Photodynamic therapy using Sulfonated Aluminium Phthalocyanine mix for the eradication of cervical cancer: an *in vitro* study

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Abstract. The use of phthalocyanines in Photodynamic Therapy (PDT) has greatly influenced the approach towards the treatment of cancer. PDT is very efficient in eradicating cancer cells but its efficacy depends on the correct choice of Photosensitizer (PS) used. This study, therefore, investigated the effectiveness of Sulfonated Aluminium Phthalocyanine mix ($AlPcS_{mix}$) in PDT of cervical cancer, which, in developing countries, including South Africa, is a common type of cancer. A working solution of $AlPcS_{mix}$ was prepared in phosphate buffered saline, PBS. Cervical cancer HeLa cells (ATCC® CCL2™) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Foetal Bovine Serum and incubated at 37 °C, 5% CO_2 and 85% humidity. The cells were treated with varying concentrations of $AlPcS_{mix}$ and irradiated using 673 nm diode laser at fluences of 5, 10 and 20 J/cm^2. Cellular responses were evaluated 24h post-irradiation to assess changes in cell structre, number and ability to survive after treatment. Results indicated that $AlPcS_{mix}$ localized in cytoplasm, mitochondria and lysosomes, and cellular responses showed dose-dependent structural changes, with decreased cell numbers and impaired ability to grow. The study presented $AlPcS_{mix}$ as an excellent choice of PS for use in PDT and the eradication of cervical cancer cells *in vitro*.

1. Introduction

Cervical cancer is a very common gynecologic malignancy in many parts of the world including South Africa. The burden of cervical cancer is due to lack of effective therapies which result in frequent cancer recurrences and migration to other organs (metastasis). Currently, surgery, chemotherapy and radiation are used for treatment. These therapies are associated with frequent recurrent rates and reduced quality of life for instance surgery, results in loss of fertility [1].

Modern advances in the field of applied physics in medicine, biomedical physics, has significantly modified methods of diagnosis and treatment of human diseases. Since antiquity, the use of light to treat skin diseases and other medical conditions has been in practice but only recently significant modern advances have been introduced to actively target specific diseases like cancer [2]. Photodynamic Therapy (PDT) is a treatment modality that uses light to activate a dye, referred to as Photosensitizer (PS) which reacts with molecular oxygen in its excited triplet state to produce singlet oxygen and other reactive oxygen species that cause damage to cancer cells [3]. This PS is administered into the body and actively localizes in the cancer tissue which upon light exposure, the cancer is eradicated. PDT has therefore gained much attention because of its specificity, noninvasiveness, minimal side effects and

tolerance to repeated doses [4]. It is therefore a potential treatment option for patients with both cervical intraepithelial lesions and advanced cases of cervical cancer

Numerous PSs have been used to treat various types of cancer since the beginning of the era of PDT. Among these PSs, Phthalocyanines have shown great potential in eradiating cancer cells in many studies. Like other PSs, the effectiveness of these PSs can however be further improved by chemical and biologic modifications e.g., addition of sulfur groups to the original chemical structure, and coupling to transition/poor metals. Metallation and sulfonation influences their cellular uptake by increasing solubility and also affect the intracellular distribution pattern, stability and pharmacokinetics, *in vivo* [5]. In this study we used Sulfonated Aluminium Phthalocyanine mix to treat cervical cancer.

2. Materials and methods

$AlPcS_{mix}$ was prepared in phosphate buffered saline, PBS, to a final working concentration of 0.01M. The excitation/activation wavelength was determined using Uv-Vis Spectrophotometry detecting the spectrum of $AlPcS_{mix}$ from 400 – 800nm wavelengths.

Cervical cancer cells were commercially purchased from the ATCC, HeLa cells (ATCC® CCL2™) and cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Foetal Bovine Serum, 1% pen-strep, 1% amphotericin B and incubated at 37 °C, 5% CO_2 and 85% humidity. For PDT experiments, 3×10^5 cells were seeded in 3.4cm^2 cell culture dishes and treated with $AlPcS_{mix}$ at concentrations of 5, 15 and 25 μM per plate. After 12 hours of incubation to allow maximum absorption of the PS by the cells, the cells were washed 3 times with pre-warmed Hank's Balanced Salt Solution, HBSS, to remove all traces of unabsorbed PS. Subcellular localization of the PS was observed using fluorescence microscopy. The treated cells were then irradiated using 673 nm diode laser (Oriel Corporation), supplied by the National Laser Center (NLC) of South-Africa. Cell groups were irradiated at fluences of 5, 10 and 20 J/cm^2 (Table 1).

