# Photochemical internalization as an adjunct to marginal surgery in a human sarcoma model

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***Received 25th November 2008, Accepted 12th March 2009***
***First published as an Advance Article on the web 20th March 2009***
**DOI: 10.1039/b821129a**



Local recurrences after inadequate surgery are a major challenge in many cancers. Photochemical internalization (PCI) is a new technology to release endocytosed macromolecules into the cytosol by a photochemical rupture of the endocytic vesicles. A recent study on an invasive human fibrosarcoma xenograft HT1080 indicated low sensitivity of the tumour periphery to disulfonated aluminium phthalocyanine ($AlPcS_{2a}$)-based photochemical treatment. The main goal of the present study was to evaluate the sensitivity of the remaining tumour after marginal resection of the HT1080 tumour to the photochemical treatment alone and PCI of bleomycin. $AlPcS_{2a}$ and bleomycin was systemically administrated 48 h and 30 min, respectively, prior to surgery and immediately followed by intra-operative light exposure at 670 nm (15 J $cm^{-2}$). PCI of bleomycin as an adjunct to surgery did significantly delay tumour growth in contrast to the photochemical treatment alone. The results indicate that PCI of bleomycin may be an efficient intra-operative technique to eradicate cancer cells in the wound bed after inadequate surgery.

## Introduction

Local recurrences are a major problem in cancer treatment and the rates vary from cancer to cancer and are in general dependent on the pathological anatomical diagnosis and local anatomical considerations. The local recurrence in breast cancer is reported to be 5–25%,[1] in rectum cancer 5–35%,[2] and soft tissue sarcomas 5–40%.[3] In other cancers, local recurrences are almost inevitable and no efficient local cure is available for malignant brain tumours[4] and malignant pleural mesothelioma.[5]

Photodynamic therapy (PDT) is an established treatment modality approved for many superficial cancers and non-malignant diseases.[6] PDT is based on compounds, named photosensitizers, that upon excitation by light form cytotoxic reactive oxygen species of which singlet oxygen is dominating. In a recent report PDT was found to be efficient for eradication of the central part of an invasive HT1080 fibrosarcoma model located in the gastrocnemius muscle.[7] Contrast enhanced magnetic resonance images (CE-MRI) two hours after PDT and histological sections seven days after PDT both indicated that the tumour periphery responded differently from the central region. The tumour periphery appeared substantially less sensitive to PDT than the central part of the tumour and regrowth after PDT was restricted to the tumour periphery only.[7]

Photochemical internalization (PCI) is a novel technology based on the PDT principle (singlet oxygen formation after excitation of a photosensitizer), but in addition to the biological effects of reactive oxygen species after PDT, the PCI technology allows for a photochemical release of endocytosed macromolecules into the cytosol.[8] In this way, PCI may facilitate the transport of biologically-active substances and hence improve the therapeutic effect. One of the advantages of the PCI technology is its ability to activate a plethora of therapeutic molecules accumulating in the endocytic vesicles.[9,10]

Bleomycin (BLM) is a chemotherapeutic agent and widely used for lymphomas, testicular cancers and germ cell tumours of children in clinical therapy. Bleomycin is taken up by endocytosis and trapped and degraded in the endocytic vesicles, unless otherwise released to the cytosol where it can reach its intracellular target. Photochemical internalization of bleomycin (PCI of BLM) was found superior to PDT in inhibiting growth of the HT1080 xenografts by more efficiently targeting the peripheral zone of the tumour.[7]

The application of PDT for sterilizing the tumour bed after cytoreductive surgery has been the focus of several clinical trials.[11–15] The results have been promising, but not convincing in achieving local control. In the present study the human HT1080 fibrosarcoma xenograft was transplanted in the lateral part of the femoral quadriceps muscle in mice. All macroscopic tumour tissue was removed surgically, leaving only microscopic residual tumour cells left in the transitional zone. The aim of the study was to evaluate the effect of PDT and PCI of BLM on the transitional zone and to assess these treatment modalities as an adjunct to surgery in a human sarcoma animal model resembling a clinical situation with inadequate surgery. The results indicate that the tumour transitional zone of an invasive cancer model is apparently more resistant to PDT, and that PCI may offer an efficient treatment alternative as an adjunct to surgery.

