

ABSTRACT

This study represents the first reported use of photodynamic therapy in bone and specifically, as a treatment for spinal metastases. A metastatic model in rat confirmed the efficacy of benzoporphyrin derivative monoacid-mediated PDT for treating lesions within the spine and appendicular bone. Fluorimetry confirmed the selective accumulation of drug into the tumor(s) at 3 hours post-injection. 48 hrs post light delivery into the vertebral body of the rat spine loss of bioluminescent signal and histological analyses of sectioned spine confirmed MT-1 tumor cell kill in vivo as previously confirmed in vitro using an established cell viability assay. Porcine vertebrae provided a model comparable to that of human for light propagation and PDT response. Light measurements were recorded at 2.5 mm increments as the detector probe was retracted out of the vertebral body away from a diffusing fiber at 70-90° planar angle to it. At 30 minutes or 1hr post BPD-MA administration (6 mg/m²), light (648 J, 150 mW/cm, 690 nm) was delivered to vertebrae L1 and/or L2. Vertebrae were harvested and sectioned for histology 48 hrs following PDT. Light propagation was plotted as distance (µm) from the emitting source. Results support the application of PDT to the treatment of primary or metastatic lesions within bone.

Keywords: photodynamic, bone, metastases, spine, dosimetry, bioluminescence, cancer.

* burchshane@hotmail.com, sbisland@uhnres.utoronto.ca; phone 1-416 946 4501-5916

1. INTRODUCTION

Over 100,000 bone metastases are identified in North America each year and of those an estimated 30-40,000 cases of metastatic breast cancer lesions occur in the spine. Yet, despite this alarming statistic, the frontline approach for treating such cancers remains irrefutably unsatisfactory and the related diagnosis is often met with poor prognosis. Presently, radiation therapy (RT) is considered the mainstay of treatment for ambulatory patients, while surgery is reserved for those experiencing collapse or neurological compromise. However, RT provides only limited relief from pain, composite to cord compression, offers no stability to the spine and can adversely affect the capacity for soft tissue to repair following treatment. This in turn translates into a 3 fold increase in morbidity and mortality following surgical intervention. Photodynamic therapy (PDT) is a novel, non-radiative therapy that can directly target interstitial, metastatic lesions. PDT works by delivering an excitation light of specific wavelength to a targeted tissue containing a photosensitizer. The photosensitizer is activated by the light, which in turn initiates a photochemical reaction that leads to the generation of reactive oxygen species (ROS), predominantly singlet oxygen. Singlet oxygen can kill cells directly or damage the neo-vasculature of targeted aberrant tissue. Specificity of treatment is facilitated by the direct placement of an optical fiber cable adjacent to or within the lesion. Pithily, PDT promises to offer targeted tumor ablation without collateral damage to nearby spinal cord and little obstruction to subsequent wound repair processes within soft tissue. To our knowledge there are no published reports of this therapy being used in bone or in the spine despite its use in lung, intraperitoneal and prostate cancer. We hypothesized that PDT could be used effectively to treat metastatic disease in bone and investigated this with both in vitro and in vivo models using the human metastatic breast cancer cell line, MT-1. A bioluminescent metastatic model in the nude rat was developed to facilitate the localization, targeting and progression of lesions prior to and following PDT treatment. The transmittance of irradiating light through porcine vertebrae was also evaluated as an approach for discerning light dosimetry in a model comparable to that in the human. These findings highlight the fact that PDT can be delivered into bone and support the notion that PDT will provide a valuable alternative and/or adjunct to RT in the treatment of bone cancer without the preclusion of subsequent percutaneous vertebropasty or kyphoplasty, either for first time treatments or repeat following metastatic recurrence.
2. MATERIALS AND METHODS

2.1 Rat Model

2.1.1 Cells. MT-1 cells, a human breast cancer cell line, were kindly provided by Dr. O. Engebraaten, Norwegian Radium Hospital, Oslo, Norway. Cells were grown and maintained in RPMI media containing penicillin and streptomycin with 10% fetal bovine serum at 37 °C. At 70-80% confluence, cells were resuspended into free RPMI and harvested using a 0.05% trypsin-0.05mM EDTA solution. A cell suspension was prepared at 2x10^6 cells/mL and plated into chambered, borosilicate coverglass slides (LabTek®, Nalge Nunc International Coorp, Naperville, IL.).