Table 1. Laser parameters

Variable	**Description/Value**
Laser type	Semiconductor diode
Wavelength	673 nm
Pulsed or continuous	Continuous
Power Output	93 mW
Power density	10.25 mW/cm^2
Spot size	9.1cm^2
Fluence	5, 10, 20 J/cm^2
Irradiation times	8, 16, 32 minutes

Included in the experiments were a set of control groups that contained cells only without treatment with PS or irradiation. Laser negative control groups comprised of the three concentrations without laser irradiation and PS negative control groups comprised of cells that were irradiated with 5, 10 and 20 J/cm^2 without addition of PS. Each PS concentration was irradiated at all three fluences. After irradiation of cells, the plates were re-incubated at 37°C, 5% CO_2 and 85% humidity. Cellular responses were evaluated 24h post-irradiation using trypan blue viability assay to assess the cells survival (viability), adenosine triphosphate (ATP) assay, to determine cell number increase (proliferation) based on cellular ATP quantification, which indicates the presence of metabolically active cells, and bright field microscopy for structural changes (morphology).

All individual sets of experiments were repeated three times (n=3). Statistical analysis was performed using SigmaPlot software version 13.0. The student t-test was performed to determine the statistical difference between the control and experimental groups. One tailed student's t-test was performed to

compare different groups. Statistical significant difference between the untreated control and the experimental groups is shown in graphs as (*) for $p<0.05$, (**) for $p<0.01$, (***) for $p<0.001$, and (ns) for no significant difference, where p denotes the probability. The standard error on all plotted graphs is represented by error bars, respectively

3. Results

Results indicated that $AlPcS_{mix}$ accumulated in the cytoplasm was significantly localized in lysosomes and mitochondria. At the end of the investigation, control cells appeared healthy and maintained their normal cell structure and size. PDT on the other hand caused significant changes in morphology seen as cell rounding, blebbing and detachment from the surface of the culture dishes as shown in Figure 1 below.

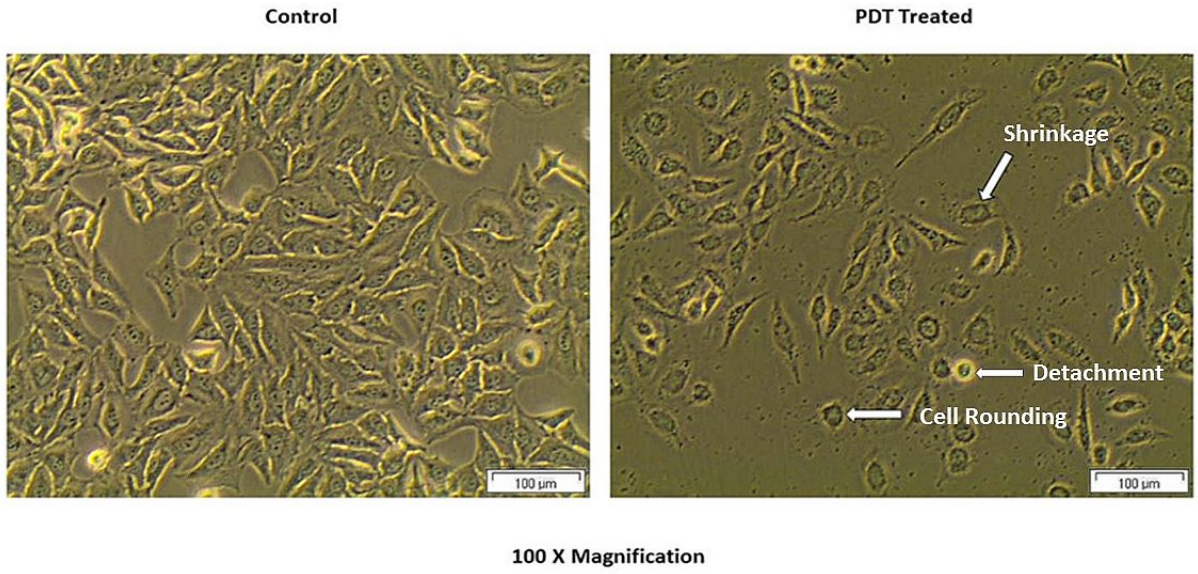

Figure 1. Cellular morphology of control and PDT treated cells seen under 100X magnification demonstrating the continuous growth of cells as a monolayer in the control group and a marked alteration of the morphology in PDT treated cells indicating cell shrinkage, blebbing and detachment from the surface.

Cellular responses showed dose-dependent structural changes, with significant ($p<0.001$) decrease in cell viability (Figure 2) and proliferation (Figure 3) compared to the control. As shown in Figure 2 below, the control group (No PS, no irradiation) presented with a high cell viability indicating that the cells remained viable at the end of the experimental period. Cells treated with PS alone and those treated with laser alone showed similar results. PDT treated cells indicated a dose dependent decrease in cell survival ($p<0.001$), with a marked decrease in viability seen in cells that received the highest dose of treatment (25 µM of PS and 20 J/cm^2).

The number of metabolically active cells was determined by detection of ATP. There was a marked decrease in cell proliferation in PDT treated cells, as compared to the control cells and untreated cells (Figure 3). The high luminescence signal seen with the control indicated increased proliferation of the cells, and the cells treated with either of the variables alone. PDT treated cells on the other hand showed a significant decrease in ATP production ($p<0.001$), in a dose dependent manner with the highest decrease observed in cells that were treated with 25 µM of $AlPcS_{mix}$ at a fluence of 20J.

