JOURNAL OF BIOMEDICAL OPTICS 3(3), 286–295 (JULY 1998)

EVALUATION OF THE SILICON PHTHALOCYANINE PC 4 FOR PHOTODYNAMIC BONE MARROW PURGING

Jan F. Keij,[†] Yajuan Jiang,[‡] Damianos A. Sotiropoulos,[‡] Ehud Ben-Hur,[*] and Jan W. M. Visser[‡]

[†]Los Alamos National Laboratory, LS-5, MS M888, Los Alamos, New Mexico 87545; [‡]New York Blood Center, New York, New York 10021; *VITEX, Audubon Center, New York, New York 10032

(Paper JBO-179 received Nov. 5, 1997; revised manuscript received Mar. 6, 1998; accepted for publication Mar. 20, 1998.)

ABSTRACT

The silicon phthalocyanine Pc 4 was tested as a photosensitizer for the selective photoinactivation of malignant cells in bone marrow transplantation samples. Using a murine model system, incubation of 1.5×10^7 cells/mL with 15 nM Pc 4 followed by exposure to red light ($\lambda > 600$ nm, fluence of 18 J/cm^2) was shown to result in a greater than 6 $\log_{10}$ reduction of the clonogenic growth for the murine cell lines ABE-8.1/2, BC3A and L1210. The clonogenic growth of WEHI-3 and P815 cells was reduced by more than 5 $\log_{10}$ and more than 3 $\log_{10}$, respectively. Late murine hematopoietic progenitor cells were less sensitive than cancer cells; the surviving fractions were 0.084 for the colony forming unit, megakaryocyte (CFU-Mk); 0.038 for the colony forming unit, granulocyte macrophage (CFU-GM); 0.0018 for the colony forming unit, mix (CFU-mix) and <0.003 for burst forming units, erythroid (BFU-E). Early hematopoietic progenitor cells, assayed by the *in vitro* cobble stone area forming cell assay, were not affected by the photodynamic treatment. Likewise, *in vivo* assays of early hematopoietic progenitor cells showed no reduction of their ability to repopulate the bone marrow. Irradiation of the samples following incubation of 1.5×10^6 cells/mL with Pc 4 resulted in increased photosensitivity of all cell types, including the early and late hematopoietic progenitor cells. Flow cytometric analysis of Pc 4 uptake by the cells revealed that the increased photosensitivity could be traced to increased Pc 4 uptake; however, Pc 4 uptake among cell types did not correlate with photosensitivity. When mixed with bone marrow (BM) cells, Pc 4 uptake in the cell lines increased as the fraction of BM increased from 0.5 to 0.95. These observations suggest that Pc 4 may be a suitable photosensitizer for bone marrow purging. © *1998 Society of Photo-Optical Instrumentation Engineers.* [S1083-3668(98)01603-7]

Keywords cobble stone area; murine; early progenitor cells; photosensitization.

1 INTRODUCTION

Low levels of contaminating malignant cells are sometimes present in autologous bone marrow of cancer patients[1] and mobilized peripheral blood stem cell grafts,[2] and in some cases these residual malignant cells have led to a relapse in transplanted patients.[3] Several techniques have been used to prevent relapse due to cotransplantation of malignant cells by selectively removing the malignant cells from the grafts.

One approach is to selectively isolate the hematopoietic progenitor cells using CD34-coupled immunomagnetic beads.[4,5] Although successful, this procedure is only applicable for grafts containing malignant cells that do not express the CD34 antigen. In one study, the CD34 antigen was reported to be expressed on 9 out of 63 breast cancer, 2 out of 11 squamous cell sarcomas of the lung and 3 out of 12 small cell lung cancers.[6]

Another approach is to selectively kill the malignant cells by either the action of antibody and complement,[7] cytotoxic drugs,[8] or a photosensitizer.[9] Photoinactivation of malignant cells has been successfully achieved using a variety of photosensitizers such as merocyanine-540 (MC 540),[10,11] dihematoporphyrin ether (DHE),[12] pyrene-containing sensitizers (P12),[13,14] and sulphonated aluminum phthalocyanine (AlSPc).[15]

The relatively low cytotoxicity of the phthalocyanines (Pc) and their resistance to chemical and photochemical degradation[16] have led to the development of new Pc photosensitizers for photobiological applications.[17] One of these is $HOSiPcOSi(CH_3)_2(CH_2)_3N(CH_3)_2$ (Pc 4),[17,18] which has been shown to effectively photoinactivate viruses[19,20] and parasites[21,22] in blood products, and mammalian cells in culture.[17] Human bone marrow

Address all correspondence to Ehud Ben-Hur. FAX: 212-923-6229; Tel.: 212-740-2268; E-mail: ehud_ben-hur@juno.com

(BM) late hematopoietic progenitors were shown to be highly resistant to photoinactivation with Pc 4 under conditions that led to a reduction of 5 $\log_{10}$ of the clonogenic growth of HL-60 (an acute promyelocytic leukemia cell line)[23] and SK-BR3 (a breast cancer cell line).[23]

The latter results led us to investigate the possibility of using Pc 4 to selectively photoinactivate malignant cells from mobilized peripheral blood and bone marrow samples for transplantation purposes. In this report, we describe the differential photoinactivation by Pc 4 of murine cell lines, and murine bone marrow progenitor subsets and stem cells as a function of cell concentration and light dose.

2 MATERIALS AND METHODS

2.1 ANIMALS

C57B1/6 (Ly-5.2) and B6SJ-cd45^{b}pep 3^b/BOY J (Ly-5.1), were obtained from Jackson Laboratories (Bar Harbor, ME) and were maintained in the animal facility of the New York Blood Center. After gamma irradiation, the animals were housed in microisolator cages and provided with autoclaved food and autoclaved acidified water (pH=3) *ad libitum*.

2.2 PREPARATION OF BONE MARROW CELL SUSPENSIONS

Ly-5.1 mice were used as the source of donor BM. Mice (8–10 weeks old) were killed and the BM was harvested by flushing the femoral shafts with Hank's balanced salt solution (HBSS), pH 7.4. The cell suspensions were centrifuged, washed once and the cells were finally resuspended in HBSS–10% vol/vol fetal bovine serum (FBS, Gemini Bioproducts Inc., Calabasas, CA).

2.3 CELL LINES AND CULTURE CONDITIONS

WEHI-3 (murine myelocytic leukemia), L1210 (murine lymphocytic leukemia) and P815 (murine mastocytoma) cells were grown in RPMI 1640 (GibcoBRL, Grand Island, NY) supplemented with 10% FBS. BC3A (murine leukemia) cells were grown in RPMI 1640 supplemented with 10 μM β-mercaptoethanol and 10% FBS; ABE-8.1/2 (murine pre-B lymphoma) cells were grown in DMEM (GibcoBRL) supplemented with 10 μM β-mercaptoethanol and 10% FBS. All cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were cultured at 37°C in a humidified 5% CO_2 incubator. Prior to incubation with Pc 4, the cell lines were centrifuged and resuspended in phosphate buffered saline (PBS) supplemented with 10% FBS, pH 7.4.

