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Authors

MAJOR, ATTILA L
TROMBERG, BRUCE J
KIMEL, SOL
[et al.](#)

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Photodynamic Therapy of the Rat Endometrium by Systemic and Topical Administration of Tin Ethyl Etiopurpurin

ATTILA L. MAJOR, M.D.,^{1,3} BRUCE J. TROMBERG, Ph.D.,¹ SOL KIMEL, Ph.D.,^{1,4}
TUAN PHAM, M.S.E.,¹ TATIANA B. KRASIEVA, Ph.D.,¹
MICHAEL W. BERNS, Ph.D.,¹ and YONA TADIR, M.D.^{1,2}

ABSTRACT

The objective of this study was to determine the optimal parameters for endometrial destruction by photodynamic therapy (PDT) using tin ethyl etiopurpurin (SnET2) as a photosensitizer in the rat model. Different application routes and different time intervals after drug administration were compared when the uterine horn was illuminated with a fixed laser light dose of 375 J/cm². Then PDT was performed 1.5 h after IV injection of 2 mg/kg SnET2 using decreasing light doses. Destruction of all endometrial glands was observed when the uterine horn was illuminated 1.5 h after IV drug administration. In contrast, weaker PDT effects were observed when light activation was delayed for 24 h following systemic or topical drug application even if the light dose was increased by a factor of 30. Endometrial fluorescence did not prove to be an effective method to predict optimal timing for photodynamic therapy, which was probably due to its binding to serum proteins. For photodynamic treatment of endometrial glands in the rat, IV administration 1.5 h before illumination was most efficient. (J GYNECOL SURG 15:71, 1999)

INTRODUCTION

PHOTODYNAMIC THERAPY (PDT) GENERALLY EMPLOYS IV ADMINISTRATION of a photosensitizing drug that is selectively retained by target tissues. Light activation results in the generation of highly reactive oxygen intermediates.¹ These intermediates, primarily singlet molecular oxygen, irreversibly oxidize essential tissue and cellular components. The resulting photodestruction ultimately causes local injury and necrosis. PDT performed in animal models has been shown to be efficient in ablating endometrial tissue following systemic administration of hematoporphyrin derivative (HPD)² and topical application of Photofrin (PII),^{3,4} aminolevulinic acid (ALA),⁵⁻⁷ and benzoporphyrin derivative monoacid (BPD).⁸ PDT is based on the localization of an administered photosensitizer in the target tissue and subsequent excitation of the photosensitizer with light at an appropriate wave length that will generate cytotoxic oxygen species. Activation of the photosensitizer by light also results in fluorescence emission, which can be used to monitor pharmacokinetics. Fluorescence microscopy studies in rats, rabbits, and humans with photosensitizers like ALA

¹Beckman Laser Institute and Medical Clinic, University of California, Irvine, Irvine, California.

²Department of Obstetrics and Gynecology, University of California Medical Center, Orange, California.

³Department of Obstetrics and Gynecology, University Hospital, Zurich, Switzerland.

⁴Department of Chemistry, Technion-Israel Institute of Technology, Haifa, Israel.

or BPD have shown specific uptake in endometrial glands compared with stroma or myometrium.^{8,9} After intrauterine (IU) administration of ALA in rats, irreversible endometrial ablation was achieved only by targeting all endometrial glands. To prevent regeneration, glands of the basal endometrial layer, where stem cells are believed to exist, had to be destroyed.^{7,10} Therefore, the method used for evaluating the efficiency of endometrial ablation was to determine the destruction of endometrial glands histologically.

The second-generation photosensitizer tin ethyl etiopurpurin (SnET2) is currently undergoing clinical trials for various applications. SnET2 is a hydrophobic compound, which is administered as an emulsion. In contrast to the first-generation photosensitizer, HPD, skin photosensitization has not been a major concern with SnET2.¹¹ Within photodynamically treated SnET2-sensitized tumors, there is a rapid decrease in blood flow with disruption of tumor microcirculation occurring soon after the initiation of PDT.¹² Similar vascular PDT effects have been demonstrated in normal tissue with HPD.¹³ It has been shown that activated SnET2 induces a high singlet oxygen yield,¹⁴ which is essential for PDT. An important advantage of SnET2 compared with HPD is that its absorption at 660 nm is five times higher than that of HPD at 630 nm.¹⁵ Increased penetration depth of light in tissue at 660 nm will result in deeper destruction of tissue. Moreover, for this wave length, small, portable diode lasers are available. In this study, we examined uptake and tissue distribution of SnET2 and then evaluated PDT effects on the rat uterine tissue, comparing (1) different routes of administration, (2) different time intervals after drug administration, and (3) different light doses at 1.5 h after IV injection of SnET2.

