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ORIGINAL ARTICLE

Hexaminolevulinate-mediated photodynamic purging of leukemia cells from BM

B Čunderlíková[1,2], V Vasovič[1], F Sieber[3], T Furre[4], JM Nesland[1,5] and Q Peng[1,6]

[1]*Department of Pathology, Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway;* [2]*International Laser Centre, Bratislava, Slovakia;* [3]*Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI, USA;* [4]*Department of Medical Physics, Institute for Cancer Research, Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway;* [5]*Faculty Division, Norwegian Radium Hospital, Medical Faculty, University of Oslo, Oslo, Norway and* [6]*State Key Laboratory for Advanced Photonic Materials and Devices, Fudan University, Shanghai, PR China*

Photodynamic therapy (PDT) with porphyrin precursors has been established for tumor treatment. This study aimed at examining applicability of hexaminolevulinate (HAL) for photodynamic purging of leukemic cells from BM grafts and evaluating the clinical relevance of *in vitro* models. The PDT dose resulting in no colony formation by leukemic cells *in vitro*, in pure form or in a mixture with BM cells, was insufficient for complete killing of the leukemic cells *ex vivo* and for the treatment of the leukemia-bearing animals *in vivo*. The efficacy of HAL–PDT in cell lines *in vitro* should be verified in clinically relevant *in vivo* models.

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Introduction

Photodynamic therapy (PDT) is a treatment modality that involves administration of a tumor-localizing photosensitizer and its subsequent activation by light of appropriate wavelength corresponding to the absorption band of the photosensitizer, resulting in photodamage to the tumor.[1] The ability of malignant cells to selectively accumulate photosensitizers offers the possibility of using PDT to eradicate tumor cells and micrometastasis from BM, inevitable for autologous BMT in cancer patients. Several exogenous photosensitizers have been suggested as agents for the purging of tumor cells from autografts, for example, merocyanine 540, Photofrin, benzoporphyrin derivative monoacid ring A and sulfonated chloroaluminum phthalocyanine. The exogenous photosensitizers in general might have 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 might, therefore, be an option to increase the selectivity of the purging effect. 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 in tumor cells possibly because of a low activity of ferrochelatase.[2,3] Moreover, the activity of porphobilinogen deaminase, another enzyme involved in heme biosynthesis, is higher in some tumors,[2,3] so that PpIX accumulates with a high degree of selectivity in these tumors. Unfortunately, ALA does not easily penetrate through cellular membranes because of its hydrophilic property. ALA esters, on the other hand, are more lipophilic and pass more easily through biological membranes.[4–6] To our best knowledge, none of the ALA esters have so far been studied for the purposes of photodynamic purging.

The efficacy of photosensitizers for BM purging purposes was in the majority of studies examined experimentally for the treatment of leukemia.[7–16] Small cell lung,[17–19] prostate[19] and breast tumor[19–21] cells were mostly analyzed from solid cancers. To mimic *in vivo* conditions, artificial cell mixture models have been used by several investigators.[7,8,22–24] In many cases, however, the *in vitro* studies evaluating phototoxicity against different cells were carried out in models of separated pure cell lines, not only in connection with BM purging but also for other applications of PDT. To what extent such *in vitro* models are relevant for *in vivo* conditions is not completely clear.

In this study, we have examined the applicability of a hexyl-ester of ALA, hexaminolevulinate (HAL), for PDT purging of tumor cells from BM grafts in the mouse L1210 leukemia model. We compared the results from *in vitro* models of individual cell types and their artificial mixtures with those from the corresponding *ex vivo* samples as well as from an *in vivo* model to evaluate the relevance of the

Correspondence: Dr Q Peng, Department of Pathology, Norwegian Radium Hospital, Oslo University Hospital, Montebello, Oslo N-0310, Norway.
E-mail: Qian.Peng@rr-research.no
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in vitro model for HAL-based photodynamic purging of BM grafts.

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 lines
The mouse lymphocytic leukemia cell line, L1210, growing in a suspension, was purchased from American Type Culture Collection (Manassas, VA, USA; ATCC no.: CCL-219). The cells, after thawing, were first maintained in an ascitic form by i.p. passaging in animals. Briefly, cells obtained from the peritoneal cavity 7–10 days after i.p. injection were washed and diluted to a cell density of 2×10^6 cells/ml. In all, 0.5 ml of the cell suspension was reinoculated i.p. into the mice. After three *in vivo* passages, the cells were cultured *in vitro*. Only passages 5–10 cultured *in vitro* were used for the study. Lower passages were omitted to minimize the number of normal cells in the culture, higher passages were avoided to keep the aggressiveness of the cell line. The cells were incubated *in vitro* in IMDM supplemented with 10% FBS (Gibco, Invitrogen), L-glutamine (Gibco, Invitrogen), penicillin and streptomycin (Gibco, Invitrogen), and subcultivated every second day at a density of 5×10^4 cells/ml.

L1210 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 on animals were conducted according to the National Ethical Committee's Guideline on Animal Welfare. Female DBA/2 mice, 6–8 weeks old at the beginning of the experiment, were purchased from Harlan (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 in 70% ethanol for approximately 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 L1210 tumor cells were mixed with BM cells to produce 5 and 50% contents of the L1210 cells in a suspension. The total cell density was kept at 8×10^5 cells/ml.

