ABSTRACT

Pancreatic cancer is an aggressive disease with a poor prognosis, usually treated with chemoradiation therapy. Interstitial photodynamic therapy is a potentially effective adjuvant treatment that is under development. In the current study, two orthotropic pancreatic cancer models (AsPC-1 and Panc-1), have been characterized with respect to growth rates, morphology and liposomal drug (Verteporfin) uptake and distribution in SCID mice. Fluorescence of Verteporfin was measured in liver and tumor in vivo using a PDT fluorescence dosimeter with measurements taken before and up to one hour after tail vein injection. Fluorescence reached a plateau by about 15 minutes and did not decrease over the first hour. At time points from 15 minutes to 24 hrs, the internal organs (kidney, spleen, pancreas, tumor, muscle, lung, liver, and skin were excised and scanned on a Typhoon imager. The ratio of fluorescence in tumor versus normal tissues was analyzed with image processing, calculated at each time point and compared to in vivo results. Tissue distribution of Verteporfin in relation to functional vasculature marked by DiOc7 was carried out on frozen sections. Final analysis will result in determination of the ideal time point to administer light to achieve maximum tumor destruction while preserving normal tissue.

Keywords: Verteporfin, photodynamic therapy, xenograft orthotopic pancreas tumors, dosimetry, contrast, ASPC-1, fluorescence, PANC-1.

1. INTRODUCTION

Pancreatic cancer is an aggressive disease with poor prognosis. Standard methods of treatment, which include chemotherapy and radiation, have proven ineffective due to the pathophysiology of the tumors. Pancreas tumors are characterized by large areas of stroma, poor vascularization and local invasion. When diagnosed, tumors are generally large and locally invasive, and therefore they are non-resectable, calcitrant to drug penetration, and present a field for radiation that includes much normal tissue. Therefore, alternative treatments and techniques are under investigation. Interstitial photodynamic therapy is a potentially effective adjuvant treatment that has shown some promise, however careful dosimetry will be required1,2. Optimal application of PDT will require knowledge of the time course and the distribution of photosensitizer localization in the tumor as well as in surrounding potentially sensitive tissues.

In this study we used two lines derived from human pancreatic cancers that show different growth curves and histopathology that impacts the success of potential treatments3,5. The choice of verteporfin as a photosensitizer for photodynamic therapy of pancreas is appealing because of its very short metabolic half-life, near-infrared absorption and the fact that it is already clinically approved for PDT for age-related macular degeneration and some cancers6. Verteporfin has had limited use for abdominal photodynamic therapy but there are two studies that tested photodynamic therapy in normal pancreas7. Yusuf et al. tested verteporfin as a photosensitizer in endoscopic ultrasound (EUS)-guided PDT of normal swine pancreas and Ayaru et al demonstrated the feasibility of using verteporfin in the normal pancreas of the Syrian golden hamster7. Minimal damage to the pancreas and surrounding normal tissue in both studies show that verteporfin PDT is a likely candidate for pancreas adenocarcinomas. Moreover, verteporfin PDT is entering a Phase I/II clinical trial at University College London Hospital, London UK2,7.
2. METHODS

2.1 Pancreatic cell lines

AsPC-1 cells, derived from a human pancreatic acinar cell adenocarcinoma (CRL-1682™, American Type Culture Collection (ATCC), Manassas, VA 20108) were cultured in RPMI 1640 with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin (P-S) prepared for a stock solution of 10,000 IU penicillin and 10,000 g/ml streptomycin (Mediatech, Herndon, Virginia), 2 mM L-glutamine (gln), 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate. PANC-1 cells, derived from a human epithelioid carcinoma of the exocrine pancreas (CRL-1469™, ATCC) were grown in DMEM/High glucose, supplemented with 10% FBS, P-S, and gln. The cells were passed by rinsing with phosphate buffer solution (PBS) without calcium and magnesium and then incubated at 37°C with 0.25% trypsin for 5-10 minutes. Trypsin was neutralized with culture medium, the cell solution was pelleted and the cells suspended in complete medium at 4x10^7 cells/ml. Cells for implantation were prepared in a 1:1 mixture of cell culture medium and Matrigel® (BD Biosciences, San Jose, CA). Matrigel® was thawed on ice in a 4°C refrigerator overnight and was kept on ice for the entire implantation procedure. AsPC-1 cells were diluted in a one to one ratio of culture medium and Matrigel® to a final concentration of 4x10^7 cells/mL for implantation.

