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ORIGINAL ARTICLE

Hexaminolevulinate-mediated photodynamic purging of marrow grafts with murine breast carcinoma

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Photodynamic therapy (PDT) with porphyrin precursors is an established therapy for certain tumors. This study aimed to explore the use of hexaminolevulinate (HAL), a porphyrin precursor, for photodynamic purging of BM grafts contaminated with cells of the 4T1 breast carcinoma cell line. The optimal PDT dose was not effective in eradicating 4T1 cells when the tumor cells were mixed with murine marrow cells *in vitro*. However, the number of pulmonary metastases was reduced, and the survival of experimental animals was prolonged substantially when they were subjected to TBI followed by transplantation of syngeneic BM containing metastasized 4T1 cells that had been treated *ex vivo* by HAL-PDT. Despite the failure of *in vitro* experiments, HAL-based photodynamic purging could be a useful modality for treating animals bearing an experimental breast carcinoma.
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Introduction

High doses of chemotherapy and/or radiation therapy in combination with allogeneic BMT are used for the treatment of a number of hematologic malignancies and selected solid tumors. However, the application of allogeneic bone marrow transplantation is still largely limited by lack of an HLA-matched donor, advanced stages of a disease, graft failure and GVHD. Autologous BMT lacking GVHD complications has thus been explored. In contrast to allografts, however, autografts may harbor residual occult malignant cells that can cause a tumor relapse after being reinfused to the patient. Various methods have recently been developed to eradicate tumor cells from the autograft, leaving the normal stem cells undamaged. They include exposure to chemotherapeutic drugs, long-term marrow cultures, immunological manipulation with immunomagnetic devices or ab/complement combination and gene therapy (including antisense therapy). According to available literature, marrow purging by mafosfamide has provided long-term remissions in certain populations of patients with leukemia.[1]

The ability of malignant cells to selectively accumulate photosensitizers offers the possibility of using photodynamic therapy (PDT) in BM purging. PDT involves administration of a tumor-localizing photosensitizer and its subsequent activation by light of appropriate wavelength corresponding to the absorbance band of the photosensitizer, resulting in photodamage to the tumor cells.[2] Several exogenous photosensitizers have been proposed as agents for the purging of tumor cells from autografts, for example, merocyanine 540 (MC 540), photofrin, benzoporphyrin derivative monoacid ring A and sulfonated chloroaluminum phthalocyanine. Their efficiency was examined experimentally, especially for the treatment of leukemia.[3–12] Small cell lung,[13–15] prostate[15] and breast tumor[15–17] cells were mostly investigated from solid tumor cancers, with uncertain results. Usually, the *in vitro* studies evaluating phototoxicity against different cells were carried out in models of separated pure cell lines, although several authors utilized artificial cell mixture models to mimic *in vivo* conditions.[3,4,18–20]

As the exogenous photosensitizers generally have a limited selective accumulation in malignant cells, and a limited selective photodynamic destruction of tumor cells therefore ensues, the possibility of using 5-aminolevulinic acid (ALA) or its esters to produce endogenous protoporphyrin IX (PpIX) in a much more selective manner could be considered. ALA is formed from glycine and succinyl CoA in heme biosynthesis. The last step of this process is the incorporation of iron into PpIX under the action of the ferrochelatase enzyme. By adding exogenous ALA, the naturally occurring PpIX may accumulate because of the limited capacity and/or low activity of ferrochelatase.[21,22] Moreover, the activity of porphobilinogen deaminase,

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another enzyme involved in heme biosynthesis, is higher in some tumors,[21,22] so that PpIX accumulates with a high degree of selectivity in these tumors. Unfortunately, ALA does not easily penetrate through cellular membrane because of its hydrophilic property. ALA esters, on the other hand, are more lipophilic and pass more easily through biological membranes.[23–25] To our best knowledge, none of the ALA esters have so far been studied for the purposes of photodynamic purging.

In the present study, we have explored the applicability of hexyl-ester of ALA, hexaminolevulinate (HAL), for PDT purging of BM grafts from tumor cells in the murine 4T1 breast carcinoma model. We compared the results from models of individual cell types and their artificial cell mixtures *in vitro* with those from their corresponding *ex vivo*/*in vivo* models of BM containing metastasized 4T1 cells.

Materials and methods

Chemicals

HAL was kindly provided by PhotoCure ASA (Oslo, Norway). The stock solution of HAL was prepared in a mixture (1:9) of ethanol and serum-free Iscove's modified Dulbecco's medium (IMDM; Gibco, Invitrogen, Oslo, Norway) to a concentration of 12 mM and kept frozen at −70 °C until use. All the chemicals used were of the highest purity commercially available.

Tumor cell line

The murine 4T1 metastatic mammary adenocarcinoma cell line, growing in monolayers, was purchased from ATCC (Manassas, VA, USA, ATCC number: CRL-2539). The cells were incubated *in vitro* in IMDM supplemented with 10% fetal bovine serum (Gibco, Invitrogen), L-glutamine (Gibco, Invitrogen), penicillin and streptomycin (Gibco, Invitrogen). Passages up to number 25 were used in the present study. For experiments, the cells were resuspended several times after trypsinization using a 1-mL syringe and a 23-gauge needle to minimize cell clustering.

The 4T1 cells for the *in vitro* experiments were, after harvesting, washed twice with serum-free IMDM, resuspended and diluted in the serum-free IMDM to a density of 8×10^5 cells/mL (if not specified).

Animals

Approval for protocols of this study was obtained from the Norwegian National Animal Research Authority, and all experiments of animals were conducted according to the National Ethical Committee's Guideline on Animal Welfare. Female BALB/c mice, 6–8 weeks old at the beginning of the experiment, were purchased from Harlan (Boxmeer, The Netherlands) and housed under specific pathogen-free conditions.

BM cells and their mixtures with tumor cells

The tibiae and femurs of the mice were separated from the surrounding muscles and other soft tissues. The intact bones were immersed into 70% ethanol for about 5 min for disinfection. Both ends of the bones were cut and the BM was flushed out with sterile serum-free IMDM using a 3-mL syringe with a 25-gauge needle. The total number of cells was counted. The cells were diluted with serum-free IMDM to a density of 8×10^5 cells/mL.

For *in vitro* studies, the 4T1 tumor cells were mixed with BM cells to produce 5 and 50% contents of the tumor cells in a suspension. The total cell density was kept at 8×10^5 cells/mL.

HAL-induced PpIX production in cells and cell cycle analysis

Flow cytometry. The aliquots of 1 mL of the cell suspensions per well were used in 12-well plates. The cells were incubated with or without HAL for 4 h in a humidified incubator at 37 °C and 5% CO_2. For the measurements of intracellular PpIX, following the incubation with HAL, the cells were first scraped off from the substratum and repeatedly resuspended, as some of the 4T1 cells were attached to the flasks during the 4-h incubation. The cell suspensions were washed to remove the drug-containing incubation medium and cell pellets were then resuspended in ice-cold phosphate-buffered saline. Samples, protected from light, were stored on ice and analyzed within 30–60 min by flow cytometry. For cell cycle distribution studies, the cells after staining, as described later, were collected into tubes and kept at room temperature.