MATERIALS AND METHODS

Tin ethyl etiopurpurin (SnET2) was provided by Miravant (formerly PDT Inc.), Santa Barbara, CA. The hydrophobic compound SnET2 was obtained as a highly scattering emulsion in a concentration of 1 mg/ml. The emulsion was stored at 4°C in the dark.

A total of 88 mature Sprague-Dawley female rats (250–350 g) were housed in a pathogen-free animal facility at the University of California, Irvine. They were given a commercial base diet and water ad libitum. The experimental protocol was approved by the Institutional Laboratory Animal Care and Use Committee at UC Irvine.

Forty-four animals were used to determine uptake and distribution of SnET2 by fluorescence microscopy (Phase I) and 44 animals for histologic evaluation of endometrial destruction after PDT (Phase II). The estrus cycle was monitored by obtaining frequent vaginal smears in order to synchronize the treatment to the day of diestrus.¹⁶ Glandular proliferation occurs only during diestrus; stromal proliferation during diestrus and proestrus.¹⁷

Phase I

Rats were divided into two groups based on the route of administration (IV or IU). In the IV group, SnET2 (2 mg/kg) was injected via a marginal tail vein as a single bolus. In the IU group, animals underwent induction anesthesia with an intramuscular injection of ketamine/xylazine (2:1). Isoflurane and oxygen were administered during surgery for continuous anesthesia. Rats were placed in the supine position, and a midline laparotomy incision was made during continued anesthesia; then, both uterine horns of the didelphic uterus were identified, and 0.15 ml of SnET2 emulsion (1 mg/ml) was injected with a 25-gauge needle into the left uterine horn. Abdominal walls were closed in two layers.

To evaluate SnET2 uptake in the uterus, tissue fluorescence was measured. The animals were killed with CO₂ 0 h (controls), 1.5 h, 3 h, 6 h, and 24 h after the administration of SnET2. Tissue samples of the uterus were retrieved immediately following euthanasia. Specimens were sectioned in the middle part of the uterine horn and placed in molds containing embedding medium for frozen sections (OCT Media, Miles, Elkhart, IN). The blocks were then snap frozen in 2-methylbutane, supercooled with liquid nitrogen, and stored at -70°C in the dark. Tissues were sectioned in low diffuse light (Cryostat microtome, AO Reichert, Buffalo, NY) to obtain 6- μ m-thick slices for fluorescence analysis. A slow-scan, thermoelectrically cooled CCD camera (model TE/CCD-576, E/UV, Princeton Instruments, Trenton, NJ) coupled to an axiovert 10 epifluorescence microscope (Carl Zeiss Inc., Germany) was used to record fluorescence images. Excitation was provided by a 100 W mercury lamp filtered with a 440 \pm 35 nm bandpass filter (Omega Engineering, Inc., Stamford, CT). The emission path was similarly isolated by a 670 \pm 40 nm bandpass filter. These wave-

lengths were chosen because SnET2 has a strong absorption peak at 440 nm and fluoresces at 669 nm. Exposure times were 2 sec, and a shutter (Uniblitz, Model T132, Vincent Associates, NY) was used to synchronize the CCD camera with the excitation source. Image acquisition, processing, and camera control were performed by a Macintosh IIfx computer with IPLab software (Signal Analytics Corporation, Vienna, VA). Tissue fluorescence (at 670 nm) and light distribution images (at 500 nm) were recorded sequentially for each sample under identical conditions. In addition, dark-noise images were acquired from blank slides for each filter. Images were corrected for nonuniform illumination and for contributing dark noise according to the following formula:

$$\text{Corrected fluorescence image} = \frac{\text{Image (670 nm)} - \text{dark noise (670 nm)}}{\text{Image (500 nm)} - \text{dark noise (500 nm)}}$$

On the SnET2 fluorescence images, intensity measurements were made in the columnar and subcolumnar cell layers, in the endometrium at 200 μm from the surface, and in the myometrium for comparative analysis.

