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In vitro toxicity testing of zinc tetrasulfophthalocyanines in fibroblast and keratinocyte cells for the treatment of melanoma cancer by photodynamic therapy

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ABSTRACT

A series of water-soluble tetrasulfonated metallophthalocyanines (MPcs) dyes have been studied to be used as a drug or photosensitizer (PS) in photodynamic therapy (PDT) for the treatment of cancers. During PDT the PS is administrated intravenously or topically to the patient before laser light at an appropriate wavelength is applied to the cancerous area to activate the PS. The activated PS will react with oxygen typically present in the cancerous tissue to generate reactive oxygen species for the destruction of the cancerous tissue. This *in vitro* study aimed at investigating the cytotoxic effects of different concentrations of zinc tetrasulfophthalocyanines (ZnTSPc) activated with a diode laser (λ = 672 nm) on melanoma, keratinocyte and fibroblast cells. To perform this study 3×10^4 cells/ml were seeded in 24-well plates and allowed to attach overnight, after which cells were treated with different concentrations of ZnTSPc. After 2 h, cells were irradiated with a constant light dose of 4.5 J/cm^2. Post-irradiated cells were incubated for 24 h before cell viability was measured using the CellTiter-Blue Viability Assay. Data indicated high concentrations of ZnTSPc (60–100 μg/ml) in its inactive state are cytotoxic to the melanoma cancer cells. Also, results showed that photoactivated ZnTSPc (50 μg/ml) was able to reduce the cell viability of melanoma, fibroblast and keratinocyte cells to 61%, 81% and 83% respectively. At this photosensitizing concentration the efficacy the treatment light dose of 4.5 J/cm^2 against other light doses of 2.5 J/cm^2, 7.5 J/cm^2 and 10 J/cm^2 on the different cell lines were analyzed. ZnTSPc at a concentration of 50 μg/ml activated with a light dose of 4.5 J/cm^2 was the most efficient for the killing of melanoma cancer cells with reduced killing effects on healthy normal skin cells in comparison to the other treatment light doses. Melanoma cancer cells after PDT with a photosensitizing concentration of 50 μg/ml and a treatment light dose of 4.5 J/cm^2 showed certain apoptosis characteristics such as chromatin condensation and fragmentation of the nucleus. This concludes that low concentrations of ZnTSPc activated with the appropriate light dose can be used to induce cell death in melanoma cells with the occurrence of minimal damage to surrounding healthy tissue.

1. Introduction

Cancer is one of the major health problems in South Africa. The Cancer Association of South Africa (CANSA) states: One in six South African men and one in seven South African women will get cancer during their lives [1]. South Africa has a high incidence for skin cancer because ambient ultraviolet radiation levels in South Africa are high throughout the year [2]. It appears that South Africa is among the top ten countries with high mortality rates for melanoma skin cancer. The other countries are New Zealand, Australia, Norway, Denmark, Sweden, Switzerland, Kazakhstan, Czech Republic and United States [3].

The standard oncology treatment for melanoma cancer is surgical excision (e.g. Moh's surgery) and adjuvant (after-surgery) treatment is sometimes offered to prevent recurrences of the cancer which solely depends on the stage of the melanoma cancer. The most common adjuvant cancer treatments are radiation therapy and chemotherapy [4]. These therapies are also used as a primary treatment when surgery is not feasible [4]. It is known that melanoma tends to resist radiation treatment so patients are given a radiation dose exceeding the healthy normal tissue tolerance [5,6]. While post-operative chemotherapy is associated with liver and kidney toxicity. It is also difficult to achieve adequate chemotherapy drug concentrations in the areas which have reduced

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perfusion due to resecting [6]. The current available adjuvant treatment options for patients at high risk of recurrences of melanoma cancer are High dose interferon (alfa, beta and gamma), Interleukin-2, Granulocytes-macrophage colony-stimulating factor and Cancer vaccine therapy [7,8]. High doses of interferon are recommended for high-risk resected melanoma since low doses are ineffective [7]. The major concern coupled with high doses of interferon during therapy is toxicity. Whilst, there has been long-term complete remission of melanoma cancer with high doses of interleukin-2 but the drawbacks of this treatment option are once again toxicity and cost [7]. The adjuvant use of granulocytes-macrophage colony-stimulating factor has also a few adverse effects of mild toxicity, transient myalgias, patient feels weak, mild fatigue and skin reactions (erythema) at the site of injection, but no detrimental long-term health effects on the patients [7].

