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Differential Response of BEAS-2B and H-441 Cells to Methylene Blue Photoactivation

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Abstract. *Background/Aim: Cancer incidence and mortalities are growing worldwide, therefore research and development of more effective and less invasive treatments, such as photodynamic therapy, are needed. Herein, we investigated the methylene blue (MB) photoactivation effects in lung epithelial cells (BEAS-2B) and lung adenocarcinoma cells (H-441). Materials and Methods: The reactive oxygen species (ROS) produced by the laser photoactivation of MB in aqueous solutions and cell cultures were measured with probes, and the cell viability was evaluated with a colorimetric assay. Results: MB up to 31.26 μM did not induce detectable effects in BEAS-2B cells. However, H-441 cells presented adverse effects below that concentration in the same range of fluencies studied. These results are in concordance with the ROS production in H-441 cells, while in BEAS-2B cells the production of ROS was less significant compared to the control. Conclusion: Photoactivation of MB at concentrations below 31.26 μM could be used for the selective treatment of H-441 cells over non-cancer cells.*

Research in photodynamic therapy (PDT) has been increasing as an alternative and promising treatment for cancer as well as unrelated diseases. PDT has been proved as a practical approach for the treatment of age-related macular degeneration (1, 2), microbial infections (3-5), atherosclerosis (6), and psoriasis (7). Additionally, research on the effectiveness of PDT in different types and locations of cancer has been reported (8-11). Methylene blue (MB) has been of great interest in PDT due to its ability to absorb light intensively within the therapeutic window, and upon radiation, it damages biomolecules by producing reactive oxygen species (ROS) (12). ROS are involved in several biological functions, but when over-produced, they can lead to cell death (13-16). ROS quantification could provide information concerning the effectiveness of PDT. Fluorescent probes, such as 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and 1,3-Diphenylisobenzofuran (DPBF), are used to determinate ROS in solutions and cell cultures due to their high sensitivity and facile determination (17-23). Previous studies of PDT using MB in squamous cell carcinoma have reported a decrease in tumor size and cell proliferation, and an increase in cytokine levels (11). Recently it was shown that the effect of photoactivated MB in non-malignant epithelial cell lines and different molecular subtypes of breast tumors had a higher impact on the malignant cell lines, without affecting non-malignant cells at a significant level (9). The objective of this study was to evaluate the MB photoactivation-induced differential response in lung epithelial (BEAS-2B) and lung adenocarcinoma cells (H-441). Our results indicate a differential response of MB photoactivation between BEAS-2B and H-441 cells.

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Materials and Methods

Determination of ROS in solutions. The photoactivation of MB was evaluated in aqueous solutions to correlate the concentration of MB and the energy fluence with the generation of ROS. Aqueous-DMSO solutions of 50 μM DPBF (Sigma-Aldrich, St. Louis, MO, USA) and 6.25 μM or 31.26 μM of MB (Química Suastes, Del Tlalpan, CMX, Mexico) were prepared. Solutions were irradiated every 2 seconds (s) with an energy fluence of 0.2 J/cm^2 using a red laser (660 nm, 100 mW). UV/VIS absorption spectra were collected using a Genesis 10S spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

