Tumor cell lines resistant to ALA-mediated photodynamic therapy and possible tools to target surviving cells

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Abstract. We isolated and characterized cell lines resistant to aminolevulinic acid (ALA)-mediated photodynamic therapy (PDT) derived from a murine adenocarcinoma and studied cross resistance with other injuries. The most resistant clones were numbers 4 and 8, which exhibited 6.7- and 4.2-fold increase in resistance respectively. Several characteristics were altered in these clones. A 2-fold increase in cell volume, higher cell spreading, and a more fibroblastic, dendritic pattern, were the morphology features that led us to think they could have different adhesive, invasive or metastatic phenotypes. The amount of porphyrins synthesized per cell in the resistant clones was similar to the parental line but, when it was expressed per mg protein, there was a 2-fold decrease, with a higher proportion of hydrophilic porphyrins. These cells were not cross-resistant to photosensitization with Benzoporphyrin derivative and Merocyanine 540, but exhibited a slight resistance to exogenous protoporphyrin IX treatment. Both clones displayed higher protein content and increased number of mitochondria, together with a higher oxygen consumption. The distinctive features found in the resistant lines led as to think how to exploit the changes induced by PDT treatment to target surviving cells. Those hypoxic cells can be also a preferential target of bioreductive drugs and hypoxia-directed gene therapy, and would be sensitive to treatment with other photosensitizers.

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Abbreviations: ALA, 5-aminolevulinic acid; ALA-PDT, ALA-based PDT; GSH, reduced glutathione; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; PDT, photodynamic therapy; PpIX, protoporphyrin IX; Rh123, rhodamine 123

Key words: photodynamic therapy, resistance, cross-resistance, aminolevulinic acid, hypoxia

Introduction

The exogenous administration of 5-aminolaevulinic acid (ALA) is a relatively new approach in PDT (1) since it is a natural precursor of protoporphyrin IX (PpIX), an effective photosensitizer. ALA is frequently applied topically or systemically in PDT of several tumours (2). ALA-induced PpIX accumulation has been shown to be preferentially greater in certain tumoral cells primarily due to the reduced activity of ferrochelatase, the enzyme responsible for the conversion of PpIX into heme (3) and a relative enhancement of deaminase activity (4), which constitutes the biological rationale for the clinical use of the so called ALA-based PDT (ALA-PDT).

ALA-PDT has been used in multiple sessions for different tumor types in several clinical studies (5-8). However, there are no studies attempting to elucidate if PDT with ALA could induce resistance.

A few cell lines resistant to PDT have been obtained using different photosensitizers such as Photofrin, phthalocyanines and Nile Blue (9-12). However, resistant cell lines have been produced after exogenous application of photosensitizers. Since ALA and PpIX are naturally produced compounds, we wondered if the cells would respond to photodamage by either lowering PpIX concentrations or increasing defence mechanisms aimed at detoxifying cytotoxic oxygen species. Now that an increasing number of PDT treatments are being applied (5) it is important to determine how many PDT cycles would be optimal and when do the cells begin to be modified by PDT treatment.

The elucidation of PDT resistance mechanisms can also help to improve combination treatments such as PDT plus chemotherapy or radiotherapy. In this regard, the elucidation of cross-resistance is essential.

By using resistant cells, we have also attempted to study the changes that ALA-PDT can induce on the cells, whether related or not to its phototoxic mechanism of damage. Such

changes could introduce modifications that can be used to selectively target the surviving cells with other treatments.

The aim of the present work was to isolate and characterize ALA-PDT-resistant clones derived from an adenocarcinoma cell line and to study cross-resistance with other injuries. The distinctive features found in the resistant lines may be useful information to target PDT-resistant cells.

Materials and methods

Chemicals. ALA, Merocyanine 540 and PpIX were obtained from Sigma, St. Louis, MO, USA. Liposomal Benzoporphyrin Derivative (Visudyne) was a gift form Novartis Argentina. Hexyl-ALA and Undecanoyl-ALA were synthesized according to Perotti *et al* (13). Rhodamine 123 (Rh123) and LisoTracker Green DND-26 were obtained from Molecular Probes, Eugene, OR, USA and the benzodiazepine analogue, FGIN-1-27-NBD, was obtained from Alexis Biochemical Corp, San Diego, USA. All chemicals employed were of analytical grade.

Cell line and cell culture. Cell line LM3 (14) derived from the murine mammary adenocarcinoma, M3, was cultured in minimum essential Eagle's, supplemented with 2 mM L-glutamine, 80 μg gentamycin/ml and 5% fetal bovine serum, and incubated at 37˚C in an atmosphere containing 5% CO_2.

Induction of resistance to ALA-PDT. LM3 cell line cultured in 25-cm^2 flasks was exposed for 3 h to 0.6 mM ALA in medium without serum and increasing light doses (0.36-5.4 J/cm^2) aimed at achieving survival levels in the 5-10% range. The initial ALA and light doses were established in previous work (13). The surviving cells were harvested 24 h after the treatment and replated. After 2 weeks of recovery, the cells were exposed to the next ALA-PDT treatment. The final population (LM3L13) received a total of 13 cycles and, afterwards, 8 clones were isolated by the limiting dilution method. LD50 was defined as the light dose to kill 50% of cells at saturating concentrations of the pro-photosensitizer. The resistance index to ALA-PDT was defined as LD50 resistant clon/LD50 LM3. The resistant clones isolated showed a stable level of resistance following *in vitro* passage (30 passages) and after recovery from frozen stocks.