HAL-induced PpIX production in cells
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. After the incubation with HAL, the cells were washed to remove the drug-containing incubation medium and cell pellets were then resuspended in ice-cold PBS. Samples, protected from light, were stored on ice and analyzed within 30–60 min by flow cytometry.

The measurements of intracellular PpIX were performed using a LSR II flow cytometer (Becton Dickinson, San Jose, CA, USA). An argon laser operating at 488 nm was used for fluorescence excitation of PpIX and propidium iodide (Sigma-Aldrich, Munich, Germany). 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 red fluorescence (PpIX, propidium iodide) passing a combination of 685 nm dichroic and 695/40 nm band-pass filters 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. Parameter 'mean' from the statistics window generated for the red fluorescence-area histogram was used to evaluate PpIX production. Propidium iodide fluorescence was used to estimate the content of dead cells in the samples not incubated with HAL.

Fluorescence spectroscopy. Fluorescence emission spectra were recorded by means of Perkin Elmer LS50B Luminescence Spectrometer (Norwalk, CT, USA). The excitation wavelength of 410 nm and 15 nm slit width were used. The fluorescence emission was scanned from 550 to 800 nm. A cut-off filter was used to remove scattered light of wavelengths shorter than 515 nm from the light reaching the detection system of the spectrometer. 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.[25]

Protein content. For measurements of protein content of the cell samples the Pierce BSA Protein Assay (Pierce Inc., 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.).

Photodynamic treatment. After incubation of cells with HAL, described under the flow cytometry subsection, the drug-containing medium was removed and IMDM supplemented with FBS 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 up to 300 s. 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, UK) 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 L1210 and BM cells. In studies on individual types of cells, 150 L1210 and 2×10^5 BM cells, respectively, were seeded in 1.1 ml of MethoCult with recombinant cytokines, with erythropoietin (M3434, StemCell Technologies, Grenoble, France). For the mixtures of L1210 with BM, cell suspension containing 150 L1210 cells were seeded in the same medium. Parallel samples were prepared in MethoCult without cytokines as well as EPO (M3231, StemCell Technologies) for comparison. 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. After that the colonies of L1210 cells were macroscopic in size and visible with the naked eye. The regular spherical shape of the colonies enabled us to scan the dishes to facilitate counting. The colonies with a diameter of $\geqslant$0.09 mm were counted. 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.

TBI. For total body γ-irradiation of healthy mice, a ^{60}Co source was used. The mice were irradiated at dose rates of 1.75–1.87 Gy/min, reaching total doses of 9.25, 9.50, 9.75, 10.00, 10.25, 10.50, 10.75, 11.00 and 11.25 Gy.

Tumor induction and animal treatment. After i.v. injection of 1×10^6 L1210 cells into DBA/2 mice, it took 15–22 days to develop late stage of leukemia with typical signs of paralysis of the hind legs. The mice were immediately killed with cervical dislocation. 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-Aldrich) was added to a concentration of 300 unit/ml to make single-cell suspension. Finally, the treated 1×10^7 L1210 leukemic marrow cells were injected i.v. to healthy, but lethally ^{60}Co-irradiated mice. The survival of the injected mice with L1210 cells was followed up until the advent of leukemic signs. The marrow cells without PDT as controls were also included.

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 L1210 cell line and pure BM. To find optimal regime for HAL-induced photodynamic extracorporeal purging of BM, experimental conditions for incubation of HAL in L1210 cell line, BM cells and their mixtures were examined. As the number of dead L1210 cells after 4-h incubation in serum-free medium was found to be dependent on cell density, the density of 8×10^5 cells/ml was chosen to keep the percentage of dead L1210 cells below 10%. The same cell density was used for BM cells as well as for cell mixtures. Production of PpIX by cells was analyzed by means of flow cytometry and fluorescence spectroscopy.

PpIX production in the L1210 cells incubated for 4 h in serum-free medium with different amounts of HAL leveled off at the concentration of 20 μM (Figure 1a). In pure mouse BM, two main populations of living cells, could be clearly separated by flow cytometric analysis, namely granulocytes and mixed population. For both populations, the mild increase in PpIX production was observed even at 100 μM HAL (Figure 1b). The fluorescence signal of the remaining BM cells did not exceed the signal of either of the two populations. The absolute amounts of PpIX produced by HAL in the tumor cell line and BM cells were measured by means of fluorescence spectroscopy relative to protein content (Figure 1c). L1210 cells produced higher amounts of PpIX than mixture of BM cells.

HAL-induced PpIX production in the mixtures of BM with L1210 tumor cells. Flow cytometric analysis of BM cell mixtures with L1210 showed that the viable tumor cell population could be rather clearly separated from both above mentioned populations of BM cells. In addition, at concentrations of HAL from 10 μM, the population of leukemic cells could be distinguished by gating on both side scatter vs forward scatter as well as PpIX signal vs forward scatter, resulting in almost no contamination by BM cells and vice versa. This gave us the opportunity to analyze production and accumulation of PpIX in individual types of cell within the cell mixtures (Figure 2).

