Dissertation for the degree of PhD

Photochemical Internalization (PCI) as a treatment modality for leiomyosarcoma. Predictive factors for treatment response

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Mærradalen, a snowy February in 2018

Simen Sellevold

ABBREVIATIONS

AI	angiogenic inhibitor
ALA	aminolevulinic acid
AlPcS _{2a}	aluminium phthalocyanine with two sulfonate groups on adjacent phthalates
AO	acridine orange
AUC ₉₀	area under the enhancement curve (over the first 90 secs. after injection of contrast agent)
BLEO/BLM	bleomycin
BSO	L-buthionine sulfoximine
BVAF	blood vessel area fraction
BVD	blood vessel density
BVS	blood vessel size
CAF	cancer associated fibroblast
CT	computer tomography
DCE-MRI	dynamic contrast enhanced magnetic resonance imaging
GPx	glutathione peroxidase
GSH	glutathione
Gy	grey
H/E	hematoxylin and eosin
HIF-1 α	hypoxia inducible factor 1 α
HR	homologous recombination
IHC	immunohistochemistry
ISC	inter-system crossing
IU	international unit
IVIS	in vivo immunofluorescence imaging system
LD ₅₀	lethal dose, required dose to kill 50% of cells

LDL	low density lipoprotein
LMS	leiomyosarcoma
MRI	magnetic resonance imaging
MVD	microvessel density
NHEJ	non-homologous end joining
NT	no treatment
PCI	photochemical internalization
PCI _{BLEO/BLM}	PCI of bleomycin
PDT	photodynamic therapy
PET-CT	positron emission tomography-computer tomography
PS	photosensitizer
PTEN	phosphatase and tensin homolog
RIP	ribosome inactivating protein
ROI	region of interest
ROS	reactive oxygen species
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
SOD	superoxide dismutase
STS	soft tissue sarcoma
T2W	T2-weighted
TPCS _{2a}	meso-tetraphenyl chlorin disulfonate
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VDA	vascular disrupting agent
VEGF	vascular endothelial growth factor
WT	wild type

LIST OF PAPERS

Paper 1: “Impact of genotypic and phenotypic differences in sarcoma models on the outcome of Photochemical Internalization (PCI) of bleomycin”

Cathrine E. Olsen, Simen Sellevold, Theodossis Theodossiou, Sebastian Patzke, Kristian Berg

Published in Photodiagnosis and Photodynamic Therapy, Aug. 2017

Paper 2: “Photochemical Internalization (PCI) of bleomycin is equally effective in two dissimilar leiomyosarcoma xenografts in athymic mice”

Simen Sellevold, Qian Peng, Ane S. V. Fremstedal, Kristian Berg

Published in Photodiagnosis and Photodynamic Therapy, Sept. 2017

Paper 3: “Leiomyosarcoma xenograft and normal tissue responses to Photochemical Internalization (PCI) of bleomycin. Vascular characteristics as predictive markers for treatment response”

Simen Sellevold, Tiril Hillestad, Ane S. V. Fremstedal, Tord Hompland, Qian Peng, Kristian Berg

Manuscript, Feb. 2018

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INTRODUCTION

The ongoing research and development of curative treatments and treatments for improving and prolonging the lives of cancer patients is tremendous. Curation or permanent inhibition of malignant disease is the ultimate goal and different approaches are made, based on increasing understanding of the mechanisms involved in carcinogenesis. According to the World Health Organization cancer represented in 2012 14.1 million new cases and 8.2 million deaths worldwide (<http://gco.iarc.fr/today/fact-sheets-cancers?cancer=29&type=0&sex=0>, accessed Feb. 2018). In the vast majority of cases, metastatic disease is responsible for the mortality, and may present even after successful local therapy. Cancer treatment is directed towards the primary tumour and/or metastatic lesions, commonly comprising surgery, chemo- or radiotherapy, each alone or in different combinations. In addition, hormonal therapy has shown to be efficient in some cancers. Emerging new treatment strategies include targeted delivery of cancer drugs, immunoactivation, and different ways of inhibiting or obliterating blood supply to tumours. Recent efforts in research on targeted and biosimilar antibody drugs show results, and more than 30 antibody drugs are approved in treatment of not only cancer, but also chronic inflammatory diseases, autoimmune diseases, cardiovascular disorders, ophthalmic diseases, osteoporosis and in transplantation surgery [1]. As in all medical treatment, the objective is to achieve maximum effects with a minimum of adverse effects, and the resources and costs associated with the development of these new therapies are staggering. Cost-effectiveness and feasibility for implementation of new cancer treatments in third world countries are demanded in a global perspective.

Photochemical internalization (PCI) is a novel principle for targeted delivery of macromolecules into cancer cells [2]. PCI has been proven to potentiate the biological activity of several macromolecules and other substances by facilitating transport across tumour cell membranes [3]. Furthermore, PCI obliterates blood supply in the tumours [4, 5] and also stimulates the immune system [6]. PCI is derived from the clinically established treatment modality photodynamic therapy (PDT) that involves administration of specific photosensitizing agents and application of light with a corresponding wavelength.

Photodynamic therapy, PDT

In 1895 the Dane Niels R. Finsen published the article "The red light treatment of small-pox" in The British Medical Journal [7]. While light in medical therapy has been in use for thousands of years, the era of modern light therapy begins with the awarded Nobel prize in medicine in 1903 to Finsen "in recognition of his contribution to the treatment of diseases, especially lupus vulgaris, with concentrated light radiation, whereby he has opened a new avenue for medical science" (http://www.nobelprize.org/nobel_prizes/medicine/laureates/, accessed Feb. 2018). Finsen and others observed positive effects of light on skin diseases and furthermore that interaction with certain chemicals, later termed photosensitizers (PSs),

could induce cell death [8]. Medical treatment by means of a photosensitizing agent and illumination with light with an appropriate wavelength is now termed photodynamic therapy (PDT) and has been widely utilized in clinical practice since the 1970's. PDT is approved for treatment of pre-malignant and malignant skin diseases [9], age-related macular degeneration [10], and cancers in cervix, lung, oesophagus and bladder [11]. Effective antimicrobial activity of PDT treatment of infections of the skin and in the mouth as well as chronic leg ulcers has been demonstrated [12-15]. In addition, the fluorescent probabilities and the affinity of the photosensitizers for tumour tissue are utilized in detection and resection of brain tumours [16] and non-invasive bladder wall cancers [17].

PDT is a targeted treatment modality requiring a specifically developed and non-toxic *photosensitizer* and an appropriate *light source*, all in the presence of oxygen in the target tissue. The photochemical reactions occurring lead to formation of highly reactive oxygen species (ROS) that directly damage or kill the target cells. Additional effects of PDT include damage to intratumoural endothelial cells that leads to vascular shutdown [18], and a significant stimulation of the immune system [19].

Photosensitizers

A photosensitizer is “a chemical entity which upon absorption of light induces a chemical or physical alteration of another chemical entity” [20]. Photosensitizers may be applied topically or administered systemically and are non-toxic. These drugs are specifically developed and patented. Common PSs in current clinical use are: Photofrin®, Foscan®, Visudyne® and Tookad®, while Levulan® and Metvix® are topically administered prodrugs for the PS protoporphyrin IX. Their chemical structures vary, but typically consist of a core: four pyrrole ring structures linked by methine bridges (a tetrapyrrole macrocycle) and additional substituents in peripheral positions. Such PSs are porphyrins and constitute the basis of PDT treatment. However, porphyrins are naturally occurring pigments that normally contain a central metal ion and are without the ability to induce photochemical reactions. To enable porphyrins to induce photochemical reactions, they may be altered by removal of the paramagnetic metal ion or replacing it with a diamagnetic metal such as Al^{3+} or Mg^{2+} . For instance, removing the central metal ion (Fe^{2+}) from heme forms protoporphyrin IX, the main PS in 5-ALA PDT. Several metallophotosensitizers with improved solubility and stability have been developed for clinical use, although they may have a lower efficacy in photoinactivation of cells (quantum yield). When synthesizing PSs, they may be tailored by altering the substituents that determine their physicochemical properties influencing on their intracellular localization, tissue distribution and pharmacokinetics, but usually with little effects on the photophysical properties. Phthalocyanines are PSs synthesized with benzene rings fused to each of the four pyrrolic subunits which are linked by nitrogen atoms instead of carbon as in traditional porphyrins. This makes them well suited for use in solid tissues due to increased absorption of light with longer wavelengths, i.e. red light with optimal tissue penetration [21]. Furthermore, in PDT and later in PCI, membrane located photosensitizers have been found most effective.

Chemical structures of commonly used photosensitizers:

When applying or administering a PS, it readily concentrates in tumour tissue due to a number of factors of which leaky intratumoural blood vessels with absence of lymph vessels are important. Further, a hydrophobic nature of the compounds eases diffusion directly across tumour cell membranes while increased expression of LDL-receptors in tumour cell membranes (PSs bind to circulating LDLs) and altered interstitial pH in tumours also contribute [22, 23].

Light application

Each of the different PSs reacts optimally to different wavelengths of light. A challenge in treatment of solid tumours, as opposed to superficial tumours, is the delivery of light in sufficient doses to excite the PS in the entire tumour. The optical properties of the target tissue are determined by the absorption and scattering of light, together with the tissue refractive index [24] and must be taken into account when planning treatment. Narrow bandwidth lasers that correspond with different PSs increase the tissue penetration when compared to conventional arc lamps [25]. Furthermore, by placing light fibers directly into the tumours guided by ultrasonography [26] or CT [27], the problem with light delivery can be circumvented. In solid tumours and *in vivo* experiments, light sources with wavelengths from 600-800 nm ensure sufficient tissue penetration and generation of ROS [28]. Light with wave lengths above 800 nm does not provide enough energy to excite oxygen to its singlet state [29]. Even so, red light from a laser loses ~50% of its energy per 1.5-2mm of tissue [30] and delivery of sufficient doses may pose a challenge. Hence, the clinical setting dictates the preferred method of light delivery. If a tumour is superficial, light may be applied directly on the overlying skin. In deep seated parenchymal tumours or in body cavities, light may be applied via endoscope. In other locations, intratumoural placement of optic fibers may be considered, as earlier mentioned. As a combined treatment approach, PDT with illumination of the surgical cavity after marginal tumour resection has been evaluated in a clinical trial with good results [31].

Photochemical reactions

Application of light with the corresponding appropriate wavelength sets forth a cascade of reactions beginning with light absorption by the PS. The resulting excited PS (1P) is very short lived and the absorbed energy may be released as non-radiative decay or emitted as fluorescence and hence be taken advantage of in diagnostics. The third possibility is conversion from 1P to the lower energy but longer-lived triplet state 3P , through inter-system crossing (ISC). The remaining energy from 3P may still be released as non-radiative decay or phosphorescence or it may take part in reactions to form ROS, which are necessary for PDT effects. In presence of oxygen, the triplet state PS may undergo transfer of an electron (or sometimes a hydrogen atom) with the target molecule (Type I mechanism) or transfer its energy to molecular oxygen (Type II mechanism). Type I reactions result in formation of free radicals, while in Type II reactions the result is formation of singlet oxygen, 1O_2 .

Basic mechanisms in PDT and PCI. A simplified Jablonski diagram where the vibrational levels are omitted. P: photosensitizer, ISC: inter-system crossing. From: Berg et al. (2010).

Cytotoxic effects

Type I and especially Type II photochemical reactions are of significance in PDT (and PCI), depending on the local environment and molecular structures of the substrates. Type I reactions are predominant in oxygen depleted environments, and the mechanisms are less specific and poorer understood than the Type II reactions. Free radicals formed in Type I reactions are known to induce oxidative damage in membrane components and amino acids leading to tumour cell death [32]. However, most important in PDT and PCI is the formation of singlet oxygen in the presence of oxygen in Type II reactions, leading to oxidation of amino acids, unsaturated fatty acids and cholesterol, thereby causing damage to tumour cell membranes by mechanisms better understood. Furthermore, singlet oxygen may damage DNA by oxidation of guanine [20, 33]. The short lifetime of singlet oxygen ($<0.04 \mu\text{s}$) limits the radius of action to less than $\sim 0.02 \mu\text{m}$ [34], underscoring the importance of PS localization for obtaining the wanted effects. A more recent report, however, suggests that the lifetime of singlet oxygen may be significantly longer, up to $\sim 3 \mu\text{s}$ [35].

Endosomes and lysosomes are intracellular vesicles that result from invagination and budding off of the cell membrane. The process is termed endocytosis and is ongoing in nearly all animal cells. The resulting intracellular spherical vesicles consist of a membrane, including membrane-located PSs in the case of PDT (and PCI), together with surface-attached molecules and hydrolytic enzymes. Photoactivation of PS in the cell membrane insufficient to kill the cell may still suffice to induce rupture of the endo- and lysosomes and release their enzymes in the cytosol. Free in the cytosol, these enzymes may lead to direct cell necrosis or induction of apoptosis. The landmark PDT publication by Dougherty et al. from 1978 showed complete or partial response in 111 out of 113 cutaneous or subcutaneous malignant lesions (85 from breast carcinoma) in 25 patients using a hematoporphyrin derivative and a modified xenon arc lamp [9].

Vascular effects

In addition to direct cytotoxic effects, PDT has also been shown to significantly reduce tumour blood flow via effects on the tumour vasculature [18, 36-40]. Tumour vasculature has several unique morphological and functional properties that make them susceptible to

targeted approaches. The vasculature in malignant tumours is highly proliferative and sprouting, leaky due to an incomplete basal membrane, overexpresses glycoproteins and has a disorganized structure with blind ends [40-42]. In early PDT publications, vascular effects were observed as a result of platelet aggregation and vasoconstriction where the tumour microvasculature was more sensitive than normal blood vessels [43]. The vascular effects of PDT are dependent on the solubility of the PS and the drug-light interval. Lipophilic PSs exert the most potent vascular effect when serum concentrations are high, i.e. when the drug-light interval is short. Hydrophilic agents are more efficient with longer drug-light intervals, due to the tissue distribution of the PS [39]. Photosensitizers accumulate to some degree in tumour endothelial cells, and photosensitizers with improved endothelial targeting have been developed [44]. By using short drug-light intervals, circulating PSs more efficiently target the tumour vasculature before it is distributed deeper in the tissues [45]. Upon photoactivation, the result is either vasoconstriction or deformation and detachment of the endothelial cells, exposing the basal membrane. An exposed basal membrane activates circulating platelets and results in thrombus formation [40]. Furthermore, damage to endothelial cells leads to increased vascular permeability and a secondary interstitial oedema. In combination with platelet aggregates, this loss of pressure gradient leads to vascular collapse and obstruction of the blood flow [46]. The resulting reduction in supply of oxygen and nutrients to the tumour contributes to the direct and indirect cytotoxic effects of PDT. The PDT induced vascular shutdown is most pronounced in the tumour centre, while the peripheral rim has been shown to be resistant [4].

As discussed above, PDT leads to disruption and occlusion of the vasculature in tumours, but this effect is often incomplete. A partial treatment response leaves hypoxic but viable tumour tissue that has been shown to respond with an upregulation of a number of pro-angiogenic factors such as HIF-1 α and VEGF [47]. This poses a challenge as the tumour rapidly recovers through effective neovascularization. Antiangiogenic therapy in combination with photochemical treatment has been demonstrated to counteract these mechanisms and improve treatment efficacy [47, 48]. Methods other than PDT have been developed with the intent to starve tumours from oxygen and nutrients by exploitation of the vulnerable tumour vasculature. This may be achieved through several different mechanisms of action, but two main drug categories have been proposed by Siemann et al.: Angiogenic inhibitors (AIs) and vascular disrupting agents (VDAs) [49]. AIs are cytostatic molecules that attack the pro-angiogenic signaling pathways and thus prevent neovascularization. VDAs, on the other hand, are small organic molecules that act, mainly via tubulin binding, on existing tumour blood vessels and induce disruption and vascular shutdown with a pattern similar to PDT, with central necrosis and a residual viable peripheral rim [50].

Anti-tumour immune effects

The fact that chemotherapy acts as an immunosuppressant via effects on the bone marrow has long been accepted [51], however this view is currently becoming more nuanced. A

review article by Hao and coworkers cites several papers describing anti-tumour immune responses after chemotherapy administered in low doses [52]. Radiotherapy has been shown to induce anti-tumour effects outside the treated area, a phenomenon termed the abscopal effect [53]. The immunological basis for the radiotherapy-induced abscopal effect is increasingly better understood [54]. After radiotherapy (and PDT), a treatment-induced tumour cell necrosis causes acute inflammation that attracts leukocytes. Fragments of dead tumour cells, via presentation on antigen-presenting cells, are recognized as antigens by circulating T-cells which in turn are upregulated. Also, cytokine release and recruitment of dendritic cells, macrophages and neutrophils contributes to the immunogenic anti-tumour effects not only locally, but also systemically [55, 56]. Significant treatment effects outside the PDT treatment area have been reported from several *in vivo* studies. Lung metastases (EMT6 tumour cells) were significantly reduced after PDT treatment of the primary tumour [57] and mice with bilateral CT26.CL25 tumours had both tumours cured after PDT treatment of only one leg [58]. Furthermore, in PDT treated and cured animals a later rechallenge with inoculation of the same tumour cells did not result in formation of new tumours, suggesting a vaccination effect. This was first shown by Korbek et al. in 1999 [59], and has later been confirmed by other groups [60-64]. In 2017, a publication by Norum and coworkers showed the same vaccination affect after PCI treatment of a mouse colon carcinoma [6].

Schematic overview of PDT effects:

From: Castano et al. (2006)

Limitations of PDT

Although PDT has become an established treatment modality in several cancers as mentioned above, there are a number of limitations regarding PDT efficacy. Varying treatment responses in different cell lines and tumour models, and varying efficacy of different PSs have led to extensive search for relevant mechanisms for resistance to PDT.

PDT resistance is a highly complex and multifactorial phenomenon, and tumours may either have innate resistance or they may acquire resistance after multiple PDT treatments [65]. Mechanisms of resistance to cancer therapy in general are also of importance in PDT. Such mechanisms may reduce the uptake or influence the localization of PSs, by lysosomal degradation [66], reduced uptake or increased efflux of PS [67], altered intracellular trafficking or reduced activation of PS precursors or increased inactivation of PSs. Cancer cells may also have effective protective antioxidant enzymes (ROS scavengers) [68] or upregulated damage repair systems [69] that counteract PDT induced cell damage. However, a report from Casas et al. [65] concludes that resistance mechanisms to PDT and chemotherapeutic drugs in many ways are dissimilar, and thus the ground is laid for development of new strategies to circumvent tumour resistance to PDT.

Photochemical internalization, PCI

Photochemical internalization takes advantage of the principles of PDT and facilitates delivery of drugs or other active macromolecules from the circulation or interstitium into the cells' cytosol. Chemotherapeutic drugs used in traditional cancer therapy are usually unselective and high doses are required because cancer cells have mechanisms for inactivating or preventing drugs from crossing the tumour cell membrane in the active form and reach their target of action. This often results in severe adverse effects, and targeted delivery of drugs administered in lower doses may circumvent this. One such cell protection mechanism is endocytosis of surface-attached cancer drugs and subsequent lysosomal degradation. This process is a key point that may be exploited with knowledge of the mechanisms of action in PDT. By selecting PSs that localize in the endo/lysosomal membranes and drugs that are subject to endocytosis, the PCI principle was coined, on the basis of PDT [2].

Principles of action in PCI:

Clockwise from left: The drug of choice (D) binds to surface receptors and is subject to endocytosis (left) and entrapment in an endosome with PS intercalated in the membrane (top left). Application of light (red arrows) results in formation of reactive oxygen species (mainly singlet oxygen, 1O_2). The resulting photo-oxidation and rupture of endocytic membranes releases the drug into cytosol (top right) instead of degradation by lysosomal hydrolases (bottom). The drug may then interact with targets in organelles in the cytosol (T_1) or in the nucleus (T_2) and induce cytotoxic effects. From: Agostinis et al. (2011).

As mentioned above, PSs have affinity for tumour tissue due to their chemical structure and the local environment in malignant tumours. Suitable PSs for PCI are amphiphilic molecules that intercalate in the outer leaflet of the lipid bilayer of the tumour cell plasma membrane and end up in the inner leaflet of the membranes of endo/lysosomes. Endocytosis is a continuous ongoing process in all cells except red blood cells, and more so in malignant cells with a high metabolic activity [70]. The cell membrane invaginates, including surface attached molecules, is budded off and becomes a separate vesicle in cytosol. The resulting endosome contains photosensitizer in the membrane and the drug of choice is trapped in the vesicle. The drug may then remain trapped in late endosomes or lysosomes, vesicles loaded with enzymes that degrade and destroy its contents. To enable release of the trapped drug by PCI, timing is of essence for optimal results. Treatment protocols may be optimized according to the pharmacokinetics of the drug and PS and the target of action. PCI treatment commences with administration of PS followed by a carefully selected time interval, allowing for location in tumour cell and endo/lysosomal membranes. This typically takes a few hours in an *in vitro* setting and a couple of days *in vivo*. The active

macromolecule (drug), once administered, also needs time to localize, attach to tumour cell surface receptors and undergo endocytosis, a process that typically takes from 30 minutes to a few hours. Finally, application of light with the appropriate wavelength is easy *in vitro* and simple lamps with blue light may be utilized. In *in vivo* models with solid tumours, sufficient light doses may be applied as described in the PDT section above. In addition to transmembrane delivery of bleomycin and other chemotherapeutics, PCI has also been proven efficient in delivery of oligonucleotides, ribosome-inactivation protein toxins (RIPs) and genes by viral and non-viral vectors [20]. The aforementioned PCI treatment sequence has been well documented, but illumination *before* administration of the active drug has also shown to yield similar treatment results [71]. Endosomal fusions have been well described [72], and the working theory for this PCI phenomenon is that remnants of ruptured endosomes fuse with drug-laden vesicles, thereby inducing rupture and release of the drug.

