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Isolation of Cellular Clones of Murine Melanoma Resistants to the Photodynamic Therapy and Characterization of Some Mechanisms Involved in the Radioresistance

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1. Introduction

In the world, the skin cancer is by far the most common of all cancers. Melanoma accounts for less than 5% of skin cancer cases but causes 75% of skin cancer deaths (ACS, 2011). 132,000 melanoma skin cancers occur globally each year (WHO, 2011). In Mexico there is an evident increase of this neoplasia, with a growth of almost 500% in the last few years (Hernandez, 2005). The malignant melanoma is the most lethal of all skin cancers, due to its great capacity to produce metastasis and its chemotherapy-resistant properties that stand as challenging barriers to successful treatment (Kim, et al., 2010). For melanoma there is not an effective treatment, in the 95% of cases surgery is the choice therapy. Other alternatives included chemotherapy, immunotherapy, radiotherapy and a combination of these. Another strategy being explored is photodynamic therapy (PDT), a non-invasive selective therapy that opens new perspectives on the treatment of cancer. PDT emerges like a hopeful treatment for cancer and other diseases (Paras, 2003), is a low invasive treatment based on photosensitizer drugs that are administrated to the patients and can be retained on a selective mode for the ill tissue more than the normal tissue (Mang, 2004). This therapeutic procedure has been applied in the treatment of many hyper proliferative diseases of keratinocytes (Bugaj et al., 2004).

The action of photodynamic therapy is based on the use of visible light or near infrared, a photosensitizer and the presence of molecular oxygen (O_2) on transformed cells (like tumor cells); light is absorbed for the photosensitizer and this action is used to activate the oxygen (Bonnett, 2000) present into cancerous cells and to generate reactive oxygen species (mainly

singlet oxygen), that are potentially cytotoxic causing lethal damage to the cell and/or inhibits angiogenesis (Bugaj et al., 2004). Various photosensitizing drugs have been developed, such as 5-aminolevulinic acid (ALA) that unlike other PDT drugs is not a photosensitizer. When taken up by cancerous cells it is converted by a naturally occurring biosynthetic process into the photosensitizer protoporphyrin IX (PpIX). It is known that PpIX is present at low concentrations in normal cells and high concentration in tumor cells, the enzyme ferrochelatase, which converts protopophyrin IX to heme, has been found to be reduced, whereas the opposite situation has been found for porphobilinogen deaminase (Van-Hillegerberg et al., 1994). ALA can be applied topically, and was approved by the FDA in 1999 for the treatment of actinic keratosis (Zhu & Finlay, 2008). In dermatology the use of ALA has had good results on superficial tumors, however, this treatment is not recommended for pigmented tumors, like melanoma (Juzenas et al., 2002). There are many hypotheses where this problem has been discussed; maybe the presence of melanin on tumoral cells absorbs the light use on PDT on the tumor (Sheleg et al., 2004) in addition it has been considered an interaction between melanin and porphyrins, this can reduce the absorbance and generate non fluorescent compounds. However, there is little information available about PDT efficiency using ALA and derivates on the treatment of melanoma (Juzenas et al., 2002).

The aim of the present study was to obtain resistant clones of murine melanome by several photodynamic treatments and to study some factors that could be involved in the PDT-resistant such as: biosynthesis capacity of PpIX stimulated by ALA, concentration melanin and mitochondrial activity.

2. Material and methods

2.1 Cell lines and culture conditions

Murine melanoma cells B16F0 (metastatic melanoma, number CRL-6322, ATCC) and B16F10 (number CRL-6475, ATCC) both isolated from the primary tumor C57BL/6J were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/mL of penicillin and 100 U/mL of streptomycin. Cells were cultured at 37 °C in 5% CO_2.

2.2 Determination of intra and extra-cellular PpIX induced by ALA

Cells were plated into 6-well plates at a cell density of 8×10^5 per well in 2 mL of DMEM supplemented with 10% FBS. After 24 h the medium was removed and cells were exposed to 0, 25, 50, 75, 100 and 150 µg of ALA/mL of serum-free medium for 4 h in darkness at 37ºC. Afterward, the medium was removed and collected in centrifuge tubes that contained 200 µL of celita in 5% saline to its subsequent analysis. The cells were washed with saline phosphate buffer (PBS, pH 7.4) and detached with 500 µL trypsin/EDTA. Following cells were spun at 200xg, supernatant was removed and cells were rinsed once in PBS, then collected by centrifugation and lysed in 0.2 mL of 5% celite. To quantify the intra and extra-cellular PpIX it was used the Piomelli spectrofluorometry assay for free erythrocyte porphyrins (Piomelli, 1973) as adapted for cultured cells was used to quantify PpIX (Ramón et al., 1999). PpIX levels were read directly as µg/cells using a Perkin Elmer LS-2B spectrofluorometer calibrated with coproporphyrin I (0.05 µg/mL; Sigma) stock solution (excitation wavelength 408 nm, emission 608 nm). All assays were performed in triplicate. A blank was prepared in parallel by replacing the cellular suspension with 40 µL of saline solution.

2.3 Photodynamic treatment

30,000 cells/well were seeded in 96 well plates and these were exposed to 0, 25, 50, 75, 100, 125, 150 µg of ALA/mL of culture medium serum free for 4h. This proceeding was applied to the parental lines, B16F0 and B16F10, and irradiated with an argon laser with 70 mW of power and with a light dose of 64.3 J/cm². The used groups were: negative control (cells without ALA and irradiation), irradiation control (irradiated cells only), and treated cells group (cells with ALA and irradiation). In all the experiments the B16F0 and B16F10 cells were conserved in darkness before and after irradiation.

