Photodynamic therapy of diseased bone.

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Abstract

Objective: Photodynamic therapy (PDT) defines the oxygen-dependent reaction that occurs upon light-mediated activation of a photosensitizing compound, culminating in the generation of cytotoxic, reactive oxygen species, predominantly, singlet oxygen. We are investigating PDT treatment of diseased bone. **Methods:** Using a rat model of human breast cancer (MT-1)-derived bone metastasis we confirmed the efficacy of benzoporphyrin-derivative monoacid (BPD-MA)-PDT for treating metastatic lesions within vertebrae or long bones. **Results:** Light administration (150 J) 15 mins after BPD-MA (2.5 mg/Kg, i.v.) into the lumbar (L3) vertebra of rats resulted in complete ablation of the tumour and surrounding bone marrow 48 hrs post-PDT without paralysis. Porcine vertebrae provided a model comparable to that of human for light propagation (at 150 J/cm) and PDT response (BPD-MA; 6 mg/m^2, i.v.) in non-tumour vertebrae. Precise fibre placement was afforded by 3-D cone beam computed tomography. Average penetration depth of light was 0.16 ± 0.04 cm, however, the necrotic/non-necrotic interface extended 0.6 cm out from the treatment fiber with an average incident fluence rate of 4.3 mW/cm^2. Non-necrotic tissue damage was evident 2 cm out from the treatment fiber. Current studies involving BPD-MA-PDT treatment of primary osteosarcomas in the forelimbs of dogs are very promising. Magnetic resonance imaging 24 hr post treatment reveal well circumscribed margins of treatment that encompass the entire 3-4 cm lesion. Finally, we are also interested in using 5-aminolevulinic acid (ALA) mediated PDT to treat osteomyelitis. Response to therapy was monitored as changes in bioluminescence signal of *staphylococcus aureus* (SA)-derived biofilms grown onto 0.5 cm lengths of wire and subjected to ALA-PDT either *in vitro* or *in vivo* upon implant into the intramedullary space of rat tibia. Transcutaneous delivery of PDT (75 J/cm^2) effectively eradicated SA-biofilms within bone. **Conclusions:** Results support the application of PDT to the treatment of primary or metastatic lesions within bone. Secondly, that ALA-PDT may be useful as a treatment for osteomyelitis. Further studies aim to optimize the parameters of delivering PDT into bone and explore imaging technologies that can be used for clinical PDT.

1. Introduction

Photodynamic therapy (PDT) defines the oxygen-dependent photochemical reaction that occurs upon light-mediated activation of a photosensitizing compound, culminating in the generation of cytotoxic, reactive oxygen species, predominantly, singlet oxygen.[1] Our

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interest in delivering PDT into bone has focused on both cancer and microbial infections. We first established the concept and feasibility of using benzoporphyrin-derivative monoacid (BPD-MA)-PDT for treating metastatic lesions within vertebrae or long bones using a small animal model.[2] 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.[3,4] 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.[5,6,7] It is therefore imperative to establish that PDT can, if it is to be considered as an alternative to radiation, offer targeted tumor ablation without collateral damage to nearby spinal cord and little obstruction to subsequent wound repair processes within soft tissue. We subsequently assessed light dosimetry and PDT effects in large animal spine[8] and confirmed the application of PDT in non-tumour bearing bone together with vertebroplasty.

