In Vitro and *In Vivo* Photosensitizing Applications of Photofrin® in Malignant Melanoma Cells

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Background: The object of the present study was to evaluate the feasibility of photodynamic therapy (PDT) for malignant melanomas through *in vivo* and *in vitro* processes.

Methods: Photofrin® (porfimer sodium) was evaluated through *in vitro* processes with human malignant melanoma cells (MMCs). The *in vitro* absorption and photosensitizing activity of Photofrin® was examined in an MMC culture system. The *in vivo* biological activity of Photofrin® applied to subcutaneous implanted melanoma (SIM) in nude mice and exposed to different total light dosages at 630 nm was studied by determining the destruction of the tumors. Subcelluar localization and binding were observed under a fluorescent confocal microscope.

Results: MMCs incubated with Photofrin® at a concentration of about 3.5 μg/ml and exposed to laser light at 630 nm with a power density of 100 mW/cm^2, showed 50% cell killing. An electron microscopic study demonstrated significant destruction of the target after PDT.

Conclusion: Detection of the photosensitizer Photofrin® was localized and its distribution fully observed. PDT-Photofrin® has the capability to destroy MMCs through *in vitro* and *in vivo* SIM treatment.

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Key words: photodynamic therapy (PDT), malignant melanoma, photosensitizer

Photodynamic therapy (PDT), the use of a photosensitizing drug specifically activated by a specific wavelength of light to cause photoreactions in biological systems, dates back to the beginning of the last century.[1] The basic concept of PDT is that certain molecules can function as photosensitizers. The presence of these photosensitizers in biological tissue makes the tissue vulnerable to light at wavelengths absorbed by the chromophore. Basic scientific studies have attempted to achieve an understanding of the mechanisms concerning tumor destruction after PDT, yet have provided limited results.[2,3] The rationale for our use of PDT is based on the fact that PDT will specifically destroy the malignant tumor without the production of heat. The resulting cell death may be a consequence of direct cell injury resulting from retention of the photosensitizer within the tumor cells, or alternatively, from intravascular activation of circulatory photosensitizers with subsequent indirect damage to the vessels.[4] Although it is

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clear that disturbances in microcirculation play a crucial role in PDT-induced tumor destruction, the exact nature of the tumor cell damage is not well known. Our study is designed to evaluate the photosensitizing capabilities of Photofrin®, a photosensitizer approved by the United States Food and Drug Administration (FDA), applied through *in vitro* and *in vivo* experiments with human malignant melanoma cells (MMCs) and subcutaneous implanted melanoma (SIM) respectively.

METHODS

Photosensitizers

Photofrin® - obtained from Quadra Logic Technologics, Inc. (Vancouver, BC, Canada) - was stored in the dark at 4°C. Photofrin® powder was prepared with 5% dextrose to a final concentration of 2.5 mg/ml. An Olympus BH_2 microscope (MD, U.S.A.), equipped with an epi-illumination system suitable for fluorescence exposure and retrofitted with a K_2BIO scanning confocal attachment (TechnicalInstrument Co., Burlingame, CA, U.S.A.) was also used to directly view the melanoma cells. Illumination was provided by a 100W-mercury DC lamp along with an exciter filter (490 nm), an Olympus dichroic mirror (DM-500 and O-515), and a barrier filter (520 nm).

The microscope was coupled to a low-light Dage MTI series 66 SIT video camera and a Sony PVM-1343 MD high-resolution television monitor (Tokyo, Japan). Subcellular localization of Photofrin® was determined three hours after incubation. The optimal wavelength, 630 nm, was selected for laser illumination for the photosensitizer because of the improved tissue penetration of light.[5]

Laser light delivery system

Laser illumination was performed with a Coherent (Palo Alto, CA, U.S.A.) Innova 20 argon-ion laser pumping a Coherent 599-01 dye-laser and was tuned to emit radiation at 630 nm for each experiment. The wavelengths were verified with a Jobin Yvon 5/354 UV monochromator (Lonjuneau, France). Illumination was transmitted from a 400 um-fused silica fiberoptic using a Spectra-Physics (Mountain View, CA, U.S.A.) Model 316 fiberoptic coupler. A microlens was attached to the end of the fiber, which served as a means to directly focus the laser into a circular field of uniform light intensity. Laser illumination emission from the fiber was monitored with a Coherent Model 210 power meter before and after treatment.