2.4 Cell survival rate by red neutral-red assay

After exposing cells to the different conditions mentioned above cell viability was measured by neutral-red spectrophotometric assay (Borenfreud and Shopis, 1985). The medium containing ALA was removed from the wells and replaced with 100 µL of fresh medium per well containing 100 µL of neutral red. The plates were then returned to the incubator for 3 h. Medium was subsequently removed, and the cultures were washed rapidly with a mixture of 40% formaldehyde and 10% $CaCl_2$ V/V (4:1). A mixture of 1% V/V acetic acid and 50% V/V ethanol (1:1) was then added to extract the neutral red. The plate was shaken for 60 s and left to stand at room temperature for 15 min. The absorbance of the solubilized dye was subsequently read at 540 nm. Quantification of the extracted dye was correlated with the live cell number. Control wells were prepared in parallel, and these cells were exposed to neutral red, but not to ALA. The percentage of viable cells in the cell population at each concentration of the test agent was calculated by means of the equation 1.

$$\% \text{ viability} = \frac{\text{Mean absorbance of treated cells}}{\text{Mean absorbance of control cells}} \times 100 \tag{1}$$

2.5 Obtainment PDT-resistant clones (PDTR)

B16F0 and B16F10 cells were seeded into each well of a 96-well plate before treatment. Cells were incubated for 24 h following administration of ALA at a concentration of 125 and 150 µg/mL respectively, in serum-free medium. After, they were exposed to PDT with a light dose of 64.3 J/cm² in one exposition. 24h after irradiation, cells were harvested with EDTA-trypsin and centrifuged for 2 min at 224xg. The supernatant was discarded and cells were resuspended in 1.0 mL of medium supplemented with FBS and plated in a culture bottle. In parallel one experiment was realized with the same conditions utilizing a light dose of 64.3 J/cm². In both cases the viability was measured with neutral red method.

2.6 Melanin determination

This quantification was performed for all cell lines. Cells were exposed to their respectively ALA concentration and incubated for 4 h. Medium was recovered for measure extracellular melanin and cells were washed with PBS and detached with trypsin-EDTA, this solution was quenched after 15-20 min with 1 mL of DMEM supplemented with 10% FBS. Cells were centrifuged at 224xg and washed with 1 mL PBS, resuspended in 1 mL NaOH 1M in DMSO 10% and incubated at 80ºC for 2h. It was centrifuged at 224xg and supernatant was recovered to measure intracellular melanin. A blank of 1M NaOH in DMSO 10% was prepared in parallel. Absorbance was measured at 420 nm, the amount of melanin was expressed in

absorbance units (a.u.) per cell number, according to the methodology described by Rad (Rad, et al., 2004).

2.7 Assessment of mitochondrial activity by MTT assay

The mitochondrial activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mossman, 1983). The cell lines B16F0, B16F10 and cellular clones PDT-resistant were exposed to 0, 25, 50, 75, 100, 125, 150 µg of ALA/mL for 4 h. Then, the medium containing ALA was removed from the wells and replaced with 100 µL of MTT reagent diluted in DMEM medium serum-free (25 µg/mL) at 37°C. After 4h, the obtained formazan products were reconstituted in 0.04 N HCl in isopropyl alcohol. The reduction of MTT by the viable cells was measured by an absorbance maximum at 540 nm.

2.8 Determination of mitochondrial abundance by MitoTracker Green FM®

In order to evaluate the mitochondrial abundance were used the MitoTracker Green FM® selective probes (Invitrogen). It was used according to manufacturer´s conditions. Fluorescence was observed in a Nikon EFD-3.

2.9 Statistical analysis

Two-way analysis of variance (ANOVA) was performed for all the assessments made. In the case of the determination of mitochondrial abundance the Mann-Whitney rank-sum test was performed. The level of significance was set at $P < 0.05$. The tests were performed with SigmaStat version 3.1 for Windows (Jandel Scientific).

3. Results

3.1 Obtainment of PDT-resistant clones (PDTR)

Before of obtaining PDT-resistant melanoma clones from parental melanoma cells it was necessary to know the ALA concentration that induces the maximum intracellular accumulation of PpIX and afterwards to find this information in the PDT-resistant melanoma clones.

3.2 Intra and extracellular PpIX accumulation induced by ALA for 24 hours in B16F0 and B16F10 with their clones PDT-resistant

It was found that all ALA concentrations tested (25, 50, 75, 100, 125 and 150 µg of ALA/mL) induced significatively the intracellular accumulation of PpIX in comparison to the basal level (0 mg of ALA/mL) (Table 1). B16F10 accumulates 43.0 times more PpIX than basal level of B16F0, in basal conditions, did not accumulate PpIX but its concentration reached up to 2 µg of PpIX/$8x10^5$ cells and when it is compared with their intracellular PpIX concentration (1.64 µg/$8x10^5$ cells) at lest ALA concentration is possible to found that B16F0 accumulates 1.4 times. From this results it was selected the ALA concentration for PDT application; for the B16F0 was 125 µg/mL and for B16F10 was 150 µg/mL. The low level of extracellular PpIX (Table 1) shows that the majority of PpIX is keep into the cells, this is important because the PpIX acts intracellularly as a response to PDT. The B16F0 cells accumulate from 2 to 9 times more PpIX than B16F10 at ≤125 µg/mL of ALA (Figure 1A).