For, the cancer vaccine therapy a melanoma vaccine is required containing antigens expressed by melanoma cells and not healthy normal cells to stimulate the immune system to attack and destroy the cancer cells [8]. Unfortunately, some melanoma antigens are also shared by healthy normal cells known as melanoma associated antigens (MAA) [9]. The cancer vaccines need to be made from the patient's own cancer cells or from cells that are grown in a laboratory, and the treatment dosage depends on the type of cancer being treated. However, there is limited availability of tumor cells and sterile laboratories for vaccine preparations [9]. The possible side effects of cancer vaccine therapy include skin reaction at the site of the injection (rash) and mild flu-like symptoms [8].

A major obstacle to an effective treatment especially in the case of cancer vaccines is tumor heterogeneity. Melanomas consist of numerous cell populations with a variety of antigens and these melanoma cells have the ability to secrete various cytokines and growth factors [9]. Future primary and adjuvant cancer treatments under investigation must address the heterogeneity of melanoma cancer [9]. Therefore, the above mentioned cancer treatments still needs considerable improvements due to the given record of toxicity, side effects and the need of high drug doses for therapeutic efficacy [10].

Photodynamic therapy (PDT) is a promising primary or adjuvant treatment for various cancers. It aims at offering a cancer treatment which is selective and localized [11]. PDT is a process that involves the laser activation of a drug/dye that is systemically or topically administrated to the patient depending on the type of cancer or disease to be treated [11–13]. This inactive drug is termed as a photosensitizer (PS) and absorbs light from a laser source at a specific excitation wavelength [11–13]. With absorption of a photon from a laser source the PS molecule is excited or activated. When the activated PS reacts with the molecular oxygen present in biological tissue it leads to the generation of reactive oxygen species (ROS) or singlet oxygen. ROS are beneficial as they act as signalling molecules of central processes such as proliferation, apoptosis (active or programmed cell death) and necrosis (passive or accidental cell death). This makes PDT a potentially emerging therapeutic method of treatment for many cancers as it can selectively cause the destruction of cancerous cells or tissue via apoptosis or necrosis, provided that the appropriate PS concentration and light dose are administrated [11–13].

In several countries, Photofrin® was the first photosensitizer to receive approval by governmental regulatory agencies for clinical treatment of lung, oesophageal, bladder, cervical and gastric cancer [11,14]. This first-generation photosensitizer achieved great clinical success in the field of PDT although it had some undesirable characteristics. For example, it is a complex mixture. Secondly, its absorption spectrum is around 630 nm and light at this wavelength penetrate tissue to a maximum depth of 5 mm. This protocol is suitable for the treatment of superficial lesions while the treatment of deep-seated or larger tumors require photosensitizers that absorb light at longer wavelengths for greater tissue penetration depth [14–17]. In addition, Photofrin® has proven to be ineffective for certain cancers such as pigmented melanoma cancer because the absorption spectra of Photofrin® and melanin in the malignant tissue overlap [18]. Finally, Photofrin is associated with the severe side effect of prolonged photosensitivity due to the fact that this photosensitizer retains in cutaneous tissue for up to 10 weeks post-injection [18]. These undesirable characteristics of Photofrin® led to the discovery of numerous pure compounds that absorbs light at longer wavelengths and these compounds are known as second-generation photosensitizers [17,18]. A promising group of second-generation photosensitizers for PDT are phthalocyanines (Pcs). In general phthalocyanines exhibit effective tissue penetration because their suitable light absorption region is between 600 nm and 800 nm. On the contrary, most of these compounds visibly aggregate in solution making them insoluble in water [18,19]. Recently, researchers have synthesized effective water-soluble phthalocyanines by incorporating tetra sulfonato groups into the compound. This helped in producing tetrasulfophthalocyanines that will not aggregate in blood (water-based medium) when administrated intravenously to patients during PDT. Thus, allowing the photosensitizer to effectively accumulate in the tumor. Tetrasulfophthalocyanines can be further modified to enhance its photodynamic action. By adding central metal ions (Al^{3+}, Zn^{2+}, Ga^{3+}) to the tetrasulfophthalocyanines compound the important photophysical properties such as high triplet quantum yield and long life-time are increased for this excited photosensitizer during PDT [18–20]. Most importantly, the metals (e.g. zinc) used for medical applications such as PDT should be biocompatible and diamagnetic [21]. These are essential properties a photosensitizer should possess as they are required for the production of singlet oxygen, which is regarded as the cytoxic species in PDT [22].