The measurements of intracellular PpIX and cell cycle distributions were performed using a LSR II flow cytometer (Becton Dickinson, San Jose, CA, USA). An argon laser operating at 488 nm and a 355 nm UV laser were used for fluorescence excitation of PpIX, propidium iodide (Sigma-Aldrich, Hamburg, Germany) and Hoechst 33342 (Sigma, St Louis, MO, USA), respectively. The rate of data acquisition was not higher than 500 counts/s. For each sample, 10 000 events were collected using Diva software. Forward scatter, side scatter and either red fluorescence (PpIX, propidium iodide) passing a combination of 685 nm dichroic and 695/40 nm band-pass filters or blue fluorescence (Hoechst 33342) passing 450/50 band-pass filter, were collected simultaneously. Forward scatter-area vs side scatter-area, side scatter-area vs side scatter-width and forward scatter-area vs red fluorescence-area dot plots were used to gate out individual populations of cells. Mean of the red fluorescence-area histogram was used to evaluate PpIX production. Histograms of Hoechst 33342 fluorescence were used for cell cycle comparisons. Propidium iodide fluorescence was used to estimate the content of dead cells in the samples not incubated with HAL.

For sorting of BM cells, FACS Diva flow cytometer was used (Becton Dickinson). Individual cell populations were sorted out using forward scatter-area vs side scatter-area dot plots. After resuspension of the cells in phosphate-buffered saline, the cells were filtered through 50 μm pore size mesh nylon filter. The sorted cells were immediately fixed and used to prepare cytospins for the hemacolor staining.

Cell cycle distribution. The cells of the 4T1 cell line were subcultured and BM cells with metastasized 4T1 cells were seeded into flasks. Following overnight incubation, the cells

were stained with Hoechst 33342 at a concentration of 8 μg/mL for 30 min at 37 °C and washed two times with phosphate-buffered saline. After scraping off from the substratum, the cells were analyzed by flow cytometry.

Fluorescence spectroscopy. Fluorescence emission spectra were recorded by means of Perkin Elmer precisely LS45 Fluorescence Spectrometer (Norwalk, CT, USA). The excitation wavelength of 407 and 10 nm slit width were used. The fluorescence emission was scanned from 550 to 750 nm. A $1.0 \times 0.4\,cm^2$ quartz cuvette was used for the measurements. The absolute amounts of PpIX in cells were measured with the method of an internal standard as described elsewhere.[26]

Protein content. For measurements of protein contents of the cell samples, the Pierce BCA Protein Assay (Thermo-Scientific, Rockford, IL, USA), based on bicinchoninic acid for the colorimetric detection and quantitation of total protein, was applied in 96-well plates. Absorbance at 570 nm was measured by means of a plate reader (Multiskan Ex, Labsystems, Helsinki, Finland). The protein content of the unknown sample was calculated by comparison with a standard curve prepared by using BSA (Pierce, Inc., Rockford, IL, USA).

Photodynamic treatment
After incubation of cells with HAL, described under the Flow cytometry subsection, the drug-containing medium was removed and IMDM supplemented with fetal bovine serum was added to each sample of *in vitro* studies. The whole plates were centrifuged for the change of medium. The cells were then illuminated with light at different exposure times as specified in the Results section. Owing to the problem with clustering of BM cells after centrifugation, the cell suspensions from *ex vivo* samples were used without the change of medium before illumination.

For light illumination of cell samples, blue light from a bank of four fluorescent tubes (model 3026, Applied Photophysics, London, United Kingdom) emitting light mainly in the region 410–500 nm with a maximum around 440 nm was used. The whole-spectrum irradiance was kept constant for all experiments at $12\,mW/cm^2$.

In vitro *clonogenic assay*
Methylcellulose-based medium MethoCult was used for clonogenic assays of BM cells. BM cells (2×10^5), were seeded in 1.1 mL of MethoCult with recombinant cytokines and EPO (M3434, StemCell Technologies, Grenoble, France). 35-mm Petri dishes were used for the clonogenic assay. Two sample dishes, placed together with one containing only 3 mL of sterile water in a 100-mm Petri dish, were cultivated at 37 °C in a humidified incubator with 5% CO_2 for 7–10 days. The colonies of BM cells were examined according to the technical manual for mouse colony-forming cell assays using MethoCult (StemCell Technologies) and counted using an inverted microscope at ×40 magnification.

For clonogenic assay of 4T1 cells, the cells after PDT were scraped off from the substratum. In the studies on the pure 4T1 cells, 400 cells were seeded in six-well plates and cultivated in IMDM or Dulbecco's modified Eagle's medium (Bio Whittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin for 7 days. In the studies on the mixtures of 4T1 and BM cells, aliquots containing also 400 4T1 cells were seeded. The colonies of cells were fixed with methanol and stained with methylene blue before counting.

Total body irradiation
For total-body γ-irradiation of healthy mice, ^{60}Co source was used. The BALB/c mice were irradiated at dose rates of 1.70–1.87 Gy/min, reaching total doses of 6.5, 7.0, 7.5 and 8.0 Gy.

Tumor induction and animal treatment
In the 4T1 tumor model after s.c. inoculation of 5×10^5 4T1 cells into the flank of BALB/c immunocompetent mice, 25–35 days were needed for the tumor to metastasize to lungs. Within this period, micrometastases existed in the BM of mice. The BM was then harvested, diluted to a concentration of 8×10^5 cells/mL and treated with PDT. After the treatment, the marrow cells were portioned into several tubes with no more than 15 mL of cell suspension to avoid too big cell pellets after centrifugation at 1400 r.p.m. for 30 min. Cell pellets were collected and DNA-se (Sigma, Hamberg, Germany) was added to a concentration of 300 unit/mL to make single-cell suspension. Finally, the treated 2×10^7 marrow cells containing metastasized 4T1 cells were injected i.v. to healthy, but lethally ^{60}Co-irradiated mice. The animal survival was monitored up to 6 months. In addition, pulmonary metastases of all mice were studied with histopathology at the end of the experiments. The marrow cells containing metastasized 4T1 cells without PDT as controls were also included.

Histological studies
Tissues sampled from lung and mediastinum of BALB/c mice receiving i.v. PDT-treated or non-PDT-treated marrow cells containing metastasized 4T1 tumor cells were fixed in 10% buffered formalin solution. The specimens were then dehydrated and embedded in paraffin. Sections were cut and stained with routine hematoxylin and eosin for light microscopy.

Statistical analysis
For statistical evaluation of data and curve fittings, Sigma plot software was used. The Mann–Whitney Rank Sum Test was applied for statistical analysis.

Results

HAL-induced PpIX production in 4T1 breast carcinoma cell line and pure BM. The conditions for HAL-induced photodynamic extracorporeal purging of BM from the 4T1 metastatic breast carcinoma cells were first examined in the pure tumor cells and their mixtures with BM cells.