## Materials and methods

### Animals and xenograft

Female BALB/c (nu/nu) nude mice were bred at the Animal department at our institute as previously described.[7] Water and

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food were given *ad libitum*. All procedures involving mice were carried out in agreement with protocols approved by the animal care unit at the Norwegian Radium Hospital HF, under control of the National Ethical Committee's guidelines on animal welfare. The human fibrosarcoma cell line HT1080 was purchased from the American Type Culture Collection (ATCC, Rodville, MD, USA). The cell line was propagated as previously described[7] and approximately 20 µl (approx. 400 000 cells) of the tumour cell suspension was injected 3 mm above the patellar tendon into the left lateral femoral quadriceps muscle.

### Chemicals

Disulfonated aluminium phthalocyanine ($AlPcS_{2a}$) with the sulfonate groups on adjacent phthalate rings was purchased from Frontier Scientific (Logan, UT, USA), dissolved in 1 ml 0.1M NaOH and diluted with 29 ml phosphate-buffered saline (PBS) to a concentration of 2 mg $ml^{-1}$. The solution was sonicated for about 1 min, diluted with 18 ml PBS to a final concentration of 1.25 mg $ml^{-1}$ and stored as previously described.[7] The $AlPcS_{2a}$ solution was injected intraperitoneally (i.p.) at a final concentration of 10 mg $kg^{-1}$. Fifteen thousand IU of bleomycin was dissolved in 1 ml 0.9% NaCl. Hypnorm-Dormicum was injected subcutaneously for anesthesia in variable doses: 20 µl for light sedation when measuring the initial tumour volume and 100 µl for sedation during surgery.

### Estimating tumour volume

The HT1080 tumour implanted in the femoral quadriceps muscle did not appear as a clearly defined tumour viewed through the skin at the time of light exposure (approximately 100 $mm^3$) and it was not possible to directly measure the tumour volume with a calliper. However, the thigh appeared swollen at an early stage (tumour volumes of approximately 50 $mm^3$) and the volume increase of the whole leg was used to estimate tumour volume assuming a spherical shape of the tumour. When the tumour increased in size it protruded out through the muscle and external measurement was feasible. Tumour volume ($V$) was calculated five times per week after treatment determined by using the formula: $V = (W \times W \times L)/2$ where $W$ (width) is the shorter and $L$ (length) is the longer of two perpendicular diameters, measured with a calliper.

### Photochemical treatment

$AlPcS_{2a}$ (10 mg $kg^{-1}$) was injected i.p. when the tumours reached a size leading to tumour volumes of 80–150 $mm^3$ at the day of illumination. Forty-eight hours after the injection of $AlPcS_{2a}$, 1500 IU BLM was injected i.p. The animals were illuminated 30 min later using a diode laser emitting at 670 nm (CeramOptec GmbH, Bonn, Germany) as previously described.[7] The tumours where exposed to 15 J $cm^{-2}$ of light at an irradiance of 90 mW $cm^{-2}$.

### Surgical treatment

Mice under anaesthesia were placed on Styrofoam to reduce heat loss, wrapped in aluminium foil with the affected left limb out through a small hole fitting closely around the leg to prevent light penetrating the abdomen. The tumour was removed with the intension to achieve a R1 positive resection margin with no macroscopic tumour tissue left (R0 = tumour free margin, R1 = microscopically involved margin, R2 = macroscopically involved margin). Curettage was performed after removal of all gross tumour tissue to ensure a minimum of residual cells left (Fig. 1a–e). The animals were then exposed to light from the diode laser before the wounds were closed with individual nylon sutures.