After obtaining cellular PDT-resistant clones the parental cells were irradiated at 64.3 J/cm² with a concentration of ALA of 125 μg/mL for B16F0 and 150 μg/mL for B16F10. Resistant cells are the ones that survive to this irradiation conditions. To determinate if PpIX accumulation were accumulated in PDT-resistant clones a similar experiment was performed (Table 2). B16F0-PDTR cells in basal condition accumulated PpIX (Tables 1 and 2) and when these cells were exposed to ALA at minimal concentration tested (25 μg/mL) accumulated 11 times more PpIX in comparison to basal level and 30 times at 125 μg/mL of ALA. B16F10-PDTR has a similar response; in basal conditions has 3 times more PpIX than B16F10, but when this is compared with ALA at 125 and 150 μg/mL intracellular PpIX concentration increases 44 and 16 times more, in comparison with the basal level. Therefore, in the PDT-resistant clones obtained (B16F0-PDTR and B16F10-PDTR) it can be seen that these when exposed to ALA accumulated the highest concentration of PpIX in comparison to parental melanoma cells, in the other hand using low ALA concentration is possible to obtain high levels of intracellular PpIX. The B16F0-PDTR cells accumulated from 2 to 8 times more PpIX than B16F10-PDTR at ≤150 μg/mL of ALA (Figure 1B).

ALA (μg/mL)	PpIX in B16F0		PpIX in B16F10	
	Intracellular (μg/8x10⁵ cells)	Extracellular (μg/mL of culture medium)	Intracellular (μg/8x10⁵ cells)	Extracellular (μg/mL of culture medium)
0	0.00± 0.001	0.011 ± 0.001	0.06 ± 0.02	0.011 ± 0.000
25	1.64 ± 0.11	0.038 ± 0.001	0.18 ± 0.02	0.041 ± 0.004
50	1.89 ± 0.10	0.051 ± 0.001	0.35 ± 0.02	0.037 ± 0.001
75	2.24 ± 0.07	0.058 ± 0.004	0.53 ± 0.00	0.045 ± 0.001
100	2.04 ± 0.06	0.058 ± 0.003	0.99 ± 0.07	0.057 ± 0.003
125	2.21 ± 0.04	0.062 ± 0.001	1.46 ± 0.13	0.057 ± 0.002
150	2.00 ± 0.19	0.071 ± 0.000	2.45 ± 0.02	0.062 ± 0.004

Table 1. Intra and extracellular PpIX in B16F0 and B16F10 cells exposed to different ALA concentration for 24h

ALA (μg/mL)	PpIX in B16F0-PDTR		PpIX in B16F10-PDTR	
	Intracellular (μg/8x10⁵cells)	Extracellular (μg/mL of culture medium)	Intracellular (μg/8x10⁵cells)	Extracellular (μg/mL of culture medium)
0	0.20 ± 0.00	0.017 ± 0.001	0.19 ± 0.00	0.022 ± 0.00
25	2.21 ± 0.02	0.046 ± 0.002	0.28 ± 0.00	0.051 ± 0.004
50	4.42 ± 0.17	0.054 ± 0.004	2.41 ± 0.1	0.073 ± 0.002
75	5.46 ± 0.35	0.054 ± 0.000	4.88 ± 0.19	0.074 ± 0.004
100	5.44 ± 0.47	0.059 ± 0.002	5.51 ± 0.47	0.086 ± 0.004
125	5.98 ± 0.09	0.057 ± 0.001	8.39 ± 0.09	0.081 ± 0.001
150	6.12 ± 0.22	0.060 ± 0.004	3.11 ± 0.09	0.084 ± 0.001

Table 2. PpIX accumulation in B16F0-PDTR and B16F10-PDTR clones exposed to different ALA concentration for 24h