The cell density of 8×10^5 cell/mL was used for both 4T1 cell line and pure BM obtained from BALB/c mice. Production of PpIX by cells was analyzed by means of flow cytometry and fluorescence spectroscopy. The saturation of PpIX production after 4-h incubation of 4T1 cells in serum-free medium with different amounts of HAL was not completed below 50 μM HAL (Figure 1a) as found by flow cytometric analysis. In pure murine BM, two main populations of living cells (granulocytes and mixed population, see Discussion) could be clearly separated in flow cytometric dot plots. There was a mild increase in PpIX production at all HAL concentrations used (up to 100 μM) in both of them (Figure 1b). The 4T1 cells produced slightly higher absolute amounts of PpIX relative to protein content than a mixture of BM cells at 20 and 50 μM HAL (Figure 1c). The difference at 50 μM HAL was found to be statistically significant ($P = 0.01$). Higher concentrations of HAL were not tested because of the PpIX saturation curve for the 4T1 cell line.

HAL-induced PpIX production in the mixtures of BM with 4T1 carcinoma cells. Flow cytometric analysis of BM with and without 4T1 cells revealed considerable overlap between tumor cell and BM cell populations, exceeding 20% in some cases. Thus, it was difficult to measure the production and accumulation of PpIX by individual cell types within cell mixtures. However, there is a reasonable indication showing same tendency for PpIX production as for pure tumor and BM cells.

Photodynamic treatment of pure 4T1 cell line. The effect of HAL-based photodynamic purging and resulting cell survival was examined by means of a colony assay in a usual, non-methylcellulose-based IMDM medium. Increasing doses of light illumination resulted in progressive killing of 4T1 cells following the 4-h incubation with 20 μM HAL in serum-free medium (Figure 2), but the 4T1 cells were still able to form few colonies even after 240-s light illumination. Increasing the drug concentration up to 50 μM resulted in no colony formation of 4T1 cells at the light doses above 120 s illumination (Figure 2). Light alone had no effect, within experimental error, on the survival of 4T1 cells.

Photodynamic treatment of mixtures of 4T1 cell line and BM cells. Colony assays for the survival of 4T1 cells in the mixtures with BM cells were performed in a usual, non-methylcellulose-based IMDM medium. Any interference from BM cells could be ruled out, as no colonies of BM cells were formed in the non-methylcellulose-based medium at cell densities used for the colony assay.

Interestingly, there was substantial difference between the survival of pure 4T1 cell line and its 5% mixture with BM cells. Although 120-s light illumination was sufficient to kill almost all the 4T1 cells in the pure form after the incubation with 50 μM HAL, increasing the time of the light illumination up to 240 s was still not enough to kill the 4T1 cells in the 5% mixture with BM cells (Figure 2).

Photodynamic purging of BM from BALB/c mice with 4T1 metastatic breast carcinoma cells ex vivo. The conditions for HAL-based photodynamic BM purging for *in vivo* purposes were tested *ex vivo*, especially because the optimal doses indicated by the cell mixture model *in vitro* (50 μM HAL, >240 s blue light) were suspected to be too high to preserve sufficient amounts of BM cells for hematopoietic

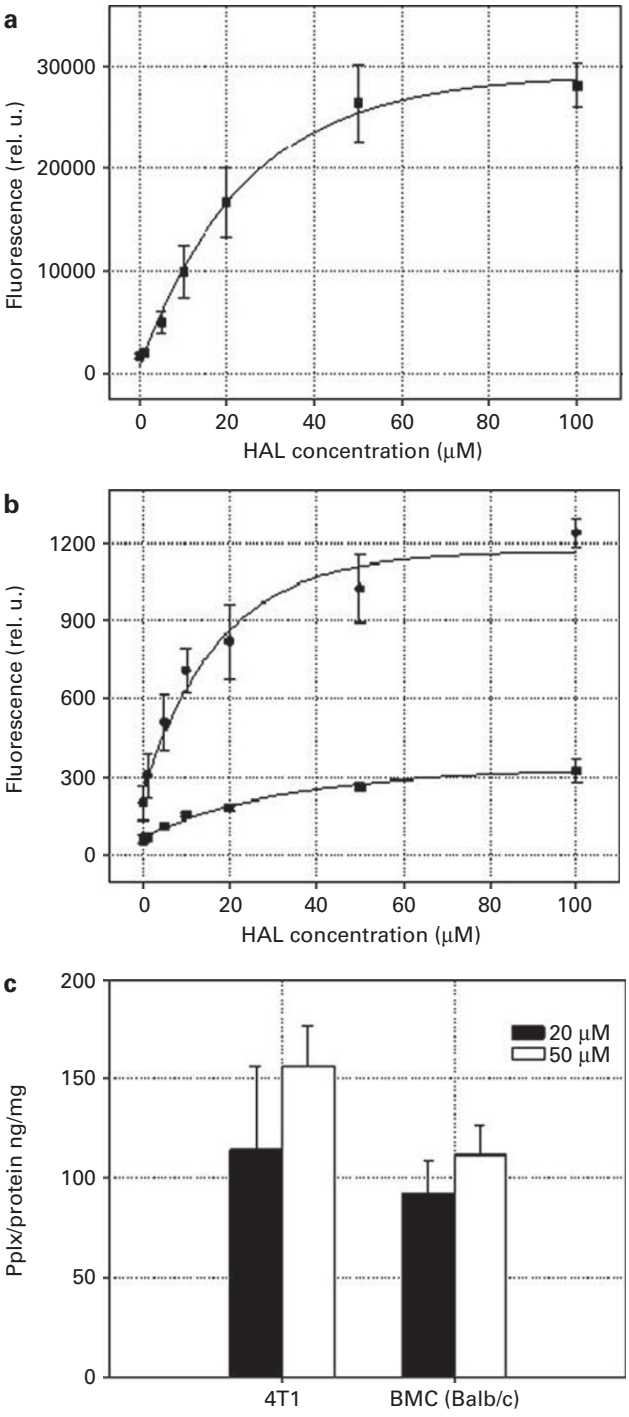

Figure 1 HAL-induced production of PpIX after incubation of the cells with different amounts of HAL. (**a**, **b**) saturation curves, (**c**) absolute amounts of PpIX relative to protein content of the cells. (**a**) 4T1 tumor cell line, (**b**) BM cells of BALB/c mice: (•) granulocytes, (■) mixed population. Cell density of 8×10^5 cells/mL was used. Each data point represents an average ± s.d. from four–eight independent measurements. The data in (**a**) and (**b**) were fitted to the exponential rise to maximum function. In (**c**), a significant difference ($P = 0.01$) was seen between 4T1 cells and BM cells incubated with 50 μM HAL.

reconstitution and thus animal revival. Differences in cellular morphology and behavior were noticed between the 4T1 cell line cultured *in vitro* and attached, epithelial cell-like cells of the BM with metastasized 4T1 cells from mice (Figure 3). The colonies of the cell line were readily formed with cells flattened out at the substratum, while the attached cells of the BM were often rounded and not capable of forming colonies.