Culture of human malignant melanoma cells (MMCs)

Skin tissue arrays of malignant melanomas were purchased readily harvested by US Biomax, Inc., (Rockville, MD, U.S.A.). MMCs were then treated with 2% dispase and directly released by brief incubation in 7.5% CO_2 for three hours at 37°C. To remove excess dispase, the tissue was washed with phosphate buffered saline (PBS). Under a microscope, the melanomas were transferred to a fresh medium where liberated MMCs were pelleted by centrifugation (1,000 rpm) for five minutes, resuspended in a proliferation medium (Cell Applications, Inc., San Diego, CA, U.S.A.), and plated on fibronectin-coated T25 flasks. One week before photosensitizer incubation, the MMCs were subcultured using a 1 ml composition of 0.3% trypsin and 1% EDTA (Cell Applications, Inc.) in each T25 flask in 7.5% CO_2 for five minutes at 37°C. The MMCs were then neutralized with the same amount of trypsin neutralizing solution (Cell Applications, Inc.) and plated on a 24-well plate coated with the attached factor solution (Cell Applications, Inc.) at a density of 2.5-3.5 x 10^4 cells/ml. On the day of photosensitizer incubation, the MMCs were incubated with Photofrin® at concentrations of 0.1, 2.5, or 5 μg/ml for three hours. The MMCs were then washed with fresh PBS for five minutes and exposed to the laser (λ = 630 nm). These cells received 10 J/cm^2 at a power density of 100 mW/cm^2.

Live/dead assay

Twenty-four hours after laser illumination, a viability/cytotoxicity kit (Molecular Probes, Inc., CA, U.S.A.) and a two-color fluorescence assay were used to determine the percentage of cell kill by counting the number of dead cells per 200 cells within the 1.5 cm diameter illumination ring under the fluorescence microscope. The differential permeability of live and dead cells to a pair of fluorescence stains allows live and dead cells to appear as fluorescent green and red, respectively. Fluorescence was excited with 470 nm of blue light and was detected through an OG 570 high pass colored glass filter

(Zeiss, Thornwood, NY, U.S.A.). Each slide was exposed to the excitation light for a period of time so that the emission from dead cells (620 nm) and live cells (520 nm) could be detected for cell calculation. For each experiment, internal control data were obtained by counting the number of dead cells that had been incubated with Photofrin® but not exposed to laser illumination. The percentage of cell kill was expressed as mean ± SD.

Lactate dehydrogenase - released (LDH) assay

The CytoTox 96™ Assay (Promega Co., Madison, WI, U.S.A.) was used to assess the percentage of LDH versus MMC cell-lysis after laser illumination with different concentrations of Photofrin®. Released LDH in culture supernatants was measured by the conversion of a tetrazolium salt into a red formazan product. The color intensity was proportional to the number of lysed cells. Visible absorbance was read at 490 nm on a THERMOmax microplate reader (Molecular Devices, Menlo Park, CA, U.S.A.). After 24 hours, a collective review of the cell kill PDT was administered. The amount of cell kill was compared between groups (0 h) and (24 h) after PDT using a paired T-test. The same calculation was carried out using the OD for LDH comparison.

Subcutaneous implanted melanoma (SIM) animal model

Twenty-four pathogen-free female nude mice, weighing 100 ± 5g, were used. The animal subjects were given a commercial diet and water. While under halothane anesthesia, the subjects were injected in the flank with 0.6 to 2×10^6 ml of melanoma cells suspended in 0.3 ml of RPMI media (Gibco Life Technologies Grand Island, NY, U.S.A.) containing 10% fetal calf serum (Gemini Bioproducts, Galabasas, CA, U.S.A.), 25 IE/ml penicillin, and 25 mg/ml streptomycin. Experiments were initiated when tumors had a maximum length and width between 14 and 22 mm (between 18 and 27 days after tumor injection). Exterior tumor growth was shown in 20 of the 24 subjects (Fig. 1). Sixteen out of 20 tumor-bearing mice were given intravenous injections of Photofrin® (2.5 mg/kg body weight). The remaining four mice received laser light without a photosensitizer and were used as controls. Among the 16 mice, 12 mice received a photosensitizer with

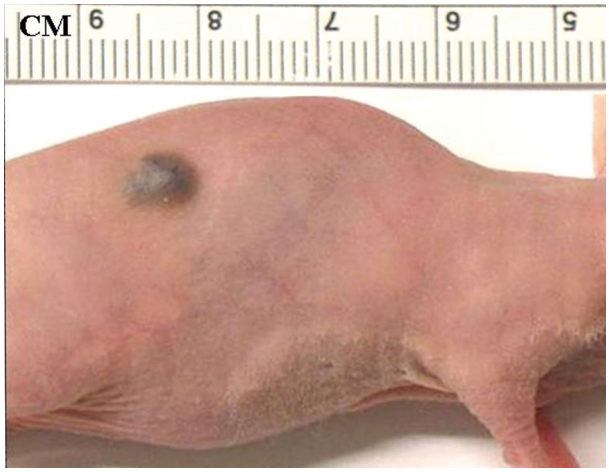

Fig. 1 Eighteen weeks after tumor implantation, tumor gets growth on the flank of a nude mouse.