The rationale for this study is that research on the photodynamic effect of ZnTSPc (synthesized by Professor Nyokong from Rhodes University, South Africa) on melanoma cancer cells, healthy normal skin fibroblast and keratinocytes have not been studied previously. However, recent reports have demonstrated the photodynamic effectiveness of a different photosensitizer namely hypericin on melanoma cells and healthy normal skin cells [23]. This study was based on the three main objectives. Firstly, to determine the optimum concentration of ZnTSPc activated with a continuous wave laser (CW) at a wavelength of 672 nm to kill approximately 50% of melanoma cancer cells with minimal toxicity in healthy normal fibroblast and keratinocyte cells. Since, toxicity and distribution of photosensitizers in cancerous tissue as well as adjacent healthy normal tissue are some of the major concerns associated with the ideal photosensitizer concentration to be administrated during PDT treatment. Secondly, to compare the efficacy of the treatment light dose of 4.5 J/cm^2 and exposure time of the CW laser by photosensitizing the melanoma, fibroblast and keratinocyte cells with the optimal ZnTSPc concentration, and exposing the cells to other light doses. Lastly, to evaluate the mechanism of cell death by the optimal photoactivated ZnTSPc concentration using melanoma cancer cells.

2. Materials and methods

2.1. Toxicity screening of ZnTSPc

2.1.1. Cell culture

Melanoma cancer cells (UACC62; Human malignant melanoma from NCI) were grown in RPMI-1640 medium (Lonza, Walkersville, USA) supplemented with 10% Foetal Bovine Serum (FBS; Gibco – Invitrogen), 1% Penicillin/Streptomycin (Lonza, Walkersville, USA) and 1% Non-essential amino acids (NEAA; Sigma, St. Louis, USA).

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Immortalized epidermal keratinocyte cells were kindly provided by Dr. Lester Davids from University of Cape Town and these cells were grown in Eagles Minimum Essential Media (EMEM – Lonza, Walkersville, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin. Primary skin dermal fibroblast cells were isolated from human skin biopsies acquired from patients at the University of Limpopo under ethical approval (MREC/M/63/2009: IR) and the isolated fibroblast cells were grown in Fibroblast Basal Medium (Lonza, Walkersville, USA). Each cell line was maintained at 37 °C in a 5% CO_2 incubator. 80% confluent cells were seeded in 24-well plates at a cell density of 3×10^4 cells/ml. Cells were allowed to attach overnight at 37 °C in a 5% CO_2 incubator.

2.1.2. Preparation of photosensitizer

The zinc tetrasulfophthalocyanines were synthesized using known methods [24]. A stock solution of ZnTSPc (100 μg/ml) was prepared in either RPMI-1640 medium without L-Glutamine, Fibroblast Basal or EMEM medium depending on whether melanoma cells, fibroblast cells or keratinocyte cells were to be treated accordingly. This stock solution was further diluted to attain concentrations of the PS in range of 10 μg/ml–100 μg/ml. The PS was prepared under light-restricted conditions.

2.1.3. Addition of photosensitizer to cells

After 24 h of cell growth the culture medium from each well was removed and the cells were washed twice with Phosphate Buffered Saline (PBS: Sigma, St. Louise, USA). The PS solutions (1 ml) of each dilution were added to the cells. Cells containing no PS and no laser irradiation were used as a control (untreated cells) during each set of experiments. Also, a negative control (medium only) was set-up. Each concentration was tested in triplicate. The plates were wrapped in aluminum foil and incubated at 37 °C in 5% CO_2 incubator for 2 h. Preliminary experiments were conducted with photosensitization incubation times of 2 h, 4 h, 18 h and 24 h. The 4 h, 18 h and 24 h incubation periods were potentially cytotoxicity to the cells without laser activation.

A dark toxicity study was conducted simultaneously to determine if ZnTSPc in its inactive state (without any laser irradiation) has cytotoxic effects on the melanoma, fibroblast and keratinocyte cells.

2.1.4. Irradiation

After 2 h, each well was irradiated with a red light diode laser (CW) emitting a wavelength at 672 nm. The output power of the laser varied for each experiment and the beam was measured using a power meter (Nova, Ophir) for each experiment. The output power was between 20 and 30 mW and the irradiation time (s) was calculated to deliver a light dose of 4.5 J/cm^2. A beam of 1 cm in diameter was used to deliver a light dose of 4.5 J/cm^2 to the cells. After irradiation the plates were incubated at 37 °C in 5% CO_2 for 24 h before cell viability was measured using the CellTiter-Blue® Viability Assay. The EC_{50} (effective concentration that reduced cell viability to ±50%) graph was constructed by calculating the cell viability (CV) percentage (%) using the data from the CellTiter-Blue Assay and the following equation:

$$\left(\frac{\text{Fluorescence Signal}_{\text{Sample}} - \text{Fluorescence Signal}_{\text{Media}}}{\text{Fluorescence Signal}_{\text{Untreated Cells}} - \text{Fluorescence Signal}_{\text{Media}}}\right) \times 100 \quad (1)$$