Sarcomas

Sarcomas are a group of rare malignancies arising in bones or soft tissue. They constitute about 1% of all cancers and account for approximately 50 new cases per million/year [73, 74]. In contrast to other cancers, there are no geographical variations or changes in incidence over time. About 10% of sarcomas arise in bones and the predominant types are osteogenic sarcoma and Ewing sarcoma in adolescents and chondrosarcoma in older patients. Bone sarcomas represent a separate diagnostic and therapeutic challenge and are not the focus of this work. Soft tissue sarcomas (STSs) usually occur in older patients, with a median age at diagnosis of 64 years [73]. Although exceedingly rare, STS accounts for about 10% of cancers in children and adolescents. In the vast majority of cases, the etiology is unknown. Even so, some factors associated with the development of sarcoma have been identified; radiotherapy [75], lymphoedema [76] and previous trauma or surgical implants [77]. Immunocompromised patients have a somewhat higher incidence of sarcomas [78] as well have patients with inherited genetic susceptibilities, for instance mutations in the p53 tumour suppressor gene (Li-Fraumeni syndrome) [79, 80].

There are more than 50 subtypes of STS based on histopathological classification, the most common are: undifferentiated pleomorphic sarcoma, liposarcoma, leiomyosarcoma, myxofibrosarcoma, synovial sarcoma and malignant peripheral nerve sheath tumours. Approximately 75% of the STSs are classified as high grade malignant lesions. Sarcomas are of mesenchymal origin and display a high degree of genotypic and phenotypic heterogeneity. Clinical presentation of a soft tissue sarcoma is usually a painless lump. Approximately 75% of the cases are in the extremities and the median size of deep seated tumours is 9 cm. About 10% of patients with STS present with synchronous lung metastases, and more than 1/3 will die from the disease [81].

Diagnostication and treatment of STS is performed by dedicated multidisciplinary teams consisting of radiologists, pathologists, medical oncologists and surgeons. In general, STSs

are relatively resistant to radio- and chemotherapy; therefore surgery with *en bloc* removal of the tumour surrounded by a cuff of healthy tissue is the mainstay of treatment. The functional outcome following sarcoma surgery may be debilitating, depending on the size and anatomical localization of the tumour. An inadequate surgical margin is associated with increased risk of relapse and death [82-84]. In superficial or low grade STS; surgery is usually the definitive treatment. High doses of ionizing radiation (50-60 Gy) are commonly used before or after resection of deep seated high grade sarcomas. Although STSs are relatively resistant to radiotherapy, such high doses have been shown to reduce the risk of local recurrence [85]. Morbidity after radiotherapy includes skin lesions, fibrosis of soft tissue, joint stiffness, fatigue and secondary malignancies. Overall, chemotherapy in STS gives relatively small benefits in terms of survival, but may be justified in selected histopathological subtypes and cases [81, 86, 87]. Adverse effects after chemotherapy can be severe and even fatal and include hair loss, mucositis, fatigue, secondary infections, damage to kidneys, lungs, cardiac muscle etc. Thus, STSs constitute a diverse and treatment resistant group of malignancies that pose a challenge to clinicians in surgical and medical sarcoma care. There is a need for a novel and more targeted therapeutic approach with the aim to improve outcomes, reduce treatment related morbidity and possibly treat or prevent metastatic disease.

Leiomyosarcoma (LMS) is one of the most common variants of STS and arises from smooth muscle cells or mesenchymal stem cells committed to this line of differentiation [88]. All parts of the body may be affected, but the most common sites of origin are uterus, retroperitoneum and extremities, often in conjunction with large vessels, for instance in the inguinal area. Although LMSs have well defined characteristics, this group of STS comprises of a number of sub-types with a variety of karyotypes [89] with different clinical presentations and responses to therapy [90, 91]. In general, the sensitivity of LMS to chemotherapeutic treatment regimens is low [92-94] and the prognosis is poor with a 40% risk of relapse despite optimal local therapy [95]. In an attempt to improve treatment results, hormone therapy has been evaluated in hormone-receptor positive uterine LMS, but with disappointing results [96]. Likewise, anti-angiogenic therapy has been evaluated in several studies with disappointing results, according to Collins et al. [97]. Novel treatment strategies in LMS are therefore needed. p53 mutations are common in LMS and associated with a poorer prognosis [98-100] and a link between p53 mutation and BRCA1 mutations which influence on DNA-repair mechanisms suggests that further studies related to p53 status are warranted [101].

PDT and sarcomas

Pre-clinical studies evaluating PDT effects in human sarcomas were initiated by a group from Pennsylvania in 1999. In need of an adjuvant in treatment of peritoneal sarcomatosis, they conducted a nice study on Photofrin-PDT of the human Ewing sarcoma cell line A673 in nude mice [102]. The authors found that relatively high doses of light resulted in curation in five out of six animals, and that the primary method of action was vascular shutdown. However,

Ewing sarcoma normally arises in bone and is considered a systemic disease highly sensitive to chemo- and radiotherapy. Thus, this study is of limited clinical relevance and not suitable for describing PDT sensitivity in more treatment resistant STSs. Synovial sarcomas are highly malignant solid tumours of the soft tissue with known resistance to chemotherapy [103]. In 2008 Takeda and coworkers published *in vitro* and *in vivo* studies on ATX-S10•Na(II)-PDT of SYO-1 synovial sarcoma cells and tumours and found a dose-dependent inhibition and eradication of cells *in vitro*, and curation of tumours in athymic mice. The authors also found a significant reduction in local recurrences when treating with ATX-S10•Na(II)-PDT after marginal resection of the subcutaneous tumours [104]. Studies by Jin et al. on angiosarcoma, an extremely lethal variant of STS, evaluated effects of Photofrin-PDT *in vitro* and *in vivo*. *In vitro* sensitivity of the ISOS-1 angiosarcoma cells was high, and *in vivo* there was observed vascular shutdown and necrosis leading to curation in three of seven animals. A marked reduction in Ki67 positively stained cells was found after PDT treatment and was in line with a growth delay observed in the tumours that were not eradicated [105]. The fibrosarcoma cell line Meth A Sarcoma was used to evaluate PDT effects using the antiangiogenic APRPG-modified liposomal BPD-MA photosensitizer. Authors Ichikawa et al. found delayed tumour growth and increased survival, but no curations [44]. In a clinical setting, AO-PDT has been evaluated in treatment of STS together with radiotherapy (AO-RDT). The studied patients underwent intralesional or marginal surgery prior to AO-PDT of the surgical cavity and then skin closure followed by 5Gy radiotherapy (AO-RDT). After a follow-up of minimum 12 months, 19 out of 21 patients were disease free after this treatment regimen [106].

Development and evaluation of different synthetic PSs with optimal characteristics for use in PDT are ongoing. In 2016, Yu et al. treated the fibrosarcoma cell lines HT1080 and S180 *in vivo* with FC₄S-PDT. FC₄S-PDT treatment was confirmed to generate ROS and resulted in an 80% reduction in tumour volume [107]. In sum, the publications so far on PDT treatment of sarcoma show variations in PDT sensitivity in the different sarcoma models, and that the optimal PS and light doses are difficult to establish. However, as vascular shutdown seems to play a major role in PDT induced anti-tumour effects, regardless of cell line or choice of PS, physiologically relevant *in vivo* studies are called upon in further work on this subject.

PCI and sarcomas

Knowledge and documentation of PCI mechanisms of action and treatment efficacy is accumulating but the vast majority of evaluated cell lines and xenograft models have been carcinomas [3]. This is not unnatural, as the incidence of carcinomas outnumbers sarcomas by a factor of about 100:1. Sarcoma models have occasionally been used, for instance the synovial sarcoma cell lines SW982 and CME-1 which were treated with PCI of Gelonin in an *in vitro* experiment, and showed treatment responses superior to PDT [108]. *In vivo*, PCI has also been proven superior to PDT in a TAX-1 sarcoma model [109], and in a HT1080 fibrosarcoma xenograft model, the latter due to increased effects in the treatment resistant peripheral zone [4]. Furthermore, PCI-based drug delivery via the CD133 receptor was shown to act inhibitory in sarcoma cell lines SW872 (synovial sarcoma) and HT1080, as well as in

SW872 xenografts [110]. The leiomyosarcoma cell lines MES-SA and the PDT-resistant MES-SA/Dx5 were both proven sensitive to PCI of Gelonin in an *in vitro* model [111]. PCI experiments on osteogenic sarcoma and Ewing sarcoma have been conducted, but may not be clinically relevant given that these tumours arise in bones where delivery of light in a clinical setting will be a major obstacle. With these sporadic experiments, the potential of PCI in treatment of the otherwise treatment-resistant STS is still to be established.

Normal tissue damage

Although PDT is a targeted tumour selective treatment, adverse effects in adjacent normal tissue have been observed and studied. Photosensitizers with affinity for tumour tissue have been demonstrated to also localize in endothelial cells, not only inside the tumours but also in normal vasculature surrounding the tumours [112] and thus exposure to light may lead to normal tissue damage. In an animal model, skin necrosis and damage to underlying muscle tissue subjected to PDT treatment has been observed due to disruption of the microvasculature [45]. Larger blood vessels have been shown to retain their structural integrity and function despite full vessel wall loss of cellularity after PDT [113]. PDT induced damage to nerves has been observed after extreme doses of PDT, resulting in demyelination but without clinical symptoms in rabbits [114] and impaired nerve conduction in dogs [115]. In clinical practice, pain during and after PDT treatment is considered the main adverse effect and appropriate measures must be taken when conducting therapy [116]. For obvious reasons, animal studies are unsuited for evaluation of treatment related discomfort and pain. In general, PDT is considered safe with a relatively low adverse effect profile, but as PDT and PCI treatment efficacy is dose-related, systematic studies on normal tissue toxicity are warranted.

AIMS OF STUDY

Overall aims

To propagate PCI into clinical practice it is necessary to increase knowledge on the mechanisms of action in general, as well as treatment effects related to tumor phenotype and genotype. In the present studies, we have chosen leiomyosarcoma, a malignancy that is highly resistant to chemo- and radiotherapy, and never before evaluated for treatment with PDT or PCI. The aim was to evaluate treatment responses and identify similarities and differences between two dissimilar but closely related leiomyosarcoma models, MES-SA and SK-LMS-1. Characterizing and analyzing treatment responses related to genotype and phenotype will aid in the selection of patients to be included in clinical trials, as well as guiding further pre-clinical studies. PDT and PCI-induced vascular shutdown has been shown in previous studies to be most pronounced in the tumour centre and to a lesser degree peripherally. This warrants further studies on vascular characteristics predictive of anti-tumour vascular effects of PCI. Further knowledge about normal tissue toxicity is also needed at a time when PCI is under clinical evaluation.

Aims, Paper 1

In this article, *in vitro* characterization and comparison of treatment effects from PDT and PCI of bleomycin in the MES-SA and SK-LMS-1 cell lines are presented with the aim to identify genotypic and phenotypic predictive markers for treatment response. In order to achieve these aims we conducted comparative analyses including growth patterns, accumulation of photosensitizer, cytotoxic effects by clonal cell viability, cell cycle effects, quantification of DNA double strand breaks and ROS formation by flow cytometry and also western blotting for addressing apoptosis.

Aims, Paper 2

In continuation with Paper 1, an *in vivo* evaluation of treatment effects after PDT and PCI of bleomycin in MES-SA and SK-LMS-1 xenografts in a mouse model was performed with the aim to identify predictive morphological markers for response to PCI of bleomycin. The analyses performed to try to answer these questions include tumour growth delay and growth rate assessments, histopathological evaluation of treatment effects at different time points, immunohistochemical evaluation of vascular density in the tumours, immunofluorescence imaging of photosensitizer uptake and MRI evaluation of intratumoural vascular treatment effects.

Aims, Paper 3

The MRI analysis presented in Paper 2 showed that vascularity differed between the two tumour models, as well as the vascular responses. MES-SA xenografts were of particular interest due to their vascular heterogeneity and varied treatment response and therefore chosen for this in-depth analysis. With the aim to identify vascular characteristics predictive of treatment response to PCI of bleomycin, we performed analysis of blood vessels and vascular responses in different layers of the MES-SA tumours, including the peripheral zone,

by DCE-MRI and immunohistochemistry. Introduction of novel treatment modalities warrants systematic studies on adverse effects, something lacking in the development of PCI towards clinical implementation. Thus, treatment effects of PCI in non-tumour bearing thigh muscles in mice were evaluated by DCE-MRI, histopathology and clinical observation. The aim was to investigate whether there was a “therapeutic window” in dosage of PCI where treatment effects are sufficient with an acceptable amount of adverse effects.

EXPERIMENTAL CONDITIONS

Cell lines and tumour models

MES-SA and SK-LMS-1 are both human derived soft tissue sarcoma cell lines purchased from American Type Culture Collection (ATCC). Their organs of origin are the uterus and vulva, respectively. Histopathologically, they are both classified as leiomyosarcoma, meaning mesenchymal malignant tumours with smooth-muscle differentiation. These two variants of leiomyosarcoma have several morphologic and clinical similarities; however they differ microscopically and in their expression of several regulatory proteins, including p53 status. The cells were stored in liquid nitrogen and upon thawing, they were cultured under standard conditions and procedures and were not included in experiments beyond their 25th passage (splitting of cells from one flask and re-seeding). Culturing the MES-SA cells in flasks in an incubator was uncomplicated; the cells were small and rounded and occupied little space. SK-LMS-1 cells were larger; they spread out with pseudopods and occupied more space. For example, a full flask (80-90% confluence) of SK-LMS-1 cells contained ~1/10 of the number of cells in an equally full flask of MES-SA cells. SK-LMS-1 cells were firmly attached to the flasks and unless carefully detached, they aggregated in large numbers that complicated injection with fine needles. Where MES-SA cells were stationary, the SK-LMS-1 cells were found in video recordings to migrate on the bottom of the culture flasks, a trait associated with metastatic potential. MES-SA cells and tumours had a highly malignant appearance in the microscope, and readily established rapidly growing tumours in nearly all inoculated animals. SK-LMS-1 tumours, on the other hand, appeared to be of intermediary malignancy grade and were more differentiated than the MES-SA tumours. Injection of SK-LMS-1 cells resulted in tumours in approximately seven out of ten animals and spontaneous regression of SK-LMS-1 tumours was observed. The animals that developed SK-LMS-1 tumours were ready for inclusion about seven weeks after injection, as opposed to MES-SA tumours which took about nine days. To increase the probability of obtaining a tumour we decided to inject approximately five million SK-LMS-1 cells, whereas approximately four million MES-SA cells were used. Injection of tumour cells was aimed intramuscularly, immediately under the muscle fascia on the lateral aspect of the thigh. Intramuscular (orthotopic) xenograft models are considered optimal due to the local physiological environment and thereby exhibit enhanced clinical relevance [117]. The tumours were measured three times weekly by caliper, and volumes estimated using the formula: $volume = \frac{width^2 \times length}{2}$. Tumour volume for inclusion was 60-200 mm³. As discussed in Paper 3, MRI-based tumour volume measurements were performed and revealed a large discrepancy with the caliper based measurements.

Animals

Female *Foxn1*^{nu} mice were bred and kept at the Department of Comparative medicine at The Norwegian Radium Hospital under pathogen-free conditions with unlimited access to water and food. They are social animals and were kept in transparent plastic cages, never alone,

and up to ten mice per cage. All handling and procedures were performed in compliance with the Norwegian Animal Research Authority Guidelines and the experiments were approved by the institutional Animal care and use committee. The mice weighed 20-30 grams at the time of inclusion and they were randomly allocated to the different treatment groups and earmarked. Euthanization by cervical dislocation was performed when tumour volumes exceeded 1000 mm³ or after the procedures were completed.

Chemicals and drugs

For the *in vitro* experiments the membrane localizing photosensitizer meso-tetraphenyl chlorin disulfonate, TPCS_{2a} (Fimaporfin®, PCI Biotech) was used. This photosensitizer absorbs light most efficiently at wavelengths of approximately 420 nm (blue light) and 652 nm (red light). The high energy blue light does not penetrate soft tissue sufficiently for *in vivo* use, but is well suited for *in vitro* assays. The SK-LMS-1 and MES-SA cells were treated with TPCS_{2a} as described in Paper 1. For PCI treatment bleomycin, a generally poorly internalized chemotherapeutic drug was administered four hours prior to illumination.

For the *in vivo* experiments, the PCI suited photosensitizer disulfonated aluminum phthalocyanine with the sulfonate groups on adjacent phthalate rings, AlPcS_{2a}, was used. A solution of AlPcS_{2a} (10 mg/kg) was injected intraperitoneally in each mouse 48 hours prior to illumination. PCI treated animals received 1500 IU of bleomycin intraperitoneally 30 minutes prior to illumination.

To avoid uncontrolled light activation, the culture flasks were kept in the dark for the duration of the experiments, and the animals for seven days after administration of the photosensitizer.

Although PDT and PCI are considered non-toxic treatment modalities, pain during light exposure is considered one of the main adverse effects. Hence, Sevoflurane® gas anaesthesia was administered by chamber during laser light exposure and by mask during MR imaging to reduce movement artifacts in the images. The anaesthetizing drug Zoletil® was injected subcutaneously in doses of 50 µL per animal, to facilitate live immunofluorescence imaging. It was also used during MR imaging for a few animals, when Sevoflurane gas was not available.

Light sources

Illumination of cells *in vitro* was performed by using LumiSource® (PCI Biotech AS, Lysaker, Norway), a lamp consisting of four 18-W Osram L 18/67 light tubes. The lamp delivers blue light ($E_{\text{max}} = 435 \text{ nm}$) with an output of 13.5 mW/cm². The irradiance of the lamp varies <10% across the illumination area (45 x 17 cm). The lamp was switched on 15 minutes prior to illumination to ensure stable irradiance. For the *in vivo* experiments a Ceralas 0.3 W red diode laser with a wavelength of 670 nm (CeramOptec GmbH, Bonn, Germany) was used for illumination of the xenografts. Light doses ranging from 5 to 40 J/cm² were used in the different experiments. The irradiance was set to 90 mW/cm² to avoid hyperthermia and the

light was applied to the skin over the tumours and approximately 1 mm of surrounding skin while the rest of the animal was covered in aluminum foil.

Evaluation assays

In vitro

The growth rates of the cell lines were determined by use of the IncuCyte ZOOM (Essen BioScience, Hertfordshire, UK) which measures plate confluence over 90 hours. Cells were seeded in six-well plates and after cellular attachment phase contrast high definition images were acquired every three hours. The data were subjected to morphological processing, due to the dissimilar cellular morphology of the two cell lines. On the basis of these data, an estimate on the cell lines' doubling times was performed. To characterize the cell lines' differences in gross morphology, migration and colony formation in culture, LiveCell microscopy was performed using a CellObserver microscope system (Carl Zeiss, Jena, Germany). Cell viability after treatment was addressed by clonal cell survival and MTT assays. In clonal cell survival assays, surviving cells forming clones after 10-14 days are counted after staining and compared between the treatment groups. The MTT assay provides an indirect way of quantifying surviving cells by measuring dehydrogenase activity, mainly from mitochondria, via spectroscopy. To compare cell size and volumes between the two cell lines, their total cell protein contents were estimated using the DC Protein Assay (BioRad), as described by the producer. SDS-PAGE western blotting was used for quantifying and comparing the expression of superoxide dismutase (SOD) and glutathione peroxidase (GPx), which are enzymes that detoxify ROS (mainly singlet oxygen). Measurement and comparison of intracellular TPCS_{2a} accumulation was performed by flow cytometry. Flow cytometry was also used for evaluation of ROS formation, by studying the cell permeable agent 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma), which upon deacetylation in the cells, converts to non-fluorescent DCFH. DCFH is rapidly oxidized to highly fluorescent DCF by ROS. The fluorescence intensity is proportional to the ROS levels in the cell's cytosol. Furthermore, flow cytometry was used for cell cycle analyses, assessment of γ H2AX phosphorylation and apoptosis (TUNEL assay).

In vivo

To study the specificity of photosensitizer uptake in the tumours, three animals with each tumour type were analyzed in the In Vivo Immunofluorescence Imaging System (IVIS) Spectrum, Xenogen (PerkinElmer, Waltham, MA, USA). Imaging was performed 48 hours after intraperitoneal injection of AlPcS_{2a}, alongside a negative control animal. Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) was performed with a Bruker BioSpec 7.05 Tesla instrument (Bruker Corporation, Billerica, MA, USA) designed for animal research purposes and provides excellent image resolution. In Paper 2, a fixed dose of the contrast agent Magnevist® was injected in a tail vein. In Paper 3, the contrast agent Dotarem® was administered according to weight, which increases the quality of the MR image analyses. Core body temperature was monitored by a rectal probe and respiration rate with a pressure sensitive abdominal probe. Presented in Paper 3 are T2 weighted (T2W)

images which are water sensitive and highlight pathological tissue such as tumours. In Papers 2 and 3, AUC₉₀ maps are presented which provide a summation of contrast agent uptake over the first 90 seconds after injection. From the AUC₉₀ maps in Paper 3 a definition of vascular shutdown was established (AUC₉₀ <38), as well as calculations of median values in different regions of interest (ROI). Standard techniques were used in preparation and staining of the tissue sections for histopathology and immunohistochemistry (IHC). Three µm thick sections were made from the centre of formalin-fixed paraffin embedded tumours or muscle tissue and stained with hematoxylin and eosin (H/E) for examination by an experienced cancer pathologist. For IHC sections with the same thickness were immunostained by incubation with primary antibodies against CD31, CD34 and Ki67. CD31 and CD34 are especially useful for detecting endothelial cells, CD 34 being somewhat less specific with detection also of mast cells, mesenchymal stem cells, umbilical cord and bone marrow cells. Ki67 is a protein expressed in the cell nucleus in all cell cycle phases except the resting phase G₀, and thus a marker for proliferative activity in each individual cell. To estimate the areas of necrosis in Paper 2, the H/E sections were reviewed in the microscope with 40X magnification and a visual estimate of the necrotic fraction in each tumour was performed, followed by calculation of average values in each treatment group. The microvessel density (MVD) of untreated tumours in Paper 2 was estimated by counting vascular structures in the area with the most vascular structures in CD31 and CD34 stained samples in a 200X magnification field in the microscope, as described by Weidner [118]. In Paper 3 computerized calculations of blood vessel density, area fraction and diameters were performed in CD31 stained sections of the MES-SA tumours.

