Photodetection and photodynamic therapy of minimal residual disease in the peritoneal cavity

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CHAPTER 6

INTRAPERITONEAL PHOTODYNAMIC THERAPY IN THE FISCHER 344 RAT USING 5-AMINOLEVULINIC ACID (ALA) AND VIOLET LASER LIGHT: A TOXICITY STUDY

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ALA-induced intraperitoneal photodynamic therapy in the Fischer 344 rat

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87
Abstract

Objective: Our study was designed to investigate 5-aminolevulinic acid (ALA) as a candidate for IP-PDT. The toxicity of IP-PDT and the effects of IP-PDT on abdominal and pelvic organs, particularly the small intestine, were investigated after ALA administration and illumination with violet laser light.

Study Design and Results: The toxicity of IP-PDT was evaluated in Fischer 344 rats in two ways. In the first part of the study local PDT effects on the intestine were analyzed histologically. Violet laser light ($\lambda$: 406-415nm) was applied as a 2 cm diameter spot on the intestine three hours after i.p. administration of 50 mg/kg ALA. (A) Histological tissue samples were taken 0 min, 6 h and one, two and three days after treatment (optical dose 3.2 J/cm$^2$). Immediately after local PDT (3.2 J/cm$^2$, 50 mg/kg ALA) showed no effect on the intestine. However, six hours post PDT there was complete destruction of the mesothelial lining and the outer (longitudinal) smooth muscle. Ganglion cells of the myenteric (Auerbach) plexus were also destroyed. The inner circular smooth muscle, the muscularis mucosa and the lamina propria were unharmed. Marked lymphectasia was present at this time. (B) To determine the threshold light dose of tissue destruction caused by PDT, different optical doses (1.6, 3.2, 6.4 J/cm$^2$) were administered and histologic analysis of tissue samples were obtained one day post treatment. Destruction of the entire external musculature, submucosal structures and muscularis mucosa of the intestine at the illumination site could be observed above 1.6 J/cm$^2$ (50 mg/kg ALA).

In the second part of the study whole peritoneal cavity PDT (WPC-PDT) was performed by illumination of the whole peritoneal cavity with 1.6 J/cm$^2$ violet light three hours after ALA administration using different drug doses (200, 100 and 50 mg/kg). WPC-PDT showed lethal toxicity with a drug dose above 50 mg/kg ALA at 1.6 J/cm$^2$. The probable cause of death in the first three days after IP-PDT was rhabdomyolysis, whereas when death occurred at longer time intervals, megaintestine associated with significant damage could be observed; however, without perforation of the intestinal wall.

Conclusion: In rats WPC-PDT with 50 mg/kg ALA, 1.6 J/cm$^2$ at $\lambda$=415 nm was the maximum tolerable light dose. This dose is likely to be above the threshold of destruction of ovarian cancer micrometastasis.

Key words: Photodynamic Therapy, ALA, NuTu-19, Ovarian Cancer, Fischer 344, Krypton-ion laser.
Introduction:

Despite a variety of surgical and chemotherapeutic interventions, ovarian carcinoma remains the fourth most common cause of cancer death in women in the United States.\textsuperscript{1} Many patients with advanced ovarian cancer (stage III and IV) initially respond to combination chemotherapy, as evidenced by negative second-look laparotomies. Unfortunately, up to 50% of these cases will recur and inevitably result in death.\textsuperscript{2} It is therefore necessary to develop improved therapeutic modalities which offer the possibility of more effective eradication of the disease. Eisenkop et al showed that a complete cytoreduction of visible peritoneal implants improved survival in patients of stage IIIC ovarian cancer.\textsuperscript{3} We have shown in an animal model of epithelial ovarian cancer that conversion of the potent photosensitizer protoporphyrin IX (PpIX) by the precursor 5-aminolevulinic acid (ALA) is specific for intraperitoneal ovarian micrometastasis.\textsuperscript{4,5} More importantly, selective eradication of ovarian micrometastases by photodynamic therapy (PDT) may be feasible.