2.4 PHOTOSENSITIZATION

Aliquots of a 1.87 mM Pc 4 (kindly provided by Dr. Kenney) stock solution in dimethyl sulfoxide were kept at −80°C in autoclaved brown glass vials. The purity of Pc 4 was 98%, determined by HPLC. Immediately prior to the photosensitization, Pc 4 was thawed and diluted in dimethyl formamide to yield a 10 μM working solution. Uptake and cytoxicity of Pc 4 were tested on murine BM samples. Using flow cytometry it was determined that uptake was maximal after 2 h. Incubation with 15 nM Pc 4 resulted in marginal losses (0%–15%) of the colony forming units, granulocyte macrophage (CFU-GM). Pc 4 was used at 15 nM throughout the experiments.

BM and cell line samples were incubated with 15 nM Pc 4 for 2 h at 37°C at cell concentrations of 1.5×10^7 cells/mL. Aliquots of 1.5 mL were transferred to 24-well plates; separate plates were used for each sample.

In a second set of experiments, the cells were incubated with 15 nM Pc 4 at cell concentrations of 1.5×10^6 cells/mL. To retain the sensitivity of the CFU assay, the sample densities were readjusted to 1.5×10^7 cells/mL by centrifugation and aspiration of 9/10 of the sample supernatant.

2.5 PHOTOINACTIVATION

The light source used to deliver red light (λ = 600–700 nm) was a filtered xenon short arc lamp (Versa-Light, model UV-R6G, Medic Lightech, Ltd., Haifa, Israel). The fluence rate was 26.6 mW/cm^2 at the surface of the cell suspension, as measured with a radiometer (model IL 1350, International Light, Newburyport, MA). Samples were placed under the light source and were stirred during irradiation. The samples were kept in the dark before and after irradiation.

2.6 COLONY FORMING ASSAYS

BM samples were plated (in duplicate) in a semisolid medium that contained IMDM (GibcoBRL), supplemented with 30% vol/vol FBS, 0.15% wt/vol deionized bovine serum albumin[24] (Sigma, St. Louis, MO), 0.8% wt/vol methylcellulose, 75 μM β-mercaptoethanol (Sigma), antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL fungizone, GibcoBRL) and a cocktail of growth factors including: murine interleukin-3 (1% vol/vol COS-cell-derived supernatant, gift from Dr. Kaushansky), murine granulocyte-macrophage colony stimulating factor (1% vol/vol COS-cell-derived supernatant, gift from Dr. K. Kaushansky), recombinant human interleukin-11 (16 U/mL, gift from Genetics Institute, Cambridge, MA), recombinant rat stem cell factor (100 ng/mL), granulocyte colony stimulating factor (10 ng/mL), recombinant human erythropoietin (20 ng/mL), gifts from Amgen (Thousand Oaks, CA), and 800 U/mL murine thrombopoietin (800 U/mL, gift from Zymonetics, Seattle, WA). Following incubation in 3.5 cm dishes at 37°C in a humidified 5% CO_2 incubator for five days, the megakaryocyte colony forming units

(CFU-MK, >3 cells) were counted. Burst forming units, erythroid (BFU-E, >40 cells), CFU-GM (>40 cells) and mixed colonies (CFU-mix) were counted after seven days. The number of colonies obtained from 10^5 control BM cells were 288±24 (CFU-GM), 47±4 (CFU-MK), 14±1 (BFU-E) and 25±3 (CFU-mix).

Serially diluted cell line samples (75–3×10^6 cells) were plated (in triplicate) in 1 ml of a semisolid medium, which consisted of DMEM supplemented with 20% vol/vol serum (2/3 horse serum, 1/3 FBS), 3 mM glutamine, 60 μM β-mercaptoethanol and 0.3% wt/vol agar. The samples were incubated in 3.5 cm dishes at 37°C in a humidified 5% CO_2 incubator for nine days, and colonies consisting of more than 50 cells were counted. The number of colonies obtained from 100 control cells were 72 ±16 (L1210), 87±19 (P815), 72±17 (BC3A), 78±12 (ABE-8.1/2) and 46±8 (WEHI-3). Three dishes were used for each dilution.

2.7 COBBLESTONE AREA FORMING CELL ASSAY

FBMD-1 stromal cells (gift from Dr. Ploemacher) were cultured at 33°C in a humidified 5% CO_2 incubator in DMEM supplemented with 3.5 mM HEPES, 2 mM glutaMax (GibcoBRL), 10^{-7} M sodium selenite, 10^{-4} M β-mercaptoethanol, 10% vol/vol fetal calf serum (Gemini Bioproducts, Inc.), 5% vol/vol horse serum and 10^{-5} M hydrocortisone 21-hemisuccinate.[25,26] Prior to each experiment, FBMD-1 cells were harvested from log-phase cultures and fresh layers were prepared by seeding 1000 cells into individual wells of 96-well flat bottom plates. These plates had been incubated overnight with 0.2% (wt/vol) gelatin to improve adherence of the stromal layer. The outer wells of the plates were filled with 100 μL 0.1 N NaOH to reduce the possibility of infection.

The FBMD-1 layers were overlaid with the bone marrow samples in a limiting dilution setup; the first wells receiving 180 000–300 000 cells.[24] Ten dilutions, twofold apart, were used for each sample with 18 replicate wells per dilution. The cells were cultured at 33°C in a humidified 5% CO_2 incubator. For each sample, the fraction of wells with at least one phase-dark hematopoietic clone of at least five cells (cobblestone area) was determined weekly and cobblestone area forming cell frequencies (CAFC) were calculated using the dedicated Poisson statistics program LIMDIL (kindly provided by Dr. Hendrikx, Erasmus University, Rotterdam). After counting, 100 μL medium was removed from the wells and replaced with 100 μL fresh medium.

2.8 BONE MARROW TRANSPLANTATION AND CHIMERISM ASSAY

Two month old Ly-5.2 mice were used as recipients in the BM reconstitution assays. Whole-body irradiation was administered in a single sublethal dose of 6.4 Gy at a dose rate of 80 cGy/min. The irradiated recipient Ly-5.2 mice were divided into six groups. The mice in these groups received HBSS, untreated control bone marrow, or Pc 4-sensitized BM that was photoirradiated with 0, 6, 12 or 18 J/cm^2, respectively. Each mouse received 250 000 donor BM cells.

Six months after transplantation, peripheral blood was obtained by tail-vein puncture and 40 μL samples were aliquoted into tubes that contained the following antibody cocktails. The first tube contained 4 μL of phycoerythrin (PE) conjugated anti-Ly-5.1 antibody (CD45.1, kind gift from Dr. Segovia) and 1 μL of fluorescein (FITC) conjugated anti-B220 antibody (B cells; Pharmingen, San Diego, CA), the second tube contained 4 μL anti-Ly-5.1^{PE} and 1 μL of anti-CD3^{FITC} (T cells; Pharmingen), and the third tube contained 4 μL anti-Ly-5.1^{PE} and 1 μL of anti-Gr-1^{FITC} (granulocytes; Pharmingen). Following a 20 min incubation at room temperature, 2 mL of FACS Lysing Solution [Becton Dickinson (BD), San Jose, CA] was added to each sample followed by gentle mixing. After 10 min the samples were centrifuged and the pellets were resuspended in 3 mL PBS. After centrifugation, the washed pellets were resuspended in 2% vol/vol formalin–PBS *p*H 7.4 and the samples were analyzed by flow cytometry within 3 h after staining.