Statistical analyses

The software programs SigmaPlot (v.12.5) and SPSS (v.21) were used. To evaluate significance a two-sided t-test (Student's t-test) was used, except when conditions dictated use of the Mann-Whitney rank-sum test. Differences in treatment results were considered significant when $p \leq 0.05$. The Kaplan-Meier plots in Paper 2 were generated with SigmaPlot. Statistical correlations were calculated by pairwise log-rank (Mantel-Cox) analyses using SPSS 21. Synergism was found when the result of a combined treatment was significantly different ($p \leq 0.05$) from the sum of the individual treatment components.

MAIN FINDINGS

Paper 1

“Impact of genotypic and phenotypic differences in sarcoma models on the outcome of Photochemical Internalization (PCI) of bleomycin”

Leiomyosarcoma is one among many different subtypes of soft tissue sarcoma. Soft tissue sarcoma is a rare and treatment resistant malignant disease that presents as a solid tumour. In evaluation of PCI as a possible treatment of soft tissue sarcoma, the leiomyosarcoma cell lines MES-SA and SK-LMS-1 with several individual features were compared. The MES-SA cells have a functioning wild type p53, while SK-LMS-1 cells have a mutated non-functioning p53 gene; in addition there are dissimilarities in molecular expression of a number of regulatory proteins. Using these different but closely related cell lines, the aim was to evaluate and compare their sensitivity to PCI of bleomycin, and possibly identify predictive markers for treatment response.

The *in vitro* studies revealed similar doubling times between the cell lines, but differences in cell shape, size, migratory properties and colony appearance. When treated with bleomycin alone, the cell lines were equally sensitive, but the sensitivity to PDT, and especially to PCI of bleomycin, was significantly higher for the MES-SA cells. Further analyses were made in an effort to elucidate the mechanisms behind this difference.

The cell lines had a similar uptake of the photosensitizer AlPcS_{2a}, but MES-SA cells were found to produce higher levels of ROS after photochemical treatment than SK-LMS-1. A possible explanation for this is that SK-LMS-1 cells were found to have higher levels of enzymes that detoxify ROS, SOD2 and GPx1. Furthermore, we found that PCI of bleomycin induced an approximately six times higher increase of DNA double strand breaks in the MES-SA cells. This is in contrast to the MES-SA cells' ~50% higher levels of GSH, a strong antioxidant and “cell protector”. By inhibiting GSH with BSO, we found a 46% reduction in double strand breaks in the MES-SA cell line, while no apparent effect in SK-LMS-1, in line with the differences in GSH expression. However, when inhibiting GSH, which is also a substrate for GPx, we found a stronger sensitizing effect for the SK-LMS-1 cells, explained by the previously found higher levels of SOD2 and GPx1. This underscores the importance of GSH in cellular response to the photochemical treatments in this study.

Apoptotic fractions were similar after PDT and PCI up to 44 hours after treatment, but the fraction was three times higher in the MES-SA cells. This was expected and in line with p53 status, and may in part explain the higher PCI sensitivity in the MES-SA cells. This also confirms that the effects of activated bleomycin by PCI are relatively slow, in line with previous publications.

In conclusion, we have found that the p53 competent MES-SA cells are substantially more sensitive to PCI of bleomycin than the p53 mutant SK-LMS-1 cells. This may be explained by a

7-8 fold higher induction of DNA double strand breaks in the MES-SA cells, in part due to a higher expression of the antioxidant enzymes GPx1 and SOD2 in the SK-LMS-1 cells.

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Paper 2

“Photochemical Internalization (PCI) of bleomycin is equally effective in two dissimilar leiomyosarcoma xenografts in athymic mice”

This study was undertaken to evaluate PCI treatment effects in a xenograft model in athymic mice using the same leiomyosarcoma cell lines as in Paper 1.

PDT treatment was found to induce no growth delay (or curations) in either tumour model. PCI induced a significant and similar increase in the time for the tumours to reach the endpoint (tumour volume $>1000 \text{ mm}^3$) in both MES-SA and SK-LMS-1 tumours, but with no curations. Growth rates in MES-SA tumours was found reduced after PCI treatment, while SK-LMS-1 tumours had a growth arrest for 12 days followed by an exponential growth similar to untreated tumours. The MES-SA and SK-LMS-1 tumours were found to have a similar and selective uptake of photosensitizer as evaluated by fluorescence imaging. MR images and histopathological samples prior to treatment showed that SK-LMS-1 tumours were better and more homogenously vascularized than the MES-SA tumours, which were penetrated with strands of fibrous tissue containing larger blood vessels. Immunohistochemistry analyses by CD31 and CD34 staining confirmed that the SK-LMS-1 tumours had a higher vascular density than MES-SA. MR images acquired 48 hours after treatment showed a marked central vascular shutdown in both tumour models. The peripheral rim of the tumours was resistant to vascular shutdown in both tumour models, but central vascular shutdown was most pronounced in the SK-LMS-1 tumours, where the MES-SA tumours had some remaining uptake of contrast agent in the tumour centre. Histopathological analyses showed central necrosis in MES-SA tumours two days after PDT and PCI, with a rapid regrowth of viable tumour cells within a week. SK-LMS-1 tumours also showed central necrosis two days after treatment, but there was no apparent regrowth after one week. Rapid regrowth of MES-SA tumours may have been facilitated by the strands of fibrous tissue containing treatment resistant larger vessels that were identified penetrating the tumour stroma. *In vitro* the MES-SA cells were shown to be more treatment sensitive, thus it seems that direct cellular toxicity accounts for more of the treatment effects in MES-SA tumours, whereas vascular effects are more important in the SK-LMS-1 tumours. Overall, PCI treatment was found equally effective in these two dissimilar leiomyosarcoma models and superior to PDT. Hence, vascularity was identified as a predictive marker for sensitivity to PCI treatment.

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Paper 3

“Leiomyosarcoma xenograft and normal tissue responses to Photochemical Internalization (PCI) of bleomycin. Vascular characteristics as predictive markers for treatment response”

Vascular shutdown is an essential component in PDT and PCI treatment efficacy in solid tumours, but has been observed to vary with pre-treatment vascularization in different tumour types. In an attempt to elucidate underlying mechanisms, we performed an in-depth analysis of tumour vascularization and PCI treatment effects on the vasculature in a MES-SA xenograft model in mice. An MRI-based definition of vascular shutdown was established. Treatment with PDT and PCI of bleomycin was administered with four different light doses from 5 to 40 J/cm². A vascular shutdown 24 hours after PCI of approximately 50% of the tumour area was observed in the DCE-MRI analysis, regardless of the applied light dose. There was remaining tumour perfusion in the peripheral zone and to some degree in the tumour centre, as previously shown. A correlation analysis showed that a high fraction of the tumours with low pre-treatment uptake of contrast agent correlated with a high degree of vascular shutdown. To further investigate the significance of pre-treatment vascularity, a layer model with four different tumour zones from the periphery towards the centre was developed. A higher blood vessel area fraction and larger blood vessels were found in the peripheral zone and were considered predictive of resistance to vascular shutdown. We also found surviving tumour cells in a layer, similar to the treatment resistant peripheral zone, surrounding larger vessels deeper in the tumour, underscoring that vascularity and in particular blood vessel size are important factors in resistance to photochemically induced vascular shutdown.

In the histopathological analysis the two highest light doses 20 and 40 J/cm² resulted in significantly more tumour necrosis than the lower light doses 5 and 10 J/cm². In tumour-free animals, the two highest light doses carried a risk of partial muscle necrosis. In accordance, limping was recorded in clinical observations, and was most pronounced after the highest light dose. There were no differences between PDT and PCI in induction of normal tissue damage and no observations of damage to large blood vessels or nerves. With the observed treatment effects and adverse effects from this study, a light dose of around 20 J/cm² is proposed to represent a safe and effective “therapeutic window” in treatment with AlPcS_{2a} - PCI of bleomycin.

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DISCUSSION

The work presented in this thesis is an extension of the knowledge acquired by fellow scientists in photochemical medicine, and is focused on the clinical development of PCI as a therapeutic modality in cancer care. Based on the fundamental principles of PDT, PCI shares many of the same advantages as PDT, but also some of the limitations. Previous studies have demonstrated that PCI is able to circumvent several of the PDT-specific and other resistance mechanisms in malignant tumours [111, 119, 120] and PCI has been proven to be far more potent than PDT [5, 109, 121]. Clinical applications of PDT in cancer care have been focused on carcinomas, while the highly treatment resistant sarcomas remain a challenge to clinicians, and also researchers due to their heterogeneity and low incidence. Development of PCI towards clinical implementation is in progression with promising results of recent and ongoing clinical trials. However, variable response rates underscore the importance of selection of suitable patients for PCI treatment. Identification of genotypic and phenotypic predictive markers for PCI sensitivity in tumours will aid in patient selection and improve clinical outcomes. Thus, to further explore the potential and also limitations of PCI, a treatment resistant soft tissue sarcoma, leiomyosarcoma, was chosen as model in this work. Leiomyosarcoma is a diagnostic entity, and one of more than 50 subtypes of soft tissue sarcoma. Leiomyosarcoma is a heterogeneous subgroup that includes a range of tumours with different genotypic and phenotypic characteristics with regards to molecular expression profiles [90], p53 status [99, 100, 122] and prognosis [123]. MES-SA is a human derived tumour originating from uterus, and there are indications that uterine leiomyosarcomas have lower survival rates than leiomyosarcomas originating in other anatomical locations [124], but they are more responsive to chemotherapy [123]. SK-LMS-1 is a human derived tumor that originates from vulva and has a known p53 mutation which is associated with lower response rates to radio- and chemotherapy [125].

Findings in relation to existing literature

Genotypic predictive factors of PCI sensitivity

In Paper 1, an *in vitro* comparison of PDT and PCI treatment responses in the human leiomyosarcoma cell lines SK-LMS-1 and MES-SA was undertaken. The cell lines differed in expression of a number of regulatory proteins, morphology and growth pattern. Both cell lines responded to PDT treatment with reduced clonal cell viability but PCI was clearly more efficient. MES-SA cells were demonstrated to be somewhat more sensitive than SK-LMS-1 to PDT but far more sensitive to PCI than SK-LMS-1, due to increased bleomycin-induced DNA double strand cleavage as seen by γ H2AX staining. PCI (and PDT) effects rely on the formation of reactive oxygen species (ROS), and one cellular mechanism of resistance is presence of ROS quenchers, which deactivate photochemically generated ROS, mainly singlet oxygen. Expression of the ROS quenchers glutathione peroxidase 1 (GPx1) and superoxide dismutase 2 (SOD2), was found higher in SK-LMS-1, and inhibiting GPx1 with BSO sensitized SK-LMS-1 cells more to PDT than MES-SA. Accordingly, expression of the

antioxidant enzymes GPx1 and SOD2 was identified as an important factor in cellular response to PDT and PCI.

The p53 status was also explored as a predictive factor to photochemical treatment response. Bax and p21 are downstream proteins from p53 and induce apoptosis. In line with wild type p53 status, activation of these proteins was only found in the MES-SA cells, but only after treatment with PCI. PDT and PCI induced the same apoptotic fraction in each cell line, indicating that the apoptotic pathways after PDT and PCI are dissimilar. Mechanisms regulating PDT-induced apoptosis may vary with p53 status but also with the PS's intracellular localization, for instance cathepsin release has been found important with the use of lysosomally localized PSs [126, 127]. However, the apoptotic fraction was found three times higher in the MES-SA cells, which may in part be explained by p53 status.

Phenotypic predictive factors of PCI sensitivity

To further compare and evaluate treatment effects of PDT and PCI, SK-LMS-1 and MES-SA xenografts were established in athymic mice. Considerable differences between the tumour models were discovered at an early stage in this work. The MES-SA cells were easy to culture in large numbers, while the SK-LMS-1 cells grew in an outspread pattern and occupied large spaces and yielded very few cells in each cell culture flask. Once the solution with MES-SA cells was injected, tumour growth occurred in nearly all animals with a time to reach the inclusion volume 100 mm^3 of about nine days. SK-LMS-1, on the other hand, resulted in tumour formation in approximately 70% of inoculated animals, and it took about seven weeks for the tumours to reach the inclusion volume. Some SK-LMS-1 tumours spontaneously regressed before reaching inclusion volume. Of the SK-LMS-1 tumours that reached the inclusion volume, 10-15% spontaneously regressed. This was believed to be unrelated to the individual treatments as it was observed in all treatment groups except that with bleomycin only. After inclusion, the untreated SK-LMS-1 tumours took almost twice as long as MES-SA tumours to grow to 1000 mm^3 (20 vs. 11 days). In Paper 1, it was shown that the doubling times were similar between the two cell lines, approximately 30 hours, therefore this was somewhat unexpected. However, given the cell and tumour appearances in the microscope, it was clear that the MES-SA tumours were highly malignant, while SK-LMS-1 tumours seemed to be of intermediate malignancy, in line with the discrepancies observed in establishment of xenografts. The underlying mechanisms for this are beyond the scope of this work, but one may speculate that tumour angiogenesis and the “angiogenic switch” [128] may play a part. As an indication of the significance of vascularity, the MES-SA tumours had strands of fibrous tissue containing larger vessels penetrating the tumour stroma that were not present in the SK-LMS-1 tumours which were more homogeneously vascularized with a larger number of small vessels. Despite these differences, the two tumour models displayed a similar and tumour specific uptake of photosensitizer in fluorescence imaging.

The primary end point for evaluation of *in vivo* treatment efficacy was the time from inclusion (treatment) to the tumour reached 1000 mm³, and with no cured animals in this study the treatment resistance of these two sarcoma models was underscored. First, it was found that ALPcS_{2a}-PDT with 30 J/cm² of light induced no or only minor growth retardation in either tumour model, even when doubling the light dose to 60 J/cm². This finding was in contrast to the *in vitro* experiments in Paper 1 showing PDT cytotoxicity in both cell lines and also to *in vivo* studies of PDT with ALPcS_{2a} and TPCS_{2a} in carcinomas and other sarcomas which showed growth tumour growth retardation or curations [4, 109, 121]. Secondly, our results showed that PCI of bleomycin with a 30 J/cm² light dose induced similar growth retardations in the two leiomyosarcoma models, also in contrast to the *in vitro* experiments where MES-SA cells were far more sensitive to PCI than SK-LMS-1. The histopathological analysis of MES-SA tumours two days after treatment with PDT and PCI revealed a similar extent of tumour necrosis, which was in line with the MRI findings and also the *in vitro* observation that PDT does have an effect. Two days after treatment of the SK-LMS-1 tumours, there was more treatment induced necrosis than in the MES-SA tumours as seen in the H/E stained histopathological samples. These findings were confirmed in the DCE-MRI analysis that showed a more pronounced vascular shutdown in the SK-LMS-1 tumours at the same time point. The treatment induced necrosis seen in the histopathological samples and the vascular shutdown seen in MR images in SK-LMS-1 tumours were somewhat more pronounced after PCI. The MES-SA tumours had remaining uptake of contrast agent in strands in the tumour centre, in line with their phenotype.

One week after PCI treatment there was a striking difference between the two tumour models in the histopathological analyses, in MES-SA tumours the necrotic areas had almost completely regrown, while SK-LMS-1 showed no regrowth. This may be attributed to the slower growth of the SK-LMS-1 tumours, whereas the MES-SA tumours' regrowth may have been facilitated by remaining tumour vasculature in the tumour centre. One week after PDT there was near complete regrowth of tumour cells in both tumour models, in line with the post treatment tumour growth analyses.

Vascular shutdown accounts for a significant part of PDT tumouricidal effects [40], and this has also been shown for PCI [4]. Vascular shutdown occurs rapidly after treatment and is fully developed well before the 48-hour time point after treatment. The bleomycin effect from PCI, on the other hand, is a slower process not fully developed after 48 hours. Thus, histopathological tumour necrosis and vascular shutdown seen in the MR images 48 hours after treatment are mainly due to vascular effects, and therefore there were no obvious differences between PDT and PCI. The more pronounced effects in SK-LMS-1 may be explained by the overall increased vascularity with lack of fibrous strands with larger vessels, leading to a more complete central vascular shutdown. One week after treatment, the full potential of PCI is unleashed, but the highly malignant and proliferative MES-SA tumours with larger central blood vessels still regrow, while the SK-LMS-1 tumours have not had enough time or the remaining central perfusion necessary for regrowth.

The direct cellular toxicity of internalized bleomycin by PCI is therefore likely to be the explanation for the observed growth retardation in both tumour models. In Paper 2, vascular shutdown was found more pronounced in SK-LMS-1 than MES-SA, while in Paper 1 the *in vitro* experiments showed increased PCI sensitivity of the MES-SA cells. When both tumour models displayed similar growth retardations after PCI, this confirms that MES-SA cells *in vivo* are still more sensitive than the SK-LMS-1 tumour cells to internalized bleomycin. The stronger bleomycin effect in the MES-SA tumours is in line with the observed reduced growth rate seen in MES-SA and not in SK-LMS-1 tumours after PCI, a finding previously reported after bleomycin monotherapy [129-131]. It is important, however, to point out that cellular sensitivity *in vitro* alone is not a reliable predictive factor for *in vivo* PCI sensitivity, as tumour vascularity emerges as an important factor. Immunostimulation via T-cell activation, the third important mechanism of PCI anti-tumour activity [6], is not considered relevant in this study due to the use of cells in culture and immunocompromised mice.

In the MRI analysis of PDT and PCI treated tumours in Paper 2 it was observed that the peripheral zone was resistant to vascular shutdown, a finding previously reported by several authors [4, 132-134]. In the MES-SA tumors there also seemed to be remaining uptake of contrast agent in areas corresponding with a high pre-treatment uptake along the strands of fibrous tissue containing blood vessels. Endothelial cells have been shown to be highly sensitive to PCI treatment [112], but it is not clear which vascular characteristics are of importance for induction of vascular shutdown. Studies on normal blood vessels have shown that large vessels retain their function even with loss of cellularity due to photochemical treatment [113, 135]. This incited further analyses of the tumour vasculature in relation to PCI treatment resistance in Paper 3.

The DCE-MRI analysis in Paper 3 was focused on vascular effects in MES-SA tumours 24 hours after PCI treatment, when the full effects of photochemical treatment on the vasculature have taken place. A definition of vascular shutdown was established with areas of the tumours with AUC₉₀ values <38 defined as non-viable due to low uptake of contrast agent. This definition was used in selection of animals for inclusion and in a quantitative analysis of photochemically induced vascular shutdown by calculation of the fraction of the tumours with AUC₉₀ <38 before and after treatment. Four different light doses were applied (5, 10, 20 and 40 J/cm²), and the first important finding was a light dose-independent vascular shutdown in approximately 50% of the tumour area. A substantial vascular shutdown after photochemical treatment with low light doses has been reported earlier [136]. When analyzing histopathological samples after the same treatment regimens, it was evident that there was increased tumour necrosis after treatment with the two highest light doses compared to the lowest light doses. However, there was no apparent increase in necrosis in the 40 J/cm² group when compared to the 20 J/cm² group, a finding in line with Paper 2 where no increase in PDT effect was found when doubling the light dose from 30 to 60 J/cm². These results indicate that the full potential for PCI induced vascular damage is

obtained at relatively low light doses, while maximum cytotoxic effects require higher light doses, but with increasing effects only up to a certain point.

AUC₉₀ is the measure for uptake of contrast agent and reflects tumour vascularity directly. The treatment induced reduction in median AUC₉₀ in each tumour was approximately the same, regardless of the applied light dose and pre-treatment AUC₉₀ value. The treatment resistant peripheral zone of the tumours appeared to have a higher pre-treatment uptake of contrast agent. This necessitated a closer look at pre-treatment AUC₉₀ value as a possible predictive factor for vascular shutdown.

A correlation analysis of pre-treatment AUC₉₀ values and vascular shutdown showed for high dose PCI that a large fraction of the tumour with AUC₉₀ values below a certain threshold value correlated with a high degree of vascular shutdown. With the observed heterogeneous uptake of contrast agent before treatment we proceeded by dividing the tumours into four layers in concentric rings from the tumour border and inwards. The outermost ring overlaps with the treatment resistant peripheral zone. The results confirmed that there was a higher median AUC₉₀ value prior to treatment in the peripheral zone, and also less vascular shutdown after treatment than in the rest of the tumour. IHC analysis of the same four zones showed that the peripheral zone had a higher blood vessel area fraction with larger blood vessels than the rest of the tumour, and these traits indicate that vascularity is an important factor in the observed treatment resistance.

A similar treatment resistant peripheral zone has also been observed after treatment with an emerging group of blood vessel targeted anti-cancer drugs, vascular disrupting agents (VDAs) [42, 137]. VDAs induce vascular occlusion mainly via tubulin binding, and in a study by Liang and coworkers it was observed that high pre-treatment vascularization (K^{trans}) was predictive of resistance to treatment induced vascular shutdown [50]. In addition to resistance to vascular shutdown in the peripheral zone, we showed in Paper 2 that there was a layer of surviving tumour cells surrounding larger vessels also in the tumour centre. This underscores the importance of vascularization in resistance to photochemical treatment. Furthermore, surviving tumour cells may actually be stimulated by vascular targeted therapies. Authors Wu et al. have described four ways stimulated angiogenesis in this setting may occur, firstly through hypoxia-induced upregulation of pro-angiogenic factors such as VEGF and HIF-1 α . In addition there will be an increase in the number of circulating endothelial progenitor cells as well as tumour-associated macrophages which are highly pro-angiogenic. Finally, activation of the coagulation cascade contributes to angiogenesis and aggressive regrowth of tumours [137]. However, not all studies point in the same direction regarding tumour vasculature and treatment effects. The treatment resistant tumour periphery was studied after PDT treatment of CT26.WT (colon carcinoma), but the authors were unable to identify blood vessels in this area after treatment and proposed that PDT only works well in vascularized tumours [134].