We also initiated studies to examine the potential application of ALA-mediated PDT as a treatment for *staphylococcus aureus* (SA)-derived infections leading to osteomyelitis.[9] Osteomyelitis refers to an acute or chronic inflammation of bone and bone marrow secondary to contamination of pathogenic microorganisms (pyrogenic bacteria, mycobacteria, fungi).[10] SA is the pathogen most commonly isolated in this and most other types of osteomyelitis, implicated in 60-90 % of childhood osteomyelitis and greater than 55% in adults.[11,12] Whether by etiology or clinical presentation, the predominant factor predicting the progression of the disease and therapeutic outcome, regardless of anatomical location, is vascularity. Trivial traumas involving skin lacerations or bruising are often the culprit leading to blood-borne bacteremia, while avascular focus resulting from peripheral vascular disease, burns or soft tissue disease are usually prone to contiguous infection of neighboring tissues. This is particularly true for diabetics or patients with sickle cell anaemia, for whom the incidence of skeletal infection is increased approximately 20-1000 times, respectively.[13,14] The results of our study in which SA-biofilm laden wires were implanted into the medullary cavities of rat tibiae were conclusive: that ALA-PDT could be effectively delivered into diseased bone providing transient abatement of infection.[9] Issues of optimization persist, however, regarding how best to orientate the maturation of PDT as a stand-alone therapy in the clinic and this is the subject of current and future studies. Particularly for bacterial infections, it will be necessary to confirm that PDT can be delivered as an effective regiment without increasing the likelihood of contiguous spread of the infection following damage to surrounding soft tissues. All the current regiments of PDT involve acute, single or repeat (fractionated) high drug and light dose. One approach that we are developing termed metronomic PDT (*m*PDT) involves a protracted, low dose drug and light delivery. Results treating glioma-derived tumours in rat brains confirmed that the

number of cells dieing by apoptosis could be increased significantly as compared with the single or fractionated acute PDT regiments.[15] We intend to establish whether a similar effect can also be achieved for bacterial infections.
There are very few reports describing the use of PDT to treat structural lesions within bone.[16,17] Similarly, PDT treatment of biofilm-producing SA *in vivo* has been described previously by other groups[18], however, thus far, the analogous studies relating to osteomyelitis *in vivo* have not been reported. In this manuscript we discuss our experimental methodologies and results thus far together with technological tools that may be symbiotic to the advancement of PDT as a viable, front-line treatment for diseases within bone.

2. Experimental models of PDT in bone.

We used a similar rat model to that first described by Engebraaten *et al.*,[19] for initiating human breast cancer-derived (MT-1) metastatic lesions within bone[8] except in our studies, the MT-1 cells were initially transformed to express the luciferase gene and resistance to neomycin antiobiotic[20] In this way, transformed MT-1 cells ($MT\text{-}1^{Luc}$) could be selectively cultured *in vitro* and visualized *in vivo* using bioluminescence. Typically, within 21 days following intracardiac injection, rats (rnu/rnu) had developed multi-focal metastatic bone disease that was subsequently treated with BPD-MA (1μg/mL i.v.) and light (690 ± 5 nm; 150 J/cm^2) 3 h later. Fig.1(i) A and C show bioluminescent lesions (arrows) prior to PDT within the spine and femur, respectively. Bioluminescence is almost entirely lost 48 h following PDT (Fig. 1(i) B and D). Light was administered via parapedicular placement of an optical fibre adjacent to the diseased vertebra. Bioluminescent lesions were visualized using the IVIS (Xenogen corp., Alameda, CA, USA) shown in Fig. 1(ii).
Fluoroscopy provides limited information discerning the extent of the tumour based on lytic degradation of the bone. By contrast, Faxitron™ (Faxitron X-ray corp., Wheeling, IL, USA) virtue of its superior contrast, can be used for this purpose or alternatively, in euthanized animals, micro-computer tomography (μCT) can provide very precise 2 or 3-dimensional reconstructed images of the osteolysis in tumour-bearing vertebrae [Fig. 1(III; see arrows)]. Fluoroscopy is, however very useful for snap-shot or real time imaging in small animal models to assist with precise placement of delivery fibre(s) onto target, bioluminescent vertebrae. In this instance a stereotactic grid was used in combination with fluoroscopy [Fig. 1(IV)]. It is interesting to observe that, as is often seen in humans, these rats display metastatic disease throughout the spine and is no doubt integral to the rapid onset of morbidity within a few weeks of injection.
Subsequent histological analysis of treated lumbar vertebrae confirmed the eradication of tumour as well as bone marrow 48 h following BPD-MA PDT with a 3 h interval been drug and light administration[2]. Of clinical importance, was the propensity (in 39 % of animals) for unilateral or bilateral paralysis following similar PDT protocols in thoracic vertebrae, highlighting the need to protect the spinal cord from PDT-induced damage at relatively short drug/light intervals, when the BPD-MA is still present within the vasculature. Paralysis was not seen when irradiating at later time points (24 hr post BPD-

MA injection) even with comparatively high light dose, 150 J/cm^2. 24 hr after injection, remnant BPD-MA is largely confined to cellular compartments.