2.9 UPTAKE OF Pc 4

Following 2 h incubation at 37°C with 15 nM Pc 4, cell line and BM samples were analyzed for Pc 4 uptake by flow cytometry. BM samples were first incubated with anti-c-kit^{PE} (Pharmingen) and anti-Sca-1^{FITC} (Pharmingen), to allow analysis of the Pc 4 uptake in the late hematopoietic progenitor fraction (c-kit$^+$, Sca-1$^-$) and the early hematopoietic progenitor cell fraction (c-kit$^+$, Sca-1$^+$).[27] Following a 20 min incubation on ice, and two subsequent washes, the samples were incubated with 15 nM Pc 4. The relative Pc 4 uptake was calculated by subtracting the mean fluorescence signals of the untreated control samples from the mean Pc 4 fluorescence signals.

In mixing experiments, aliquots of Ly-5.2 BM and cell lines samples were mixed at different ratios. The combined cell concentration of each sample was 1.5×10^7 cells/mL. Prior to the mixing of the samples, the bone marrow cells were first stained with anti-c-kit^{PE} to allow analysis of the Pc 4 uptake in the late progenitor subset (c-kit$^+$). The cell lines were stained with anti-Ly-5.1^{FITC}. This treatment allowed clear resolution of the relatively small ABE-8.1/2 and BC3A cells from the bone marrow granulocytes. Immediately prior to the analyses, propidium iodide (PI, 0.5 μg/mL) was added to the samples to allow exclusion of dead cells (PI$^+$) from the analyses. The samples were analyzed in a FACStar^{PLUS} (BD) that was equipped with an argon

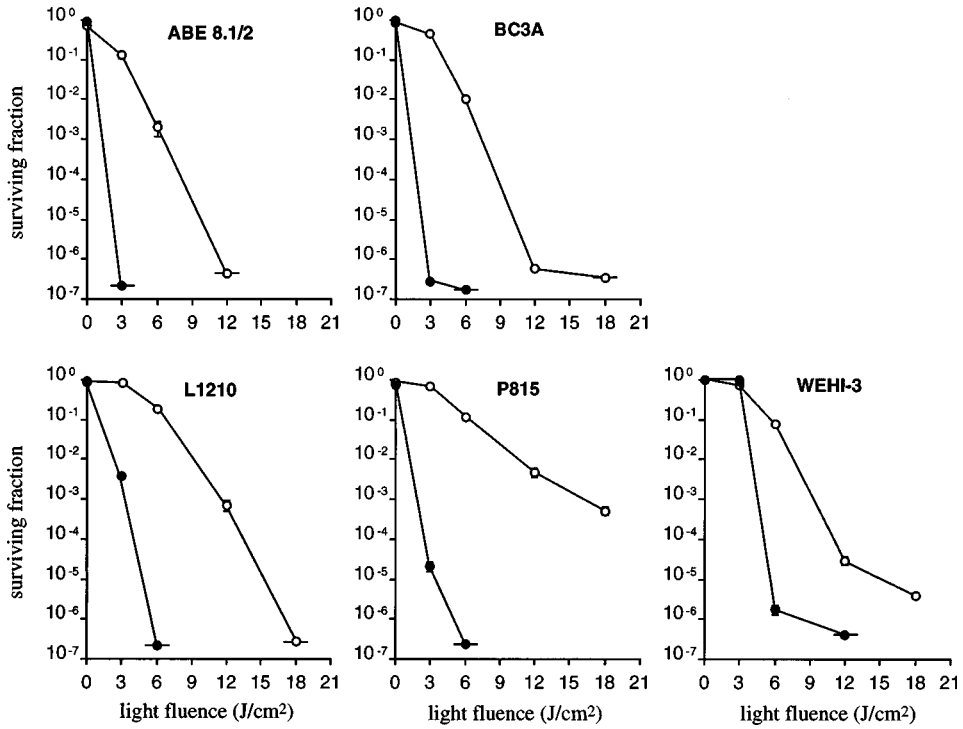

Fig. 1 Dose response of murine cell lines incubated with 15 nM Pc 4 at concentrations of 1.5×10^7 cells/mL (open circles) and 1.5×10^6 cells/mL (solid circles). The survival data represent the means ± SEM of three experiments; the survival of the untreated controls was standardized to 1.0. Symbols with horizontal lines indicate the threshold levels of detection.

ion laser tuned to 488 nm (200 mW) and a dye laser tuned to 610 nm (300 mW). FITC, PE, PI and Pc 4 fluorescence emissions were collected through 530/30, 575/42, 660/10, and 670/10 nm band pass filters, respectively.

2.10 STATISTICS

All assays were performed in triplicate unless mentioned otherwise. Data are expressed as mean values ± standard error of the mean (SEM). Survival data are presented as surviving fractions relative to the surviving fractions of the untreated controls. The significance of the differences between experimental values was assessed by means of the Student's t-test.

2.11 CELL SIZE

Microscopic measurements of at least 20 cells were performed using a calibrated micrometer. To determine the cell size of the BM progenitor subsets, the c-kit$^+$, Sca-1$^-$ and c-kit$^+$, Sca-1$^+$ fractions were sorted. The sorted cells were centrifuged and the pellets were resuspended in a 20 μl of HBSS. Samples were deposited onto microscope slides and covered with a cover slip. Cell diameters of 7.7±0.2 (c-kit$^+$, Sca-1$^+$), 8.4±0.3 (c-kit$^+$, Sca-1$^-$), 10.9±0.2 (BC3A), 11.1±0.2 (ABE-8.1/2), 13.0±0.3 (L1210), 14.2±0.3 (P815), 15.0±0.4 μm (WEHI-3) were obtained.

3 RESULTS

3.1 PHOTOSENSITIVITY OF CELL LINES

The efficiency of photoinactivation by Pc 4 of murine cells was tested using a variety of cell lines. Because it had previously been reported that the cell density could affect the efficiency of photoinactivation,[13,27,28] it was decided to test the efficacy of the procedure at two cell concentrations. After incubation with 15 nM Pc 4 at 1.5×10^7 cells/mL, the surviving fractions of the cell lines ranged from 0.73 to 1.00. Irradiation of the sensitized cells with increasing fluences of red light resulted in a progressive loss of the colony forming abilities of the cell lines (Figure 1). For samples that were incubated with Pc 4 at 1.5×10^7 cells/mL, the BC3A and ABE 8.1/2 cells were photoinactivated to below the detection levels (surviving fractions $<5\times10^{-7}$) after receiving fluences of 12 J/cm^2, while the L1210 cells required 18 J/cm^2. This latter fluence was insufficient to photoinactivate P815 and WEHI-3 cells below detection levels; surviving fractions were $5.3\pm2.6\times10^{-4}$ and $3.8\pm0.07\times10^{-6}$, respectively.

