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Potential of the Anti-Tumor Effect of Merocyanine 540-Mediated Photodynamic Therapy by Amifostine and Amphotericin B

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Abstract

Leukemia and lymphoma cells are much more sensitive to Merocyanine 540 (MC540)-mediated photodynamic therapy (PDT) than normal pluripotent hematopoietic stem cells and normal granulocyte/macrophage progenitors (CFU-GM). By contrast, most solid tumor cells are only moderately sensitive to MC540-PDT. The limited activity against solid tumor cells has detracted from MC540's appeal as a broad-spectrum purging agent. We report here that non-cytotoxic concentrations of amifostine (Ethyol, Ethiofos, WR-2721) and amphotericin B used either alone or in combination potentiate the MC540-sensitized photoinactivation of leukemia cells, wildtype small cell lung cancer cells, and cisplatin-resistant small cell lung cancer cells. Amphotericin B also enhances the MC540-sensitized photoinactivation of normal CFU-GM whereas amifostine protects CFU-GM against the cytotoxic action of MC540-PDT. The yield of CD34-positive normal hematopoietic stem and progenitor cells is only minimally diminished by pretreatment with amifostine, amphotericin B, or combinations of amifostine plus amphotericin B. Purging protocols that combine MC540-PDT with amifostine or with amifostine plus amphotericin B may offer a simple and effective approach to the purging of autologous stem cell grafts that are contaminated with solid tumor cells or the purging of stem cell grafts from heavily pretreated leukemia patients that contain reduced numbers of normal stem and progenitor cells and, therefore, can ill afford additional losses caused by purging.

INTRODUCTION

The photosensitizing dye, Merocyanine 540 (MC540), has been used in preclinical models and in phase I/II clinical trials for the extracorporeal purging of autologous stem cell grafts (1-10). Combinations of dye and light that reduce the concentration of leukemia and lymphoma cells by 5 to 8 orders of magnitude typically preserve about 50% of normal human pluripotent hematopoietic progenitor cells (CFU-mix) and normal human granulocyte/macrophage progenitors (hCFU-GM) (1-10). In bone marrow cells from most mouse strains, the same combinations of dye and light deplete granulocyte/macrophage progenitors (CFU-GM) and the radioprotective capacity of bone marrow about 10-fold (2,8,9).

In the first phase I/II clinical trial of the MC540-mediated purging, most evaluable patients engrafted late, requiring between 23 and 67 days to achieve absolute neutrophil counts of 500 (8,9). For lack of a control arm, it was not possible to determine to which extent engraftment delays reflected poor marrow quality and to which extent they were attributable to purging related losses of stem and progenitor cells. Since most grafts were harvested from heavily

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pretreated patients, poor marrow quality undoubtedly was a factor. Based on our preclinical data, it also is reasonable to assume that purging related depletions of already marginal numbers of stem and progenitor cells contributed to engraftment delays. Any measure that reduces purging-related losses of normal stem and progenitor cells while maintaining adequate depletions of tumor cells would thus be of considerable practical interest, especially as it pertains to the purging of stem cell grafts from heavily pretreated patients.

In marked contrast to leukemia and lymphoma cells, most solid tumor cells are only moderately sensitive to MC540-mediated photodynamic therapy (PDT) (2,3,8,9). This limited activity against solid tumor cells has detracted from MC540's appeal as a broad-spectrum purging agent.

We report here on attempts to improve the therapeutic index of MC540-mediated purging by combining MC540-PDT with the radioprotective agent, amifostine (Ethyol, WR-2721) and/or the polyene antifungal antibiotic, amphotericin B. The rationale for combining MC540-PDT with these two drugs was three-fold. 1) Both drugs are fast acting. Therefore, their inclusion in a purging procedure was unlikely to prolong processing times for stem cell grafts beyond acceptable limits. 2) Both drugs have been in clinical use several years (albeit for different purposes), and we have by now a good understanding of the risks associated with their use. 3) Based on the previous experience with amifostine and amphotericin B used in conjunction with cytotoxic drugs, we saw a realistic chance that amifostine would reduce MC540-mediated damage to normal hematopoietic stem and progenitor cells while amphotericin B would enhance toxicity to tumor cells.