2.1.5. Controls

A negative control containing culture media (without cells) was used for each set of experiments to detect for background fluorescence signals that contribute to the fluorescence signal readings of the PDT treated samples. The untreated cells contained 0 μg/ml of the PS and were not irradiated. This control would be an indication of the amount of viable cells present before PDT treatment. Cells (in the absence of photosensitizer) exposed to the laser served as another control (laser control) and this control was compared to the untreated cells to rule out the possibility that the laser in the absence of the PS is responsible for the decrease in cell viability of the PDT treated cells. The laser control and untreated control were also used to indicate the uptake of the photosensitizer (dye) by cells by comparing them to the photosensitizered cells and PDT treated cells under an inverted microscope.

2.2. Determining the effective treatment light dose

The optimal concentration of ZnTSPc (50 μg/ml) was prepared using RPMI-1640 medium, Fibroblast Basal or EMEM medium for treating melanoma, fibroblast or keratinocyte cells accordingly. The cells were grown in 24-well plates and washed with PBS as described previously. Cells were photosensitized with ZnTSPc for 2 h before cells were irradiated with a diode laser emitting a wavelength of 672 nm. The output power of the laser varied between 20 and 30 mW and the irradiation time was calculated to deliver light doses of 2.5 J/cm^2, 7.5 J/cm^2 or 10.5 J/cm^2. An additional set of controls were prepared for each treatment light dose experiment, control cells that were irradiated in the absence of the photosensitizer. After irradiation the plates were placed at 37 °C in a 5% CO_2 incubator for 24 h before cell viability was measured using the CellTiter-Blue® Viability Assay.

2.3. Cell death mechanism induced by photoactivated ZnTSPc – transmission electron microscopy (TEM)

Melanoma cancer cells were grown in T-25 culture flask containing 5 ml of RPMI-1640 medium supplemented with 10% FBS, 1% Penicillin/Streptomycin and 1% NEAA. 80% confluent cells were washed with PBS before photosensitization with ZnTSPc (50 μg/ml). A flask of untreated cells were prepared simultaneously. After 2 h, the ZnTSPc treated flask was irradiated with diode laser emitting a wavelength of 672 nm. The output power of the CW laser was 28.71 mW and the output power was measured using a power meter (Nova, Ophir). A beam of 1.5 cm in diameter was used to deliver light doses of 4.5 J/cm^2 in 4 min 38 s. After irradiation the flasks were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Samples were processed for TEM by detaching cells from the flask with a cell scraper and centrifuging at 1000 rpm for 5 min. The pellet for each sample was sealed in microcapillary tubes before placing them into gold coated chambers. Then samples were immediately loaded on a high pressure freezing device (EMPACT 2, Leica). Fixed samples were embedded in resin before thin sections were cut to be placed onto copper grids. The sections were stained with uranyl acetate and lead citrate Post-stained copper grids were examined using a JEOL 2100F (200 kW) TEM and digital images were captured for examination.

3. Results

3.1. Dark toxicity assay – photosensitization of cells with no light activation

In Fig. 1 the cell viability percentage for each cell line photosensitized with the different concentrations of ZnTSPc was calculated using a negative control (background fluorescence that may be present in fluorescence signal readings of the samples) and the untreated cells. There is a significant difference (Fig. 1; $P < 0.001$) in the cell viability of melanoma cancer cells, healthy normal fibroblast and keratinocyte cells photosensitized with ZnTSPc under

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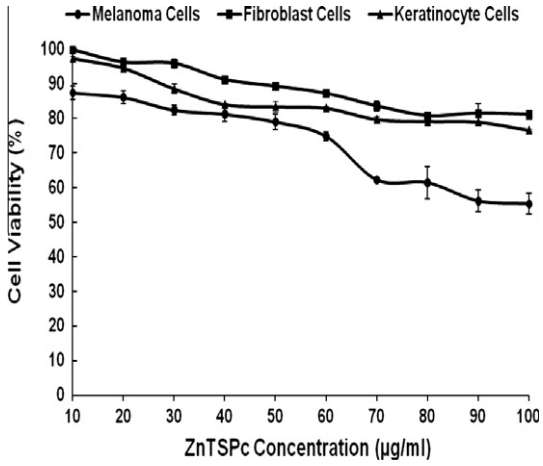

Fig. 1. Changes in the cell viability (%) of melanoma, fibroblast and keratinocyte cells photosensitized with different concentrations of ZnTSPc without laser treatment. Cell viability was measured using the CellTiter-Blue® Viability Assay and original cell viability is expressed as a percentage of the untreated cells. Data points represent the mean ± standard deviation, $n = 3$.

experimental conditions in the absence of light activation. High concentrations of ZnTSPc (without laser activation) such as 60 μg/ml, 70 μg/ml and 100 μg/ml were able to reduce the cell viability of melanoma cancer cells to 75%, 62% and 55% respectively. Negligible cytotoxicity was observed in fibroblast and keratinocyte cells photosensitized with the different concentrations of ZnTSPc.

