The photodynamic therapy effect of aluminum and zinc tetrasulfophthalocyanines on melanoma cancer cells

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ABSTRACT

Photodynamic therapy (PDT) represents a novel treatment that uses a photosensitizer (PS), light source (laser) of an appropriate wavelength and oxygen to induce cell death in cancer cells. The aim of this study was to investigate the photodynamic effects of aluminum tetrasulfophthalocyanines (AlTSPc) and zinc (ZnTSPc) tetrasulfophthalocyanines activated with a 672nm wavelength laser on melanoma cancer, dermal fibroblast and epidermal keratinocyte cells. Each cell line was photosensitized with either AlTSPc or ZnTSPc for 2 h before using a diode laser with a wavelength of 672nm to deliver a light dose of 4.5 J/cm^2 to the cells. The cell viability of melanoma cells were decreased to approximately 50% with concentrations of 40 µg/ml for AlTSPc and 50 µg/ml for ZnTSPc. These PS concentrations caused a slight decrease in the cell viability of fibroblast and keratinocyte cells. Both photosensitizers in the presence of high concentrations (60 µg/ml-100 µg/ml) showed cytotoxicity effects on melanoma cells in its inactive state. This was not observed in fibroblast and keratinocyte cells. Cell death in PDT treated melanoma cells was induced by apoptosis. Therefore, AlTSPc and ZnTSPc exhibit the potential to be used as a PS in PDT for the treatment of melanoma cancer.

Keywords: Photodynamic Therapy, Photosensitizer, Melanoma, Cancer, Aluminum Tetrasulfophthalocyanines, Zinc Tetrasulfophthalocyanines

1. INTRODUCTION

Photodynamic therapy (PDT) is a promising oncology treatment which aims at offering treatment that is selective and localized. PDT is a very simple principle which requires three essential components a photosensitizer (PS), visible light (laser source) and molecular oxygen present in the biological tissue. The combination of these components will result in the destruction of tumor cells.[1] This oncology treatment is advantageous because it is an invasive, low-cost treatment that can be applied repeatedly at the same site if necessary.[2]

Porfimer sodium was the first PS to receive approval. It is now licensed for the use in the oesophagus, lung, stomach, cervix and bladder treatments. A light wavelength of 630 nm is needed for the activation of porfimer sodium and the absorption band at this wavelength is weak. The efficiency of energy transfer from light to cytotoxic products is moderate and tissue penetration is limited. It could not be used to treat deep-seated or larger tumors. The therapeutic dose of porfimer sodium resulted in skin photosensitivity which persisted for many weeks.[1]

First-generation photosensitizers encouraged the search for novel, chemically well-defined photosensitizers with improved biological properties generally referred to as “second generation photosensitizers”.[1] Phthalocyanines (Pc) are one of the most promising second-generation PS in PDT for the treatment of cancer.[3] Also, the high triplet state quantum yields and long triplet lifetimes of metallophthalocyanines can be enhanced by the addition of sulfonated derivatives.[3] Literature states that tetra-substituted phthalocyanines are usually more soluble in water than octa-substituted phthalocyanines.[4]

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Water soluble phthalocyanines are desirable photosensitizers for PDT because phthalocyanines can easily be absorbed by blood, when administrated intravenously.[5] Fabris (2006) has shown that topical formulation of phthalocyanines can penetrate sufficiently into the epidermal layers. This makes phthalocyanine an ideal photosensitizer for the treatment of skin diseases or cancers. The use of phthalocyanines in PDT for the treatment of cutaneous cancers needs to be explored in detail.[6]

Most importantly phthalocyanines are also efficient Type II reaction pathways sensitizers[7], which is the heart of photo-initiated cell death. In Type II reaction pathways, the activated PS in its triplet state will transfer its energy directly to molecular oxygen (a triplet form in the ground state), to form an excited state singlet oxygen. This highly reactive form of oxygen (reactive oxygen species, ROS) will react with many biological molecules (e.g. lipids, proteins or nucleic acid). Type I and Type II reactions can occur simultaneously. ROS can initiate a large number of reactions with biomolecules, including amino acids residues in proteins and these interactions cause damage and potential destruction to cellular membranes and enzymes resulting in cell death of cancer cells.[8]

The aim of the study was to determine the cytotoxicity effect of different concentrations of AlTSPc and ZnTSPc activated with continuous wave laser at a wavelength of 672 nm for the for the destruction of melanoma cancer cells as well as normal healthy cells (fibroblast and keratinocyte cells) using an *in vitro* system.