The PpIX saturation pattern of BM cells after the incubation in mixtures with L1210 cells was similar to that found in pure BM cells. The amount of PpIX in BM cells seemed to be dependent on the total content of tumor cells in the mixture. The higher the amount of L1210 cells in the mixture, the higher the PpIX signal found in both populations of BM cells (Figures 2a and b). The amount of PpIX in L1210 cells seemed to be slightly affected by the presence of BM cells, with the lowest signal corresponding to the samples with the highest amount of BM cells (Figure 2c).

In another study, the L1210 and BM cells were initially incubated separately with the same amounts of HAL and then mixed shortly before measurement. The PpIX signals

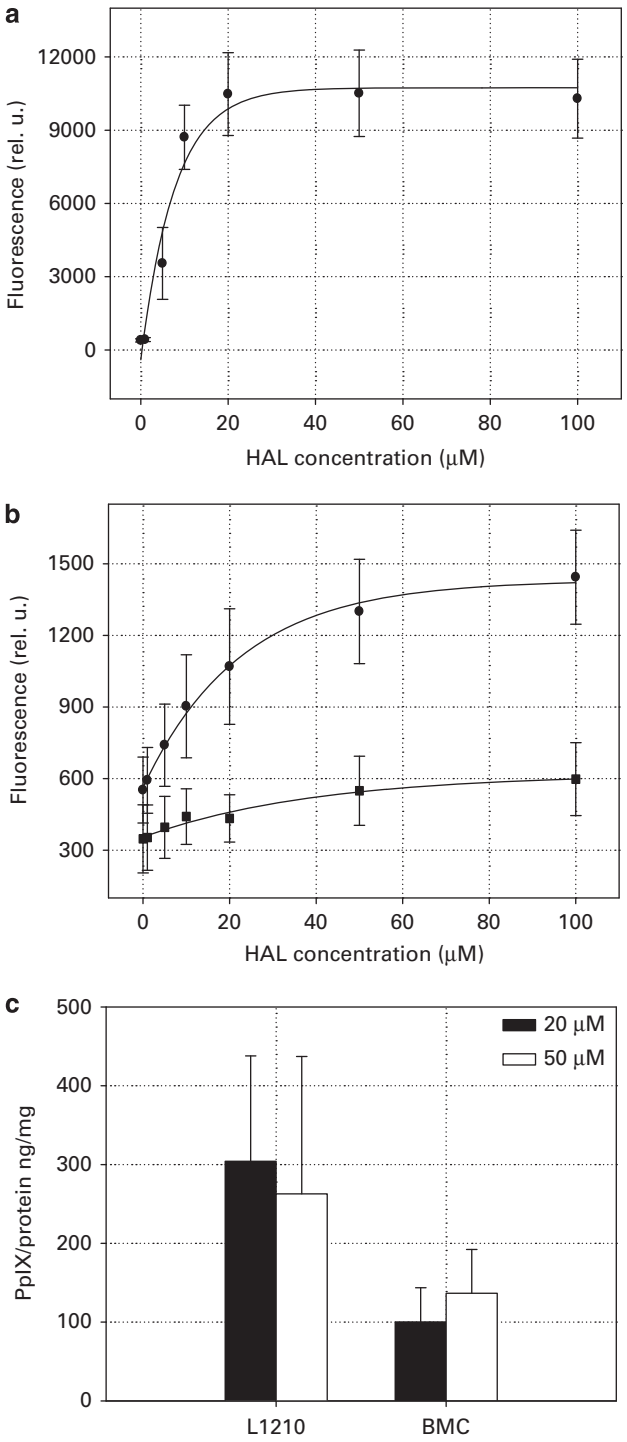

Figure 1 HAL-induced production of PpIX after the incubation of the cells with different amounts of HAL. (**a**, **b**) saturation curves, (**c**) absolute amounts of PpIX relative to protein content of the cells. (**a**) L1210 cell line; (**b**) BM cells: (●) granulocytes, (■) mixed population. Cell density of 8×10^5 cells/ml was used. L1210 cell line had been passaged *in vivo* before it was used for *in vitro* experiments. Each data point represents an average ± s.d. from four to eight independent measurements. The data were fitted to the exponential rise to maximum function.