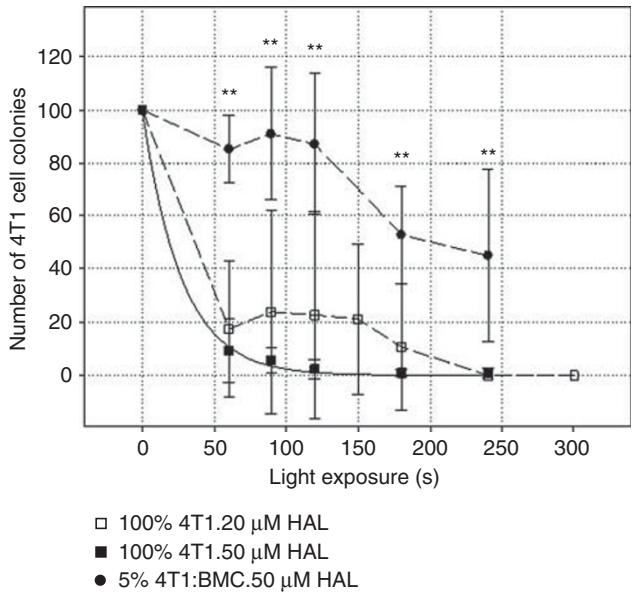

Figure 2 Cell survival after HAL-based photodynamic treatment of 4T1 cell line and cell mixtures. Light-dose dependence of relative numbers of 4T1 cell colonies. (□, ■) pure 4T1 cell line, (●) mixture of BM cells from BALB/c mice containing 5% 4T1 cells. (□) 20 μM HAL, (●, ■) 50 μM HAL. Total cell density was kept at 8×10^5 cells/mL. Each data point represents an average ± s.d. from at least four independent measurements. The full line is a fit to the exponential decay function. The dashed lines are shown to indicate the tendency. ** denote significant difference at $P<0.01$ between the pure 4T1 cell line and mixture of BM containing 5% 4T1 cells both incubated with 50 μM HAL. Two sets of experiments were performed with the following colony counts at zero time point: (a) 47 ± 33 (100%, 20 μM), 51 ± 21 (100%, 50 μM), (b) 80 ± 21 (100%, 50 μM), 105 ± 30 (5%, 50 μM). The normalized data points for the 100% 4T1 cell suspension incubated with 50 μM HAL from both experiments were the same within experimental error.

Variations in the number of attached cells from animal to animal were obvious. Majority (>90%) of the BM colonies were colonies of granulocytes (colony-forming unit-granulocyte), colony-forming unit-macrophages and mixed colonies of granulocytes and macrophages in accordance with the data for C57BL/6 mouse BM reported in the technical manual for mouse colony-forming cell assays using MethoCult. After incubation with 20 μM followed by 240-s light illumination only 2–3% of BM cell colonies compared with control samples were formed (Figure 4). Therefore, the doses indicated by *in vitro* results (>20 μM HAL, >240 s light) were too high for the *in vivo* purging purposes.

*BMT ('*in vivo*' purging).* A range of doses from 6.5 to 7.5 Gy for TBI was examined to find an optimal irradiation dose for HAL-based photodynamic purging. Total-body doses of 7 Gy and lower were sublethal with animals surviving without any sign of the disease for several months after γ-irradiation. Although γ-irradiation at a dose of 7.5 Gy resulted in death of all animals with median survival time of 15 days (11–15, $n=5$), a higher dose of 8.0 Gy was used for the TBI in *in vivo* experiments to ensure a sufficient dose to be delivered. BM was harvested from the donors injected s.c. with 0.5×10^6 4T1 cells 30–35 days before. The purging procedure consisted of 4-h incubation of BM contaminated with 4T1 cells in serum-free medium with 50 μM HAL followed by 120-s illumination with light. Two criteria were used to judge the outcome of the photodynamic purging, namely animal survival and number of pulmonary metastases. i.v. injection of 2×10^7 cells treated with the purging procedure into lethally irradiated animals without 4T1 tumor (recipients) resulted in the death of only one mouse ($n=10$) after 31 days (Figure 5), whereas 10 out of 12 recipient mice (in the control group) injected with the same amount of non-PDT-treated cells died within 16–29 days (Figure 5). Further, the pulmonary metastases in PDT-treated and non-PDT-treated animals were inspected. In one set of experiments, substantially lower numbers of metastases in the thoracic cavity (lung and mediastinum) were found in six out of seven surviving recipients than those in the control group (with one exceptional case) after 6 months (Figure 6). Histopathological findings confirmed the metastasized tumors

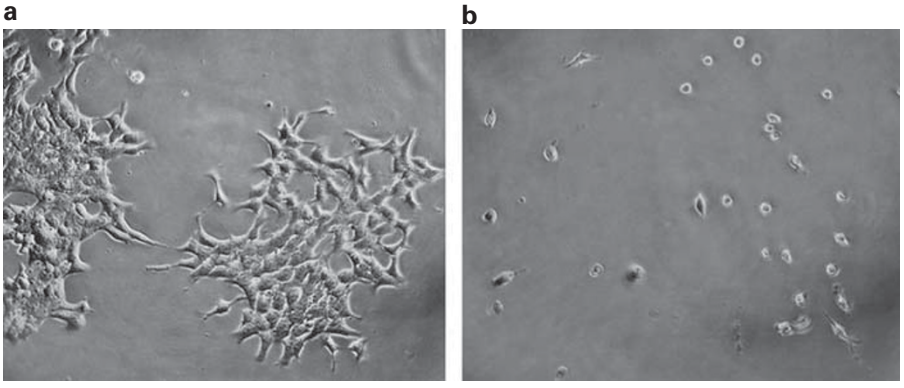

Figure 3 Growth patterns of 4T1 cells. (**a**) 4T1 cell line passaged *in vitro* (passage 7), (**b**) attached cells of the BM with metastasized 4T1 cells. 4T1 cells (0.5×10^6) were injected s.c. into BALB/c mouse and BM was harvested after 35 days. BM cells containing 4T1 cells (about 4×10^7 cells in total) were cultivated 7 days and subcultured. The cells in both pictures were seeded on the same day at about the same cell density and the phase-contrast images were taken 4 days after subcultivation. Invererted microscope with ×20 objective was used.

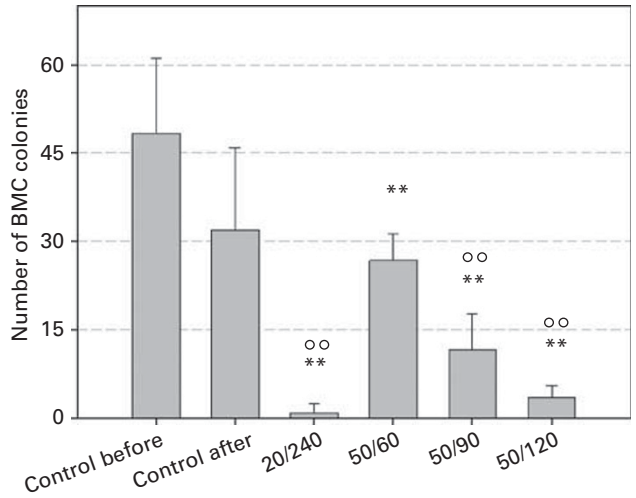

Figure 4 Cell survival after HAL-based photodynamic treatment of BM with metastasized 4T1 cells. PDT dose dependence of numbers of BM cell colonies grown in methylcellulose-based medium with cytokines. The bars represent averages ± s.d. from four–seven independent measurements. ** and °° denote significant difference at $P<0.01$ compared with control samples with no incubation or incubated for 4 h in serum-free medium, respectively.

