra of PC and HpD (10 μg/ml in physiological saline).

**Laser source** GVL[19, 22] (Model AU-10) was developed by Oxford Lasers Ltd., Oxford. The instrument delivered pulsed monochromatic light at 627.8 nm. The pulse width at base was 80 ns and the pulse repetition rate was 10 kHz. With the average power of 4.5 W, the peak power was 10 kW. The light beam was coupled to a 600 μm quartz fiber.

***In vitro* PDT** KK-47 cells were harvested by trypsinization, resuspended in fresh medium and plated in a 96-well microplate (Microtest III™ Tissue Culture Plate, Becton Dickinson & Company, NJ). Eight thousand cells in 200 μl of medium in each well were incubated for 24 h before drug exposure. The cells were incubated with each of the photosensitizers at various concentrations. The drug-containing medium was sucked off, and the cells were washed gently three times with PBS. These cells were treated with graded doses of UVA (Dermaray, Model M-DMR-1, mounting Toshiba FL20S. BLB, 300-430 nm, Eisai Co., Ltd., Tokyo) or red light of >600 nm, >640 nm or >660 nm in 50 μl of PBS. The red light sources used in the present study were prepared by using the visible light from a slide projector lamp (Kodak Ektagraphic, 375–750 nm with a peak at 615±10 nm) and passing it through a sharp low-cut filter (R-60, R-64 or R-66, Hoya Co., Ltd., Tokyo). The MTT assay was performed to determine the surviving fractions according to the method of Mosmann[23] with a modification as reported by Carmichael *et al.*[24] In brief, immediately after the light exposure, PBS was sucked off and 25 μl of a 2 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) with a 200 μl of fresh medium was added to each well. Plates were incubated for 6 h with MTT at 37°C. MTT solution and the medium were sucked off and 200 μl of dimethyl sulfoxide was added. The plates were placed in a microplate shaker for 2–3 min to dissolve the dark blue crystals and were read on a multiwell scanning spectrophotometer at 540 nm. The surviving fraction was calculated as follows: Surviving fraction (%) = 100× (mean absorbance of the treated wells/mean absorbance of the control wells). The cytotoxicity of each photosensitizer without any light exposure was determined by using the same method as mentioned above after incubating cells with each drug continuously in the dark.

***In vivo* PDT** When tumors reached approximately 6×6×4 mm in size, usually 7 to 10 days after tumor transplantation, the mice were given a single intraperitoneal injection of a photosensitizer in 0.2 ml of

physiological saline (the three doses used were 0.1, 1.0 and 10.0 mg/kg body weight) or 0.2 ml of physiological saline as a control injection. Each tumor was irradiated 24 h later by GVL at 200 mW/cm$^2$ adjusted output power at the tumor surface and 200 J/cm$^2$ total light dose. The irradiation time was 16.7 min. The tumor volume was estimated using a slide caliper on the 30th day after treatment by applying the following formula. Tumor volume$=\pi/6 \times a \times b \times c$, $a$: length, $b$: width, $c$: thickness. The relative tumor volume after a 30-day follow-up period (tumor volume on day 30/tumor volume on day 0) in various treated groups was compared by using Kruskal-Wallis' nonparametric ANOVA followed by Hollander-Wolfe's multicomparison test. Complete tumor remission (CR) was defined as no palpable tumor at 60 days after treatment. The CR rate was compared among the treated groups by using Fisher's exact probability test.

**Skin photosensitivity** Various doses of PC and HpD were intraperitoneally injected into C3H/He mice 24 h after depilation of the back skin. Twenty-four hours after the drug injection (0.1, 1.0, 5.0 and 10.0 mg/kg body weight), the back skin was irradiated with UVA or visible light. Various doses of each light were obtained by changing the irradiation time. The back skin thickness was measured by using a dial thickness gauge[25-27] (Ozaki Mfg. Co., Ltd., Tokyo), before, and 12, 24, 48, 72 and 96 h after light irradiation. The results obtained from each group of 5 mice were expressed as percent change in dorsal skin fold thickness over that of the pre-irradiation controls.