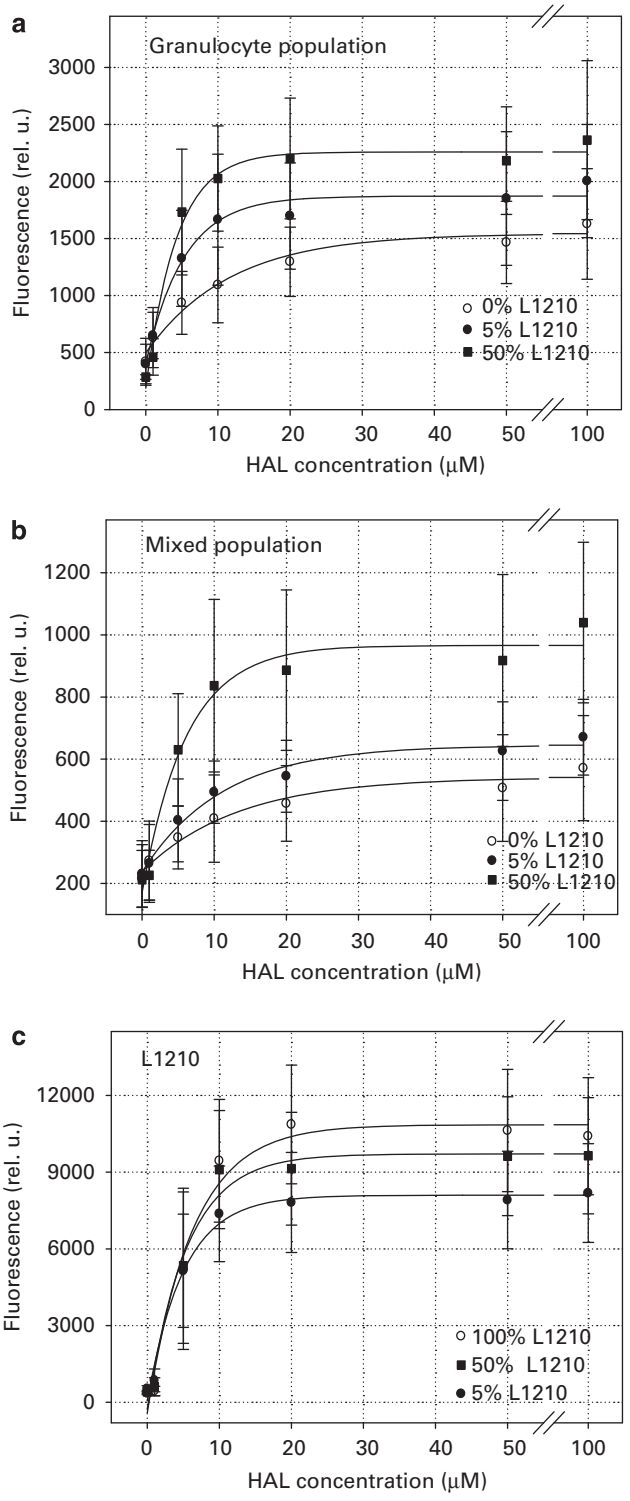

Figure 2 HAL-induced production of PpIX after the incubation of the cell mixtures with different amounts of HAL. Population of granulocytes (**a**) and mixed population (**b**) in BM of DBA/2 mice containing (○) 0%, (●) 5% and (■) 50% L1210 cells; (**c**) population of L1210 cells in the mixture of BM containing (●) 5% and (■) 50% L1210 cells and (○) pure L1210 cells. Total cell density was kept at 8×10^5 cells/ml. Each data point represents an average ± s.d. from three to five independent measurements. The data were fitted to the exponential rise to maximum function.

in both populations of separately incubated BM were similar to the signal of PpIX produced in pure BM cells, but lower than those of the BM cell population, which had

been mixed with L1210 tumor cells before HAL incubation (Figure 3). When comparing corresponding samples mixed before and after incubation with HAL, the difference (Figure 3) between signals of BM populations was found in the case of 50% L1210 containing samples, no matter which HAL concentration was used. The signal of PpIX in L1210 cells was lower when mixtures of BM cells containing 5% L1210 cells were incubated with 50 μM HAL compared with the signal of the corresponding sample mixed just before the measurement (Figure 3c). No difference was found for the sample containing 50% leukemic cells.

Photodynamic treatment of L1210 cell line and BM. The effect of HAL-based photodynamic purging and its resulting cell survival were examined by means of a colony assay. On the basis of the saturation curves of PpIX production found in pure L1210 cells and their mixtures with BM cells, the concentration of 20 μM HAL was chosen for photodynamic treatment. Increasing doses of blue light illumination from 0 to 120 s resulted in progressive killing of L1210 cells. At the light dose of 90 s, no colonies of L1210 cells were formed (Figure 4a).

The photodynamic effect of light illumination on survival of healthy BM cells under the same conditions was only negligible up to 180 s (Figure 4b).

Photodynamic treatment of mixtures of L1210 cell line with BM cells. Survival of L1210 cells treated with HAL–PDT in the mixtures with BM cells could be examined in methylcellulose-based medium without cytokines, because the plating efficiency of L1210 cells in a medium without cytokines was linear in the range of 1–1500 cells seeded, while no colonies of BM cells were formed in the medium without cytokines. A plating efficiency of 31% was found for L1210 cells. Parallel samples were also incubated in a medium supplemented with cytokines to check the survival of BM cells as well as to confirm the purging effect on tumor cells in a complete medium.

No difference in the survival curves of L1210 cells after 4-h incubation in serum-free medium with 20 μM HAL was found between 5% mixture with BM cells and pure tumor cell line (Figure 4a). At the light dose of 90 s, no colonies of L1210 cells were formed in either medium (with or without cytokines). There was no photodynamic effect on the number of BM cell colonies grown in medium with cytokines up to 150 s light dose after the incubation with HAL under the same conditions (data not shown).