2.1.2 Benzoporphyrin derivative (BPD-MA; Verteporfin®) uptake into cells. The uptake of BPD-MA (1 µg/mL in phosphate buffer solution [PBS; 45mM Na2HPO4, 5mM NaH2PO4 and 0.15 M NaCl, pH 7.4]) into MT-1 cells was visualized using epi-fluorescent microscopy (Zeiss AxioVert 200M; λex/λem = BandPass 485/20 nm and LongPass 590 nm respectively) and recorded using a CCD camera (CoolSnap HQ; Photometrics, Roper Scientific, MD, USA) attached to the microscope.

2.1.3 BPD-MA detection in vivo. BPD-MA (0.25 mg/Kg in PBS) was administered intravenously (i.v.) to 10 athymic, nude rnu/rnu rats (150-180 g, Harlan Sprague Dawley Indianapolis, IN.) through the tail vein. Animals (including controls) were then euthanized upon CO2 inhalation at 15 minutes, 3 hrs, 6 hrs and 24 hours post-injection and samples of serum, spine and spinal cord were harvested. The spine was fixed in 10% formalin for 7 days, then decalcified in 10% formic acid for a further 7 days before conducting quantitative analysis of BPD-MA fluorescence using fluorescent microscopy. Control studies confirmed that BPD-MA and its corresponding fluorescence were unaffected by the formic acid treatment. Spectrofluorimetric (Photon Technology International, London, ON. Canada) measurements of BPD-MA uptake into blood serum and spinal cord was performed on solubilized samples using a technique previously described in our laboratory.14

2.1.4 Cell viability assay in vitro. The PDT-induced cell kill was assayed in 96 well microplates using the Sulphorhodamine B (SRB) viability assay.200 µL of a 2x10^6 MT-1 cells/ml suspension was added to each well. Once attached, BPD-MA was added to give a final concentration of 1 µg/mL or 10 µg/mL and 8 hours later, cells were irradiated with 150 mW of 690 nm laser (Model LFI 4532, Wavelength Electronics, London, ON, Canada) light for a total light dose of 100 J/cm^2 or 25 J/cm^2. At 24 hours following PDT treatment, the growth media was removed from the wells and the cells rinsed with sterile PBS to remove the dead cell population. The remaining cells were fixed in 10% trichloroacetic acid (TCA; 100%) at 4°C for 1 hr. After fixation, the wells were left to dry at room temperature before adding 50µL of 0.4% (w/v) SRB solution (in 1% acetic acid). Cells were stained for 30 min at RT prior to rinsing (x5) with 1% acetic acid and dried. The cell bound SRB was finally precipitated out using 100 µL unbuffered Tris solution (Sigma; Trizma base; pH 10.5 and the resulting absorbance read at 540 nm (with 690 nm background subtraction) using a microplate spectrophotometer (Titertek Multiskan® MCC/340). Results are expressed as normalized values to the controls (cells exposed to light without BPD-MA).

2.1.5 Spinal Metastases Model. Ten nude rnu/rnu (Harlan Sprague Dawley, Indianapolis, IN) female rats (4-6 weeks of age) were anaesthetized using halothane/O2 (2%/3.5 L respectively) and then injected with MT-1 cells (2x10^6 in 200 µL) into the left ventricle using a 1ml syringe with a 26g needle. Pulsatile blood within the injection syringe confirmed that the needle was in the left ventricle. Following the injection, animals were immediately recovered and returned to their cages with free access to food and water. Animals were examined 14 and 21 days post injection for signs for paralysis and/or cachexia and the progression of metastases assessed using fine detail radiography (faxitron). By 21 days, most animals displayed profuse boney metastases within the spine, upper femur, tibia lower mandible, and occasionally in the shoulder or humerus. Some animals were excluded from the study due to the development of extensive tumor burden outside the spine (most notably the pericardium) or due to a debilitating loss of body weight (>50%). By day 23-30 animals had to be euthanized by CO2 inhalation for compassionate reasons. After PDT treatment, vertebrae and long bones were harvested and fixed in 10% formalin for 7 days. Micro-CT images of lytic lesions within the spine and/or femur were obtained prior to decalcification in 10% formic acid for 7 days and the presence of tumor within these sites confirmed histologically from haematoxylin and eosin-labelled paraffin sections using light microscopy.