Pc 4 photoinactivation proved more efficient when the cells were incubated at 1.5×10^6 cells/mL. The cell lines could be photoinactivated to below detection levels with fluences of 3 J/cm^2 (ABE-8.1/2), 6 J/cm^2 (BC3A, L1210, P815) and 12 J/cm^2 (WEHI-3), respectively.

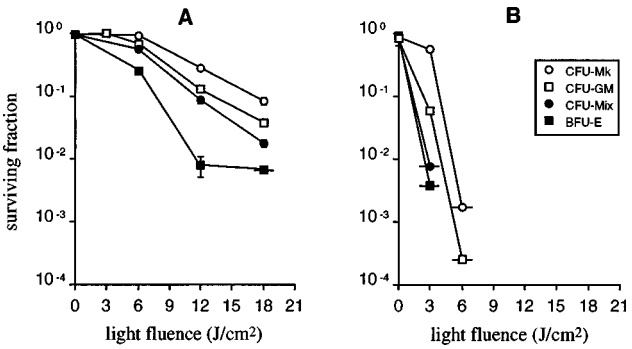

Fig. 2 Dose response of late murine bone marrow progenitor subsets after incubation with 15 nM Pc 4 at concentrations of 1.5×10^7 cells/mL (A) and 1.5×10^6 cells/mL (B). The survival data represent the means ± SEM of three experiments; the survival of the untreated controls was standardized to 1.0. Symbols with a horizontal line indicate the threshold levels of detection.

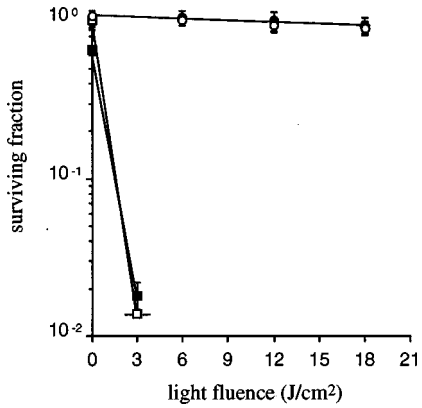

Fig. 3 Dose response of murine early hemopoietic progenitor cells, as measured by cobble stone forming cell (CAFC) assay, after incubation with 15 nM Pc 4 at concentrations of 1.5×10^7 BM cells/mL (circles) and 1.5×10^6 cells/mL (squares). The data represent the means ± SEM of four individual experiments; the survival of the untreated controls was standardized to 1.0. The symbols with a horizontal line indicate the threshold levels of detection. The CAFC frequencies of the samples that were incubated with Pc 4 at 1.5×10^7 cells/mL and irradiated with 6 J/cm^2 ($p=0.58$), 12 J/cm^2 ($p=0.40$), and 18 J/cm^2 ($p=0.26$) were found to be not significantly lower than CAFC frequencies of the untreated samples.

3.2 PHOTOSENSITIVITY OF LATE HEMATOPOIETIC PROGENITOR CELLS

For BM samples incubated with Pc 4 at 1.5×10^7 cell/mL, all late progenitor subsets proved resistant to a fluence of 3 J/cm^2. However, after photoirradiation with a maximum fluence of 18 J/cm^2, the colony forming abilities of the CFU-Mk, CFU-GM, CFU-mix, and BFU-E were reduced to $8.4\pm2.2\times10^{-2}$, $3.8\pm0.7\times10^{-2}$, $1.8\pm5.0\times10^{-3}$ and $<3.0\times10^{-3}$ of the untreated controls [Figure 2(A)], respectively. The late progenitors were also found to be more sensitive to Pc 4 photoinactivation after incubation with 15 nM Pc 4 at 1.5×10^6 cells/mL [Figure 2(B)]. Photoinactivation to below the detection levels was found for the fluences of 3 J/cm^2 (CFU-mix and BFU-E) and 6 J/cm^2 (CFU-Mk and CFU-GM), respectively.

3.3 PHOTOSENSITIVITY OF EARLY PROGENITOR CELLS—*IN VITRO* ASSAY

To evaluate the photosensitivity of the most immature cells in the BM, two different assays were performed: the *in vitro* CAFC assay and the analysis of chimerism in peripheral blood leukocytes after bone marrow transplantation. The results from two CAFC assays (Figure 3) revealed that CAFC frequencies of the photosensitized samples (incubated with Pc 4 at 1.5×10^7 cells/mL) were not affected after irradiation. The relative CAFC frequencies on day 28 (CAFCd28) of samples containing Pc 4 that were irradiated with 18 J/cm^2, were 0.91 ($p=0.69$) and 0.92 ($p=0.78$), indicating that there was no significant photoinactivation of the early progenitor cells.

However, the survival of BM samples was greatly reduced after incubation with Pc 4 at 1.5×10^6 cells/mL. Following irradiation with a fluence of 3 J/cm^2, the relative CAFCd28 frequencies were 0.018 and below the detection limit of 0.014.

3.4 PHOTOSENSITIVITY OF EARLY PROGENITOR CELLS—*IN VIVO* ASSAY

The competitive repopulation (CR) ability of photoirradiated immature BM progenitor cells was tested using Ly-5.1 BM cells that were transplanted into irradiated Ly-5.2 mice. In a pilot experiment, lethally irradiated recipients (8.5 Gy total body gamma irradiation) received either 70 000 or 250 000 untreated or treated donor BM cells (18 J/cm^2, 15 nM Pc 4, 1.5×10^7 cells/mL) per group ($n=3$). All mice that received 70 000 cells died within one month, while most of the mice that received 250 000 donor cells developed high levels of Ly-5.1 chimerism (>70% after four months) in the peripheral blood granulocytes, T cells and B cells (data not shown).

In another experiment, sublethally irradiated Ly-5.2 mice were transplanted with 250 000 photoirradiated Ly-5.1 bone marrow cells. Two months after the transplants, several mice from different groups died: 1/5 in the control group, that received untreated BM cells, and 1/6 in the groups that received BM cells treated with Pc 4 and fluences of 0, 6 and 18 J/cm^2, respectively. Six months after transplantation, significant levels of chimerism were found in the peripheral leukocytes of all recipient mice (Figure 4). The differences between the chimerism levels of the group that received untreated BM cells and the group that received BM cells that were treated with Pc 4 and 18 J/cm^2 were found to be not significant, indicating that (1) the CR ability of the early progenitor cells was not affected by the photoinactivation procedure, and (2) the loss of 2–3 logs of late progenitor cells did not significantly af-