MATERIALS AND METHODS

Reagents. Merocyanine 540 (MC540) was from Kodak (Rochester, NY), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) hemisodium salt (HEPES) from Research Organics (Cleveland, OH), methylcellulose (4000 cPs) from Fluka (Buchs, Switzerland), monochlorobimane from Molecular Probes (Eugene, OR), and ethanol (spectroscopic grade) from Aldrich (Milwaukee, WI). Fluorescently labeled annexin V (FITC-annexin V) and recombinant granulocyte/macrophage colony stimulating factor (GM-CSF; murine or human sequence) were from R&D Systems (Minneapolis, MN). FITC-labeled anti human CD34 antibody and isotype control antibody were from BD Biosciences Pharmingen (San Diego, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cells. Female C57BL/6J x DBA/2J mice (10-25 weeks old) from Jackson Laboratories (Bar Harbor, ME) served as a source of normal murine bone marrow cells. Normal human bone marrow cells (mononuclear cell fraction) were obtained from Poietic/BioWhittaker (Gaithersburg, MD) or from healthy volunteer donors for allogeneic bone marrow transplants after informed consent had been obtained under protocols approved by the institutional review board.

L1210 murine leukemia cells (CCL 219) were obtained from the American Type Culture Collection (Manassas, VA). Wild-type H69 and cisplatin-resistant H69/CDDP human small cell lung cancer cells were gifts from Dr. N. Saijo (11). All tumor cells were propagated in alphamodified Dulbecco's medium (alpha-medium) supplemented with 10% fetal bovine serum and were harvested in exponential growth phase. Aliquots of a frozen stock were thawed at regular intervals to prevent the inadvertent selection of mutants.

Dye-sensitized reactions. The MC540-sensitized photoirradiation of tumor and normal bone marrow cells was performed as described previously (1-3). In brief, cells were suspended at a density of 1×10^6 cells/mL in HEPES-buffered (10 mM, pH 7.4) alpha-medium supplemented with fetal bovine serum (12%) and subsequently distributed as 2 mL-aliquots into clear 15 mL-

polybutadiene styrene tubes (Nunc, Naperville, IL). The large air space above the cell suspension insured an adequate supply of oxygen during photosensitization experiments. MC540 was added from a freshly prepared 1 mg/mL stock solution in 50% ethanol to a final concentration of 15 μ g/mL. The tubes were mounted on a Plexiglas disk that rotated at approximately 30 rpm between two banks of tubular white fluorescent lights (5 lights per bank; F20T12.CW; General Electric, Cleveland, OH). The fluence rate at the sample site was 27 W/m² as determined by a S351A power meter (United Detector Technology, Hawthorne, CA) equipped with a model 262 detector and a radiometric filter number 1158. Cell suspensions were irradiated for up to 90 min. The reaction was terminated by transferring the cells to the dark and by washing them twice with dye-free HEPES-buffered alpha-medium supplemented with 5% fetal bovine serum (wash medium). Cells that were exposed to dye in the dark served as controls. In selected experiments, two additional controls were included; 1) cells that were exposed to light but no dye, and 2) cells that were exposed to no dye and no light. All three controls produced very similar results.

Treatment with amifostine and amphotericin B. To pretreat cells with amifostine, cells were suspended at 10⁶/mL in HEPES-buffered alpha-medium supplemented with 12% fetal bovine serum. Amifostine was added from a 10-fold concentrated stock solution in alpha-medium (supplemented with 12% fetal bovine serum) to a final concentration of 3 mg/mL (12,13). Cells were incubated at 37 °C for 30 min, washed twice with wash medium, and then processed without delay for MC540-PDT.

Where indicated, amphotericin B (1 mg/mL stock solution) was added to the cell suspensions to a final concentration of 30 μ g/mL immediately before the addition of MC540. At the end of the photoirradiation period, excess amphotericin B and MC540 were removed by washing the cells twice with wash medium.

In vitro clonal assays. Four hundred untreated or up to 80,000 treated tumor cells (cell concentrations adjusted to compensate for the lower plating efficiency of treated cells) were plated in 35 mm Petri dishes (Greiner, Nürtingen, Germany) containing 1 mL alpha-medium supplemented with 0.8% methylcellulose and 32% (H69 and H69/CDDP cells) or 20% (L1210 cells) fetal bovine serum. After incubating clonal cultures for 7 (L1210 cells) or 10 (H69 and H69/CDDP cells) days at 37 °C in a humidified atmosphere of 5% CO₂ in air, colonies consisting of $\geq$ 30 cells were scored *in situ* with the aid of an inverted microscope (1-3).