In the present MES-SA xenograft model, the fibrous strands penetrating the tumour tissue consist of cancer-associated fibroblasts which previously have been shown to stimulate tumour progression and metastasis when exposed to ROS [138]. Thus, this phenotype represents a separate resistance mechanism to photochemical treatment.

Normal tissue damage

In conjunction with the presented evaluations of PCI treatment efficacy in MES-SA tumours, assessment of normal tissue toxicity from the same treatment regimens was undertaken. The aim was to investigate whether there was a therapeutic window in dosage of PCI treatment, with potent anti-tumour effects and acceptable levels of adverse effects. Photosensitizers and therapeutic drugs used in PCI have been shown to have selective uptake in endocytic vesicles in tumour tissue [109] but there is also some uptake in surrounding normal tissue [4]. Application of light during PCI treatment includes a cuff of the normal tissue surrounding the tumour, to ensure sufficient light delivery. Thus, there is potential for PCI induced normal tissue damage and systematic studies in this respect have, to our knowledge, not yet been published. Normal tissue toxicity from PDT has been investigated, especially effects on blood vessels and nerves. In 1994 and 1995, Grant et al. published studies on vascular effects of PDT in tumour-free rats and rabbits and found no obstruction of blood flow in large vessels, despite full vessel wall loss of cellularity [113, 135]. Damage to nerves from high doses of PDT has been documented in dogs [115] and in rabbits [114]. In the work presented in Paper 3, there was found no damage to large blood vessels and nerves, even within areas with extensive muscle necrosis after treatment. Muscle cell necrosis, however, was observed in conjunction with haemorrhage. Necrosis of muscle cells may occur by direct effects of the photochemical treatment or indirectly via disruption of supplying blood vessels. The photosensitizer ALPcS_{2a} has been shown to preferentially locate in endothelial cells, and PDT and PCI to exert cytotoxic effects in the endothelial HUVEC cell line [112]. Therefore, it is likely that PCI induced muscle cell necrosis is secondary to disrupting effects on intramuscular capillaries and small blood vessels. The findings from the study of normal tissue toxicity are in accordance with the vascular effects found in PCI treated MES-SA tumours, where only small blood vessels are sensitive to PCI treatment. Of importance is the fact that the two lowest PCI light doses (5 and 10 J/cm²) that induced significant vascular shutdown in the tumours did not result in disruption of blood vessels in normal tissue. An increased blood vessel sensitivity in tumours to photochemical treatment is not surprising, given the abnormal morphological and functional properties of tumour vasculature [40]. There were no indications of increased normal tissue toxicity by addition of bleomycin to PDT (i.e. PCI).

Methodology and limitations

Medical research, and particularly cancer research, is resource demanding, time-consuming and tedious. Not uncommon is a cost of >500 million dollars and a time frame of 10 years (or

more) from concept to completion of clinical trials [139]. In cancer research, the establishment of commercially available cell lines derived from human malignant tumours is of paramount importance. In a controlled laboratory environment, studies of cellular effects from different cancer treatments can be conducted efficiently, repeatedly and thoroughly. Such *in vitro* studies provide essential information and are a pre-requisite for further studies involving the use of research animals. Naturally, there are a number of limitations in the transfer of knowledge acquired from *in vitro* studies to research animals and ultimately to human physiology and pathology [140]. Even though the cells in the experiments are actual human cancer cells, the important interactions with the normal microenvironment are absent. Novel *in vitro* techniques have been developed for studies of microvessel-interactions [141], but have not yet been implemented in PCI experiments. Likewise, the interaction with the immune system is important in cancer therapy, and this is not possible with conventional *in vitro* study designs. However, techniques involving co-culturing of cancer cells and immune cells have been developed [142]. The subculturing of cells from sarcomas with cellular heterogeneity is likely to result in selection of only a subset of the different cell types, resulting in reduced heterogeneity when compared to the original tumour. This represents another limitation of pre-clinical cancer research models.

From the above-mentioned commercially available cell lines, or by direct transplantation of tumour tissue, *in vivo* tumour models in research animals may be established. In 1969, the first report of a human tumour grown in an immunodeficient mouse was published [143]. Since then the method has been validated in development of cytotoxic drugs with more than 300 different xenograft models described [139]. Even with the great value these studies have proven, they may be of limited predictive value when transferred to clinical medicine [144]. By transplantation of tumour tissue directly, the heterogeneity of the tumour is better preserved than after inoculation of a cell suspension from cells cultured *in vitro*. On the other hand, the properties of the transplants may be more difficult to control over the duration of an experiment involving a large number of research animals. The site of tumour implantation also matters, with orthotopic (e.g. intramuscular, as opposed to subcutaneous) localization offering the most relevant physiological environment, but this deeper localization offers challenges regarding tumour size measurements with a caliper. In Paper 2 the SK-LMS-1 xenografts were difficult to establish, they took several weeks to become palpable and some spontaneously regressed. These are inconsistencies considered unfavourable in *in vivo* work, and may inflict results. Spontaneous necrosis was seen in MES-SA xenografts over a certain size as described in Paper 3. We were not aware of this during the analyses in Paper 2, and this may have had an impact on the results. In our xenograft models, there were sometimes large discrepancies in the results that may be attributed to the administration of the drugs. For instance, high doses of PCI occasionally resulted in no oedema or tumour necrosis which may be explained by injection of PS or bleomycin into the intestine instead of the peritoneal cavity. Even though significant treatment responses discovered in pre-clinical studies lay the ground for clinical testing, such studies provide little

information about dosage due to differences in drug metabolism between humans and research animals.

In evaluation of results after pre-clinical trials, the choice of end point is important and a source for making erroneous conclusions. For instance, in Paper 2 it was found that PDT induced significant tumour necrosis as evaluated by histopathology, while no tumour growth delay was seen in the Kaplan-Meier plots.

In Paper 1, the *in vitro* experiments had a limited number of variables and were repeated several times, thereby the results may be considered reliable. In Paper 2, the different treatment groups consisted of about 10 animals, and therefore the results may be interpreted with some degree of certainty of their quality. In Paper 3, the presence of spontaneous necrosis in the larger tumours led to the exclusion of a number of animals, resulting in a low number of animals included in the final analyses. We have taken this into account by performing statistical analyses with p-value calculations, but the results could be more detailed and convincing with a larger number of included animals.

Animal experiments are under strict regulations by Norwegian law (The Animal Welfare Act), but there are important ethical considerations to be made. Researchers are obligated to practice under the 3 R-principle of Reduction (of number of research animals), Refinement (of models, methods and environment) and Replacement (with *in vitro* experiments where possible), formulated by Russell and Burch in 1959. With the comfortable living conditions, thoughtful care of the research animals and precautions taken when conducting experiments, the importance of the goals of the work justifies the costs at the animals' expenses, in my opinion.

Conclusions

The *in vitro* experiments showed that the cytotoxic effects of PCI of bleomycin were superior to PDT in both leiomyosarcoma cell lines. MES-SA cells were proven more sensitive to treatment than SK-LMS-1, and the difference in sensitivity was larger for PCI than PDT. SK-LMS-1 cells had higher levels of the ROS-quenching enzymes SOD2 and GPx1, which are though to act protectively. The wild type p53 status of MES-SA was also considered as a factor in induction of apoptosis after treatment. The *in vivo* experiments, however, showed that the treatment induced increase in time for the MES-SA and the SK-LMS-1 tumours to reach the end point volume was similar. Increased vascular shutdown was observed in MR images of the SK-LMS-1 tumours, and seemed to balance the increased cellular sensitivity of the MES-SA cells observed in Paper 1. Thus, cellular responsiveness and tumour vascularity were identified as predictive markers for PCI sensitivity. The resistance to vascular shutdown in the peripheral zone of the tumours was in Paper 3 shown to correlate with a high blood vessel area fraction, and in particular the presence of blood vessels with a larger diameter. The only clinically significant adverse effect identified in the study of normal tissue toxicity

was limping of the treated hind limb. PDT and PCI treatment with a 20 J/cm² light dose involved a 50% risk of moderate limping, while the anti-tumour effects were significant. Thus, there seems to be a therapeutic window in dosage of PCI treatment that provides potent anti-tumour effects with an acceptable profile of adverse effects.

Future perspectives

The findings presented in this thesis indicate that future clinical trials should emphasize cellular responsiveness and tumour vascularity in selection of the types of tumour to treat with PCI. Further exploration of the properties of tumour cells and blood vessels in the peripheral tumour zone is needed, as vascularity is likely to be only one amongst several mechanisms of resistance to photochemical treatment. To improve treatment outcomes, combination therapies targeting the peripheral zone, for instance radiotherapy administered before PCI treatment to ensure optimal synergism, are called upon. Combinations of PCI with other drugs targeting tumour vasculature, such as anti-angiogenic drugs or vascular disrupting agents may also be explored. Modern cancer therapy aims towards patient specific rather than diagnose-specific treatment regimens, and therefore the search for predictive markers for treatment efficacy must continue. Identification of predictive markers from a biopsy will guide individually tailored treatment strategies and result in improved outcomes and reduced adverse effects.

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PAPERS

Impact of genotypic and phenotypic differences in sarcoma models on the outcome of photochemical internalization (PCI) of bleomycin

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ABSTRACT

The low curative response to current treatment regimens for most soft tissue sarcomas indicates a strong need for alternative treatment strategies and predictive markers for treatment outcome. PCI (photochemical internalization) is a novel treatment strategy to translocate drugs into cytosol that otherwise would have been degraded in lysosomes. Two highly geno- and phenotypically different uterine and vulvar leiomyosarcoma cell lines, MES-SA and SK-LMS-1, were treated with bleomycin (BLM) activated by PCI (PCI_{BLM}). The MES-SA cells were much more sensitive to PCI_{BLM} than the SK-LMS-1 cells and the treatment induced a 7–8 fold higher increase in DNA double-strand breaks at the same dose of light as measured by γ H2AX staining. A 3-fold higher induction of apoptosis and stronger activation of Bax and p21 was also measured in the P53^{WT} MES-SA cells, compared to the P53^{mut} SK-LMS-1 cells. The basal formation of reactive oxygen species (ROS) was 3-fold higher in SK-LMS-1 cells than in the MES-SA cells and SK-LMS-1 cells expressed glutathione peroxidase 1 (GPx1) and more superoxide dismutase 2 (SOD2) than the MES-SA cells. Glutathione depletion with the glutathione synthetase inhibitor buthionine sulfoximine increased the cytotoxic effect of the photochemical treatment (PDT) most strongly in the SK-LMS-1 cells, and reduced PCI_{BLM}-induced H2AX activation in the MES-SA cells, but not in the SK-LMS-1 cells. The results indicate PCI_{BLM} as a potential novel treatment strategy for soft tissue sarcomas, with antioxidant enzymes, in particular GPx1, and the P53 status as potential predictive markers for response to PCI_{BLM}.

1. Introduction

Soft tissue sarcomas constitute a rare group of solid cancers. They are generally difficult to treat, and surgery remains the first line treatment, often supplemented by radiation therapy. Surgery does, however, bear the risk of contamination of healthy tissue, leading to cases of tumour recurrence. Radiotherapy improves local control after surgery [1] and chemotherapy increases survival in a metastatic setting [2], but most important are adequate surgical margins. Surgery of high grade sarcomas has often been combined with cytostatics such as doxorubicin and ifosfamide in hope of improving long term survival. The long term results are, however, still not convincing, and adjuvant chemotherapy remains a subject of debate. Thus, there is a need for new treatment strategies to improve the survival rate and reduce the need for debilitating surgery to obtain local control.

Photochemical internalization (PCI) is a novel treatment modality that may be considered for treatment of soft tissue sarcomas. PCI is a

technology where a drug accumulated in endocytic vesicles becomes released into cell cytosol due to photodynamic destruction of the endosome and lysosome membranes. The PCI technology is based on amphiphilic photosensitizers, such as disulfonated tetraphenyl porphyrine with the sulfonate groups on adjacent phenyl groups (TPCS_{2a}), which localizes to endosomal membranes, and upon light activation at appropriate wavelengths, generates reactive oxygen species (ROS) that rupture the membrane of the endocytic vesicles and thereby releasing the entrapped drug. A phase I clinical trial, including one sarcoma patient, showed highly promising results, and 13 out of 16 patients showed complete [11] or partial [2] response 28 days after treatment [3]. The highly heterogeneous phenotypic and genotypic characteristics of soft tissue sarcomas even within the same subgroup, result in different responses to radio- and chemotherapy [4]. It is therefore of great importance to reveal the phenotypic and genotypic characteristics of soft tissue sarcomas that may predict the response to PCI.

Soft tissue sarcomas are classified into over 40 subgroups, according

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to their histological appearance. With a pure smooth-muscle differentiation, leiomyosarcomas are among the most common soft tissue sarcomas. Leiomyosarcomas are a heterogeneous group of tumours with a range of molecular expression profiles including *P53* status [4]. They may arise in any location, but occur most often in the retroperitoneum and pelvis, and in large vessels or extremities [5]. The most important prognostic factors are tumour localization and size, and 5 year survival rates, overall, are 50–60% [2]. MES-SA [6] and SK-LMS-1 [7] are two heterogeneous uterine-and vulvar leiomyosarcoma cell lines, respectively, exhibiting dissimilar phenotypic and genotypic characteristics [8]. MES-SA cells express p53^{WT}, HER2, pAKT, c-Met, but no PTEN or EGFR. In contrast, SK-LMS-1 cells express p53^{mut}, do not express HER2, but express PTEN, EGFR, pAKT and c-Met [9–11]. On this basis these cell lines appeared ideal to reveal differences in sensitivity to PCI treatment and to initiate studies of predictive markers for treatment response. In this study PCI was utilized to translocate bleomycin (BLM) into the cells since BLM is a poorly internalized chemotherapeutic that has been used in clinical PCI trials. The results indicate that the MES-SA cells are more sensitive to PCI of BLM (PCL_{BLM}) than the SK-LMS-1 and provide insight into the mechanistic causes for this difference in cell sensitivity.

2. Materials and methods

2.1. Cell lines and culturing

The human uterine sarcoma cell line MES-SA (ATTC CRL-1976) and human vulvar leiomyosarcoma cell line SK-LMS-1 (ATCC HTB-88) were obtained from the American Type Culture Collection (LGC Standards AB, Borås, Sweden) and subcultured 2–3 times a week in McCoy's 5a medium (Sigma-Aldrich, St. Louis, MO, USA) and DMEM (Sigma-Aldrich), respectively, supplied with 10% fetal calf serum (FCS; PAA Laboratories, Pasching, Austria), 100 U/mL penicillin (Sigma-Aldrich), and 100 µg/ml streptomycin (Sigma-Aldrich). Detachment of the cells for subcultivation was performed at 37 °C with 0.25% (w/v) trypsin-0.53 mM EDTA solution (Sigma-Aldrich). The cells were kept at 37 °C in a humidified tissue culture incubator with 5% CO₂.

2.2. Cell growth densities

MES-SA cells (2×10^5 , 4×10^5 and 6×10^5) and SK-LMS-1 cells (0.75×10^5 , 1.25×10^5 and 1.75×10^5) were seeded in 6 well plates. After cellular attachment the plates were analysed in the IncuCyte ZOOM (Essen BioScience, Hertfordshire, UK) by measurement of plate confluence over 90 h. Phase contrast high definition images were acquired every 3 h. The data were subjected to morphological processing, due to the dissimilar cellular morphology of the two cell lines.

2.3. Live cell microscopy

For live cell microscopy cells were seeded (3000 cells/cm²) in ibiTreat µ-culture 8-well slides (Ibidi, Munich, DE), allowed to attach overnight and imaged using a Cell Observer microscope system (Carl Zeiss; Jena, DE) equipped with a 20×/0.8 PlanApo Phase 2 lens, an AxioCam MRm camera, a temperature controlled XL-chamber, a temperature, humidity and CO₂ controlled stage incubator, a motorized coded X,Y-stage, and a Definite Focus system. Cells were imaged for 20 h at 10 min intervals in 2 × 2-fields with 10% overlap and image tiles aligned using the MosaiX-module (AxioVision 4.2; Carl Zeiss). For documentation of gross growth morphology cells were seeded in 6-well plates (Nunc), grown to near confluence and imaged in 5 × 5 fields with 10% overlap using a 10x/0.25 Phase 1 N-Achroplan lens. Image tiles were aligned using the MosaiX-module (AxioVision 4.2; Carl Zeiss).

2.4. Light source

Illumination of cells was performed by using LumiSource[®] (PCI Biotech AS, Lysaker, Norway), a lamp consisting of four 18-W Osram L 18/67 light tubes. The lamp delivers blue light ($E_{\text{max}} = 435 \text{ nm}$) with an output of 13.5 mW/cm². The irradiance of the lamp varies < 10% across the illumination area (45 × 17 cm).

2.5. PDT and PCI_{BLM} treatment

Two hundred thousand cells were allowed to attach in 6-well plates (Nunc) over night, and then incubated with 0.2 µg/ml TPCS_{2a} (PCI Biotech, Lysaker) for 18 h. Cells were then washed twice with PBS, and either incubated with 0.3 IU/ml BLM (Bleomycin, Baxter AS, Oslo) for 4 h prior to illumination. The cells were then illuminated with blue light (435 nm) using the LumiSource[®] (PCI Biotech AS) lamp.

2.6. Assessment of cell viability

Assessment of cell viability was addressed by clonal cell survival and the MTT assay. Based on plating efficacy, an appropriate number of cells were seeded to obtain approximately 100 colonies in 6-well-plates. Cells were treated as described above, and colonies counted manually after fixation in ethanol and methylene blue staining 10–14 days after treatment. Only colonies with more than 50 cells were counted. The MTT assay was performed by incubation with 0.4 mg/ml MTT (Sigma-Aldrich) for 4 h. The medium was then removed, and the formazan crystals were dissolved in DMSO (Sigma-Aldrich). Absorbance was read at 570 nm using a PowerWave XS2 microplate spectrophotometer (BioTek, Winooski, VT, USA).

2.7. Intracellular TPCS_{2a} accumulation and ROS formation

For addressing TPCS_{2a} accumulation, 3×10^5 cells were seeded in 6-well plates, allowed to attach, and incubated with 0.2 µg/ml TPCS_{2a} for 18–20 h. Then the cells were washed twice in PBS, and incubated in drug free medium for 4 h. Cells were analyzed for photosensitizer accumulation by flow cytometry as described below. Relative ROS formation was addressed by studying the cell permeable agent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma), which upon deacetylation in the cells, converts to non-fluorescent DCFH. DCFH is rapidly oxidized to highly fluorescent DCF by ROS. The fluorescence intensity is proportional to the ROS levels in the cell cytosol. Three hundred thousand cells were seeded in 6-well plates, allowed to attach, and incubated with 0.2 µg/ml TPCS_{2a} for 18–20 h. Then the cells were washed twice with PBS, and incubated with 0.3 IU/mL BLM or drug free medium for 4 h. Immediately after illumination, cells were washed once with PBS, detached with trypsin and resuspended in medium. After 30 min cells were incubated with 0.1 mM DCFH-DA in medium for 1 h, and analyzed by flow cytometry. Cells were analyzed based on forward and side scattering (area) to gate live cells, and side scatter (area and width) to gate singlets. Fluorescence analyses were performed in LSRII flow cytometers (Becton-Dickinson, San Jose, CA, USA). TPCS_{2a} was excited by either a 405 nm laser (25 mW) or a 407 nm laser (100 mW) and collected through a 660/20 nm emission filter combined with either a 505 nm or 635 nm longpass dichroic filter, respectively. DCF was excited by a 488 nm laser (50 mW), and collected through a 530/30 nm or a 525/50 nm emission filter combined with a 505 nm dichroic filter.

2.8. Cell protein content

To compare cell protein contents, the DC Protein Assay (BioRad, Hercules, CA, USA) was used as described by the producer. Cell lysates were obtained by lysing 2×10^6 cells with 100 µL 0.1 M NaOH.

2.9. Western blotting

Cells were treated as described above, and harvested at 4 h and 20 h after treatment by washing the cells with PBS, and placing the plates on ice. Then the cells were lysed with RIPA buffer (added 10 μ L/ml Protease Inhibitor Cocktail (Sigma-Aldrich), 10 μ L/ml Phosphatase Inhibitor Cocktail I and II (Sigma-Aldrich), 10 μ L/ml 2 M β -glycerol phosphate, 5 μ L/ml 10 mM Na_3VO_4 , 5 μ L/ml 200 mM PMSF and 5 μ L/ml 200 mM NaF) or Lane Marker Sample Buffer (Thermo Scientific), sonicated for 10–15 s and heated (95 °C for 5 min) before the lysates were applied on 4–20% Mini-PROTEAN[®] TGX[™] 15 well Gels (BioRad) together with Precision Plus Protein Kaleidoscope[™] Standard (BioRad) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (200 V, 30 min). Then all the proteins were blotted (100 V, 2 h) onto methanol-activated polyvinylidene difluoride (PVDF) transfer membranes (Amersham Hybond-P, GE Healthcare). The Transblot Turbo system was also used, as recommended by the producer. The membranes were washed in tris-buffered saline with 0.1% tween 20 (TTBS) and blocked in 5% milk for 1 h at room temperature. Then, the membranes were washed in TTBS and incubated with antibodies against p21 #sc-397, GPx4 #50497 and SOD2 #sc30080 (1:200) (Santa Cruz) or Bax #2772 (Cell Signaling) (1:1000) in 5% bovine serum albumin (BSA), GPx1 #3206 and SOD1 #4266 (Cell Signaling) (1:1000) in 5% milk over night at 4 °C. The membranes were then washed thrice in TTBS and incubated with secondary antibodies against rabbit #7074 (1:1000) or mouse #7076 (1:1000) (Cell Signaling) in 5% milk at room temperature. The membranes were further washed thrice in TTBS before incubation with SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) and luminescence measured with ChemiDoc (BioRad). Loading was controlled by anti- γ -tubulin antibody; clone GTU-88 #T 6557 (Sigma-Aldrich) or incubating the membranes with Ponceau S. Stain (Sigma-Aldrich).