PDT is based on the accumulation of a photosensitizer in the targeted tissue and activation of the photosensitizer with light at an appropriate wavelength, which results in the production of cytotoxic oxygen species. The first successful intraperitoneal PDT study in a murine ascitic tumor model utilized the photosensitizer hematoporphyrin derivative (HPD).\textsuperscript{6} This was followed by a human clinical trial with disseminated intraperitoneal malignant neoplasms.\textsuperscript{7,8} In these studies, illumination of large surface areas such as the peritoneum caused transient toxicity in multiple organs. However, small bowel perforations were seen in three patients. In a rat study, utilizing Photofrin or mesotetrahydroxyphenylchlorin (mTHPC) and red light, intestinal organs were the most photosensitive intra-abdominal structures and intestinal perforation was the common cause of death after PDT.\textsuperscript{9} Therefore, PDT of the peritoneal cavity may be feasible provided the small intestine can be protected during treatment.

Exogenously applied ALA results in endogenous production of the potent, fluorescent photosensitizer PpIX.\textsuperscript{10} ALA, in itself, is not a photosensitizing agent, but is the precursor of PpIX in the biosynthetic pathway of heme. Physiologically, the production of heme regulates the synthesis of ALA via a mechanism of negative feedback. Administration of exogenous ALA bypasses this mechanism and induces accumulation of PpIX in certain cells of normal organs like the liver and the skin. ALA-mediated PpIX synthesis allows selective cancer treatment if 1) photosensitizer is preferentially accumulated in malignant tissue (e.g. ovarian cancer), and 2) excitation of PpIX with light at an appropriate wavelength results in production of cytotoxic species in a sufficient amount to eradicate tumor without significant injury to normal tissue. As shown in a previous study,\textsuperscript{4} no significant conversion of PpIX occurred in the muscular layers of the intestine after ALA administration, as evidenced by minimal fluorescence using fluorescence microscopy. Therefore, converted PpIX may show substantially less intestinal damage compared to previously utilized photosensitizers. Indeed, homogeneous distribution of PpIX in tumors was observed, suggesting that photodynamic destruction of these nodules could be successful. The fact that small volume tumors on the serosal surface of the peritoneum and of the small bowel showed conversion to PpIX may lead to improvement of adjuvant treatment of intraperitoneal micrometastic disease.

PpIX absorbs at different wavelength bands with peaks at 630, 575, 540, 505 and 405 nm. At 405 nm, absorption is about 30x larger than at 630 nm. Since the PDT effect is proportional to the number of photons absorbed by the photosensitizer accumulated in the
cell, it was suggested that illumination at 405 nm is more effective in cell killing than red light.\textsuperscript{11} In comparison, with HPD (40 mg/kg) violet light has a lower threshold for tumor damage (0.8 J/cm\textsuperscript{2}) compared to green light (2.5 J/cm\textsuperscript{2}) and red light (4.2 J/cm\textsuperscript{2}).\textsuperscript{12} The fact that radiation around 400 nm is better absorbed by the sensitizer is compensated by the difficulty of producing such radiation especially in a complex geometric organ as the peritoneal cavity. However, compared to red light, violet light has a lower penetration depth in tissue because violet light is also absorbed by hemoglobin and is highly scattered in tissue. Violet light was shown to be more effective only at depths less than 0.2 mm. In whole peritoneal cavity PDT (WPC-PDT) this is an advantage since the thickness of the intestinal wall in the rat is about 0.5 mm. In this case a low penetration depth of light is important, because surrounding tissue will be spared. So, using violet light may protect the small intestine, which is the most sensitive organ to PDT, from damage which could result in perforation.

Effective PDT (of peritoneal micrometastasis) with minimal side effects could be achieved by using a photosensitizer, or a photosensitizer precursor, having minimal uptake in muscle and connective tissue, and by exciting the converted photosensitizer (in ovarian cancer cells) with violet light. Because of the small penetration depth in tissue this is expected to result in effective surface treatment.

In this article, we report a toxicology study of IP-PDT with laser light ($\lambda=415$ nm) after i.p. administration of ALA in the Fischer 344 rat. PDT effects on the small intestine were particularly analyzed.