The ALA dose that induced the highest accumulation of intracellular PpIX in the cell line B16F0-PDTR was of 150 μg/mL (6.12 ± 0.22μg / 8×10^5 cells). Two-way ANOVA statistical analysis showed a significant difference in PpIX content between the lines B16F10 and B16F10-PDTR ($p<0.05$) starting from 25 μg/mL of ALA (Fig. 1-A). With regard to B16F10-PDTR PpIX levels obtained were higher compared with the B16F10 (Fig. 1-B) and also with those obtained in B16F0 or B16F0 PDTR. The highest level of accumulation of PpIX in B16F10-PDTR was 8.39 ± 0.09 with 125 μg/mL of ALA, this level is four times higher than that obtained with 150 μg/mL in B16F10.
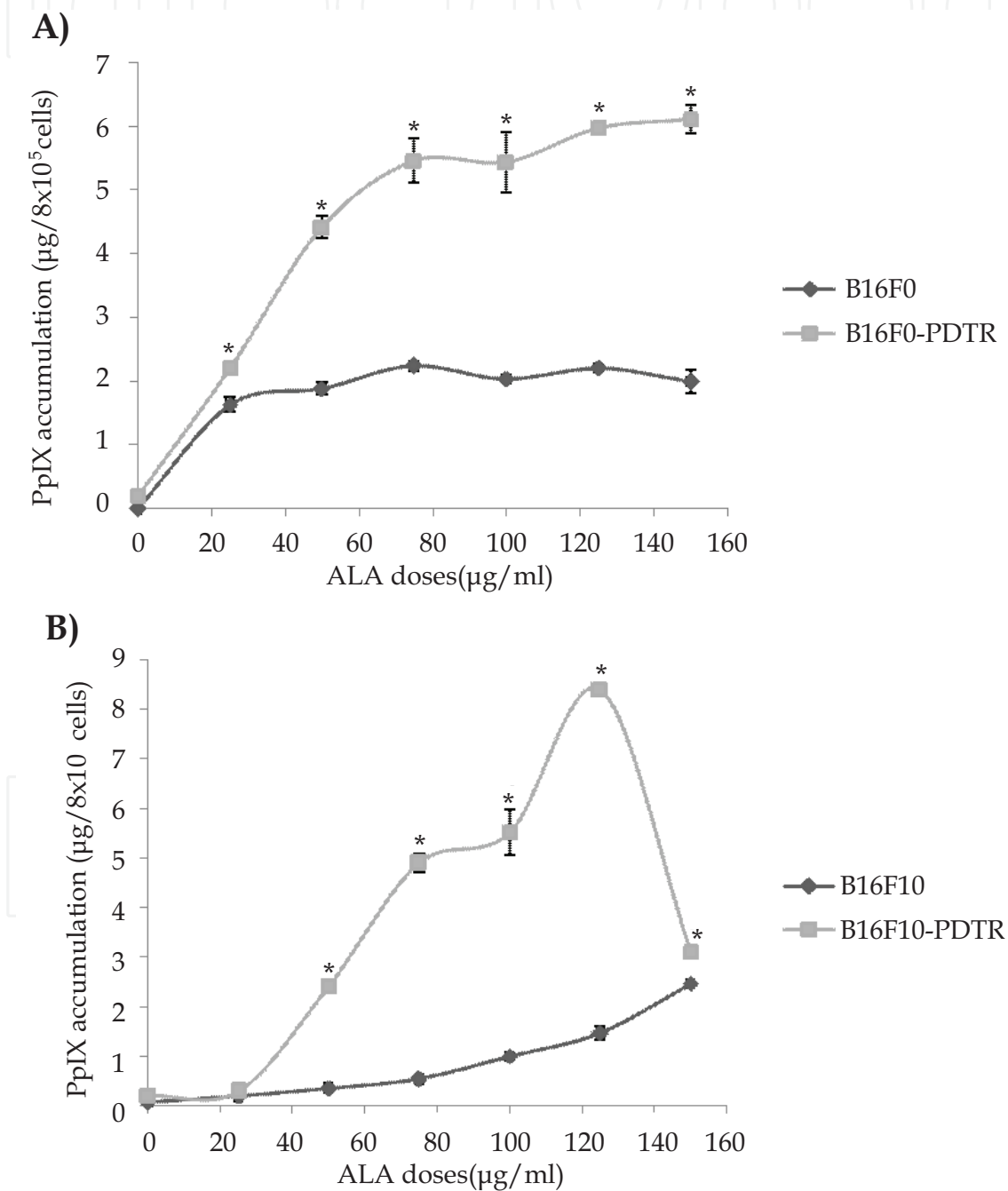
Fig. 1. Comparison of the accumulation of PpIX between B16F0 and B16F0-PDTR (A) and between B16F10 and B16F10-PDTR (B), * p < 0.05

3.3 PDT effect on cell mortality in different cell lines

The evaluation of cytotoxicity of PDT in the four cell lines was evaluated by neutral red technique. The results are expressed as percentage of mortality at different doses of ALA (125 and 150 µg/mL) exposed to a light doses of 64.3 J/cm^2 of irradiation. For B16F0 and B16F0-PDTR clone the mortality values ranged from 95-97%. B16F0-PDTR had a higher sensitivity to PDT in comparison with B16F0 ($p < 0.05$). With regards to B16F10 and B16F10-PDTR the mortality index, as well as the previous cell lines, was higher (96±0.3% to 100%) in B16F10-PDTR. The PDT had a higher phototoxic effect when it was compared with B16F10, B16F0 and B16F0-PDTR (Fig. 2).
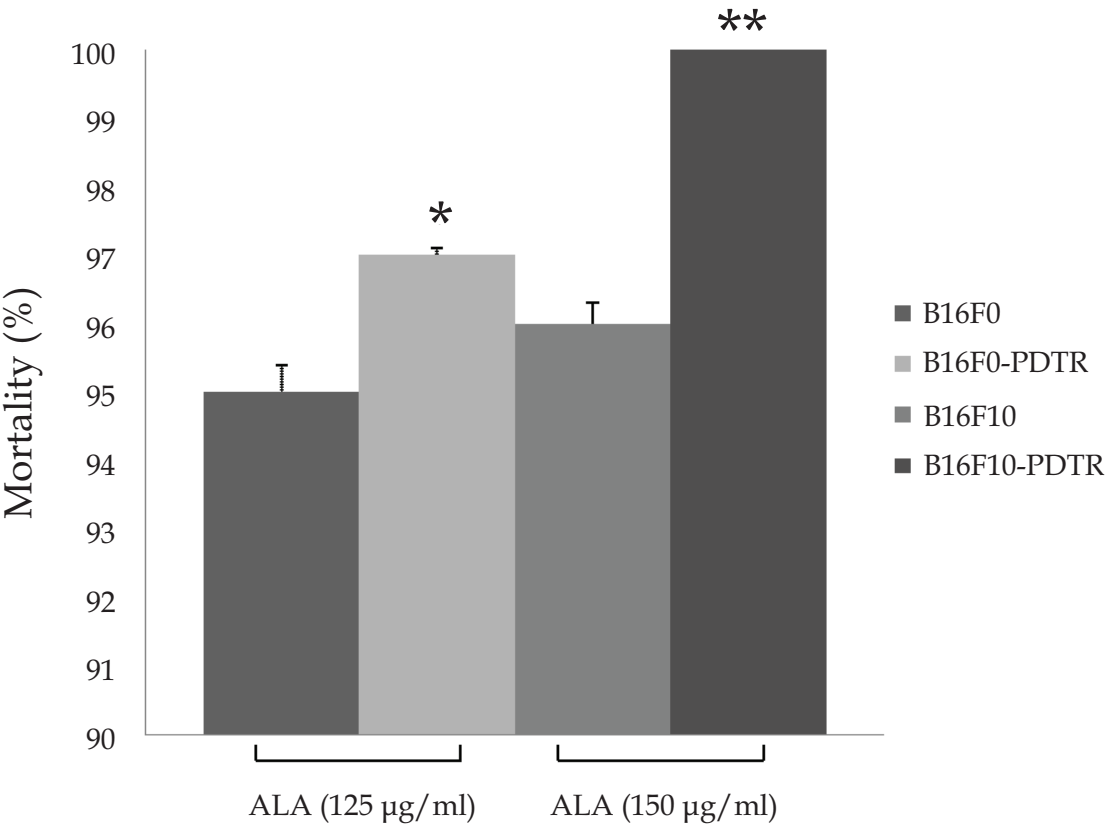