Photodynamic purging of leukemic BM of DBA/2 mice ex vivo. The conditions of HAL-based photodynamic BM purging for *in vivo* purposes were first tested *ex vivo*. Incubation of leukemic BM from L1210-bearing animals with 20 μM HAL followed by 90-s illumination with blue light resulted in considerable but not complete reduction of the number of L1210 cell colonies compared with non-purged samples. Considerable variations of various samples were noticed, most probably because of different amounts of leukemic cells in BM of individual mice. The PDT dose resulting in no colony formation of L1210 cells in the *in vitro* model system was not sufficient to kill completely

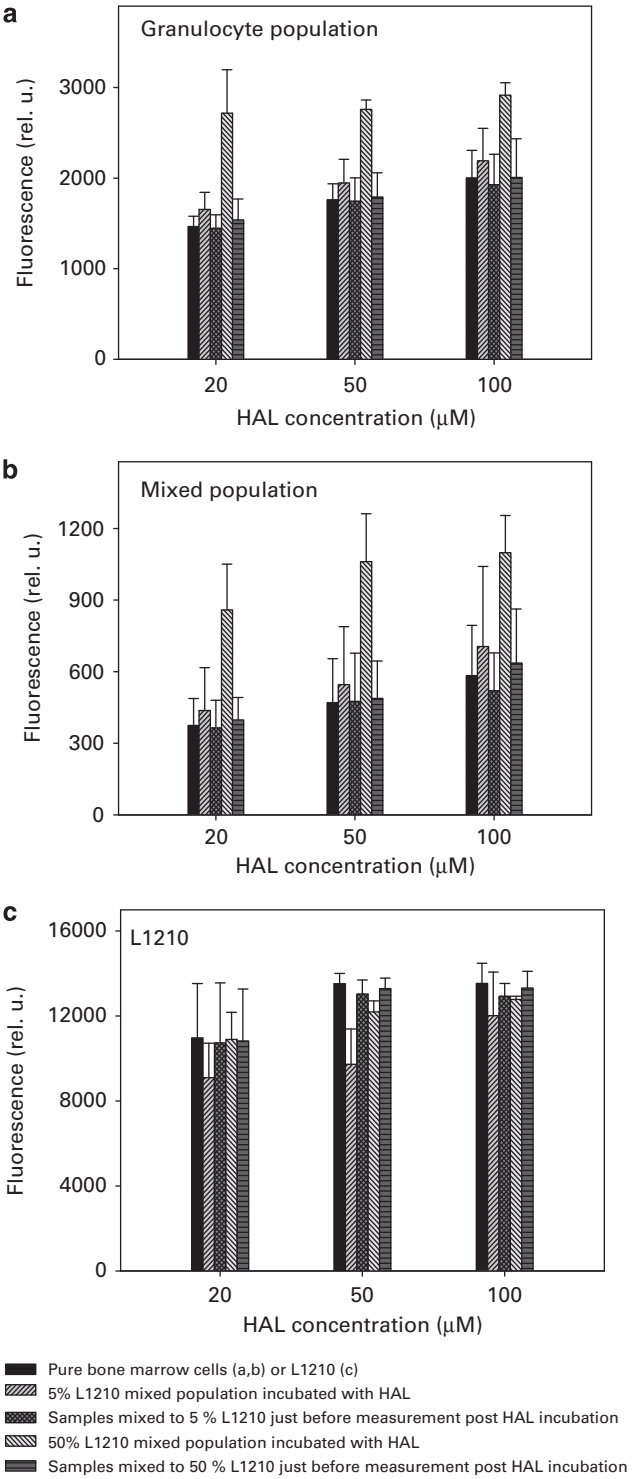

Figure 3 HAL-induced production of PpIX after the incubation of the cells with different amounts of HAL. Incubation of the samples mixed before and after the HAL incubation. Population of granulocytes (**a**) and mixed population (**b**) of the BM of DBA/2 mice containing 0, 5 or 50% L1210 cells and (**c**) population of L1210 cells. Total cell density was kept at 8×10^5 cells/ml during the incubation with HAL. Each data point represents an average ± s.d. from three independent measurements.

the L1210 cells in the *ex vivo* samples. Further increase in illumination time up to 240 s, and/or drug concentration from 20 to 50 μM HAL, still did not lead to complete killing

