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Permalink

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Journal

Journal of the National Cancer Institute, 82(10)

ISSN

0027-8874

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Publication Date

1990-05-16

DOI

10.1093/jnci/82.10.868

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animals also received light treatment (630 nm for TPPS$_4$ and/or 658 nm for Photofrin II). The results show that the approach using both drugs and the corresponding therapeutic wavelengths enhanced the effectiveness of PDT. This approach achieved a cure rate of up to 100%, which was, depending on the light intensity used, as much as 40% greater than the rate achieved by the approach using one drug and one wavelength. The results also show that lesser amounts of drug and/or light may be required if both drugs and wavelengths are used, thus lowering the chances of side effects common to PDT. Furthermore, the results indicate that the increased tumor kill is due to a synergistic effect of the two photosensitizers that was tested on the tumor microvasculature in the first few hours after PDT. [J Natl Cancer Inst 82:868–873, 1990]

Use of Multiple Photosensitizers and Wavelengths During Photodynamic Therapy: A New Approach To Enhance Tumor Eradication

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Several studies have examined the synergism of hyperthermia or chemotherapy agents in combination with photodynamic therapy (PDT) to enhance tumor eradication. In our unique approach to treatment, multiple photosensitizers and wavelengths were used: two photosensitizers, Photofrin II and meso-tetra-(4-sulfonatophenyl)-porphine (TPPS$_4$), irradiated at the appropriate therapeutic wavelength for each photosensitizer. EMT-6 mammary tumors were induced in the flanks of BALB/c mice. The mice were assigned to a control group (50 mice) or treatment group (150 mice). All treatment animals and some control animals received photosensitizing drug (5 mg/kg of TPPS$_4$, 5 mg/kg of Photofrin II, or 2.5 mg/kg of both TPPS$_4$ and Photofrin II). All treatment animals and some control

Photodynamic therapy (PDT), which uses a photosensitizing drug specifically activated by a certain wavelength of light to cause photoreaction in biological systems, dates back to the beginning of this century (*1*). Since that time, numerous examples of the "photodynamic effect" have been reported for a wide range of photosensitizers, both in vitro and in vivo (*2*). During the photodynamic reaction, the photosensitizer is excited by a specific wavelength of light. The excited photosensitizer subsequently transfers its energy to a molecular substrate, such as oxygen, to produce highly reactive singlet oxygen that causes irreversible oxidation of some essential cellular component (*3*). The exact structures targeted by the excited intermediates responsible for cell death have

not been identified, although damage to the cell membrane, mitochondria, lysosomes, microsomes, and nuclear material have all been reported. -- -- -- -- ----- - -

During the past several years, interest in the use of PDT for malignant tumor therapy has increased. Porphyrins have received more attention since the observation by Policard in 1924 (4) that certain malignant tumors in animals and humans demonstrated a reddish fluorescence on light exposure. This fluorescence has been attributed to the accumulation of endogenous porphyrins resulting from secondary infection by hemolytic bacteria. Several porphyrins are known to localize in malignant tumors. In 1975, Dougherty et al. (5) showed that systemic hematoporphyrin derivative (HpD), activated by light from a xenon-arc lamp, could cause complete eradication of a transplanted mouse mammary tumor without appreciable damage to the overlying skin. Concentration of the "active ingredient" in HpD led to the introduction of commercially available Photofrin II (also called dihematoporphyrin ether/ester) into clinical trials in 1983. This drug, which is currently approved for clinical use, consists of an 80%–90% mixture of the ether and ester linkages of diporphyrins.

It is now estimated that, worldwide, more than 3,000 patients with malignant tumors have received PDT, including patients with cancers of the skin, female genital tract, esophagus, bladder, eye, breast, or lung. At recent symposia, the overall response rate reported for large patient series is greater than 70% (6–8).

Although Photofrin II is the only photosensitizing agent approved for clinical use, it is far from ideal. The selectivity of Photofrin II for malignant tumors does not apply to all organs. The long half-life of Photofrin II retained in the skin is clinically significant. Such retention can lead to phototoxic reactions arising from exposure to sunlight or even bright, artificial light. This side effect is not trivial and may result in complications ranging from slight erythema to extensive skin sloughing and necrosis. Other clinical disadvantages include (a) a relatively weak Photofrin II absorption band resulting in inefficient phototoxicity, and (b) a low tissue transparency at 630 nm. Because of all of these drawbacks, considerable effort is being devoted to developing new and more efficient tumor localizing photosensitizers

with greater extinction coefficients at longer wavelengths.