2.10. Cell cycle analysis, assessment of H2AX phosphorylation and apoptosis (TUNEL assay)

After treatment, cells were detached with trypsin, washed in PBS, and resuspended in 500 μ L ice cold methanol and stored at –20 °C. Methanol fixed cells were washed once with PBS, and added 35 μ L TdT reaction mix (3.5 μ L TdT Reaction Buffer, 2.1 μ L 25 mM CoCl_2 , 0.35 μ L Biotin-16-dUTP, 0.35 μ L 10 mM DTT, 0.14 μ L TdT enzyme, 28.58 μ L ddH₂O) (Roche) and incubated for 30 min at 37 °C. Then the cells were washed once with PBS, and incubated with 50 μ L of primary antibody mix (mouse α - γ H2AX 1:500 (Millipore) in 5% milk in PBS) for 30 min at room temperature. Then the cells were washed once with PBS, and incubated with 50 μ L secondary antibody mix (goat α -rabbit-PE 1:50 (Invitrogen), rabbit α -mouse-FITC (Dako) and streptavidin-Cy5 1:400 (GE Healthcare) in 5% milk in PBS) for 30 min at room temperature. Then the cells were washed once with PBS and resuspended in 500 μ L Hoechst 33258 (1.5 μ g/ml) in PBS, and incubated overnight at 4 °C. The cell cycle analyses were performed in an LSRII flow cytometer (Becton-Dickinson, San Jose, CA, USA). PE, FITC and Cy5 were excited by 561 nm (40 mW), 488 nm (50 mW) and 640 nm (40 mW) lasers, and collected through 582/15 nm, 525/50 nm and 670/14 nm emission filters, the former combined with 570 nm and 505 nm longpass dichroic filters, respectively. Single cells were gated either based on side scatter (area and width) or on side scatter (area) and Hoechst (width). Cell cycle analysis was performed by FlowJo Software (Tree Star Inc, Ashland, Oregon, U.S.A.) on Hoechst 33258-stained cells, based on Hoechst area, and analyzed by fitting the Dean-Jett-Fox model to define cell cycle distribution.

2.11. Metabolic analysis

Fifteen thousand cells were inoculated onto special 96 well plates for the XF[®]96 Seahorse metabolic analyzer. The cells were incubated

overnight in their normal media at 37 °C, 5% CO₂ humidified atmosphere. One hour prior to the experiments, the cells were placed on Seahorse Assay media supplemented either with 2 mM pyruvate, 10 mM glucose and 2 mM L-glutamine or 2 mM L-glutamine but no pyruvate and glucose. The cells were incubated for 1 h in a 37 °C humidified atmosphere (non CO₂) and then assayed for their oxygen consumption rate (OCR) and lactate induced extracellular acidification rate (ECAR), using the XF[®]96 Seahorse metabolic analyser (Agilent, Santa Clara, CA, USA). The cells were assayed for their respiration and extracellular acidification in i) media only i.e. basal respiration; ii) in the presence of 1 μ M oligomycin which inhibits the F₀F₁ ATP synthase. The drop of these rate values from the basal ones indicate the amount of respiration required for ATP production; iii) in the presence of 1 μ M carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma-Aldrich), which at that concentration maximize mitochondrial electron transport (and hence respiration). The rate increase from the corresponding basal value shows the maximal respiratory capacity of the cells; iv) in the presence of 1 μ M Rotenone (Sigma-Aldrich) and Antimycin A (Sigma-Aldrich) which in combination completely inhibit electron transport by ubiquinone; The OCR values at these conditions reveal the residual oxygen consumption at zero mitochondrial respiration. Extracellular acidification following the addition of FCCP cannot be taken into consideration due to the free shuttling of protons which falsely add to acidification higher by lactate. Any rise in the ECAR following the addition of oligomycin however, signifies the maximal glycolytic capacity of the cells. The rates (OCR and ECAR) were determined by 3 min measurements, followed by 3 min of media mixing to replenish oxygen and also to reinstate physiological pH. Three measurements were performed in each of the four conditions described above.

2.12. Statistical evaluations of data

To evaluate significance the *t*-test (student's *t*-test) was used if not otherwise stated. The cell cycle distributions were statistically analyzed by two-tailed paired *t*-tests. Results were considered significant when *p* < 0.05. The statistical analyses were performed by the software SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA, USA).

3. Results

3.1. Different cell growth patterns between uterine and vulvar leiomyosarcomas, but no difference in growth rates

Cell growth assessed by the IncuCyte ZOOM is shown in Fig. 1A and B, and revealed insignificant overall growth rate differences between the cell lines. The calculated doubling times were 31.3 ± 1.2 (S.D.) and 32.2 ± 1.4 (S.D.) hrs for the MES-SA and SK-LMS-1 cells, respectively. The cell density curves were used to calculate the appropriate number of cells to seed in each experiment, based on the experiment duration and plate type, to maintain optimal growth conditions. Live cell microscopy showed, however, phenotypic differences in growth pattern, migration and the formation and appearance of colonies of the two cell lines (Fig. 1C and D, and Suppl. Video 1). The MES-SA cells appear round, only rarely found with pseudopods and express low mobility and form dense colonies. In contrast, the SK-LMS-1 cells appear flat with long pseudopods and high mobility. Accordingly, the MES-SA and SK-LMS-1 cells form dense and scattered colonies, respectively.

3.2. Insignificant differences in sensitivity to BLM

Therapeutic effects of increasing doses of BLM were addressed by the clonal cell viability assay, as described in Material and Methods (Fig. 2A). The cells were treated with BLM for 4 h. The clonal cell viability decreased with increasing doses of BLM for both cell lines. The

Fig. 1. Cell confluence in percent at increasing time after seeding of 200 000, 400 000 and 600 000 cells (MES-SA, Fig. A) and 75 000, 125 000 and 175 000 cells in 6-well plates (SK-LMS-1, Fig. B). Cell confluence was calculated from images obtained by the IncuCyte ZOOM (Essen BioScience) from 16 individual areas in the wells every 3 h (Error bars = S.D.). Representative images of live MES-SA (C) and SK-LMS-1 (D) cells obtained by live cell Microscopy in 1 μ -Slide 8 well ibiTreat Microscopy Chambers.

LD₅₀ values were found similar (2.75 IU/ml) for both cell lines as calculated by the exponential decay formulas.

3.3. PCI_{BLM} increases the therapeutic effect of BLM

Studies on response to TPCS_{2a} and light (photodynamic therapy (PDT)) showed that the TPCS_{2a}-treated MES-SA cells were slightly more sensitive to light than the SK-LMS-1 cells (Fig. 2B). In both cell lines PCI_{BLM} reduced the cell viability in a synergistic manner (Fig. 2C and D). However, the MES-SA cells were found more sensitive to increasing doses of BLM in cells treated with PCI than the SK-LMS-1 cells. In the MES-SA cells the LD₅₀ dose of BLM was reduced from approx. 3 IU/ml to approx. 0.1 IU/ml, 0.03 IU/ml and 0.03 IU/ml in cells treated with 40 s, 70 s and 100 s of light, respectively, corrected for the effect of PDT alone. In SK-LMS-1 cells LD₅₀ dose of BLM was reduced from approx. 3 IU/ml to 1 IU/ml and 0.3 IU/ml in cells treated with 70 s and 100 s of light respectively. All the other experiments with BLM are based on using 0.3 IU/ml BLM.

3.4. PCI_{BLM} increases DNA double strand breaks

BLM exerts cytotoxic effects by induction of DNA strand breaks, including double-strand breaks [12]. The induction of DNA double strand breaks is usually indirectly detected by analyzing the phosphorylation of the histone H2AX, described as γ H2AX. A major increase in DNA double strand breaks addressed by measurements of γ H2AX was found when MES-SA cells were treated with PCI_{BLM} (Fig. 3). The γ H2AX staining was increased more than 20-fold 1 h after PCI_{BLM}, while only a 3–4 fold increase was seen in the SK-LMS-1 cells. In both cell lines BLM alone or PDT induced no increase in γ H2AX staining. The analyses of

γ H2AX staining by flow cytometry were confirmed by fluorescence microscopy which showed that the staining was nuclear and appeared almost homogenous in the PCI_{BLM}-treated MES-SA cells and less intense and more punctuate in the SK-LMS-1 cells (Fig. 4).

The γ H2AX staining of untreated G2/M phase cells was approximately 2-fold higher than in G1 cells of both cell lines, reflecting the doubling of the DNA content of cells in G2/M phase (Fig. 3C and D). However, the γ H2AX staining was similarly strong in both cell cycle phases 1 h after PCI_{BLM}. The γ H2AX staining was found decreasing with time after treatment in both cell lines, but was still high 44 h after treatment in MES-SA cells (Fig. 3A and B). The repair kinetics appear similar after high (300 s) and low doses (90 s) of light, indicating that the repair mechanisms are not influenced by the photochemical treatment (data not shown). Interestingly, the γ H2AX staining appeared to decline slightly faster in G1 cells than in the G2/M cell population in both cell lines (Fig. 3C and D). Twenty hours after PCI_{BLM} treatment of MES-SA cells the γ H2AX staining in G1 cells had declined to $17.7 \pm 11.2\%$ ($n = 5$) of that at 1 h after treatment while in the G2/M population γ H2AX declined to $30 \pm 8.0\%$ ($n = 5$). The difference in rate of reduction of γ H2AX staining had a one-tailed p -value = 0.0381, and a two-tailed p -value = 0.0763.

The untreated SK-LMS-1 cells exhibited a 3–4-fold higher γ H2AX staining than the MES-SA cells (Fig. 3). This is, however, partly reflecting the approximately 2-fold higher DNA content in the SK-LMS-1 cells (data not shown).

The differences in cellular response to PDT and PCI_{BLM} as described above may be due to differences in cellular uptake of photosensitizer, differences in rate of ROS formation, cellular protection mechanisms against ROS or differences in expression of oncogenic signals. The following are attempts to reveal the mechanistic parameters of importance

Fig. 2. Clonal cell viability of MES-SA and SK-LMS-1 cells treated with BLM and PCI_{BLM} : (A) Cell treated with increasing concentrations of BLM for 4 h. The curves are fit to exponential decay curves for MES-SA cells following the equation $f(x) = 22 \times e^{-4.2x} + 57 \times e^{-0.23x} + 22 \times e^{-0.059x}$ ($r^2 = 0.998$) and for the SK-LMS-1 cells following $f(x) = 86 \times e^{-0.92x} + 14 \times e^{-0.040x}$ ($r^2 = 0.998$) where x is the BLM dose; (B) The cells were treated with PDT, BLM (0.3 IU/ml) or PCI_{BLM} with 90 or 180 s of light as indicated on the figure and described in the Materials & Methods; (C) and (D) MES-SA and SK-LMS-1 cells were treated with PDT and PCI_{BLM} with increasing doses of BLM and light as indicated in the figure. The data are the mean of 3 individual experiments. Error bars = S.E.

Fig. 3. H2AX activation by PCI_{BLM} : Median γH2AX was analyzed in untreated (NT) cells and cells treated with either 0.3 IU/ml BLM (BLM) for 4 h, TPCS_{2a} without light (PS) for 18 h, PS + BLM without light, 150 s PDT and PCI of 0.3 IU/ml BLM (MES-SA (A) and SK-LMS-1 (B)). Cells were harvested 1 and 20 h (and MES-SA 44 h) after PDT/ PCI_{BLM} . The data are the average of one (PS and PS + BLM) and at least four individual PDT/ PCI_{BLM} experiments, and are normalized to the median of NT cells at each harvest time point (Error bars = S.E.). PS and PS + BLM were not measured at the 44 h time point. Merged representative flow cytometry dot plots of γH2AX vs Hoechst staining are also shown for the 1 h time point, with separate histograms of Hoechst and γH2AX staining. γH2AX status of G1 and G2/M cells 1 and 20 h (and MES-SA 44 h) after PCI_{BLM} and 150 s light in MES-SA (C) and SK-LMS-1 (D) cells, representative from the data in (A) and (B).

Fig. 4. γ H2AX staining of MES-SA and SK-LMS-1 cells treated with PCI_{BLM} : epi-fluorescence microscopy images of untreated (NT) cells and cells harvested 1 h after 150 s PCI_{BLM} , subsequently subjected to methanol-fixation. DAPI shows nuclear staining, FITC shows γ H2AX-staining, DIC shows phase contrast, while Merge shows the overlaid DAPI-and FITC-micrographs. The experiment was performed once to confirm the localization of the γ H2AX staining.

for the differences in response to PDT and PCI_{BLM} between MES-SA and SK-LMS-1 cells.

3.5. Cellular uptake of TPCS_{2a}

Studies of TPCS_{2a} accumulation, at a time point corresponding to the time of light exposure of TPCS_{2a} – sensitized cells, revealed that the SK-LMS-1 cells accumulated more photosensitizer than the MES-SA cells (Fig. 5A). The median fluorescence after subtracting the background fluorescence, in the SK-LMS-1 cells was found to be $3.3 \pm 0.15(\text{S.E.})$ -fold higher than in the MES-SA cells. However, the SK-LMS-1 cells are larger than the MES-SA cells. The protein content was found $3.7 \pm 0.28(\text{S.E.})$ -fold higher per cell in SK-LMS-1 cells. Thus, the accumulation of TPCS_{2a} was 12% lower per cell volume in the SK-LMS-1 cells than in the MES-SA cells.

3.6. ROS generation in MES-SA and SK-LMS-1 cells after treatment

ROS formation was studied after the various treatments (Fig. 5B and C). In both cell lines all photochemical treatments induced detectable increases in ROS. The increase in ROS formation after normalization to the control levels was higher per cell in the MES-SA cells than in the SK-LMS-1 cells (B). The increase in ROS-formation was close to proportional to the light doses in the MES-SA cells, while doubling of the light dose caused only an approximately 60% increase in ROS formation in the SK-LMS-1 cells. Treatment with PCI_{BLM} did not induce any additional ROS formation compared to PDT.

The ROS assay is based on flow cytometric single cell analyses. ROS

formation in untreated cells was $12.5\text{-fold} \pm 6.5 (\text{S.E.})$ higher in SK-LMS-1 cells than in the MES-SA cells. Taking into account the absolute relative formation of ROS per mg protein it appears that the generation of ROS is 3.3-fold higher in untreated SK-LMS-1 cells than in the MES-SA cells (Fig. 5C), and the total ROS formation in the PDT/ PCI_{BLM} treated cells is approximately twice as high in the SK-LMS-1 cells than in the MES-SA cells (range 1.8–2.6-fold).

3.7. SK-LMS-1 cells exhibit higher expression levels of superoxide dismutase and glutathione peroxidase than MES-SA cells

The enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) are essential in detoxification of ROS generated by the cells and by treatment-induced ROS. The basal levels of mitochondrial SOD2 generally involved in catalysing the dismutation of superoxide radical into H_2O_2 , was found to be more strongly expressed in the SK-LMS-1 cell line compared to MES-SA cells, while cytosolic and peroxisomal SOD1 was found equally expressed in both cell lines (Fig. 6A). Cytosolic GPx1 was found expressed only in the SK-LMS-1 cell line, while GPx4 was hardly detectable in both cell lines.

3.8. The impact of GSH on cell survival and double-strand breaks after PDT and PCI_{BLM}

Glutathione (GSH) is a strong antioxidant as well as substrate for GPx enzymes. Measurements of the basal levels of GSH showed an approximately 50% higher level in MES-SA cells than in the SK-LMS-1 cells (Fig. 6B). Treatment with L-Buthionine-S,R-Sulfoximine (BSO),

Fig. 5. (A) Relative TPCS_{2a} accumulation at the time point for PDT/PCI_{BLM}. The data are representative from three individual experiments. (B) Relative ROS generation per cell after treatment as indicated in the figure. The data are the mean of three individual experiments normalized to untreated (NT) cells of both cell lines. (C) Absolute relative ROS generation per cell protein normalized to the untreated (NT) MES-SA cells. (Error bars = S.E.).

which is an inhibitor of γ -glutamylcystein synthetase, lead to a $\geq 80\%$ decreased GSH level in both cell lines after treatment with 50 μM BSO for 24 h (Fig. 6B). BSO induced an incubation time-dependent toxicity that excluded utilization of the clonogenicity assay. The highest non-toxic concentration of BSO was initially determined (72 h treatment at 50 μM for MES-SA and 100 μM for SK-LMS-1) by the MTT assay (Fig. 6C and D). Both cell lines were found more sensitive to PDT in the presence of BSO, but the effect appeared much stronger in the SK-LMS-1 cells (Fig. 6E and F). The cytotoxicity of PCI_{BLM} is usually analyzed by clonogenic cell viability assay due to the slow response to BLM. Three days of incubation was found too short to detect a significant synergistic effect of PCI_{BLM} in the MTT assay and the impact of BSO on the cytotoxicity of PCI_{BLM} could therefore not be determined.

GSH and GPx activities have been shown to influence on chromosomal DNA damage and repair in cells treated with ionizing radiation and BLM [13,14]. In MES-SA cells treated with PCI_{BLM} the γH2AX staining was lowered by 46% (0.54 ± 0.24) as seen by a slightly reduced peak value and a longer tail towards low γH2AX staining by pretreatment with BSO, while BSO treatment had no apparent effect in the SK-LMS-1 cells (0.98 ± 0.25 of that in the absence of BSO, Fig. 7). BSO had no detectable effect on γH2AX staining in either cell line treated with BLM only or PDT.

3.9. Treatment-induced effects on cell cycle regulations and induction of apoptosis

The cell cycle distribution in response to PDT and PCI_{BLM} was studied by flow cytometry of Hoechst stained methanol fixed cells. A two-fold increase of MES-SA cells in G2/M phase was seen 20 h after treatment with PCI_{BLM} and PDT with 150 s of light ($p < 0.03$, Fig. 8). Concurrently, PDT reduced the fraction of cells in S phase by almost 50% ($p = 0.064$) and less by means of PCI_{BLM} (PDT vs PCI_{BLM}, $p < 0.05$). In SK-LMS-1 cells subjected to PDT the G2/M fraction was lower by 40% compared to untreated control cells 20 h after treatment ($p < 0.01$), while after PCI_{BLM} the cell cycle distribution was only slightly modified with a 25% increase in the G2 M phase ($p = 0.06$). BLM treatment resulted in no statistically significant effect in either cell line.

MES-SA cells express wild type p53 while p53 is mutated in SK-LMS-1 cells [11,15]. This difference may influence on the cell cycle regulation and induction of apoptosis. Apoptosis, addressed by identification of DNA fragmentation (TUNEL assay,) showed that neither PDT nor PCI_{BLM} induced apoptosis 1 h after treatment. Twenty hours after PDT and PCI_{BLM}, apoptosis appeared in both cell lines (Fig. 9A and B). There were no differences in the apoptotic fraction between the cells treated with PDT and PCI_{BLM}, but the apoptotic fraction was 3-fold

Fig. 6. (A) Western blots of SOD1, SOD2, GPx1 and GPx4 with respective γ -tubulin blots in SK-LMS-1 (SK) and MES-SA (M) cells. (B) GSH content (nmol) per μ g cell protein in untreated cells (NT) and cells treated with 50 and 500 μ M BSO for 20 h. The data are the mean of two individual experiments (Error bars = S.E.). (C) and (D) MTT of MES-SA and SK-LMS-1 cells subjected to 24, 48 and 72 h incubation with increasing concentrations of BSO. The data are the triplicates from one experiment (Error bars = S.D.). (E) and (F) MTT of MES-SA and SK-LMS-1 cells 48 h after PDT with and without co-incubation with 50 μ M and 100 μ M BSO, respectively. The data are triplicates from a representative experiment out of three and two individual experiments, respectively. (Error bars = S.D.).

higher in MES-SA cells than in SK-LMS-1 cells. Additionally, when PDT and PCI_{BLM} light doses were increased (from 150 s to 300 s of light) on the MES-SA cell line, an increase in DNA fragmentation with time was observed (data not shown). The highest doses tested at the latest time points after treatment revealed approximately 30% apoptosis (300 s of light, 44 h after irradiation, data not shown) in the MES-SA cell line.

The apoptotic fraction did not change between 20 and 44 h after illumination (Fig. 9B). In the procedure for analysis of apoptosis, floating cells are collected together with the cells bound to the substratum. It has previously been shown that apoptotic cells floating in the medium after PDT with a structurally similar photosensitizer (TPPS_{2a}) remain detectable in the medium for more than 60 h after illumination [16]. In accordance with the cell line differences in induction of apoptosis after PCI_{BLM} , Bax was induced 20 h after illumination in the MES-SA cells, but not in the SK-LMS-1 cells (Fig. 9C). Activation of the p53 downstream protein p21 was also seen after

PCI_{BLM} , but only in the MES-SA cells, in accordance with the p53 status. However, no activation of Bax or p21 was seen after PDT in either cell line.

