Effects of photodynamic therapy on A375 Melanoma cells using aluminium phthalocyanine photosensitizer

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Abstract. Metastatic Melanoma (MM) is highly aggressive and is among cancers causing major global deaths annually. It is imperative to find therapies that can eliminate MM and has become a major concern due to the potential for cancer relapse and metastasis, as well as the disease being accounted to be resistant to multiple forms of therapy. This *in vitro* study explores the effect of Photodynamic Therapy (PDT) using an Aluminium Phthalocyanine Photosensitizer ($AlPcS_4Cl$) at 673 nm and a fluency of 5 J/cm^2, in targeting Melanoma cells (A375). Dose dependent response of $AlPcS_4Cl$ was studied on both A375 and fibroblast (WS1) cell lines and the IC_{50} calculated from this. Significant post-irradiation signs of cell death were detected using microscopy and biochemical assays. An increased release of Lactate Dehydrogenase (LDH) content due to cytotoxicity with increasing doses of $AlPcS_4Cl$ was measured. Cell viability testing showed increased damaged cells taking up Trypan Blue Dye. The study suggested an effective treatment against Melanoma cells. Enhanced capabilities of PDT for MM could possibly be achieved through gold nanoparticle (AuNP) activated increased uptake of $AlPcS_4Cl$ photosensitizer, targeting their quiescent cancer stem cells.

1. Introduction

Melanoma typically arises in the skin and is a malignant tumour of melanocytes that is highly life-threatening with an increasing incidence worldwide [1]. Advanced or Metastatic Melanoma (MM) treatment can be difficult as patients are highly prone to cancer relapse and disease progression despite treatment due to developing resistance to treatment [2].

PDT uses the combination of a photosensitizer, intracellular molecular oxygen, and light for the destruction of cancer cells [3]. Phthalocyanines (PS) retain a vigorous absorbance near the infrared region and maintain high chemical levels in tissue and photo-stability with deeper tissue region in vivo activation [4]. Additional properties are longer absorption wavelengths in the region of 650–750 nm with high extinction coefficients, and elevated singlet oxygen quantum yields [5].

Intracellular localisation of a PS into targeted cells is one of the factors that determine the success of PDT as it assists in establishing the system and effectiveness of photo-induced cell death [6]. The localisation of a PS in the mitochondria commonly initiates apoptosis due to the high levels of ROS production as the mitochondria are sites of high oxygen levels and cytochrome c which causes the activation of caspases [7,8]. Lysosomal PS localisation leads to the leakage of catalytic hydrolases such as cathepsin D post PDT and membrane destabilisation [9], while in the endoplasmic reticulum (ER) implications are calcium instability and the accumulation of misfolded proteins [10].

2. Methods and materials

2.1. Cell culture and maintenance

Human Malignant Melanoma cell line A375, commercially purchased from the European Collection of Authenticated Cell Cultures (ECACC no: 88113005), was grown in a complete liquid medium DMEM (Dulbecco's Modified Eagle Medium). Fibroblast WS1 cells, obtained from the American Type Culture Collection (WS1, ATCC®, CRL-1502™), were grown in a complete liquid medium MEM (Minimum Essential Medium) and both cell lines were incubated at 37°C, 5% CO_2 and a humidity of 85%. Cell

confluent monolayers were detached and cellular suspensions seeded at 5 x 10^5 in 3.4 cm diameter cell culture dishes and incubated for a further 24 hours to adhere to the surface.

2.2. Aluminium phthalocyanine and irradiation dose response

A low-intensity diode laser (Oriel Corporation, USA, LREBT00-ROITHI) emitting at a wavelength of 673 nm, fluency 5 J/cm^2 supplied by the National Laser Centre of South Africa was used for this study. Cells were incubated for 4 hours with different $AlPcS_4Cl$ concentrations (dose 1: 4 µM, dose 2: 10 µM, dose 3: 15 µM, dose 4: 20 µM) and controls. Post-irradiation signs of cell death were determined after 24 hours of incubation. The lowest inhibitory concentration (IC_{50}) was established for A375 through calculation, using biochemical assay data.