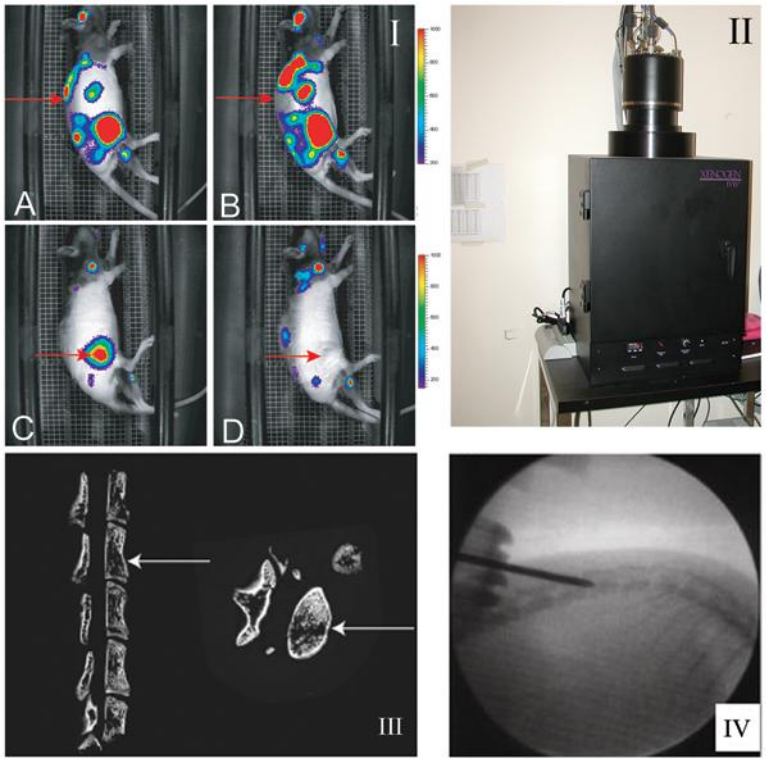


Fig. 1. (i) Photographs of bioluminescent MT-1Luc lesions within spine and femur of rnu/rnu rats (see arrows) before (A and C and after (B and D) BPD-MA-PDT. (ii) The IVIS bioluminescence detector. (iii) Micro-CT images provide vivid assessment of osteolytic lesions secondary to tumor infiltration were evident along the rat spine. (IV) Target vertebrae were irradiated upon precise paraventricular placement of an optical fiber using fluoroscopy.

Large animals were used to assess light dosimetry and the effects of BPD-MA PDT on normal, non-tumor-bearing vertebrae.[8]

Mature Landrace swine were used to address the technical feasibility of fibre placement and photodynamic response using BPD-MA in normal cancellous bone. This model also allowed better appreciation of advanced imaging technologies that could be used in conjunction with PDT to allow accurate transpedicular placement of fibers inside the bone. We used a cone beam CT unit with C-arm (Siemens PowerMobil) and flat panel detector to allow large (256x256x192), high resolution (2048 x 1536 pixels at 194 μm pixel pitch), 3-dimensional (D) reconstructions from 2D projections[21] with numerous fields of view (see Fig. 2 a-f). Light dosimetry revealed a local fluence of 9.3 J/cm^2 at the necrotic/non-necrotic interface using 150 mW/cm delivered dose. Histology revealed a necrotic radius of 0.59±0.02 cm out from the delivery fiber. These results are very encouraging and confirm the feasibility of delivering light into trabecular bone, not surprising given its highly scattering, cavernous network. Imaging tools that provide intra-operative navigation for placement of fibers, validation of response and registration for re-location upon follow-up will be pivotal for PDT acceptance into clinic. Cone beam

CT appears to be a very strong candidate. It will be important to integrate modalities for measuring response, perhaps magnetic resonance imaging (MRI), at least for soft tissues (tumours) and bone marrow. We are currently focused on providing a multi-modal platform for treatment planning based on physiological parameters governing photodynamic effect such as photosensitizer concentration, tissue oxygenation and light together with co-registration of pre- and intra-operative imaging to allow accurate fibre placement.