3.10. Metabolic activity characteristics of MES-SA and SK-LMS-1 cells

The reduced dependency on oxidative phosphorylation and enhanced use of glycolysis have been described as characteristic for cancer cells [17]. Recent studies indicate that increased utilization of glycolysis correlates not only with survival benefits in a hypoxic environment but also increased drug resistance [18]. Addressing the mitochondrial respiration showed that the SK-LMS-1 cells consumed oxygen at approximately the same rate (OCR/min) as the MES-SA cells when corrected for the difference in cell protein (3.7: 1) (Fig. 10A). When removing the glucose and pyruvate from the media, the basal respiration levels slightly increased for both cell lines, but the

Fig. 7. Representative dot plots of γ H2AX vs Hoechst staining in untreated cells (NT) and cells treated with BLM, 150 s PDT or 150 s PCI_{BLM} , with and without the combination of 50 or 100 μM BSO present throughout the experiment. Cells fixed in methanol 1 h after PDT/ PCI_{BLM} . The data is one representative experiment out of three or more individual experiments.

maximum respiration capacity upon treatment with the respiration uncoupler FCCP remained the same (Fig. 10B).

When, however, the glycolytic profiles of the two cell lines were assessed, MES-SA cells were found to demonstrate an approximately 2.5-fold higher glycolytic activity (ECAR/min) than SK-LMS-1 cells when corrected for cell volume (Fig. 10C). ATP synthase inhibition, by adding 1 μM oligomycin, only slightly increased the glycolytic activity in both cell lines, without much affecting the ratio of the respective activities before oligomycin administration. When removing glucose and pyruvate from the medium, the glycolytic activity in the cell two lines was in general lowered (Fig. 10D), with the MES-SA cells being most affected by the glucose and pyruvate absence. This resulted in a profound decrease in the difference between the glycolytic activities of the two cell lines.

4. Discussion

In the present study the two pheno- and genotypically diverging soft tissue sarcoma cell lines MES-SA and SK-LMS-1 have been evaluated for treatment response to PCI_{BLM} . The MES-SA cells were found slightly more sensitive to PDT and substantially more sensitive to PCI_{BLM} than the SK-LMS-1 cells. The cell line difference in treatment response is related to the observation that PCI_{BLM} induced more double-strand breaks and apoptosis in MES-SA cells than in SK-LMS-1 cells. The GSH-

based protection mechanisms appear also to be involved in the sensitivity to PCI_{BLM} and PDT.

The induction of DNA double strand breaks as measured by phosphorylation of the histone H2AX (γ H2AX) was seen in both cell lines after PCI_{BLM} although much stronger, e.g. 7–8-fold at 150 s of light, in the MES-SA cells than in the SK-LMS-1 cells. BLM alone induced hardly any double-strand breaks, confirming that PCI_{BLM} induces the translocation and activation of BLM in the nucleus. The biological consequences of the double-strand breaks are the main cause of the BLM- and ionizing radiation-induced tumour necrosis [19]. The difference in PCI_{BLM} – induced cytotoxicity between the two evaluated cell lines may therefore to a high extent be related to the formation of double strand breaks and/or differences in the efficiency of the corresponding repair mechanisms.

BLM is regarded as a radiomimetic and the effects on cells by BLM and ionizing radiation are highly similar. Glutathione is a radio-protector and depletion of glutathione by inhibiting the enzyme γ -glutamylcysteine synthetase with BSO increases cellular radio-sensitivity and increases the formation of radiation-induced chromosomal aberrations [20]. This is in contrast to the attenuation of H2AX activation found after PCI_{BLM} in BSO treated MES-SA cells. However, it has been reported that glutathione can reactivate oxidized BLM so that it can undergo repeated cycles of chromosome cleavage [21,22]. It was unfortunately not possible to evaluate the impact of glutathione

Fig. 8. Cell cycle of non-apoptotic cells harvested 20 h after BLM, PDT or PCI_{BLM} exposed to 150 s of light. The figures in (A) are representative for responses to treatments as indicated in the figure. In (B) the cell cycle distribution in controls and treated cells are shown based on analyses as described in Materials & Methods. Bars, SD $n = 5$ for MES-SA cells, $n = 4$ for SK-LMS-1 cells.

depletion on the cytotoxicity induced by PCI_{BLM}. The importance of glutathione to protect against ROS, mainly singlet oxygen, induced by the photochemical treatment was, however, substantial and in accordance with previous reports [23]. The stronger sensitizing effect of BSO in the SK-LMS-1 than the MES-SA cells correlates well with the expression of GPx1 only found in the SK-LMS-1 cells. The sensitizing effect of BSO in the MES-SA cells may be due to a direct antioxidant effect of glutathione or the conjugation of glutathione to photooxidized biomolecules by glutathione-S-transferase followed by detoxification through expulsion from the cells [24]. Altogether, these results indicate that glutathione is an important factor in cellular response to PDT and PCI_{BLM}.

The basal level of double-strand breaks is higher by a factor of 2 corrected for the amount of DNA per cell, in the SK-LMS-1 than in the MES-SA cells. Darzynkiewicz and coworkers have shown that ROS generated by metabolic activity may lead to formation of double-strand breaks, indicating a higher rate of basal ROS formation in the SK-LMS-1 than in the MES-SA cells [25,26]. This is also in accordance with the 3-fold higher ROS formation detected in untreated SK-LMS-1 cells than in the MES-SA cells. The elevated ROS formation observed in cancer cells is largely due to a defective mitochondrial electron transport chain [27] leading to activation of antioxidant enzymes such as SOD and GPx [28]. The SK-LMS-1, but not the MES-SA, cells express GPx1 and SOD2, while both cell lines express SOD1 and almost no GPx4. It is thus tempting to speculate that an elevated rate of ROS formation stimulates expression of GPx1 and SOD2 in the SK-LMS-1 cells. The expression of GPx1 and SOD2 is expected to provide improved protection against ROS generating treatment modalities such as PDT, BLM and PCI_{BLM}.

PCI_{BLM} induced a more than 20-fold increase in γ H2AX staining in the MES-SA cells compared to the 3-fold increase in SK-LMS-1 cells. GPx1, that was found expressed in SK-LMS-1 cells, has been shown to

be located in cytosol and the mitochondria, but also in the nucleus [29]. In contrast, SOD2, that also was found mainly expressed in the SK-LMS-1 cells, has not been found to be located in the nucleus and is expected to influence on the dismutation of $O_2^{\cdot-}$ to H_2O_2 in the mitochondria, but is not likely to be involved in quenching BLM-induced double-strand breaks. Thus, the dissimilar expression of GPx1 and inverse correlation with double-strand breaks may indicate that GPx1 attenuates the level of double-strand breaks. This is in accordance with the reduced γ H2AX staining of BLM-treated colon cancer cells pretreated with selenium, enhancing GPx1 activity 5–8 fold [14]. However, GPx1 transfection of GPx1-negative MCF-7 cells enhanced γ H2AX staining after BLM treatment and reduced the cytotoxicity, while the selenium pretreatment had no effect on survival of the colon cancer cells [14]. The influence of GPx1 on H2AX activation and cell survival is therefore not fully understood.

The repair kinetics of DNA double-strand breaks appeared to not depend on the light dose used in PCI_{BLM}-treatment. However, the results indicate that the repair in G1 phase of the cell cycle is faster than in the G2/M phase. The repair of double-strand breaks in G1 is based on non-homologous end joining (NHEJ), while the repair in S and G2/M phase is based on homologues recombination (HR) [30]. The current results thus indicate that double-strand breaks induced in G1 are more rapidly repaired by the NHEJ mechanism than cells in later stages of the cell cycle. Studies based on knock-out mutant cells with defective NHEJ repair mechanisms become hypersensitive to ionizing radiation when treated in G1 [31,32]. Similarly, HR knock-out cells become hypersensitive when treated in the S-G2/M phase [31]. Dysfunctional DNA repair mechanisms may influence sensitivity to PCI_{BLM} and contribute to predict treatment response. E.g. deficiency in DNA-PK, a downstream kinase in the repair of double-strand breaks by the NHEJ pathway, influences sensitivity to ionizing radiation [33,34]. Similar

Fig. 9. Induction of apoptosis after PDT and PCI_{BLM} treatment: (A) Dot plot of cells stained by the TUNEL-assay, indicating apoptosis, based on flow cytometry of untreated (NT) cells and cells treated as in Fig. 8. The data is representative from 4 and 5 individual experiments. (B) The quantification of apoptosis as in (A), after treatments as indicated in the figure. Error bars = S.E. PS and PS + BLM without light were not measured at the 44 h timepoint. (C) Western blots of Bax and p21 measured 20 h and 4 h, respectively, after 90 and 150 s PDT or PCI_{BLM} , representative of three individual experiments.

results have not been reported after treatment with BLM.

MES-SA cells are known to express wild type p53. The p53 downstream proteins p21 and Bax were not activated by PDT in the MES-SA cells, but strongly activated by PCI_{BLM} . Interestingly, PDT induced a similar induction of apoptosis as PCI_{BLM} without induction of Bax, indicating that the pathways for induction of apoptosis by PDT and BLM are different. BLM alone has previously been shown to induce Bax and reduce Bcl-2 expression, induce cytochrome C release and caspase 8/9/3 activation [35]. PDT has also been shown to induce apoptosis, which includes enhanced Bax expression [36] utilizing non-lysosomal photosensitizers [37]. However, lysosomal rupture by PDT with the photosensitizer Pc13 was followed by mitochondrial disruption, cytochrome C release and caspase activation, but did not increase Bax expression. Instead Bax was shown to translocate from cytosol to the mitochondria [38]. Similarly, Bax was found to be translocated from cytosol to mitochondria without a concomitant enhanced expression of Bax in ASTC-a-1 lung adenocarcinoma cells treated with the lysosomally located photosensitizer NPe6 and light [39]. Thus, current literature indicates that PDT based on lysosomally located photosensitizers induces release of cathepsins into the cytosol causing cleavage of Bid to t-Bid. This further causes mitochondrial release of cytochrome C supported by translocation (but not enhanced expression) of Bax to the mitochondria [40,41].

The activation of apoptosis in MES-SA cells and to a much lower extent in SK-LMS-1 cells is expected to be related to p53 status and may contribute to the higher sensitivity of MES-SA cells to PCI_{BLM} than the SK-LMS-1 cells. However, the fraction of cells entering apoptosis as measured by the TUNEL assay is relatively low. The induction of apoptosis by lysosomally located photosensitizers appears generally less pronounced than with photosensitizers located in other compartments and may instead induce a stronger autophagic response [37]. As pointed out above, the release of cathepsins into the cytosol is regarded as the main apoptosis pathway after PDT with lysosomally located photosensitizers [40,41]. The expression of cathepsin activity after such PDT has, however, been shown to be relatively low due to cytosolic cathepsin inhibitors (steffins), high sensitivity of cathepsins to PDT and only partial rupture of the lysosomal fraction [42]. Without these protection mechanisms the PCI technology would not have been able to rupture endocytic vesicles without inducing complete cell death.

The similar levels of apoptosis induction after PDT and PCI_{BLM} up to 44 h after treatment may indicate that activation of bleomycin by PCI does not result in an increased induction of apoptosis. However, our experience with PCI-induced BLM activation shows that the cytotoxic effects of BLM are relatively slow. This is seen by the need for clonogenicity assays to reveal the full treatment effect of PCI_{BLM} as compared to PDT. Viability assays such as MTT performed 2 days after light

Fig. 10. Metabolic characterization of MES-SA and SK-LMS-1 cells: Relative oxygen consumption rate per minute (OCR/min) per cell and relative extracellular acidification rate (ECAR/min) per 15 000 cells seeded in 96 well plates, measured by the Seahorse XFe Analyzer with (A and C) and without (B and D) the presence of glucose and pyruvate in the media. (A and C) is the average of the means from four individual experiments (Error bars = S.E.). (B and D) is the average of the means from two independent experiments (Error bars = S.E.). The arrows show the addition of 1 μ M oligomycin, 1 μ M FCCP and 1 μ M antimycin A and 1 μ M rotenone. The scales have been adjusted to provide a direct correlation of the metabolic activity per mg of cell protein.

exposure reveal little or no effect of the PCI-activated BLM. This is also in accordance with the inability to detect enhanced ROS formation in PCI_{BLM}-treated cells as compared to the PDT response despite the well documented ROS formation by BLM treatment [12]. Therefore, we cannot rule out that BLM-induced apoptosis is a significant downstream cause of the strong cytotoxic effect of PCI_{BLM} in MES-SA cells initiated by DNA double-strand breaks.

The loss of p53 has been postulated to lead to a metabolic switch, also named the Warburg effect, as p53 is thought to suppress glycolytic activity and promote oxidative phosphorylation [43]. In this respect it was expected that the glycolytic activity would dominate in the SK-LMS-1 cells, but surprisingly the glycolytic activity was found highest in the MES-SA cells. The glycolytic activity was however almost non-responsive to the respiratory inhibitor oligomycin meaning that during oligomycin inhibition the cells' ATP requirements were met by glycolysis. Given the fact that the two cell lines exhibited similar OCR profiles, the MES-SA cells appear to express a profile closer to Warburg metabolism than the SK-LMS-1 cells. It should be pointed out that the Warburg effect correlates with drug resistance and should be taken into account when searching for predictive markers for treatment response [43].

In conclusion, the uterine MES-SA cells responded slightly more to PDT than the vulvar soft tissue sarcoma SK-LMS-1 cells. In contrast, the MES-SA cells were substantially more sensitive than the SK-LMS-1 cells to PCI_{BLM} which correlates well with the 7–8-fold higher rate of double-strand breaks formation as measured by γ H2AX staining. One may hypothesize that SK-LMS-1 have more leaky mitochondria since they express a similar respiratory activity, but a 3-fold higher rate of ROS

formation, presumably resulting in increased expression of GPx1 and SOD2. The expression of these antioxidant enzymes correlates with the attenuated induction of double-strand breaks in the SK-LMS-1 as well as the strongly enhanced PDT sensitivity in glutathione depleted cells. Expression of GPx1 and the P53 status should be considered as predictive markers of response to PCI_{BLM}.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.pdpdt.2017.08.010>.

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Supplementary video, Paper 1

Live MES-SA and SK-LMS-1 cells imaged every 10 minutes in a time course of 23 hours. The images are combined and show approximately 45 minutes time-duration per second video.

Photochemical internalization (PCI) of bleomycin is equally effective in two dissimilar leiomyosarcoma xenografts in athymic mice

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ABSTRACT

Background: Photochemical internalization (PCI) is a novel technique for delivery of active macromolecules into cancerous cells, via light activation of a specific photosensitizer and a low dose systemic drug. Numerous pre-clinical studies and one clinical trial have confirmed the treatment potential in carcinomas. Soft tissue sarcomas are rare and generally resistant to radio- and chemotherapy. Due to treatment resistance and surgical morbidity in sarcoma care, we seek to increase knowledge on PCI effects in sarcomas by studying two different, but closely related leiomyosarcomas.

Methods: MES-SA and SK-LMS-1 tumours were established in the leg muscles of athymic mice. Treatment effects after AlPcS_{2a}-PCI of bleomycin, PCI with no drug (photodynamic therapy, PDT) and control groups were evaluated by: 1) assessment of tumour growth, 2) uptake of contrast agent during MRI and 3) histopathology. **Results:** PCI of bleomycin induced a similar and significant increase in time to reach the end point in both tumour models, while neither responded to AlPcS_{2a}-PDT. In the MES-SA tumours PCI reduced the growth rate, while in the SK-LMS-1 tumours the growth was blocked for 12 days followed by exponential growth close to that of untreated tumours. SK-LMS-1 tumours were more homogeneously and better vascularized than MES-SA. After PCI the vascular shutdown was more complete in the SK-LMS-1 tumours than in the MES-SA tumours.

Conclusions: AlPcS_{2a}-based PCI, but not PDT, induced significant tumour growth delay in the evaluated sarcomas. Cellular responsiveness to bleomycin and tumour vascularity are identified as predictive markers for PCI treatment effects.

1. Introduction

Transmembrane delivery of active macromolecules into cancer cells by photochemical internalization (PCI) has been proven effective in pre-clinical investigations [1], has shown promising results in a clinical trial [2] and is currently under evaluation in treatment of cholangiocarcinoma (<https://clinicaltrials.gov/ct2/show/NCT01900158>) as well as for stimulating antigen presentation in cancer vaccination (<https://clinicaltrials.gov/ct2/show/NCT02947854>). In PCI systemic administration of a tumour localizing photosensitizer is followed by administration of the active drug. Illumination of the tumour with a light source with the proper wavelength results in formation of reactive oxygen species inducing endosomal rupture and escape of drugs accumulated in endocytic vesicles into cytosol. Tumoural effects include direct tumour cell death by apoptosis, autophagy [3] and necrosis, vascular shutdown, inflammatory reactions and specific T-cell immunoactivation [4]. PCI has been investigated in *in vitro* and *in vivo* models with a

number of different therapeutic macromolecules for a variety of malignancies [1]. Bleomycin, a poorly internalized cancer drug, has proven well suited for light-directed delivery by PCI and has been used in several preclinical and clinical studies [5–10]. The vast majority of tested cell lines have been carcinomas, which in clinical practice outnumber the rare and mesenchymal sarcomas by a factor of about 100:1. The results are generally good and carry promise for PCI as an alternative or adjunct to surgery and radiotherapy in for example cancers of head and neck, cholangiocarcinoma and other soft tissue malignancies. Only two human sarcomas, HT1080 (fibrosarcoma) and TAX-1 (malignant fibrous histiocytoma) have so far been evaluated in pre-clinical *in vivo* PCI models. Both responded well to PCI of bleomycin, but the orthotopic HT1080 tumours all recurred [9], whereas a complete response was found in 3 out of 5 (60%) animals with TAX-1 tumours [10]. Thus, the potential of PCI in treatment of otherwise treatment resistant mesenchymal tumours, sarcomas, is yet to be established.

Soft tissue sarcomas are rare and constitute about 1% of all cancers

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[11]. They comprise more than 50 subgroups with heterogenous genotypical and phenotypical characteristics. The clinical behaviour differs widely, and the sensitivity to chemotherapy is generally low [12]. Leiomyosarcoma is the most frequent subgroup and accounts for approximately 20% of soft tissue sarcomas [13]. They are mesenchymal neoplasms, with no curative potential from radio- or chemotherapy. Adequate surgery is therefore the mainstay of treatment, but may be debilitating, depending on tumour size and localization. In general adjuvant radiotherapy improves local control in deep seated high grade soft tissue sarcoma, but not survival [14]. Leiomyosarcomas are relatively resistant to all kinds of chemotherapy [15]. The overall 5 years survival rate for leiomyosarcomas is 43–55%, lowest in uterus and higher in other soft tissue locations [13]. On this basis there is a clear need for new treatment modalities for treatment of leiomyosarcoma.

Variations in PCI treatment responses in different cell lines and xenograft models have been observed and deeper understanding of the mechanisms involved is warranted. p53 mutations are common in human cancers and are predictive for unfavourable responses to cancer treatments, such as radio- and chemotherapy [16]. In well differentiated soft tissue sarcoma, mutant p53 expression is associated with decreased survival [17]. Leiomyosarcoma is a heterogenous subgroup of sarcoma with respect to molecular expression profiles [18]. p53 mutations are detected in 15–50% of leiomyosarcoma, but the clinical implications for this particular entity remain unclear [19–21]. Furthermore, there are indications that uterine leiomyosarcomas are more responsive to chemotherapy than the extra-uterine variant [22]. Molecular profiling is a rapidly growing field, also in sarcoma research, and ever increasing amounts of information about gene alterations is available [23]. Mapping of genotypical and phenotypical tumour characteristics that can predict treatment response may be used to guide treatment selection, and improve outcomes. Analysis of treatment response related to molecular expression profiles and morphological properties will aid in development of precision therapeutics.

We aim to investigate treatment responses to AlPcS_{2a}-PCI of bleomycin (PCI_{BLEO}) in athymic mice for two closely related orthotopic sarcoma models that are different with regards to p53 status and expression of a number of regulatory proteins such as PTEN and AKT (Table S1). We compare the two human soft tissue sarcoma xenografts, MES-SA [24], a uterine leiomyosarcoma with wild type p53 and SK-LMS-1 [24], a vulvar leiomyosarcoma with known p53 mutation. A recent *in vitro* study shows that these two cell lines respond differently to PCI_{BLEO} as well as to photodynamic treatment (PDT), with SK-LMS-1 displaying relative treatment resistance [25]. Limitations of *in vitro* studies with regards to recently reported endothelial and vascular PCI effects call for more physiologically relevant *in vivo* studies [26]. Evaluating and comparing PCI treatment effects in these two different leiomyosarcoma xenografts will increase knowledge on PCI treatment efficacy and resistance, and contribute in the development of PCI towards clinical application.

2. Material and methods

2.1. Animals

Female *Foxn1^{nu}* mice were bred and kept under pathogen-free conditions with water and food *ad libitum*. All handling and procedures were performed in compliance with the Norwegian Animal Research Authority Guidelines. Experiments were approved by the institutional Animal care and use committee. The mice weighed 20–30 g at the time of inclusion. They were randomly allocated to different treatment groups and earmarked. The tumours were measured three times per week by digital calliper and volumes calculated with the formula $Volume = (width^2 \cdot length) / 2$, approved by the US National Cancer Institute. Tumour volume for inclusion was 60–200 mm³. Animals were euthanized by cervical dislocation when tumour volumes exceeded 1000 mm³ or after completed procedures.

2.2. Cell lines

MES-SA (Catalog No. CRL-1976) is a human uterine leiomyosarcoma cell line obtained from American Type Culture Collection (ATCC). The cells were subcultured 2–3 times per week in McCoy's 5A medium (Sigma Aldrich, St. Louis, MO, USA) with 10% foetal calf serum and 100 U/mL penicillin (Sigma Aldrich) and 100 µg/mL Streptomycin (Sigma Aldrich). Cells were grown at 37° C with 5% CO₂. A suspension of approximately 4 million cells in phosphate buffered saline with Ca²⁺, Mg²⁺ and 5% glucose was injected superficially in the muscle in the left leg.