3.2. Toxicity screening of ZnTSPc with laser activation

Results in this experiment indicate activated ZnTSPc with a light dose of 4.5 J/cm^2 can effectively kill melanoma cancer cells as illustrated in Fig. 2. It is significantly evident that ZnTSPc in its activated state was more successful in reducing the cell viability of melanoma cancer cells than ZnTSPc photosensitization without laser activation ($P < 0.001$). PDT is shown to be a concentration-dependent treatment because as the ZnTSPc concentration increased the cell viability of each cell line proportionally decreased as illustrated in Fig. 2. Results also clearly show that melanoma cancer cells photosensitized with a ZnTSPc concentration of 60 μg/ml, 70 μg/ml and 100 μg/ml in combination with a laser light dose of 4.5 J/cm^2 at a wavelength of 672 nm was able to decrease cell viability of melanoma cancer cells to 55%, 50% and 3% respectively. For this study, ZnTSPc used at a concentration of 50 μg/ml was chosen to be optimum concentration although it reduced the cell viability of melanoma cancer cells to 61% and not 50% because the dark toxicity associated with high concentrations of ZnTSPc (60–100 μg/ml) which was seen in Fig. 1 was taken into consideration.

In the case of fibroblast cells treated with photoactivated ZnTSPc concentrations of 50 μg/ml, 60 μg/ml, 70 μg/ml and 100 μg/ml there was accordingly a 81.32%, 79.32%, 61.57% and 43.46% cell viability. For keratinocyte cells treated with photoactivated ZnTSPc at concentrations of 50 μg/ml, 60 μg/ml, 70 μg/ml and 100 μg/ml the cell viability was 83.32%, 72.23%, 72.05% and 62.68% respectively.

The post-irradiated melanoma, fibroblast and keratinocyte cells that were not exposed to the photosensitizer but treated with the laser (laser control) showed an average cell viability of 96% when compared to the untreated cells and this helped to exclude the possibility that the laser was responsible for the decrease in cell viability without the photosensitizer.

3.3. Treatment light dose study

Fig. 3 shows the effect of different light doses (2.5 J/cm^2, 4.5 J/cm^2, 7.5 J/cm^2 and 10.5 J/cm^2) delivered from a CW laser at a wavelength of 672 nm on the cell viability of melanoma cancer cells and healthy normal cells (fibroblast and keratinocyte cells) photosensitized with ZnTSPc (50 μg/ml). The greatest reduction in cell viability of melanoma cells was achieved by exposure of photosensitized melanoma cells to a light dose of 4.5 J/cm^2. It was observed that the photoactivation of fibroblast cells treated with 50 μg/ml of ZnTSPc, with a treatment dose of 2.5 J/cm^2 killed less healthy normal fibroblast cells in comparison to a light dose of 4.5 J/cm^2. Thereafter, the cell viability of fibroblast cells decreased as the treatment light dose increased. The cell viability of the post–irradiated keratinocyte cells indicated that a treatment light dose of 2.5 J/cm^2 killed more keratinocyte cells in comparison to a treatment light dose of 4.5 J/cm^2. A further decrease in cell viability with a treatment light dose of 7.5 J/cm^2 was observed. There was a slight increase in cell viability with a treatment light dose of 10.5 J/cm^2 in comparison to treatment light doses of 4.5 J/cm^2 and 7.5 J/cm^2.

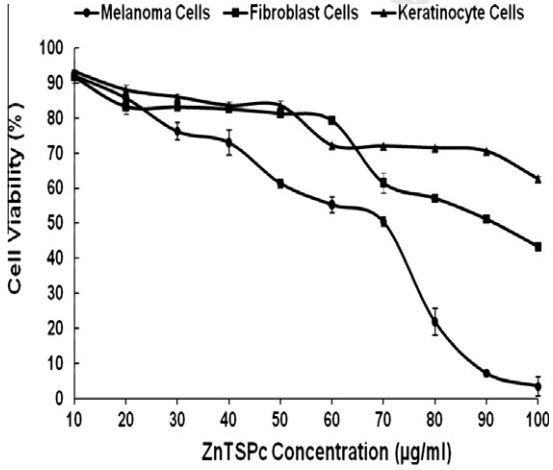

Fig. 2. The dose-dependent effect of the different ZnTSPc concentrations photoactivated with a light dose of 4.5 J/cm^2 from a CW laser on the cell viability of melanoma, fibroblast and keratinocyte cells. Data points represent the mean ± standard deviation, $n = 3$.