### Material and methods

Pathogen free F344 female rats (160-180 grams) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and housed in a pathogen free animal facility at The University of California, Irvine. They were given unlimited access to commercial basal diet and water. The experimental protocol (# 95-1646) for the use of these animals for these studies was approved by the Institutional Laboratory Animal Care and Use Committee at U.C. Irvine.

Animals underwent induction anesthesia with an intramuscular injection of ketamine (13 mg/kg) and xylazine (87 mg/kg). Isoflurane and oxygen were provided during surgery for continuous anesthesia. Crystalline 5-aminolevulinic acid hydrochloride (DUSA Pharmaceuticals, Inc., Denville, NJ) was diluted to 50 mg/ml in sterile water and titrated with 10 N sodium hydroxide to pH 6.5. ALA was administrated i.p. in each animal with a dose of 50 mg/kg in the first part of the study, whereas in the second part of the study different drug doses (200, 100 and 50 mg/kg) were used.

In the first part of the study, PDT effects on the intestine were evaluated histologically. The intestine was illuminated locally to assure accuracy of light doses delivered. Three hours after drug injection the rats were placed in the supine position and a midvertical incision was made under continued anesthesia. PDT was performed by illuminating a 2.0 cm diameter spot of the exposed small intestine. Violet light ($\lambda=406.7 - 415.4$ nm) was provided by a multiline krypton-ion laser (Coherent, model 90-K) coupled to a microlens-terminated optical fiber (100 mW/cm\textsuperscript{2}, spot size 2 cm). Part A: In order to evaluate the time course of morphologic effects, rats were sacrificed 0 min, 6 h and one, two and three days after PDT (optical dose 3.2 J/cm\textsuperscript{2}). Part B: To determine the threshold light dose for tissue destruction, PDT was performed with increasing optical doses (0.8, 1.6, 3.2 J/cm\textsuperscript{2}) and tissue samples were retrieved one day after treatment. Specimens of the treated small intestine were retrieved immediately via laparotomy and placed in
buffered 10% formalin. Samples were paraffin embedded, sectioned transversely and stained with hematoxylin/eosin.

In the second part of the study, WPC-PDT of the whole peritoneal cavity was performed three hours after i.p. injection of different ALA dose (200, 100 and 50 mg/kg) with the previously defined threshold light dose of 1.6 J/cm². To improve homogeneous light distribution, the peritoneal cavity was filled, under anesthesia, with 40ml Intralipid® 0.02% (Kabi Pharmacia Inc., Clayton, NC 27520) scattering medium, diluted with 0.9% NaCl. With the rat supine, the peritoneal cavity was punctured with a 14 gauge i.v. catheter (Jelco Laboratories, Raritan, N.J., Johnson & Johnson Company). This was performed bilaterally in the middle between the costal arc and the crest of the iliac bone. The stainless steel catheter was removed and a quartz optical fiber, terminated with a 1.2 mm diameter, 3 cm long cylindrical diffusing applicator (model 4420-A02, PDT Inc., Santa Barbara, CA), was inserted into the sheath. The krypton-ion laser power was adjusted to 100 mW/cm cylindrical diffusing applicator (300 mW total power coupled into the fiber). The abdomen was divided into 5 regions: right upper, left upper, middle, right lower and left lower. During therapy each region was treated for 5 minutes for a total elapsed lasin g time of 25 minutes per rat. At half time of the treatment, 10 ml of 0.9% saline was administered i.p. to replace leaked fluid. The estimated total treated peritoneal surface, which is approximately equivalent to the body surface, was 280 cm² for a 170g rat. For 25 minute illumination time this results in an optical dose of 1.6 J/cm².

The optimal Intralipid concentration was determined in vitro by adding 20% Intralipid in a 5 liter beaker filled with 0.9% NaCl. The light intensity was measured with a detector "hidden" from the laser light source (415 nm), both placed in the beaker. This experimental set-up mimics the treatment of the peritoneal cavity, which includes difficult to illuminate intraperitoneal organs and recesses of the peritoneal cavity.

