Preferential photoinactivation of leukemia cells by aluminum phthalocyanine

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Abstract

The efficacy of chloroaluminum phthalocyanine (AlPc) for photodynamic therapy (PDT) has been evaluated in vitro on acute myeloid leukemia (AML) cells, normal peripheral blood leukocytes (PBL) and mobilized peripheral blood stem cells (mPBSC). The selectivity of the treatment has been evaluated by mixing PBL and TF-1, an erythroleukemic cell line. Upon photoradiation, this photosensitizer leads to a significant and preferential photokilling of leukemia cells in comparison to normal cells. The use of stimulated lymphocytes in PBL/TF-1 mixtures instead of resting cells also leads to a preferential killing towards TF-1 although activated PBL are more affected than resting PBL. The analysis of AlPc intracellular emission by flow cytometry shows that the uptake of the dye by leukemia cells is faster. This good efficacy towards AML and the observed lower phototoxicity towards normal cells (PBL, normal progenitors) suggest that this phthalocyanine is a potential bone marrow purging agent. © 1998 Elsevier Science S.A. All rights reserved.

Keywords: Photodynamic therapy (PDT); Phthalocyanines; Selectivity; Bone marrow purging; Leukemia; Progenitors

1. Introduction

Age and lack of a histocompatible donor restrict allogeneic bone marrow transplants to a low percentage of adults with acute myeloid leukemia (AML) [1]. If no compatible donor is found, autologous bone marrow transplantation (ABMT) is an alternative treatment. However, leukemia cells that contaminate the graft can contribute to relapse after autologous transplantation [2,3]. Selective elimination of AML cells from bone marrow using monoclonal antibodies is not easy because of the heterogeneity of the leukemia phenotype and its similarity to normal progenitor cells. Chemical purging agents such as mafosfamide derivatives have been used [4,5], but these compounds are known for their lack of selective toxicity for clonogenic AML blasts [6-8]. This lethal effect towards normal clonogenic progenitors may cause delayed hematopoietic reconstitution [9]. A variety of methods have been developed to purge malignant cells. Among them is photodynamic therapy (PDT) [10], which combines the application of light and light-activated dyes [11]. It involves the uptake of the photoactive sensitizer into the target tissue, and the generation of toxic oxygen species through direct activation of the photosensitizer with red light that corresponds to the maximum of penetration of visible light into human tissues [12].

The majority of PDT clinical applications have centered on the use of hematoporphyrin derivatives such as Photofrin® in the treatment of solid tumors [13]. Photofrin® recently received an approval for use in advanced esophageal cancer treatment from the Food and Drug Administration. However, porphyrins do not absorb sufficiently in the therapeutic red-light wavelengths (600–800 nm). By contrast, phthalocyanines are second-generation photosensitizers which absorb strongly at 675 nm within the 'therapeutic window' [14].
More than 60 different metal or metalloid atoms can be inserted into the macrocycle of phthalocyanines. The photosensitizing properties of phthalocyanines in vitro have been described [15,16] for a variety of tumors, and aluminum phthalocyanine (AlPc) was found to be more efficient than hematoporphyrin derivatives [17,18]. Most phthalocyanines are not soluble in water. This problem can be solved by synthesizing sulfonated derivatives, but sulfonation significantly decreases the photosensitizing effect, and reproducible synthesis of these sensitizers is difficult. Moreover, sulfonated phthalocyanines are present as dimers and possibly higher aggregates in aqueous media, even at very low concentrations [19]. Aggregation reduces the lifetime of the excited phthalocyanines, thus diminishing their photosensitizing properties [20]. Unsubphated AlPc is in a monomeric form when dissolved in DMSO [21].

Sieber et al. [22] have pioneered the use of photosensitizers as potential bone marrow purging agents using merocyanine 540 (MC-540), a lipophilic dye. MC-540 [22] was found to reduce the concentration of in vitro clonogenic human neuroblastoma cells, while preserving hematopoietic progenitor cells from normal bone marrow. Similar experiments have shown that the use of MC-540 as a photosensitizer leads to 4 logs cell killing of leukemia cells (Daudi, K-562, Raji and HL-60), whereas 80% of the normal bone marrow cells and 40% of granulocyte macrophage colony forming units (CFU·GM) survive the treatment [23].