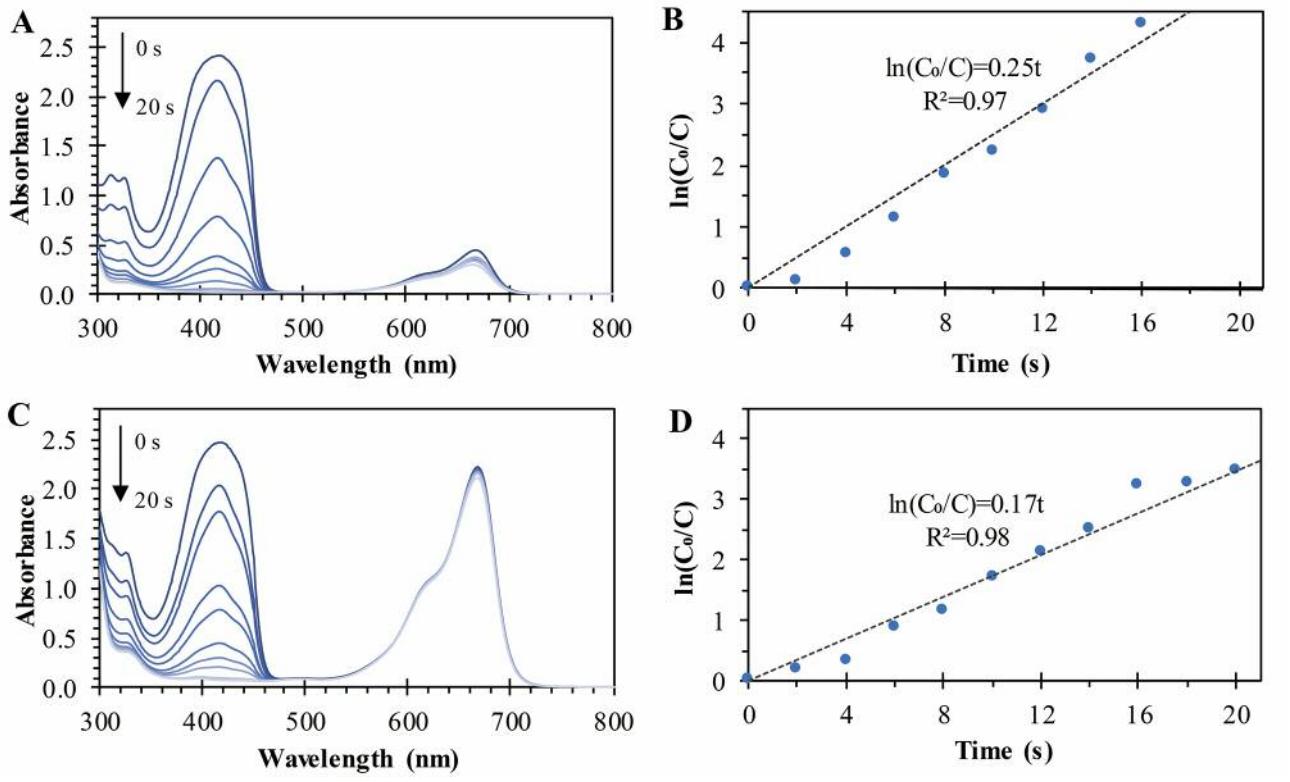

Figure 1. *DPBF decay following irradiation in the presence of 6.25 μM of MB (A) and 31.26 μM of MB (C). Proportionality between DPBF absorbance decay and the irradiation time in the presence of 6.25 μM of MB (B) and 31.26 μM of MB (D).*

Cell culture. BEAS-2B cells and H-441 cells were cultured in LHC-9 serum-free media and RPMI complete media (Thermo Fisher Scientific, Waltham, MA, USA), respectively, and were incubated at 37˚C in a 5% CO_2 environment. Cells were seeded (43,750 cells/cm^2) in 96-well plates 24 hours before experimentation. Cells were treated with MB solution in LCH-9 and serum-free RPMI media, respectively for each cell line, at different concentrations (0-156 μM), and were incubated for 3 hours. Then, media was removed, and cells were washed three times, twice with Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific, Waltham, MA, USA) and once with media.

In vitro PDT treatment. Fresh LCH-9 and serum-free RPMI media were added to BEAS-2B and H-441 cell cultures, respectively, for PDT treatment (5, 24, 25). Cells were irradiated with energy fluence ranging between 0 and 36 J/cm^2.

Determination of intracellular ROS. Cells were cultured as described previously and were treated with HDFC-DA (Biotium) at a final concentration of 5 μM. Then, cells were incubated at 37˚C for 40 minutes followed by *in vitro* PDT treatment as described before. Two hours following PDT treatment, fluorescence intensity was quantified by excitation at 485 nm and emission at 528 nm using a cell imaging reader (BioTek, Winooski, VT, USA).

Determination of cell survival. Following PDT treatment cells were incubated for 36 hours, and a colorimetric 3-(4, 5-dimethyle thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was carried to quantify cell survival (25-27).

Statistical analysis. All the experiments for the determination of intracellular ROS and cell survival produced for the applied treatments were compared between groups using paired Student's *t*-tests assuming equal variances. When the *p*-values were minor than 0.05, the differences were considered statistically significant.

Results and Discussion

ROS production by MB photoactivation in aqueous-DMSO solutions was verified following the DPBF absorbance decay at 417 nm, as presented in Figure 1A and C. This decay is caused by ROS produced in the form of singlet oxygen. DPBF absorbance peak was maintained constant in irradiated solutions free of MB (results not shown here), confirming that irradiation by itself does not produce ROS in the range of fluence studied. When MB was applied, a proportionality between DPBF absorbance decay and the irradiation time was observed (12, 28). These proportionalities are presented in Figure 1B and D for MB concentrations of 6.25 μM and 31.26 μM, respectively. These figures were plotted by considering the first-order decay of DPBF (28-31). DPBF decay constants were 0.25 s^{-1} and 0.17 s^{-1} for the respective concentrations of MB of 6.25 μM and 31.26 μM. These values are proportional to the ROS generation for each