SK-LMS-1 (Catalog No. HTB-88) is a p53-mutated human vulvar leiomyosarcoma cell line obtained from ATCC. Cells were subcultured 2–3 times per week in Dulbecco's Modified Eagle's medium (Sigma Aldrich) with 10% foetal calf serum and 100 U/mL penicillin (Sigma Aldrich) and 100 µg/mL Streptomycin (Sigma Aldrich). Cells were grown at 37° C with 5% CO₂. A suspension of approximately 5 million cells in phosphate buffered saline with Ca²⁺, Mg²⁺ and 5% glucose was injected superficially in the muscle in the left leg.

2.3. Drugs

The photosensitizer disulfonated aluminum phthalocyanine with the sulfonate groups on adjacent phthalate rings (AlPcS_{2a}) was purchased from Frontier Scientific (Logan, UT, USA). Sixty milligrams of AlPcS_{2a} was dissolved in 1 mL 0.1 M NaOH and diluted with 29 mL phosphate buffered saline (PBS) to a concentration of 2 mg/mL. The solution was sonicated for about 1 min, diluted with 18 mL PBS to a final concentration of 1.23 mg/mL, aliquoted and stored at –20° C. According to the formula $weight(g) \cdot 10 / 1.23 \text{ mg/mL}$, approximately 200 µL of the stored AlPcS_{2a} solution was injected intraperitoneally 48 h prior to illumination, to a final concentration of 10 mg/kg. The animals were kept in the dark for 7 days after administration.

Bleomycin® (Baxter, Deerfield, IL, USA) 1500 U in 100 µL 0.9% NaCl was injected intraperitoneally 30 min prior to illumination.

Sevofluran® (Baxter) gas anaesthesia was administered by chamber during laser light exposure and mask during MR imaging.

Zoletil® (Virbac, Carros, France) stock solution with 19 mg/mL Xylasin, 0.48 mg/mL Butorphanol, 11.9 mg/mL Zolazepam and 11.9 mg/mL Tiletamin was diluted 1:5 in sterile water. 50 µL was injected subcutaneously as an anaesthetic to facilitate fluorescence imaging. Zoletil was also used in a few animals during MR imaging when Sevofluran gas was not available.

2.4. Light source

A Ceralas 0,3 W, 670 nm diode laser (CeramOptec GmbH, Bonn, Germany) was used. A light dose of 30 J/cm² (333 s) at an irradiance of 90 mW/cm², was applied to the tumours and approximately 1 mm of surrounding skin, unless otherwise stated.

2.5. Fluorescence imaging

To study the distribution and specificity of the uptake of photosensitizer in the tumours, fluorescent images were acquired using the IVIS Spectrum In Vivo Imaging System (PerkinElmer, Waltham, MA, USA) with a 675 nm excitation filter and a 720 nm emission filter. Three animals of each tumour type were imaged 48 h after intraperitoneal injection of AlPcS_{2a}, alongside a negative control animal.

2.6. Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI)

MRI was performed using a 7.05 T MR system (Bruker BioSpin GmbH, Ettlingen, Germany, software ParaVision 6.0) equipped with a volume transmit resonator and a receive surface coil. The animal was placed on the side in the scanner, with the receive coil placed on top of

Table 1

Median time (days) for the MES-SA and SK-LMS-1 tumours to reach 1000 mm³ after the indicated treatments. The increase as well as the statistical difference (pairwise log-rank) in time to reach the endpoint as compared to the untreated tumours.

Treatment	MES-SA				SK-LMS-1			
	No. in group	Median $\pm$ SE (days)	% increase vs. NT	p-value vs. NT	No. in group	Median $\pm$ SE (days)	% increase vs. NT	p-value vs. NT
No treatment (NT)	32	11 $\pm$ 0,4	–	–	33	20 $\pm$ 1,1	–	–
Light 30 J/cm ²	11	15 $\pm$ 1,7	36%	0,044	11	20 $\pm$ 2,1	0%	0,932
AlPcS _{2a}	10	12 $\pm$ 3,2	9%	0,056	10	24 $\pm$ 2,4	20%	0,270
Bleomycin	11	17 $\pm$ 0,9	55%	0,001	12	25 $\pm$ 2,9	25%	0,863
PDT 30 J/cm ²	15	12 $\pm$ 0,6	9%	0,158	10	24 $\pm$ 5,3	20%	0,082
PDT 60 J/cm ²	10	14 $\pm$ 1,0	27%	0,026	10	30 $\pm$ 3,1	50%	0,296
PCI _{BLEO} 30 J/cm ²	12	30 $\pm$ 2,2	173%	0,000	15	49 $\pm$ 9,7	145%	0,001

the tumour. During MRI mice were given 0.5 L/min O₂ containing ~4.0% Sevofluran. An abdominal pressure sensitive probe was used for monitoring respiration rate. The core body temperature was kept at 37 °C by using automatically adjusted hot air flow and monitoring with a rectal temperature probe. DCE images were acquired using a 2-dimensional SPGR pulse sequence (2D-FLASH). The contrast agent Gd-DTPA (Magnevist® 469 mg/mL (0,5 mmol Gd/mL), Bayer, Leverkusen, Germany) was diluted 1:10 in 0,9% NaCl and 180 μ L was administered in the tail vein using an automated infusion pump (Harvard Apparatus, Holliston, MA, USA) during imaging. Images were acquired immediately before and 48 h after treatment. The MRI control groups (untreated and 40 J/cm² light only) were scanned one day before, 2 and 72 h after treatment. Initial Area Under Curve (AUC₉₀) maps were generated by integrating the concentration curve the first 90 s after injection of the contrast agent. The calculations were performed using in-house-made software developed in Matlab (MathWorks, Natick, MA, USA). The DCE-MRI series were analyzed on a voxel-by-voxel basis.

2.7. Histopathology and immunohistochemistry

Three μ m thick sections were made from formalin-fixated paraffin embedded tumour tissue and stained with hematoxylin and eosin (H/E) for histological examination. For immunohistochemistry (IHC) sections with the same thickness were immunostained using the Dako EnVision⁺ system (K8012, Dako Cooperation, CA, USA) and Dako Autostainer. Deparaffinization, rehydration and target retrieval was performed in a Dako PT-link and EnVision Flex target retrieval solution with high pH for the antibodies. Endogenous peroxidase was blocked using Dako blocking reagent for 5 min, followed by incubation with primary antibodies against CD31 (rabbit polyclonal antibody, ab28364, 1:500 dilution, abcam, Cambridge, UK) and CD34 (rabbit polyclonal antibody, PAB18289, 1:500 dilution, Abnova, Taipei, Taiwan). Thereafter, the sections were incubated with Dako EnVision FLEX⁺ rabbit linker for 5 min followed by incubation with Dako EnVision FLEX/HRP for an additional 30 min. For visualization of the staining the sections were treated with 3'-3'-diaminobenzidine tetra-hydrochloride, counterstained with hematoxylin and mounted in Richard-Allan Scientific Cytoseal XYL (Thermo Scientific, Waltham, MA, USA).

To estimate the area of necrosis in the H/E sections post treatment, all tumour samples were examined in a microscope with an objective lens with 40 $\times$ magnification. A visual estimate of the proportion of complete necrosis in each tumour was performed, followed by calculation of average values in each treatment group.

The microvessel density (MVD) of untreated tumours was determined by counting vascular structures in the area with the most vascular structures in CD31 and CD34 stained samples in a 200 $\times$ field in the microscope, as described by Weidner [27].

2.8. Statistical analyses

Animals with tumours that never reached the end point volume

1000 mm³ were observed until no further changes were seen (37–173 days). Due to the differences in observation times, the times to reach the end point in the seven treatment groups are presented as median values. In addition, data excluding animals with tumours that never reach the end point volume are presented with both median and mean values (Supplementary tables). Data from all animals are included in the Kaplan-Meier plots using SigmaPlot version 12.5. Statistical correlations are calculated by pairwise log-rank (Mantel-Cox) analyses using SPSS 21, significance defined as $p < 0.05$. In tumour growth and survival assays, the various treatments delayed tumour growth as monotherapies, and documentation of synergism required more detailed analyses as previously described [10]. Synergy is regarded as statistically significant when the additional interaction variable, β_3 , is significantly different ($p < 0.05$) from the sum of the individual survival variables, $\beta_{12} = \beta_1 + \beta_2$. The synergistic effect can be described by $R = \beta_3/(\beta_1 + \beta_2)$, where $R = 0$ describes additivity and significant values of $R < 0$ and $R > 0$, describes sub-additivity and synergy (or supra-additivity), respectively.

3. Results

3.1. In vivo evaluation of photochemical activation of bleomycin

Photochemical activation of bleomycin was evaluated in superficial intramuscular MES-SA and SK-LMS-1 tumors in the left leg of athymic mice. The time (median) for the tumors to reach 1000 mm³ was selected as end point.

MES-SA tumours required on average 9 (5–14) days after injection of the cell suspension to reach the inclusion volume 100 mm³ (inclusion range: 60–200 mm³). Tumours appeared in more than 90% of animals injected with MES-SA cells. Untreated tumours grew rapidly and reached the end point of 1000 mm³ 11 days after inclusion (Table 1). In Fig. 1A the times for the MES-SA tumours to reach 1000 mm³ after the various treatments are presented in a Kaplan-Meier plot. The initial growth curves after treatment, shown in Fig. 1B, indicate strong growth inhibition after PCI_{BLEO}, but only minor effects of all the other treatments. Fig. 1C presents the results from Fig. 1B in a semi-logarithmic plot, showing exponential growth in all treatment groups with a volumetric doubling time of 9,4 days for PCI_{BLEO} compared to 4,8 and 2,9 days for bleomycin only and untreated tumours, respectively. The growth curves can only be presented for the time period from treatment until the first tumour in each treatment group reaches the end point. As can be seen in Table 1, treatment with PCI_{BLEO} prolonged the time from inclusion to the end point to 30 days. This equals an increase in time to reach the end point of 173% compared to untreated controls ($p < 0.001$) and was also significantly different from all the other treatment groups ($p \leq 0.013$, Table S2). Treatment with photosensitizer and light (PDT) resulted in a time to reach the end point of 12 days, not significantly different from the untreated tumours. In an attempt to estimate the difference in response between PDT and PCI_{BLEO}, the light dose in another PDT cohort was doubled. PDT with a

Fig. 1. Treatment response to PCI_{BLEO} on MES-SA xenografts. A: Kaplan-Meier plots of response to PCI_{BLEO} and control treatments as indicated in the figure with a tumour volume of 1000 mm³ as end point. B: Average tumour volume shown on a linear scale after treatment, followed until the first animal in each treatment group has reached the end point. C: Average tumour volumes on a logarithmic scale after treatment, based on results from Fig. B.

60 J/cm² light dose showed a time to reach the end point of 14 days, a 23% increase ($p = 0.026$) when compared to untreated controls. However, the time to reach the end point after PDT with 60 J/cm² of light was not significantly different from treatment with PDT 30 J/cm², bleomycin, ALPcS_{2a} or light only (Table S2). Treatment with 30 J/cm² of light alone resulted in a time to reach the end point of 15 days, a 36% increase compared to untreated controls ($p = 0.044$), but not significantly different from other treatment groups, except PCI_{BLEO}. Tumours treated with bleomycin alone showed a time to reach the end point of 17 days, 55% longer than untreated controls ($p = 0.001$), although not significantly different from other treatment groups, except PCI_{BLEO}. Included in this dataset were two MES-SA tumours that spontaneously regressed after an initial growth phase and never reached the end point (Table S3).

In order to calculate the synergistic effect of the various treatment combinations, we used a log-logistic survival function and β_n as the efficiency variables of the various treatments, as determined by the statistical calculations described by Berg et al. [10]. The variable values and significance levels are listed in Table 2. The calculations indicate that the combination of bleomycin and PDT, i.e. PCI_{BLEO}, acts synergistically in the MES-SA tumours. PCI_{BLEO} induced a tumour growth delay that was 2.1 times the sum of the individual treatments.

SK-LMS-1 tumours reached the inclusion volume 100 mm³ (inclusion range: 60–200 mm³) about 7 (6–8) weeks after injection of the cell suspension (approx. 5×10^6 cells). The growth was less predictable than for the MES-SA tumours, and tumours appeared in less than 70% of injected animals. Once the tumours reached the inclusion volume, the time (median) to reach the end point of untreated controls was 20 days (Table 1). In Fig. 2A the times for the SK-LMS-1 tumours to

Table 2
Results of the time accelerated survival analysis in the log-logistic regression model for the treatments of MES-SA and SK-LMS-1 xenografts as presented in Figs. 1 and 2. The interaction group (β_3) indicates the supra-additive effect observed experimentally.

Treatment groups	MES-SA		SK-LMS-1	
	$\beta_1 \pm SE$	Significance level (p)	$\beta_1 \pm SE$	Significance level (p)
No treatment	2,40 $\pm$ 0,045		3,10 $\pm$ 0,088	
Bleomycin	0,38 $\pm$ 0,088	< 0,001 ^a	−0,015 $\pm$ 0,19	0,94 ^a
PDT 30 J/cm ²	0,11 $\pm$ 0,078	0,15 ^a	0,41 $\pm$ 0,18	0,026 ^a
Interaction	0,54 $\pm$ 0,13	< 0,001 ^b	0,49 $\pm$ 0,27	0,069 ^b

^aCompared to No treatment.

^bCompared to treated groups.

reach 1000 mm³ after the various treatments are presented in a Kaplan-Meier plot. As for the MES-SA tumours PCI_{BLEO} induced a clear growth delay shortly after treatment (Fig. 2B and C). In addition, a moderate growth delay appeared after PDT. No initial effects in the other treatment groups were seen. In contrast to the MES-SA tumours PCI_{BLEO} induced a biphasic growth response seen as a 12 days long tumour growth arrest, followed by exponential growth with a volumetric doubling time of 7,2 days, compared to 5,6 days for untreated control tumours and 5,0 days for bleomycin treated tumours. These results were in accordance with the Kaplan-Meier plots. The time to reach the end point after treatment with PCI_{BLEO} was 49 days, an increase in time

Fig. 2. Treatment response to PCI_{BLEO} on SK-LMS-1 xenografts.

A: Kaplan-Meier plots of response to PCI_{BLEO} and control treatments as indicated in the figure with a tumour volume of 1000 mm^3 as end point. B: Average tumour volume shown on a linear scale after treatment, followed until the first animal in each treatment group has reached the end point. C: Average tumour volumes on a logarithmic scale after treatment, based on results from Fig. B.

of 145% compared to untreated controls ($p = 0.001$, Table 1). PDT resulted in a time to reach the end point of 24 days, a 20% increase compared to untreated controls ($p = 0.082$). Similarly to the treatment of the MES-SA tumours the light dose was doubled to estimate the difference in response between PDT and PCI_{BLEO} . PDT with a 60 J/cm^2 light dose resulted in 30 days from inclusion to the end point, only marginally longer than PDT 30 J/cm^2 , and still not statistically different from untreated controls ($p = 0.296$). The other treatment groups light alone, AlPcS_{2a} and bleomycin showed times to reach the end point of 20, 24 and 25 days, respectively, neither statistically different from untreated controls (Table S4). The synergy calculations showed that the combination of bleomycin and PDT, i.e. PCI_{BLEO} , acted almost synergistically in the SK-LMS-1 tumours ($p = 0.069$, Table 2). PCI_{BLEO} induced a growth delay that was 2.2 times the sum of the individual treatments.

In line with the low fraction of animals inoculated with SK-LMS-1 cells that obtained palpable tumours, a relatively large fraction (4 out of 33, 12%) of the untreated control tumours spontaneously regressed after inclusion. This was also observed in all the other treatment groups except bleomycin only, where all animals reached 1000 mm^3 . In the other treatment groups, six tumours disappeared and two stabilized, in an apparently random pattern. The observation time for these animals varied from 51 to 173 days. The fractions of regressions in animals treated with PCI_{BLEO} and PDT (10–20%) were similar to those in the other groups (Table S3). It was therefore reasonable to assume that the observed regressions were not treatment initiated. Henceforth, it was of interest to see whether exclusion of the tumours that never reach 1000 mm^3 had an impact on the conclusions to be drawn from the

treatment results. For the SK-LMS-1 tumours it was found that treatment with PDT 30 and 60 J/cm^2 both significantly increased the time to reach 1000 mm^3 when compared to untreated controls, but neither were significantly different from the other treatment groups, except PDT 30 J/cm^2 vs. light alone ($p = 0.038$, Tables 3 and S5). When excluding animals that never reached 1000 mm^3 from the MES-SA cohorts, the only difference that appears was that bleomycin alone showed significantly more effectiveness than PDT 30 J/cm^2 ($p = 0.025$, Table S6). Excluding tumours that never reached the end point allowed for data analysis using mean values. However, the mean values differed insignificantly from the median values (Table S7).

3.2. In vivo localization of AlPcS_{2a}

Fluorescence imaging of the distribution of the photosensitizer AlPcS_{2a} showed selective uptake in the tumours in the left leg (Fig. 3). The uptake of AlPcS_{2a} appeared similar in the MES-SA and the SK-LMS-1 tumours.

3.3. Vascular response after PDT and PCI of bleomycin assessed by DCE-MRI

Previous studies have shown that PCI_{BLEO} induces vascular shut-down in HT1080 fibrosarcoma [9]. In order to evaluate the treatment effect on the vasculature in the MES-SA and the SK-LMS-1 tumours, the tumours were analysed by DCE-MRI immediately before and 48 h after treatment. In addition 3 MES-SA and 3 SK-LMS-1 untreated control tumours were analysed by DCE-MRI one day before and 2 h and 72 h

Table 3

Median time (days) for the MES-SA and SK-LMS-1 tumours to reach 1000 mm³ after the indicated treatments. The increase as well as the statistical difference (pairwise log-rank) in time to reach the endpoint as compared to the untreated tumours. Only animals that reached 1000 mm³ are included (see text).

Treatment	MES-SA				SK-LMS-1			
	No. in group	Median ± SE (days)	% increase vs. NT	p-value vs. NT	No. in group	Median ± SE (days)	% increase vs. NT	p-value vs. NT
No treatment (NT)	32	11 ± 0,4	–	–	29	20 ± 1,3	–	–
Light 30 J/cm ²	11	15 ± 1,7	36%	0,044	10	20 ± 1,5	0%	0,892
AlPcS _{2a}	10	12 ± 3,2	9%	0,056	9	24 ± 1,5	20%	0,023
Bleomycin	10	17 ± 0,9	55%	0,002	12	25 ± 2,9	25%	0,086
PDT 30 J/cm ²	14	12 ± 0,6	9%	0,404	8	24 ± 0,7	20%	0,014
PDT 60 J/cm ²	10	14 ± 1,0	27%	0,026	9	30 ± 2,8	50%	0,029
PCI _{BLEO} 30 J/cm ²	12	30 ± 2,2	173%	0,000	12	42 ± 3,4	110%	0,000

after the tumours reached inclusion volume. There was no change in uptake of contrast agent in the untreated tumours during this time frame in either tumour model (data not shown). Similarly, no effect on uptake of contrast medium was seen after treatment with light only (40 J/cm²).

Ten animals implanted with MES-SA tumours were scanned immediately before and 48 h after treatment, 5 treated with PCI_{BLEO} and 5 with PDT. In all AUC90 maps the MES-SA tumours showed a moderate and scattered uptake of contrast agent centrally and in most cases a high peripheral uptake prior to treatment (Fig. 4). Forty-eight hours post PCI_{BLEO} and PDT all MES-SA tumours displayed a marked reduction in uptake of contrast agent. This vascular effect was most pronounced in the tumour centre, but also evident in the peripheral zone, especially after PCI_{BLEO}. Areas and strands with uptake of contrast agent were visible centrally after both treatment modalities, indicating intact tumour vasculature (Fig. 4).

Eight animals implanted with SK-LMS-1 tumours, 4 treated with PCI_{BLEO} and 4 with PDT were analysed as the MES-SA tumours. Prior to treatment the SK-LMS-1 tumours displayed a more homogenous and often a higher uptake of contrast agent than the MES-SA tumours (Fig. 5). In SK-LMS-1 tumours treated with PCI_{BLEO} a total vascular shutdown in the tumour centre was observed. A thin peripheral rim of the tumours still displayed uptake of contrast agent in two out of four tumours. In PDT treated SK-LMS-1 tumours small areas with uptake of contrast agent were frequently observed centrally (not shown), in addition to uptake in the tumour periphery (Fig. 5). This indicates a less complete vascular shutdown after treatment with PDT than after PCI_{BLEO}.

In the adjacent tissue outside the tumours (reactive zone) there was high uptake of contrast agent prior to treatment in both tumour models. Post treatment, enhanced uptake of contrast agent in the adjacent connective tissue was observed, corresponding with treatment induced inflammation and oedema.

3.4. Histopathological evaluation of treatment response

The peripheral rim of tumours has been shown to be especially resistant to PCI (and PDT) treatment in HT1080 and other xenograft models [9]. As we found no cured mice in either tumour model, it was of interest to see whether the MES-SA and SK-LMS-1 xenograft models displayed the same treatment resistance in the periphery, and shed light on possible differences. Three untreated MES-SA tumours were excised for histopathological characterization. The MES-SA tumours appeared highly malignant with nuclear pleomorphism and poor differentiation with no resemblance to smooth muscle cells (Fig. 6). There were strands of fibroblasts with blood vessels penetrating the tumour tissue. Several mitoses were seen and the tumours were quite well vascularised. Small areas with spontaneous necrosis, haemorrhage and inflammatory cells (neutrophils) were occasionally seen.

Two days post PDT 3 out of 4 evaluated MES-SA tumours displayed focal necrotic areas in the superficial part of the tumours. In the fourth tumour there was widespread central necrosis. The average area of necrosis was estimated to 1/3 of the total tumour area (Fig. 7A). Seven days post PDT there were scattered traces of earlier treatment effects in all 4 evaluated tumours, with some remaining necrosis, haemorrhage and inflammation. The average area of necrosis was estimated to 1/10 of the total tumour area (Fig. 7B).