2. METHODOLOGY

2.1 Cell Culture

The melanoma cancer cells (UACC-62) were grown in T-75 cell culture flask containing 15 ml of RPMI-1640 medium supplemented with 10% FBS, 1% Penicillin/ Streptomycin and 1% NEAA. Keratinocyte cells were grown in T75 tissue culture flask containing 15 ml of EMEM supplemented with 10% FBS and 1% Penicillin/ Streptomycin. The isolated primary skin dermal fibroblast cells were maintained aseptically in T75 cell culture flasks containing 15 ml of Fibroblast Basal Medium. The cells were maintained at 37ºC in a 5% CO_2 incubator.

2.2 PDT Experiments

The 80% confluent cells were trypsinized, counted using a haemocytometer and cells were seeded in 24 well-plates at a density approximately 20 000 of cells/ml. Cells were allowed to attach overnight in a CO_2 incubator.

2.2.1 Preparation of Photosensitizer

The PS or drugs (ZnTSPc and AlTSPc) used in this study were synthesized by Professor Tebello Nyokong from Rhodes University (Department of Chemistry). Stock solutions of ZnTSPc and AlTSPc (100 µg/ml) were prepared using RPMI-1640, Fibroblast Basal or EMEM medium for treating melanoma, fibroblast or keratinocyte cells respectively.

2.2.2 Addition of photosensitizer to cells

The culture medium from each well was removed before washing the cells twice with PBS. Cells in triplicate wells were photosensitized by adding a specific amount of the stock solution of AlTSPc or ZnTSPc and diluting it with fresh culture medium to the desired concentrations of 10 µg/ml - 100 µg/ml. Untreated cells with 0 µg/ml of the PS was used as a control. Plates were wrapped in aluminum foil before placing in a CO_2 incubator for 2 h.

2.2.3 Irradiation

After the two hour incubation period, cells as monolayer cultures were irradiated with a diode laser emitting a wavelength at 672 nm. The laser set-up is shown in Figure 1. The output power of laser varied for each experiment and the beam was measured using a power meter for each experiment. The output power was in the range of 20 – 30 mW and the irradiation time (s) was calculated to deliver a light dose of 4.5 J/cm^2. A beam of 1cm in diameter was used to deliver a light dose of 4.5 J/cm^2 to the cells.

2.2.4 Cell Viability

The number of viable cells was measured using the CellTiter Blue® Reagent from Promega Corporation. 100 µl CellTiter Blue® reagent was added to each well containing 1ml of fresh culture medium without FBS and plates were placed in a 5% CO_2 incubator for 4 h. Each plate was kept protected from the light by keeping each plate wrapped in aluminum foil. The fluorescence signal was measured using a plate reader (FLUOstar, OPTIMA, BMG LABTECH) with an excitation filter at 544 nm and an emission filter at 620 nm filters.

2.3 Dark Toxicity

A Dark Toxicity Assay is a study which is carried out simultaneously with the laser irradiation study using the same experimental conditions as described above but omitting the laser irradiation step in the protocol.

2.4 Inverted Microscopy

Cells were seeded in 24-well cell culture plates at a density of approximately 20 000 cells/ml per well. Cells were allowed to attach overnight in a 5% CO_2 incubator. Cells in triplicate wells were photosensitized by adding 1ml of the stock solution of AlTSPc (40 µg/ml) or ZnTSPc (50 µg/ml). After a two hour incubation period, cells as monolayer cultures were irradiated with a diode laser emitting a wavelength of 672 nm and delivering a light dose of 4.5 J/cm^2 to each well. After irradiation the plates were placed in a 5% CO_2 incubator for 24 h. Changes in cell morphology was analyzed by viewing plates under an inverted microscope and photographed.