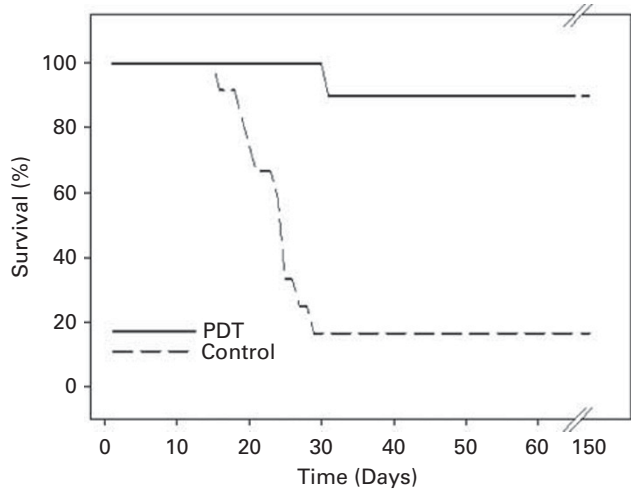

Figure 5 Survival plots of lethally irradiated BALB/c mice injected with BM contaminated with 4T1 cells. Recipient mice were i.v. inoculated with BM from animals bearing 4T1 breast carcinoma that was previously treated by HAL-induced photodynamic purging (PDT, $n=10$) or untreated (control, $n=12$). The recipient mice had been previously irradiated with a lethal dose of 8.0 Gy. Two animals in the control group were paralysed before death probably due to tumor metastases into the spinal nerves.

in the lungs and their surrounding tissues (Figure 6). In the remaining set of experiments, no pulmonary metastases were found in any of PDT-treated animals.

Discussion

Photodynamic treatment has been proposed for the purging of BM autografts from residual tumor cells. In the present study, we provide an investigation of HAL-based photodynamic purging of murine BM to indicate a potential for the use of the modality for the murine 4T1 breast carcinoma.

We started with the simplest model of individual cells in order to find 'preliminary' conditions for the treatment. The difference in the absolute amounts of PpIX produced by the pure 4T1 cell line as compared with its mixture with BM cells was higher (and significant) at 50 μM HAL than at 20 μM concentration, indicating more favorable selectivity of the purging at the higher concentration. Moreover, the lower concentration with 20 μM HAL turned out to be insufficient for optimal killing of the 4T1 cells *in vitro* (Figure 2). We did not check the PDT doses for BM from healthy animals as more relevant information, concerning our *in vivo* testing, could be obtained using BM from animals infected with 4T1 cells (this was not possible for the 4T1 cells, see below). Furthermore, the same survivals, within experimental error, were found when comparing leukemic BM with BM from healthy animals at a regime combining 50 μM HAL with 90-s light exposure in another model.[2] HAL-induced PpIX production and its subsequent PDT effect were compared between the pure 4T1 cell line and its artificial mixture of BM cells with 5% tumor cells *in vitro*. Although several authors have used higher ratios of tumor to normal cells,[3,18,19] low amounts of tumor cells were indicated by flow cytometry in the BM of the animals with an advanced stage of leukemia.[27] We used low contents of tumor cells in our artificial mixture model because we believe that the number of breast carcinoma cells harboring in BM do not exceed those of leukemic cells.

The results on photodynamic inactivation of pure tumor cells were not consistent with those in their mixtures with BM. Prolongation of light illumination from a dose sufficient for killing pure 4T1 tumor cells (>120 s) up to 240 s was still not enough for complete inactivation of the tumor cells in the mixtures (Figure 2). As shown in Figure 1, PpIX production in the BM cells had not leveled off at a concentration used for photodynamic treatment (50 μM) and the transfer of PpIX from tumor cells might occur to reach the saturation. Although our data indicated PpIX transfer from L1210 leukemic cells to BM cells,[27] the confirmation of the PpIX transfer by flow cytometric analysis for the 4T1 carcinoma cells turned out to be impossible because of a considerable overlap among various cell populations. The BM itself is a complex mixture of different types of cells. Two major populations of BM cells, as shown on side vs forward scatter dot plots, were analyzed by flow cytometry in our study. Both populations were clearly separated from the area typical for dead cells. Granulocytes were identified by light microscopy after being sorted to be the main entity of the population with the highest forward scatter signal. Damage to cells during the sample preparation for microscopic analysis precluded identification of the second cell population, but this population is usually believed to be a mixture of various types of cells such as monocytes, lymphocytes and stem cells. Substantial part of the 4T1 cell population has overlapped the populations of BM cells. Identification of 4T1 cells in the