2.2 Orthotopic implantation of pancreatic tumors

All animals were used in accordance with an approved protocol from the Institutional Animal Care and Use Committee (IACUC) at Dartmouth College. Orthotopic pancreatic cancer tumors were implanted into 6 week old male SCID mice (~22 g) by the following method. Ketamine/xylazine (100/10 mg/kg) was used for anesthesia. The left side of the mouse was shaved and the fur completely removed by the use of a depilatory cream for approximately 3 minutes, or until skin was entirely free of fur. The surgical area was sterilized with an iodine solution (Povidone-Iodine, Novaplus, Irving, TX) and a small incision (< 1 cm) was made through the skin and abdominal wall at the base of the spleen. The spleen was gently pulled through the incision, exposing the tail of the pancreas. The cell-Matrigel® solution (1x10^6 cells in 50 microL) was injected into the tail of the pancreas. The Matrigel® was allowed to set (~ 10 sec) and the needle gently removed from the pancreas and the area swabbed with iodine to kill any stray cells in the injection site. The pancreas and spleen were replaced in the abdomen and the incision site closed with 3-4 sutures (Ethilon 5-0 PS-3, Ethicon, Piscataway, NJ). The Matrigel® method of orthotopic tumor implantation has resulted in 100% tumor uptake with little evidence of extrapancreatic leakage. ASPC-1 tumors were studied two weeks after implantation, while PANC-1 tumors were used at five weeks. Average tumor volume at the time of experiment, was approximately 60 mm^3 in each line as previously determined by MRI.

2.3 Photosensitizer

The liposomal formulation of verteporfin-for-injection (Visudyne™, Novartis, New York) was used as the contrast agent in this study. The photosensitizer was obtained from QLT (Vancouver, Canada) as a gift. A stock saline solution of verteporfin (VP) was reconstituted in water according to the manufacturer’s instructions, using 2.5% as the active component and used immediately.

2.4 In Vivo dosimetry

VP concentration measurement was achieved using a customized commercial fluorimeter system (Aurora Optics, Inc., Hanover, NH) fitted with a 6mm diameter probe with 1 source and 6 detector fibers. The fluorimeter system employs a 405-nm diode laser, a 660-nm long-pass filter, and a photomultiplier tube for detection and has been previously described. The probe was placed alternately on the surface of tumor and liver, sampling ten times at each site. Measurements were made at about 1 minute intervals for 10-15 minutes before and repeated for 15 minutes after injection, then periodically for up to 1 hr. The average of 10 data points for each time point was used to represent the VP uptake by the individual animal in the liver and the tumor. All the measurements were recorded within 1 hr after VP administration.
2.5 Ex Vivo fluorescence

To determine the localization of the dye in the abdominal organs, a mouse was injected with the verteporfin (1mg/kg) in water. After the desired time elapsed, during which the mouse was held in subdued light, the mouse was injected with 1 mg/kg DiOc-7 (Invitrogen) to visualize functional blood vessels and sacrificed immediately. DiOC7-stained vessels emit green fluorescence when excited by blue light and can be visualized in the same tissue slices as verteporfin. The tumors were dissected away from normal pancreas and other organs then sliced in half, with half embedded in Tissue Tek and flash frozen in a methylbutane bath. The other half of the tumor was sliced and placed cut side down on a 100mm plastic dish, with slices of skin, muscle, and other abdominal organs including kidney, pancreas, tumor, liver, lung, spleen. White light images were obtained with a digital camera. All ex vivo imaging was performed on a Typhoon 9410 Variable Mode Imager (GE Healthcare Systems) equipped with a 685 LP filter and 633nm excitation source. Dilutions of the stock verteporfin solution were used to quantify fluorescence for comparison of verteporfin fluorescence in tissues. Post-image analysis was performed with ImageJ (National Institutes of Health, Bethesda, MD).

3. RESULTS AND DISCUSSION

Throughout these results we compared the two pancreatic human xenograft tumors that differ significantly in growth rate, vascular density, origin and reputedly, metastatic potential. ASPC-1 is aggressive, grows rapidly and reaches treatment size two weeks after injection into the pancreas. PANC-1 grows more slowly reaching treatment size only after five weeks. Uptake of verteporfin was followed by two methods. In vivo measurement of fluorescence in tumor and liver was carried out at short time points using the Aurora dosimeter. Ex vivo assessments confirmed in vivo assessments at short time points and also were made at longer time points up to 24 hrs.

3.1 Ex vivo fluorescence quantification

Mice were anesthetized lightly with isoflurane, injected with verteporfin, and allowed to recover and kept in subdued light. At 3, 8 or 24 hrs mice were sacrificed, the tumor dissected from surrounding tissues and sliced in half. Fifteen minute and one hour time points were obtained by sacrificing animals after in vivo assessments (see section 3). Background fluorescence standards were used to normalize fluorescence means of the ROI drawn on each organ, so that mice could be compared from day to day.

![Verteporfin Standards](image-url)
The uptake of verteporfin as shown in these ex vivo assessments followed a similar time course in each of the organs in each tumor model. Liver and kidney were always the most intense fluorescence and muscle and skin were the least intense. The peak of fluorescence was at 1 hr in the ASPC-1 tumor although intensity remained high at 3 hr (Figure 2). The peak of fluorescence was at 3 hr in the PANC-1 tumor suggesting that this time point would be optimal for PANC-1 (Figure 3).