Phase II

To evaluate endometrial PDT after SnET2 application, the acute morphologic effects were measured by histology. Therefore, rats were killed 48 h after light treatment. Uterine specimens were retrieved via laparotomy and placed in buffered 10% formalin immediately following euthanasia by asphyxiation with CO_2 gas. A sample of the middle portion of the uterine horns was paraffin embedded and sectioned transversely. After staining with hematoxylin and eosin, the thickness of the entire uterus was measured in length and width using a light microscope with a calibrated ocular scale.

In the first part of the treatment study, PDT was performed with a fixed light dose of 375 J/cm^2 . In order to evaluate the optimal time point of PDT, different time intervals after SnET2 administration were chosen: for IU administration, 3, 6, and 24 h, and for IV administration, 1.5 and 24 h. Time intervals for IU administration were determined in accordance with fluorescence results. Since no fluorescence was detected in the IV group, the time intervals were selected on the assumption that the effects of PDT are stronger at short time intervals following systemic application. PDT was performed by intrauterine illumination of the left uterine horn. The light source was a 0.5 W diode laser (Model 1050; Miravant, formerly PDT Inc., Santa Barbara, CA) operating CW at 665 nm. Light was delivered to the uterine cavity via a 3-cm-long, 1.2-mm diameter Flex Diffuser (Model FX1; Miravant). The light applicator was snugly fitted into the left uterine horn superior to the bifurcation of the uterus. The right uterine horn (control) was shielded from light with gauze soaked with physiologic saline kept at body temperature. The source power was adjusted to 100 mW/cm diffusing fiber (300 mW total power into the light applicator). Initially, exposure time was set at 1500 sec, resulting in an incident optical dose at the surface of the uterus of 375 J/cm^2 , which was reported to be the PDT threshold dose for complete endometrial tissue destruction.¹⁸ In control animals, the uterus was illuminated without drug priming.

After optimal drug delivery conditions had been established, PDT was performed with decreasing optical doses (375, 150, 50, 25, 12.5, 6.25 J/cm^2) 1.5 h after IV injection in order to determine the light dose threshold for PDT. This time interval was chosen because it showed consistent destruction of all endometrial glands with the 375 J/cm^2 light dose.

Statistical analysis

For statistical analysis, differences in uterine thickness between treated and control horns were examined at different time points for each administration route, using the Kruskal-Wallis test. If a significant overall difference was present, multiple comparisons were performed using the Mann-Whitney test with Bonferroni correction. Statistical significance was taken as $p \leq 0.05$. Data are presented as mean \pm standard error (SE).

RESULTS

Figure 1 shows the mean fluorescence of the columnar epithelium, of the area immediately underlying the columnar epithelium (subcolumnar), of the endometrium 200 μm away from the surface, and of the