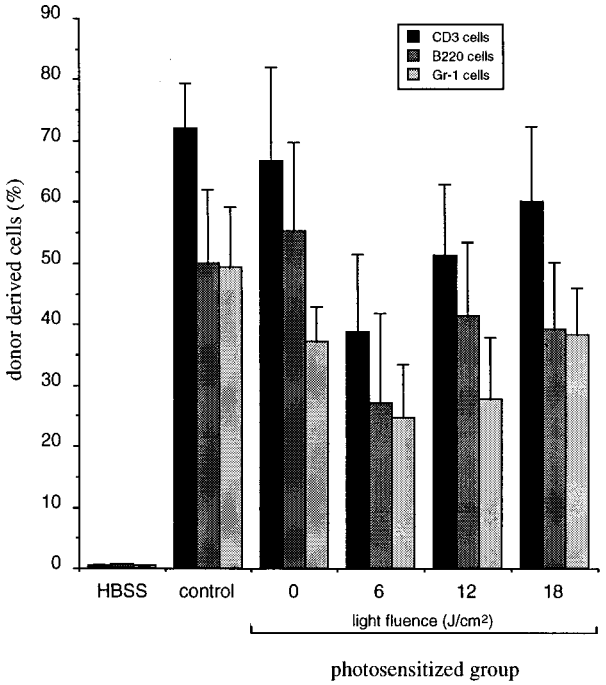

Fig. 4 Chimerism levels of leukocytes subsets in recipient Ly-5.2 mice six months after transplantation with 2.5×10^5 Ly-5.1 donor BM cells. The data represent mean ± SEM. The recipient mice were nonlethally irradiated with 6.4 Gy. Mice were injected with HBSS, untreated BM cells, or BM cells incubated with 15 nM Pc 4 at 1.5×10^7 cells/mL followed by graded light doses. The CD3, B220 and Gr-1 positive cells represent donor stem cell derived T cells, B cells and granulocytes, respectively.

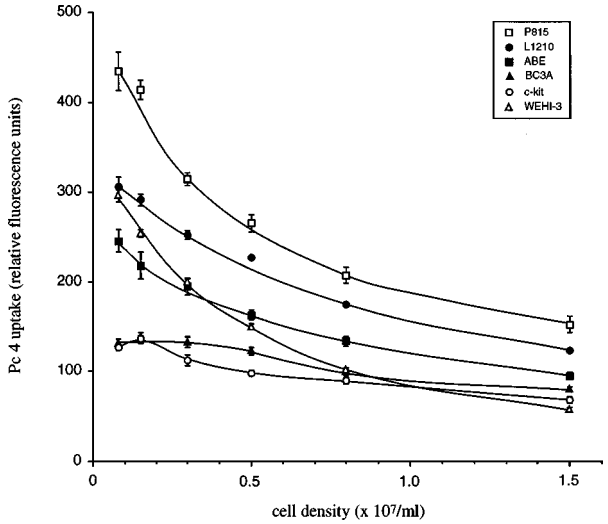

Fig. 5 Relative Pc 4 uptake as a function of cell concentration after incubation with 15 nM Pc 4. The fluorescence data represent the means ± SEM of the three experiments; the lines were manually fitted to the data points. Cells were incubated at concentrations ranging from 8.0×10^5 to 1.5×10^7 cells/mL. The Pc 4 values represent the relative mean fluorescence values of the Pc 4-treated cells minus the mean fluorescence values of the untreated cells. BM cells that stained positive with the anti-c-kit antibody were regarded as late BM progenitor cells.

fect engraftment. The chimerism levels for the groups that received untreated control bone marrow cells and bone marrow cells that were photoinactivated with 18 J/cm^2 were 0.72±0.07, 0.60±0.12 (T cells, $p=0.42$), 0.50±0.12, 0.39±0.11 (B cells, $p=0.53$) and 0.49±0.01, 0.38±0.08 (granulocytes, $p=0.42$), respectively.

3.5 DIFFERENTIAL UPTAKE OF Pc 4 BY CELL LINES AND BONE MARROW PROGENITOR SUBSETS

The relative Pc 4 contents of the cell lines and bone marrow progenitor subsets were analyzed by flow cytometry (Figure 5). For all cell types, the uptake of Pc 4 was inversely correlated with the cell concentration during incubation. The Pc 4 uptake in the BC3A and the BM progenitor cells reached a plateau at cell concentrations of 8.0×10^5 cells/mL. Increasing the cell concentrations from 8.0×10^5 to 1.5×10^7 cells/mL resulted in a 1.6-fold (BC3A), 1.8-fold (BM progenitors), 2.5-fold (ABE-8.1/2, L1210), 2.8-fold (P815), and 5.1-fold (WEHI-3) reduction of Pc 4 uptake, respectively.

The cellular Pc levels for cells incubated at 1.5×10^7 and 1.5×10^6 cells/mL (Table 1) revealed a close correlation with the cell size, the WEHI-3 cells being the exception. Despite the significant differences in photosensitivity, the difference in Pc 4 content (at 1.5×10^7 cells/mL) between the early progenitor cells and the late progenitors was found to be not significant ($p=0.26$), and the differences between the late progenitors and the BC3A and ABE-8.1/2 were found to be not significant ($p=0.29$) and not quite significant ($p=0.07$), respectively.

When the data were corrected for the cell volume, it was found that the differences in cellular Pc 4 concentration among the BC3A, ABE-8.1/2, L1210 and P815 cells were marginal. The BM fractions containing the late progenitors and the early progenitor cells were found to contain almost twice the Pc 4 per cell volume unit compared to the cell lines.

For each cell type, the photosensitivity correlated with the cellular Pc 4 fluorescence as a function of cell concentration. However, when comparing cell types, there appeared to be no correlation between photoinactivation and photosensitizer uptake. In fact, the cells with highest Pc 4 fluorescence based on cell volume, i.e., the c-kit$^+$, Sca-1$^+$ BM fraction containing the early progenitor cells, were found to be the least sensitive to Pc 4 photoinactivation.

Addition of verapamil, a known inhibitor of the multidrug resistance (mdr) pump to the BM samples during incubation with Pc 4 did not increase the uptake of Pc 4 (data not shown). This result suggested that Pc 4 may not be a substrate for the multidrug resistance pump, which is expressed in murine early progenitor cells.[29]

Table 1 Uptake of Pc 4 as a function of cell type and cell concentration.

	Pc 4 content Mean fluorescence/cell±SEM		Pc 4 content (Mean fluorescence/μm^3)		
	Cells/ml during incubation		Cell volume	Cells/ml during incubation	
Cell type	1.5×10^7	1.5×10^6	(μm^3)	1.5×10^7	1.5×10^6
Early progenitor cells[a]	54±6.9	109±6.5	239	0.23	0.50
Late progenitor cells[b]	68±8.2	136±7.8	310	0.22	0.44
BC3A	80±5.5	137±11.3	678	0.12	0.20
ABE-8.1/2	95±7.6	218±29.0	716	0.13	0.30
L1210	124±4.2	291±14.0	1150	0.11	0.25
P815	152±17.2	414±21.5	1499	0.10	0.28
WEHI-3	57±4.7	296±8.5	1767	0.03	0.17

[a] Early progenitor cells were defined as kit$^+$, Sca-1$^+$ bone marrow cells.

[b] Late progenitor cells were defined as kit$^+$, Sca-1$^-$ bone marrow cells.

3.6 UPTAKE OF Pc 4 IN CELL MIXTURES

BM and cell lines were mixed to simulate clinical samples and Pc 4 uptake was also used to evaluate the differential Pc 4 photoinactivation (Table 2), using the results described in the previous paragraphs. The cellular uptake of Pc 4 by the cell lines and the BM progenitors (c-kit$^+$) was found to increase as the fraction of BM cells in the mixtures was increased from 0.5 to 0.95.