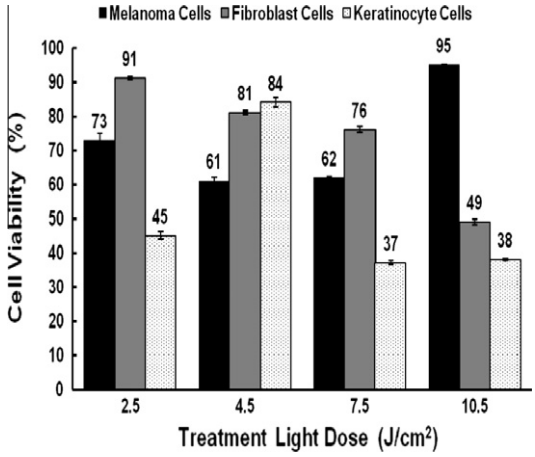

Fig. 3. The graph comparing the cell viability (%) of melanoma, fibroblast and keratinocyte cells treated with the optimal ZnTSPc concentration (50 μg/ml) and a treatment light dose of 4.5 J/cm^2 to other treatment light doses of 2.5 J/cm^2, 7.5 J/cm^2 and 10.5 J/cm^2. Data points represent the mean ± standard deviation, $n = 3$.

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3.4. Cell death mechanism: TEM

A representative TEM micrograph of an untreated melanoma cancer cell with an intact plasma membrane and nucleus is seen in Fig. 4. The treated melanoma cancer cell which was photosensitized with 50 μg/ml of ZnTSPc and exposed to laser light from CW diode laser (Fig. 5) shown characteristics of apoptosis such as nucleus fragmentation and chromatin condensation. Also, Fig. 6 shows a disintegrated nucleus and chromatin condensation from a melanoma cancer cell after PDT treatment. At higher magnification super aggregation of the cells chromatin can be seen in the PDT treated melanoma cancer cells (Fig. 7).

4. Discussion

This study demonstrates that photosensitization using ZnTSPc following PDT is able to kill melanoma cancer cells using an *in vitro* system, and confirms a similar study demonstrating that these metallophthalocyanines synthesized at Rhodes University are effective in killing human oesophageal carcinoma cells [25,26]. In addition, this study demonstrated that death occurs by an apoptotic mechanism.

The dark toxicity studies indicated that high concentrations of ZnTSPc ranging from 60 μg/ml to 100 μg/ml exhibit cytotoxic effects on melanoma cancer cells without laser light activation. In the case of fibroblast and keratinocyte cells photosensitized with ZnTSPc an insignificant decrease in cell viability was revealed when compared with the control (untreated cells). The amount of photosensitizer accumulated in melanoma cancer cells must have be greater than that accumulated in fibroblast and keratinocyte cells for the photosensitizer to kill the melanoma cancer cells without laser activation, especially when higher concentrations of the photosensitizer were used. The use of ZnTSPc at low concentrations could be the solution to minimize or eradicate any dark toxicity that can be caused by the PS in its inactive state before light activation to cancerous and healthy surrounding tissues. Also, the incubation time with ZnTSPc before light activation or irradiation was 2 h in this experiment and this incubation time can be decreased to minimize the effect of dark toxicity on cells.

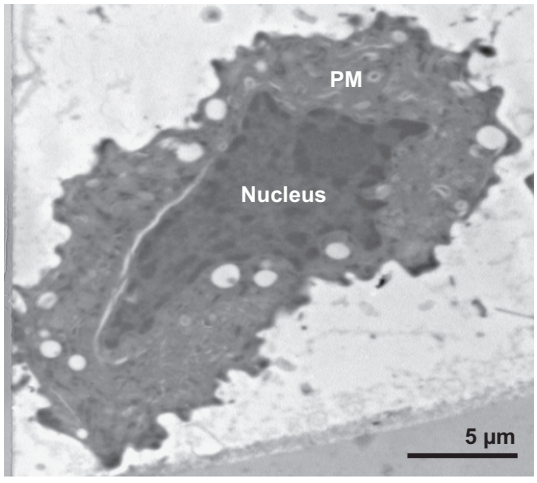

Fig. 4. A TEM micrograph at a magnification of 12,000× showing an intact nucleus bounded by a nuclear membrane and intact plasma membrane (PM) of an untreated melanoma cancer cell.