For the assay of CFU-GM, between 40,000 and 200,000 bone marrow cells (cell concentrations adjusted to compensate for the lower plating efficiency of treated cells) were plated in 35 mm Petri dishes containing 1 ml of alpha-medium supplemented methylcellulose (0.8%), fetal bovine serum (15%), and a saturating concentration of human (1 ng/mL) or murine (3 ng/mL) GM-CSF. Cultures were incubated for 7 (murine cells) or 14 (human cells) days at 37 °C in a humidified atmosphere of 5% CO₂ in air. Colonies consisting of $\geq$ 30 cells were scored *in situ* with the aid of an inverted microscope (14,15).

Assay of CD34-positive cells. Treated and untreated human bone marrow mononuclear cells were stained with FITC anti human CD34 antibody as recommended by the manufacturer. Cells stained with the FITC-conjugated isotype control antibody served as controls. CD34-positive cells were subsequently identified with a flow cytometer.

Flow cytometric analyses. Flow cytometric analyses were performed as described previously using a FACScan (Becton-Dickinson, Mountainview, CA) flow cytometer equipped with an argon-ion laser (15 mW, 488 nm line) (16). Fluorescence emission was measured through a 525/30 nm (FITC-annexin V, FITC-antibodies) or a 573/34 nm (MC540) band pass filter and expressed in arbitrary units (channel number) on a logarithmic scale. Data were analyzed using the CellQuest (Becton-Dickinson) software package.

Dye binding measurements. The binding of MC540 to cells is known to be light dependent (16-20). Cells that are incubated with MC540 in the dark bind only small amounts of dye, and the amounts of dye bound are generally quite similar regardless of the cells' sensitivity to MC540-PDT. However, when cells are simultaneously exposed to MC540 and light, the amounts of dye bound by highly sensitive cells (e.g. leukemia cells) increase sharply as a function of light exposure. By contrast, cells that are only moderately sensitive or resistant to MC540-PDT (e.g. lung cancer cells) show little or no increase in dye binding. Some MC540-resistant cells may even show an apparent decrease in dye binding due to photobleaching. For these reasons, comparative dye binding experiments are performed preferably with cells that have been exposed to moderate doses of light. For the present study, cells were exposed to fluences of 0, 48.6 and 97.2 kJ/m². Higher light doses were avoided because the binding of dye to intracellular structures of severely photodamaged cells would have made the interpretation of results very difficult. No attempt was made to correct data for fluorescence losses due to photobleaching.

Two methods were used to quantify cell-bound MC540. Dye binding to intact cells was quantified by flow cytometry as described previously (16). Alternatively, stained cells were pelleted by low-speed centrifugation, washed once with wash medium and twice with cold phosphate-buffered saline and then extracted with a small volume (1 mL per 10⁷ cells) of 2% Triton X-100. Cell debris were pelleted by centrifugation, and the dye content of the clear supernatant was determined with a fluorescence spectrophotometer using appropriate dilutions of authentic dye in 2% Triton X-100 as standards.

Annexin V binding experiments. To detect phosphatidylserine on the surface of intact cells, we used the Apoptosis Detection Kit of R&D Systems according to the manufacturer's instructions. The binding of fluorescein-conjugated annexin V was quantified by flow cytometer (16).

Quantitation of free dye. To distinguish between free dye and dye bound to serum macromolecules, dye solutions were filtered through Centricon YM-30 ultrafiltration devices (Millipore, Bedford, MA) with a molecular weight cut-off of 30,000 daltons (21). Dye concentrations in original solutions and the low molecular weight filtrates were determined spectrofluorometrically. To avoid artifacts due to self-quenching or to solvent-dependent differences in fluorescence quantum yields, all samples were diluted 10- to 1000-fold in 2% Triton X-100 prior to spectrophotometric analysis.

Assay of intracellular glutathione. The concentration of intracellular glutathione (GSH) was determined with the thiol specific probe, monochlorobimane (22). Cells were suspended at a density of 10⁶/mL in wash medium, and monochlorobimane was added from a 5 mg/mL stock solution in ethanol to a final concentration of 0.1 mg/mL. Cells were incubated for 15 min at 37 °C in the dark, pelleted, and extracted with a 1:99 mixture of acetic acid and ethanol. The concentration of GSH-monochlorobimane adducts in the solvent extract was measured with a fluorescence spectrophotometer with excitation at 395 nm and emission at 460 nm. To determine the effects of amifostine and amphotericin B on the GSH levels of tumor cells, tumor cells were treated as described above except that MC540-PDT was omitted. That is, cells treated with amifostine only were washed free of excess drug after the 30 min incubation period and then stored at room temperature for 60 min prior to being processed for the GSH assay. Cells treated with amphotericin B were incubated for 60 min at room temperature, washed free of excess drug, and then processed without delay for the GSH assay.