2.3. Post-irradiation analyses

Cellular morphological changes were observed at 200× magnification using an inverted microscope (Wirsam, Olympus CKX41) with an attached digital camera.

A Lactate dehydrogenase (LDH) kit (CytoTox96® Non-Radioactive Cytotoxicity Assay - Promega G1780) was used for cytotoxicity determination. The LDH excreted from the cytosol into the cell culture media upon membrane destruction was evaluated by measuring the calorimetric compound spectrophotometrically at 490nm using a microplate reader (Perkin Elmer, Victor^3).

Trypan blue dye viability exclusion assay measured the percentage of viable cells present in the categorised cell suspensions. Equal parts of 0.4% (w/v) trypan blue dye (Invitrogen, Trypan Blue Stain Thermo Fisher-T10282) was added to cell suspensions and counted on an automated cell counter.

2.4. Subcellular localisation of $AlPcS_4Cl$ PS in A375 cells

A375 cells seeded at 2.5 x 10^5 cells/ml were incubated with added $AlPS_4Cl$ PS for 4 hours in the dark for PS localisation. Post incubation and washing with PBS (Phosphate-buffered saline), cells were stained with an ER-tracker, Mitotracker, and Lysotracker for each respective group. Cells were washed with PBS and counterstained with 4′-6-diamidino-2-phenylindole (DAPI). After washing again with PBS, coverslips were mounted onto slides and viewed for PS organelle localisation under a fluorescent microscope.

2.5. Statistical analysis

Graphs represent the mean, and standard error of biochemical assays using Sigma Plot version 14.0. The Dunnett's method was used for non-normally distributed data, whereas the one-way analysis of variances (ANOVA) was used for normally distributed data. These tests were used to determine the significant difference between control and experimental groups where values in the 95% confidence interval ($p < 0.05$*, $p < 0.01$** or $p < 0.001$***) were accepted as statistically different.

3. Results and discussion

3.1. Light microscopy cell morphological changes

Dark toxicity WS1 and A375 cells, denoted as C - F in Figure 1 and 2 respectively, that received doses 5 µM – 20 µM $AlPcS_4Cl$ photosensitizer alone without laser irradiation did not display significant morphological changes. This group resembled that of the cells only control group that received no treatment, shown in A. No morphological changes were seen in B, indicating irradiation of cells alone had no effect. Cells that received doses 5 µM – 20 µM PS and laser suggested morphological changes with increasing doses; however, non-significant cell death was seen in WS1 cells receiving dose 20 µM. A375 cells in Figure 2: J were noted to show the most significant cytotoxic effects. Signs of blebbing, vacuolisation, and cell shrinkage could be seen.