## RESULTS

***In vitro* PDT** Incubation with each of the photosensitizers for 24 h, with concentrations up to 200 $\mu$g/ml, resulted in no cytotoxicity. PC was less toxic than HpD after continuous drug exposure for 72 h without light irradiation (Fig. 3). In addition, neither UVA nor red light showed cytotoxicity to the cells in the absence of a drug. With UVA irradiation, the photodynamic cell killing effect of HpD was significantly higher than that of PC (Fig. 4). The cell killing effect was dependent on the UVA dose as well as the drug dose as shown in Fig. 4 and Fig. 5. After red light irradiation at >600 nm, no difference in photodynamic cell killing effect was demonstrated between the cells treated with PC and those treated with HpD (Fig. 6a), whereas red light irradiations at >640 nm and >660 nm showed a superior photodynamic cell killing effect on the cells treated with PC compared with those treated with HpD (Fig. 6b and Fig. 6c). The cell killing effects of PC and HpD were dependent on the red light irradiation dose as well as UVA dose.

***In vivo* PDT** No macroscopic change was observed immediately after the PDT. About 24 to 36 h after the PDT the skin on the tumor became necrotic. Shrinkage of the tumor was recognized usually 36 to 48 h later. Regrowth of the tumor occurred apparently 7 to 10 days after the treatment. Table I summarizes the results of measurement of relative tumor volume on the 30th day after the PDT and the rate of complete remission in each group. No tumor-eradicating ability was seen in the animals injected with PC or HpD without laser irradiation. The group treated with GVL alone showed percep-

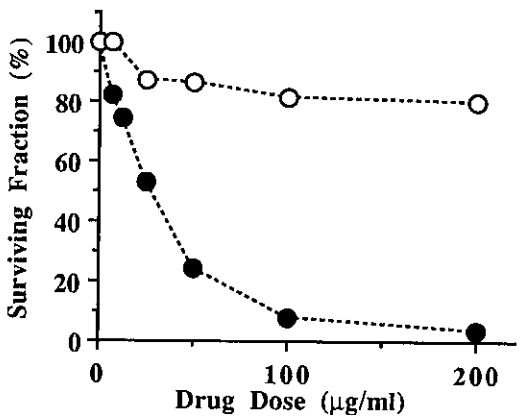


Fig. 3. Cytotoxicity of PC (○) and HpD (●) on KK-47 cells. KK-47 cells were incubated with each photosensitizer for 72 h without any light exposure. The surviving fraction was determined by means of the MTT assay.

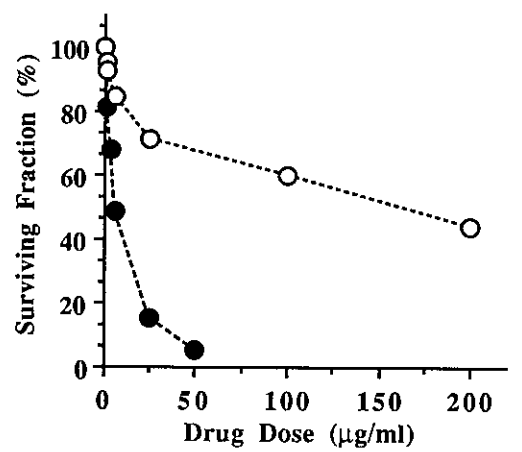


Fig. 4. Photodynamic cell killing effect of PC (○) and HpD (●) on KK-47 cells with UVA irradiation. The cells were incubated with graded doses of each drug for 24 h and exposed to UVA (2 J/cm$^2$).