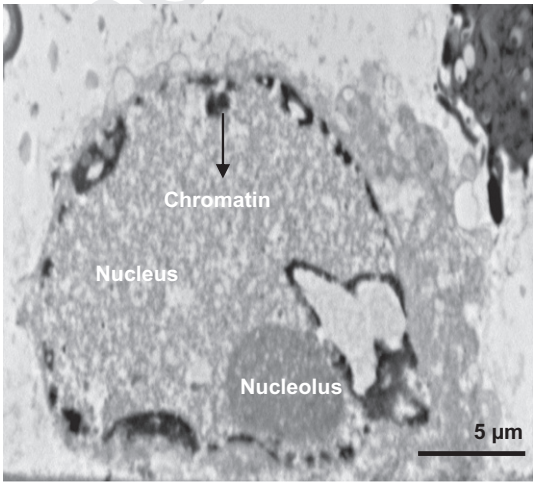

Fig. 6. A TEM micrograph at a magnification of 10,000× showing the disintegration of the nucleus and chromatin condensation from a melanoma cancer cell after PDT treatment with ZnTSPc at a photosensitizing concentration of 50 μg/ml and a light dose of 4.5 J/cm^2.

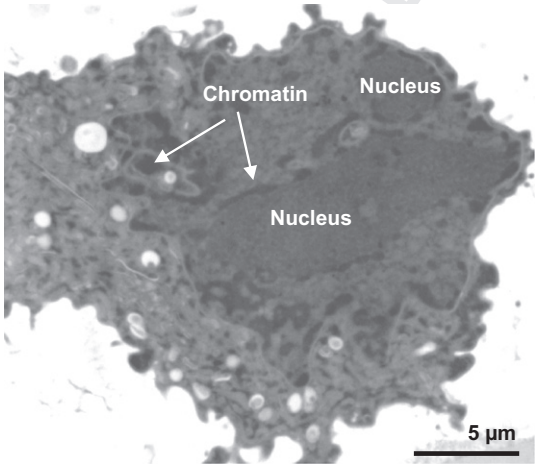

Fig. 5. A TEM micrograph at a magnification of 12,000× showing the condensed chromatin and fragmentation of the nucleus of a PDT treated melanoma cancer cell with ZnTSPc at a photosensitizing concentration of 50 μg/ml and a light dose of 4.5 J/cm^2.

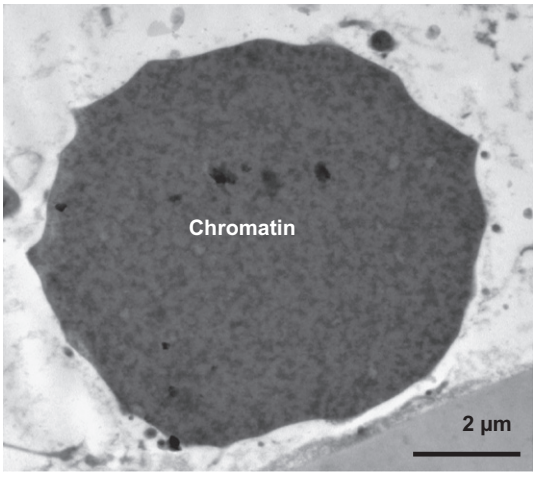

Fig. 7. A TEM micrograph at a magnification of 20,000× showing the aggregation of chromatin from a melanoma cancer cell exposed to ZnTSPc at a photosensitizing concentration of 50 μg/ml and a light dose of 4.5 J/cm^2.

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The exposure of photosensitized melanoma cancer cells to red light from continuous irradiation at a wavelength of 672 nm resulted in a further decrease in cell viability for each of the different PS concentrations in comparison to the dark toxicity data [27]. For effective PDT treatment the photosensitizer in combination with laser light is necessary. Results demonstrated that as the ZnTSPc concentration increased the cell viability of melanoma, fibroblast and keratinocyte cells proportionally decreased. For this study, the use of 50 μg/ml of ZnTSPc in combination with a laser light dose of 4.5 J/cm^2 at a wavelength of 672 nm are optimum conditions for the effective killing of melanoma cancer cells. The dark toxicity results and the adverse killing effects on normal healthy cells after PDT treatment for each of the different ZnTSPc concentrations were taken into consideration before regarding the ZnTSPc of concentration 50 μg/ml the EC_{50} value or the optimal photosensitizing concentration for this *in vitro* study.

Statistically, in this study there is a significant difference in the cell viability between absence of laser activation and laser activation for each cell line photosensitized with ZnTSPc, ($P < 0.0001$) and adjusted for all variables. It is more likely that melanoma cells ($P = 0.0016$), fibroblast cells ($P < 0.0001$) or keratinocyte cells ($P < 0.0001$) would be killed with laser exposure than without laser exposure.