2.1.6 Co-transfected of MT-1 cells for stable expression of the luciferase gene. MT-1 cells were co-transfected using dioctadecylamidoglycylspermine-trifluoroacetate salt (Transfектant; E1231, Promega) with plasmid DNA containing the luciferase gene of the fire-fly (Photinus pyralis, pGL3 control vector E1741, Promega) and a plasmid containing the neomycin resistance gene (PCI-Neo, E184, Promega). Luciferin; Beetle, Potassium salt anhydrous (E1603) Neomycin antibiotic (G-418 sulphate) were also purchased from Promega. Stable transfecnts of Luciferase-expressing MT-1 cells
(MT-1<sup>Lac</sup>) were confirmed by growing cells in the presence of G-418 antibiotic (100 µg/mL) for 10 days, at which time those colonies expressing the highest bioluminescent signal were isolated and cultured. Bioluminescent signal was analyzed using the IVIS Bioluminescent Imaging system from Xenogen corp, Alameda, California, USA. Bioluminescence of the luciferase gene was initiated following the addition of Luciferin substrate (25 µM) to cells in vitro or 30 mg/Kg i.p. in vivo. In vitro, 10 µL of Luciferin stock (0.5 mM in PBS) was added to MT-1<sup>Lac</sup> cells containing 190 µL of growth media (without phenol red). The plates were gently agitated and placed into the IVIS. In vivo, MT-1<sup>Lac</sup> (2 x 10<sup>6</sup> in 200 µL) were injected intracardially (see above) and the resulting bioluminescent signal analysed at timepoints post-treatment (0-48 hr). Bioluminescent signal was captured as the absolute total flux (# of photons emitted per steradian cm<sup>2</sup>) emitted within a 5 min integration time using the Living image™ software and plotted against time.

### 2.1.7 PDT treatment of bone metastases

At day 21 post injection, tumor-bearing animals (n = 30; rn/Lnu, 4-6 weeks of age) were anesthetized with 2% halothane / air mixture and placed into a custom made radiolucent, stereotactic jig in the left lateral decubitus position. Because lesions within the vertebral bodies could not be detected by fine detail radiography, histological analysis of bone taken from 10 animals used in establishing the metastatic model (see above) was used to confirm that for those animals with tumor, by 21 days most vertebrae contained metastases and T12 and L4 vertebrae were selected as representative levels for treatment. An 18g needle was placed onto the cortex of the targeted vertebrae or long bone with the use of a mini C-arm image intensifier. BPD-MA was administered intravenously at a dose of 2mg/kg prior to the administration of the light dose. Drug light intervals included 1 hour, 3 hours and 24 hours. 690 nm laser light (150mW output) was delivered via an optical fiber (200 µm diameter) with the use of a cylindrical diffusing tip, 400 µm in diameter, and the resulting bioluminescent signal analysed at timepoints post-treatment (0-48 hr). Bioluminescent signal was captured as the absolute total flux (# of photons emitted per steradian cm<sup>2</sup>) emitted within a 5 min integration time using the Living image™ software and plotted against time.

### 2.1.8 Statistical Analysis

Statistical significance between treatment and control groups was tested using one-way analysis of variance (ANOVA) for 95 % confidence intervals with Bonferroni correction for multiple comparisons of group means.

#### 2.2 Porcine Model

##### 2.2.1 Animal Model

Five female, Landrace pigs (46-53 Kg body weight) were used. Animals were taken off solid food 18 hr prior to surgery. Anaesthesia was induced following intravenous (i.v.) bolus injection of Ketamine (Ketalean®, 15 mL; 30 mg/Kg, BiMeda-MTC, Animal Health Inc., Cambridge, Canada), the animals were intubated and maintained under anaesthetic (2 % isoflurane (Abbott Laboratories Ltd., Saint-Laurent, Quebec) /0.4 FIO<sub>2</sub>) with assist-controlled ventilation (12 breaths/min, 600 cc tidal volume) throughout the surgery. Physiological measurements included MAP, HR and PO<sub>2</sub> saturation and rectal temperature. Animals were hydrated throughout the procedure with 0.9 % sodium chloride solution (10 mL/min; Baxter Corporation, Toronto, Canada). Once stable, the animals were placed prone onto a custom-designed, radiolucent gurney to allow free access for circumferential C-arm CT imaging. Two laparoscopic incisions (4 cm) were made using a scalpel through the skin and underlying muscle proximal to the transverse processes (left and right) of vertebrae L1 and L2. A channel (~2 mm diameter) was made through each pedicle of both the left and right transverse processes into the vertebral bodies of L1 or L2. Channels within the left and right pedicles were orientated approximately 70-90% planar angle to each other through which closed-ended, optically translucent catheters were placed. The fluorescence detector probe(s) (400 µm silicon with spherical, isotropic tip) and emitting fiber(s) (2 cm cylindrical diffusing tip, 400 µm diameter silicon) were subsequently inserted into the catheters.