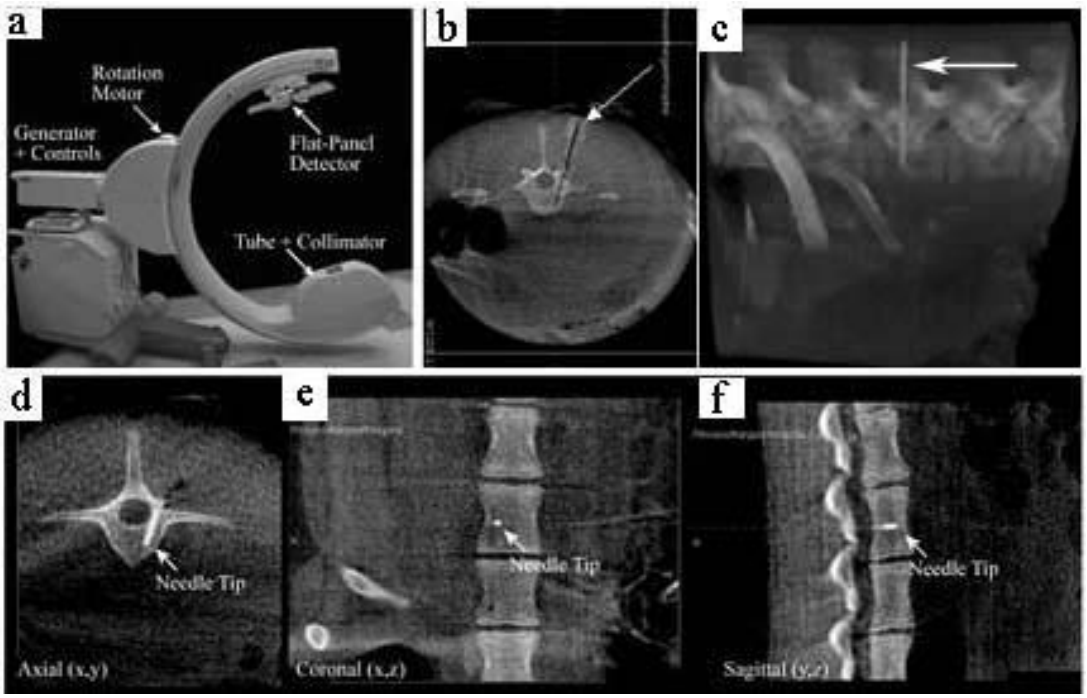
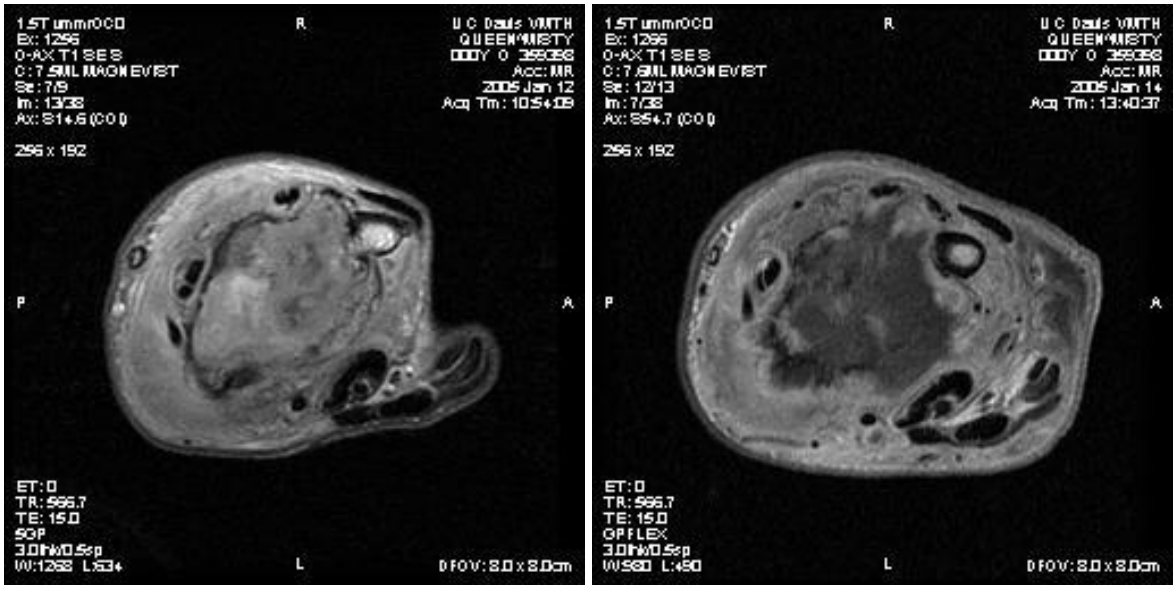