Spectroscopy. Absorbance spectra were recorded on a Perkin-Elmer (Norwalk, CT) Lambda 4C UV-VIS or a Hitachi (Tokyo, Japan) U-3310 spectrophotometer. Corrected fluorescence emission spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer.

RESULTS

Effects of amifostine and amphotericin B on the MC540-sensitized photoinactivation of cells

Pretreating bone marrow cells with amifostine protected normal granulocyte/macrophage progenitors against the cytotoxic action of MC540-PDT (Fig. 1 and 2). The protective effect of amifostine was more pronounced in murine (mCFU-GM; Fig. 2) than in human (hCFU-GM; Fig. 1) progenitor cells because murine progenitor cells were more sensitive to MC540-PDT under the chosen experimental conditions. Amifostine also caused a modest (19% in human, 17% in murine bone marrow) increase in granulocyte/macrophage colony formation in cultures of bone marrow cells that were not subjected to MC540-PDT. In Figs. 1c and 2b the small increases in granulocyte/macrophage colony formation by amifostine treated but not photosensitized (closed circles, 0 kJ/m²) bone marrow cells results are barely visible because data are plotted on a logarithmic scale.

The same concentration of amifostine that protected normal granulocyte/macrophage progenitors against the cytotoxic effects of MC540-PDT potentiated the MC540-sensitized photoinactivation of L1210 murine leukemia cells, wild-type H69 human small cell lung cancer cells, and cisplatin-resistant H69/CDDP human small cell lung cancer cells (Fig. 1 and 2). Performing MC540-PDT in the presence of a non-toxic concentration (30 µg/mL) of amphotericin B also enhanced the MC540-sensitized photoinactivation of tumor cells (Fig. 1 and 2). Unlike amifostine, amphotericin B also enhanced the depletion of normal granulocyte/macrophage progenitors, albeit to a lesser degree than the depletion of tumor cells (Fig. 1 and 2).

The triple combination, MC540-PDT plus amifostine plus amphotericin B, depleted more tumor cells than any of the two-agent combinations (MC540-PDT plus amifostine or MC540-PDT plus amphotericin B). An added advantage of the triple combination was that it depleted fewer normal granulocyte/macrophage progenitors than the two-agent combination, MC540-PDT plus amphotericin B (Fig. 1 and 2).

Drug-resistant mutant H69/CDDP cells were less sensitive to MC540-PDT than wildtype H69 cells (Fig. 1). This was expected because the drug-resistance of H69/CDDP cells is based on elevated levels of intracellular glutathione (GSH), glutathione-S-transferase- π , and metallothionein (11). High levels of intracellular GSH are known to protect cells against the cytotoxic action of MC540-PDT (23). However, with regard to their response to amifostine and amphotericin B there was little difference between wild-type H69 and mutant H69/CDDP cells; indicating that purging protocols that combine MC540-PDT with amifostine and amphotericin B are effective against at least some multidrug-resistant tumors.

Since CFU-GM are committed progenitors that cannot insure long-term engraftment, we also assessed the effect of combination purging on the yield of normal human CD34-positive stem and progenitor cells. As Table 1 shows, both 2-agent combinations and the triple combination were well tolerated by normal CD34-positive bone marrow cells. Purging related losses amounted to only 15% and 16%, respectively.

Effects of amifostine and amphotericin B on intracellular glutathione levels and dye binding

There are two known determinants of a cell's sensitivity to MC540-PDT. One is the cell's capacity to bind dye molecules (16-20). The second one is the cell's capacity to defend itself against MC540-mediated oxidative damage (19,23,24). Cells that bind large quantities of dye are more likely to be photoinactivated than cells that bind less dye under identical experimental conditions. Cells with low levels of intracellular glutathione (GSH; a key component of a cell's defense against oxidative damage) tend to be more susceptible to MC540-PDT than cells with high levels of intracellular GSH (23).