Results

Histology of local PDT on the intestine

Figure 1 shows the time course of morphological tissue effects after administration of ALA (50 mg/kg) and 415 nm laser light (3.2 J/cm²). Control animals demonstrated no microscopic signs of tissue injury to the small intestine after treatment with laser light alone. Similarly, no effects could be noted immediately after PDT. In contrast, significant intestinal injury involving mesothelial cells, ganglion cells of the myenteric (Auerbach) plexus and longitudinal smooth muscle was noted as early as 6 h following PDT. The smooth muscle layer of the inner circular muscular layer was slightly thinner, but the remaining cells appeared healthy. The submucosa, muscularis mucosa, and lamina propria all appeared to be histologically intact. While submucosal vessels (arterioles and venules) were also unharmed, marked dilatation of submucosal lymphatics was evident in the treated areas. One day after PDT, near complete destruction of all smooth muscle layers (including the outer longitudinal, inner circular and muscularis mucosa) was noted. The bowel wall consisted only of thin wisps of connective tissue containing scattered lymphatic spaces and occasional vessels. Lymphatics showed marked dilatation. All ganglion cells were destroyed. Two and three days after PDT, the histologic pictures were similar; marked dilatation of both submucosal and mucosal lymphatics with evidence of lymphocyte sludging and congestion. Lymphatic obstruction resulted in such massive lymphedema that the intestinal villi because distorted, lost viability and sloughed into the lumen. In general, the epithelium of the lamina propria showed preservation of all cell types and marked proliferative activity of the basal reserve cells. Because the major
Histologic changes were noted as early as 1 day after PDT, subsequent evaluation of the effects of PDT at different dosages was performed at 24 h after treatment.

The PDT (50 mg/kg ALA) effect with decreasing optical doses 1 day after treatment is shown in fig. 2. PDT damage occurred down to 1.6 J/cm²; with morphological changes as described for 3.2 J/cm²; at 0.8 J/cm², the histology of the intestine was normal.

WPC-PDT (whole cavity): threshold of lethal PDT dose (Table I)

Toxicities of whole cavity PDT (WPC-PDT) were related to both the dosages of ALA and light. Using 50 mg/kg ALA, light doses above 1.6 J/cm² at λ = 415 nm resulted in lethal toxicity (50% of the animals died). However 1.6 J/cm² or less was not lethal. Therefore the threshold level for WPC-PDT include 50 mg/kg i.p. of ALA and light dose of ≤1.6 J/cm². Similar untoward effects were noted if the photosensitizer doses was increased above 50 mg/kg. Deaths occurred in two different patterns, early (1 - 3 d) and late (>10 d). Histological analysis of tissues obtained at autopsy revealed the reasons for these deaths. The histology from animals that died within the first three days (Table I) was consistent with extensive rhabdomyolysis in both the abdominal wall and diaphragm. Thus, a renal failure secondary to myoglobinemia was likely to be the immediate pathogenetic mechanism of death. If death occurred after 10 days, the pathology was notable for severely dilated loops of intestine with complete or near complete destruction of the mucosa and smooth muscle. However none of the animals studied (early or late) had evidence of intestinal perforation.

Table I. Toxicity: Number (percentage) of animals per group that died after PDT (3 hours time interval)

<table>
<thead>
<tr>
<th>Drug and light dose</th>
<th>Died / Number of animals (time)</th>
</tr>
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<tbody>
<tr>
<td>200 mg/kg (1.6 J/cm², 25min)</td>
<td>4/4 (1 at 5.5h, 2 at 1D, 1 at 3D)</td>
</tr>
<tr>
<td>100 mg/kg (1.6 J/cm²)</td>
<td>2/4 (1 at 2D, 1 at 18D)</td>
</tr>
<tr>
<td>50 mg/kg (1.6 J/cm²)</td>
<td>0/4</td>
</tr>
<tr>
<td>50 mg/kg (3.2 J/cm²)</td>
<td>2/4 (1 at 3D, 1 at 13D)</td>
</tr>
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Intralipid concentration

When the beaker wall was covered with aluminum foil or with black paper, the light intensity at 415 nm measured by the "hidden" detector, reached a maximum at a dilution of 0.02% Intralipid. At higher concentrations the output voltage decreased again. The light intensity in the aluminum covered beaker peaked at 0.016% and was nearly 4 times higher than in the black beaker.