Fig. 2. (a) Cone beam CT unit with C-arm and flat panel detector. (b) Axial reconstruction showing transpedicular placement of the delivery fiber held in place using a custom-designed bone screw device (arrow). (c) 3D reconstruction showing a cannula in vertebral body of L1. (d-f) alternate, 2D views of inserted cannula.

Ultimately, one would hope that on-line treatment could be provided with real-time feedback of response. Despite the many software packages that exist for finite element interpolation and intra-operative navigation, few reports have yet to demonstrate unequivocally the application of intra-operative feedback parameters for optimizing therapy.

Recent studies have demonstrated the use of BPD-MA-mediated PDT to treat primary osteosarcomas in the radii of dogs. Patient dogs were treated at the University of California Veterinary College in Davis, USA with a single acute PDT regiment. BPD-MA (0.4 mg/Kg) was administered as an intravenous infusion over 10 mins followed 5 mins later by laser light (690 +/- 5nm). Light was delivered into the intramedullary located tumour using an optical fibre (0.94 mm diameter) with cylindrically diffusing terminals 5 cm in length. The total delivered fluence was 600 J at a rate of 200 mW/cm. The response of tumours to PDT was assessed at 48 hr post treatment using MRI. Patient

dogs were also scheduled for limb amputation allowing histological analysis of the treatment site. An example of MR images taken before and after PDT treatment is shown in Figure 3 (a and b). The osteosarcoma fills the entire circumference of the medullary cavity extending from the lower-radius to the wrist. Following treatment (Fig. 3b) the margins of treatment conform very closely with the pre-operative MR of the tumour. Although, these studies are still ongoing, we are very confident that the results will corroborate the substantial tumoricidal effects of PDT in these large bulk tumours. It is also clear that PDT can provide remarkable tumour control even for these large, bulk tumours and if used in combination with surgical resection and/or repeat PDT regiments, could provide complete tumour eradication with what is essentially a minimally invasive approach. Ultimately PDT may be able to offer limb salvage for similar large primary lesions in bone. A number of studies are currently on going to address the safety and efficacy of using PDT for the treatment of bone cancers and despite the fact that our initial focus has been breast cancer-derived metastatic disease in spine, the epidemiological and etiological will undoubtedly encompass a large number of potential cancer-related indications including, multiple myeloma, chondrosarcoma, hemingioma, and Ewing's sarcoma to name but a few.



a b

Figure 3. (a) Axial T1 weighted gadolinium enhanced MR scan of a canine radius before PDT treatment. The osteosarcoma complete fills the medullary cavity extending from the lower radius to the wrist. (b) An MR scan 48 hr following a single PDT treatment using BPD-MA reveals a vast area of PDT-induced cell death spanning the entire breadth of the tumor.