Light source. Cells were exposed during different time periods from below to the expanded homogeneous beam of 514 nm Argon laser irradiation (Coherent Inc., Santa Clara, CA, USA) at a dose rate of 0.03 W/cm^2 as measured by a Coherent Lasermate power meter model 200.

MTT assay. Cell viability was documented by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) assay (15), a method based on the activity of mitochondrial dehydrogenases, which has been shown to correlate well with other established measures of cytotoxicity such as colony formation (16).

Clonogenic assay. Immediately after ALA-PDT, cells were detached using trypsin/EDTA and suspended in complete medium. Diluted suspensions were plated on 100-mm dishes and incubated for 10 days. Cells were fixed with 0.2% buffered formalin in methanol and stained with 0.1% aqueous crystal violet. Colonies of more than 50 cells were counted.

Cell growth and cell doubling time. $7x10^4$ cells/ml were seeded in triplicate in 24-well plates. Every 2, 24, 48 and 72 h, cells were counted in a hemocytometer. Doubling time was estimated at the midpoint of the exponential phase of the growth curve. The lag time was defined as the time taken for the cells to exceed their initial plating density.

Cell volume measurement. A cell suspension was placed on a microscope slide using 0.02-mm-thick distance holders to prevent compression. Transmission images were photographed in a Leika confocal laser microscope (Leica Mikroskopie und System GmbH, Wetzlar, Germany) using a magnification x63. The mean diameter of cells was calculated from 20 different observations (two diameters per cell). The cells were assumed to be spherical after estimation of cell depth and cell diameter.

ALA-PDT. For ALA-PDT treatment, cells plated in 35-mm dishes were incubated for 3 h with ALA in medium without serum and then illuminated. MTT assay was performed after 19 h. For ALA-PDT of cells in suspension, illumination was performed under shaking, which was also continued for 2 h after treatment. For PDT of cells attached to fibronectin, the dishes were incubated overnight with human fibronectin (25 μg/ml) dissolved in 0.1 M carbonate buffer pH 9.7, blocked with 1% bovine seroalbumin and then the cells were plated. ALA-PDT treatment was performed as described above.

Cellular protein determination. After 3 washings with PBS, cells were kept overnight in the presence of NaOH 1 N. Afterwards, the Lowry assay method (17) was performed.

Electron microscopy. Cells were fixed for 4 h at 4˚C in 3% glutaraldehyde in sodium phosphate-buffer 440 μOsm pH 7.3, post-fixed in 2% osmium tetroxide in 0.1 mM sodium cacodylate buffer pH 7.2, dehydrated in ethanol and embedded in epon 812. Thin sections (750-900 Å) were cut after staining with uranyl acetate 2% Reynolds lead citrate and then observed in a Zeiss EM109 transmission electron microscope.

Measurement of porphyrin synthesis after chemical extraction. Intracellular porphyrins were extracted twice with 5% HCl. These conditions proved to be the optima for total porphyrin extraction. The media were acidified and measured directly fluorometrically. The excitation and emission wavelengths producing the highest fluorescence in PpIX, Uroporphyrin and Coproporphyrin solutions in 5% HCl were 406 and 604 nm respectively. These wavelengths were employed to measure the samples in a Shimadzu spectrofluorophotometer Model RF-510. PpIX was used as a reference standard.

Fluorescence analysis in living cells and confocal microscopy. Cells were grown in 60-mm dishes and, after addition of the fluorochrome, they were covered with coverslips. A confocal laser scanning microscope was used at magnification x40 or x63. The excitation source was an argon laser (488 nm) and the fluorescence signal was separated into a green range and red range using a 580-nm dichroic mirror plus 530-nm bandpass

filter and 590-nm longpass filter respectively. Fluorescence images were displayed in green and red false color and electronically overlaid.

Cells were incubated for 3 h with 0.6 mM ALA or 4 h with 2.5 μM PpIX to evaluate photosensitizer localization. For colocalization studies, organelle fluorescent probes (10 nM Rh 123, 25 nM LysoTracker Green and 10 μg/ml of FGIN-1-27-NBD) were introduced in the final 30 min.

Flow cytometry for porphyrin determination. Fluorescence of individual cells was quantified using flow cytometry (FACScalibur, Becton-Dickinson, San Jose, CA, USA) interfaced with Macintosh Cell Quest software (Becton-Dickinson). ALA-treated cells were incubated in trypsin-EDTA and suspended in PBS after washing. Prior to analysis, cells were passed through a 37-μm nylon filter. Using excitation at 488 nm from a single-beam argon laser, red fluorescence emission of 10,000 cells was recorded through a 670-nm longpass filter. Cell debris were eliminated by gating cells based on their forward scatter versus side scatter properties. Fluorescence intensity was quantified by use of arbitrary units and was depicted on logarithmic scale.