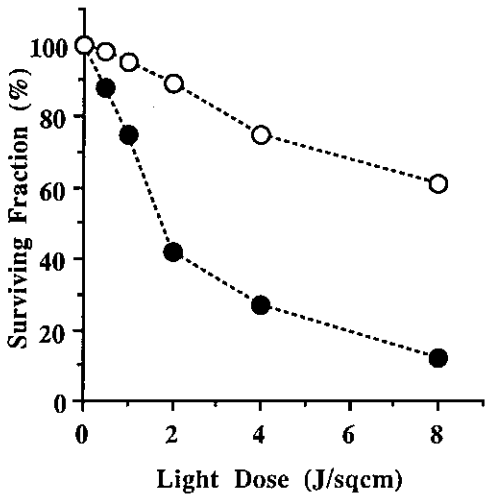


Fig. 5. Photodynamic cell killing effect of PC (○) and HpD (●) on KK-47 cells with UVA irradiation. The cells were incubated with 3.125 μg/ml of each drug and exposed to graded doses of UVA.

tible tumor regression. Compared with the control group, only three of the treatment groups (HpD: 10.0 mg/kg body weight+GVL, PC: 1.0 mg/kg body weight+GVL and PC: 10.0 mg/kg body weight+GVL) demonstrated a statistically significant difference in relative tumor volume. Among the treated groups, however, there was no statistically significant difference in tumor volume. The PDT effect seemed to be dependent on the drug dose. Concerning the complete remission rate, only one treatment group (PC: 10.0 mg/kg body weight + GVL) demonstrated a statistically significant CR rate (3/6) compared with the control group (0/10). Intratumor temperature measured during the laser irradiation was elevated to 45.0 to 46.0°C, 5 min after the irradiation started and was maintained at over 44.0°C until the end of the treatment.

**Skin photosensitivity test** There was no significant skin edema or erythema in the control groups receiving physiological saline injection or no light irradiation. The maximum degree of skin edema was obtained 12 to 24 h after UVA or visible light irradiation. In severe edema cases, a marked skin necrosis was observed approximately 72 to 96 h after the light exposure (Fig. 7). With UVA irradiation, a stronger skin reaction was observed in the HpD-injected group compared to the PC-injected group (Fig. 8a). In addition, as shown in Figs. 8a and 8c, the degree of skin edema due to HpD and UVA was dependent on both the drug and light doses. With visible light irradiation, the degree of skin reaction induced by the two drugs was equivalent and dependent on the drug dose (Fig. 8b).