Discussion

Although substantial work has been performed in PDT with porphyrins, such as HPD and Photofrin, the unique characteristics of ALA-mediated PpIX synthesis for selective treatment of tumors are just beginning to be explored. Moreover, skin phototoxicity -the main side effect of photosensitizers- after systemic ALA administration lasts for about one day which represents an important improvement compared to HPD phototoxicity which lasts for up to six weeks. We have previously shown selective ALA-induced photosensitization of ovarian cancer micrometastasis in the peritoneal cavity. This is due to a higher conversion rate of Pp IX in cancer cells, than in cells of the muscle and the connective tissue, which normally constitute the surrounding tissue of tumors. ALA induces direct photosensitization of ovarian cancer cells, whereas other photosensitizers
localize mainly in the vascular stroma of the tumor. Intraperitoneal administration of ALA leads to a higher photosensitizer concentration in ovarian tumor infiltrating the peritoneal surface. This is likely to enhance selective ovarian micrometastasis destruction using PDT. In the same matter photosensitization of cancers of the skin, the bronchi and the breast occurs after topical ALA application. Various mechanisms have been suggested for the intracellular increase of PpIX in specific cells after exogenous administration of ALA.

The most sensitive organ in WPC-PDT after administration of Photofrin or m-THPC is the small intestine and intestinal perforation was the most serious complication and cause of death. In a recent study we demonstrated that fluorescence in the small intestines after i.p. administration of ALA, was confined to the inner layer of the mucosa, whereas no fluorescence could be observed in the musculature. Based on this, we expected that smooth muscle cells would be resistant to ALA PDT. Interestingly, PDT with 3.2 J/cm² violet light on the small intestine showed rapid damage to the muscle wall without affecting the epithelium of the lamina propria. The mechanism of action of the damage in the small bowel musculature appears therefore to be a direct cytotoxic effect of PDT, which is in conformity with the fact that ALA has to be first converted intracellularly to the potent photosensitizer PpIX. As shown in our previous study, peritoneal ovarian cancer nodules strongly emit fluorescence. We expect therefore that ovarian cancer will be more sensitive to PDT than muscle. However, smooth muscle cells were sensitive to PDT without exhibiting PpIX fluorescence. This means either that PpIX in muscle cells undergoes efficient fluorescence quenching, or that a small amount of ALA was converted to PpIX, which was still sufficient to cause cellular destruction. In line with this explanation is that smooth muscle cells received the highest light dose because of their direct exposure to light, which resulted in increased generation of cytotoxic species (e.g. singlet oxygen) by the activated photosensitizer. It is well known that light intensity decreases exponentially in tissue and in deeper tissue layers fewer cytotoxic species are generated despite a higher photosensitizer concentration. Immediately after PDT the mucosa was not affected, in contrast to outer muscle cells. One day after PDT, however, extensive destruction of the smooth muscle wall, submucosa and muscularis mucosa were noted. There was marked lymphectasia with lymphocyte sludging and lymphedema of the lamina propria. We suggest that the damage to the epithelium of the mucosa was mainly secondary to the non-specific effects of severe lymphedema. Lymphectasias and lymphedema is likely due to damage of lymphatic drainage in the muscle layer causing severe congestion in the well-developed lymphatic network of the intestinal mucosa. This, in turn, resulted in edema of the lamina propria eventually resulting in sloughing of mucosal villi by mechanical eruption. The observed edema was so widespread that it may have induced a compartment syndrome by capillary collapse and ischemia, thus increasing damage to the mucosa. At one day after PDT, regenerative activity of the epithelium was high with numerous mitotic figures present in the basal reserve cell layers. It is important to note that by 3 days the entire mucosa had regenerated. In general renewal of the complete mucosal epithelium occurs within about three days.