HPLC analysis. Intracellular porphyrins were extracted with HCl as explained above. Reverse-phase HPLC of porphyrins was carried out according to the method of Lim and Peters (18) employing acetonitrile in methanol and acetonitrile in 1 M ammonium acetate buffer, pH 5.16. The samples were run immediately after extraction in a Shimazdu HPLC model RF-10A model employing a RF-10A spectrofluorometric detector and a Hypersil column (250x5, i.d., 5 μm particle size). A porphyrin kit was used as standard (Frontier Scientific Ltd., Palo Alto, USA).

Oxygen consumption. A LICOX oxygen partial pressure monitor (Gesellschaft fur medizinische Sondentechnik mbH, Kiel-Mielkendorf, Germany) was used to monitor oxygen consumption in a suspension of cells in PBS in a glass cuvette with stirring. The signal from the Clark electrode was captured by LICOX software via an RS232 interface. The initial rates of oxygen consumption were plotted against the cell number to obtain pO_2/min/10^9 cells.

Glutathione measurement. Intracellular reduced glutathione (GSH) was determined by a modification of the Hissin & Hilf Method (19). Briefly, the cells were resuspended in phosphate-EDTA buffer, sonicated and centrifuged. The supernatant was mixed with of *o*-phthalaldehyde and fluorescence was measured using excitation of 350 nm and emission of 420 nm. GSH intracellular levels were calculated from a calibration curve.

Flow cytometry analysis of apoptotic cells. Cells were exposed for 3 h to 0.6 mM ALA and illuminated with a light dose killing 50% or 20% of the cells. At different times after PDT, cells were trypsinized, mixed with the detached cells, and fixed with 70% ethanol. After washing, they were incubated with Propidium iodide 40 μg/ml and RNase A 100 mg/ml. After washing, the cells were passed through a 37-μm nylon mesh and analyzed on a FACScalibur flow cytometer using excitation at 488 nm. Red fluorescence emission of 10,000 cells was recorded through a 670-nm longpass filter. Cell debris was eliminated by gating cells based on their forward scatter versus side scatter properties. Cell cycle distribution was analyzed using Cell Quest software.

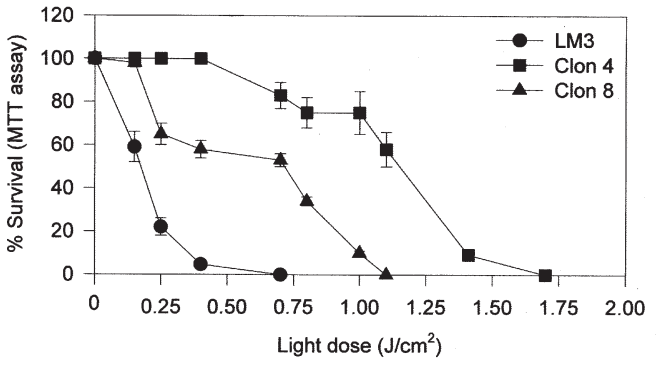

Figure 1. Cell survival after PDT with different light doses. Cells, parental LM3 line, Clon 4 and Clon 8 were incubated 3 h with 0.6 mM ALA and irradiated with different light doses and after 19 h the MTT assay was performed. Cell survival was expressed as percentage of the non-irradiated control incubated with ALA. Points, average of 3 experiments performed in triplicate; bars, SE.

Cross-resistance. For hyperthermia, cells were incubated for different times at 45˚C. For UV irradiation, cells received different time exposures to a Germicydal lamp (G30T8, 30 W, General Electric). For ALA derivatives-PDT, cells were exposed to Hexyl-ALA and Undecanoyl-ALA (0.015-1.2 mM). PDT was performed with 0.05 to 1.5 J/cm^2. For Benzoporphyrin derivative, cells were exposed for 2 h to Verteporfin (0.02-0.18 μg/ml) and were irradiated with 0.1-0.6 J/cm^2. For Merocyanine 540, cells were exposed to 100-μM Merocyanine 540 for 1, 2 or 3 h, and exposed to 0.1-0.6 J/cm^2 light. For PpIX, cells were exposed for 4 h to PpIX (2.5-10 μM) and exposed to 0.5-10 J/cm^2 light. MTT assays were always performed 19 h after treatment.

Statistical treatment. The values in the figures and tables are expressed as mean ± standard deviations of the mean. A two-tailed Student's t-test was used to determine statistical significance between means. $p<0.05$ are considered significant.

Results

Resistance to ALA-PDT. Porphyrin synthesis was studied as a function of ALA dose for the eight clones isolated from the heterogeneous resistant LM3L13 line. Afterwards, light dose curves were performed exposing the cells to ALA under saturating conditions and then we calculated the resistance indexes (data not shown). Most of the clones did not show resistance to ALA-PDT and we selected the most resistant clones, which were called Clon 4 and Clon 8 (6.7 and 4.2 resistance indexes respectively) to perform a thorough characterization.