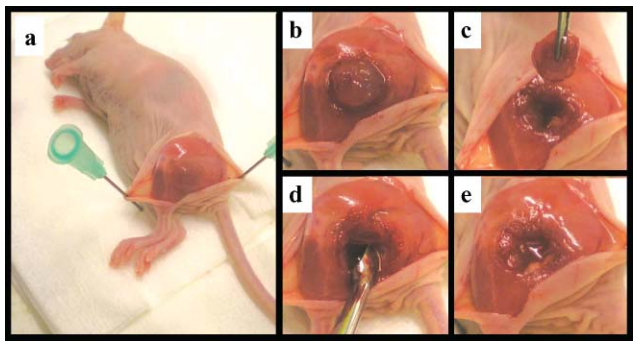


**Fig. 1** The surgical procedure. After administration of adequate anaesthesia the skin was opened and the lateral femoral quadriceps muscle was exposed (a). An incision was made in the muscle fibers at the macroscopic interface between the tumour tissue and the adjacent normal tissue (b). The tumour was removed by partly sharp and partly blunt dissection (c). After tumour removal, a curette was used in the wound bed (d) and hemostasis was obtained with compression over 30 s (e). The mice recovered rapidly after surgery and showed normal behavior usually within 3–4 h.

### Statistical analysis of the data

The tumour growth data are subjected to survival analysis, using the day when the tumour volume supersedes the volume $V_{crit}$ = 1000 $mm^3$ as the failure time, and the duration of the experiment as the censoring time, if the tumour does not obtain this volume. Statistical differences in treatment response were evaluated by pairwise log-rank analyses.

The documentation of synergy is based on a log-logistic survival function as described in ref. 7 and 9.

## Results

### Tumour model and measurements of initial tumour volume

The photosensitizer $AlPcS_{2a}$ was administrated 48 h prior to light exposure. At the time of light delivery, when the initial tumour volume was approximately 100 $mm^3$, only an intramuscular swelling was present in the muscle at the injection site in the animals. The estimated tumour volume (see Materials and methods) was compared to the surgically removed and weighed tumours (wet weight) in a pilot study and the method was found sufficiently valid for tumour volume estimation with a regression coefficient equal of 0.88.

### Response to PDT, BLM and PCI of BLM

In order to avoid curative response by PCI alone or by PDT in combination with surgery, the total light dose was reduced by 50% of that in the study on HT1080 in the gastrocnemius muscle,[7] where the intact skin represented a natural light barrier and no surgery was performed. In the present study, PDT and

**Table 1** Mean time for the tumour HT1080 xenograft to reach the endpoint of 1000 $mm^3$. Mean time (days) for the HT1080 tumour to reach the endpoint of 1000 $mm^3$ after various specified treatments based on Kaplan-Meier survival analysis in SPSS 15.0 (SPSS Inc.) where all animals in the groups are included. SE represents standard error

| Group | No. of animals | Mean time (days) to reach 1000 $mm^3$ | SE |
|---|---|---|---|
| Control | 11 | 6.9 | 0.4 |
| PDT | 10 | 8 | 1.0 |
| BLM | 10 | 8.5 | 0.5 |
| PCI | 11 | 16.7 | 1.0 |
| Surgery | 10 | 7.9 | 0.5 |
| Surg. + PDT | 10 | 8.5 | 0.7 |
| Surg. + BLM | 10 | 12.5 | 0.8 |
| Surg. + PCI | 10 | 23.9 | 1.4 |

BLM alone induced only a 1–1.5 day delay of tumour growth as compared to the untreated tumours (Fig. 2, Table 1). PCI of BLM was found to delay the tumour growth by 9 days compared to PDT and 10 days compared to the control group (Table 1). PCI of BLM caused a significantly longer growth delay than all the other non-surgical treatment regimens (Fig. 2, Table 2) and was found to act in a synergistic manner ($p < 0.001$) in inhibiting tumour growth in accordance with previous PCI reports on subcutaneous tumour models.[7,9] From previous studies $AlPcS_{2a}$ is known not to influence tumour growth in the absence of light and was therefore not evaluated in the present study.