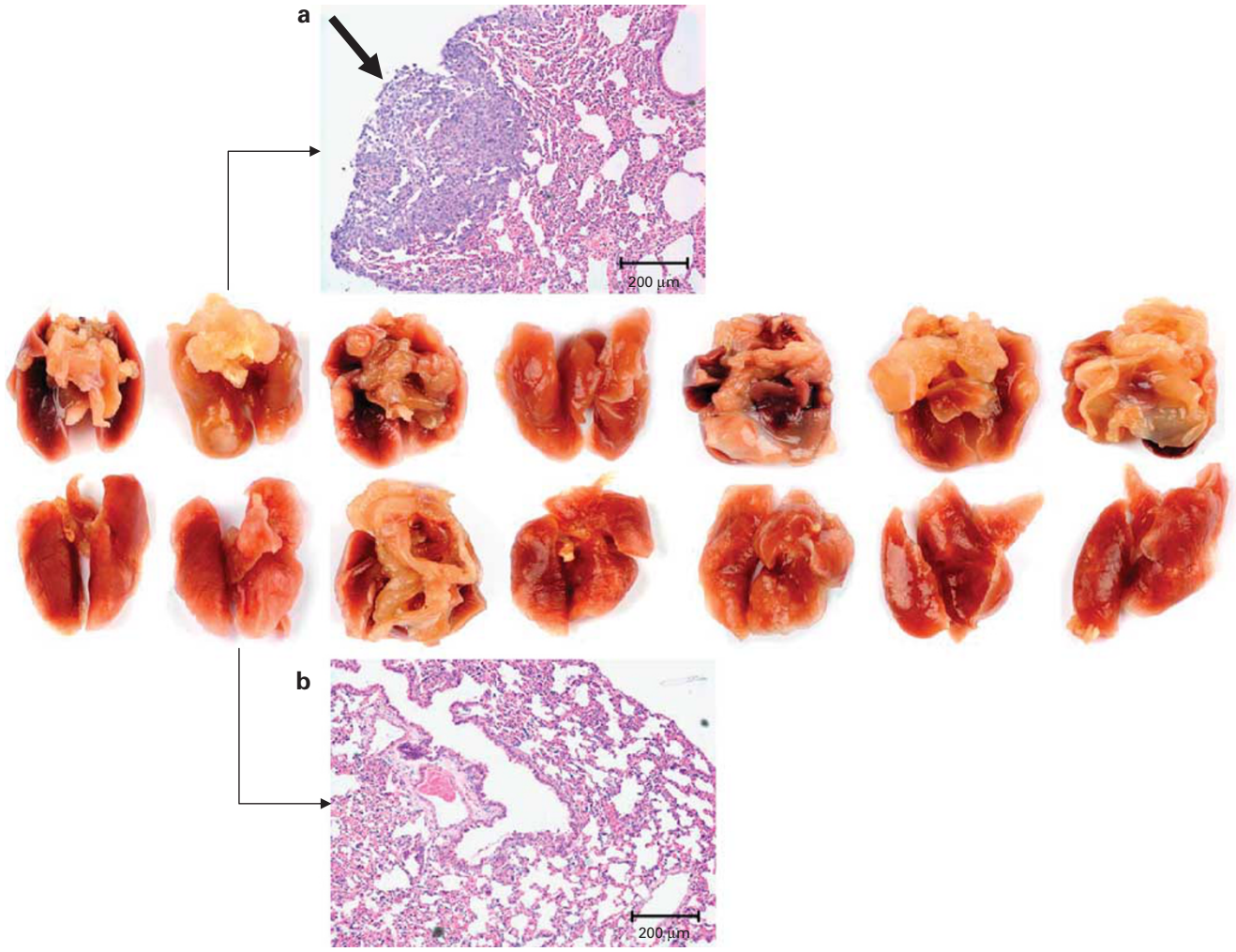

Figure 6 Metastases in the thoracic cavity developed in the lethally irradiated BALB/c mice i.v. injected with PDT-treated (lower row) and non-PDT-treated (upper row) BM from the donors with metastasized 4T1 tumors. (**a**, **b**) Histopathology of 4T1 pulmonary metastasis. Transmission microphotographs of lung tissues of BALB/c mice i.v. injected with non-PDT-treated (**a**) and PDT-treated (**b**) BM from the donors with metastasized 4T1 tumors. The arrow in (**a**) shows a metastasized tumor.

mixture, therefore, requires marking of the cells with, for example, staining by specific antibodies, which, however, was technically not feasible for simultaneous evaluations of PpIX production in the cells.

Despite discouraging results found in an artificial model of BM mixed with 4T1 tumor cell line, we decided to continue with a testing of the purging conditions. We intended to compare *in vitro* samples with a clinically relevant model of BM with metastasized 4T1 cells. The comparison between *in vitro* and *ex vivo* results was not feasible for the 4T1 cells due to the impossibility of morphologically distinguishing the 4T1 cells from 'plastic adherent' cells in BM (Figure 7). BM of BALB/c mice was reported to yield the highest number of the latter cells among five most commonly used mouse strains.[28] These cells have fibroblast-like morphology and are capable of both forming colonies and culturing for several passages. Their cultures consist of a heterogeneous mixture of morphologically distinct cell types.[28] Real-time PCR detecting *FOXC2* gene, a transcription factor reported to be overexpressed in breast metastatic cancer cell lines, specifically in the 4T1 cells,[29] was found not to be overexpressed in the 4T1 cells used in our study. Applications of immunocytochemistry and flow cytometry after staining with several antibodies, such as monoclonal anti-cytokeratin 8, monoclonal anti-cytokeratin 16, pan-anti-cytokeratin and monoclonal AE1-AE3 anti-cytokeratins, failed to identify the population of 4T1 tumor cells. This failure in our *ex vivo* samples was surprising and could be because of the possibility of expressing little keratins in our 4T1 cells. Even though the *ex vivo* samples could not be used to search for the PDT dose necessary for complete eradication of tumor cells, they provided us with crucial information for animal revival, namely the lethal doses for PDT-induced inactivation of BM cells, as colony-forming unit-macrophages, colony-forming unit-granulocyte and granulocytes and macrophages constituted majority of the colonies in the BM colony assay used. A concentration of

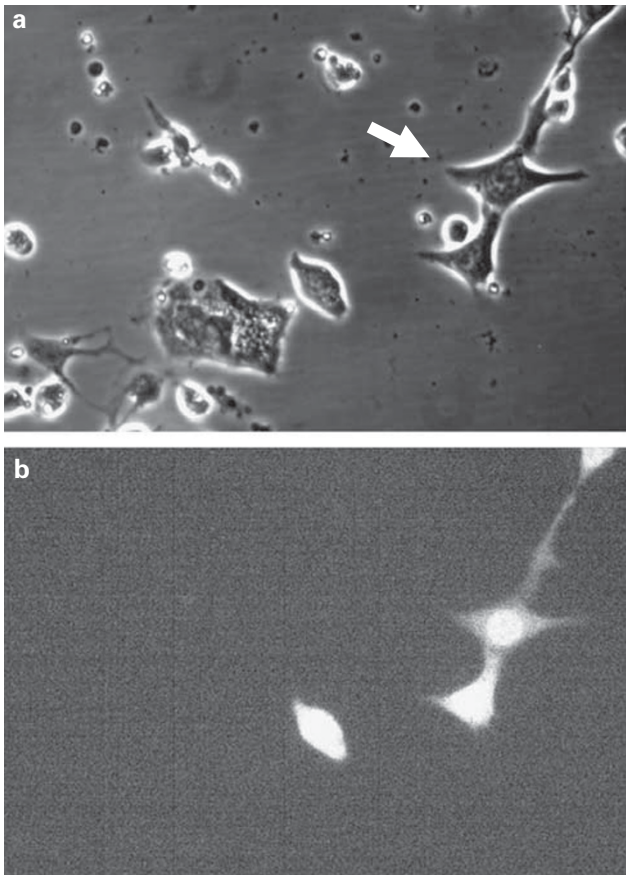

Figure 7 Microscopic images of the mixture of BM cells with the 4T1 cell line stained with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate), Invitrogen, Molecular Probes, Eugene, OR, USA). (**a**) phase contrast, (**b**) fluorescence. BM cells were seeded shortly after harvesting from BALB/c mouse. Next day, 4T1 cells were added after they had been stained as follows: cells of the 4T1 cell line attached at the bottom of the culture flask were washed with serum-free medium. Afterwards, serum-free medium containing CellTracker (1 μM) was added and cells were incubated in the incubator at 37 °C. After 30 min incubation, the cells were harvested by trypsinization and single-cell suspension was prepared. A small drop of the suspension was added to the flasks with BM cells. Images were taken after overnight incubation by means of a Zeiss Axioplan epifluorescence and phase contrast microscope (Oberkochen, Germany) with a HBO/100 W mercury lamp using a ×20 objective. The fluorescence images were made with a cooled charge-coupled device camera (Quantix CCD camera, Photometrics, Tucson, AZ, USA). The filter combination consists of a 450–490 nm band-pass excitation filter, a 510 nm beam splitter and a 510–540 nm band-pass emission filter. The AnalySIS Soft Imaging System software (Olympus Deutschland, Hamburg, Germany) was used to process the images.