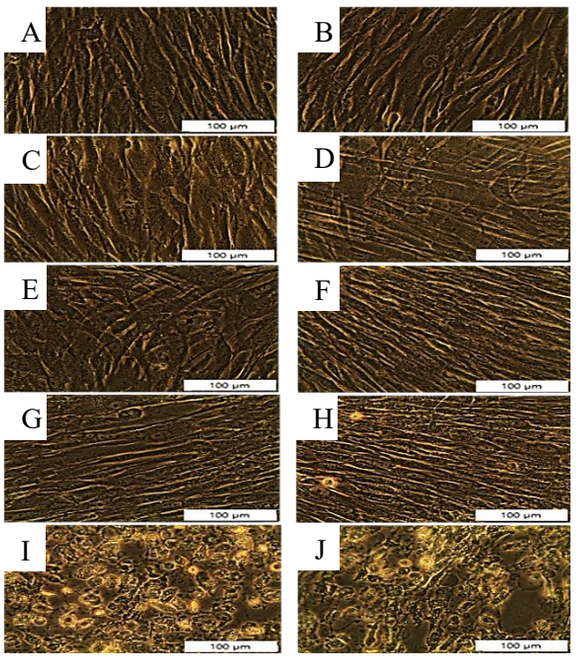

Figure 1. Morphological analysis post irradiation of WS1 cells at 200x magnification: A - Cells only; B - Cells + irradiation; C - Cells + $AlPcS_4Cl$ dose 1 (4 µM); D - Cells + $AlPcS_4Cl$ dose 2 (10 µM); E - Cells + $AlPcS_4Cl$ dose 3 (15 µM); F - Cells + $AlPcS_4Cl$ dose 4 (20 µM); G - Cells + $AlPcS_4Cl$ dose 1 (4 µM) + PDT; H - Cells + $AlPcS_4Cl$ dose 2 (10 µM) + PDT; I - Cells + $AlPcS_4Cl$ dose 3 (15 µM) + PDT; J - Cells + $AlPcS_4Cl$ dose 4 (20 µM) + PDT.

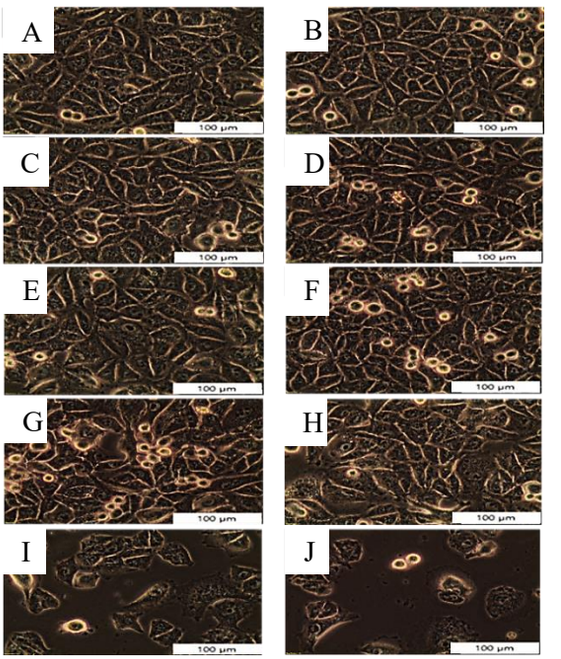

Figure 2. Morphological analysis post irradiation of A375 cells at 200x magnification: A - Cells only; B - Cells + irradiation; C - Cells + $AlPcS_4Cl$ dose 1 (4 µM); D - Cells + $AlPcS_4Cl$ dose 2 (10 µM); E - Cells + $AlPcS_4Cl$ dose 3 (15 µM); F - Cells + $AlPcS_4Cl$ dose 4 (20 µM); G - Cells + $AlPcS_4Cl$ dose 1 (4 µM) + PDT; H - Cells + $AlPcS_4Cl$ dose 2 (10 µM) + PDT; I - Cells + $AlPcS_4Cl$ dose 3 (15 µM) + PDT; J - Cells + $AlPcS_4Cl$ dose 4 (20 µM) + PDT.

3.2. Cytotoxicity

A non-significant increase in LDH was observed for dose dependant dark toxicity groups for both WS1 and A375 cell lines, indicating that $AlPcS_4Cl$ PS dispensation to cells without light exposure has no cytotoxic effects. WS1 treatment groups showed no statistically significant results as seen in Figure 3. Treatment groups for A375 cells as seen in Figure 4, with doses 4, 10, 15 and 20 µM indicated statistically significant results of $p < 0.001$ (***).