laser illumination. The remaining four of that group received a photosensitizer without laser application, and each implanted melanoma tumor was excised and analyzed thereafter. The excised area was bisected and one half was utilized immediately for fluorescence measurements. It was then placed, but not immersed, in a petri dish with saline at physiologic temperatures and kept warm. Measurement of tissue fluorescence was performed using a krypton ion laser (Coherent, Irvine, CA, U.S.A.) (407 nm). The tissue was irradiated at 100 m/sec. During excitation, induced fluorescence was determined using a cooled CCD camera with a tunable filter (Princeton Instruments Model ST-130, Trenton, NJ, U.S.A.). Images were recorded and transferred to a Macintosh Quadra-based image analysis and processing workstation (Cupertino, CA, U.S.A.) with specialized software. Fluorescence from both the tumor and adjacent healthy tissue was analyzed in this manner to investigate for preferential PpIX biosynthesis in the tumor tissue. Cryosections of the other halves of each tumor sample were utilized, permitting measurement of the depth of fluorescence, and thus, Photofrin® penetration within the tissue.

Three hours after photosensitizer injection, the mice were exposed to the laser ($\lambda = 630$ nm). The total laser energy density was 100 J/cm^2 with a power of 100 mW. The experimental group was divided and euthanized at varying times: immediately, 0.5, 1, and 24 hours after PDT. The tumors were

then processed for histological evaluation. In addition, tumors were examined for ultrastructural evidence of damage after laser illumination which was documented by a transmission electron microscope.

RESULTS

MMC culture

Under a fluorescence confocal microscope, subcellular localization of Photofrin® in concentrations of > 3.5 μg/ml in MMCs showed positive uptake throughout the cytoplasm in a diffused pattern three hours after application of the photosensitizer (Fig. 2). MMCs treated with light (630 nm) plus Photofrin® at different concentrations showed percentages of cell kill immediately after PDT as follows: 1.03 ± 0.17 (0 μg/ml), 7.51 ± 0.94 (1 μg/ml), 11.24 ± 1.67 (2.5 μg/ml), 14.97 ± 1.55 (5 μg/ml), and 28.42 ± 5.32 (10 μg/ml). MMCs at different concentrations showed percentages of cell kill 24 hours after PDT as follows: 14.28 ± 1.72 (0 μg/ml), 18.34 ± 6.71 (1 μg/ml), 35.41 ± 6.73 (2.5 μg/ml), 79.15 ± 8.45 (5 μg/ml), and 98.81 ± 10.29 (10 μg/ml). The results showed a 50% cell kill with the approximate dosages of 3.5 μg/ml. All controls showed negligible cell kill. The CytoTox 96™ assay for LDH release from MMCs showed the following optical densities immediately after PDT: 0.21 ± 0.07 (0 μg/ml), 0.36 ± 0.05 (1 μg/ml), 0.42 ± 0.04 (2.5 μg/ml), 0.73 ± 0.08 (5 μg/ml), and 0.84 ± 0.06 (10 μg/ml). The CytoTox 96™ assay results for MMCs 24 hours after PDT were as follows: 1.10 ± 0.31 (0 μg/ml), 1.17 ± 0.45 (1 μg/ml), 1.29 ± 0.52 (2.5 μg/ml), 1.53 ± 0.73 (5 μg/ml), and 1.68 ± 0.69 (10 μg/ml). A 50% optical density obtained for each concentration of the photosensitizer corresponded to 50% cell kill after laser illumination (Table 1). The resulting cell kill viewed immediately after PDT and that 24 h thereafter produced a significant difference statistically (p = 0.046). The same descriptive pattern was also obtained for the LDH findings, both immediately following PDT and after 24 h (p = 0.001).