A pyrene-linked short-chain alcohol was also found to be an efficient photosensitizer with selectivity for leukemia cells. It causes a 99% reduction in leukemia clonogenic cells under conditions that keep almost intact the clonogenic potential of normal hematopoietic progenitors [24]. Jamieson et al. [25] demonstrated that leukemia cells and cell lines take up more benzoporphyrin derivatives (BPD) than normal bone marrow cells and peripheral blood leukocytes (PBL). Few reports comparing the phototoxicity of phthalocyanines against leukemia and normal cells have been published, except the two by Singer et al., who studied the potential value of the hydrophilic sulfonated aluminum phthalocyanine (AlSPc) as a purging agent for bone marrow autografts in AML [26,27].

In this paper, we investigated the phototherapeutic potential of unsubphated AlPc towards a series of AML and normal cells. Mobilized peripheral blood stem cells (mPBSC) obtained from non-leukemia patients were used as a source of normal progenitors, because peripheral blood stem cells have been substituted for bone marrow cells at many transplant centers [28]. We studied the effects of photosensitization by AlPc towards mPB-CD34+ derived CFU-GM and burst forming unit-erythroid (BFU-E) by in vitro clonal assay. The selectivity of the treatment was evaluated by mixing TF-1 [29], an erythroleukemic cell line, with normal resting or stimulated PBL in an equal ratio. The TF-1 ultrastructural changes induced by photosensitization were analyzed with electron microscopy.

2. Materials and methods

2.1. Chemicals

AlPc was obtained as a powder (Eastman Kodak, Rochester, NY). Stock solutions were prepared in DMSO and kept in the dark at 4°C.

2.2. Cell preparation and culture

Blood samples were obtained from non-leukemia cancer patients and from patients with AML after informed consent. PBL and AML mononuclear cells were obtained by centrifugation on Ficoll (Pharmacia, Uppsala, Sweden) and washed twice with phosphate buffered saline (PBS, Biowhittaker, Walkersville, MD). PBL were incubated overnight in RPMI 1640 containing 10% fetal calf serum (FCS) at 37°C-5% CO2 to deplete monocytes. T lymphocytes were stimulated in vitro with 0.5 μl/ml phytohemagglutinin (PHA, Sigma, Saint-Louis, MO) and 50 U/ml interleukin 2 (IL-2) for five days. Cells were cultured in IMDM (Biowhittaker, Walkersville, MD) supplemented with 20% decomplemented FCS, 10 U/100 mg penicillin/streptomycin per ml. TF-1 cells were grown with 10 ng/ml human recombinant granulocyte macrophage colony stimulating factor (GM-CSF) [29]. Culture of mPBSC was supplemented with interleukin 3 (IL-3), IL-6 (R and D System, Inc., Minneapolis, MN), GM-CSF (PeproTech Inc., Rocky Hill, NJ), granulocyte colony stimulating factor (G-CSF, 10 ng/ml, rhG-CSF, Amgen, Thousand Oaks, CA), erythropoietin (EPO, 2 U/ml, Boehringer Mannheim, Germany) and eKit ligand (100 ng/ml, R and D System, Inc.). Cells were incubated at 37°C-5% CO2 in a fully humidified incubator. The culture medium was changed every 48 h.