New compounds have recently been reported as photosensitizers for selective tumor necrosis in animal models. These compounds include meso-tetra-(4-sulfonatophenyl)-porphine (TPPS₄) (9), mono-L-aspartyl chlorin e₆ (MACE) (10), chloroaluminum sulfonated phthalocyanine (CASPc) (11), and purpurins (12). While studies on all these new compounds appear promising, they are still at an early stage. It is hoped that future investigations will define the role of these and other potential photosensitizing compounds in the management of cancer.

In this report, we suggest that the most successful application of PDT may be the use of two photosensitizers and two wavelengths. This approach certainly has a successful parallel in the treatment of leukemias and lymphomas that uses combination chemotherapy. Our method may require smaller amounts of drug and/or decreased amounts of light for optimal response, thus making PDT a much more sophisticated approach, with minimal side effects, for the treatment of selected cancers. The objectives in this study were (a) to compare the photosensitizers Photofrin II or TPPS₄ alone against a combined regimen of these two compounds irradiated at the wavelength specific for each drug, and (b) to determine the mechanism of tumor destruction resulting from laser light therapy with either Photofrin II or TPPS₄ alone or both Photofrin II and TPPS₄.

Materials and Methods

Animal and Tumor System

EMT-6 mammary tumors were induced in the flanks of BALB/c mice. All mice were 6–8 weeks old and weighed 30–35 g. When the tumors reached a diameter of 1–2 cm, they were excised and minced with fine scissors in phosphate-buffered saline. The resulting suspension of tumor cells was filtered through sterile gauze, washed twice in phosphate-buffered saline, and resuspended in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) at a concentration of 5 × 10⁵ viable cells/mL. Cell viability was assessed by the ability to resist cell lysis and to exclude trypan blue dye (GIBCO Laboratories).

Tumors were initiated by injecting 0.1

mL of fresh tumor inoculum into the right flanks of mice. The tumors were generally palpable at 5 days and reached a size of 5–7 mm by 10–14 days, at which time treatment was started. At this size, the small tumors were homogeneously white and spontaneous tumor necrosis was minimal or absent.

Photosensitizers

Photofrin II was obtained from Photomedica, Inc. (Raritan, NJ), as an aqueous solution at a concentration of 2.5 mg/mL and stored in the dark at −70 °C until used. For in vivo experiments, Photofrin II was diluted 1:4 with 0.9% NaCl solution and injected intraperitoneally.

TPPS₄ was obtained from Porphyrin Products, Inc. (Logan, UT), as a dark-red powder, reconstituted in Dulbecco's phosphate-buffered solution to a final concentration of 2.5 mg/mL, and stored in the dark at −70 °C until used. Prior to injection, TPPS₄ was diluted 1:4 with 0.9% NaCl solution and injected intraperitoneally.

Absorption spectra of Photofrin II and TPPS₄ were obtained prior to in vivo experiments with a spectrophotometer (model DV-7, Beckman, Fullerton, CA).

Laser Light Delivery System

Laser irradiation was performed with an argon-pumped dye laser (model 770 DL, Cooper Lasersonics, Santa Clara, CA). The dye used was DCM premixed laser dye (Cooper Lasersonics) with a tuning range of 610–690 nm. The dye laser was tuned to emit radiation at 630 nm for Photofrin II and 658 nm for TPPS₄. A Clinical Hartridge Reversion spectroscope (Ealing Electro-Optics, South Natick, MA) was used to verify these wavelengths to ±1 nm.

The radiation was then coupled into a 400-μm fused, silica fiberoptic with a fiberoptic coupler (model 316, Spectra-Physics, Mountain View, CA). The output end of the fiber terminated with a microlens that focused the laser radiation into a circular field of uniform light intensity. Laser irradiation emanating from the fiber was monitored with a power meter (model 210, Coherent, Palo Alto, CA) before, during, and after treatment.

Experimental Procedure

Mice bearing EMT-6 tumors were divided into control and treatment groups.