##### 2.2.2 Imaging acquisition

Guidance for placement of the probes was afforded by fluoroscopy and 3-D cone beam computer tomography (CBCT at 125 KVp, 4.3 mA; Varian 43030 A flat panel imager mounted on a Siemens PowerMoBIL Isocentric C-arm) imaging of the lateral and anterior/posterior aspects. The precise coordinates (x, y, z) for each probe within the vertebrae were discerned from reconstructed CT images. We acquired over 200 projections across 180° in 60 s with high resolution (1024 x 768 pixels) per projection at 388 µm/pixel that were reconstructed by cosine-
reconstruction, medium filter with 0.5 mm resolution (512 x 512 x 384; 400 µm voxels) using a Pentium 4 processor and acquisition time of approximately 6 minutes. Fluoroscopy involved spot film imaging of orthogonal pair images at 125 KVp, 4.3 mA at 0.5 resolution projections (1024 x 768 pixels).

2.2.3 Light dosimetry and analysis. Once in place, irradiating light (150 mW/cm; 690 nm) was transmitted into each of the vertebrae, L1 and L2 and light dosimetry studies were conducted by retracting the isotropic fluence detector probe and catheter out of the vertebral body towards the pedicle in 10 incremental movements each 2.5 mm apart. Measurements of fluence rate (mW/cm²) at each of the ten points within the vertebrae were made using a 4-channel PMT system as the probe was retracted and the total fluence rate approximated using both N point theory and actual values for 20 individual point sources representing the 2 cm cylindrical fluence fiber geometry. Calculations of point source fluence rate and conversion to cylindrical geometry were according to the equation devised by S. Jacques et al., 1998.

2.2.4 Preparation of BPD-MA. BPD-MA (Verteporfin®) was supplied to us by QLT Inc., Vancouver, Canada as a dry powder. The powder was reconstituted into 7 mL of sterile de-ionized water, filtered (0. 45 µm Millipore filter) and added into 23 mL of 5 % dextrose solution (Baxter Corporation, Toronto, Canada). Preparation was conducted on the day of the experiment.

2.2.5 BPD-MA-PDT in the spine. In order to negate the inflammatory/allergic reaction of the pigs to the photosensitizing agent, benzoporphyrin derivative-monoacid (BPD-MA; Verteporfin®, QLT Inc., Vancouver, Canada) benadryl® was administered (2 mg/Kg i.v. bolus) 5 mins before BPD-MA and similarly, to avoid precipitation of the BPD-MA out of solution, the saline i.v. infusion was flushed with 5 % dextrose solution. BPD-MA (0.33 mg/Kg; ~6 mg/m²) was subsequently co-injected as a slow infusion (1.5 mg/min for 10 min) together with the dextrose. At 30 mins or 1hr post BPD-MA administration, light (648 J, 150 mW/cm, 690 nm) was delivered to the L1 and/or L2 vertebral bodies for a total duration of 36 mins/treatment.

2.2.6 Post-operative. After PDT was given, the probes were removed and the small incisions through the muscle and skin sutured closed (4.0 braided vycril; Ethicon Inc., Johnson and Johnson Co., Somerville, NJ and 2.0 prolene; Ethicon Inc., Johnson and Johnson Co., Somerville, NJ, respectively). The animals were recovered to the point of being able to stand unaided before being given buprenorphine analgesic (Buprenex®, 0.01 mg/Kg, i.m. bolus) and antibiotic (2.5 mL i.m. bolus of 1500 I.U. Duplocillin® LA; Intervet Canada Ltd., Whitby, Canada). Repeat injection of analgesic were given twice daily.