2.5 Apoptotic DNA Ladder Assay: Agarose Gel Electrophoresis

80% confluent melanoma cells in T25 flask were photosensitized with 40 µg/ml of AlTSPc or 50 µg/ml of ZnTSPc for 2 h. After 2 h cells as monolayer cultures were irradiated with a diode laser emitting a wavelength of 672 nm. The flask was divided into sections and each section was given a light dose of 4.5 J/cm^2. After exposure to light the flasks were placed in a 5% CO_2 incubator for 24 h. DNA was isolated from the PDT treated samples using the Apoptotic DNA Ladder Kit (Roche Diagnostics, Germany). The purified DNA samples were quantified using a Nanodrop ND-1000 Spectrophotometer. 15 µl (209.1 µg/ml) of the purified DNA for each sample and positive control with loading dye were loaded on a 1% agarose gel containing ethidium bromide. The gel was run in 1 x TBE (Tris – Borate EDTA) buffer at 75 V for 1.5 h (RT). Gel was analyzed under UV illumination for a visual DNA laddering.

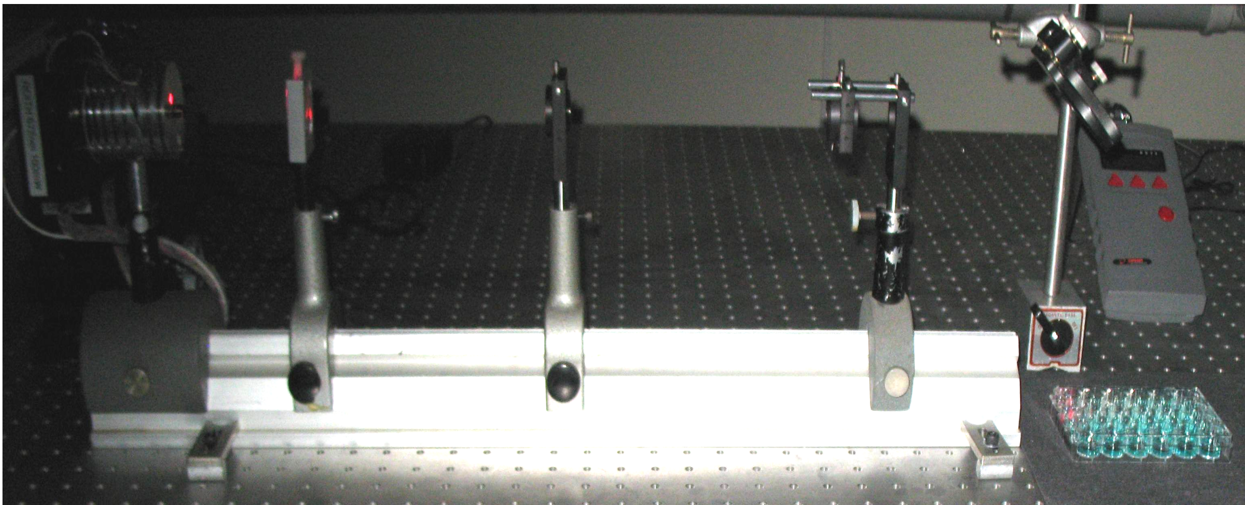

Figure 1 Set-up of the red light diode laser at a wavelength of 672 nm.

3. DATA

The cytotoxic effect of two PS (AlTSPc and ZnTSPc) in its inactive state on the cell viability of melanoma cancer cells as well as healthy normal fibroblast and keratinocyte cells is presented in Figure 2A and Figure 2B respectively.