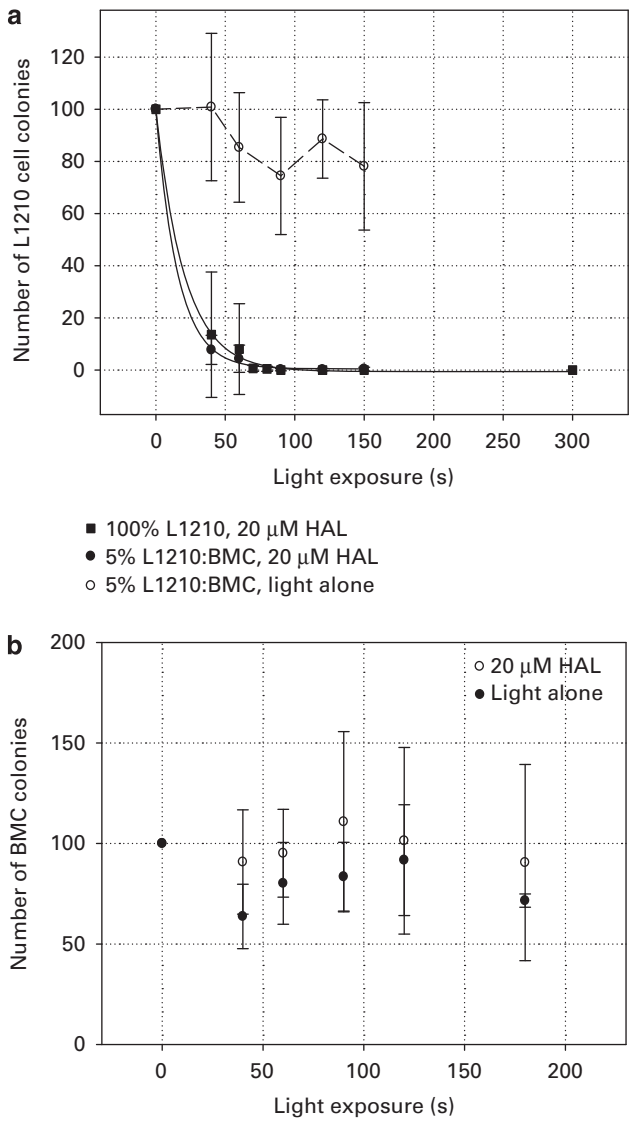

Figure 4 Relative cell survival after HAL-based photodynamic treatment of L1210 cell line and BM cells from healthy animals. Light dose dependence of number of (**a**) L1210 and (**b**) BM cell colonies. (**a**) (●,○) BM cells containing 5% L1210 cells, (■) pure L1210 cell line. (●,■) 20 μM HAL, (○) no drug. (**b**) Pure BM (●) 20 μM HAL, (○) no drug. Colonies of L1210 from cell mixtures were grown in a methylcellulose-based medium without cytokines to prevent growth of BM colonies. Total cell density was kept at 8×10^5 cells/ml. Each data point represents an average ± s.d. from four to five independent measurements. In (**a**) the full lines are fits to the exponential decay functions. The dashed line is shown to indicate tendency. Colony counts at zero time point were: (**a**) 103 ± 14 (100%, HAL), 91 ± 16 (5%, HAL), 95 ± 23 (5%, light alone); (**b**) 23 ± 8 (HAL), 28 ± 10 (light alone).

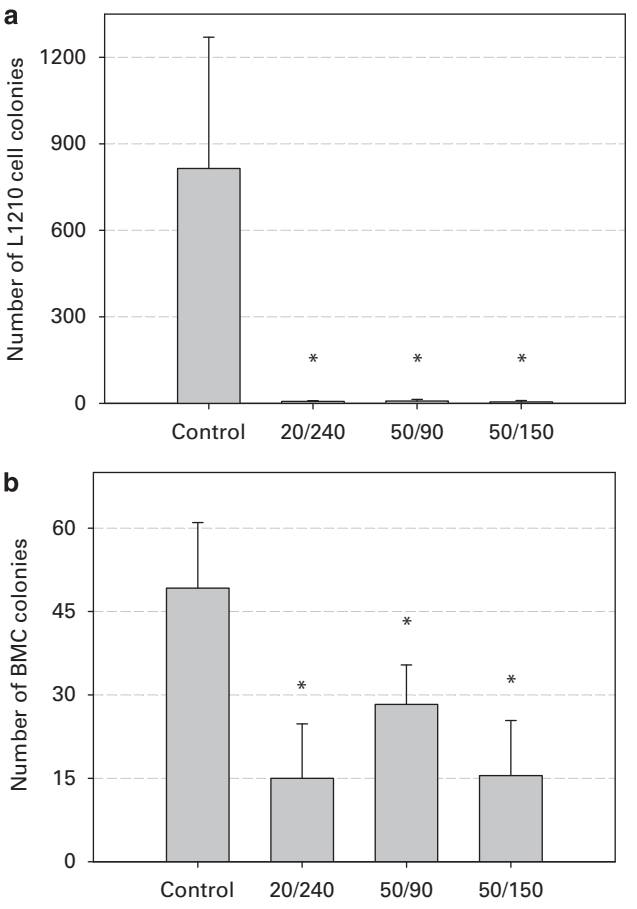

Figure 5 Cell survival after HAL-based photodynamic treatment of leukemic BM. PDT dose dependence of numbers of (**a**) L1210 and (**b**) BM cell colonies. Data shown are number of colonies of BM and L1210 cells counted after the growth in methylcellulose-based medium with and without cytokines, respectively. Different total numbers of cells were seeded to different types of medium. Numbers of L1210 colonies growing simultaneously with BM cell colonies in the medium with cytokines were also counted. The same relative cell survival, within experimental error, was found (not shown). The bars represent averages ± s.d. from four to five independent measurements. Asterisks denotes significant difference at $P<0.05$ with regard to control.

of L1210 cells (Figure 5a). Approximately 1% of the total L1210 colonies compared with non-treated samples was still formed, that roughly corresponds to 6.0×10^3–1.6×10^4 leukemic cells if 31% plating efficiency of L1210 cells is assumed. At the same time, doses of 20 μM or 50 μM HAL followed by 240 or 150-s light illumination, respectively, gave approximately 70% killing of BM cells (Figure 5b).