3. RESULTS

3.1 Rat model

3.1.1 Sensitivity of MT-1 cells to BPD-MA PDT in vitro. A differential contrast interference (DIC) image of MT-1 cells superimposed with a fluorescent image taken 45 minutes post BPD-MA administration (Fig. 1a) confirms that uptake of BPD-MA is ubiquitous, rapid and predominantly targeted to perinuclear organelles within the cell cytosol. Moreover, once inside the cells, the effect of BPD-MA mediated PDT in MT-1 cells was demonstrated in vitro using the SRB assay (Fig. 1b). ANOVA statistical analysis confirmed a significant difference (p < 0.001) between the mean absorbance of the untreated cells versus treated (light and drug) with no statistical difference (p > 0.1) between untreated wells and wells treated with light or drug only. The administration of BPD-MA at a final concentration of 10ug/mL or 1ug/mL followed by a light dose of either 100J/cm² or 25J/cm² resulted in comparable cell viability suggesting that the PDT effect was maximal at 1 µg/mL BPD-MA and 25 J/cm² light dose.

3.1.2 BPD uptake in the serum and spinal cord. Fluorimetry was used to assay the specific uptake of BPD-MA into the spinal cord and blood serum at 15 minutes, 3 and 24 hours post injection. Fluorescence intensity versus time (not shown) revealed a rapid increase in serum drug concentration within 15 minutes post injection followed by a steady decline over the next 6 hours and returning to base line levels by 24 hours. Uptake into the spinal cord was less obvious with a slight increase in intensity at 3 hours that cleared by 6 hours post injection. The presence of BPD-MA within the neuronal cell bodies of the spinal cord and the bone marrow of the vertebrae was also evident using fluorescent microscopy at 15 minutes and 3 hours respectively (not shown). BPD-MA related fluorescence was not evident in either structure at 24 hours post injection.
Fig. 1 (a) An overlay of differential interference contrast and fluorescent microscopy images showing the presence of BPD-MA predominantly localized to perinuclear sites within the cytosol of cells after 45 minutes incubation at 37°C, 95% O₂. Magnif. 63x. (b) SRB assay confirms the sensitivity of MT-1Luc cells to BPD-MA-PDT using 1 µg/mL BPD-MA and 25 J/cm² (lane 4) or 100 J/cm² (lane 6) which was not significantly altered using 10 µg/mL BPD-MA and 25 J/cm² (lane 5) or 100 J/cm² (lane 7). Results are expressed as % viability of untreated cells (lane 1) with 10 µg/mL BPD-MA (Lane 2) or 100 J/cm² (Lane 3) light treatment alone included as controls.

3.1.3 The spinal metastases model and quantitative assessment of the BPD-MA induced PDT effect using MT-1Luc cells. 7 out of 10 animals developed metastatic disease. The mean survival for animals with tumor was 25 days. Four of the animals showed palpable tumors in the femur and tibias as well as the lower mandible. Two animals developed hind leg paralysis secondary to metastatic disease. All animals with tumors became cachexic. The affected animals appeared well until day 18 after which the animals developed rapid weight loss and overt tumors. Faxitron indicated lesions within the humerus, femur and tibia as early as day 14 in some animals (Fig. 2a). However, lesions could not be detected in the vertebrae of any animals by day 21 using Faxitron. Micro-CT analysis of the thoracic and lumbar spines of these animals showed multiple lytic lesions within the vertebrae (Fig. 2b) and tibia (not shown). The mean area of the lytic lesions within the lumbar and thoracic vertebrae was 2.92 mm² and 2.14 mm² respectively. The lesions approximated 1/3 of the vertebral body size in both of the lumbar and thoracic vertebrae that were imaged.
Fig. 2 Osteolytic lesions secondary to tumour infiltration were evident in a number of sites throughout the rat. The loss of radio-opacity at the proximal head of the femur (a; defined by arrow) is clearly evident using high definition radiograph (Faxitron®) of rat vertebrae and left femur at 21 days post i.c. injection of MT-1 cells. Lesions within spinal vertebrae were less obvious to demarcate using this technique. Corresponding images using micro-computer tomography x-ray of rat vertebrae, with sagittal and transverse views (b) clearly define the metastatic lesions within the bone.