When tumors reached 5–7 mm, the tumor area was shaved, and if the animal was in an appropriate control or treatment group, intraperitoneal injection of photosensitizer was administered. The remainder of the experiment, including housing of the animals, was performed in the dark.

Control mice were divided into four groups: 20 mice received light (10 received 80 J/cm² of 630 nm and 10 received 80 J/cm² of 658 nm) but no photosensitizer; 10 received Photofrin II (5 mg/kg) but no light; 10 received TPPS₄ (5 mg/kg) but no light; and 10 received both Photofrin II and TPPS₄ (2.5 mg/kg each) but no light. Treated animals were divided into five groups (numbered 1–5) of 30 mice each. In each group, 10 mice received Photofrin II (5 mg/kg), 10 received TPPS₄ (5 mg/kg), and 10 received both Photofrin II and TPPS₄ (2.5 mg/kg each) (table 1). All animals in the treated groups received the light treatment appropriate for the photosensitizer(s) administered.

Twenty-four hours after the injection of photosensitizer(s), both control and treated animals receiving light were exposed to the laser light delivery system described above. The animals were restrained without anesthesia and positioned underneath an aperture that restricted the area of light illumination to 1 cm² on the tumor site. Each of the five groups of treated animals received a different total laser light dose. The doses varied from 10 to 80 J/cm² of 630 nm, 658 nm, or both wavelengths, as appropriate (table 1), with a power density of 100 mW/cm². After PDT, animals were returned to the dark

and examined daily for a period of 4 weeks to determine the percentage of cured animals.

An additional 45 animals were divided into three groups of 15 animals each. The first group received Photofrin II (5 mg/kg), the second received TPPS₄ (5 mg/kg), and the third received both Photofrin II and TPPS₄ (2.5 mg/kg each). All 45 animals were exposed to a total light dose of 80 J/cm². Biopsy specimens were taken from tumors in animals that were killed immediately, 30 minutes, 1 hour, 2 hours, and 4 hours after laser light treatment. These drug and light doses were predetermined to ensure complete tumor kill in all animals in an attempt to document the mechanism of tumor destruction (i.e., direct cytotoxic effect on tumor cells vs. vascular disruption with subsequent tumor cell death). Biopsy tissue was excised immediately and fixed in 3% glutaraldehyde–5% formaldehyde in phosphate buffer (pH 7.4). Samples were then dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Six-micrometer sections were cut, stained in hematoxylin and eosin, cleared of paraffin in xylene, and dried. Sections were examined with an Olympus microscope and photographed with Panatomic-X film (Eastman Kodak Co., Rochester, NY).

Results

The effects of Photofrin II or TPPS₄ alone or in combination on irradiated tumors in mice are summarized in table 1. Cure is defined as no palpable mass at least

4 weeks after treatment; partial response is defined as any necrosis in the 4 weeks following treatment. For animals in group 1, the cure rate was 100%. The entire tumor mass disintegrated into a nonpalpable scab within a few days after PDT, and eventually, the skin completely healed, with varying degrees of hair regrowth.

In group 2, the cure rate was 100% only in those animals that received the combined regimen. Animals that received either Photofrin II or TPPS₄ alone had cure rates of 50% and 60%, respectively. However, regrowth of the noncured tumors, which showed partial necrosis, generally was apparent within 7 days and usually occurred around the periphery of the original tumor. These tumors subsequently grew as rapidly as tumors in the nontreated controls, and many animals died of tumor bulk.

In group 3, 70% of the animals that received both photosensitizers were cured. Animals that received Photofrin II or TPPS₄ alone had cure rates of only 20% or 30%, respectively. In group 4, 30% of the animals that received both photosensitizers were cured, but none of the animals that received either photosensitizer alone was cured. In group 5, partial necrosis was apparent in the tumors of 20% of the animals receiving both photosensitizers, but no cures were observed. Animals that received either Photofrin II or TPPS₄ alone showed no response. None of the control animals showed response, regardless of the photosensitizer or light parameters used.