Fig. 2. Mortality percentage in the four cell lines exposed to a light doses of 64.3 J/cm^2. The four cell lines were treated with ALA at different doses; afterwards they were irradiated with 64.3 J/cm^2. *($p < 0.05$) when comparing B16F0-PDTR with B16F0 and B16F10-PDTR with B16F10

ALA (µg / mL)	Mortality (%)							
	15 J/cm^2				64.3 J / cm^2			
	B16F0	B16F0-PDTR	B16F10	B16F10-PDTR	B16F0	B16F0-PDTR	B16F10	B16F10-PDTR
0	0	0	0	0	0	0	0	0
125	44.39±3.6	85.31±0.43	_	_	95.0±0.4	97±0.1	_	_
150	_	_	91.00±0.51	96.82±0.24	_	_	96±0.3	100

Table 3. Percentage of mortality in the four cell lines with a light dose of 15 and 64.3 J/cm^2
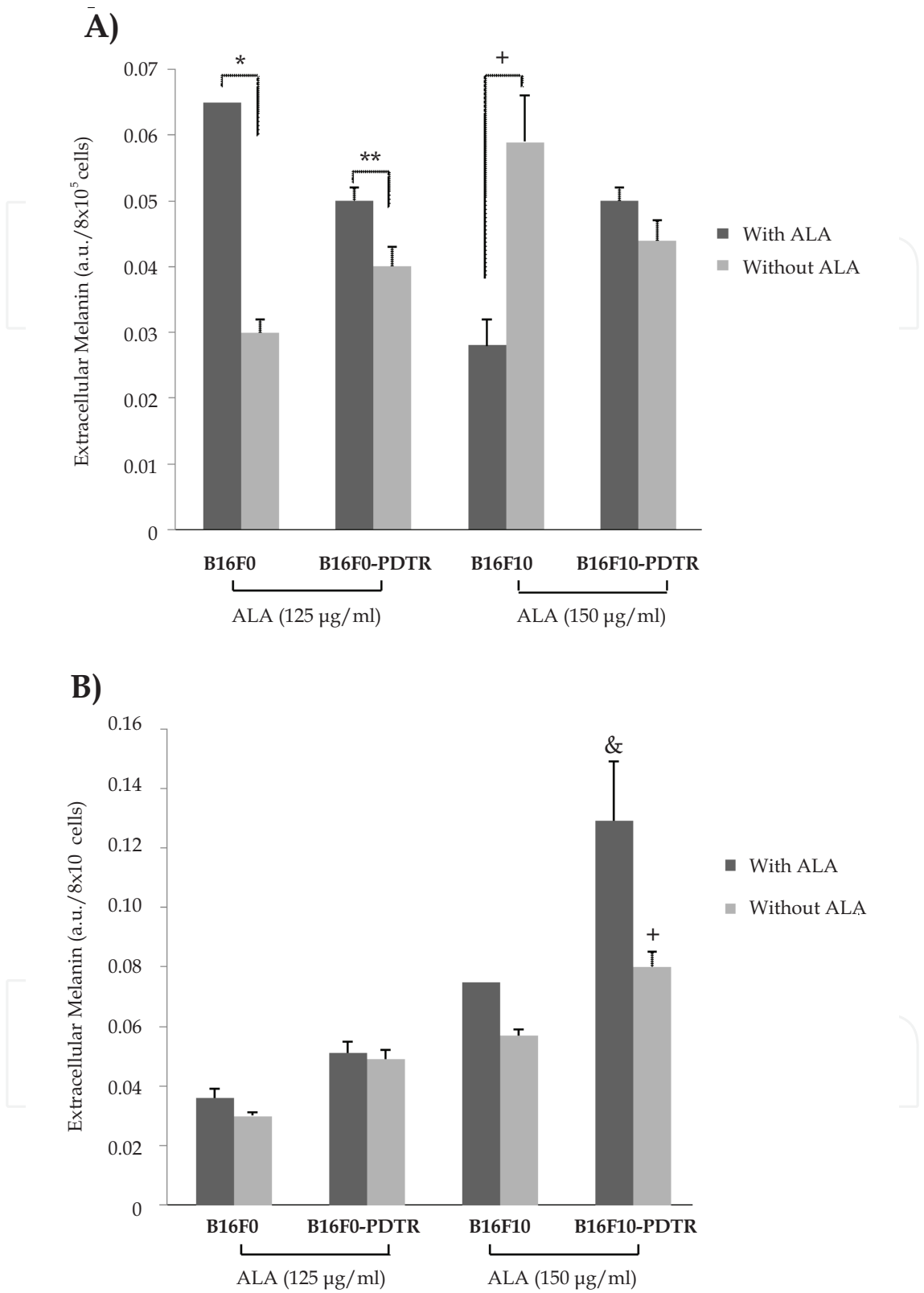
Fig. 5. Melanin concentration in four cell lines with and without ALA. A) Extracellular,
B) Intracellular *(p<0.05) when comparing B16F0 without ALA, ** (p<0.05) when comparing
B16F0-PDTR with B16F10-PDTR without ALA, +(p<0.05) with B16F10 with ALA, &(p<0.05)
with B16F0, B16F0-PDTR and B16F10

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The effectiveness of PDT on *in vitro* murine melanoma lines have been previously reported with a light dose of 15 J/cm^2 or minor (Vena, et al, 2004), thus in this work it was decided to find if the four lines used suffer changes in its sensitivity to PDT using a ALA dose of 125 and 150 µg/mL with a irradiation of 15 J/cm^2 (Table 3).