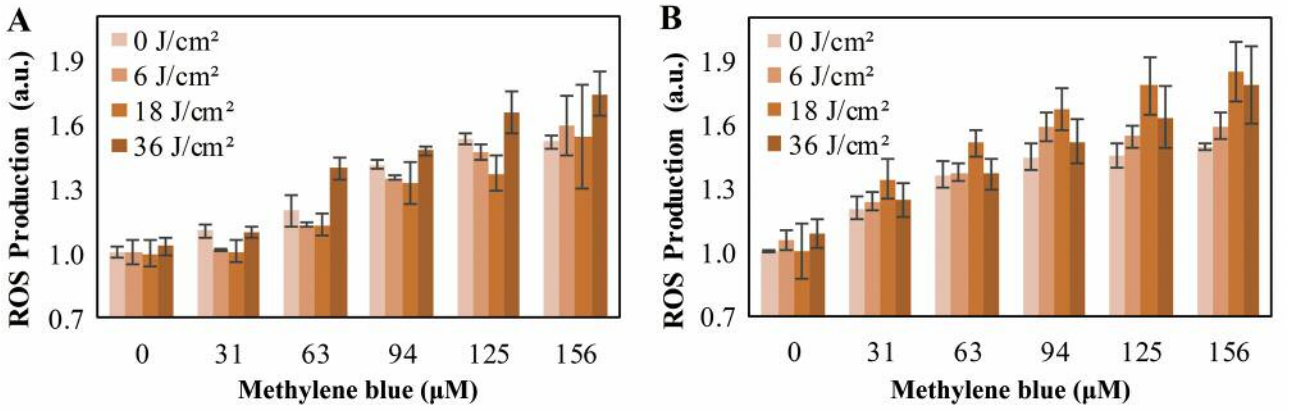

Figure 2. *ROS production in (A) BEAS-2B and (B) H-441 cells after treatment with MB concentrations ranging from 0 to 156 μM in combination with energy fluence of 0 J/cm², 6 J/cm², 18 J/cm², and 36 J/cm². Data presented with respect to sample controls (value of 1). Data represent the mean±standard deviation for n=3. Arbitrary units (a.u).*

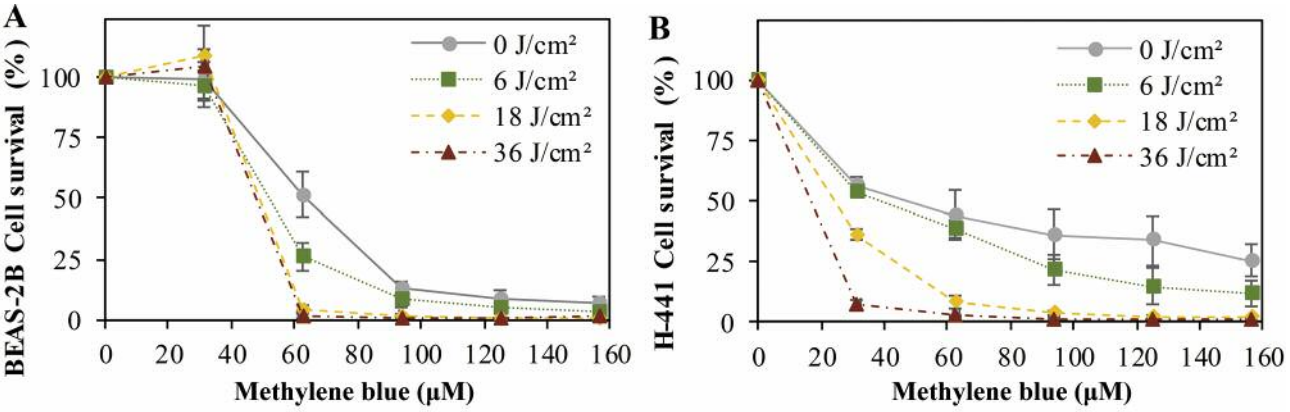

Figure 3. *Cell viability of (A) BEAS-2B and (B) H-441 cells after PDT with MB (0-156 μM) in combination with energy fluence of 0 J/cm² (●), 6 J/cm² (■), 18 J/cm² (◆), and 36 J/cm² (▲). Data represent the mean±standard deviation for n=3.*

experiment; it is noted that for lower concentration of MB, more ROS were produced. This effect could be explained by the formation of dimers, which are less effective generators of ROS (12, 28). Also, MB absorbance peak at 665 nm decayed with irradiation time, as could be observed in Figure 1A and C, indicating a degree of aggregation of MB and confirming the presence of dimers (12, 32).

ROS generated in the cell cultures were measured by their reaction with DCFH-DA, producing the fluorescent molecule 2,7-dichlorofluorescein (DCF). Figure 2 shows DCF fluorescence increments for the different concentrations of MB and the different levels of energy fluence evaluated. ROS were produced in BEAS-2B and H-441 cells without MB and irradiation. This result could be attributed to some oxidative stress caused by the cell culture process itself, facilitating the generation of reactive species (33). Fluorescence results are presented in arbitrary units (a.u) by adjusting to the controls (without MB and irradiation). BEAS-2B and H-441 cell survival at different MB concentrations and different levels of energy fluence are presented in Figure 3A and B, respectively.