Histological analysis of the vertebrae confirmed the presence of osteolytic tumor within the long bones and vertebrae of the affected animals. All animals showed localization of bioluminescent signal to the spine or long bones by day 21. However, the bioluminescent signal intensity was quite variable. Nine of the twenty animals had either gross visible tumors or cachexia. Bioluminescent imaging of these animals showed a similar pattern of metastases among these animals. High signal was obtained from the lumbar and thoracic spine, the humerus, lower mandible, femur and tibia in addition to the lung. The use of a custom made stereotactic radiolucent jig facilitated localization and targeted treatment of bioluminescent metastases. Targeted lesions treated with 25 J of light with a 3 hour drug light interval showed a decrease in tumor growth of 66% compared to that of the control lesions. No effect was seen when light was administered at a 24 hour drug light interval or in control animals with light or drug alone. Targeted lesions treated with 150 J of light with a 3 hour drug light interval reduced the signal from the targeted site by 87 % as compared to control lesions 48 hours following treatment (Fig. 3). Hind leg paralysis was seen in animals when treated at the 3 hour drug light interval at T12 with light doses of between 50 J and 150 J but not 25 J. Furthermore, no paralysis was seen at the 24 hour drug light time interval in animals treated with 150J at T12 or L5 level of the spine and no paralysis was seen in hind legs of rats following treatment with 150 J directed at the distal femur. Four of the animals had high signals within the chest cavity and gross dissection revealed large metastatic tumors within the lung and pericardium. Six animals showed a diffuse weak bioluminescent signal localized to the thoracic and lumbar spine.
Fig. 3 Photographs of bioluminescent MT-1\textsuperscript{Luc} lesions in the spine and femur of rmu/rmu rats (see arrows) before (a and c) and 48 hours after (b and d) PDT with 1 μg/mL, i.v. BPD-MA, followed 3 hours later with 150 J/cm\textsuperscript{2} irradiance. The bioluminescent signal from lesions is considerably reduced and in some cases the signal is lost entirely.

3.1.4 The Effect of PDT in Vertebrae with Metastases. Subsequent histological staining with H&E, keratin and immunohistochemical staining for human EGF-r (Fig. 4a-f) confirmed the presence of human breast cancer cells within the thoracic and lumbar spine.

Fig. 4 MT-1 tumour cells (T) are highly conspicuous from surrounding bone marrow (BM) within the vertebrae using haemotoxylin and eosin histological stains (a; magnif; x10) and display high specificity for keratin staining (b; magnify; x5). MT-1 cells also express high levels of endothelial growth factor receptor (EGFr) which can be immunohistochemically visualized using a human-
derived EGFr antibody labeled with horseradish-peroxidase (c; magnify; x20). The effects of BPD-MA (2 mg/mL; i.v.)-PDT are clearly evident upon histological analysis with nuclear condensation and blood pooling within the surrounding bone marrow (d and e).

Histological confirmation of tumors within the spine was verified in only two of these animals with one animal having metastases identified in the spinal cord. Of the thirty animals injected 5 succumbed to attrition relating to tumor inoculation or drug administration. Light doses ranging from 25 J to 150 J had an ablative effect on both normal bone marrow and tumor tissue. The region of effect ranged from 2.5mm to 22mm in the rostral-caudal dimension. The effect varied in direct proportion to the amount of light given with the greatest effect being seen with 150J. However, a 75J light dose administered at a 1 hour drug light interval produced a similar effect. Histological analysis of contiguous slices through the rat spine confirmed that BPD-MA-PDT with 150 J and 24 hr drug/light interval induces wide spread apoptotic cell death (not shown) within the MT-1 metastatic breast cancer 48 hr post treatment.

3.2 Porcine Model
3.2.1 Light transmittance in vertebrae. Figure 5 shows a reconstructed sagittal CT image through the pig spine (L1) which clearly reveals the bi-transpedicular placement of treatment and detector probes into the vertebral body. The angle of insertion was critical in order to avoid damaging the spinal cord and to approximate a 90° planar angle between them. Transverse and coronal reconstructions were also analyzed to ensure close approximation between the probe tips in 3-D geometry.

Fluence rate for the cylindrical diffusing fiber implanted into the pig vertebra (L1 level) was derived as 20 individual point source values along its 2cm length.

![Fig. 5 A CBCT reconstruction (512x512x384 @396um voxels) showing the transpedicular trajectory of the probes into the vertebral body of the pig.](image)

![Fluence rates vs distance](image)

Fig. 6 Plot of decrease in fluence rates in pig vertebra as a function of distance between the detector probe and cylindrical treatment fiber displayed as normalized actual measurements (symbols) and theoretical N points matrix (line).
Each of these measurements were repeated as the isotropic detector probe was retracted out of the vertebral body in 10 increments of 2.5 mm and plotted in Figure 6 as a function of distance between detector and cylindrical diffuser (mm). The decrease in fluence rate appears to be linear and there is a close correlation (p < 0.05) between the N theory approximation values as defined by the line and the actual values measured using the detector, shown as symbols. This is particularly true for distances of ≤ 1 cm. At 1 cm distance fluence rate is decreased by 2 log values and at 2 cm the fluence rate is < 0.01 mW/cm².