Through the use of the additional group

Table 1. Combined photosensitizer dose-response in treated groups of mice

Group	Dose of light (J/cm²)		Dose of drug (mg/kg)		Cure	Partial response	No response	% Cured
	630 nm	658 nm	Photofrin II	TPPS₄				
1	80		5.0		10			100
		80		5.0	10			100
	40	40	2.5	2.5	10			100
2	60		5.0		5	5		50
		60		5.0	6	4		60
	30	30	2.5	2.5	10			100
3	40		5.0		2	5	3	20
		40		5.0	3	7		30
	20	20·	2.5	2.5	7	3		70
4	20		5.0			3	7	0
		20		5.0		5	5	0
	10	10	2.5	2.5	3	7		30
5	10		5.0				10	0
		10		5.0			10	0
	5	5	2.5	2.5		2	8	0

of 45 mice, this study also attempted to determine the mechanism of tumor destruction resulting from laser light therapy with either Photofrin II or TPPS$_4$ alone or both Photofrin II and TPPS$_4$. Control (either drug or light alone) slides showed the usual tumor architecture with easily discernible patent vessels (fig. 1). No significant structural changes were noted in biopsy samples taken immediately or 30 minutes after PDT.

In animals treated with either Photofrin II (fig. 2). or TPPS$_4$ (fig. 3), the first structural change, noted 1 hour after PDT, was occlusion of the tumor capillary lumen with tightly packed erythrocytes. This observation was based on histopathologic examination of all sections and tumors in treated and control mice and was not attributable to the way a particular section was cut.

At 2 hours after PDT in these animals, the capillaries were further engorged, and over time, the capillary wall broke down with extravasation of erythrocytes into the surrounding perivascular tumor stroma. Additionally, tumor cells closer to the hemorrhage showed more signs of cell membrane damage and lysis. However, the cell membranes of tumor cells distant from the microvasculature in the center of the tumor appeared to be structurally intact even 4 hours after PDT. The tumor ultimately became a mass of erythrocytes and amorphous granular debris.

For animals treated with both Photofrin II and TPPS$_4$, the specimens obtained immediately after PDT demonstrated widespread vascular occlusion with hemorrhage throughout the tumor (fig. 4). This finding was consistent with observations 2–4 hours after PDT in tumors treated with either Photofrin II or TPPS$_4$ alone. Furthermore, it was noted that, in tumors treated with both photosensitizers, cells closest to the hemorrhage showed more signs of cell membrane damage and lysis than those treated with either Photofrin II or TPPS$_4$ alone.
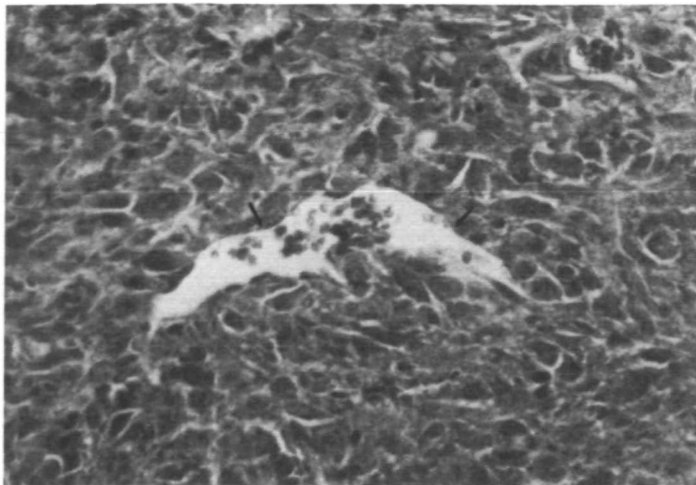

Figure 1. Photomicrograph of control (no photosensitizer; no light) EMT-6 tumor. *Note* normal patent tumor capillaries at the tips of the arrows ($\times$ 600).
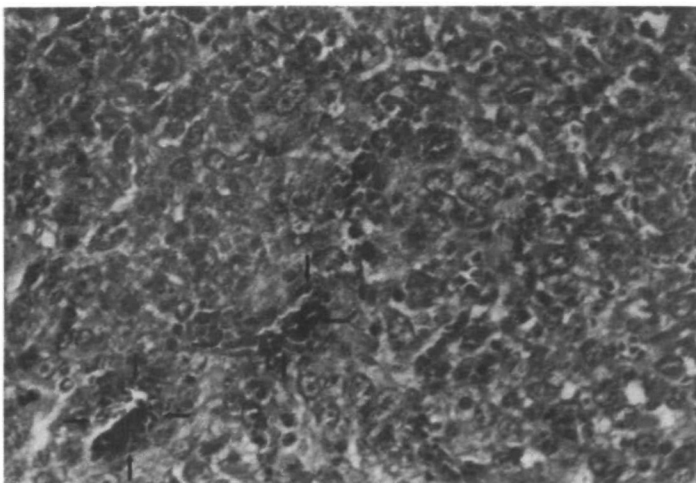

Figure 2. Photomicrograph of EMT-6 tumor treated with Photofrin II and light. Specimen was removed 1 hr after phototherapy. *Note* occluded tumor capillaries with tightly packed erythrocytes at the tips of the arrows ($\times$ 600).