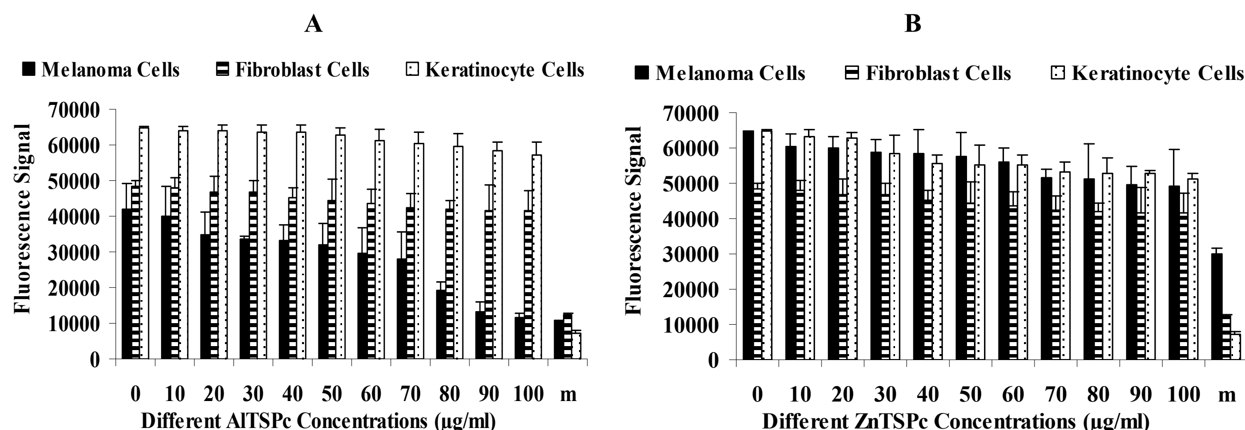

Figure 2 The effect of different AlTSPc (A) and ZnTSPc (B) concentrations in its inactive form on the cell viability of melanoma, fibroblast and keratinocyte cells were measured using the CellTiter-Blue® Viability Assay. 0 = untreated cells, m = media Fluorescence signal (544nm/630nm) is proportional to the number of viable or live cells.

Each cell line (melanoma, fibroblast and keratinocyte cells) was photosensitized with different concentrations AlTSPc or ZnTSPc before irradiation with continuous wave laser (CW, diode laser) at a wavelength of 672 nm. Post-irradiated cells were incubated for 24 h before cell viability was measured (Figure 3A and Figure 3B) respectively.

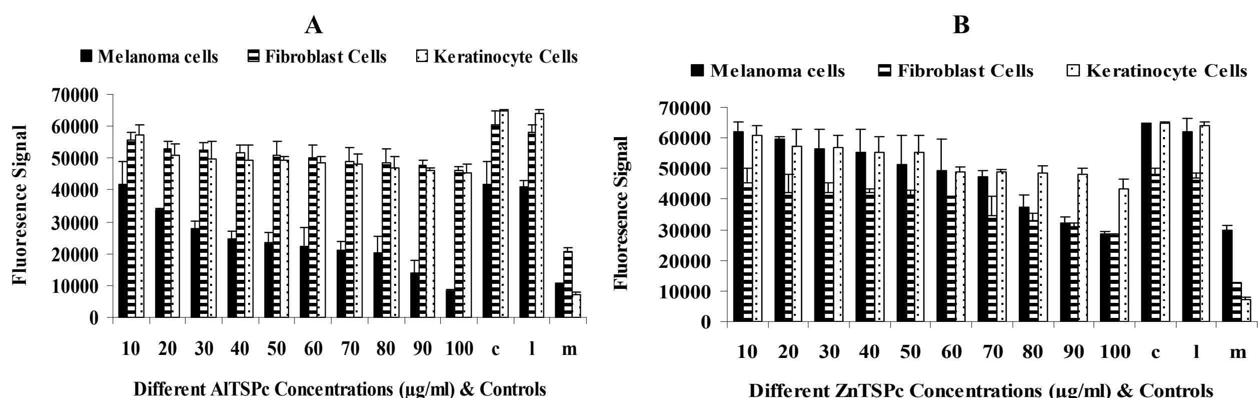

Figure 3 The effect of different AlTSPc (A) and ZnTSPc (B) concentrations activated with diode laser on the cell viability of melanoma, fibroblast and keratinocyte cells were measured by using the CellTiter-Blue® Viability Assay, c = untreated cells, l = laser irradiated cells without PS, m = culture media.