It is perhaps even more tempting to consider the application of PDT not only for cancers within bone but also as a therapy for bacterial infections within bone. There have been a number of reports describing the application of PDT as an anti-microbial therapy *in vitro* and *in vivo*.[22] We are however the first group to describe the treatment of SA biofilms

within bone directly, although similar implants have been described previously.[23] We again adopted fluoroscopy and bioluminescence as our primary imaging tools in a rat model of osteomyelitis (see Fig. 4 a-d, below). A bioluminescent strain of gram +ve SA (Xen29) was grown as biofilm onto lengths (0.5 cm) of wire and implanted into the medullary cavity of rat tibia. 10 days following implant PDT was administered involving ALA (300 mg/Kg, i.p.) followed 4h later by exposure to light (635 ± 10 nm; 75 J/cm^2). An acute regiment of PDT resulted in a substantial decrease in % (of pre-treatment value) bioluminescence at 24 and 48 h post treatment as compared to contra-lateral tibia that received no light (Fig. 4d). In all cases, however, an increased bioluminescence was evident within days of treatment indicating the SA infection had recurred and this often occurred in regions well beyond the initial infection site. Despite this however, the actual number of viable SA colony forming units (CFU/mL) surviving within the isolated tibia was usually reduced indicating that the recurrent infection was largely confined to the surrounding muscle and tissues and not the bone. In order to determine the CFU/mL, tibia were excised and cleared of soft tissues before being mashed into slurry containing bacterial growth medium using a mortar and pestle. It is likely, therefore, that the preponderance for re-infection is to some extent determined by the PDT-induced damage to neighbouring soft tissues. We hypothesize that we can perhaps minimize the extent of collateral damage to neighboring tissues by delivering a *m*PDT regimen directly onto the infection. The rational for ALA-mediated PDT extends from the elevated levels of porphyrin that can result, namely coproporphyrin (III) in Gram positive strains and protoporphyrin IX (PpIX) in Gram negative strains.[24] Indeed, many bacteria virtue of their rapid metabolism, display heightened endogenous porphyrins that could potentially facilitate specificity using PDT without the addition of exogenous substrate although, this remains to be seen. ALA is an appropriate compound for *m*PDT as it can be administered chronically via the drinking water at therapeutic doses without side effects and its mechanism of action is governed by the metabolic state of the cells. It is not clear yet whether our ability to treat SA biofilms within bone offers any selectivity toward the bacterial cells versus the resident bone marrow cells since established biofilms are known to be largely senescent when compared to planktonic cultures.

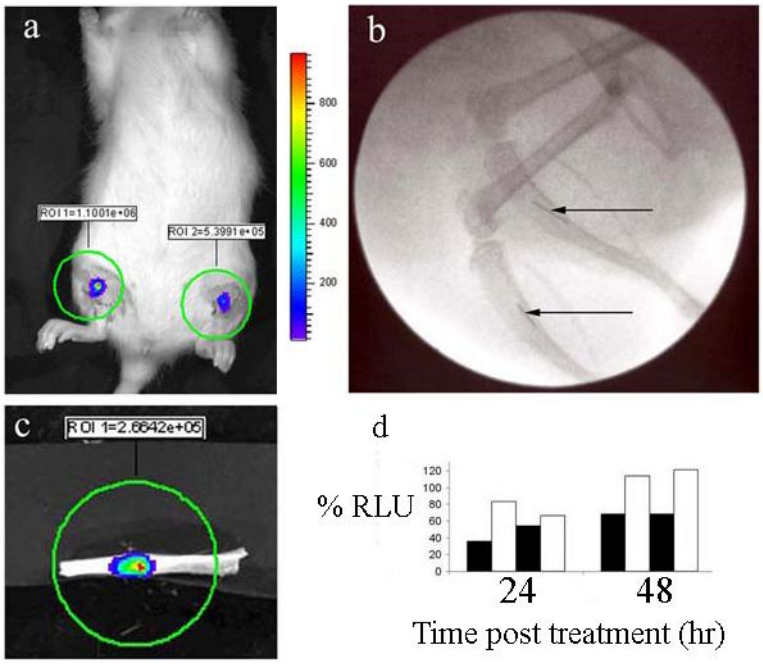


Fig. 4. a) IVIS image of bioluminescent Xen-29 biofilms 24 hr post implantation into the right and left tibia of a rat. b) Fluoroscopy confirmed the precise location of the same biofilm-coated wires within the tibia (see arrows). c) An excised tibia clearly demonstrates the presence of viable bacterial infection confined to the wire. d) Relative light units (RLU; % of bioluminescence before treatment) revealed a considerable difference between untreated (□) and treated (■) tibia of the same animal (n=2).