Figure 3. Photomicrograph of EMT-6 tumor treated with TPPS$_4$ and light. Specimen was removed 1 hr after phototherapy. *Note* occluded tumor capillaries with tightly packed erythrocytes at the tips of the arrows. ($\times$ 600).
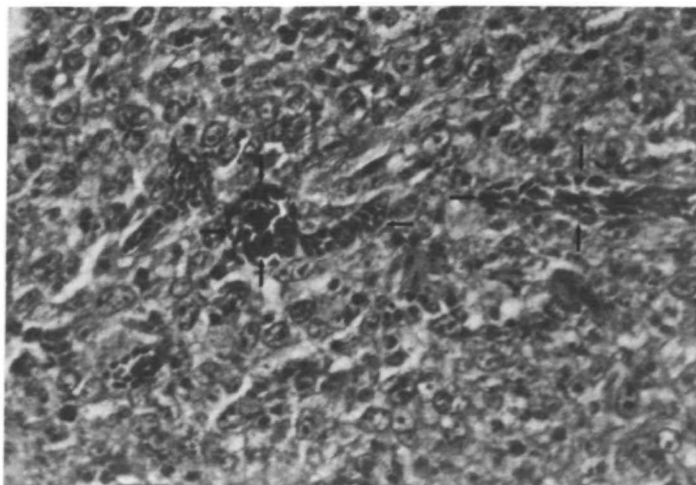
Discussion

With the exception of acute leukemia, malignant tumors in advanced stages were treated with single-agent chemotherapy until 1970. The control of neoplasms by this method was based mainly on the judicious rotation of available drugs. Once the therapeutic potential of conventional drugs had been exhausted, the tumor was treated with experimental drugs. When single agent chemotherapy is used, complete remission occurs in no more than 30% of the most responsive neoplasms. In solid tumors, complete remission does not exceed 10%–15% even when the most effective drugs are used and prognostic factors are the most favorable (*13*).

The biologic and pharmacologic basis of combination chemotherapy has been widely studied in laboratory animals (*14*). From a clinical point of view, the combination of several antineoplastic drugs is aimed at:

Figure 4. Photomicrograph of EMT-6 tumor treated with both Photofrin II and TPPS₄ and light. Specimen was removed immediately after phototherapy. *Note* widespread vascular occlusion with hemorrhage into the surrounding perivascular stroma at the tips of the arrows (× 600).
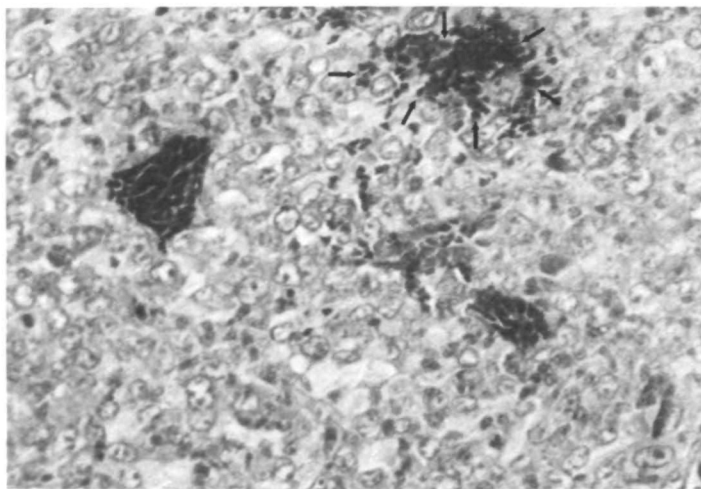
(a) increasing therapeutic synergism by exploiting the different mechanisms of action with subsequent improvement of therapeutic activity;

(b) preventing or delaying the emergence of resistant cell clones through the mechanism of action of the drugs used;

(c) increasing patient tolerance to the toxic effects of the drugs used by properly varying their dosage; and

(d) making use of differing pharmacologic characteristics of the various compounds to achieve rapid, complete regression without simultaneously producing a high degree of toxicity, so that long-term remission or cure can be achieved (15–20).