In all mixtures, the Pc 4 uptake of the BM progenitor fractions was lower, though not significantly ($p>0.07$), than when the BM samples were incubated without the cell lines. In contrast, BC3A, L1210, P815 and WEHI-3 cells contained more Pc 4 in the mixtures than when these cells were incubated at 1.5×10^7 cells/mL without BM. The Pc 4 uptake in the ABE-8.1/2 cells in mixtures that contained a BM fraction <0.9 was lower than the uptake of ABE-8.1/2 cells that were incubated without BM. These data indicated that ABE-8.1/2 would be less effectively photoinactivated in mixtures that contained a BM fraction <0.9 and that the other four cell lines would be photoinactivated more effectively in mixtures with BM cells. Whether this would lead to complete photoinactivation of the P815 and WEHI-3 cells could not be predicted from the data in Table 1 as the Pc 4 concentrations in the mixed samples were lower than in the cells after incubation at 1.5×10^6 cells/mL without BM.

4 DISCUSSION

The data presented in this report indicate the preferential photoinactivation of murine cancer cells over early and late hematopoietic progenitors, confirming the data reported for human cells.[23] In ad-

Table 2 Uptake of Pc 4 in mixtures of BM and cell lines. Mixtures of bone marrow and cell line cells were incubated with 15 nM Pc 4 at a cell concentration of 1.5×10^7 cells/ml.

	Pc 4 content (mean fluorescence/cell) in BM/cell line mixtures				
	Fraction of BM in mixtures				
Cell mixture	0.50	0.70	0.80	0.90	0.95
c-kit[a]/BC3A	48/104	53/126	61/138	68/153	64/164
c-kit[a]/ABE-8.1/2	48/74	52/86	54/91	55/95	62/105
c-kit[a]/L1210	35/153	43/189	48/210	54/242	60/269
c-kit[a]/P815	42/205	51/241	56/270	63/312	64/337
c-kit[a]/WEHI-3	30/106	38/140	46/164	58/204	65/226

[a] Bone marrow progenitor cells were identified as c-kit$^+$ cells.

dition, they indicate that the early progenitor cells, which are of the utmost importance for transplantation, can be spared during Pc 4 phototreatment. After incubation with 15 nM Pc 4 at 5×10^6 cells/mL and irradiation with 8 J/cm^2, the surviving fractions of human cells were reported to be 1.0 (CFU-GM), 0.9 (BFU-E), and $<10^{-5}$ (SK-BR3, HL-60), respectively.[23] A direct comparison of the human and murine survival data was not possible due to the different cell concentrations that were used during the incubation with Pc 4. However, from the data in Fig. 1 it could be assumed that the murine survival data for incubations at 5×10^6 cells/mL would be similar to the human data.

Used under conditions (15 nM Pc 4, 1.5×10^7 cells/mL, 18 J/cm^2) that resulted in 3–6 $\log_{10}$ photoinactivation of all malignant cells tested, Pc 4 reduced the surviving fractions of murine late hematopoietic progenitors subsets, CFU-GM and BFU-E, to 0.038 and <0.003, respectively. In comparison, the surviving fraction of human CFU-GM was reduced to 0.44 in AlSPc photoinactivation experiments, that resulted in more than 2 log depletion of K562 cells.[15] Furthermore, P12 photoinactivation reduced the surviving fractions of human CFU-GM and BFU-E to 0.6 and 0.7, while survival of HL60 and U-937 was reduced more than 4 logs.[13] MC-540 and DHE were also shown to be less phototoxic towards human late progenitors: photoinactivation after incubation with MC-540 at 20 μg/ml (Ref. 30) or at 25 μg/ml (Ref. 10) resulted in surviving fractions of 0.18–0.21 and 0.14–0.18, respectively, for the murine CFU-GM and BFU-E, while surviving fractions of HL60,[30] Daudi[30] and Reh cells[10] were reduced more than 5 log. Used at concentrations that yielded more than 4 log photoinactivation of HL60, Reh and SK-DHL-2 cell, DHE phototoxicity to human late progenitors was found to depend on the DHE concentration: CFU-GM and BFU-E surviving fractions were 0.29 and 0.47, respectively, at [DHE]=12.5 μg/ml.[28] Finally, photoinactivation after incubation with benzoporphyrin derivative monoacid ring A (BPD-MA) yielded surviving fractions of human CFU-GM=0.04 and BFU-E=0.05, while survival of HL-60 and SK-DHL-2 cells was reduced more than 4 log.[28] Although the cited studies were performed under different experimental conditions, with cell concentrations varying from 1×10^6/ml to 2×10^7/ml, it can be concluded that Pc 4 was more phototoxic towards late hematopoietic progenitors when used in protocols that resulted in >4 $\log_{10}$ malignant cell death. Another comparison between Pc 4 and other photosensitizers used for BM purging can be made with regard to the concentrations used. With the exception of BPD-MA all other sensitizers were used at ~1000-fold higher concentration than Pc 4.

No adverse effect on the survival of early hematopoietic cells, assayed *in vitro* or *in vivo*, was found after photoirradiation (at 1.5×10^7 cells/mL) with a fluence of 18 J/cm^2, while this resulted in a selective photoinactivation of >6 $\log_{10}$ for ABE-8.1/2, BC3A and L1210, of 5 $\log_{10}$ for WEHI-3 and >3 $\log_{10}$ for P815. Incubation at 1.5×10^6 cells/mL resulted in a photoinactivation of the early progenitor cells (CAFCd28) by approximately 2 $\log_{10}$, compared to about 5 $\log_{10}$ for ABE-8.1/2 and BC3A, 4 $\log_{10}$ for WEHI-3, 3 log for P815, and <1 $\log_{10}$ for L1210. Thus, under the described conditions, incubation at 1.5×10^7 cells/mL appeared superior for selective photoinactivation. The reasons for the enhanced selectivity of Pc 4 for killing cancer cells at higher cell concentrations are not known. The effective light exposure per cell is expected to be reduced as cell concentration increases due to light scatter. This effect, however, should be the same for all cell types in the cell suspension.

The attempts to explain the selectivity of Pc 4 photoinactivation by differential uptake of Pc 4 (Table 1) failed. However (with the exception of WEHI-3), the correlation between cell size and photosensitizer uptake that was reported for hematoporphyrin derivative (HPD)[31] was confirmed. Furthermore, increased uptake resulted in increased photosensitivity for each cell type as was reported for Photofrin.[32] In the latter study, uptake had been found to linearly correlate with cell death when low fluences were used and cell survival ranged from 100% to 1%.