To determine if the protective and sensitizing effects of amifostine and amphotericin B were mediated by GSH, intracellular GSH levels were determined in treated and untreated tumor cells. As Table 2 shows, amifostine had no effect on intracellular GSH levels. Tumor cells that were pretreated with amphotericin B or amifostine plus amphotericin B showed moderate reductions of intracellular GSH. Since amphotericin B is a colored (yellow) compound, we were concerned about a potential interference between amphotericin B and the monochlorobimane-based GSH assay. As a control, we therefore mixed a constant concentration of GSH (2 mM) with graded concentrations of amphotericin B (0, 5, 10, or 30 $\mu\text{g/mL}$), incubated the mixture for 30 min at room temperature, and then determined GSH levels by the monochlorobimane-based assay. In the absence of interference, all samples should have given identical results. However, the spectrophotometric analysis showed that in samples containing amphotericin B, fluorescence intensities were reduced 10%, 13%, and 57%, respectively. This suggested that the results shown in Table 2 might possibly overestimate the effect of amphotericin B on intracellular GSH levels. Since the concentration of amphotericin B in extracts of amphotericin B-treated was not known, it was not possible to use the results of the simple cell-free system to calculate a correction factor.

We considered using the o-phthalaldehyde-based assay by Hissin and Hilf (25) instead of the monochlorobimane-based assay to determine GSH levels in amphotericin B-treated cells. However, pilot experiments indicated that the o-phthalaldehyde-based assay was as susceptible if not more susceptible to interference by amphotericin B.

The effects of amifostine and amphotericin B on the intracellular GSH levels of normal hematopoietic stem and progenitor cells were not determined as techniques for the large-scale isolation of pure populations of stem and progenitor cells are not yet available.

Flow cytometric analyses of MC540-stained H69 cells showed that cells treated with amphotericin B or with amphotericin B plus amifostine bound more dye than untreated cells or cells that had been treated with amifostine only (Fig. 3a). The same patterns were also observed in H69/CDDP and L1210 cells (data not shown). Sodium deoxycholate, which is commonly added to commercial preparations of amphotericin B to improve solubility in water, had no effect on dye binding.

The fluorescence quantum yield of MC540 is known to be sensitive to the environment (26-29). Therefore, we had to consider the possibility that flow cytometric dye binding data reflected changes in fluorescence quantum yield rather than genuine differences in dye binding. Dye measurements performed on detergent extracts of MC540-stained cells avoid solvent-related artifacts because all samples are in essentially the same environment. Fluorescence spectrophotometric dye measurements performed on Triton X-100 extracts of MC540-stained cells confirmed that cells treated with amphotericin B or with amphotericin B plus amifostine bound more dye than untreated cells or cells that had been treated with amifostine only. Fig. 3b shows data obtained with H69 cells. Similar results were also obtained with H69/CDDP and L1210 cells.

There are several mechanisms by which amphotericin B could potentially enhance the binding of MC540 to tumor cells. 1) Amphotericin B may displace MC540 from serum macromolecules, thereby enhancing the number of free dye molecules available for binding to cells. 2) MC540 may form complexes with amphotericin B that bind to plasma membrane domains normally accessible only to amphotericin B but not to MC540. 3) Amphotericin B may induce a loss of transmembrane asymmetry, thereby enhancing the number of dye binding sites on the cell surface. 4) Amphotericin B may make the plasma membrane permeable to MC540, thereby making intracellular dye binding sites accessible to the dye. 5) Amphotericin

B may enhance dye binding by altering the transmembrane potential of cells. Three of the five hypotheses were tested in the context of this study.

Displacement from protein carrier

MC540 is known to bind to serum albumin and, to a lesser extent, serum lipoproteins (17,18, 30). Under conditions commonly used for stem cell purging, only a small fraction ($\leq 5\%$) of the dye is present as free dye. Salicylate and related drugs have been shown to displace MC540 from albumin, thereby enhancing the concentration of free dye molecules that are available for binding to cells (21). Amphotericin B is also known to bind to serum macromolecules (31-33), but it is not known if amphotericin B and MC540 compete for the same binding sites. To assess the capacity of amphotericin B to displace MC540 from albumin, we dissolved MC540 (15 $\mu\text{g/mL}$) in HEPES-buffer (10 mM, pH 7.4) supplemented with bovine serum albumin (1.75 mg/mL) and mixed it with graded concentrations (0, 30, and 60 $\mu\text{g/mL}$) of amphotericin B. The mixtures were then loaded onto Centrifree ultrafiltration devices with a 30 kDa molecular weight cut-off, and dye concentrations in the low molecular weight filtrates (free dye) were determined by fluorescence spectroscopy. All samples were diluted with 2% Triton X-100 prior to spectroscopic analysis to rule out artifacts due to self-quenching or solvent-dependent differences in fluorescence quantum yield. In control samples that contained no amphotericin B, $2.58 \pm 0.02\%$ of the total amount of dye was recovered in the low-molecular weight filtrate. In samples containing a low (30 $\mu\text{g/mL}$) or a high (60 $\mu\text{g/mL}$) concentration of amphotericin B, $2.53 \pm 0.11\%$ and $2.83 \pm 0.01\%$, respectively was recovered in the low-molecular weight filtrates (data presented as means of 3-4 determinations $\pm$ standard errors). The presence of amphotericin B thus had no significant effect on the concentration of free dye, indicating that amphotericin B did not displace MC540 from serum albumin.