The degree of resistance to ALA-PDT as a function of the light dose was tested in Clones 4 and 8 by MTT viability assay (Fig. 1) and confirmed by colony formation assay. The LD50

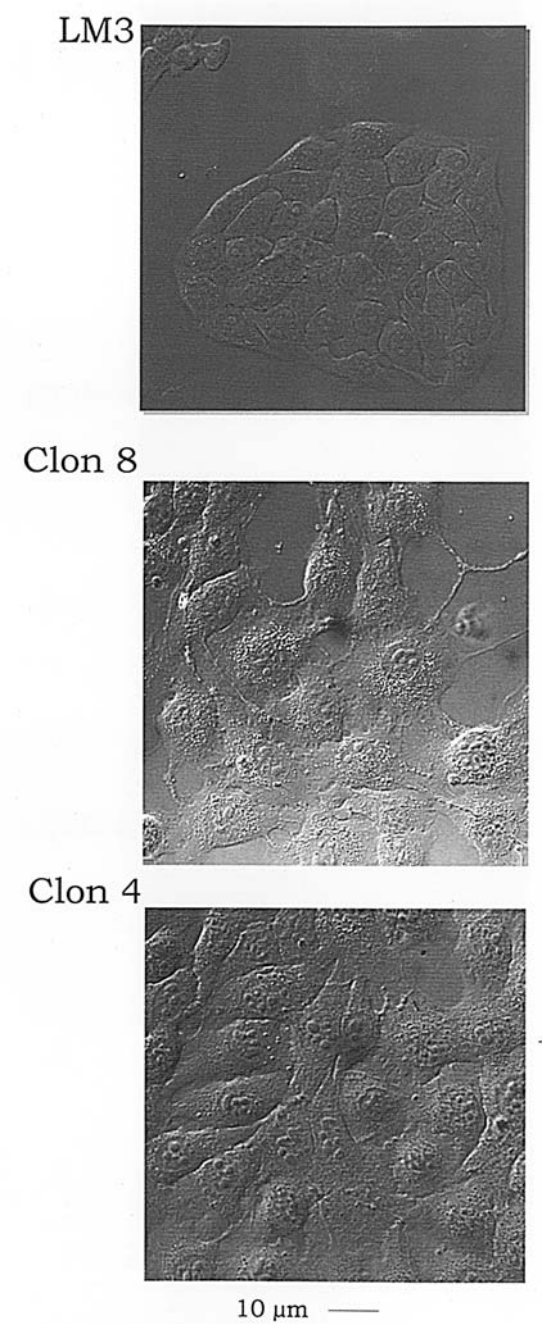

Figure 2. Transmission confocal microscopy of LM3, Clon 4 and Clon 8 cells.

calculated according to survival data were as follows: LM3 = 0.17 J/cm^2, Clon 8 = 0.73 J/cm^2 and Clon 4 = 1.15 J/cm^2.

The LD50s calculated from the clonogenic assay were similar to the formers for LM3 and Clon 4, whereas LD50 for Clon 8 was substantially lower (0.38 J/cm^2) (data not shown).

Cell morphology. Clon 4 and Clon 8 exhibited a more fibroblastic, dendritic pattern, and higher cell spreading than the parental line, LM3. LM3 cells exhibited a more polyhedric shape, grew in clusters and were smaller (Fig. 2).

At the subcellular level, electron microscopy showed that there were no noticeable differences on lysosomes and membranes among the three lines. On the contrary, the mitochondrial number was increased per cell and per area in both resistant clones, although of similar size (Fig. 3).

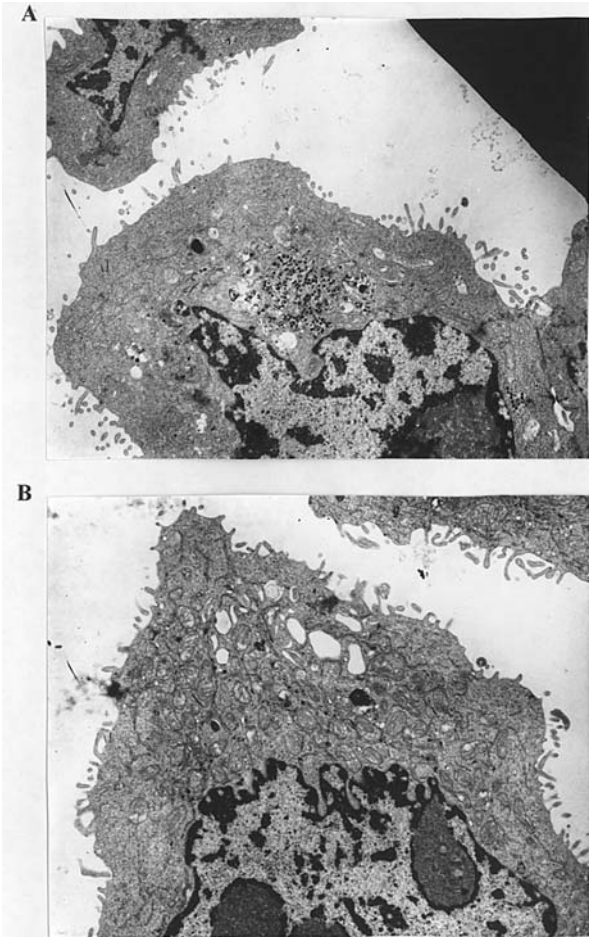

Figure 3. Transmission electron micrographs. (A) LM3; (B) Clon 4. (x4400). Similar features were observed for Clon 8.

Table I. Characteristics of parental LM3 and ALA-PDT-resistant clones.