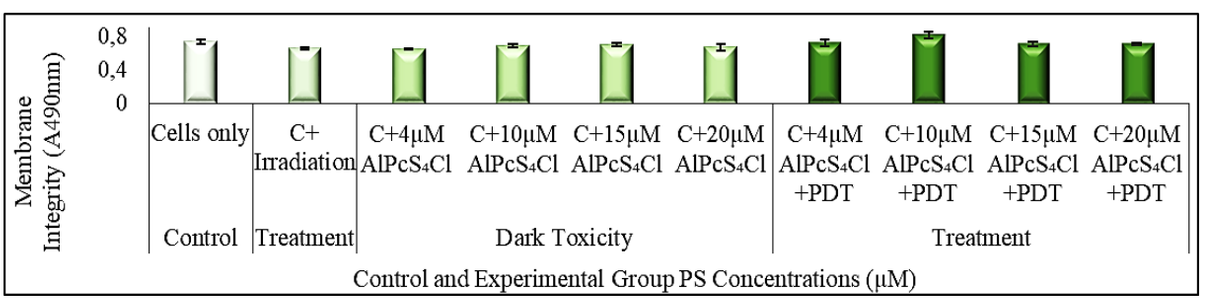

Figure 3. Post-irradiation cytotoxicity analysis on WS1 using LDH assay for control, treatment, and dark toxicity cell groups with increasing $AlPcS_4Cl$ dose and laser irradiation at 673nm, fluency: 5 J/cm^2. No significant results from sample groups.

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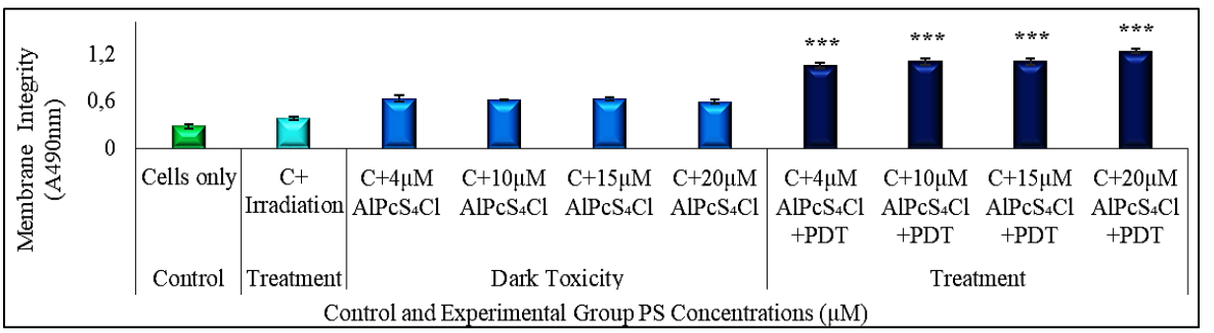

Figure 4. Post-irradiation A375 cells LDH cytotoxicity analysis for control, treatment, and dark toxicity cell groups with increasing $AlPcS_4Cl$ dose and laser irradiation at 673nm, fluency: 5 J/cm^2. A high significance of $p < 0{,}001$ (***) was established from treatment doses 4, 10, 15 and 20 μM.

3.3. Cellular viability

Trypan blue dye viability exclusion assay showed no statistically significant results for WS1 treatment group cells when compared to the control in Figure 5. This suggested that PDT was non-lethal at these doses. Treatment groups were compared to cells only (control) and cells with laser only groups that showed a high viability count. The dark cytotoxicity group showed insignificant decreased in viability for cells subjected to the dose of 15 μM. Significant results for A375 (Figure 6) cells when collated against the control were $p < 0.05$ (*) for the treatment group cells at doses 4 μM and $p < 0{,}01$ (**) for dose 15 μM and 20 μM. The A375 dark toxicity group showed no significant decrease in viability.

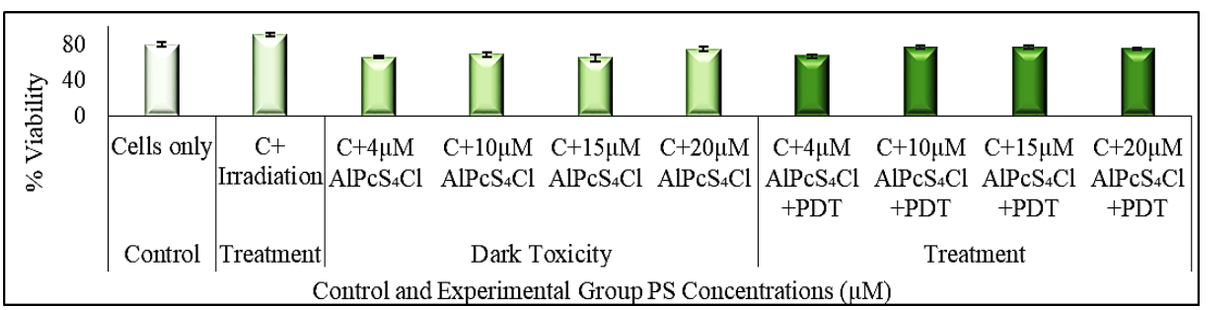

Figure 5. WS1 Trypan blue dye viability exclusion assay results for control, treatment and dark toxicity groups of increasing $AlPcS_4Cl$ dose and laser irradiation at 673nm, fluency: 5 J/cm^2. No significant results were noted.