A number of investigators have examined the synergism of hyperthermia (21,22) and chemotherapeutic agents both in vitro and in vivo (23,24) in combination with PDT to enhance tumor eradication. However, no studies have been conducted using two photosensitizers and two wavelengths for treatment. The objectives of this study were (a) to compare the photosensitizers Photofrin II or TPPS₄ alone against a combined regimen of these two compounds irradiated at the wavelength specific for each drug, and (b) to determine the mechanism of tumor destruction resulting from laser light therapy with either Photofrin II or TPPS₄ alone or both Photofrin II and TPPS₄.

Our study suggests that the combination of the photosensitizers Photofrin II and TPPS₄ can enhance the effectiveness of PDT. The results show that this combined approach produces a larger percentage of cured animals than a method that uses only a single photosensitizer and wavelength. Treatment with a single photosensitizer and wavelength often produces only a partial remission, which is usually of short duration. If, on the other hand, an effective combination of multiple photosensitizers and wavelengths is administered in a responsive tumor, especially one that is not large, an increased reduction in the number of neoplastic cells may be obtained, leading to cure.

An equally significant implication from our study is that lesser amounts of drug and/or light may be required during PDT if the combined approach is used. This finding is of considerable clinical importance because the only known drawback to the use of PDT is the potential for damage to normal tissue resulting from the drug-induced effect of ultrasensitivity to sunlight. Patients receiving PDT are warned to avoid exposure to sunlight for at least 4–6 weeks. Light exposure during that time may result in symptoms ranging from mild erythema to extensive skin sloughing and necrosis. Presently, the doses of drug used clinically are high enough to have these deleterious effects. If lesser amounts of photosensitizer and light are required, PDT would become a much more sophisticated approach. Higher therapeutic efficacy and minimal toxic effects in the treatment of selected malignant tumors would result.

Our interest in the combination of multiple photosensitizers and wavelengths in PDT, using Photofrin II and TPPS₄, was based on recent studies concerning the mechanism of tumor death resulting from the use of these photosensitizers. Our group has demonstrated that the effects of PDT with Photofrin II leading to rapid necrosis of tumor tissue are not the result of direct tumor cell kill, but are secondary to destruction of the tumor microvasculature (25). Another study has suggested that TPPS₄ induces a preferential necrosis of the neoplastic cells (26). It was hoped that by exploiting the different mechanisms of action of these two photosensitizers, we might produce a beneficial effect with a consequent improvement in the percentage of animals cured by PDT.

The results of this study demonstrate that using two photosensitizers, irradiated at the appropriate wavelength for each drug, may enhance the therapeutic efficacy of PDT by inducing a synergistic effect. However, the study also suggests that the possible synergistic effects of Photofrin II and TPPS₄ during PDT are not the result of differing mechanisms of action of the two photosensitizers on different tumor loci. Instead, they are secondary to destruction of the tumor microvasculature by both compounds.

Binding of both photosensitizers, with subsequent destruction of important structural elements in the tumor capillary wall, appears to be the key feature of the dye-sensitized photodynamic reaction with Photofrin II and TPPS₄. This observation contrasts sharply with that reported by Milanesi et al. (26) on the mechanism of tumoricidal activity resulting from the use of TPPS₄. However, since both the power density (300 mW/cm²) and the total light dose (300 J/cm²) used in that study were very high, it is possible that a nonspecific effect was produced due to hyperthermia.

Another explanation of the apparent contradiction is that our histologic examinations were performed at earlier times after treatment (immediately, 30 min, 1 hr, 2 hr, and 4 hr) than those of Milanesi et al. (15 hr) (26). This is an important consideration since it is well known that the first observable signs of tumor destruction occur in the first few hours after PDT. As a result, this study was considered more likely to detect the important ultrastructural changes produced by PDT. At present, the authors are extending their ultrastructural studies with TPPS₄ to irradiated tumor tissues examined with the electron microscope.

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