The data observed in table 3 are similar to the ones obtained with an irradiation of 64.3 J/cm^2. B16F10-PDTR was the most sensitive cell line with a mortality rate of 96.82± 0.24 and B16F0 had the highest survival capacity (55.61%).

3.4 Concentration of extra and intracellular melanin

One of the possible mechanisms involved in the resistance to PDT is the melanin content in the cells, therefore a comparative analysis was performed between original lines and resistant clones at two levels (intracellular and extracellular). Results showed significant differences in melanin content (Fig. 5). It was observed a major concentration of intracellular melanin. The extracellular melanin content showed that B16F0 accumulates higher concentration in comparison with B16F0-PDTR (0.065 and 0.050±0.002 a.u./8x10^5 cells), in addition B16F10-PDTR accumulates a higher concentration of melanin than its parental line (0.050±002 and 0.028±0.004 a.u./8x10^5 cells) (Fig. 5-A). Resistant cell lines accumulate a higher intracellular concentration than their parental cell line. B16F10-PDTR was the cell line that accumulates the highest intracellular melanin concentration (Fig. 5-B). The presence of ALA does not interfere with the intracellular melanin contain.

3.5 Determination of mitochondrial activity by MTT assay

Another variable to be analyzed was the determination of mitochondrial activity in order to study functional changes in the mitochondria caused by ALA through MTT assay. The
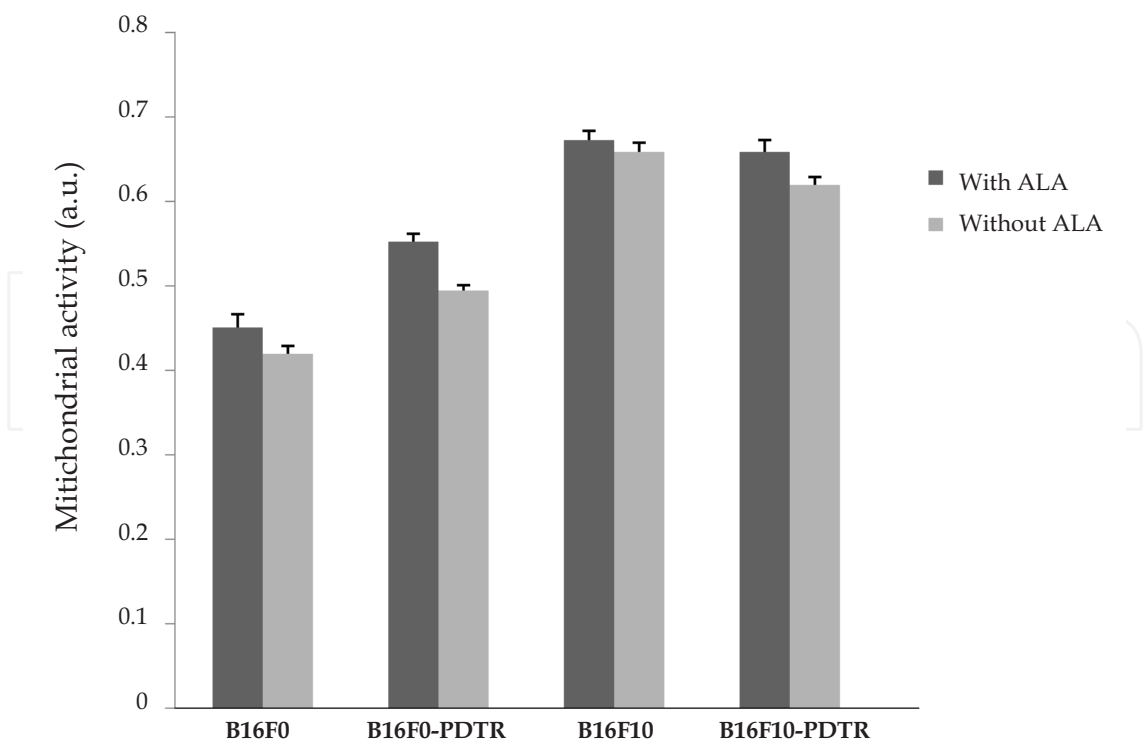

Fig. 6. Mitochondrial activity evaluated by MTT assay for B16F0, B16F0-PDTR, B16F10 and B16F10-PDTR

mitochondrial activity was evaluated in the four cell lines. The results showed an increment in the level of mitochondrial activity in the four lines when exposed to ALA, being more evident for B16F10 and its clone in comparison with B16F0 or B16F0-RPDT (Fig. 6).

The statistical analysis of mitochondrial activity between B16F0 and B16F0-RPDT showed significant differences (p<0.05). B16F0-PDTR showed a higher mitochondrial activity than B16F0 with or without ALA treatment. With regard to B16F10 and B16F10-RPDT lines, statistical analysis by two-way ANOVA only showed a significant difference between them without ALA (p<0.05), in addition B16F10-RPDT had a higher mitochondrial activity than B16F10, however they did not show significant differences in the treatment with ALA.

3.6 Mitochondrial abundance determination using fluorescent probes

As can be observed in table 4 B16F0 showed a higher mitochondrial light intensity (47.14±1.40) than the other three cell lines follower by B16F10-PDTR (45.01±1.04), B16F10 (34.75±0.71) and finally B16F0-PDTR (31.23±0.77) (Table 4).