3.2.2 The Effect of PDT in normal pig vertebrae. Interestingly, the effects of BPD-MA-mediated PDT were evident on histological slices through the vertebrae up to 2 cm out from the cylindrical fluence fiber. The areas of dead cells within bone marrow are clearly distinguishable from live tissue (Fig. 7) with mass areas of cellular debris and/or acellularity. This implies that lower fluence rates may be advantageous when treating lesions within bone close to critical structures such as peripheral nerve roots and spinal cord.

Fig. 7 Histological slice through the pig vertebra pre (left side) and post (right side) BPD-MA PDT. The bone marrow, including osteoclasts, are destroyed with only a few remaining cells in amongst large fat deposits.

4. DISCUSSION

Breast cancer continues to be the most prevalent cancer accounting for an estimated 211,300 new cases in 200317 and at least 30 % of patients with systemic malignancies will develop spinal metastases during their disease18,19,20. Cancer in bone is often accompanied by very poor prognosis for long-term survival beyond 1-2 years, depending on the primary malignancy. Moreover, the chronic morbidity associated with spinal metastases, specifically, can be the defining factor predicting survival with intractable back pain due to lesions in one or more vertebral bodies encroaching onto the spinal cord, loss of bowel and bladder function, paresis and paralysis. As a consequence, the patients’ quality of life is often markedly depreciated and declines sharply with increasing longevity. Yet, despite this alarming statistic for mortality and chronic morbidity, the treatment options for such lesions are limited and disturbingly inadequate. In the ambulatory patient the mainstay of treatment is currently radiation therapy (RT), however, results of RT for the treatment of spinal metastases have shown that only one third have complete relief of their back pain21. The shortfalls of RT are largely two-fold, firstly, that RT is a non-discerning modality, affecting both normal supporting tissues and tumor tissue alike, which can lead to myelopathy and pronounced fragility within the spine. As a result, the number of treatments as well as the range of radiation dose that can safely be administered for treating spinal tumors is limited. Secondly, that cells and tissues can rapidly develop resistance to radiation upon subsequent re-treatment(s) thus making the initially prescribed dose quickly ineffective with little option for elevating the dose due to the impending risk of myelopathy and/or bone fracture. Both of these factors contribute to the high incidence of recurrence, an estimated 33 %, of patients with spinal metastases12,22, which is further exacerbated by an increased longevity of patients with spinal metastases (average survival 2 years with breast cancer, mean 1 year survival of 78%)23. As recurrence intensifies and lesions become increasing radio-resistant, so does the need for spinal surgery. Unfortunately, RT is known to increase the morbidity of surgical intervention by as much as three fold, a procedure that is already associated with a 30 – 40% risk of morbidity and a 7-16% risk of mortality. Ultimately, alternate approaches for treating tumors within the spine and other boney metastases are urgently needed and one such potential candidate is photodynamic therapy (PDT).
In order for PDT to be a successful treatment for spinal metastatic lesions, the drug and light combination must be locally targeted to the tumor. In this study we chose BPD-MA as our photosensitizing agent given its propensity to target vasculature and/or tumor cells directly. There are several reports of BPD-MA-PDT in soft tissue tumors in the murine model as well as in an orthotopic chondrosarcoma and fibrosarcoma tumor models. Results from these studies showed a significant effect at both the 15 minute and 3 hour drug light interval with 33% of the lesions being completely ablated at 4 weeks post treatment. To our knowledge there have been no reports until now of PDT use in an in vivo metastatic breast cancer model affecting bone and the pharmacokinetics of BPD-MA into bone and the light scattering properties of bone are poorly defined. A number of studies do exist though and include Takeuchi et al., 1997, who, reported light is attenuated significantly more through cortical bone as compared to cancellous bone, which in itself may prove favourable if the intention is to deliver PDT to a single vertebra without causing collateral damage to the adjacent structures including the spinal cord. This study encompasses the transmittance of light through pig vertebrae as a model for human spine and describes the delivery of PDT in this model as well as in a small animal, rat model of human metastatic breast cancer. The size of the latter model precludes the placement of optical fibers trans-pedicularly and para-pedicular insertion would also be difficult. Consequently, fibers were placed adjacent to the targeted spinal vertebrae and our results confirm that light transmission through metastatically involved rodent vertebrae is impeded minimally by surrounding cortical bone. As such this study reinforces our hypothesis that PDT can be administered both safely and effectively providing tumor ablation without damaging