![Figure 2](http://spiedigitallibrary.org/)

**Figure 2.** Fluorescence of Verteporfin in ex vivo organs of ASPC-1 tumors as a function of time after injection. Verteporfin concentration is in mg/ml based on mean fluorescence of ROIs for each organ, normalized by fluorescence of stock solutions of verteporfin. There were 3 animals per group for a total of 15 animals per tumor model. Error bars are SEM, n=3.

To investigate whether there is selective uptake in the tumors versus surrounding normal tissue, we calculated the ratio of the mean fluorescence and standard error of mean, of tumor versus the normal pancreas and tumor versus the muscle sample (Table 1). There was no significant difference between the ratios of fluorescence of these organs. These data reinforce the conclusion that delivery of light specifically to the tumor will be necessary to enhance the therapeutic effect on tumor while minimizing normal tissue damage. Magnetic resonance imaging was shown to be useful for orthotopic pancreas tumors\(^\text{10}\). The work of Samkoe, et al\(^\text{3-5}\) has shown the techniques and utility of MRI to determine tumor volume and to monitor treatment response in ASPC-1 and PANC-1 tumors. Therefore, the tools are in place to determine tumor location, and boundaries with normal tissue so that precise delivery of light can be accomplished.
Figure 3. Fluorescence of Verteporfin in ex vivo organs of PANC-1 tumors as a function of time after injection. Verteporfin concentration is in mg/ml based on mean fluorescence of ROIs for each organ, normalized by fluorescence of stock solutions of verteporfin. There were 3 animals per group for a total of 15 animals. Error bars are SEM, n=3.

Table 1. The ratio of mean and standard error of the mean was calculated for each tumor line versus normal pancreas at each time point. There is high variability and no selectivity of fluorescence in tumor versus the surrounding normal tissue. There was no significant difference between the ratios at any of the time points in either tumor line.

<table>
<thead>
<tr>
<th>Time</th>
<th>ASPC-1/ Pancreas</th>
<th>SEM</th>
<th>ASPC-1/ Muscle</th>
<th>SEM</th>
<th>Panc-1/ Pancreas</th>
<th>SEM</th>
<th>PANC-1/ Muscle</th>
<th>SEM</th>
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<td>1.0</td>
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<td>1.2</td>
</tr>
<tr>
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<td>0.3</td>
<td>2.0</td>
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<td>0.1</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
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<td>1.7</td>
<td>1.0</td>
<td>0.8</td>
<td>0.2</td>
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<td>0.8</td>
</tr>
<tr>
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<tr>
<td>24 hr</td>
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<td>0.3</td>
<td>1.1</td>
<td>0.9</td>
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3.2 In vivo fluorescence measurement

Subsets of animals were studied in vivo, by exposing tumor and liver under anesthesia and placing the probe on the organ of interest. First, precontrast readings were collected on each organ by averaging 10 measurements at each site, over about 5-10 minutes before verteporfin injection. After i.v. injection of the verteporfin, measurements were begun immediately and collected periodically for up to one hour. Measurements were made by placing the probe alternately on tumor and liver. The fluorescence in liver (data not shown) was more intense and followed a similar time course to that in tumor in each model. There was a rapid rise in fluorescence immediately after injection, reaching a plateau at about 15
minutes in ASPC-1 tumors. The fluorescence in PANC-1 tumors continued to rise during the 1 hr post injection. The pattern in the two tumors is shown in Figure 3.

![Graph of fluorescence over time for ASPC-1 and Panc-1 tumors](image)

**Figure 3.** In vivo measurement of fluorescence following i.v. injection of 1mg/kg verteporfin, in both tumor models. Each point represents the mean of 10 measurements. These measurements are mean values for 3 animals per tumor model.

4. **SUMMARY**

It was shown here that the uptake of verteporfin could be monitored in the orthotopic pancreas cancer mouse model both in vivo over short time periods after injection and over longer time periods in excised organs. *Ex vivo sections* were imaged at up to 24 hr after injection, and fluorescence in tumor and normal pancreas was shown to follow a similar time course to that of the liver and other organs. No selectivity of localization was shown in either tumor line at time points studied. It was shown *in vivo*, that fluorescence in tumor can be followed before injection, immediately after injection and over the first hour before sacrifice. In both tumor and liver there was a rapid rise in fluorescence within the first minutes with a plateau reached at about 15 minutes in the ASPC-1 tumor, while the PANC-1 tumor fluorescence continued to rise until sacrifice at 1 hr.

Due to the apparent lack of selectivity of porphyrin localization, it will be extremely important to deliver light precisely to the target tumor. These preliminary studies indicate that the mouse orthotopic pancreas cancer model is reproducible, and is a good candidate for further studies on the improvement of pancreatic cancer treatment through photodynamic therapy.

**ACKNOWLEDGEMENTS**

This work has been funded by NIH grant P01CA84203.

**REFERENCES**