	LM3	Clon 8	Clon 4
Plating efficiency (%)[a]	95±8	87±10	91±5
Cell doubling time (h)[a]	22±4	24±5	23±3
Lag time (h)[a]	18±1	24±2[c]	24±2[c]
Protein content (pg/cell)[a]	22.6±2.5	44.2±4.2[c]	49.9±4.5[c]
Diameter (μm/cell)[b]	10.04±1.98	12.72±2.26	12.79±1.57
Volume (fl/cell)[b]	523±4	1077±6[c]	1092±4[c]

Mean ± SD. [a]n=4, [b]n=20, [c]p<0.005 compared to LM3 cells.

Cell characteristics. Both parental and resistant cells showed similar characteristics in terms of plating efficiency and cell doubling time, but the lag time was significantly larger in the resistant cells. Protein content as well as cell volume was increased 2-fold in the resistant clones (Table I).

Table II shows that MTT activity, which correlated with mitochondrial dehydrogenase activity, was 2-fold increased in the resistant cells expressed per cell number, but was not

Table II. Biochemical characterization of parental LM3 and ALA-PDT resistant clones.

	LM3	Clon 8	Clon 4
ng intracellular porphyrins/10^5 cells[a]	40±16	32±10	36±12
ng intracellular porphyrins/μg protein[a]	17.69±6.80	7.23±2.25[c]	7.21±2.40[c]
ng porphyrins released/10^5 cells[a]	9.3±1.1	6.4±0.9	6.15±1.4
ng porphyrins released/μg protein[a]	4.11±0.79	1.44±0.65[c]	1.23±0.28[c]
μg GSH/10^5 cells	10.23±2.15	19.73±2.53[c]	22.15±4.37[c]
μg GSH/μg protein	4.52±0.86	4.46±0.57	4.43±0.94
MTT activity[b]/10^5 cells	0.109±0.016	0.191±0.016[c]	0.217±0.025[c]
MTT activity[b]/μg protein	0.047±0.006	0.043±0.003	0.043±0.005
pO_2 consumption (mm Hg/min/10^9 cells)	14.9±2.15	41.98±4.28[c]	58.27± 4.32[c]
pO_2 consumption (mm Hg/min/mg protein)	0.65±0.09	0.94±0.09[d]	1.16±0.08[d]

Mean ± SD; n=4. [a]Porphyrin synthesis after 3 h-exposure to 0.6 mM ALA, [b]A_{560}, [c]P<0.005 compared to LM3 cells, [d]P<0.05 compared to LM3 cells.

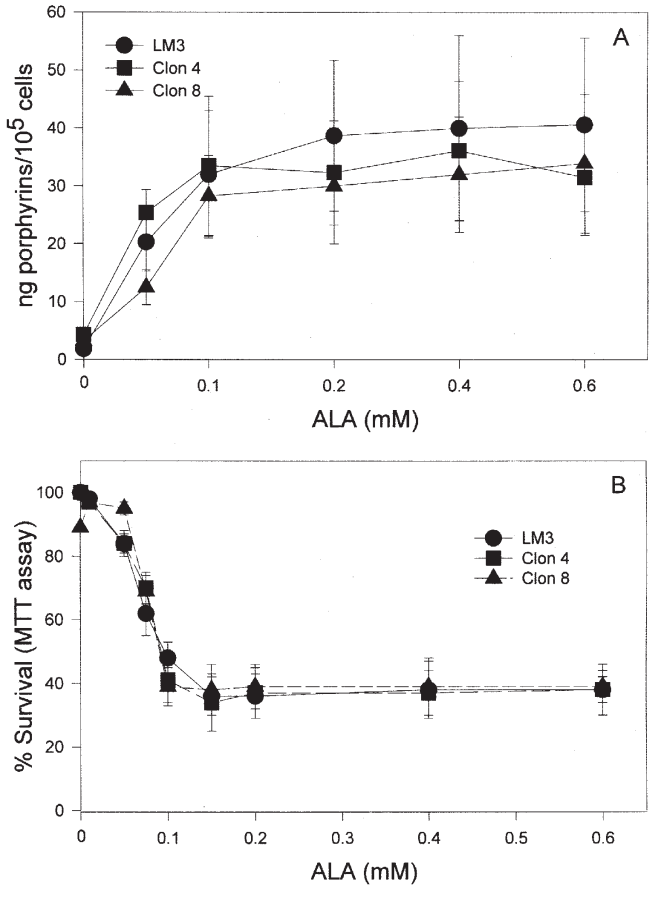

Figure 4. Porphyrin synthesis and survival after PDT as a function of ALA concentration. Cells were incubated with different concentrations of ALA during 3 h. (A) Porphyrins were quantified. (B) Cells were irradiated with the LD50 light dose and cell survival was calculated after 19 h by the MTT assay. Points, average of 4 experiments performed in duplicate; bars, SE.

significantly different when expressed per μg protein. Oxygen consumption was increased 3- and 4-fold in Clones 8 and 4 respectively expressed per cell number. On a per mg protein basis, oxygen consumption was significantly increased in both clones. GSH content expressed on the basis of cell number was 2-fold increased in the resistant clones, but similar when expressed per μg protein.

Porphyrin synthesis in parental and resistant clones. Fig. 4A shows the dependence of porphyrin synthesis on ALA concentration. The saturation for the three lines occurred in the 0.15-0.2 mM ALA range. Fig. 4B shows survival data of cells exposed to ALA and illuminated with LD50 doses. Under non-saturating concentrations of ALA, PDT efficacy increased with ALA concentration, with identical survival rates for the three lines.

