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Photolon™, a chlorin e6 derivative, triggers ROS production and light-dependent cell death via necrosis

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Abstract

Photolon™ is a photosensitiser with demonstrated potential as an anti-tumour agent. In this study, an *in vitro* investigation was performed to determine the mechanism of Photolon™-induced cell death. Cell killing was observed in a light-dependent manner and light-activated Photolon™ resulted in a significant production of reactive oxygen species (ROS), which could be blocked by type I ROS scavengers. Inhibition of ROS production using Trolox prevented Photolon™-induced cell death. Light-activated Photolon™ caused no increase in caspase-3/7 activity, but a rapid increase in lactate dehydrogenase (LDH) release suggesting a loss of membrane integrity and subsequent cell death by necrosis. We conclude that the mechanism of Photolon™-induced cell death involves the induction of ROS via a type I mechanism, which is ultimately responsible for cell killing by necrosis.

Keywords: Photodynamic therapy; Photolon™ (Fotolon); Cancer; ROS production; Necrosis

1. Introduction

Photosensitisers are light-absorbing compounds that are activated by exposure to light of a specific wavelength. These activated compounds are capable of inducing cell death, and are thus being established as a cancer treatment regimen. Their cytotoxicity is mediated through the production of reactive intermediates that damage cellular components, leading to cell death (Dolmans, Fukumura, & Jain, 2003). Chlorin e6 is a naturally occurring chlorin derivative that has shown promise as a cancer therapeutic (Kostenich, Zhuravkin, & Zhavrid, 1994). The chlorin e6 molecule has been adapted to improve its clinical efficacy, and the conjugation of a trisodium salt of chlorin e6 to polyvinylpyrrolidone (PVP), a water-soluble polymer, has been reported (Parkhots et al., 2003). This formulation (Ce6-PVP) is also known as Photolon™. Photolon™ has two absorption peaks at approximately 420 and 660 nm (Ulatowska-Jarza et al., 2005) and its activation at 665 nm has been shown to induce tumour necrosis in mice (Chin, Heng, Bhuvaneswari, Lau, & Olivo, 2006). This long wavelength of activation means that Photolon™ can be used for deep tissue excitation, and thus the destruction of deep-seated tumours.

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Photolon™ has several advantages as a photosensitiser compared to chlorin e6 alone. The complex of chlorin e6 with PVP increases its stability and improves its solubility in water, thereby increasing its bioavailability and enhancing its photosensitising effect (Chin, Heng, et al., 2006). Moreover, the covalent attachment of chlorin e6 to a polymeric carrier enables a prolonged circulation of the drug in the plasma due to its increased molecular weight (Kaneda et al., 2004). This leads to passive tumour targeting by the "enhanced permeability and retention" (EPR) effect (Duncan, 2003). This effect is based on the fact that tumour vasculature is hyper permeable, and thus allows preferential diffusion of circulating drugs into the tumour tissue (Duncan, 2003). This explains the higher selectivity of Photolon™ accumulation in malignant tissues compared to chlorin e6 alone (Chin, Heng, et al., 2006). A study of the effect of Photolon™ on human nasopharyngeal carcinoma cell lines, xenografted on nude mice, found that Photolon™ fluorescence after 6 h of uptake was in a ratio of 28:1 in tumour versus normal tissue (Ramaswamy, Manivasager, Chin, Soo, & Olivo, 2005).

To date, Photolon™ has been used successfully in the treatment of head, neck and skin melanomas, where the cancerous growths disappeared in 95% of cases (Petrov, Trukhacheva, Isakov, Turyn, & Kravchenko, 2004). Photolon™ is also favourable as a therapeutic agent as it accumulates rapidly in tumour tissue (within 1 h of intravenous administration) and has a faster clearance rate from the body compared to chlorin e6 alone; thus minimising the problem of prolonged skin photosensitisation (Chin, Heng, et al., 2006). Photolon™ has been shown to clear from the body as early as 12 h post-drug administration (Petrov et al., 2004). In addition to the therapeutic benefits, Photolon™ shows promise as a detection tool for cancer. Its emission of fluorescence upon light excitation and its selective accumulation in tumour tissue make it a promising photosensitiser for fluorescence imaging (Chin, Lau, Heng, Bhuvaneswari, & Olivo, 2006).

Although Photolon™ has clinical advantages and appears to be proficient in the killing of cancer cells *in vivo* (Chin, Heng, et al., 2006), little is known regarding its photobiological effects. Different photosensitisers induce different mechanisms of cell death, and both apoptotic and necrotic mechanisms have been proposed for different photosensitisers (Calzavara-Pinton, Venturini, & Sala, 2007). For example, an apoptotic mechanism of cell death has been demonstrated for the second-generation photosensitiser PAD-S31 (Date et al., 2004), while necrosis-like death has been observed in Photogem-treated cells (Ahn et al., 2004). In this study we investigated the mechanism through which Photolon™-induced cell death occurs using cancer cell lines.

2. Materials and methods

2.1. Materials

Photolon™, was obtained from Belmedpreparaty (Minsk, Belarus) and dissolved in sterile water at a stock concentration of 10 mg/ml. DCF-DA and DHR were both obtained from Sigma, as were the ROS scavengers Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 1,3-dimethyl-2-thiourea (DMTU), butylated hydroxyanisole, (BHA) and 1,4-diazabicyclo(2,2,2)octane (DABCO), and the singlet oxygen enhancer deuterium oxide (D_2O). 1,3-Diphenylisobenzofuran (DPBF) was obtained from Aldrich.