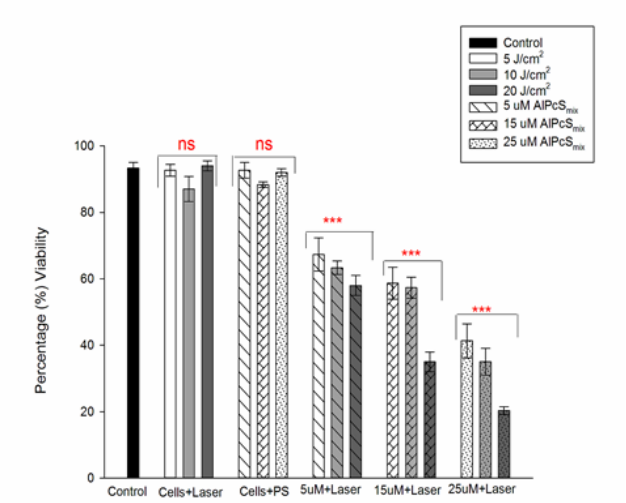

Figure 2. Post-irradiation cell viability showing a dose dependent decrease in cell survival. The control group and groups treated with either of the variables alone showed no significant decrease in viability (ns). All PDT groups compared to the control, indicated significant decrease in cell viability (p<0.001) after 24 h incubation post treatment.

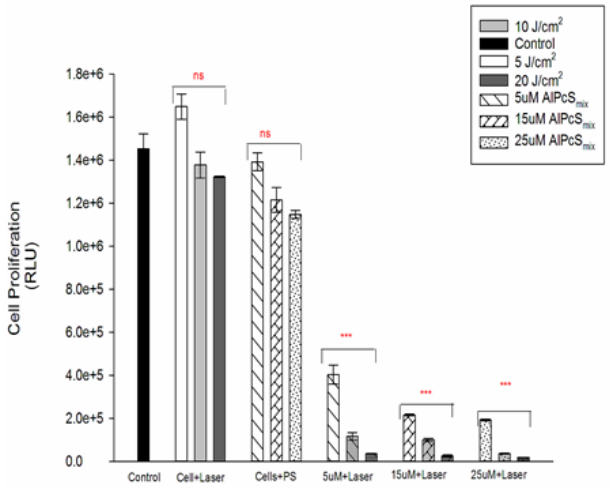

Figure 3. Post-irradiation ATP Luminescent signal of cells indicating proliferation. Control cells and groups treated with either of the variables alone did not show significant decrease in cell proliferation (ns). There was significant decrease in proliferation of PDT treated cells (p<0.001) after 24 h.

4. Discussion and conclusion

Singlet oxygen and other reactive oxygen species are produced by the PS in its activated state. These highly reactive oxygen species directly cause cell death by interactions with cellular components and oxidization of biomolecules causing the observed cell damages [6]. In simple terms, PDT induces cell death by virtue of photochemical interactions of light and a PS which in the presence of molecular oxygen, yields a set of chemical reactions that generate ROS and other free radical species causing membrane damage. In this set up, the different concentrations of the PS caused a significant dose dependent damage upon activation by laser light, with the extent of damage directly proportional to the concentration used. Similarly, the different fluences activated the PS at different rates with higher fluences causing more damage to the cells. Decreased cell viability in PDT treated cells was observed using Trypan Blue viability assay which adequately represented the ability of PDT to cause cell death through ROS generation. To further confirm this observation, ATP proliferation assay significantly presented decreased proliferation of the treated cells. After 24 hours, PDT treated cells also completely lost their morphological characteristics and died.

$AlPcS_{mix}$ has shown desired therapeutic effects in many solid tumours in previous studies [7, 8]. Zharkova et al used $AlPcS_{mix}$ to treat patients with various types of cancer, *in vivo*, from which the majority showed complete regression of the tumours. In another study by Kresfelder et al [8], it was shown that $AlPcS_{mix}$ induced sufficient cell death in oesophageal cancer cells with significant alteration of the post-irradiation cell proliferation. It was also demonstrated in the same study that $AlPcS_{mix}$ had the most prominent effect when compared to a different PS, $GePcS_{mix}$.

Results obtained indicate the effectiveness of $AlPcS_{mix}$ in PDT and the eradication of cervical cancer cells *in vitro*. Observable phenotypic changes with significant decrease in viability and proliferation were demonstrated. We therefore suggest in our study that $AlPcS_{mix}$ can be considered for use as a photosensitizing agent for cervical lesions and cervical cancer cells. Future studies should consider checking this effect on normal cervical cells to adequately determine the specificity of PSs in treating cancer. Further research is now expected to examine the therapeutic effectiveness of $AlPcS_{mix}$ *in vivo*.

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