HAL (50 μM) for maximal production of PpIX in the 4T1 cells (Figure 1a) and its subsequently complete photodynamic killing of the tumor cells *in vitro* (Figure 2) seemed to be too high for BM cells when light exposures longer than 120 s were used (Figure 4). Moreover, only one out of three animals survived when light exposure was increased to 180 s at the same HAL concentration (data not shown). The *in vitro* results at lower doses, however, indicated inefficiency of HAL-based PDT purging for BM infected with the carcinoma cells. Surprisingly, the modality with a regime combining 50 μM HAL concentration with 120-s light exposure turned out to be promising *in vivo* according to the endpoints of both the animal survival and the number of lung metastases. Owing to inability to identify tumor cells in BM samples, we were not able to estimate the numbers of tumor cells escaping from the photodynamic treatment, but from comparison of the numbers of attached cells in the BM from healthy and tumor-bearing animals treated with and without PDT, we suspect that certain numbers of 4T1 cells survived. The number of those surviving 4T1 tumor cells may be so low that they may be managed by the host immune system of the animals, as there is an increasing number of studies reporting that photosensitization triggers antitumor immunity.[30]

HAL-induced PpIX for photodynamic purging has shown promising results in the clinically relevant 4T1 breast carcinoma model. Lethally irradiated recipients received PDT-treated BM, with the metastasized 4T1 carcinoma cells from s.c. transplanted local tumors that had undergone potential biological modifications *in vivo* instead of artificially prepared mixtures of BM cells with the tumor cell line. We have noticed the difference (Figure 3) between the 4T1 cell line and the attached cells of BM that certainly contains 4T1 tumor cells as pulmonary metastases have developed in the mice receiving such cells. The majority of the latter cells failed to divide and had the morphological appearance of G_0/G_1 phase (Figure 3b). Flow cytometric analysis of DNA content in the *ex vivo* samples (contamination with BM cells was unavoidable as discussed above) also confirms this finding (Figure 8). Even in such model, the results of the animal survival and number of 4T1 lung metastases show that HAL-based PDT purging allows not only eradication of the 4T1 tumor cells from BM, but spares sufficient amounts of progenitor BM cells to ensure hematopoietic reconstitution. In the present study with a murine model, the cell density of 8×10^5 cell/mL was used. Although such density is not applicable to human BM grafts that usually contain $\sim 10^8$ cells/kg body weight, it would provide valuable information if this modality would work or not in the murine tumor model. Further, experience with other photochemical purging protocols suggests that stem cell grafts can be processed at substantially higher concentrations and varying the concentration of human cells has only a moderate effect on the efficiency of photochemical purging.[31,32] In any case, if this treatment procedure would be applied in the clinic, the conditions suitable for the human situation need to be thoroughly investigated.

Finally, application of autologous BMT and BM purging in the treatment of solid tumors seems to have declined in the past years. Nevertheless, high-dose chemotherapy in combination with auto-SCT is still actively pursued for small subsets of breast cancer patients and has obtained encouraging results.[33,34] Benefits of BM purging are still in a debate, and some authors show no significant benefit; whereas others demonstrate lower tumor recurrences.[35,36] These inconsistent results suggest that a successful protocol of autologous BMT combined with BM purging in the treatment of solid tumors may be achieved provided that all factors affecting the efficacy of the modality are optimized including strict selection of suitable patients.[33,34,37]