The change in cell morphology as a result of PDT mediated cell death was observed using an inverted microscopy and is shown in Figure 4-5.

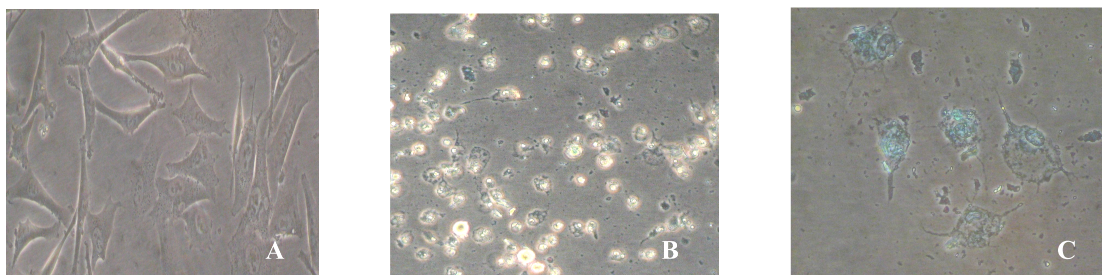

Figure 4 Micrographs showing the cell morphology of untreated (A) and post–irradiated melanoma cancer cells treated with 40 µg/ml of AlTSPc (B); 100 µg/ml of AlTSPc (C); (Magnification = 20 x).

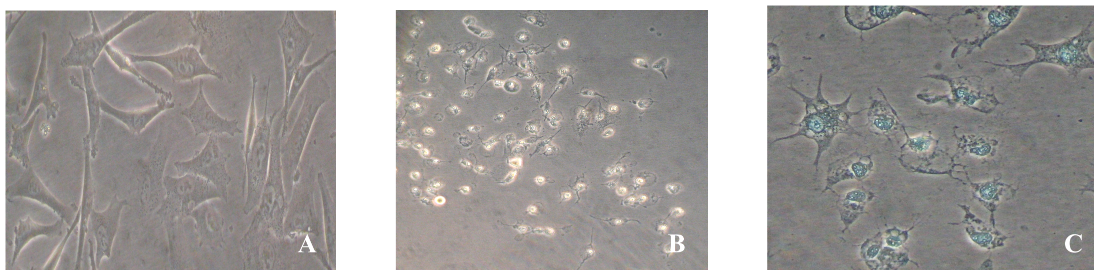

Figure 5 Micrographs showing the cell morphology of untreated (A) and post–irradiated melanoma cancer cells treated with 40 µg/ml of ZnTSPc (B); 100 µg/ml of ZnTSPc (C); (Magnification = 20 x).

Results of a gel electrophoresis of AlTSPc and ZnTSPc photosensitized melanoma cells after irradiation showed degradation of the chromatin DNA (Figure 6).