*BMT ('*in vivo*' purging).* A range of doses from 9.25 to 11.25 Gy for total body ^{60}Co-irradiation was examined to find a lethal dose with suitable length of animal survival for HAL-based photodynamic purging. Lethally irradiated mice were used to closely mimic clinical situation when high doses of radiotherapy are combined with BMT. Total body doses of 9.25–10.25 Gy were sublethal with a number of animals surviving for several months after the γ-irradiation. In contrast, the animals irradiated at doses 11.0 Gy and higher died within <9 days, the period being too short for accomplishing animal revival after BM purging. A lethal dose of 10.5 Gy, with median survival time of 11 days (9–11, $n=5$), was thus chosen for TBI for *in vivo* purging experiments. Leukemic BM was harvested from donors i.v. injected with 10^6 L1210 cells 15–22 days before. The purging procedure consisted of 4-h incubation of leukemic BM in serum-free medium with 20 μM HAL

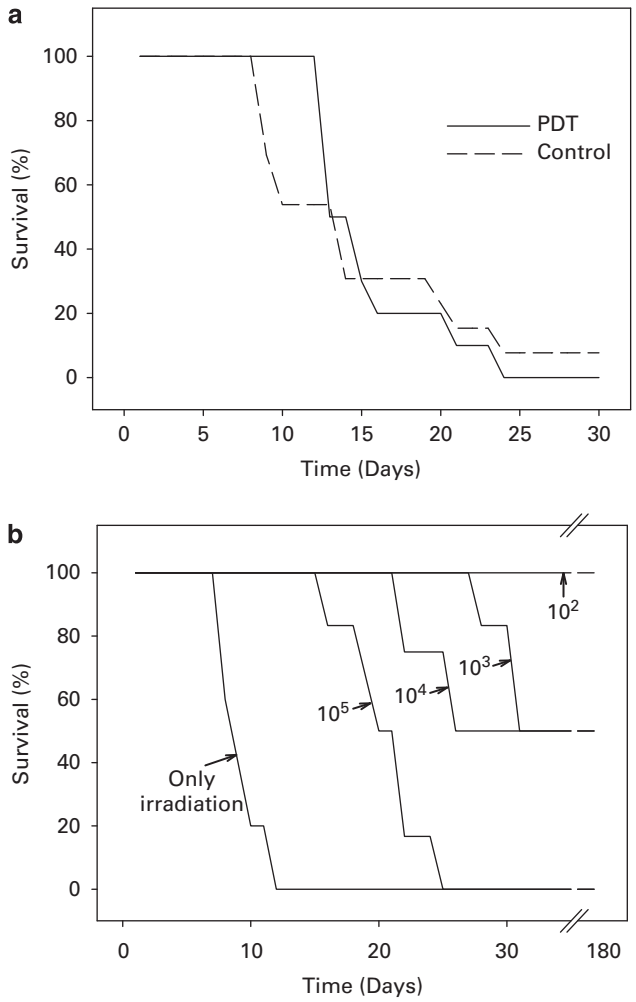

Figure 6 Survival plots of lethally irradiated DBA/2 mice injected with BM contaminated with L1210 cells. (**a**) Mice i.v. inoculated with BM from animals bearing L1210 leukemia treated by HAL-induced photodynamic purging according to the Table 1 (PDT) or untreated (Control); (**b**) mice i.v. inoculated with BM from healthy donors contaminated with 0–10^5 L1210 cells. Total number of injected cells was 1×10^7, five to six animals were used in each group. In the group that had received 10^4 L1210 cells, one animal had no signs of paralysis before its death probably because of the effect of irradiation and has not been included in calculations. The recipient mice were previously irradiated with a lethal dose of 10.75 Gy.

followed by 150-s illumination with blue light. I.v. injection of 1×10^7 purged leukemic BM cells into lethally irradiated recipient animals without leukemia resulted in death of mice within 13–24 days (Figure 6a and Table 1), all with signs of paralysis. The recipients injected with the same amount of non-purged leukemic marrow cells died within 9–24 days (Figure 6a and Table 1). To find a minimal amount of leukemic cells necessary for development of leukemia, lethally irradiated mice (10.75 Gy) were i.v. injected with a mixture of BM from healthy donors containing different numbers (10^2–10^5) of L1210 cells (Figure 6b). All animals ($n = 6$) injected with 10^2 leukemic cells survived without any sign of disease, whereas the animals injected with 10^5 leukemic cells ($n = 6$) died within 16–25 days after injection.

Table 1 HAL-induced photodynamic purging of BM of animals bearing L1210 leukemia

Animal	*DBA/2*
Cell line	L1210
γ-irradiation	10.5 Gy
PDT dose	
HAL concentration	20 μM
Light dose	150 s
Number of injected cells	1×10^7
PDT	
Mortality/total number of animals	10/10
Median (range) survival time	14 (13–24) days
Control	
Mortality/total number of animals	12/13[a]
Median (range) survival time	12 (9–24) days

Abbreviations: HAL = hexaminolevulinate; PDT = photodynamic therapy.
[a]One animal did not develop leukemia.

Discussion

Photodynamic treatment has been suggested for the purging of BM autografts from residual tumor cells. In the present investigation of HAL-based photodynamic purging of BM grafts, we have compared results from *in vitro* models of BM cells, tumor cell lines and their mixtures with those from *ex vivo* and *in vivo* models. Our results have shown limited relevance of the *in vitro* models to similar clinical situation of *ex vivo–in vivo* models.