Complex formation

There is at least one precedent for complex formation between MC540 and an antibiotic. As Lavie and Sonenberg (34) have shown, the ionophore antibiotic, valinomycin forms complexes with MC540 as evidenced by a blue-shift of the fluorescence emission spectrum of MC540 and an approximately 40% reduction of the fluorescence emission peak of MC540. The presence of valinomycin also enhances the binding of MC540 to cell membranes, most likely as the result of an efficient incorporation of MC540-valinomycin complexes into lipid bilayers. To determine if a similar interaction occurred between MC540 and amphotericin B, solutions of MC540 (15 $\mu\text{g/mL}$) were mixed with graded concentrations (0, 30 or $\mu\text{g/mL}$) of amphotericin B and subsequently analyzed with a fluorescence spectrophotometer. No red-or blue-shifts of the spectrum were noted, and neither the shape nor the peak height of the fluorescence emission spectrum was changed by the addition of amphotericin B. Peak heights of fluorescence emission spectra, for example, were 2419 (arbitrary units) for control samples, and 2298, and 2397, respectively for samples containing a low (30 $\mu\text{g/mL}$) or a high (60 $\mu\text{g/mL}$) concentration of amphotericin B. This argued against a complex formation between MC540 and amphotericin B.

Loss of plasma membrane asymmetry

Several studies have shown a good correlation between loss of plasma membrane asymmetry (as indicated by an increased surface exposure of phosphatidylserine) and the binding of MC540 or annexin V to the outer leaflet of the plasma membrane (35-37). Since amphotericin B can enhance the transbilayer mobility of phospholipids (38), we had to consider the possibility that the enhanced binding of MC540 to amphotericin B-treated tumor cells was the result of a loss of plasma membrane asymmetry (increased surface exposure of phosphatidylserine). Aliquots of amphotericin B-treated (30 $\mu\text{g/mL}$) and untreated L1210 leukemia and H69 small cell lung cancer cells were therefore probed with either MC540 or

FITC-annexin V. While amphotericin B-treated cells consistently bound more MC540 than untreated cells, both treated and untreated cells bound very similar amounts of FITC-annexin V (Table 3). This suggested that the enhanced binding of MC540 by amphotericin B-treated cells was not the result of an amphotericin B-induced loss of plasma membrane asymmetry.

DISCUSSION

The main finding of this study is that amifostine or amifostine combined with amphotericin B potentiates the MC540-sensitized photoinactivation of leukemia and small cell lung cancer cells while reducing or only minimally enhancing damage to normal granulocyte/macrophage progenitors (CFU-GM) and normal CD34-positive hematopoietic stem and progenitor cells. Combining MC540-PDT with amifostine or with amifostine plus amphotericin B may thus prove useful for the purging of autologous stem cell grafts from heavily pretreated patients that contain reduced numbers of normal stem and progenitor cells and can ill afford additional purging related depletions. The same combinations of MC540-PDT and amifostine or MC540-PDT and amifostine plus amphotericin B also show promise for the purging of stem cell grafts that are contaminated with solid tumor cells, including multidrug-resistant tumor cells that are only marginally sensitive to MC540-PDT. Both amifostine and amphotericin are approved drugs and freely available. Since both drugs are fast acting, their inclusion in a PDT-based purging regimen does not significantly prolong processing times. In our preclinical model, pretreatment with amifostine increased overall graft processing times by less than one hour. The inclusion of amphotericin B did not affect processing times because the drug was applied during rather than before or after PDT.

Amphotericin B is a polyene antifungal antibiotic from *Streptomyces* that has been used clinically for several decades (39). It binds to ergosterols of fungal cell membranes and - with lower affinity - to sterols of mammalian plasma membranes (40-42). Amphotericin B forms discrete pores that make membranes permeable to ions and small molecules such as doxorubicin, cisplatin, and carboplatin (43-49). Amphotericin B has been used successfully to overcome drug-resistance in preclinical models of cancer (43-49). The results of clinical trials, however, have been disappointing with amphotericin B failing to reverse drug resistance in the majority of cases (50).