2.3. Preparation of CD34+ cells

Cell samples were obtained after informed consent, from aphereses in patients primed with chemotherapy or recombinant-human granulocyte colony stimulating factor (rhG-CSF, Amgen, Thousand Oaks, CA). 300 or 600 μg rhG-CSF was administered daily subcutaneously when patient blood had recovered from previous chemotherapy (i.e., white blood cell count >3×109/l). Patients were leukapheresed when CD34+ cells exceeded 20/μl in their peripheral blood. Mononuclear cells (low-density cells <1.077 g/ml) were obtained by centrifugation over Ficoll (Pharmacia, Uppsala, Sweden) and washed twice in PBS (Biowhittaker, Walkersville, MD). PBL were depleted of adherent cells by overnight incubation in RPMI 1640 with 10% FCS at 37°C in a 5% CO2 atmosphere. Viability was checked with trypan blue, and was always superior to 95%. Monocyte-depleted PBL were then incubated for 30 min at 4°C with purified antihuman CD34 monoclonal antibody (1 μl/106 cells, Immu 133, Immunotech SA, Marseille, France or HPCA-2, Becton Dickinson, Mountain View, CA), in PBS-0.5% human polyvalent...
Ig (Inter Transfusion, Strasbourg, France) at 10^7 cells/100 µl. The cells were washed once in PBS–0.5% bovine serum albumin (BSA)–5 mmol/l EDTA (Sigma, St Louis, MO) and were incubated for 30 min with ferritin microbeads conjugated with rat-antimouse IgG1 monoclonal antibody (MoAb) (Miltenyi Biotec, Bergish-Gladbach, Germany). Cells were washed once with PBS–0.5% BSA–5mmol/l EDTA and suspended at 2 X 10^6 cells/ml. Immunoselection of CD34+ cells was performed according to the manufacturer's recommendations. The efficiency of separation was evaluated by flow cytometry, using a FACScan (Becton Dickinson) and phycoerythrin (PE)-conjugated anti-CD34 MoAb (HPCA-2; Becton Dickinson).

2.4. Phototoxicity assays

The same procedure as described by Fiedorowicz et al. [30] was used. Briefly, 0.5 X 10^5 cells/ml were incubated 60 or 30 min with fresh dye-containing medium at specified concentrations. Cells were washed twice with medium to eliminate excess dye. After centrifugation, the pellets were resuspended in culture medium at original concentrations, 2 ml aliquots were transferred into clear polystyrene 12 mm X 75 mm round-bottom tubes. A photoreactor equipped with eight 110 W incandescent light tubes (Aric France) was used for irradiation. The photoreactor had a cooling system in the center. Cells were irradiated at 25°C. 10 mW/cm² were delivered to cells. After illumination, 1 ml aliquots of each sample were placed into separate wells of a 12-well plate (Costar). 1 ml of fresh culture medium was added to each sample and cells were incubated at 37°C–5% CO_2.

2.5. Fluorescence-activated cell sorter (FACS) analyses

Light-induced cytotoxicity was evaluated by flow cytometry with propidium iodide (PI). Surviving fractions (SFs) were calculated as follows: SF = living cells after irradiation / living cells before irradiation. Cell surface antigens were labeled for flow cytometry analyses. In experiments where TF-1/PBL were involved, TF-1 cells (CD34+ ) were discriminated from PBL using CD34-FITC MoAb. Briefly, 0.1–0.5 X 10^6 cells were incubated for 30 min on ice with CD34-FITC monoclonal antibody. Each cell sample was washed twice with ice-cold PBS, then 1 µg/ml PI solution was added. After 5 min, samples were analyzed on a FACScan (Becton-Dickinson) flow cytometer using an excitation source of 488 nm, and fluorescence was detected at 525 nm for FITC-labeled probe. PI fluorescence was detected at 610 nm. Iso-type matched irrelevant antibodies were used as negative controls. Data were collected and analyzed with the Lysis II software (Becton-Dickinson). Each sample was analyzed in triplicate and results were the average of at least two independent experiments.

2.6. FACS measurements of dye uptake

Cells incubated with AlPc were washed twice with PBS and analyzed by FACS. The excitation wavelength was 350 nm and a 650 nm emission (longpass) filter was used to detect AlPc red fluorescence.