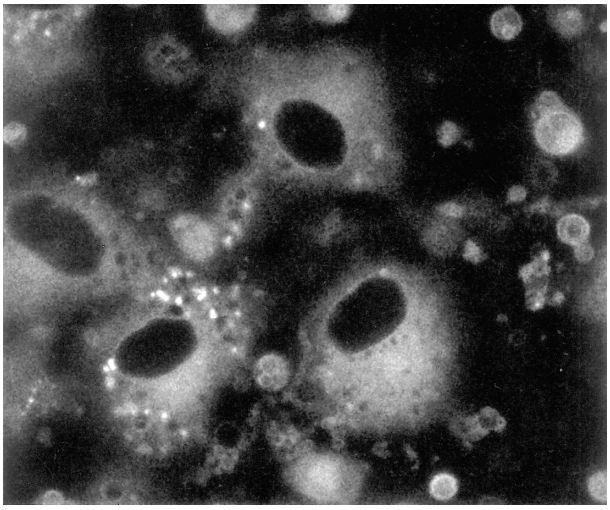

Fig. 2 Under the fluorescence confocal microscope, subcelluar localization of Photofrin® shows uptake throughout the cytoplasm in a diffused pattern.

Animal histopathology

The histopathological findings of SIM were evaluated by senior pathologists in the Department of Pathology at Chang Gung Memorial Hospital. Under electron microscopy, the treated tumors showed intracellular vacuole formation (Fig. 3). In the control animal subjects (receiving only laser application or photosensitizer without laser application) no significant histopathological findings were seen under fluorescence microscopy (Fig. 4).

DISCUSSION

A photosensitizer absorbs photons of the appropriate wavelength and is elevated to an excited state.

Table 1. The CytoTox 96™ Assay Assessing MMC Killing after PDT.

Photofrin Concentration (μg/ml)	0	1	2.5	5	10
% Cell Kill (0 h)	1.03 ± 0.17	7.51 ± 0.94	11.24 ± 1.67	14.97 ± 1.55	28.42 ± 5.32
% Cell Kill (24 h)	14.28 ± 1.72	18.34 ± 3.71	35.41 ± 6.73	79.15 ± 8.45	98.81 ± 10.29
O.D. (0 h)	0.21 ± 0.07	0.36 ± 0.05	0.42 ± 0.04	0.73 ± 0.08	0.84 ± 0.06
O.D. (24 h)	1.10 ± 0.31	1.17 ± 0.45	1.29 ± 0.52	1.53 ± 0.73	1.68 ± 0.69

Abbreviation: O.D.: optical density.

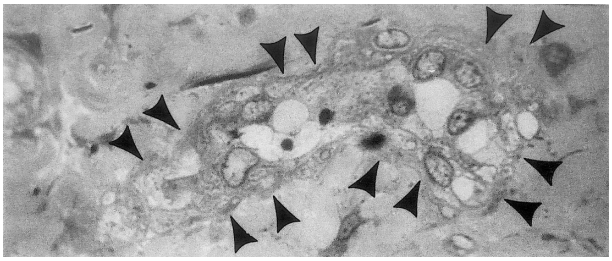

Fig. 3 Under electron microscopy, the treated tumors show intracellular vacuole formation (arrow heads).

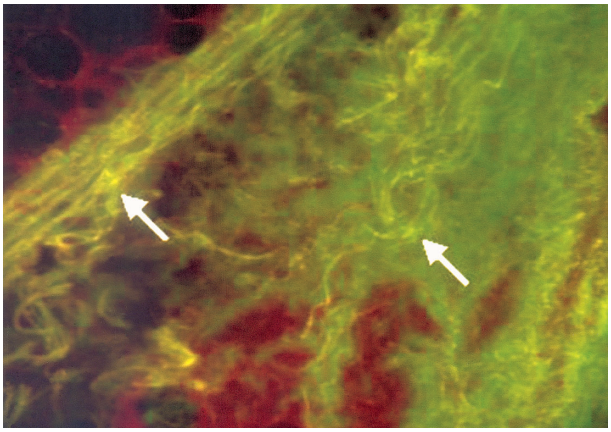

Fig. 4 In the control animal subjects (receiving only laser application or photosensitizer without laser application) no significant histopathological findings are seen under fluorescence microscopy (arrow heads).

The excited photosensitizer subsequently reacts with a substrate, such as oxygen, to produce highly reactive singlet-molecular oxygen that causes irreversible oxidative damage to biologically important molecules.[6,7] The phototoxic reaction is a local phenomenon that takes place within the same cell on a time scale of microseconds. Illumination at the appropriate wavelengths absorbed by the photosensitizer provides the energy to drive photodynamic reactions without the generation of heat provided the incident power density is kept below 100 mW/cm^2.[8]

Efforts to define the mechanism(s) of PDT action have led to controversial discussions that attribute cytotoxicity to vascular-mediated events, (i.e. indirect cell kill) or to cellular targets (i.e. direct cell kill) of photochemically produced mono oxygen (O_2) or other oxygen radicals.[9,10] It has been hypothesized that if systemic Photofrin® treatment could produce effective photosensitization, the most likely target would be microvascular and malignant cells.[11,12] Therefore, our experiments were performed to ascertain the time course and dose relationship of Photofrin® in MMCs. In our study, the results after PDT were monitored by calculating the percentage of cell kill of MMCs and the optical density of the CytoTox 96™ assay. This approach to defining the mechanisms of PDT action has enabled us to design an animal model for PDT treatment for malignant melanomas.