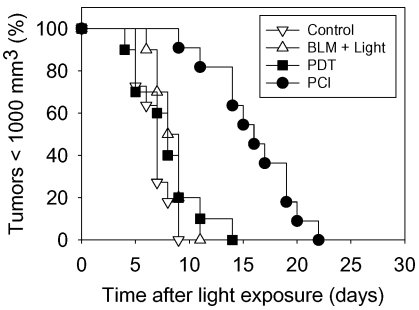


**Fig. 2** Tumour growth assessment (Kaplan-Meier plot) after photochemical treatment with and without bleomycin (PDT and PCI of BLM) of HT1080 fibrosarcoma xenografts. The various treatments are indicated on the figure. The endpoint was the time after treatment when the individual tumours reached a tumour volume of 1000 $mm^3$. The results are based on interpolations of the measured tumour volumes. For clarity, not all data points are shown.

### Response to surgery alone and in combination with PDT and PCI

The results of the treatment effect of surgery followed by PDT or PCI of BLM are shown in Fig. 3. The surgical treatment alone did not induce a significant tumour growth delay ($p = 0.211$, Table 2) compared to the control group. The light exposure used for PDT and PCI was performed on the tumour bed immediately after the tumour was resected. Thus, although the applied light dose was the same as in the treatment groups not subjected to surgery, the fluence received by the tumour cells remaining in the tumour bed was much higher. Interestingly, with PDT following surgery little was achieved and the growth delay was not significantly different from the control and surgery groups ($0.07 < p < 0.90$, Table 1 and 2, Fig. 3a). Bleomycin in combination with surgery induced a

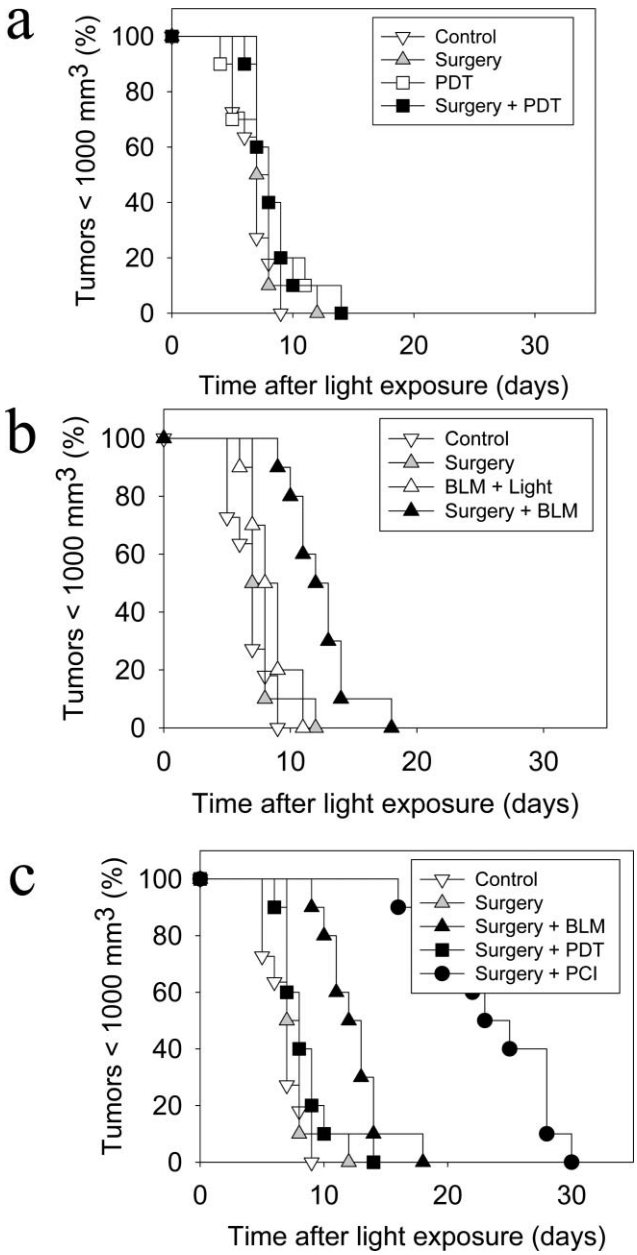


**Fig. 3** Tumour growth assessment after photochemical treatment with and without bleomycin (PDT and PCI of BLM) of HT1080 fibrosarcoma xenografts alone or after surgery. (a) Kaplan-Meier plot of PDT after surgery, (b) BLM after surgery and (c) PCI of BLM after surgery. The various treatments are indicated on the figures. The endpoint was the time (days) after treatment when the individual tumours reached a tumour volume of 1000 $mm^3$. The results are based on interpolations of the measured tumour volumes. For clarity, not all data points are shown.