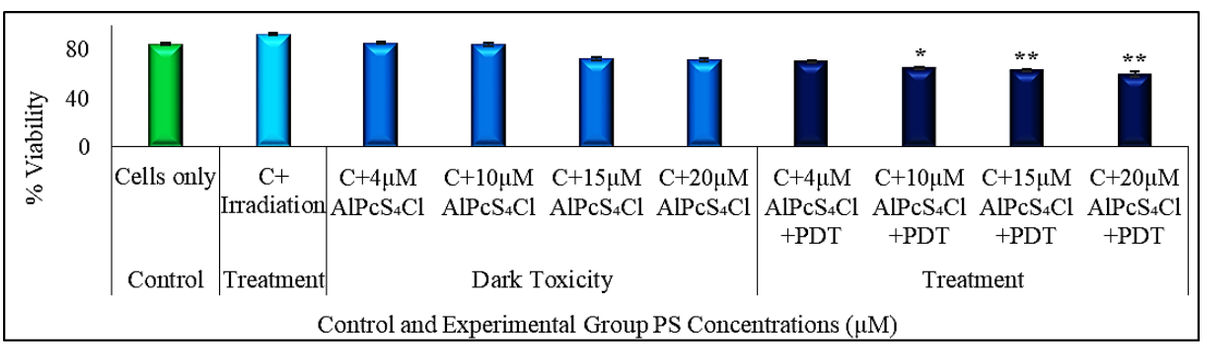

Figure 6. A375 Trypan blue dye viability exclusion assay results for control, treatment and dark toxicity groups of increasing $AlPcS_4Cl$ dose and laser irradiation at 673nm, fluency: 5 J/cm^2. Significant results were noted for dose 10 μM: $p < 0{,}05$ (*), dose 15 μM, and 20 μM: $p < 0{,}01$ (**).

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3.4. IC_{50} Calculation

The lowest inhibitory concentration (IC_{50}) was established by calculations, using data from the Trypan Blue viability biochemical assay conducted. The optimal IC_{50} concentration of $AlPcS_4Cl$ after PDT treatment that induced approximately 50% cytotoxicity was found to be 35 µM.

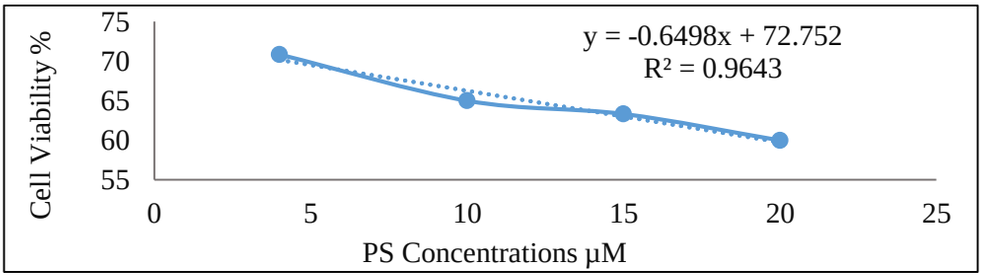

Figure 7. Trypan Blue viability IC50 calculation: 35,01385 µM.

3.5. Subcellular Localisation of $AlPcS_4Cl$ in A375 cells

Findings in Figure 8 suggest that there is passive uptake and localisation of $AlPcS_4Cl$ PS in the mitochondria and lysosomes of cultured A375 cells. Intermediate yellow-orange is seen in image D and H, where the merged green fluorescence from the mitochondrion and red fluorescence from the PS are seen overlapping. No overlapping is seen for the ER in image L which fluoresces blue-white when merged with the PS that fluoresces red.