2.7. In vitro clonal assays

Methyl cellulose assays were performed with Methocult 4230 (Stem Cell Technology Inc., Vancouver, Canada). 400 CD34+ cells were plated in 0.5 ml of methyl cellulose supplemented with IL-3, IL-6, GM-CSF, G-CSF (10 ng/ml), EPO (2 units/ml) and cKit ligand (100 ng/ml). Cells were incubated at 37°C–5% CO_2, CFU-GM and BFU-E were scored 14 days later according to published criteria [31].

2.8. Electron microscopy

The cell suspension was centrifuged (900g, 5 min); the pellet was fixed in situ by immersion in 3% glutaraldehyde (Sigma, Saint-Louis, MO) in Sorensen phosphate buffer (0.1 M, pH 7.4) for 5 min, at 4°C. The pellet was post-fixed by immersing in the same fixative for 20 min at 4°C. The fixed pellet was then washed for 60 min in phosphate buffer 0.1 M (1 vol.) containing sucrose (3 vol.) and post-fixed in 2% osmium tetroxide (Sigma, Saint-Louis, MO) in Millonig phosphate buffer 0.1 M, 30 min at 48°C. The cells were then washed for 60 min in the same Millonig phosphate buffer, dehydrated in ethanol and embedded in Epon (Fluka, Buchs, Switzerland). The ultrathin sections were double-stained with uranyl acetate and lead citrate (Fluka, Buchs, Switzerland). Sections were examined and photographed at 80 kV on a Phillips 400 electron microscope.

3. Results

3.1. Preliminary evaluation of AML photosensitivity

Six AML samples were photosensitized with AlPc. The post-treatment surviving fractions are shown in Fig. 1 (a) and (b) for dye concentrations of 2 X 10^-9 and 4 X 10^-9 M, respectively. Incubation with 2 X 10^-9 M followed by only 10 min photoradiation induced more than 95% PI positive events (after 24 h incubation post-treatment) in all samples. 4 X 10^-9 M AlPc led to a cell reduction of 1.5 to 2.8 logs with the same irradiation time. These surviving fraction values were in the practical range of the PI method which covers cell depletion of 2–3 logs.

3.2. Phototoxicity towards leukemia and normal cells

3.2.1. Phototoxicity towards TF-1 and PBL

We have previously reported [32] that low concentrations of AlPc are able to produce a significant lethal effect towards
TF-1 or DAUDI cell lines upon light irradiation. However, the toxicity of AlPc towards normal cells and progenitors was unknown. TF-1 cells were mixed with normal PBL obtained from healthy donors. The mixture was treated by PDT. Following incubation for 1 h [15] and photoradiation, the results indicated that 2 × 10^{-9} M AlPc initiated a faster photokilling effect of TF-1 cells than of PBL. Fig. 2 (histograms) indicates that more than 99% of TF-1 cells were killed by the treatment, while more than 45% of PBL remained intact. Fig. 2 (curves) shows that 30 min incubation with dye followed by 45 min irradiation efficiently killed TF-1 cells (> 99% dead cells) while preserving > 92% of PBL, thus increasing the therapeutic index of AlPc. The uptake of AlPc by cells was determined by measuring the cells associated fluorescence using flow cytometry. After incubation with the photosensitizer, the cells' fluorescence intensity was recorded by FACS. Fig. 3 (a) shows that after 1 h incubation, the mean fluorescence intensity of TF-1 cells increases as a function of AlPc concentration. There was a 2 logs variation in fluorescence intensity within this cell line but no discrete subpopulations could be distinguished. The same technique was also used to determine if there was a difference between normal and leukemia cells with regard to AlPc uptake (Fig. 3 (b)). FACS analyses (UV excitation) performed on the TF-1/PBL mixture (Fig. 3 (b)) showed that the ratio I_{flu} (TF-1)/I_{flu} (PBL) was higher after incubation than before incubation (time zero). This observation means that TF-1 cells took up more dye than PBL. From these experiments, the higher value of the ratio I_{flu} (TF-1)/I_{flu} (PBL) after 30 min incubation than after 60 min brought the conclusion that dye uptake was faster in TF-1 cells than in PBL. Since we obtained a better selective efficacy with an incubation time of 30 min, the following experiments were carried out with this incubation time.