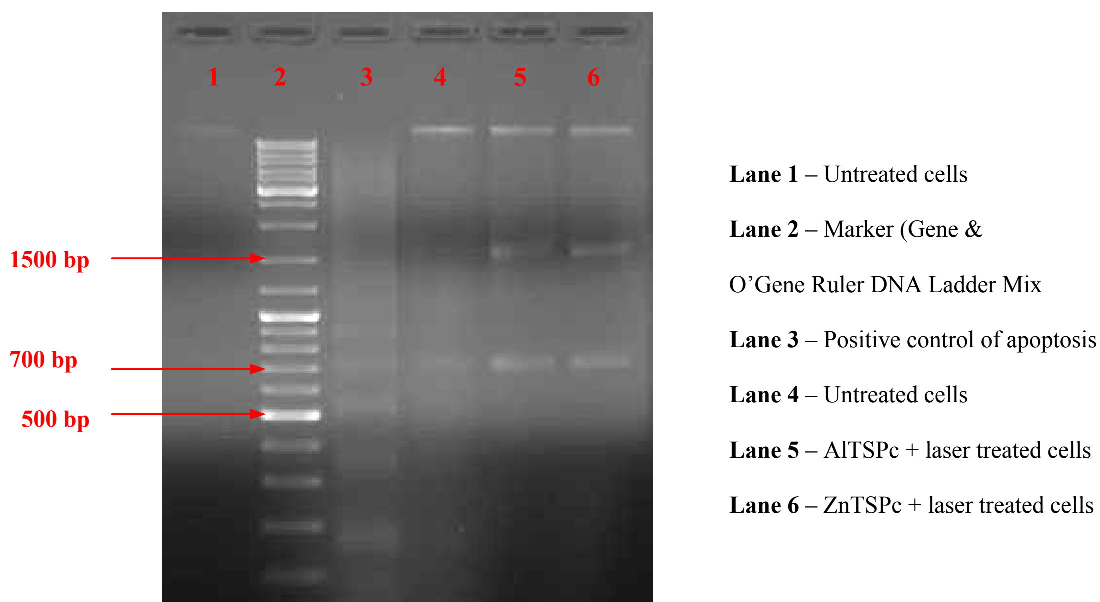

Figure 6 Agarose gel electrophoresis of DNA from untreated melanoma cells and treated melanoma cells after PDT. Bands were produced at 1500 bp (base pairs) and 700 bp.

4. DISCUSSION

Dark toxicity studies were performed to determine the cytotoxicity effects of AlTSPc and ZnTSPc in its inactive state on the melanoma cancer, as well as normal healthy fibroblast and keratinocyte cells. The data in Figure 2 indicated that high concentrations of AlTSPc and ZnTSPc ranging from 60 µg/ml – 100 µg/ml when compared to the untreated melanoma cells exhibit cytotoxic effects on melanoma cancer cells without laser light activation. In the case of fibroblast and keratinocyte cells photosensitized with either AlTSPc or ZnTSPc revealed an insignificant decrease in cell viability when compared to with the control (untreated cell). By using lower concentrations of both these photosensitizers the possibility of any dark toxicity can be eliminated The exposure of photosensitized melanoma, fibroblast and keratinocyte cells to red light from diode laser resulted in decrease in cell viability (Figure 3). Results demonstrated that as the AlTSPc and ZnTSPc concentrations increased the cell viability of each cell line proportional decreased. The viability studies have shown the use of 40 µg/ml of AlTSPc and 50 µg/ml of ZnTSPc in combination with a laser light dose of 4.5 J/cm^2 at a wavelength of 672 nm is the optimum conditions in this *in vitro* study for the effective killing of approximately 50% of melanoma cancer cells.

Under the inverted microscopy morphological changes of cell shrinkage and cell swelling is easily recognized in samples. The micrographs in Figure 4 and 5 showed that under experimental conditions the PDT treated melanoma cancer cells endured changes in morphology. Morphology changes like a reduction in cell volume (Figure 4B and 5B), condensation of the nucleus, blebbing (Figure 3C and 4C) and cell fragmentation giving rise to apoptotic bodies (Figure 4C and 5C) that were phagocytised by neighboring cells, which are characteristics of predominantly apoptosis. A band pattern depicting DNA degradation was noted in melanoma cancer cells receiving PDT with the optimum AlTSPc and ZnTSPc concentrations (Figure 6). Literature states that not all apoptotic cells will display the typical ladder formation.[9] This could occur due to various reasons such as not all cultured cell *in vitro* may accurately reflect the processes occurring *in vivo*. For example, cells grown *in vitro* may be prevented from exhibiting certain apoptotic characteristics through the loss of signal transduction pathways or metabolic components. Also, several features of apoptosis such as morphological characteristics or internucleosomal DNA cleavage may be inhibited. Therefore, DNA degradation remains a good indicator of apoptosis, particularly when ascertained with morphological characterization. [10]

5. CONCLUSION

It has been shown by this study that AlTSPc and ZnTSPc can be used as photosensitizers in PDT for the treatment of malignant melanoma cancer.

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