spinal cord and/or peripheral neurovascular structures. Histology clearly demarcates the boundaries of PDT response revealing substantial ablation of tumor and surrounding bone marrow tissue. The incidence of apoptosis was directly correlative to the metastatic dispersion of MT-1 cells within vertebrae. Furthermore, the influence of PDT on tumor growth kinetics involving differing regiments of BPD-MA and light, was convincingly demonstrated using bioluminescence imaging of MT-1Luc cells in vivo. Bioluminescence represents a measure of cellular metabolism. The relative intensity of signal is dependent on the energetic status of the cell and reflects the availability of ATP and molecular oxygen within the cell. Indeed we have recently reported on the use of bioluminescence to monitor the tumor response following PDT. Results in this study reveal a 99.8% decrease in tumor growth 48 hours following an acute PDT treatment of 150 J delivered over the course of 16 minutes. The relative size of response area was correlative to the number of joules of light delivered with a 66% reduction in the tumor growth at 25J. It was important not to measure the bioluminescent signal during or immediately following PDT as both bioluminescence and PDT are oxygen-dependent and therefore a decrease in bioluminescent signal could be interpreted as increased cell death or a reduction in available of oxygen due to PDT. The target location on the spine and the drug light interval were the major defining factors predicting outcome and response. BPD-MA uptake into the spinal cord was negligible and time delayed by > 1-2 hour as compared to the uptake into vertebrae (15 minutes) suggesting that a therapeutic window exists during which PDT can be delivered safely without damage to the spinal cord. However, the onset of neurologic sequelae including unilateral and/or bilateral paralysis was only evident following PDT (50-150 J at 3 hours) to lesions within the thoracic spine and was not seen following similar treatment in the lumbar region. This observation is important clinically and reiterates the importance of light/drug dosing and the effects of PDT on peripheral nerve roots in addition to spinal cord. Optical properties within rat vertebrae cannot be extrapolated to that for human vertebrae. For this we chose the pig vertebrae. The pedicular anatomy in pig spine is comparable to that of human thus affording us the opportunity for developing and assessing the transpedicular placement of treatment (and detecting) fibers into trabecular bone of the vertebral body. The transmittance of light through trabecular bone was measured as fluence with increasing distance between emitting fiber and the detector probe being retracted at 2.5 mm increments out of the vertebral body. The idea that light can travel whether by reflection, scattering or direct transmission, through trabecular bone is not surprising given its cavernous network. Our results imply that 150 J/cm of 690 nm laser light can travel beyond 2 cm from the source fiber, although it is conceivable that this will depend on the extent of blood pooling within the fiber tract and the age of bone. It is also clear that the transmittance of light through bone will not be the same for tumor and dosimetry for treating diffuse metastatic lesions within the spine may require higher energies than predicted from non-lesion vertebrae. It is also encouraging to note that unlike the rat, the outer layer, cortical bone in pig vertebrae serve as very good barriers to light raising the potential for treatment targeted to a single vertebra without risk of damage to adjacent structures. The PDT response in pig was consistent with that in rat with respect to bone marrow ablation and clearly demarcated boundaries of cell death surrounding the treatment fiber. The destruction of bone marrow is clinically advantageous as bone-degrading osteoclasts within the marrow display symbiotic interaction with the growing tumor cells. Given that 150 J/cm was implemented as a sub-maximal, non-thermal light regiment, it is probable that higher energies, perhaps administered at the same intensity, could be adopted to provide even larger target response without damage to spinal...
cord. The practicalities of fiber placement in this current large animal study were critical and rigorous and many of the potential hazards integral to this procedure were quickly realized. Current clinical intervention provides tumor debulking by radiation followed by surgical resection when applicable. It is likely that PDT could be added to this arsenal either in adjunct to surgery or as stand alone therapy with vertebroplasty to provide mechanical stabilization post treatment. The option for fractionated, repeat and/or metronomic PDT regimes can also be considered upon permanent percutaneous implant of optical fibers.

In conclusion, the feasibility of a minimally invasive surgical approach to target spinal metastases using photodynamic therapy has been established in this pre-clinical study. The evaluation of light transmittance through the vertebral body in a pig model reinforces the potential for fiber-based, non-thermal light delivery into bone for targeting lesions up to 2 cm or greater from the treatment fiber without damaging the spinal cord.

5. REFERENCES