3.2.2. Phototoxicity towards TF-1 and activated PBL

The relation between phototoxicity and cell cycle activity was evaluated using lymphocytes stimulated with PHA and...
Fig. 4. Photosensitization of TF-1 and activated PBL mixture (50:50) following 30 min incubation with 2 × 10^{-9} M AlPc. Averages and standard deviations (n=3). Viability was determined by PI exclusion assay. TF-1 cells were stained with anti-CD34-FITC prior to analysis.

IL-2. We studied the viability of stimulated T-lymphocytes/TF-1 mixtures in response to light-activated AlPc sensitizer. Experiments were performed five days after activation. The treatment of the mixture with 2 × 10^{-9} M AlPc and light led to a reduction of TF-1 surviving fraction by a factor close to 150 (Fig. 4). The stimulated PBL were still more resistant than TF-1 (surviving fraction = 0.27 against 0.007 for TF-1) but were more sensitive than resting PBL (SF = 0.27 against 0.92). However, the preferential killing was still observed.

3.2.3. Photosensitization of normal mPBSC

The effect of AlPc and light on hematopoietic progenitors was checked using mPBSC. The phototoxicity was evaluated by measuring the ability of these cells to develop CFU-GM- and BFU-E-derived colonies following photosensitization. Under conditions previously used in this work (30 min incubation), the phototoxicity towards mPBSC, based on CFU-GM and BFU-E recoveries, was low. At dye concentrations of 2 × 10^{-9} and 4 × 10^{-9} M, respectively (Fig. 5(a) and (b)), less than 1 log of normal clonogenic CD34+ progenitors (10 min irradiation time) were eliminated.

3.3. Ultrastructural study of TF-1 treated with AlPc and light

TF-1 cells (Fig. 6(a)) were typically rounded and characterized by a large and often polylobed nucleus which contains one to three nucleoli. Some cells were immature with a dense cytoplasm, containing numerous ribosomes and polysomes. Other more differentiated cells presented long ergastoplasmic lamellae sometimes dilated, numerous mitochondria, one Golgi apparatus containing four to five saccules, as well as lysosomes (Fig. 6(b)). The plasma membrane displays villous surface with few blebs. Mitotic figures were also observed. We analyzed ultrastructural changes appearing after the photodynamic treatment with AlPc. The cells were characterized by numerous blebs of the plasmalemma (Fig. 6(c), arrow). The plasma membrane no longer had microvilli, and cytoplasmic dilatations disappeared resulting in a cellular desegregation. The earliest changes were a marked dilatation of the Golgi apparatus sacculi and inflation of mitochondria (Fig. 6(d)). Ultrastructural changes observed in our study suggested that phthalocyanine PDT initially affected the Golgi apparatus and plasma membrane.

4. Discussion

We evaluated the in vitro differential sensitivity of TF-1, AML, PBL and mPBSC to phthalocyanine-mediated photosensitization. The tumor selectivity was assayed by mixing TF-1 cells and normal leukocytes. The relation between cell cycle activity and phototoxicity was determined using stimulated and resting leukocytes. One key point of this study concerns the in vitro preferential phototoxicity of AlPc towards leukemia cells. In mixtures containing TF-1 and normal cells, TF-1 leukemia cells were more susceptible to the tumoricidal effect of PDT than PBL. In a preliminary experiment using the exclusion of PI as a viability evaluation test, AML were highly sensitive as indicated by the number of PI positive events and debris produced by the exposure to AlPc and light. In TF-1/PBL-containing mixtures, depletion of the erythroleukemic cell line was preferential. Depletion of CFU-GM and BFU-E progenitors was in an acceptable range. This preferential phototoxity may have been due to a better ability of normal cells to repair their oxidative damages. This phenomenon is known in radiotherapy and could also operate...
in PDT. Yamazaki et al. have shown [33] that a CFU-GM repairs photodynamic damage more rapidly than leukemic cell lines. A second explanation could be a more rapid photosensitizer uptake by leukemia cells. Photoradiation following an incubation time of 30 min instead of 60 min did not change the antileukemic effect and reduced the PBL death rate. These results are in good agreement with a faster dye uptake by TF-1 cells. Although activated lymphocytes exhibited a higher sensitivity to PDT than resting cells, a significant proportion of these cells still survived, thus suggesting that cell cycle activity is not the only parameter that governs this selective killing effect. Other mechanisms may be involved to explain the high cytotoxicity observed with TF-1 cells or fresh leukemia cells. In many cases, lipophilic sensitizers are delivered to tumors via a lipoprotein receptor-mediated pathway [34]. This is by no means the only route: passive diffusion and scavenger receptors could also contribute to the dye uptake. These other ways may explain why a fraction of normal cells is always and inevitably killed during PDT assays.