In our study, amphotericin B-treated tumor cells bound more MC540 than untreated cells. This offers a plausible (if not necessarily complete) explanation for the accelerated photoinactivation of amphotericin B-treated cells. Doxorubicin and MC540 have similar molecular weights, 527.5 and 569.7, respectively. It is thus conceivable that amphotericin B increased the binding of MC540 to target cells by allowing MC540 access to intracellular binding sites. Such a mechanism would explain why pretreatment with amphotericin B enhanced binding of MC540 but not annexin V. While both MC540 and annexin V bind to phosphatidylserine, only MC540 would have been small enough to pass through transmembrane pores formed by amphotericin B. The 36 kDa protein, annexin V, would have remained restricted to cell surface binding sites.

An alternative explanation for the effect of amphotericin B on dye binding is that amphotericin B interferes with the cation homeostasis of cells which in turn affects the transmembrane potential, a known regulator of dye binding (51-53).

In comparison to most other antibiotics amphotericin B is a very toxic drug. Most patients on systemic amphotericin B therapy experience significant adverse effects such as acute infusion reactions or nephrotoxicity. When used in the context of extracorporeal purging, the toxic side effects of amphotericin B would not be a major concern as excess antibiotic would be washed out before the graft is reinfused.

Amifostine (WR-2721, Ethiofos, Ethyol) was originally developed as a radioprotective agent. It has received regulatory approval for use in reducing cumulative renal toxicity in patients treated with cisplatin for advanced ovarian cancer or non-small-cell lung cancer, and for use in reducing the incidence of xerostomia in patients undergoing post-operative radiation treatment for head and neck cancer (54-56). On an experimental basis, amifostine has also been used in the treatment of myelodysplastic syndrome and a wide range of cancers (57), including the ex vivo purging of autologous hematopoietic stem cell grafts (12,13,58-60). The protective effect of amifostine is remarkably selective. That is, amifostine protects normal tissue but not tumor tissue against the cytotoxic effects of radiation and chemotherapy. In some situations, amifostine not only fails to protect tumor tissue but also actually potentiates the anti-tumor effect of chemotherapeutic agents (12,13,59). In our purging model, amifostine consistently showed such a dual effect. It protected normal CFU-GM against the cytotoxic action of MC540-PDT but potentiated the cytotoxic effect against all three tumor cell lines. Of the three other laboratories that have evaluated amifostine in the context of MC540-based purging, two (including the one that had collaborated with us and had used PDT equipment that was identical to ours; 59, 60) have reported similar dual effect (protection of normal stem and progenitor cells and potentiation of the anti-tumor effect) although they used different tumor cells and somewhat different experimental conditions (59-62). The one laboratory that failed to observe a dual effect found a potentiation of the photoinactivation of small cell lung cancer cells but no protection of normal hematopoietic progenitor cells (12). It is not clear if the discrepant finding was caused by the use of different cell lines or different experimental conditions. It is worth noting in this context that we might have dismissed the protective effect of amifostine on hCFU-GM as insignificant if we had limited our studies to human progenitor cells or if we had not used amifostine in combination with amphotericin B (Fig. 1c). On the other hand, the protective effect of amifostine on human progenitors most likely would have been more if PDT conditions had been modified to inflict more damage on normal progenitors.

Amifostine is a prodrug that is dephosphorylated by alkaline phosphatases to the pharmacologically active free thiol metabolite (WR-1065) (54,56,62). The cytoprotective effect of WR-1065 has been attributed to the thiol's ability to scavenge free radicals, participate in chemical repair through the donation of hydrogen atoms, induce cellular hypoxia as a result of auto-oxidation, induce glutathione biosynthesis, catalytically inactivate topoisomerase II, and inhibit apoptosis by the activation of NF- κ B/Rel transcription factors (54,56,62-65).

The selectivity of amifostine's protective effect has been explained by low alkaline phosphatase levels, low pH, and poor vascularity of tumor tissue as well as a selective exclusion of amifostine derivatives by the polyamine transporter (66,67). In a purging situation where both normal cells and tumor cells are present as single cell suspensions, differential drug or metabolite uptake offers the most plausible explanation for a selective protection of normal hematopoietic stem and progenitor cells. Unfortunately, it is difficult to test the hypothesis, as methods for the large-scale isolation of pure populations of normal stem and progenitor cells are not yet available.