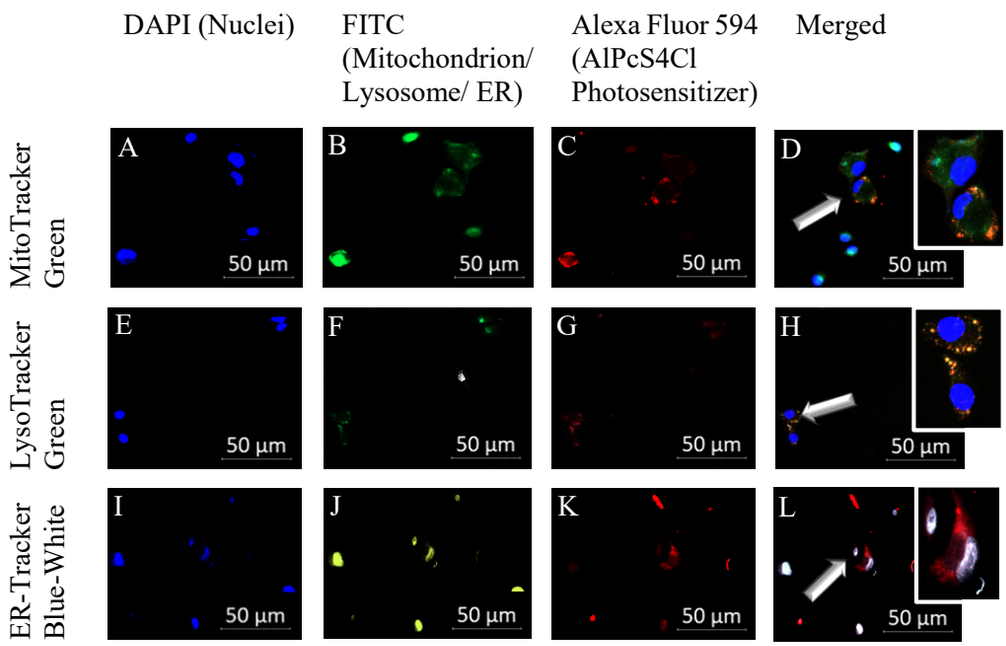

Figure 8. Subcellular localisation live cell imaging of $AlPcS_4Cl$ PS in A375 cells: A, E, I - Control stained blue with DAPI (nuclei); B, F, J - Mitochondrion/ Lysosome fluoresces green and ER fluoresces blue-white (FITC); C, G, K - $AlPcS_4Cl$ fluoresces red (A594). (200x magnification).

4. Conclusion

The efficiency of $AlPcS_4Cl$ PDT on A375 cells was realised through the successful cellular uptake and accumulation of the PS in the mitochondria and lysosomes with subsequent tumour cell cytotoxicity. The cell viability and cytotoxicity study results attained from treated A375 cells demonstrated a decrease

in cell viability with an IC_{50} of 35 μM dose of $AlPcS_4Cl$ irradiated at 673 nm with fluency of 5 J/cm^2. An increasing dose concentration of the PS renders high phototoxicity for the *in vitro* PDT of melanoma A375 cells. Cell death or cytotoxic indications were not observed from the WS1 cells, those treated with PS doses and incubated in the dark as well as those without PS treatment exposed to laser irradiation. Characteristics of a good photosensitizing agent are denoted to have no dark toxicity, (Zeng et al., 2011). This can be attributed to $AlPcS_4Cl$, which may however show toxicity to WS1 fibroblast (normal) cells at higher dose concentrations. Moreover, the results have indicated an idea on the dose optimization of $AlPcS_4Cl$ PS and agents that can deliver targeted therapy to accomplish future research objectives. Strategies developing and enhancing multiplex photosensitizer drug targeting systems that will deliver desired concentrations only in precisely targeted cells are progressing with an alarming interest.

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Conflicts of interest
The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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