The ultrastructural changes observed following photosensitization initially affected the membrane. These observations are in good agreement with the lipophilic properties of the sensitizer, which can photosensitize oxidizing reactions towards unsaturated cell membrane components. Upon irradiation, AlPc generates cytotoxic singlet oxygen via triplet-triplet energy transfer with a quantum yield value of 0.29 (type II cytotoxic process) [35]. Superoxide (O$_2^-$) production, a potential source of conjugated base 'O$_2$H then highly cytotoxic hydroxyl radical (OH') via a Fenton process has also been reported [36] (type I mechanism). All these species are able to initiate membrane phospholipid chain oxidation. In both type I and type II reactions, the formation of oxygenated cytotoxic species strongly depends on the excited triplet lifetime [37] of the sensitizer. For AlPc, we have previously reported that this parameter is doubled when the compound is bound to a protein matrix mimicking the cellular environment [32]. A difference in the cell membranes of the leukemia clone (lipid content) may result in a more rapid uptake of photosensitizer, leading to a greater phototoxicity towards these cells. Triplet quenching may also occur during the metabolic processes of mPBSC, reducing the lifetime of the excited triplet state of AlPc. The dye-photosensitized photoactivation of tumor cells in autologous bone marrow grafts has already been explored with a few sensitizers including MC540, which has been evaluated in a phase I/II clinical trial [38]. Concerning the metallophthalocyanines, only sulfonated chloroaluminum phthalocyanine (AlSpc), a hydrophilic derivative, has undergone limited pre-clinical testing as a marrow purging agent. When this compound is used
under conditions that eliminate 0.2 log CFU-GM, the concentration of AML blasts is reduced by 1.2 log. Unfortunately, one third of AML samples are refractory to this compound [26].

Purging agents, 4 HC or ASTA Z-7557, deplete CFU-GM and show no selectivity [8]. Although CFU-GM are delayed to eliminate leukemia cells preferentially when mixed with normal cells and the low toxicity towards CFU-GM progenitors, work is now in progress to evaluate the impact of AIPc-mediated photosensitization towards leukemia progenitors.

5. Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>ABMT</td>
<td>autologous bone marrow transplantation</td>
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<td>AIPc</td>
<td>chloroaluminum phthalocyanine</td>
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<td>AML</td>
<td>acute myeloid leukemia</td>
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<td>BFU-E</td>
<td>burst forming unit-erythroid</td>
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<td>BPD</td>
<td>benzoporphyrin derivatives</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CFU-GM</td>
<td>colony forming unit-granulocyte macrophage</td>
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<td>EDTA</td>
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<td>EPO</td>
<td>erythropoietin</td>
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<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
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<td>FCS</td>
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<td>FITC</td>
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<td>GM-CSF</td>
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<td>MC-540</td>
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<td>mPBSC</td>
<td>mobilized peripheral blood stem cells</td>
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<td>PBL</td>
<td>peripheral blood leukocytes</td>
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<td>phosphate buffered saline</td>
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<td>photodynamic therapy</td>
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<td>propidium iodide</td>
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<tr>
<td>SF</td>
<td>surviving fraction</td>
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