In general, an increment in the ROS production concerning the MB concentration was observed, as depicted in Figures 2A and 2B, for BEAS-2B and H-441 cells, respectively. Also, the energy fluence by itself does not contribute to the ROS production, with no significant statistical differences, as shown in Figure 2A and B. Figure 2A for BEAS-2B cells shows no significant statistical differences related to the energy fluence between 31 μM and 63 μM of MB, except at the set of values of 36 J/cm2 and 63 μM of MB. At this set of values, the BEAS-2B cell survival drops remarkably from 100% to 0%, as observed in

Figure 3A. The ROS values obtained in BEAS-2B at concentrations above 63 μM of MB for all the energy fluence used displayed similar magnitudes to those obtained at the set of values at 36 J/cm^2 and 63 μM of MB. In consequence, the survival rates drop substantially for all the energy fluence used, as shown in Figure 3A.

Non-irradiated BEAS-2B and H-441 cells at different MB concentrations present significant statistical differences, as shown in Figures 2A and 3A. This result indicates that MB itself is inducing ROS generation in both cell lines. However, the laser irradiation in BEAS-2B contributes to significant differences compared to H-441 with regards to the ROS generation at concentrations above 31 μM of MB and energy fluence of 36 J/cm^2. Besides, the laser irradiation in H-441 cells does not contribute significantly in the ROS production at any concentration of MB. In contrast, H-441 cell survival drops significantly at concentrations of 31 μM of MB, and above, when the laser irradiation increases, as observed in Figure 3A. This outcome could be explained by the formation of dimers, as observed in the experiments for the determination of ROS in solutions. MB dimers are less effective generators of ROS (28).

The half maximal inhibitory concentration (IC_{50}) of BEAS-2B cells with MB and energy fluence of 0 J/cm^2, 6 J/cm^2, 18 J/cm^2, and 36 J/cm^2, resulted in values of 63.5 μM, 52 μM, 49 μM, and 48 μM of MB, respectively (Figure 3A). The results for H-441 cell survival show decrements for all the concentrations of MB analyzed, including the samples not irradiated. H-441 cells treated with MB and energy fluence of 0 J/cm^2, 6 J/cm^2, 18 J/cm^2, and 36 J/cm^2, resulted in values of IC_{50} of 47 μM, 39 μM, 24 μM, and 17 μM of MB, respectively (Figure 3B). This means that lung adenocarcinoma H-441 cells are more sensitive to PDT with MB compared to lung epithelial BEAS-2B cells. This result is consistent with other works reporting that MB was more toxic in erythroleukemic cells compared to normal peripheral blood mononuclear cells (34). Studies of PDT using MB in lung adenocarcinoma A549 cells have shown an enhancement of apoptosis associated with down-regulation of anti-apoptotic proteins, reducing the mitochondrial membrane potential and increasing phosphorylation of the mitogen-activated protein kinase and the generation of ROS (8). Also, other studies of PDT with MB in B16F1 melanoma cells shows mitochondria-related apoptosis through a series of steps beginning with the photochemical generation of ROS that activate the caspase-9/ caspase-3 apoptosis pathway (35). Similarly, PDT with MB in HeLa tumor cells triggered apoptotic cell dead by a mitochondria-dependent apoptotic pathway (36). Other works report the enhancement of ROS production and cell death (CHO) by using a combination of light and ultrasound activation of MB (37). In addition, derivatives of MB have been reported in the literature, where longer alkyl chains substituted the methyl groups of MB, resulting in more phototoxic effects than MB in RIF-1 murine fibrosarcoma cells. This effect was explained by the accumulation of the derivatives in the mitochondria (25).

This work presents the differential response of BEAS-2B and H-441 cells to MB photoactivation. The increments of ROS produced by MB photoactivation are directly related to BEAS-2B and H-441 cell survival. Results suggest that oxidative stress caused by ROS could be adjusted by modifying MB concentrations or different levels of energy fluence. Concentrations of MB above 31.26 μM are required to decrease BEAS-2B cell survival, while H-441 cell survival was similarly affected at a lower MB concentration. These results are in concordance with ROS production in BEAS-2B and H-441 cells, where larger production was obtained in H-441 cells. Therefore, selective cell damage could be achieved by using MB in PDT of lung cancer.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

RJRC and CAGV performed all experiments. PB, RE, PH, KSR, and RGZ contributed with reagents/materials/analysis tools and discussion for all experiments. ALA conceived and designed the experiments and contributed to the writing of the manuscript.

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