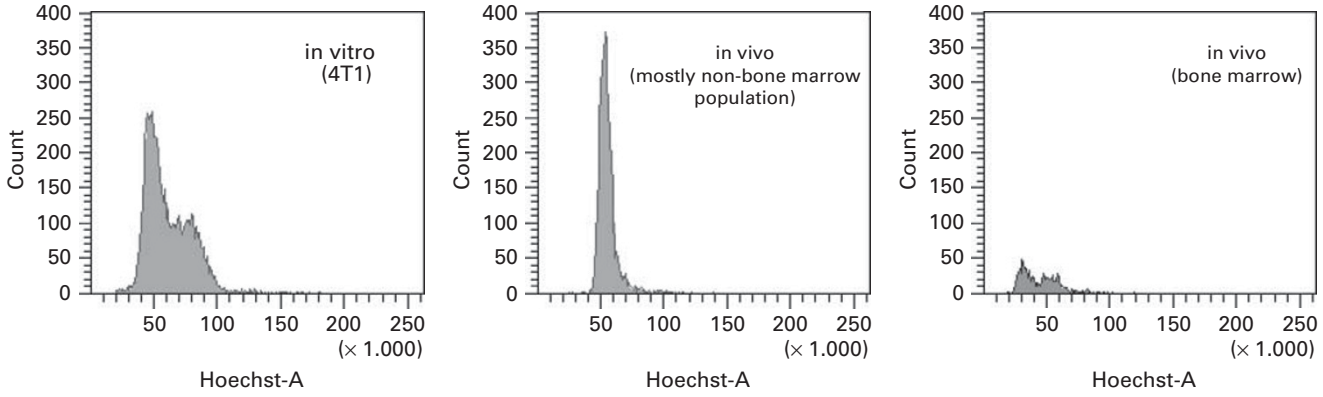

Figure 8 Flow cytometric cell cycle histograms. The cells were stained with Hoechst 33342 as described under Materials and methods. Left figure shows DNA distribution of 4T1 cells cultured *in vitro*. In the case of *in vivo* samples (middle and right figures), a population (presumably tumor cells) appearing only in BM of animals with 4T1 tumors was gated out on forward scatter vs side scatter dot plots indicating most cells in the G_0/G_1 phase. Contribution from BM cells of healthy animals found in the same gate is shown in the right figure.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Abdallah A, Egerer G, Weber-Nordt RM, Korbling M, Haas R, Ho AD. Long-term outcome in acute myelogenous leukemia autografted with mafosfamide-purged marrow in a single institution: adverse events and incidence of secondary myelodysplasia. *Bone Marrow Transplant* 2002; **30**: 15–22.
2 Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M *et al*. Review: photodynamic therapy. *J Natl Cancer Inst* 1998; **90**: 889–905.
3 Daziano JP, Humeau L, Henry M, Mannoni P, Chanon M, Chabannon C *et al*. Preferential photoinactivation of leukemia cells by aluminum phthalocyanine. *J Photochem Photobiol B* 1998; **43**: 128–135.
4 Huang HF, Chen YZ, Wu Y. Experimental studies of the effects of ZnPcS2P2-based-photodynamic therapy on bone marrow purging. *Chin Med J (Engl)* 2005; **118**: 105–110.
5 Huang HF, Chen YZ, Wu Y, Chen P. Purging of murine erythroblastic leukemia by ZnPcS2P2-based-photodynamic therapy. *Bone Marrow Transplant* 2006; **37**: 213–217.
6 Danilatou V, Lydaki E, Dimitriou H, Papazoglou T, Kalmanti M. Bone marrow purging by photodynamic treatment in children with acute leukemia: cytoprotective action of amifostine. *Leuk Res* 2000; **24**: 427–435.
7 Kapsokalyvas D, Dimitriou H, Skalkos D, Konstantoudakis G, Filippidis G, Stiakaki E *et al*. Does Hypericum perforatum L. extract show any specificity as photosensitizer for HL-60 leukemic cells and cord blood hemopoietic progenitors during photodynamic therapy? *J Photochem Photobiol B* 2005; **80**: 208–216.
8 Qiu K, Sieber F. Merocyanine 540-sensitized photoinactivation of leukemia cells: effects of dose fractionation. *Photochem Photobiol* 1992; **56**: 489–493.
9 Yamazaki T, Sieber F. Effect of hypothermia on the merocyanine 540-mediated purging of hematopoietic cells. *J Hematother* 1997; **6**: 31–39.
10 Yamazaki T, Sieber F. The alkyl-lysophospholipid, ET-18-OCH3 synergistically enhances the Merocyanine 540-mediated photoinactivation of leukemia cells: implications for the extracorporeal purging of autologous hematopoietic stem cells. *Bone Marrow Transplant* 1997; **19**: 113–119.
11 Yamazaki T, Sato Y, Sieber F. Role of cytoprotective mechanisms in the photochemical purging of autologous bone marrow grafts. *Exp Hematol* 1997; **25**: 629–637.
12 Hrkal Z, Cajthamlova H, Grebenova D, Bartosova J, Klamova H, Marinov J. Selective photodynamic destruction of leukemic cells. *Cas Lek Cesk* 2000; **139**: 148–154.
13 Tsujino I, Anderson GS, Sieber F. Postirradiation hyperthermia selectively potentiates the merocyanine 540-sensitized photoinactivation of small cell lung cancer cells. *Photochem Photobiol* 2001; **73**: 191–198.
14 Tsujino I, Miyagi K, Sampson RW, Sieber F. Potentiation of the antitumor effect of Merocyanine 540-mediated photodynamic therapy by amifostine and amphotericin B. *Photochem Photobiol* 2006; **82**: 458–465.
15 Miyagi K, Sampson RW, Sieber-Blum M, Sieber F. Crystal violet combined with Merocyanine 540 for the *ex vivo* purging of hematopoietic stem cell grafts. *J Photochem Photobiol B* 2003; **70**: 133–144.
16 Anderson GS, Miyagi K, Sampson RW, Sieber F. Anti-tumor effect of Merocyanine 540-mediated photochemotherapy combined with Edelfosine: potential implications for the *ex vivo* purging of hematopoietic stem cell grafts from breast cancer patients. *J Photochem Photobiol B* 2002; **68**: 101–108.
17 Brasseur N, Ménard I, Forget A, el Jastimi R, Hamel R, Molfino NA *et al*. Eradication of multiple myeloma and breast cancer cells by TH9402-mediated photodynamic therapy: implication for clinical *ex vivo* purging of autologous stem cell transplants. *Photochem Photobiol* 2000; **72**: 780–787.
18 Atzpodien J, Gulati SC, Strife A, Clarkson BD. Photoradiation models for the clinical *ex vivo* treatment of autologous bone marrow grafts. *Blood* 1987; **70**: 484–489.

19 Atzpodien J, Gulati SC, Clarkson BD. Comparison of the cytotoxic effects of merocyanine-540 on leukemic cells and normal human bone marrow. *Cancer Res* 1986; **46**: 4892–4895.
20 Porcellini A, Talevi N, Marchetti-Rossi MT, Palazzi M, Manna A, Sparaventi G *et al*. Limiting-dilution analysis for the determination of leukemic cell frequencies after bone marrow decontamination with mafosfamide or merocyanine 540. *Blood* 1987; **70**: 1543–1549.
21 Peng Q, Berg K, Moan J, Kongshaug M, Nesland JM. Review: 5-Aminolevulinic acid-based photodynamic therapy: principle and experimental research. *Photochem Photobiol* 1997; **65**: 235–251.
22 Peng Q, Warloe T, Berg K, Moan J, Kongshaug M, Giercksky K-E *et al*. Review: 5-Aminolevulinic acid-based photodynamic therapy: clinical research and future challenges. *Cancer* 1997; **79**: 2282–2308.
23 Gaullier JM, Berg K, Peng Q, Anholt H, Selbo PK, Moan J. The use of esters of 5-aminolevulinic acid to improve photodynamic therapy on cells in culture. *Cancer Res* 1997; **57**: 1481–1486.
24 Peng Q, Warloe T, Moan J, Heyerdahl H, Steen HB, Giercksky K-E *et al*. ALA derivative-induced protoporphyrin IX build-up and distribution in human nodular basal cell carcinoma. *Photochem Photobiol* 1995; **61**: 82S.
25 Peng Q, Moan J, Warloe T, Iani V, Steen HB, Bjørseth A *et al*. Build-up of esterified aminolevulinic-acid-derivative-induced porphyrin fluorescence in normal mouse skin. *J Photochem Photobiol B* 1996; **34**: 95–96.
26 Peng Q, Evensen JF, Rimington C, Moan J. A comparison of different photosensitizing dyes with respect to uptake C3H-tumors and tissues of mice. *Cancer Lett* 1987; **36**: 1–10.
27 Čunderlíková B, Vasovič V, Sieber F, Furre T, Nesland JM, Peng Q. Hexaminulevulinate-mediated photodynamic purging of bone marrow with leukemia. *Bone Marrow Transplant* 2010; **45**: 1553–1561.
28 Phinney DG, Kopen G, Isaacson RL, Prockop DJ. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. *J Cell Biochem* 1999; **72**: 570–585.
29 Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N *et al*. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc Natl Acad Sci USA* 2007; **104**: 10069–10074.
30 Castano AP, Mroz P, Hamblin MR. Photodynamic therapy and anti-tumour immunity. *Nat Rev Cancer* 2006; **6**: 535–545.
31 Sieber F. Phototherapy, photochemotherapy, and bone marrow transplantation. *J Hematother* 1993; **2**: 43–62.
32 Sieber F, Stuart RK, Rowley SD, Sharkis SJ, Sensenbrenner LL. Dye-mediated photolysis of normal and neoplastic hematopoietic cells. *Leukemia Res* 1987; **11**: 43–49.
33 Kurian S, Qazilbash M, Fay J, Wolff S, Herzig R, Hobbs G *et al*. Complete response after high-dose chemotherapy and autologous hemopoietic stem cell transplatation in metastatic breast cancer results in survival benefit. *Breast J* 2006; **12**: 531–535.
34 Nieto Y, Shpall EJ. High-dose chemotherapy for high-risk primary and metastatic breast cancer: is another look warranted? *Curr Opin Oncol* 2009; **21**: 150–157.
35 Nieto Y, Jones RB, Shpall EJ. Stem-cell transplantation for the treatment of advanced solid tumors. *Springer Semin Immunopathol* 2004; **26**: 31–56.
36 Gorin NC. Autologous stem cell transplantation in hematological malignancies. *Springer Semin Immunopathol* 2004; **26**: 3–30.
37 Kyriakou C, Canals C, Sibon D, Cahn JY, Kazmi M, Arcese W *et al*. High-dose therapy and autologous stem-cell transplantation in Waldenstrom macroglobulinemia: the Lymphoma Working Party of the European Group for Blood and Marrow Transplantation. *J Clin Oncol* 2010; **28**: 2227–2232.