While differential drug or metabolite uptake can readily explain a preferential protection of normal cells, it does not readily explain why amifostine can have opposite effects on normal cells and tumor. List and others (66-69) have pointed out a striking homology between the dephosphorylated form of amifostine (WR-1065) and its symmetrical disulfide oxidation product (WR-33278) on the one hand and spermidine and spermine, respectively. They have suggested that proliferating tumor cells maintain high levels of intracellular polyamines by ornithine decarboxylase-controlled biosynthesis and block the influx of amifostine derivatives by the induction of antizyme. Passive inward diffusion of WR-1065 could accelerate polyamine degradation by the induction of spermine/spermidine acetyl transferase, which would result in a lowered apoptotic threshold (69). By contrast, in normal cells, polyamine synthesis is not

upregulated, and the disulfide (WR-33278) is actively imported into cells where it can participate in the defense against oxidative stress, thereby preventing the induction of apoptosis (69).

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Abbreviations

GM-CSF, granulocyte/macrophage colony stimulating factor; GSH, glutathione; hCFU-GM, human colony forming unit-granulocyte/macrophage; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) hemisodium salt; mCFU-GM, murine colony forming unit-granulocyte/macrophage; MC540, Merocyanine 540; PDT, photodynamic therapy..

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Figure 1.

Potentiating effects of amifostine, amphotericin B, and amifostine combined with amphotericin B on the the MC540-sensitized photoinactivation of wild-type H69 human small cell lung cancer cells (a), cisplatin-resistant H69/CDDP human small cell lung cancer cells (b), and normal human granulocyte/macrophage progenitors (c). Data represent mean colony counts of 4 culture dishes $\pm$ standard errors. Most error bars are smaller than the data symbols. While the survival of hCFU-GM was better in the presence of amifostine at all four fluences tested, only 2 data points (0 kJ/m², 146 kJ/m²) showed differences that were statistically significant with *P*-values of 0.008 and 0.02, respectively (unpaired t-test.) Untreated H69 cells, H69/

CDDP cells, and human bone marrow cells (surviving fraction 1.0) formed 111.75, 172.25, and 163 colonies per plate, respectively.

Figure 2. Potentiating effects of amifostine, amphotericin B, and amifostine combined with amphotericin B on the MC540-sensitized photoinactivation of murine L1210 leukemia cells (a) and normal murine granulocyte/macrophage progenitors (b). Data represent mean colony counts of 4 culture dishes $\pm$ standard errors. Most error bars are smaller than the data symbols. Cultures of untreated L1210 and normal murine bone marrow cells (surviving fraction 1.0) formed 197 and 83.5 colonies per plate, respectively.

Figure 3. Effects of amifostine, amphotericin B, and amifostine combined with amphotericin B on the binding of MC540 to L1210 leukemia cells. Analysis was done by flow cytometer (a) or by fluorescence spectrophotometer after extraction with detergent (b). Data represent means of 2 determinations $\pm$ standard error.

Table 1.

Survival of normal human CD34-positive hematopoietic stem and progenitor cells after exposure to MC540 and light or MC540 and light combined with amifostine and/or amphotericin B

Treatment	Surviving Fraction
MC540-PDT (15 µg/mL, 146 kJ/m ²)	0.97 ± 0.044
MC540-PDT + amifostine (3 mg/mL, 30 min)	0.84 ± 0.006
MC540-PDT + amphotericin B (30 µg/mL, 90 min)	0.85 ± 0.071
MC540-PDT + amifostine + amphotericin B	0.85 ± 0.032

Data represent means of 4 determinations ± standard errors.

Table 2.

Intracellular glutathione levels in treated and untreated L1210 leukemia cells

Treatment	Glutathione (% of control)
Amifostine (3 mg/mL)	99.3 ± 4.46
Amphotericin B (30 µg/mL)	78.5 ± 2.54
Amifostine (3 mg/mL) + amphotericin B (30 µg/mL)	83.8 ± 1.42

Data represent means of 4 determinations ± standard errors.

Table 3.

Effect of amifostine and amphotericin B on the binding of FITC-annexin V to L1210 leukemia cells and H69 small cell lung cancer cells

Treatment	FITC-annexin V bound (% of control)	
	L1210	H69
Amifostine (3 mg/mL)	105.9 ± 10.5	103.6 ± 4.2
Amphotericin B (30 µg/mL)	103.7 ± 1.7	110.3 ± 3.0
Amifostine + amphotericin B	94.4 ± 5.0	105.8 ± 5.9

Binding of FITC-annexin V was determined with a flow cytometer. Data represent means of 4 determinations ± standard errors.