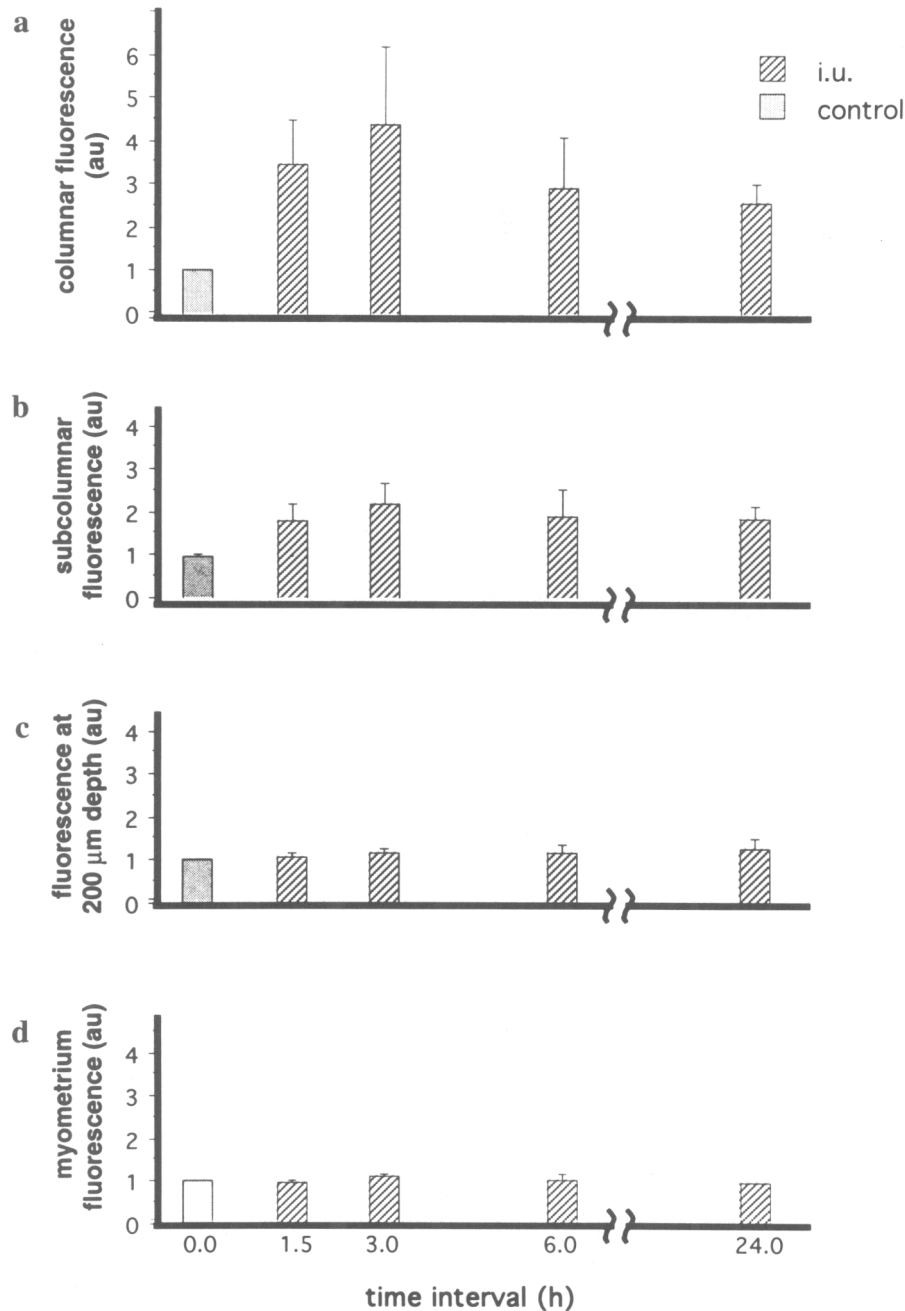


FIG. 1. Fluorescence microscopy at different time points after IU (0.15 ml, 1 mg/ml) administration of SnET2: mean fluorescence of the columnar epithelium of the endometrium (a), of the subcolumnar area (b), of the area 200 μ m away from the surface of the endometrial cavity (c), and of the myometrium (d). Four rats per time point; bars represent mean \pm SE.

myometrium as a function of the time interval between IU SnET2 injection and retrieval of the uterine horn. High fluorescence yield is observed in the columnar epithelium at all time points, peaking at 3 h. Rapid decrease of fluorescence occurs in the upper 200- μ m layer of the endometrium. Glands and myometrium exhibited no fluorescence above autofluorescence levels (Fig. 2). Surprisingly, no fluorescence could be detected after IV injection of SnET2 despite the fact that endometrial destruction was observed by performing PDT after IV administration. In an effort to understand this phenomenon, we evaluated whether the detection of SnET2 by fluorescence is altered by its binding to serum protein. We compared the absorption and