Damage and congestion of the lymphatic network seen in this study after PDT may explain the poorly understood finding that peripheral lymphocyte count decreased after WPC-PDT. We suggest that the normal physiological lymphatic drainage was damaged by WPC-PDT and that lymphocytes were trapped in lymphatic vessels. Since the rate of production of lymphocytes in bone marrow is low, trapped lymphocytes may have caused transient peripheral lymphopenia.

Since PDT with ALA affected the outer muscle layers of the small intestine, the penetration depth is a major concern in the treatment of the whole peritoneal cavity. Violet
light will restrict possible damage to a depth of 0.2 mm, at the same time being more efficient in activating photosensitizer molecules on the surface compared to other wavelengths.12

Interestingly, no intestinal perforation could be observed in the animals after WPC-PDT of the whole peritoneal cavity with violet light even by increasing the light or drug dose. However, enlargement of the small intestine and of the cecum occurred in two animals 2 weeks after PDT (Table 1). Histology of the intestine revealed a complete loss of the outer muscle layers and ganglion cells (Fig.3). The cause of death in these animals was probably intestinal dysfunction. Thus, dysfunctional bowel syndrome and megaintestine without perforation are significant complications of ALA induced WPC-PDT. It was shown that ALA-PDT on the mucosa of the colon with red light resulted in deep destruction involving muscle tissue but without causing perforation.16 It was suggested that ALA-PDT, in contrast to other treatments like electrocautery, preserved connective tissue, which is important for the regeneration of the mucosal wall. The death of the other animals occurred in less than 3 days after PDT. In these animals, areas of the abdominal wall showed unexpectedly rhabdomyolysis, even though fluorescence data showed that muscle was not mediating PpIX conversion. While every effort feasible was made to avoid uneven illumination, the diffusing fiber may have been in direct contact with the abdominal wall, causing a high uncontrolled local irradiance. This irradiance may have produced a higher penetration depth of destruction through the effect of PDT and certainly not through a thermal effect since the fluence rate was kept under 100 mW/cm² at the surface of the diffusing fiber.

To our knowledge, these studies are the first to document the clinical and histological parameters associated with ALA induced PDT of the peritoneal cavity. These results demonstrate that WPC-PDT is feasible but limited to both the dosage of ALA and light. The major complications observed resulted from direct phototoxicity as well as indirectly, secondary to lymphatic obstruction. Since the conversion of PpIX from ALA is known to be much higher in ovarian cancer cells than surrounding normal tissue, suggests PDT will hold new possibilities for the adjuvant treatment of peritoneal cancer.

The authors would like to thank Jeff J. Andrews and Glen A. Profeta for excellent technical support throughout these studies and to John C. Hiserodt Hiserodt for his help in interpretation of the histological slides.

Reference List


Figure 1. Histological changes (x400) in the small intestine of F344 rats following Photodynamic therapy (Violet laser light, \( \lambda \): 406-415nm, 3.2 J/cm\(^2\)) with ALA (50 mg/kg i.p.). A) control animal. MC = Mesothelial Cells; MPE = Muscularis Propria Externa; MPI = Muscularis Propria Interna; MM = Muscularis Mucosa; GC = Ganglion Cells; LVS = Lymphovascular space (x400); B) small intestine immediately after PDT; C) 6 hours after; D) 24 hours after PDT; E) 48 hours after PDT; F) 72 hours after PDT.
Figure 2. Histological changes (x400) in the small intestine of F344 rats one day following Photodynamic therapy (Violet laser light, $\lambda$: 406-415nm) with ALA (50 mg/kg i.p.). A) small intestine of animal treated with 1.6 J/cm$^2$ light dose. B) small intestine of animal treated with 3.2 J/cm$^2$ light dose; C) small intestine of animal treated with 6.4 J/cm$^2$ light dose
Figure 3. Histological changes (x400) of F344 rats following Whole Peritoneal Cavity Photodynamic therapy (Violet laser light, \( \lambda \): 406-415nm, 1.6 J/cm\(^2\)) with ALA (100 mg/kg i.p.). A) rhabdomyolysis B) small intestine.