Biochemical experiments suggest that Photofrin® is a derivative of hematoporphyrin. This photosensitizer, after dispersal throughout the cytoplasm, is highly susceptible to functional inhibition by PDT.[13,14] Meanwhile, it has been proposed that cells containing Photofrin®, found primarily in the plasma membrane, can be killed by an apparently non-apoptotic (necrosis) as well as an apoptotic mechanism.[15-17] In our previous studies, administration of Photofrin® resulted in intracellular accumulation of protoporphyrin IX (PpIX) in microvascular endothelial cells (MEC) for PDT.[18-21] Under fluorescence microscopy, subcellular localization of Photofrin® within the cytoplasm and mitochondria was detected. Therefore, PpIX is an effective photosensitizer particularly for those reactions involved in oxidative photophorylation and ATP synthesis associated with the skin phototoxicity seen in porphyria patients.[15-21] For Photofrin®, it was predictable that lysosomal membranes could be destabilized by protoporphyrin photosensitization. In support of these hypotheses, destabilization of lysosomes and release of acidic proteinases have been shown by CytoTox 96™ assay in monitoring the percentage of lactate dehydrogenase (LDH) released from MMCs after laser illumination. Significant cell rupture assessed by the optical densities of the CytoTox 96™ assay was seen at the concentration where a 50% cell kill was obtained by viability/cytotoxicity assay. From our control group, we reached the conclusion that there will be minimal cell destruction as a result of single element photosentizer or laser application, which further supports our findings in previous experiments. This, in turn, supports our theoretical results for photodynamic therapy. The results of the *in vivo* SIM model demonstrated marked PDT-induced destruction of malignant melanomas. Based on the electron microscopy findings, we propose that PDT damage to plasma membranes, and lysosomes,

as well as mitochondria, could be the major factor responsible for the effectiveness of PDT.[15] Our preliminary study demonstrated that Photofrin® has the ability to destroy human MMCs through both *in vitro* and SIM *in vivo* applications. Continued improvement of our knowledge in these fields will be feasible for us to use PDT on patients with deep, large malignant tumors.[21-29] In subsequent studies, the relationship between PDT and the apoptosis of MMCs should be considered.

Conclusion

Our preliminary study demonstrated that Photofrin® has the ability to destroy MMCs with *in vitro* and SIM *in vivo* applications. However, continued improvement in the treatment of patients with malignant melanoma after PDT will depend on the ability to cause selective destruction of only the targeted melanoma without the production of heat by non-thermal mechanisms. In addition, the greater tissue penetration of the longer wavelengths used in PDT should make it ideal for treatment of large, deep malignant tumors, thus substantially expanding the population of patients expected to benefit from PDT.

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光顯劑 Photofrin® 於惡性黑色素瘤運用之評估

張承仁　余兆松[1]　魏福全

背　景： 本實驗之目的在探討利用光顯劑 Photofrin®，配合光動力療法來治療惡性黑色素瘤，而運用試管內及試管外模型以爲評估。

方　法： 試管內光顯劑能力的評估，乃以培養之人類惡性黑色素瘤細胞爲主，以共軛焦螢光顯微鏡檢視光顯劑被細胞吸收後之位置。而黑色素瘤細胞經光動力療法後死亡與存活之比例，可比較 Photofrin® 之各種濃度及波長 630 奈米之雷射光照射後而得知。同時，亦可運用此法於裸鼠之動物模型，觀察裸鼠皮下植入惡性黑色素瘤細胞在經光動力療法後破壞之程度。

結　果： 惡性黑色素瘤細胞於 Photofrin® 濃度 3.5 微克／毫升時，接受 630 奈米雷射光，強度 100 毫瓦／平方公分之照射，顯示約百分之五十細胞的致死。電子顯微鏡下顯現出細胞死亡之狀態。

結　論： Photofrin® 有足夠能力破壞惡性黑色素瘤及裸鼠皮下植入黑色素瘤細胞。然於體內不同組織被吸收破壞之程度及其間相互之影響關係，則是吾人應進一步探討的課題。
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關鍵詞： 光動力療法，惡性黑色素瘤，光顯劑

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