Two days post PCI_{BLEO} tumour responses varied as evaluated in 5 treated tumours. Two tumours displayed complete central necrosis along with haemorrhage and inflammation. Two other tumours had no obvious treatment response, while the last one showed scattered treatment-induced necrosis. In general, treatment effects were most pronounced in the superficial part of the tumours, with viable tumour cells in the periphery. The average area with necrosis was estimated to 1/3 of the total tumour area, as for PDT (Fig. 7C). Seven days post PCI_{BLEO} remnants of necrosis were found in all 4 evaluated tumours, in a similar pattern to that of the PDT treated tumours, but somewhat

Fig. 3. Fluorescence Images 48 h after intraperitoneal injection of AlPcS_{2a}. The left animal in each group has been injected with 10 mg/kg AlPcS_{2a}.

Fig. 4. Area Under Curve (AUC90) maps for the first 90 s after injection of contrast agent of MES-SA xenografts, acquired immediately before and 48 h after treatment with PDT and PCI_{BLEO} , as indicated on the figure. Scale bar indicates relative signal intensity, arbitrary units.

more pronounced. The average area with necrosis was estimated to 1/5 of the total tumour area (Fig. 7D).

Three untreated SK-LMS-1 tumours were excised for histopathological characterization. The SK-LMS-1 tumours displayed an intermediate malignancy grade with some resemblance to smooth muscle cells and a growth pattern also suggestive of their origin. Few mitoses were seen and the nuclear pleomorphism was less pronounced as compared to the MES-SA tumour (Fig. 8). Capillaries and vessels were abundant, and no necrosis or haemorrhage was found.

Two days post PDT treatment a marked central necrosis was found in 4 out of 6 evaluated SK-LMS-1 tumours (Fig. 9A). In the remaining two tumours smaller necrotic areas were scattered in the tumour centre. The average area with necrosis was estimated to 1/2 of the total tumour area. Eight days post PDT the tumours generally appeared viable with only small areas of coagulation necrosis (Fig. 9B). The average area with necrosis was estimated to $< 1/10$ of the total tumour area.

Two days post PCI_{BLEO} treatment there was widespread central

necrosis with haemorrhage in 6 out of 7 evaluated tumours (Fig. 9C). Viable tumour cells were dominating in the periphery. One tumour had no obvious treatment effect. The average area with necrosis was estimated to 2/3 of the total tumour area. Eight days post PCI_{BLEO} there were still large areas with coagulation necrosis in the tumour centre of all 4 evaluated tumours, with viable tumour cells in the periphery (Fig. 9D). The average area with necrosis was estimated to 1/2 of the total tumour area.

3.5. Vascular characteristics of MES-SA and SK-LMS-1 tumours assessed by immunohistochemistry (IHC)

As previously pointed out, vascular shutdown is proven to play a part in the tumouricidal effects of photochemical treatment. Differences in vascular response to PCI between tumour types, as indicated by MRI studies described above, may depend on the density or quality of intratumoural vessels. Evaluation of microvessel densities after IHC with

Fig. 5. Area Under Curve (AUC90) maps for the first 90 s after injection of contrast agent of SK-LMS-1 xenografts, acquired immediately before and 48 h after treatment with PDT and PCI_{BLEO} , as indicated on the figure. Scale bar indicates relative signal intensity, arbitrary units.

Fig. 6. Histopathological staining of MES-SA tumours.

MES-SA tumour tissue (T), stained with hematoxylin and eosin (H/E), showing small areas of spontaneous necrosis (N), a thin layer of overlying muscle (M) and skin (S) (40× magnification). At high magnification (200×) there is no smooth muscle cell differentiation or organisation, and a highly malignant appearance.

staining of CD31 and CD34 proteins, showed that SK-LMS-1 tumours had somewhat more abundant vascular structures than MES-SA [27]. The average number of CD31 positive vessels in SK-LMS-1 tumours was 76% higher than in the MES-SA tumours ($p = 0.05$). The average number of CD34 positive vessels was 24% higher in the SK-LMS-1 tumours ($p > 0.05$) (Table S8). While the appearance of the vessels in the tumour centre was similar in the two xenograft models, the vessels in the periphery of the SK-LMS-1 tumours had larger diameters and open lumina compared to MES-SA (Fig. 10). The distribution of the vessels seemed heterogenous in both tumour models as the average vascular density in the peripheral part of the tumours was somewhat higher than in the centre (Figs. 11 and 12). Furthermore, the MES-SA tumours displayed penetrating strands of fibrous tissue with larger vessels in the tumour centre, in contrast to the homogenous distribution pattern of vessels in the centre of the SK-LMS-1 tumours.

4. Discussion

In the present study we report superior efficacy of ALPcS_{2a}-PCI of bleomycin in two dissimilar leiomyosarcoma xenograft models, the human uterine MES-SA and the human vulvar SK-LMS-1 tumours, compared to all other treatment groups including PDT. However, there were no cures after PCI_{BLEO} and neither xenograft model responded significantly to treatment with PDT with time to reach the termination volume 1000 mm³ as end point. Doubling the PDT light dose to 60 J/cm² did not change this, underscoring that photodynamic therapy with

ALPcS_{2a} is not efficient in these xenograft models. The lack of PDT effect is in contrast to previous *in vivo* findings where both photosensitizers used for PCI treatments *in vivo* (ALPcS_{2a} and TPCs_{2a}) in combination with light always have induced tumour growth delay. These studies include WiDr colon carcinoma, CT26 mouse colon carcinoma, TAX-1 undifferentiated pleomorphic sarcoma and orthotopic HT1080 fibrosarcoma xenografts [9,10,28]. In the same tumour models PCI induced a stronger tumour growth delay and also remission in a large fraction of the tumours, except in the HT1080 fibrosarcoma where no cure was documented. This indicates a relative treatment resistance in the leiomyosarcoma xenografts evaluated in the current study, not surprising with the known chemotherapy resistance of soft tissue sarcomas in clinical practice [12,29]. This treatment resistance in human patients is proposed to be a consequence of several factors, among them a vast intratumoural cell heterogeneity, and the presence of cancer stem cells in sarcomas [30].

The leiomyosarcoma xenografts MES-SA and SK-LMS-1 have previously been reported to differ in molecular expression profiles (Table S1) and were also found in this study to differ in vascularity, growth pattern, histological differentiation and malignancy grade. Accordingly, *in vitro* studies have shown superior PCI_{BLEO} cytotoxicity in MES-SA cells compared to SK-LMS-1 cells [25]. Yet, the present *in vivo* study showed a similar growth retardation after treatment with PCI_{BLEO} and no significant response to PDT or any other treatment with time to reach 1000 mm³ as end point (Figs. 1 A and 2 A). Thus, these results indicate that *in vitro* response to PCI_{BLEO} cannot be used as an indicator

Fig. 7. Histopathological staining of MES-SA tumours treated with PDT or PCI_{BLEO}.

The tumours were excised and sectioned 2 and 7 days after treatment as indicated on the figure. The sections were H/E-stained and necrosis (N) and viable tumour tissue (V) are indicated.

Fig. 8. Histopathological staining of SK-LMS-1 tumours.

SK-LMS-1 tumour tissue (T), stained with H/E, shows a thin layer of overlying muscle (M) and no spontaneous necrosis (40× magnification). The tumour cells are poorly differentiated, but more uniform than the MES-SA cells, with a growth pattern and organisation suggestive of their origin, smooth muscle (200× magnification). The tumour appearance is of intermediate malignancy grade.

of therapeutic outcome *in vivo*.

The growth rate curves after treatment with PCI_{BLEO} were found to differ between the MES-SA and SK-LMS-1 tumours. PCI_{BLEO} resulted in an exponential but reduced growth rate in the MES-SA tumours while the SK-LMS-1 tumours stopped growing for approximately 12 days, followed by an exponential growth similar to that of the untreated tumours. The growth pattern post non-curative PDT usually resembles that seen after PCI_{BLEO} in the SK-LMS-1 cells (initial growth delay followed by exponential growth similar to that of untreated tumors) and not the continuously reduced growth rate as seen after PCI_{BLEO} in the MES-SA tumors [28,31–33]. A major cause of bleomycin cytotoxicity is the formation of DNA double-strand breaks [34]. We have recently found that PCI_{BLEO} induces a stronger cytotoxic effect and a much stronger induction of DNA double-strand breaks as seen by γ -H2AX staining, in MES-SA compared to SK-LMS-1 cells [25]. In this work it was further found that the cell death process induced by photochemical activation of bleomycin is slow acting and could not readily be detected by a viability test 2–3 days after treatment, but required 10–12 days and utilization of the clonogenicity assay. It is therefore reasonable to assume that the substantial change in growth rate as seen only in the MES-SA tumours is due to a stronger activation of bleomycin in the MES-SA cells than in the SK-LMS-1 cells. The reduced growth rate after PCI_{BLEO} in MES-SA tumours is also in accordance with reports on monotherapy with higher doses of bleomycin [25,35–37].

The histological evaluations one week after PCI_{BLEO} showed massive necrosis in the SK-LMS-1 tumors and virtually no remaining necrosis in

the MES-SA tumours. These differential effects of PCI_{BLEO} may be explained by a stronger vascular effect in the capillary rich SK-LMS-1 tumours. SK-LMS-1 tumours may require more time to heal and regrow than the MES-SA tumors where neutrophils and monocytes may be recruited through remaining intact intratumoural vasculature. Surviving larger blood vessels in the centre of the MES-SA tumours also supply proliferating tumour cells with oxygen and nutrients and propagate tumour regrowth.

The MRI data in the current study show, as in previous studies, that PDT and PCI_{BLEO} both induce a vascular shutdown. The photosensitizer AlPcS_{2a} has been proven to also accumulate in endothelial HUVEC cells, as well as endothelial cells *in vivo*, and furthermore that PDT and PCI_{BLEO} exert cytotoxic effects in the endothelial cell line HUVEC *in vitro* [26]. The SK-LMS-1 tumors were found better vascularized than the MES-SA tumors and with a more homogenous vessel distribution, while the MES-SA tumours were penetrated with strands of fibrous tissue containing larger vessels. Such vessels have previously been shown to be resistant to PDT [38]. This is in accordance with the histopathological pattern of necrosis after treatment with PDT and PCI_{BLEO} and the MR images showing remaining strands of contrast agent uptake in the tumour tissue after PCI_{BLEO} in the MES-SA tumors. Vascular shutdown has been shown to induce vasculogenesis based on circulating endothelial progenitor cells, as well as progenitor cells released from the bone marrow [39]. Thus, the growth kinetics of the SK-LMS-1 tumors after PCI_{BLEO} may indicate that the vasculature was severely damaged, the PCI -induced activation of bleomycin in the tumour cells

Fig. 9. Histopathological staining of SK-LMS-1 tumours treated with PDT or PCI_{BLEO} .

The tumours were excised and sectioned 2 and 8 days after treatment as indicated on the figure. The sections were H/E-stained and necrosis (N), viable tumour tissue (V) and coagulation necrosis (CN) are indicated.

Fig. 10. Immunohistochemical staining of CD31 and CD34 for identification of vascular structures in MES-SA and SK-LMS-1 tumours, from the most highly vascularised areas. Blood vessels are stained brown.

Fig. 11. Immunohistochemical staining of CD31 and CD34 in MES-SA tumours indicates a somewhat higher vascular density in the tumour periphery than in the centre.

less efficient and that vasculogenesis rescued the SK-LMS-1 tumours from a significant tumour growth delay. Based on the more complete vascular shutdown after PCI_{BLEO} in the SK-LMS-1 tumours, the differences in growth kinetics in the two tumour models and the *in vitro* studies indicate PCI_{BLEO} to be more efficient on the tumour parenchymal cells in MES-SA tumors and more efficient on the vasculature in the SK-LMS-1 tumours. One may speculate that a combination of tumour cell sensitivity to PCI-induced bleomycin activation and vascular characteristics may be used together as predictive markers for therapeutic outcome, e.g. cells sensitive to PCI_{BLEO} *in vitro* forming tumours with a treatment sensitive capillary-rich stroma being the most

sensitive tumours to PCI_{BLEO} .

MES-SA tumours were found to have strands of fibroblasts and vessels penetrating the tumour tissue that were accessible by MRI contrast agents. Vascular effects of photochemical therapy are mostly on capillaries, the larger vessels in these strands are therefore likely to be more resistant to vascular collapse. Furthermore, cancer associated fibroblasts (CAFs) are known to have an important regulatory role in the microenvironment and also to stimulate tumour growth [40]. CAFs have been shown to stimulate tumour progression (and metastasis) when subjected to increased levels of reactive oxygen species (ROS) [41], and may play a part in the MES-SA tumours' resistance towards

Fig. 12. Immunohistochemical staining of CD31 and CD34 in SK-LMS-1 tumours shows a somewhat higher vascular density and wider vessel lumina in the tumour periphery than in the centre.

this photochemically induced vascular effect, as observed in the MR images. Rapid regrowth of tumour cells in the tumour centre, as shown histologically in MES-SA samples harvested one week post treatment, may be facilitated by the strands of fibrous tissue with CAFs and undamaged larger blood vessels.

PCI *in vivo* was found to effectively eradicate cancer cells in the tumour centre nourished by capillaries, while tumour cells in the better vascularized peripheral zone were found treatment resistant. This pattern of treatment effects resembles that of an emerging group of cancer therapeutics, vascular disrupting agents (VDAs) [42]. VDAs disrupt tumour vasculature via several different molecular targets, most importantly via tubulin binding. The VDAs induce central necrosis, incite and promote proangiogenic substances such as HIF-1 α , VEGF and SDF-1 α , thereby stimulating recruitment of endothelial progenitor cells resulting in surviving peripheral cells that may propagate and metastasize [43]. Development of treatment with VDAs is therefore mainly performed as combination therapies with chemotherapeutics, other anti-angiogenic therapies or radiotherapy. Likewise, future development of PCI should probably include combinations with other treatment modalities that better target the tumour periphery. Norum and coworkers investigated radiotherapy as an adjunct to PCI, and administered radiotherapy post PCI with favourable results [32]. However, as Liang and coworkers suggest for VDAs, it may be more effective to give radiotherapy before PCI, because PCI induced oxygen depletion and acidosis prevents optimal radiotherapy-induced DNA damage [43].

In conclusion we have, for the first time, demonstrated the *in vivo* treatment efficacy of PCI of bleomycin in leiomyosarcoma. The MES-SA and the SK-LMS-1 tumours differ genotypically and morphologically, but their responses to PCI_{BLEO} and PDT *in vivo* are comparable. *In vitro*, the MES-SA cells are substantially more sensitive to PCI_{BLEO} than the SK-LMS-1 cells [25]. Differences in vascularity, both in terms of vessel size and distribution, as well as tumour cell response to bleomycin activation between the two xenograft models may explain the discrepancies in treatment responses *in vitro* and *in vivo*. The similar treatment efficacy *in vivo* for these dissimilar xenograft models proves the unselective potential of PCI as a targeted treatment modality of solid malignant tumours. To improve efficacy further, refinement of the

PCI technique is needed. Combining PCI with other existing therapies, higher light doses, different drugs to internalize, e.g. immunotoxins, are possible areas for future research. For further pre-clinical evaluation of PCI it is necessary to investigate in depth the vascular effects, as this emerges as an important factor for success.

Competing interests

None of the authors have competing interests regarding publication of this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.pdpdt.2017.08.015>.

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Table S1

	MES-SA	SK-LMS-1
p53	Wild type	Mutated
PTEN	-	+
pAKT	++	+
EGFR	-	+
HER2	+	-
C-met	-	+
IGF-1R	++	++

Table S1

Comparison of molecular expression profiles between MES-SA and SK-LMS-1. References, MES-SA: p53 [44]; PTEN, pAKT, HER2, C-met [45]; EGFR [3]; IGF-1R [24]. References, SK-LMS-1: p53 [46]; PTEN, pAKT, HER2, C-met [45]; EGFR [47]; IGF-1R [24].

Table S2

Treatment	NT	Light 30 J/cm ²	AlPcS _{2a}	Bleomycin	PDT 30 J/cm ²	PDT 60 J/cm ²
NT						
Light 30 J/cm ²	0,044					
AlPcS _{2a}	0,056	0,601				
Bleomycin	0,001	0,121	0,366			
PDT 30 J/cm ²	0,158	0,594	0,812	0,070		
PDT 60 J/cm ²	0,026	0,523	0,896	0,343	0,614	
PCI _{BLEO} 30 J/cm ²	0,000	0,000	0,000	0,013	0,002	0,000

Table S2

Statistical significance analyses (p) to reveal differences in therapeutic effect between various treatment regimens on the MES-SA tumours.

Note: The values are based on paired log-rank analyses of the time for the MES-SA tumours to reach 1000 mm³.

Table S3

Treatment	MES-SA		SK-LMS-1	
No treatment	0/32	0%	4/33	12%
Light 30 J/cm ²	0/11	0%	1/11	9%
AlPcS _{2a}	0/10	0%	1/10	10%
Bleomycin	1/11	9%	0/12	0%
PDT 30 J/cm ²	1/15	7%	2/10	20%
PDT 60 J/cm ²	0/10	0%	1/10	10%
PCl _{BLEO} 30 J/cm ²	0/12	0%	3/15	20%

Table S3

Fractions of regressions in each treatment group (number of animals that never reached 1000 mm³/number of animals in each group).

Table S4

Treatment	NT	Light 30 J/cm ²	AIPcS _{2a}	Bleomycin	PDT 30 J/cm ²	PDT 60 J/cm ²
NT						
Light 30 J/cm ²	0,932					
AIPcS _{2a}	0,270	0,972				
Bleomycin	0,863	0,761	0,298			
PDT 30 J/cm ²	0,082	0,089	0,502	0,077		
PDT 60 J/cm ²	0,296	0,245	0,806	0,214	0,476	
PCI _{BLEO} 30 J/cm ²	0,001	0,002	0,012	0,000	0,154	0,017

Table S4

Statistical significance analyses (p) to reveal differences in therapeutic effect between various treatment regimens on the SK-LMS-1 tumours.

Note: The values are based on paired log-rank analyses of the time for the SK-LMS-1 tumours to reach 1000 mm³.

Table S5

Treatment	NT	Light 30 J/cm ²	AlPcS _{2a}	Bleomycin	PDT 30 J/cm ²	PDT 60 J/cm ²
NT						
Light 30 J/cm ²	0,892					
AlPcS _{2a}	0,023	0,120				
Bleomycin	0,086	0,179	0,584			
PDT 30 J/cm ²	0,014	0,038	0,695	0,383		
PDT 60 J/cm ²	0,029	0,064	0,763	0,458	0,594	
PCI _{BLEO} 30 J/cm ²	0,000	0,000	0,000	0,000	0,001	0,001

Table S5

Statistical significance analyses (p) to reveal differences in therapeutic effect between various treatment regimens on the SK-LMS-1 tumours. Only animals that reached 1000 mm³ are included (see text).

Table S6

Treatment	NT	Light 30 J/cm²	AlPcS_{2a}	Bleomycin	PDT 30 J/cm²	PDT 60 J/cm²
NT						
Light 30 J/cm²	0,044					
AlPcS_{2a}	0,056	0,601				
Bleomycin	0,002	0,245	0,853			
PDT 30 J/cm²	0,404	0,236	0,281	0,025		
PDT 60 J/cm²	0,026	0,523	0,896	0,817	0,182	
PCI_{BLEO} 30 J/cm²	0,000	0,000	0,000	0,000	0,000	0,000

Table S6

Statistical significance analyses (p) to reveal differences in therapeutic effect between various treatment regimens on the MES-SA tumours. Only animals that reached 1000 mm³ are included (see text).

Table S7

	MES-SA					SK-LMS-1				
Treatment	No. in group	Mean $\pm$ SE (days)	% increase vs. NT	p-value vs. NT		No. in group	Mean $\pm$ SE (days)	% increase vs. NT	p-value vs. NT	
No treatment (NT)	32	11,5 $\pm$ 0,5	-	-		29	19,9 $\pm$ 1,0	-	-	
Light 30 J/cm ²	11	13,9 $\pm$ 1,0	21%	0,044		10	20,2 $\pm$ 2,1	2%	0,892	
AIPcS _{2a}	10	13,9 $\pm$ 1,6	21%	0,056		9	25,3 $\pm$ 2,8	27%	0,023	
Bleomycin	10	15,6 $\pm$ 0,9	36%	0,002		12	22,8 $\pm$ 2,6	15%	0,086	
PDT 30 J/cm ²	14	12,4 $\pm$ 0,7	8%	0,404		8	27,1 $\pm$ 2,9	36%	0,014	
PDT 60 J/cm ²	10	14,2 $\pm$ 1,3	23%	0,026		9	26,2 $\pm$ 2,8	32%	0,029	
PCI _{BLEO} 30 J/cm ²	12	31,3 $\pm$ 2,7	172%	0,000		12	44,7 $\pm$ 3,9	125%	0,000	

Table S7

Mean time (days) for the MES-SA and SK-LMS-1 tumours to reach 1000 mm³ after the indicated treatments. The increase as well as the statistical difference (pairwise log-rank) in time to reach the endpoint as compared to the untreated tumours. Only animals that reached 1000 mm³ are included.

Table S8

MES-SA			SK-LMS-1		
Animal no.	CD31	CD34	Animal no.	CD31	CD34
1	75	100	1	144	124
2	51	79	2	111	68
3	108	125	3	155	183
Average	78 ± 16*	101 ± 13*	Average	137 ± 13*	125 ± 33*

* Standard error, SE

Table S8

Number of vascular structures per 200x field in the microscope based on the area with the highest vessel density in each sample, method as described by Weidner, Semple et al. 1991 [27].