No correlation was found between photosensitivity and Pc 4 content among the tested cell types. This result is in agreement with the results from photoinactivation experiments with P12 (Ref. 33) and Photofrin.[34] In these studies marginal differences in photoinactivation were found between cell types despite significant differences in photosensitizer uptake. When corrected for cell volume, there also was no apparent correlation between intracellular Pc 4 concentration and photosensitivity. Most strikingly, the early progenitor cell fraction was found to be the least photosensitive while these cells contained the highest concentration of Pc 4. This finding was in conflict with the assumption that during low power irradiation, a higher photosensitizer concentration should lead to a higher concentration of triplet state photosensitizer molecules which in turn translates to higher concentrations of reactive oxygen species and subsequently to more photodamage.[35] Taken together, the data indicate that other factors, such as localization of photosensitizer molecules at specific damage-sensitive sites, the capacity to repair photodamage,[36] and the ability to undergo apoptosis determine photosensitivity among different cell types. With regard to the latter, it should be noted that several cancer cell types have a profound apoptotic response shortly after photodynamic treatment with Pc 4.[37,38] If early progenitor cells lack such a response this could explain their resistance.

When mixed with BM, previous studies with AlSPc (Ref. 15) (BM fraction = 0.99) had shown that cell lines were photoinactivated as effectively as unmixed cell lines, while studies with MC540[39] (BM fraction = 0.95–0.99), indicated that cell lines were more effectively inactivated in mixtures. Uptake data from the mixing experiments (Table 2), revealed that Pc 4 uptake by the cell lines was proportional to the fraction of BM cells in the mixtures. Cell line fractions in the mixtures took up more Pc 4 than cell lines incubated at 1.5×10^7 cells/mL; ABE-8.1/2 being the exception. In contrast, Pc 4 uptake by late progenitor cells was inversely proportional to the BM fraction in the mixtures and uptake was equal or lower than when unmixed BM cells were incubated alone. A possible explanation for the effect of mixing is that as the BM fraction is increased the fraction of cancer cells is decreased and more Pc 4 is available per cancer cell. It can, therefore, be concluded that in BM mixtures the cell lines would be more effectively photoinactivated and that progenitor cells would suffer less photodamage.

Comparison of the effect of Pc 4 photosensitization and other photosensitizers on early hematopoietic progenitors cell survival is not straightforward, since, to the best of our knowledge, this is the first report in which the *in vitro* CAFC assay was used to assess stem cell photosensitivity. However, the CAFCd28 results were previously found to correlate with the phenotype of the most immature progenitor cells[25,26] and with the results from long term culture initiating cell (LTC-IC) assays.[25,26] Thus, it can be concluded that Pc 4, when used at a BM density of 1.5×10^7 cells/ml, yielded results equivalent to DHE and BPD-MA.[28] All our studies were done at room temperature. Since cytoprotective mechanisms that are temperature dependent appear to play a role in the outcome of photochemical purging of bone marrow grafts,[36] the temperature control during and after treatment could further enhance the selectivity of the treatment.

In conclusion, Pc 4 was shown to be an effective photosensitizer for the selective photoinactivation of all murine malignant cells tested compared to BM cells, particularly early progenitor cells. Based on these results, further studies involving human bone marrow samples are in progress.

Acknowledgments

This work was supported in part by Award No. 2RO1-HL41221 from the National Heart, Lung and Blood Institute and in part by The Starr Foundation. Thanks are due to Dr. M. E. Kenney for a gift of Pc 4.

REFERENCES

1. S. J. Simpson, M. Vachula, M. J. Kennedy, H. Kaizer, J. S. Coon, R. Ghalie, S. Williams, and D. Van Epps, ''Detection of tumor cells in bone marrow, peripheral blood, and apheresis products of breast cancer patients using flow cytometry,'' *Exp. Hematol.* **23**, 1062–1068 (1995).
2. W. Brugger, K. J. Bross, M. Glatt, F. Weber, R. Mertelsmann, and L. Kanz, ''Mobilization of tumor cells and progenitor cells into peripheral blood of patients with solid tumors,'' *Blood* **83**, 636–640 (1994).
3. D. R. Rill, V. M. Santana, W. M. Roberts, T. Nilson, L. C. Bowman, R. A. Krance, H. A. Heslop, R. C. Moen, J. N. Ihle, and M. K. Brenner, ''Direct demonstration that autologous bone marrow transplantation for solid tumors can return a multiplicity of tumorigenic cells,'' *Blood* **84**, 380–383 (1994).
4. R. J. Berenson, W. I. Bensinger, R. S. Hill, R. G. Andrews, J. Garcia-Lopez, D. F. Kalamasz, B. J. Still, G. Spitzer, C. D. Buckner, I. D. Bernstein, and E. D. Thomas, ''Engraftment after infusion of $CD34^+$ marrow cells in patients with breast cancer or neuroblastoma,'' *Blood* **77**, 1717–1722 (1991).
5. E. A. De Wynter, L. H. Coutinho, X. Pei, J. C. W. Marsh, J. Hows, T. Luft, and N. G. Testa, ''Comparison of purity and enrichment of $CD34^+$ cells from bone marrow, umbilical cord and peripheral blood (primed for apheresis) using five separation methods,'' *Stem Cells* **13**, 524–532 (1995).
6. G. Köhler, W. Brugger, H. E. Schaefer, and L. Kanz, ''Expression of CD34 stem cell antigen on tumor cells,'' *Blood* **86**, 497a (1995).
7. I. C. M. Slaper-Cortenbach, L. C. Admiraal, E. F. Van Leeuwen, J. M. Kerr, A. E. G. K. Von dem Borne, and P. A. T. Tetteroo, ''Effective purging of bone marrow by a combination of immunorosette depletion and complement lysis,'' *Exp. Hematol.* **18**, 49–54 (1990).
8. F. M. Uckun, K. Gajl-Peczalska, D. E. Meyers, N. C. Ramsay, J. H. Kersey, M. Colvin, and D. A. Vallera, ''Marrow purging in autologous bone marrow transplantation for T-lineage acute lymphoblastic leukemia: Efficacy of *ex vivo* treatment with immunotoxins and 4-hydroperoxycyclophosphamide against fresh leukemic marrow progenitor cells,'' *Blood* **69**, 361–366 (1987).
9. J. G. Levy, C. Dowding, D. Mitchell, R. Sorrenti, S. Yip, and C. Jamieson, ''Selective elimination of malignant stem cells using photosensitizers followed by light treatment,'' *Stem Cells* **13**, 336–343 (1995).
10. J. Atzpodien, S. C. Gulati, and B. D. Clarkson, ''Comparison of the cytotoxic effects of merocyanine-540 on leukemic cells and normal human bone marrow,'' *Cancer Res.* **46**, 4892–4895 (1986).
11. F. Sieber, ''Elimination of residual tumor cells from autologous bone marrow grafts by dye-mediated photolysis: Preclinical data,'' *Photochem. Photobiol.* **46**, 71–76 (1987).
12. J. Atzpodien, S. Gulati, A. Strife, and B. D. Clarkson, ''Photoirradiation models for the clinical *ex vivo* treatment of autologous bone marrow grafts,'' *Blood* **70**, 484–489 (1987).
13. E. Fibach, S. Gatt, and E. A. Rachmilewitz, ''Selective photosensitization of human leukemic cells by a pyrene-containing fatty acid,'' *Exp. Hematol.* **18**, 89–93 (1990).
14. E. Fibach, A. Rabia, and S. Gatt, ''Pyrene-butanol—An efficient, selective and non-metabolized photosensitizing agent for human myeloid leukemia cells,'' *Leukemia Res.* **16**, 453–462 (1992).
15. C. R. J. Singer, D. C. Linch, S. G. Bown, E. R. Huehns, and A. H. Goldstone, ''Differential phthalocyanine photosensitization of acute myeloblastic leukaemia progenitor cells: A potential purging technique for autologous bone marrow transplantation,'' *Br. J. Haematol.* **68**, 417–422 (1988).
16. E. Ben-Hur, ''Basic photobiology and mechanisms of action of phthalocyanines,'' in *Photodynamic Therapy: Basic Principles and Clinical Application*, T. J. Dougherty and B. W. Henderson, Eds., pp. 63–77, Marcel Dekker, New York (1992).
17. N. L. Oleinick, A. R. Antunez, M. E. Clay, B. D. Rihter, and M. E. Kenney, ''New phthalocyanine photosensitizers for photodynamic therapy,'' *Photochem. Photobiol.* **57**, 242–247 (1993).
18. S. I. A. Zaidi, R. Agarwal, G. Eichler, B. D. Rihter, M. E. Kenney, and H. Mukhtar, ''Photodynamic effects of new silicon phthalocyanines: *In vitro* studies utilizing rat hepatic microsomes and human erythrocyte ghosts as model membrane sources,'' *Photochem. Photobiol.* **58**, 204–210 (1993).
19. E. Ben-Hur, M. M. Zuk, S. Chin, D. Banerjee, M. E. Kenney, and B. Horowitz, ''Biodistribution and virus inactivation ef-