Fluorescent probe	Light Intensity (%)			
	Cell line			
	B16F0	B16F0-PDTR	B16F10	B16F10-PDTR
MitotrackerGreen FM	[a]47.14±1.40	[a] 31.23±0.77	[b]34.75±0.71	[b] 45.01±1.04

Table 4. Mitochondrial abundance in cell lines measured by fluorescent probes. [a]26 fields, [b]30 fields
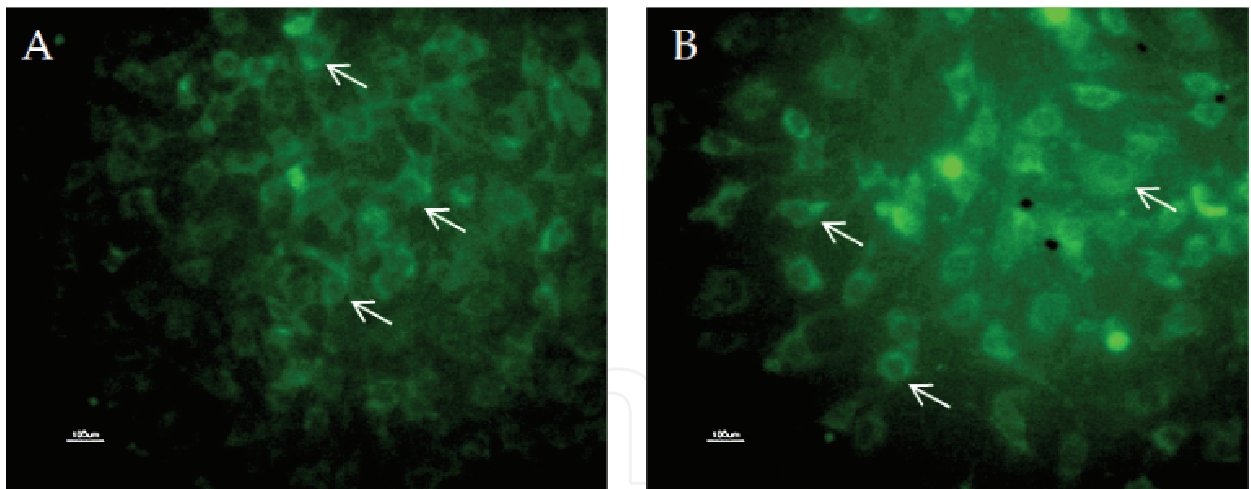

Fig. 7. Shows a representative image of the mitochondrial abundance in B16F10 (A) and B16F10-PDTR (B). The resistant cells B16F10-PDTR show a high light intensity compared with original cells, this suggests that there are a great number of mitochondria in these cells that could be involved in the process of resistance to PDT. Arrows indicate cells with fluoresce

3.7 Global analysis between the Photodynamic effect on parental melanoma cells and their resistant clones and the PpIX, melanin concentration, mitochondrial activity and mitochondrial concentration

A global analysis of the results is shown in table 5. The photodynamic effect can be observed by means of cellular death using two light doses (16 and 64.3 J/cm²) and by observing that

the cells that survive the first irradiation (B16F0-PDTR and B16F10-PDTR) when are irradiated for the second time become more sensitive; probably this effect is not clearly seen when it is used the wave length of 64.3 J/cm^2, but when it is used a wave length of 16 J/cm^2 the mortality in the resistant cells of the line B16F0 exposed to µg ALA/mL doubles in the resistant line, it as from 44.39 % of mortality to 85.31 %, this correlates with the concentration of extracellular melanin, it increases significantly when cells are exposed to ALA. Since it is so high the mortality in the line B16F10 this only increases 2 % and the extracellular melanin has no change. The mitochondrial activity increased significantly in the resistant clona B16F0-PDTR, whereas in the other lines though there is an increase from 10 to 20 % this one is not significant. In addition it can be observed that the concentration of PpIX increased in resistant cells.

Cell line	ALA (µg ALA/mL)	Intracell PpIX µg/8x10^5 cel	PpIX ratio	Photodynamic effect (% Mortality)		Melanin concentration (a.u./8x10^5 cells)		Mito-chondrial activity	Mito-chondrial abundance
				16 J/cm^2	64.3 J/cm^2	Intra-cellular	Extra-cellular		
B16F0	0	0 [d]	-	0	0	0.03	0.03 [a]	0.43	47
	125	2.21 [d]	1.2	44.39	95	0.039	0.07 [a]	0.45	-
B16F0-PDTR	0	0.20 [e]	-	0	0	0.048	0.04 [b]	0.48 [c]	31.23
	125	5.98 [e]	30	85.31	97	0.05	0.06 [b]	0.65 [c]	-
B16F10	0	0.06 [f]	-	0	0	0.06	0.06	0.65	34.75
	150	2.45 [f]	40	95	96	0.65	0.03	0.68	-
B16F10-PDTR	0	0.19 [g]	-	0	0	0.08	0.045	0.63	45
	150	3.11 [g]	16	97	100	0.12	0.05	0.65	-

PpIX ratio=[PpIX at 125 or 150 µg ALA/mL]/[PpIX at 0 µg ALA/mL]. [a,b,c,d,e,f,g] $p<0.01$

Table 5. Global analysis between the Photodynamic effect on parental melanoma cells and their resistant clones

4. Discussion

Malignant melanoma is one of the most aggressive cancer, in addition is resistant to the ordinary therapies (chemotherapy, immunotherapy and radiotherapy) (Gray et al., 2007), thus is necessary to look for new treatments; photodynamic therapy is a hopeful therapy that presents some advantajes over surgery and radiotherapy, however clinical results have been variable. The aim of this work was to evaluate the possibles mechanism involved in resistance to PDT of two differents resistant melanoma cell lines (B16F0-PDTR, B16F10-PDTR) derived from a invasive weakly and strong metastatic melanoma cell lines (B16F0 and B16F10).