significant growth delay of 5.6 days compared to the control group, and 4.6 and 4 days compared to the surgery and the BLM alone group, respectively ($p < 0.001$, Table 1 and 2, Fig. 3b). Bleomycin in combination with surgery was found to act in a synergistic manner ($p < 0{,}001$) as compared to the expected sum of the individual treatments. PCI in combination with surgery induced a strong growth delay of 17 days compared to the untreated animals and 11 days compared to surgery plus BLM ($p < 0.001$, Table 1 and 2, Fig. 3c). PCI of BLM in combination with surgery was found to

 

**Table 2** Statistical significance analyses ($p$) to reveal differences in the response to various treatment regimens. The values are based on paired log-rank analyses of the time for HT1080 tumours to reach 1000 $mm^3$

| Treatment | Control | BLM | PDT | PCI | Surgery | Surg. + PDT | Surg. + PCI | Surg. + BLM |
|---|---|---|---|---|---|---|---|---|
| Control | | | | | | | | |
| BLM only | 0.037 | | 0.885 | <0.001 | 0.483 | 0.883 | <0.001 | <0.001 |
| PDT | 0.187 | 0.885 | | <0.001 | 0.591 | 0.905 | <0.001 | 0.009 |
| PCI | <0.001 | <0.001 | <0.001 | | <0.001 | <0.001 | <0.001 | 0.004 |
| Surgery | 0.211 | 0.483 | 0.591 | <0.001 | | 0.437 | <0.001 | <0.001 |
| Surg. + PDT | 0.071 | 0.883 | 0.904 | <0.001 | 0.437 | | <0.001 | 0.006 |
| Surg. + PCI | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | | <0.001 |
| Surg. + BLM | <0.001 | <0.001 | 0.009 | 0.004 | <0.001 | 0.006 | <0.001 | |

act in a synergistic manner ($p < 0{,}001$) compared to the expected sum of the individual treatments (BLM and surgery + PDT and surgery). After surgery alone, or surgery in combination with PDT or BLM, the local recurrence was detectable on day 2–3, while after PCI of BLM they were not detected until 10 days after treatment (data not shown).

## Discussion

The infiltrative growth of many cancers frequently results in local recurrences as well as metastases and is therefore a major challenge in cancer treatment. In the present study PDT and PCI of BLM have been evaluated for treatment of the tumour cavity after inadequate surgical resection (R1 resection) of an infiltrative human sarcoma. The results indicate that PCI of BLM, but not PDT under the current conditions, inhibits the growth of the remaining fibrosarcoma cells following surgery. The results indicate an effective delivery of BLM in the tumour periphery illustrating an advantage of PCI over PDT induced by the release of endocytosed therapeutic agents.

All the animals subjected to surgical resection alone showed local recurrence and the time to reach a volume of 1000 $mm^3$ was delayed by only one day as compared to the control group (Table 1). The high rate of local recurrence and the lack of significant benefit from surgery (Table 2) is in accordance with earlier reports on marginal surgery (which may be regarded as R1 resections) on intramuscular sarcoma models in animals[16,17] as well as in clinical practice.[3] Administration of a chemotherapeutic agent prior to surgery is known to increase the relapse free time in animal models.[16] In the present study the combination of BLM and surgery was found to act in a synergistic manner (Fig. 3c, Table 2). The results indicate further that BLM is taken up and exerts a therapeutic effect on the tumour remaining in the cavity after surgery.