The PDT effect at the tumor site depends on the PS concentration and the radiant energy density at the site which together determines the energy absorbed per unit volume at the target site. Knowledge of light dose and PS concentrations is therefore essential for the safe and effective treatment. Therefore, the influence of different light doses (2.5 J/cm^2, 7.5 J/cm^2 and 10.5 J/cm^2) on the cell viability of melanoma, fibroblast and keratinocyte cells photosensitized with the optimum ZnTSPc concentration was evaluated during the light dose study. It was noted that the optimum ZnTSPc (50 μg/ml) concentrations and the light dose of 4.5 J/cm^2 was the most lethal for the melanoma cancer cells in comparison to the other light doses (2.5 J/cm^2, 7.5 J/cm^2 and 10.5 J/cm^2). The cell viability of healthy normal fibroblast cells photosensitized with ZnTSPc (50 μg/ml) decreased with the increase in light dose. Keratinocyte cells photosensitized with ZnTSPc (50 μg/ml) showed the highest cell viability with the light dose of 2.5 J/cm^2 and 4.5 J/cm^2. This indicates that the low light doses of 2.5 J/cm^2 and 4.5 J/cm^2 using the output power between 20 and 30 mW would be a better combination with the optimum PS concentrations than 7.5 J/cm^2 and 10.5 J/cm^2 for the killing of melanoma cancer cells.

During this study the light doses 7.5 J/cm^2 and 10.5 J/cm^2 with the output power between 20 and 30 mW required irradiation times in range of 3–7 min. Many photosensitizers can undergo a process called photobleaching (photodegradation) during prolonged irradiation. Photobleaching or photodegradation is the degradation of the photosensitizer for the production of photoproducts by specific photochemical reactions. Studies have shown that second-generation photosensitizers (e.g. Foscan®) can be more readily bleached than first-generation photosensitizers (e.g. Photofrin) [15,28,29]. Patients treated with Foscan® demonstrated that 75% of the photosensitizer in the tumor is bleached at the end of an irradiation light dose treatment with only 10 J/cm^2. Photobleaching has its advantages of theoretically helping to increase the therapeutic effects of PDT providing that the photosensitizer levels are higher in the tumors than surrounding healthy tissue [15,28,29].

In this study, the possibility of the photosensitizer (ZnTSPc) photodegrading with the irradiation times associated with the light doses of 7.5 J/cm^2 and 10.5 J/cm^2 could be the reason for the ineffective killing of the melanoma cancer cells. Unfortunately, healthy normal fibroblast and keratinocyte cells were not affected by PS degradation during long irradiation times because as the light dose increased cell viability decreased in most cases. Therefore, the most effective light dose would be 4.5 J/cm^2 for the killing of most of the melanoma cancer cells while sparing most of the healthy normal fibroblast and keratinocyte cells.

Lastly, ultrastructural features of apoptosis were clearly identified in post-irradiated melanoma cancer cells treated with ZnTSPc after 24 h. Chromatin condensation (Figs. 5 and 6), nucleus fragmentation (Fig. 5), nucleus disintegration (Fig. 6) and chromatin aggregation (Fig. 7) are detectable morphological changes of late apoptosis. The TEM can also provide some information on the nature of the biochemical pathways because the morphological or ultrastructural changes in apoptotic cells are initiated by certain specific apoptosis proteins or factors [30,31]. For example, the TEM image in Fig. 6 shows characteristics of caspase-independent apoptosis, namely lumpy incomplete chromatin (electron dense regions or matter) condensation and disintegrated nucleus with tightly packed partially condensed micronuclei [30–33]. Parallel DNA fragmentation and morphological studies were conducted, which reported that apoptosis was induced in melanoma cancer cells after PDT treatment with ZnTSPc [34].

5. Conclusion

This *in vitro* study has shown that ZnTSPc mediated photodynamic therapy is an effective treatment option for melanoma cancer. 50 μg/ml of ZnTSPc with the treatment light dose of 4.5 J/cm^2 from a CW diode laser source with a wavelength of 672 nm was adequate to destroy melanoma cancer cells via apoptosis with low killing effects on healthy normal skin cells. There are still several questions on the detailed effects of photobleaching that still needs to be answered in order to understand its role in PDT. PDT as a primary treatment and an adjuvant therapy with either surgery or other treatment modalities for melanoma cancer needs to be further investigated in a clinical setting.

6. Abbreviations

ZnTSPc	zinc tetrasulfophthalocyanine
PDT	Photodynamic therapy
PS	photosensitizer
Pcs	phthalocyanines
CW	continuous wave

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