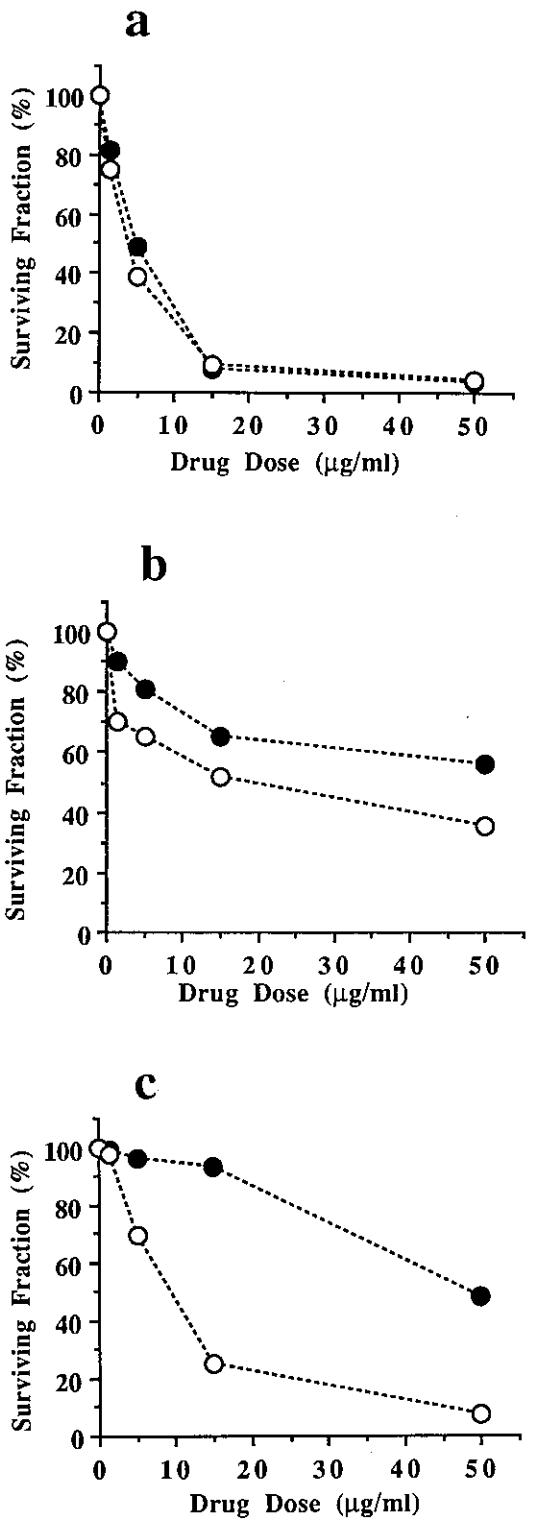


Fig. 6. Photodynamic cell killing effect of PC (○) and HpD (●) on KK-47 cells with red light of three different wavelengths. (a) >600 nm, 8 J/cm$^2$, (b) >640 nm, 8 J/cm$^2$, (c) 660 nm, 8 J/cm$^2$.

Table I. Effect of PDT in C3H/He Mice Tumor after Sensitization with PC and HpD

| Treatment group | Relative tumor volume[a] at 30 days follow up | CR[b] rate |
|---|---|---|
| Control[c] | 219.6±64.7 | 0/10 |
| CVL[d] | 55.7±23.6 | 0/6 |
| PC (0.1 mg/kg body weight)+GVL | 14.2±11.3 | 1/6 |
| PC (1.0 mg/kg body weight)+GVL | 8.6±8.9[e] | 2/6 |
| PC (10.0 mg/kg body weight)+GVL | 1.6±1.8[f] | 3/6[h] |
| HpD (0.1 mg/kg body weight)+GVL | 22.2±12.2 | 0/6 |
| HpD (1.0 mg/kg body weight)+GVL | 12.2±10.7 | 1/6 |
| HpD (10.0 mg/kg body weight)+GVL | 7.1±6.5[g] | 2/6 |

*a*) As described in "Materials and Methods." Values are mean±SD.
*b*) CR: complete remission at 60 days.
*c*) Physiological saline was injected.
*d*) GVL: gold vapor laser; 627.8 nm, 200 mW/cm$^2$, 200 J/cm$^2$.
*e, f, g*) Significant regression compared with the control group ($P<0.05$, Kruskal-Wallis' nonparametric ANOVA followed by Hollander-Wolfe's multicomparison test).
*h*) Significant CR rate compared with the control group ($P<0.05$, Fisher's exact probability test).

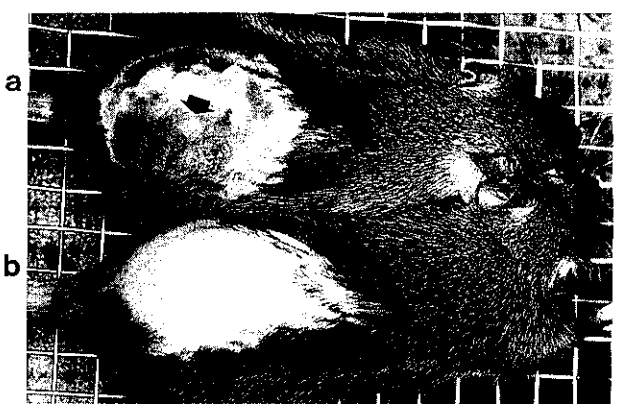


Fig. 7. Macroscopic aspects of acute skin response on back skin of C3H/He mice. (a) Marked skin necrosis (⬇) was observed 96 h after light (UVA: 10 J/cm$^2$) exposure following sensitization with 10 mg/kg body weight of intraperitoneal HpD. (b) No remarkable change was observed 96 h after the same dose of UVA irradiation following physiological saline injection.