3. Conclusions

Our studies demonstrate that the application of PDT as treatment for diseased bones including metastatic lesions and microbial infection is possible and therapeutically efficacious. Moreover, PDT is not hampered by the limitations associated with current regiments of treatment including collateral damage following radiation and the increased resistance of microorganisms to antibiotics. The use of PDT to treat primary and secondary cancerous lesions within bone via minimally invasive procedures offers great potential and the rational for developing PDT, certainly as a palliative therapy for patients recalcitrant to current regimens, seems very promising indeed. It will be important, however, to establish safe and reproducible outcomes regarding fibre placement into the spine and response to treatment. Both of these will require choreographed pre-treatment planning in which optimized fibre(s) placement is calculated according to the light dosimetry of the vertebrae and tumour. Currently, we are initiating our photodynamic action while the photosensitizer, BPD-MA is still within the vasculature. It is proposed that given the high vascularity of bone and tumour alike, the PDT-induced lesion will be maximal and since preservation of the surrounding bone marrow is not a prerequisite, and

its eradication together with the tumour may actually prove to be more efficacious[8] it is unlikely that a cellular targeted therapy could be as effective. We are, however, conducting extensive studies of the pharmacokinetics related to BPD-MA in tumour-bearing vertebrae in order to optimize the interval of time between drug and light delivery. Ultimately, patients with metastatic disease seldom present with localized lesions, it is more common that the disease is both diffuse and multi-focal. It is imperative therefore to develop strategies that allow for prolonged or repeat treatments. One strategy that we are investigating is *m*PDT. It could be proposed that implantable light sources that deliver low dose drug and light into the tumor may be utilized in such a way as to allow the patient greater management of his or her disease without the draw backs of debilitating side effects.

Anti-microbial PDT has been conducted since the early 1970s, however, only recently has there been renewed interest in the concept of using endogenous porphyrins to treat bacteria.[22,25] A number of studies have now confirmed the anti-microbial action of ALA-PDT and is becoming increasing evident that distinct differences exist between Gram positive and Gram negative strains as far as how the ALA is metabolized.[24] Indeed, the fact that coproporphyrin is largely responsible for the photoinactivation of Gram positives like staphylococcus aureus and is not accumulated within eukaryotic cells, could be a means of increasing therapeutic selectivity. Furthermore, the excitation spectra for PpIX and coproporphyrin III at the near infra-red region may be sufficiently distinct to allow selective excitation of one metabolite and not the other.[24] The incidence of osteomyelitis in humans is increasing particularly in accordance with increased use of prosthetic implants. Our *in vivo* studies address the use of ALA-PDT to treat bone implnated SA-derived biofilms grown onto a small length of metallic wire in an attempt to mirror the prosthesis in patients. A number of issues relating to these studies remain to be verified, including the levels of coproporphyrin III that result following ALA adminstration in the SA biofilm, the penetration of ALA into cells within the biofilm layers as compared with cells in planktonic cultures. Finally, the penetration of light (at 615-635 nm) into long bones and the biofilms must be calculated before optimization of this procedure can be considered such as the use of *m*PDT to reduce the risk of recurrence and minimize damage to surrounding tissues.

It will be critical for future studies to confirm that *m*PDT is effective at treating bacterial infections within bone without augmenting the spread of infection into soft tissues, as appears to be the case following the acute PDT regimens. Moreover, although the endpoints of our current study do not assess the progression of osteomyelitis per se, but rather the persistence of bacterial infection, it will be important to for future studies to substantiate directly that PDT using ALA can prevent or significantly reduce the likelihood of osteomyelitis in this model. We have recently acquired a digital Faxitron™ unit (MX-20 digital; Faxitron x-ray corp.) that will allow us to follow the progression of osteolytic degradation within the bone. It will be important for future studies to gear the maturation of PDT in bone toward the clinic with further integration of modern imaging and navigational technologies that allow for co-registration of pre- and intra-operative treatment planning strategies with real-time feedback of therapeutic response.

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