ficacy of a silicon phthalocyanine in red blood cell concentrates as a function of delivery vehicle,'' *Photochem. Photobiol.* **62**, 575–579 (1995).
20. E. Ben-Hur, J. Oetjen, and B. Horowitz, ''Silicon phthalocyanine Pc 4 and red light causes apoptosis in HIV-infected cells,'' *Photochem. Photobiol.* **65**, 456–460 (1997).
21. P. Gottlieb, L. G. Shen, E. Chimezie, S. Bahng, M. E. Kenney, B. Horowitz, and E. Ben-Hur, ''Inactivation of *Trypanosoma cruzi* trypomastigote forms in blood components by photodynamic treatment with phthalocyanines,'' *Photochem. Photobiol.* **62**, 869–874 (1996).
22. S. Lustigman and E. Ben-Hur, ''Photosensitized inactivation of *Plasmodium falciparum* in human erythrocytes by phthalocyaines,'' *Transfusion* **36**, 543–546 (1996).
23. R. A. Pretti, M. Fitzgerald, J. F. Keij, J. W. M. Visser, and E. Ben-Hur, ''Photolytic purging of SK-BR3 and HL-60 tumor cells with the phthalocyanine, Pc 4,'' *J. Hematotherapy* **4**, 246 (1995).
24. G. Migliaccio and A. R. Migliaccio, ''Cloning of erythroid progenitors (BFU-E) in the absence of fetal bovine serum,'' *Br. J. Haematol.* **67**, 129–133 (1987).
25. D. A. Breems, E. A. W. Blokland, S. Neben, and R. E. Ploemacher, ''Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay,'' *Leukemia* **8**, 1095–1104 (1994).
26. J. C. M. Van der Loo and R. E. Ploemacher, ''Marrow- and spleen-seeding efficiencies of all murine hematopoietic stem cell subsets are decreased by pre-incubation with hematopoietic growth factors,'' *Blood* **85**, 2598–2606 (1995).
27. C. L. Li and G. R. Johnson, ''Murine hematopoietic stem and progenitor cells: I. Enrichment and biological characterization,'' *Blood* **85**, 1472–1479 (1995).
28. R. M. Lemoli, T. Igarashi, M. Knizewski, L. Acaba, A. Richter, A. Jain, D. Mitchell, J. Levy, and S. C. Gulati, ''Dye-mediated photolysis is capable of eliminating drug-resistant (MDR^+) tumor cells,'' *Blood* **81**, 793–800 (1993).
29. J. M. Zijlmans, J. W. M. Visser, K. Kleiverda, P. M. Kluin, R. Willemze, and W. E. Fibbe, ''Modification of rhodamine staining allows identification of hematopoietic stem cells with preferential short-term of long-term bone marrow-repopulating ability,'' *Proc. Natl. Acad. Sci. USA* **92**, 8901–8905 (1995).
30. K. S. Gulliya and S. Pervaiz, ''Elimination of clonogenic tumor cells from HL-60, Daudi, and U-937 cell lines by laser photoirradiation therapy: Implications for autologous bone marrow purging,'' *Blood* **73**, 1059–1065 (1989).
31. R. M. Böhmer and G. Morstyn, ''Uptake of HPD by normal and malignant cells: Effects of serum, *p*H, temperature and cell size,'' *Cancer Res.* **45**, 5328–5334 (1985).
32. R. D. Williams and T. C.Runge, ''Photodynamic therapy of human bladder cancers *in-vitro* correlated with cellular fluorescence levels of photofrin,'' *Photochem. Photobiol.* **46**, 733–737 (1987).
33. E. Fibach, E. A. Rachmilewitz, and S. Gatt, ''Analysis of cellular heterogeneity in the response of human leukemic cells to photosensitization induced by pyrene-containing fatty acid,'' *Leukemia. Res.* **13**, 1009–1017 (1989).
34. J. Blais, C. Amirand, J. P. Ballini, P. Debey, M. T. Foultier, and T. Patrice, ''Photofrin-induced fluorescence in progressive and regressive murine colonic cancer cells: Correlation with cell photosensitivity,'' *J. Photochem. Photobiol. B: Biol.* **27**, 225–231 (1995).
35. J. F. Keij, J. T. H. Jansen, F. W. Schulz, and J. W. M. Visser, ''Reduced photoinactivation of 10-dodecyl acridine orange-sensitized yeast cells at high fluences rates: Measurements and computer simulations,'' *Photochem. Photobiol.* **60**, 503–509 (1994).
36. T. Yamazaki, Y. Sato, and F. Sieber, ''Role of cytoprotective mechanisms in the phototchemical purging of autologous bone marrow,'' *Exp. Hemotol.* **25**, 629–637 (1997).
37. S. I. A. Zaidi, N. L. Oleinick, M. T. Zaim, and H. Mukhtar, ''Apoptosis during photodynamic therapy-induced ablation of RIF-1 tumors in C3H mice: Electron microscopic, histopathologic, and biochemical evidence,'' *Photochem. Photobiol.* **58**, 771–776 (1993).
38. R. Agarwal, N. J. Kormam, R. R. Mohan, D. K. Feyes, S. Jawed, M. T. Zaim, and H. Mukhtar, ''Apoptosis is an early event during phthalocyanine photodynamic therapy-induced ablation of chemically induced squamous papillomas in mouse skin,'' *Photochem. Photobiol.* **63**, 547–552 (1996).
39. R. C. Meagher, S. A. Rothman, P. Paul, P. Koberna, C. Willmer, and P. A. Baucco, ''Purging of small cell lung cancer cells from human bone marrow using ethiofos (WR-2721) and light-activated merocyanine 540 phototreatment,'' *Cancer Res.* **49**, 3637–3641 (1989).