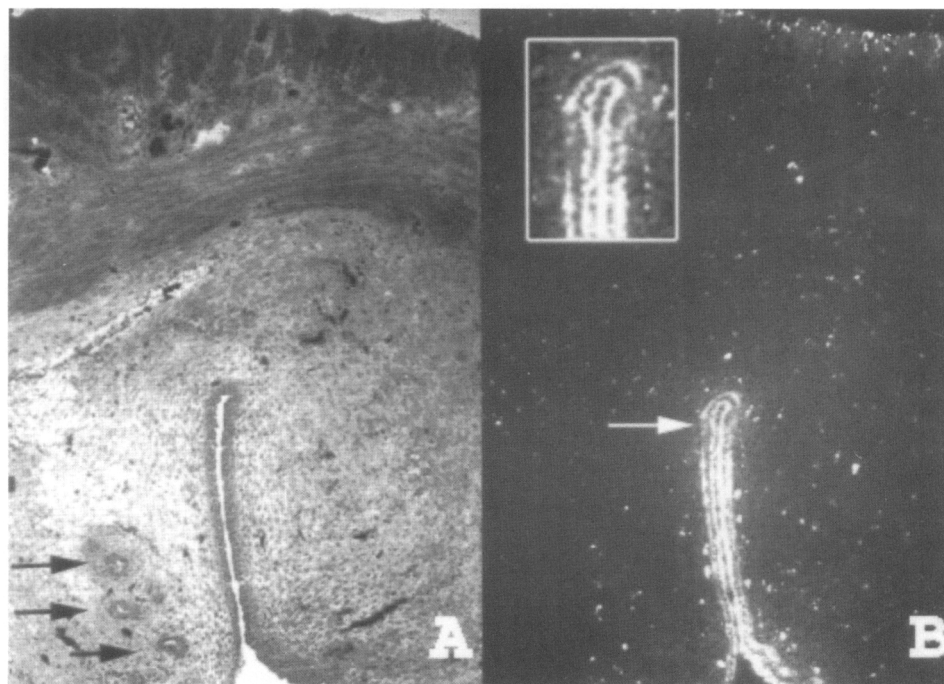


FIG. 2. Fluorescence micrograph of endometrium following 3 h after IU administration of SnET2 (B) and corresponding hematoxylin and eosin staining (A). Fluorescence is mainly in the columnar layer of the endometrium and not deeper in the endometrium, especially not in the endometrial glands. Electronic magnification (two times over optical) of the top of the columnar epithelium (arrow) shows fluorescence confined to the monolayer of columnar epithelium cells (B, insert).

the emission spectrum in vitro of SnET2 emulsion diluted in water at a concentration of 1 $\mu\text{g/ml}$ to SnET2 emulsion diluted in fetal bovine serum (FBS) at the same concentration. Interestingly, the fluorescence maximum of SnET2 in FBS (669 nm in water, 669.5 nm in FBS) decreased by nearly 50%. At this wavelength, FBS showed no autofluorescence. The absorption maximum of SnET2 in water and in FBS was 662.7 and 663.5 nm, respectively.

Figure 3 summarizes the results of the difference in thickness of the left uterus 48 h after PDT (375 J/cm^2) to the corresponding value of the right (control) uterus. Uterine thickness increased significantly in all groups compared with controls (laser illumination of the left uterine horn at 375 J/cm^2 without drug administration) except for the group in which PDT was performed 3 h after IU administration of the drug.

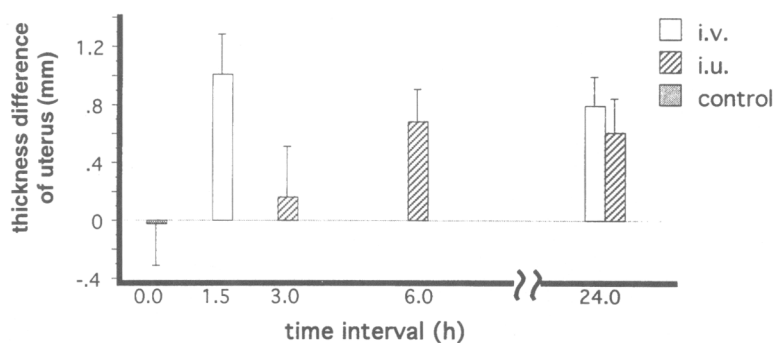


FIG. 3. Differences in thickness of the left uterus to the corresponding value of the right uterus, measured 2 days after photodynamic therapy, performed at different time points following IU and IV administration. Bars represent mean \pm SE; 4 animals per time point.