Table II shows that the amount of porphyrins synthesized by LM3 cells normalized by cell number was not significantly different from the resistant sublines. However, when expressed on a per μg protein basis, the tetrapyrrole synthesis was 2-fold increased in the parental line. A similar feature was observed on the amount of porphyrins released to the medium.

Flow cytometry dot plots (Fig. 5A) illustrate a heterogeneous distribution of fluorescence in the three lines. In addition, Clon 4 and Clon 8 showed a subpopulation that represents ~5% of the total, exhibiting lower fluorescence intensity. Fig. 5B shows the histogram plot representation. Mean ± SD fluorescence values of cells exposed to ALA were as follows: LM3 = 413±392, Clon 8 = 252±196 and Clon 4 = 241±200. A significantly higher mean of porphyrin fluorescence was observed in the LM3 line (p<0.005). Control resistant cells non-exposed to ALA exhibited higher autofluorescence than LM3 cells.

HPLC analysis. Both parental and resistant clones mainly accumulated Uroporphyrin and PpIX (Table III). A small percentage of Heptaporphyrin was also present, but the rest of the intermediate porphyrins were under the limit of detection. LM3 accumulated twice the percentage of PpIX compared to the resistant clones and significantly less Uroporphyrin and Heptaporphyrin compared to the clones.

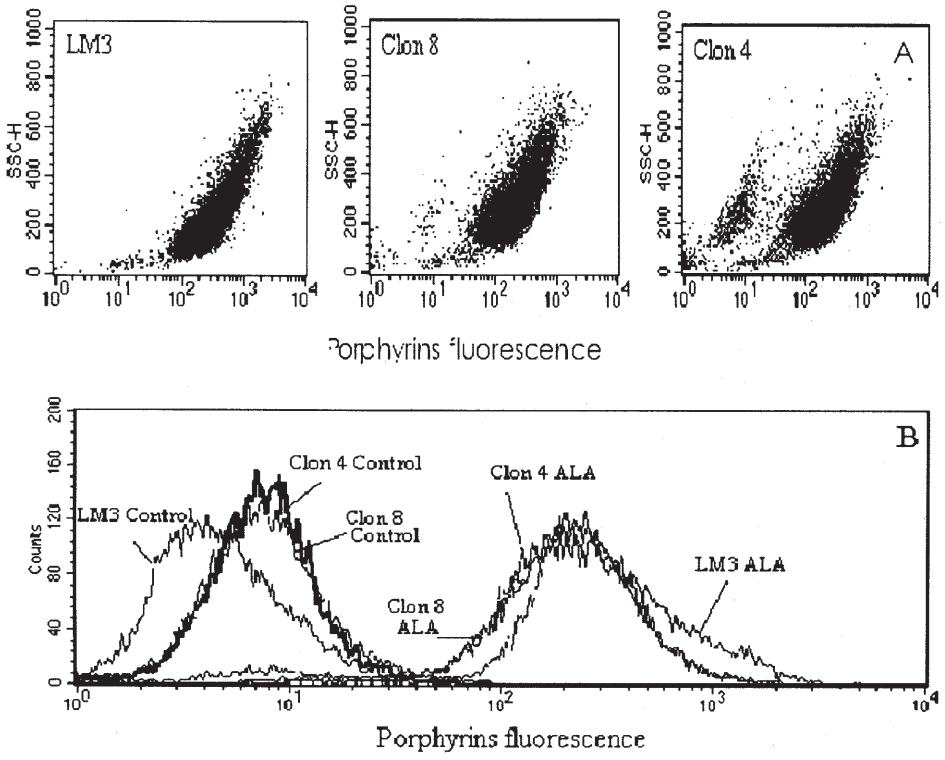

Figure 5. Flow cytometry dot plot of cells exposed for 3 h to ALA 0.6 mM (A) and histogram plot including control cells (B). Number of events: 10,000.

Table III. HPLC analysis of LM3 cells and resistant clones exposed to ALA.

	LM3	Clon 4	Clon 8
Uroporphyrin	53±3.2	66.2±4.1[a]	74±5.4[a]
Heptaporphyrin	3.6±0.1	8.5±0.6[a]	4.7±0.2[a]
Hexaporphyrin	ND	ND	ND
Pentaporphyrin	ND	ND	ND
Coproporphyrin	ND	ND	ND
Protoporphyrin	43.4±2.6	25.3±1.3	21.3±1.5

Porphyrins synthesized after 3 h of 0.6 mM ALA exposure. Expressed as percentage of the total porphyrins. ND, non-detectable. Mean ± SD; n=3. [a]P<0.05 compared to LM3.

Subcellular localization of endogenously synthesized PpIX. Fig. 6 shows colocalization of endogenous porphyrin from ALA and Rh123. The three cell lines exhibited a similar and widespread cytoplasmatic PpIX localization, including mitochondria, lysosomes, cell membrane and Golgi apparatus. LM3 cells appear to have higher porphyrin content.

The distribution of porphyrin and rhodamine is uneven in the resistant lines, with areas of porphyrin fluorescence that do not correlate with Rh123 fluorescence and vice versa.