## DISCUSSION

Phthalocyanines, which can be regarded as azaporphyrins,[16] are expected to be more useful photosensitizers than HpD because they are relatively easy to synthesize and are resistant to chemical and photochemical degradation. They produce long-lived triplet states and have absorption peaks in the wavelength region of red light with a large molecular coefficient.[16] As a result of *in vitro* screening of phthalocyanines with various metal components, Chan *et al.* showed that most of the dyes have little inherent cytotoxicity, as was confirmed by the present *in vitro* data, in accordance with the concept that the optimal photosensitizer should be nontoxic to cells.[15]

In practical PDT with HpD, even though HpD has a very low absorption peak in the red region (630 nm), laser light at 600 to 630 nm in wavelength is used because of its optimal tissue penetration.[13] In this regard, PC having a strong absorption in the red wavelength region, as shown in Fig. 2, is a more suitable photosensitizer than HpD. In fact, after PC sensitization, a significant photodynamic cell killing effect was obtained with red light (>640 nm and >660 nm) irradiation *in vitro*.

Using an *in vivo* tumor model, both photosensitizers gave a dose-dependent photodynamic tumor regression after GVL irradiation. GVL with a fixed wavelength of 627.8 nm can emit pulsed light with very high peak power of short duration. This laser light has a longer excitation length, which results in deeper tissue penetration, than the argon dye laser emitting a continuous beam.[19] In addition, GVL can deliver a therapeutic hyperthermic effect in an experimental tumor model.[19] The tumor regression effect on the group treated with GVL alone in this study can be explained as a consequence of laser-induced hyperthermia. There was no significant difference in relative tumor volume between PC- and HpD-treated groups at corresponding drug doses. However, the most beneficial effect appeared in the CR rate of one