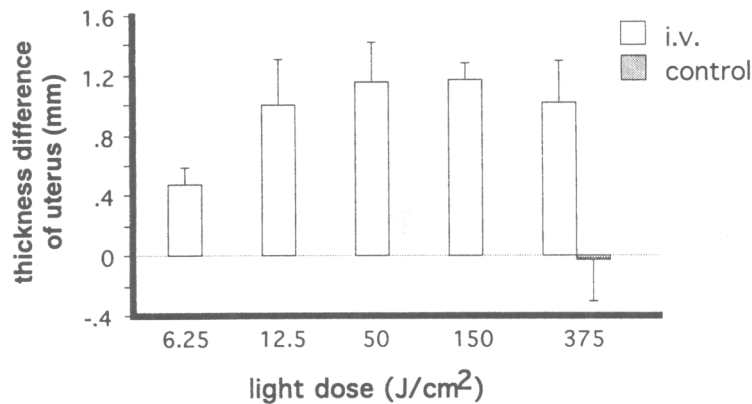


FIG. 4. Differences in thickness of the left uterus to the corresponding value of the right uterus for different optical doses applied 1.5 h after IV administration, measured 2 days after photodynamic therapy. Bars represent mean \pm SE; 4 animals per light dose.

The increase in uterine thickness was attributed to edema of the stroma. In the 1.5-h IV group (Fig. 4), vessel disruption with hemorrhage contributed to the additional increase in uterine thickness. This change in uterine thickness was also present in the 24-h IV group.

Table 1 summarizes the acute morphologic changes observed in uterine horns 48 h after PDT (Fig. 5B,C) compared with untreated control horns (Fig. 5A). Substantial endometrial edema and necrosis of the endometrium and the myometrium were observed in all treated animals. In the IU group, the PDT effects were less pronounced when PDT was performed 3 h after drug administration (Fig. 5B). Stromal cells were absent (ghost cells) or were diminished in number and size. Consistent destruction of the glands, however, was observed only in the animal group in which PDT was performed 1.5 h after IV administration (Fig. 5C).

Figure 4 shows the thickness increase of the uterus, 48 h after PDT, when different optical doses (375, 150, 50, 12.5, 6.25 J/cm²) were applied 1.5 h after systemic administration of SnET2. The thickness of the uterus increased significantly compared with controls even when the uterus was illuminated with 12.5 J/cm² (50 sec exposure time). Microscopic structural analysis of the uterine wall showed a transmural response (Table 2) with destruction of all glands (Fig. 5C). In contrast, illumination with 6.25 J/cm² resulted in less increase of the uterine thickness and incomplete damage of the endometrial glands.

DISCUSSION

Hysterectomy is one of the most common surgical procedures in the United States, and dysfunctional uterine bleeding is one of the main reasons for this operation. Endometrial ablation/coagulation is seen as

TABLE 1. MORPHOLOGIC CHANGES OBSERVED 48 H AFTER PHOTODYNAMIC THERAPY (LIGHT DOSE: 375 J/cm²)

Route ^a /time interval	No. of rats group	No. of rats with positive response				
		SnET2 concentration	Endometrial glands absent or necrotic	Endometrial glands reduced \geq 50%	Stroma necrosis \geq 50%	Necrosis of myometrium \geq 50%
Controls	4	0	0	0	0	0
IV 1.5 h	4	2 mg/kg	4	0	4	3
IV 24 h	4	2 mg/kg	1	3	4	3
IU 3 h	4	1 mg/ml	0	2	2	1
IU 6 h	4	1 mg/ml	2	2	4	4
IU 24 h	4	1 mg/ml	1	1	4	4

^aRoute: IV (intravenous), IU (intrauterine).