Comparison between pure tumor cells and their mixtures with BM cells

In the L1210 leukemic model used in this study, consistency between results on pure tumor cells and their mixtures with BM cells was obtained. Similar cell mixtures have been used in several studies on photodynamic purging of stem cell grafts:[7,8,22–24] The selectivity of the phthalocyanine-mediated photodynamic inactivation of erythroleukemic cells was studied in the mixtures with normal peripheral blood leukocytes in an equal ratio.[7] The same ratio was used to compare the cytotoxic effect of merocyanine 540 on leukemic cells and normal human BM[22,23] (supposedly mimicking a rather advanced stage of the disease). Lower amounts of leukemic cells were indicated by flow cytometry in the BM of the animals with an advanced stage of the disease in this study. Therefore, the mixtures with lower percentages of tumor cells should be more relevant for real *in vivo* situations in our case, similar to the studies, in which tumor cells were mixed with BM cells at ratios of 1:10, 1:19 and 1:100.[22,24] Even lower ratios, 1:100 and 1:1000, in the mixtures of leukemic cells with normal mononuclear cells were examined by Huang *et al*.[8] Unfortunately, the application of flow cytometric analysis for the mixtures without tumor-specific markers in this study prevented us from using the mixtures containing $<5\%$ tumor cells.

It follows from our *in vitro* results that relative amounts of tumor cells in BM graft might affect the content of PpIX

in the cells incubated with HAL and supposedly also the purging efficiency as a result. One of the reasons, indicated by dependence of PpIX fluorescence signal on L1210 cell content in the mixtures with BM cells (Figure 2), could be a transfer of PpIX from tumor cells to BM cells. Comparison of the PpIX fluorescence of samples incubated separately with that found in the mixtures (Figure 3) supports our concern that such transfer occurs. As shown in Figure 1, PpIX production in the BM cells is not leveled off at concentrations used for photodynamic treatment (20 μM) and the transfer of PpIX from tumor cells might occur to reach saturation. It is reasonable to believe that the less tumor cells in the mixture, the more PpIX would be transferred from tumor cells to reach equilibrium in the mixture. Therefore, the concentration of PpIX in tumor cells will drop below the value optimal for PDT to a greater extent in the mixtures containing low numbers of tumor cells. This explanation is consistent with our data shown in Figure 3. However, no difference in the L1210 cell killing was found between the pure leukemic cell line and its mixture with BM cells, although the PpIX transfer between the two types of cells did occur according to our data. This may be explained by various degrees of sensitivities of different kinds of cells to ALA/HAL–PDT.[26–29] The transfer of the drug indicated for HAL-induced PpIX is probably not so important in the case of exogenous photosensitizers, although the extent of drug binding might differ among different kinds of cells.

Comparison between the in vitro *and* ex vivo *results*

The morphological differences between colonies of BM cells and leukemic cells gave us opportunity to study simultaneously the survivals of different types of cells in artificial cell mixtures, that is, mixtures of BM from healthy animals with L1210 cell line and of *ex vivo* samples, that is, leukemic BM from animals with leukemia. The numbers of leukemic cells inoculated for the colony assays could differ between *in vitro* and *ex vivo* samples because the lack of the specific marker for L1210 cells makes it impossible to distinguish the entire population of the leukemic cells from BM cells. Nevertheless, the non-treated *ex vivo* samples have shown considerable spread in the number of colonies formed, indicating large animal-to-animal variations. The numbers of leukemic cells inoculated for colony assay supposedly included those used for *in vitro* experiments.

The effective treatment protocol for the L1210 cells in either pure form or the mixture with BM cells *in vitro* was not enough to completely kill tumor cells in the leukemic BM. The reason might be related to a change of biological behavior of the tumor cells in an *in vivo* environment compared with tumor cell line cultured *in vitro*. The fact that L1210 cells had to be passaged *in vivo* before they were capable of inducing leukemia in mice is consistent with the occurrence of cell modification.

Comparison between the in vitro *and* in vivo *results*

On the basis of the *in vitro* results for the L1210 leukemia model, one could conclude that HAL-based PDT purging is a promising treatment modality. Doubts from *ex vivo* experiments requested verification of this conclusion in an animal model. Surprisingly, the treatment turned out to be rather inefficient *in vivo*. All treated animals died of the disease in the same time period as control mice without PDT purging (Table 1). The number of injected tumor cells escaping from the treatment judged by the *ex vivo* experiments was in the range efficient for the development of leukemia by the L1210 cells (Figure 6b). In addition, HAL-induced PpIX used in thist study is an endogenous photosensitizer synthesized in the cells during heme biosynthesis. Therefore, the cells at the resting phase could produce much less endogenous PpIX and possibly escape from an inefficient PDT. This is supported by a report that shows human bladder cancer cells in G_1 resting phase to produce less PpIX after incubation with ALA than those in S or G_2 phase.[30] At the same time, there is an indication that many of the cells in BM appear in the resting phase.

In conclusion, this study clearly shows that the efficiency of HAL–PDT based on the results obtained from pure tumor cell lines *in vitro* should be taken with caution and always verified in clinically relevant *in vivo* models. If *in vitro* models are to be used, they have to be designed in such a way that they reliably mimic the *in vivo* clinical situation. In spite of encouraging *in vitro* data, this therapy does not appear to be suitable for the treatment of the L1210 leukemia *in vivo*.

Conflict of interest

The authors declare no conflict of interest.

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