In this work were obtained clonas of murine melanoma resistant to PDT as well as previous studies have obtained resistant clonas of colon adenocarcinome HT29-P14 to PDT (Hanlon et al., 2001) and mouse fibrosarcome Rif-8A (Di Prospero et al., 1997).

One of the mechanisms evaluated in this project was the concentration of intracellular PpIX because the amount of photosentizer induces the generation of reactive oxygen species (ROS) that leads to the increment cellular death. The results obtained showed that the PpIX is concentrated intracellularly and not extracellularly, also it was observed an accumulation of PpIX highly significant ($p<0.05$) in B16F0-PDTR compared with B16F0 in all ALA doses

evaluated. A similar behavior was observed in B16F10-PDTR from 50 µg/mL. The higher concentration of PpIX of isolated cells with regard to parental lines could be due to different factors such as the difference in the expression of enzymes involved in the hemo biosynthesis (Ruiz et al., 2007), which showed differences between retinoblastoma cell lines Y-79 and WERI-Rb-1, mediated RT-PCR found that protoporphyrinogen oxidase, uroporphyrinogen synthase, and 5-aminolevulinate synthase were over expressed, these enzymes favored the synthesis of PpIX. On the other hand, it has been reported that ferrochelatase enzyme is decreased in its activity in cancer cells; therefore this contributes to the intracellular accumulation of PpIX (Li et al., 2001). Another factor that allows the biosynthesis of PpIX is the assimilation of ALA like as it was observed in murine fibrosarcoma cells MethA (Ohgari et al., 2005). Acording with the results obtainned in this work the chossen concentration of ALA was 125 µg/mL to B16F0 and 150 µg/mL to B16F10. Another important factor that is involved in PDT is the light intensity, previous studies evaluated PDT with murine melanoma using 15 J/cm² or low doses (Inuma et al., 1994; Vena et al.2004), in this work it was compared the efficency of PDT using 15 J/cm² and 64.3 J/cm², our results showed that the use of a higher light dose increases cells mortality in the 4 cell lines tested, but the higher mortality was observed in isolated clones.

In this study was eliminated between 95-100% of melanoma cells and its clones, the most susceptible cells were the B16F10-PDTR, highly metastatic melanoma cells resisted the first application of the PDT, but were totally sensitively to the second irradiation.

Other parameter analyzed in the resistant to PDT was the melanin content. The results showed a higher intracellular accumulation of melanin. In relation with this its has been reported that PpIX can induced melanogenesis since PpIX is one activator of melanin synthesis through guanilate ciclase activation (Soo-Kyeong et al., 2005). Previous studies showed that pigmented melanoma does not has a good efficiency using PDT because the melanin interfere with the light absorption, as melanin absorbs and disperse light and decrees the production of ROS (Dea-Seon et al., 2004). Nevertheless the melanin that seems to be involved in the resistance in the melanoma cells studied in this work is extracellular, nevertheless the melanin that seems to be involved in the resistance of the melanoma cells studied in this work is the extracellular, probably this effect is not clear when it was used the density of energy of 64.3 J/cm², but when it was used a density of 16 J/cm² the cellular death in the resistant cells of the line B16F0 exposed to 125 µg ALA/mL doubles in the resistant line, goes from 44.39 to 85.31% of mortality.

With regard to the analysis of the concentration and mitochondrial activity, only the latter appears to be involved, for example in the table 5 it can be observed that this activity only increases significantly in the resistant clone B16F0-PDTR, while in other lines though there is an increase from 10 to 20% it is not significant ($p > 0.05$). Consequently, it could be carefully inferred that the increase in mitochondrial activity makes the cells susceptible to PDT only if there are significant amounts of PpIX and extracellular melanin.

5. Conclusion

The resistant clones (B16F0-PDTR, B16F10-PDTR) accumulate more PpIX than the parental lines (B16F0 and B16F10).

The factors involved in the resistance of melanoma cells are extracellular melanin (the higher the concentration of melanin the bigger the resistant to PDT), mitochondrial activity (the decrease of mitochondrial activity promotes resistance) and the concentration of PpIX

(the smaller PpIX there is less cell death). It seems that the irradiation stimulates changes in cellular metabolism related with the mitochondria that diminishes the resistance of melanoma cells, thus it would be recommendable irradiate the cells several times and at densities of 64.3 J/cm² or more. With the implementation of a laser of argon and irradiating to 64.3 J/cm² it was achieved a mortality between 95-100% of PDTR B16F0, B16F10-PDTR, B16F0 and B16F10 cells.

6. Acknowledgment

7. References

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Breakthroughs in Melanoma Research

Edited by Dr Yohei Tanaka

Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

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Ramón-Gallegos E., Rosas-Flores L., Barrera-Mendoza C.C., Herrera-Carrillo Z., González-Agüero G., Estrada-Manrique G. and Cruz-Orea A. (2011). Isolation of Cellular Clones of Murine Melanoma Resistants to the Photodynamic Therapy and Characterization of Some Mechanisms Involved in the Radioresistance, Breakthroughs in Melanoma Research, Dr Yohei Tanaka (Ed.), ISBN: 978-953-307-291-3, InTech, Available from: http://www.intechopen.com/books/breakthroughs-in-melanoma-research/isolation-of-cellular-clones-of-murine-melanoma-resistants-to-the-photodynamic-therapy-and-character

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