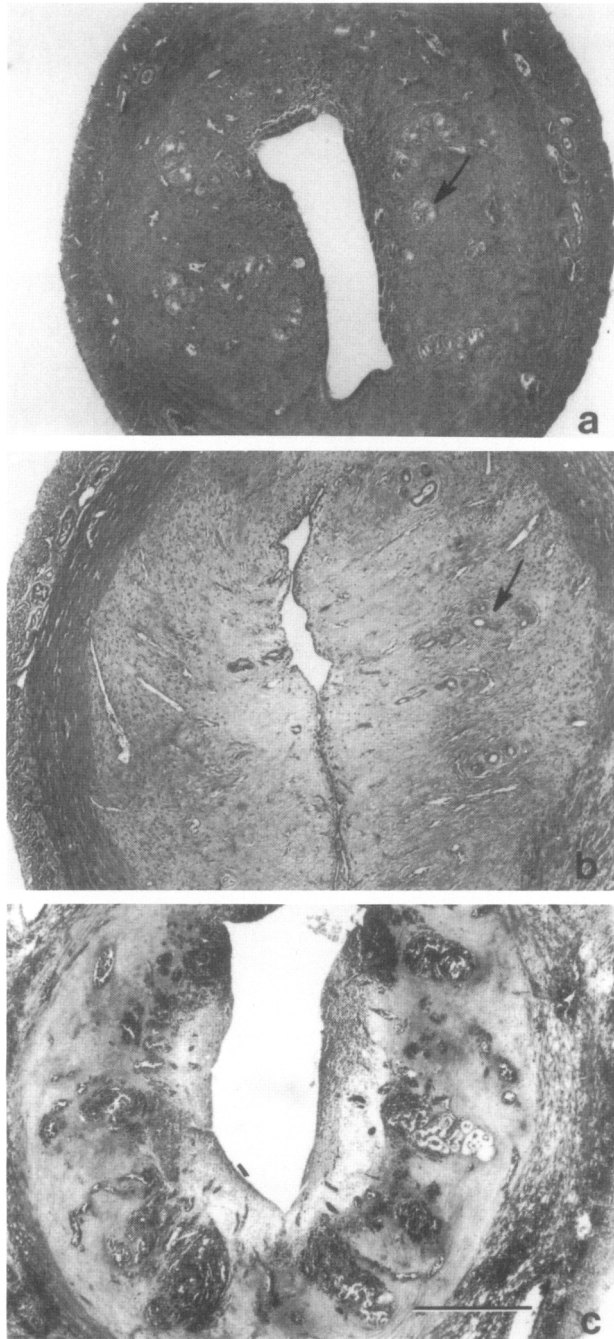


FIG. 5. Hematoxylin and eosin stained sections of left uterine horn 2 days after illumination: control uterine horn (a), incomplete destruction of endometrial glands (3 h after IU) (b), and successful PDT of endometrial glands (1.5 h after IV) (c). In panel (c), edema of the entire uterine wall, complete loss of cellularity, and absence of endometrial glands are evident. Length scale represents 0.5 mm.

an alternative to hysterectomy in patients for whom medical treatment is not effective. Minimally invasive procedures aimed at endometrial removal, whether by resection, coagulation, or ablation, were developed in order to avoid major surgery. Various different techniques and complications are summarized by Valle and Baggish.¹⁹ PDT offered potential advantages as an outpatient procedure because the device needed to activate the photochemical effects is much smaller than all other available devices,²⁰ and there are no thermal changes during this procedure.

TABLE 2. MORPHOLOGIC CHANGES OBSERVED 48 H AFTER PHOTODYNAMIC THERAPY (IV) ADMINISTRATION OF 2 MG/KG BODY WEIGHT (1.5 H PRIOR TO ILLUMINATION)

Light dose (J/cm ²)	No. of rats	No. of rats with positive response			
		Endometrial glands absent/necrotic	Endometrial glands reduced $\geq 50\%$	Stroma necrosis $\geq 50\%$	Necrosis of myometrium $\geq 50\%$
≥ 12.5	16	16	0	16	16
6.25	4	1	3	3	1

PDT is proposed as a simple, cost-effective, noninvasive treatment for dysfunctional uterine bleeding. To perform endometrial PDT, it is desirable for the drug to be administered with minimal patient discomfort. Topical drug administration requires repeated vaginal procedures, possibly involving anesthesia. Systemic administration of SnET2 may be considered because skin photosensitization will disappear within hours.¹¹ In humans, the thickness of the endometrium ranges from 2 to 7 mm.²¹ To target tissue at this depth, a photosensitizer with a strong absorption band in the red spectral region, like SnET2, or in the near infrared, is required. The drug is activated at 664 nm by a simple, portable light delivery system¹⁸; diode lasers, which are relatively small and inexpensive, are available for this wave length. PDT of the endometrium should preferably be achieved with one short light exposure of the uterine cavity.