PDT is of interest as an adjuvant to surgery when a safe surgical resection margin cannot be obtained. However, in this study PDT did not cause any delay in the tumour growth of surgically treated animals (Fig. 3b, Table 2b). $AlPcS_{2a}$ is the most phototoxic phthalocyanine photosensitizer[18] and the therapeutic effect could possibly be increased by increasing the PDT dose. Principally, the surgery removed the central part of the tumour, leaving only cells from the transitional zone in the surgical cavity. Recent reports indicate that the tumour periphery is less sensitive to PDT than the tumour centre[7,19] and is in accordance with the present study. Earlier reports on PDT after marginal surgery in animal models have shown significant benefit of PDT applied to the surgical cavity, but residual growth has been seen in all cases[17,20,21] indicating a survival fraction of tumour cells in the periphery. The invasive and aggressive properties of the HT1080 fibrosarcoma model may be a greater challenge for obtaining good local control and treatment effects of PDT after surgery could most possibly be obtained in other tumour models or by increasing the PDT dose.

The anticipated low sensitivity to PDT of the remaining HT1080 cells in the tumour cavity after surgery should be viewed in accordance with multiple Photofrin and Foscan phase I and phase II clinical trials with PDT after debulking surgery (mostly R2 resections).[11–15] In general, the maximum tolerated dose in these clinical trials is limited by treatment related side effects on normal tissue. In PDT treatment after debulking of peritoneal carcinomatosis excessive fluid shift with volume overload has been a problem.[13] In PDT after debulking of non-small cell lung carcinoma and malignant pleural mesothelioma in thoracic surgery there are reports of oesophageal perforations and bronchopulmonal fistula.[11,12,15] Regarding neurosurgery, the post-treatment oedema, the direct cytotoxic effect on brain tissue and the vascular occlusion after PDT of brain tumours limits the PDT dose.[22] The clinical side effects and challenges in these reports have been tentatively overcome by giving fluid resuscitation, anti-inflammatory drugs, adjusting the drug light interval, and most of all by reducing the light and drug doses. In general, the clinical trials have not been successful in achieving long lasting local control and the studies conclude that there is a need of a more specific treatment effect either by adjusting current treatment regimes, by modifying the existing photosensitizers or developing new and more specific photosensitizers. Our results indicate that the limited clinical success of PDT as an adjuvant to surgery may be due to lower PDT sensitivity of the peripheral and the transitional zone of the residual tumour tissue remaining after debulking surgery. This has driven the clinicians to administer the highest possible PDT doses resulting in dose-limiting toxicity due to normal tissue damage.

In contrast to the low PDT effect on the residual tumour cells after R1 resection, PCI of BLM was found to induce a significant delay in tumour growth (Fig. 3c). The observed effect of PCI of BLM in combination with surgery is significantly stronger than the expected sum of the two treatments (BLM + surgery) and (PDT + surgery). In this study we have documented that, under the described conditions, PDT after surgery did not have an adequate effect on tumour growth. However, it cannot be excluded that with higher light doses PDT would also be able to eradicate the tumour periphery in our model. The PCI technology in this study acts as

an effective drug delivery system to the cells in the transitional zone in which BLM is released to cytosol from where it can reach its nuclear targets and inhibit tumour growth. The observed PCI effect also indicates that the photosensitizer is taken up by the remaining tumour after surgery, but for reasons that are not fully understood is not able to induce detectable photocytotoxicity in the absence of BLM.

In conclusion we have shown PCI of BLM acts as an effective drug delivery system to the transitional zone after inadequate surgery. The PCI technology may therefore be useful in future clinical applications in combination with surgery and elucidates some of the challenges observed with PDT as an adjunct to surgery. One of the advantages of the PCI technology is its ability to activate a plethora of therapeutic molecules accumulating in the endocytic vesicles.[9,10] Other therapeutics such as targeted macromolecules may therefore be useful in combination with PCI for optimizing the therapeutic effect on the transitional zone remaining after inadequate surgery.

## List of abbreviations

| | |
|---|---|
| $AlPcS_{2a}$ | Disulfonated aluminium phthalocyanine with the sulfonate groups on adjacent phthalate rings |
| BLM | Bleomycin |
| PCI | Photochemical internalization |
| PDT | Photodynamic therapy |

## Acknowledgements

Financial support from the Norwegian Radium Hospital Research Foundation and the Norwegian Cancer Society. We would like to thank Marie-Therese Roppestad Strand for skilful technical assistance.

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