We performed also colocalization studies of endogenous PpIX coincubated with lisotracker and the benzodiazepine analogue, FGIN-1-27-NBD, which attaches to the mitochondrial benzodiazepine receptor localized in the outer mitochondrial membrane, postulated to bind porphyrins (20). No major differences among the cells were found in either case (data not shown).

Table IV. Cross-resistance.

	Clon 4	Clon 8
UV	Non-resistant	More sensitive
Hyperthermia	More sensitive	More sensitive
PDT-ALA derivatives	Cross-resistant	Cross-resistant
PDT-Benzoporphyrin derivative	Slightly more sensitive	Slightly more sensitive
PDT-Merocyanine 540	Slightly more sensitive	Slightly more sensitive
PDT-PpIX	Slight cross-resistance	Slight cross-resistance

Degree of resistance to several treatments compared to LM3 parental line.

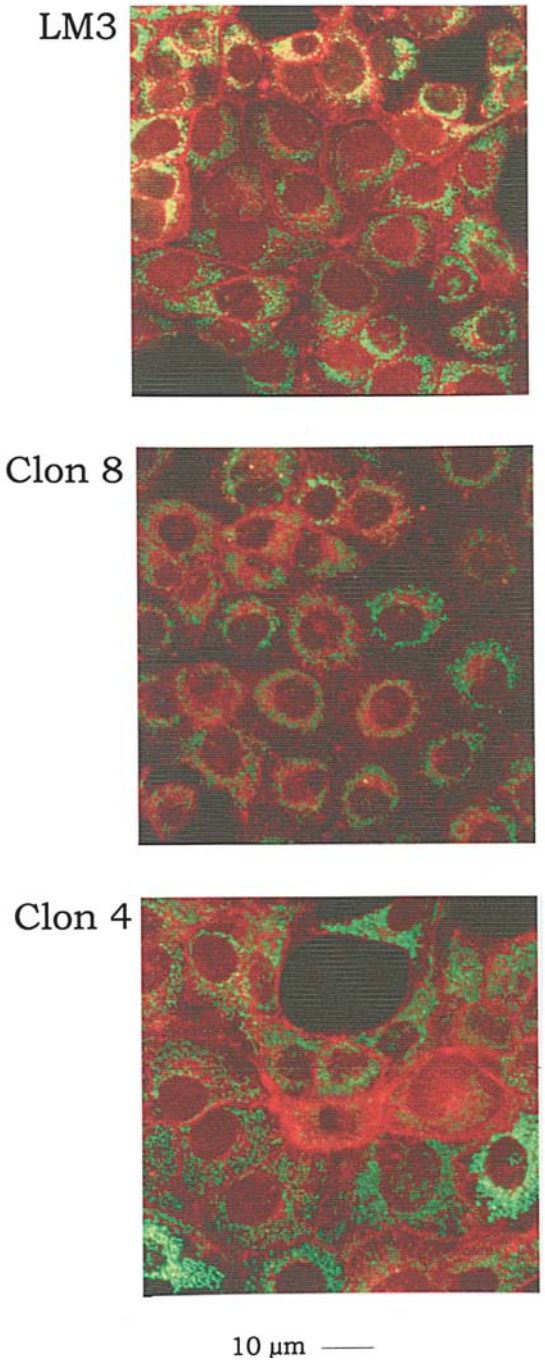

Figure 6. Two-color colocalization confocal micrographs of cells exposed for 3 h to 0.6 mM ALA (ALA-PDT conditions) and for 30 min to 10 nM Rh123. Red channnel, porphyrin fluorescence. Green channel, Rh123 fluorescence. These micrographs are representative of those obtained from three independent experiments. Scale bar, 10 μm.

Apoptosis. We looked into the percentage of apoptotic (Pre-G_1) cells after ALA-PDT exposed to a light dose to produce either 50% or 20% cell death in the three cell lines. Under these conditions, we did not find any significant differences between the 3 lines (data not shown).

Cell adhesion-mediated drug resistance. Based on the observation that resistant clones spread more than the parental line, we tested the hypothesis that when ALA-PDT is applied to cells in suspension, the resistant cells would lose resistance. We found that for the three lines, the light dose necessary to induce cell killing was half of the dose that killed attached cells. However, no differences in the resistance indexes were found (data not shown). We also performed ALA-PDT in cells attached to fibronectin, but again we did not find differences in the resistant indexes of the clones (data not shown).

Cross-resistance. The ALA-PDT resistant clones were challenged against different treatments (Table IV). They were more sensitive to UV and hyperthermia, as well as to PDT with Benzoporphyrin derivative and Merocyanine 540.

On the other hand, a slightly higher degree of resistance was found when exogenous PpIX was employed. Resistance indexes were 1.46 and 1.6 respectively employing 2.5 μM PpIX. When ALA derivatives such as Hexyl-ALA and Undecanoyl-ALA were employed, the resistance indexes did not change as compared to ALA.

Intracellular accumulation of Benzoporphyrin derivative, Merocyanine 540 and exogenous PpIX expressed per protein was equal in both parental and resistant clones. In addition, fluorescence microscopy of cells exposed to exogenous PpIX revealed colocalization with Rh123 and Lisotracker for the three lines, and no differences among cells were found (data not shown).