No fluorescence was detected in uterine tissue after IV injection of SnET2. This may suggest that there is no significant uptake of SnET2 from blood into rat uterine tissue after the drug is distributed in the blood circulation. SnET2 fluorescence was observed after IU application, although it was restricted to the endometrial surface (Fig. 2A,B). Specifically, endometrial glands did not exhibit SnET2 fluorescence. Uptake of SnET2 in glands is important to destroy the glands and to avoid endometrial regeneration. Surprisingly, strong PDT response was observed in deeper endometrial layers with both routes of administration (Table 1) despite the fact that the same tissue showed no SnET2 fluorescence (Fig. 1). Probably a different environment modifies the fluorescence properties of SnET2, making it impossible to detect small quantities of SnET2 in tissue. As also described qualitatively by others,²² we observed that the fluorescence intensity decreased by 60% when SnET2 was bound to protein (SnET2 in FBS). Since no spectral shift was detected in fluorescence and in absorption, there is apparently no strong interaction. It is possible that because of nonradiative interactions, SnET2 weakly bound to protein has a low fluorescence yield compared with autofluorescence. This might explain why fluorescence microscopy was not a useful tool for studying SnET2 distribution in the endometrium or for predicting PDT effects in this study.

Forty-eight hours after PDT, the endometrium and the myometrium showed significant structural changes, such as edema and necrosis accompanied by hemorrhage. Even though the endometrial stroma of all animals was damaged considerably, endometrial glands were preserved in many animals. As previously reported by our group using ALA in the same animal model, uterine cells do not have uniform photosensitivity.⁷ The relative PDT resistance of glandular cells observed in this study may be due to superior oxidative stress repair mechanisms²³ or to a lower supply of oxygen, which is crucial for PDT. This is important because if endometrial glands survive, regeneration of the endometrium will occur. In human endometrium, regeneration results from epithelial proliferation in basal glands. Therefore, endometrial PDT may be performed successfully only by direct destruction of deep endometrial glands or by complete damage of the stroma because endometrial glands cannot exist without endometrial stroma.

Acute PDT response was characterized by edema and hemorrhage. This resulted in thickening of the uterine wall, which was used to quantify the acute PDT response. Weak PDT response was elicited when illuminated 3 h after IU drug administration. Microscopic analysis showed that all endometrial glands were preserved. In contrast, the 6-h, 24-h IU, and IV groups showed a strong PDT response of the uterine wall. However, consistent destruction of all endometrial glands could be observed only in the 1.5 h IV group. This indicates that measurement of the uterine wall thickness can serve as a first diagnosis, but complete information must be obtained, in addition, by microscopy of endometrial gland destruction.

In order to compare PDT after IV versus IU administration, a light dose of 375 J/cm² was applied to the uterine horn. This light dose produced a good PDT response at longer time intervals (6 h, 24 h) following IU administration. As previously mentioned, consistent endometrial destruction was observed with PDT

1.5 h following IV injection. At this time point, the major target of PDT was the vasculature, as evidenced by blood congestion and hemorrhage. The mechanism of PDT action with SnET2 was by disruption of vessels, thereby causing tissue necrosis. Interestingly, the strongest PDT effect occurred at short time intervals when the drug reached a high concentration in blood vessels. This indicates that PDT specificity may have been obtained by blood perfusion of the targeted tissue and by selective light application. The light applicator was placed in the uterine cavity and targeted the surface area. The highest photon density will therefore be in the endometrium. In addition, because of backscattering, radiant exposure in the surface layer will be higher than the incident radiant exposure.⁷ Since light intensity decreases exponentially in tissue, PDT damage is limited to surface structures.

At 1.5 h following IV drug administration, illumination for as little as 50 sec (12.5 J/cm^2) was sufficient to cause complete destruction of endometrial glands, which is essential to prevent endometrial regeneration. After longer intervals between drug administration and illumination, only partial damage to the glands could be achieved even after an exposure time of 24 min (375 J/cm^2).

In conclusion, as a photosensitizer for endometrial PDT in a rat model, SnET2 exhibited efficient destruction of endometrial glands at lower light doses than other photosensitizers. At short time intervals after IV administration of SnET2, strong endometrial PDT effects were observed at light doses 30 times less than those previously reported. If indeed a clinical study corroborates these results, SnET2 may well be considered as a serious candidate for PDT of the endometrium in humans.

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Address reprint requests to:

Yona Tadir, M.D.

Beckman Laser Institute and Medical Clinic

1002 Health Sciences Rd. E.

University of California, Irvine

Irvine, CA 92612