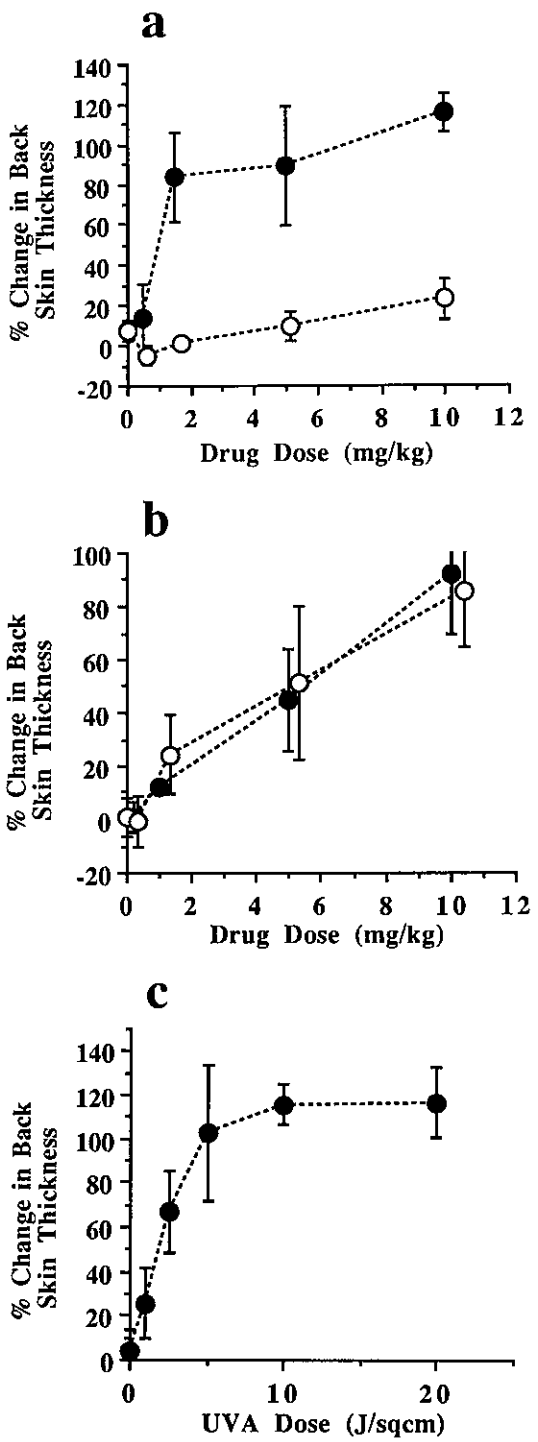


Fig. 8. Changes in back skin thickness on C3H/He mice 24 h after light irradiation following drug sensitization. The mice were injected with various doses of PC (○) or HpD (●) and exposed to 10 J/cm$^2$ of UVA delivered at 5 mW/cm$^2$ (a), or to visible light for 10 min (b). The mice were injected with HpD (10 mg/kg body weight) and exposed to various doses (0–20 J/sm$^2$) of UVA delivered at 5 mW/cm$^2$ (c).

PC-injected group (10.0 mg/kg body weight): 3 of 6 treated animals in this group had no evidence of tumor 60 days after PDT. As Chan *et al.* reported that the destructive ability of PC-PDT within a solid tumor mass confers a selective survival advantage on tumor-bearing hosts,[14] the present *in vivo* result was also encouraging. A further study needs to be made to determine the optimal light dose and drug dose for achieving a higher rate of complete remission.

From the clinical point of view, skin hypersensitivity caused by retention of photosensitizers in the skin for several weeks is a major disadvantage of PDT. In 1913 a famous experiment on himself by Meyer-Bets revealed that injected hematoporphyrin and light exposure could induce an acute skin reaction involving edema, erythema and skin necrosis.[28] Recently it has been shown that the peak of the action spectrum of this reaction is in the UVA region, which corresponds to the absorption peak of porphyrins.[12] Apart from studies to find new photosensitizers, several authors have attempted to avoid this photosensitivity by intratumoral injection of HpD,[29] skin protection using $\beta$-carotene,[27,30] reducing the drug dose[31] and photobleaching.[10] Besides the present *in vitro* data suggesting that, even though PC as well as HpD has strong absorption in the UVA region, PC provoked less photodynamic effect with UVA irradiation than HpD, the photodynamic skin reaction induced by PC and UVA proved to be weak compared with that by HpD on mice back skin models. This result represents an advantage of PC.

On the other hand, animals receiving visible light irradiation after sensitization with PC or HpD showed almost the same degree of skin edema in the present study. Tralau *et al.*, however, reported that dihematoporphyrin ether, an active component of HpD, caused greater skin photosensitivity than PC to simulated solar radiation in mice.[11] This discrepancy can be explained by the following considerations. (1) The spectrum of simulated solar rediation used in Tralau's experiment is similar to that of sunlight between 300 to 600 nm, with peaks at 450 to 500 nm. At 600 to 800 nm, the spectral power falls more repidly than that of sunlight. But the visible light source used in the present study showed peaks at 615$\pm$10 nm, shifting to a longer-wavelength region. (2) Dihematoporphyrin ether may cause severe phototoxicity compared with HpD.

In conclusion, PC may be a useful photosensitizer in PDT of cancers based on the present *in vitro* and *in vivo* results, i.e., the superiority in cell killing effect with red light irradiation and reduced toxicity to cells in the dark, the photodynamic tumor-eradicating ability with GVL (627.8 nm) irradiation (comparable to that of HpD), and the mild skin reaction to UVA exposure.

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