Discussion

The differences in porphyrin synthesis between parental and resistant lines expressed per protein may at least partially account for the resistance. This means that less porphyrins are available to target the same amount of proteins and, as it has already been demonstrated, proteins are a target for PDT (21). If the amount of porphyrins and not the target molecule is the limiting factor in photodamage, this feature can lead to development of resistance. Similarly, plasma membrane is the main target for PDT damage (22) and since larger cells have a greater surface area, the treatment could be less effective in the resistant clones.

Although the increase in cell size is not a common feature for induced resistance to chemotherapy, this has occasionally been observed (23). However, it might be a common feature in PDT resistance, as two reports on Photofrin-PDT resistance, describe an increase in cell volume (17), greater cellular and nuclear areas, increased cell spreading (24) and increased protein content (17,24).

As LM3 cells grow in clusters, with less spreading, their membrane contacts increase. The bystander effect already described for ALA-PDT (25) should be more marked for LM3 more packed cells.

In addition, the type of porphyrin appears to be crucial, since we also found a decreased content of PpIX in the resistant clones together with an increased proportion of hydrophilic porphyrins. It is believed that the hydrosoluble porphyrins are not such good photosensitizers as PpIX, although this is difficult to evaluate because they are poorly taken up by the cells (26). In addition, different subcellular localization of these hydrophilic porphyrins may also contribute to the resistance.

Exogenous PpIX induces similar accumulation on a per protein basis in resistant and parental lines but the clones remain partially resistant to exogenous PpIX. This suggests

that the photosensitizer:protein ratio and the type of porphyrin are not the only parameters involved in the resistance.

Flow cytometry analysis as well as confocal microscopy of cells exposed to ALA, in contrast to porphryin extraction data, showed that LM3 has higher porphyrin fluorescence. To analyze these differences, we performed excitation spectra of Uroporphyrin and Coproporphyrin solutions at pH 7.4 and found that 488 nm excitation induces a weak fluorescence signal, whereas PpIX fluorescence was almost maximal. This means that we are only looking at PpIX content and not total porphyrins when exciting the sample with 488 nm for confocal microscopy or flow cytometry.

It has already been demonstrated that ALA derivatives are incorporated into the cells by different transport mechanisms (27). The fact that the resistance was not overcome when pro-ALAs were employed reveals that ALA uptake into the cell is not related to the resistance mechanism.

Detoxification by glutathione conjugation has been correlated with drug resistance in cancer (28). In the present study, it is difficult to evaluate the impact of GSH due to the different protein content of the resistant lines. Since GSH is a non-specific protector, the same degree of protection should be exerted with any photosensitizer, but this did not happen. However the ratio of GSH:endogenous porphyrins is higher in the resistant clones, and so is the ability to detoxify cytotoxic species per molecule of sensitizer.

The activity of mitochondrial dehydrogenases can be functionally affected by PDT *in vitro* (21). Cells with a higher mitochondria number could be selected in the development of resistance. The higher mitochondrial area in our resistant cells probably correlates with a higher energy requirement and a consequent higher oxygen consumption. It is noteworthy that, although the number of mitochondria is higher in the resistant cells, PpIX production is not increased.

Preliminary *in vivo* results show that, after subcutaneous injection of clones to mice, tumor growth is delayed compared to LM3, and both clones show early necrosis; factors that can be related to their increased oxygen consumption. According to our results, not only PDT can induce *in vivo* chronic hypoxia due to vascular shut-down but, also, surviving cells may be hypoxic by an independent mechanism.

These data are relevant in terms of which combination therapy one should choose. Since tumor hypoxia may impair the efficacy of cytostatic drugs but particularly radiotherapy (29), those hypoxic cells can be also preferential targets of bioreductive drugs and hypoxia-directed gene therapy (30,31).

ALA-PDT-resistant cells are slightly more sensitive to PDT with Mercocyanine 540 and Benzoporphyrin derivative and they were, in general, more sensitive to hyperthermia and UV treatment. We have not addressed the reason for the increased sensitivity to these unrelated therapies; however, this aspect can be exploited for combining ALA-PDT with local hyperthermia.

We demonstrated that resistance to PDT can be induced even when employing an endogenous compound as photosensitizer. The tightly controlled heme pathway can be altered by exposing the cells to a selective pressure, thus leading to a different pattern of porphyrin synthesis, whereas the total amount of porphyrins per cell was not changed. We have thoroughly characterized these ALA-PDT-resistant clones and concluded that PDT resistance is a complex phenomena and that multiple factors appear to be involved. The lack of cross resistance to most exogenous photosensitizers suggests that the selective pressure may be almost exclusively related to the photosensitizer and not to unspecific free radical reactions.

An application of ALA-PDT-resistant clones will be in examining their different metastatic potential. Isolated clones exhibited a more fibroblastic, dendritic pattern, and higher cell spreading, which lead us to think that they could present different adhesive, invasive or metastatic phenotypes. A reduction of metastasis has been reported *in vivo* after PDT compared to after surgery (32). In addition, Momma *et al* (33), employing an orthopic prostate tumor model, showed that PDT combined with surgery produced a significant reduction in frequency and number of distant metastasis. Future studies will include characterization of metastatic potential of Clones 4 and 8 and cross resistance to cytostatic drugs and to ionizing radiation.

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