2.2. Cell culture

ME180, MS751 and CaSki cells were obtained from American Type Culture Collection (ATCC). WHCO5 was acquired from Rob Veale at the University of Witwatersrand (Jones & Veale, 2003). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were maintained at 37 °C in 95% air and 5% CO_2.

2.3. Cell survival and proliferation assays

Cell survival assays: 100 000 cells were plated in 1 ml of media on a 24-well tissue culture plate and incubated at 37 °C until confluent. Varying concentrations of Photolon™ were added and the cells incubated for 2 h. Cells were exposed to light from an X-ray film box by placing the culture plate onto the surface of the box for 15 min, as described previously (Cavanaugh, 2002). The light dose was approximately 0.7 mW/cm^2. Cells were then returned to the dark and incubated O/N at 37 °C. This was followed by washing with 1× PBS to remove dead (floating) cells, fixing with methanol, staining with crystal violet and washing three times with water. The plates were scanned using a Cannon 4200F digital colour scanner. After scanning, 200 μl of 50% acetic acid was added to each well to solubilise the violet stain, and the absorbance was measured at a wavelength of 595 nm.

IC_{50}s with 95% confidence intervals were calculated in GraphPad Prism version 4.00.

Cell proliferation assay: the MTT cell proliferation assays were performed using the Cell Proliferation Kit I from Roche, according to the manufacturers' instructions. Experiments were performed in quadruplicate in 96-well tissue culture plates and repeated at least 2–3 times. Briefly, cells were incubated in media containing 10 μg/ml of Photolon™ at 37 °C for 2 h and either kept in the dark or exposed to light for 15 min. Growth was measured over a period of 5 days. Absorbance was measured at 595 nm.

2.4. Reactive oxygen species (ROS) assays

ROS was assayed using dichlorofluorescin diacetate (DCF-DA) (Sigma). DCF-DA reacts with intracellular H_2O_2 as well as with other peroxides to emit fluorescence (LeBel, Ischiropoulos, & Bondy, 1992). Cells were plated in 60 mm tissue culture dishes and grown to 70% confluency and incubated with 10 μg/ml Photolon™ for 2 h. Cells were then exposed to light for 15 min, washed with 1 ml of pre-warmed Krebs–Ringer's buffer and 50 μM DCF-DA in KR buffer was added (in the dark) and incubated for an additional 15 min at 37 °C. The cells were harvested and the cell pellet was resuspended in 300 μl KR buffer. One hundred microlitres of the cell suspension was aliquoted into two wells of an opaque 96 well plate and the fluorescence read at 485 nm. The remainder of the solution was used for protein quantification using the bicinchoninic acid (BCA) assay (Pierce). For the scavenging of ROS, 50 μM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma) was added to the cells for 30 min at 37 °C prior to the addition of Photolon™. To screen the effect of specific ROS scavengers and enhancers, cells were incubated for 30 min prior to Photolon™ treatment, with each reagent diluted in culture media at the following concentrations: 10 mM DMTU, 100 μM BHA, 1 mM DABCO and 0.05 mM DPBF. These concentrations were those found to be optimal in other published reports (Sparrow et al., 2002; Uslu et al., 2000), and were found to be non-toxic to the cells. For the light treatment of cells in D_2O, D_2O was prepared at a concentration of 90% in PBS and substituted for culture media just before the 15 min light exposure. ROS assays using scavengers were performed using 50 μM dihydrorhodamine (DHR), a non-fluorescent probe that can be converted into fluorescent rhodamine-123 (R-123) and measure hydrogen peroxide, singlet oxygen and peroxynitrite (at an excitation wavelength of 485 nm) (Costa et al., 2007).

2.5. Caspase-3/7 assays

The Caspase-Glo™ 3/7 Assay kit (Promega) was used as a measure of apoptosis. Cells were plated in quadruplicate in a 96 well microplate and grown O/N. Photolon™ was added to a final concentration of 10 μg/ml and the cells incubated for 2 h. After a 15 min light incubation, cells were assayed for caspase-3/7 activity after 0.5, 1, 2, 4 and 8 h. At each time point cells from the "light" and "dark" plates were processed. Sixty microlitres of Caspase-Glo™ 3/7 Reagent was added to each well, and the plates were then shaken at 300 rpm for 30 s and incubated at RT for 30 min. One hundred microlitres of the cell solution was aliquoted into an opaque microplate and left for 30 min at room temperature. Caspase-3/7 was then assayed using the Veritas™ Microplate Luminometer (Turner BioSystems, Inc.).

2.6. Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is released from cells with damaged membranes and this is indicative of necrosis. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay Kit (Promega) was used to measure LDH release. Protocols were followed according to the manufacturers' instructions.

3. Results

3.1. Effect of Photolon™ on cell survival and proliferation

Photodynamic therapies have primarily been shown to be effective in the treatment of cancers that are readily accessible to a light source. In this study, we used cervical and oesophageal cancer cell lines, as these are representative of the cancer types that fulfil this criteria and photodynamic therapy is proving to have potential in the treatment of these cancers. To determine whether Photolon™ is taken up by these cell lines, cells were incubated with the photosensitiser and visualised under fluorescent microscopy. Results shown are fluorescent and phase-contrast images of CaSki and WHCO5 cells incubated with Photolon™ after optimisation of the time taken for the photosensitiser to enter cells (Fig. 1). The minimum time for Photolon™ to be taken up was found to be between 1 and 2 h. Similar results were found for a number of different cell lines (data not shown). The concentration of Photolon™ required to induce optimal killing of cancer cells was determined by incubation with varying concentrations of Photolon™ (1–40 μg/ml) for 2 h, and surviving cells monitored 24 h later by crystal

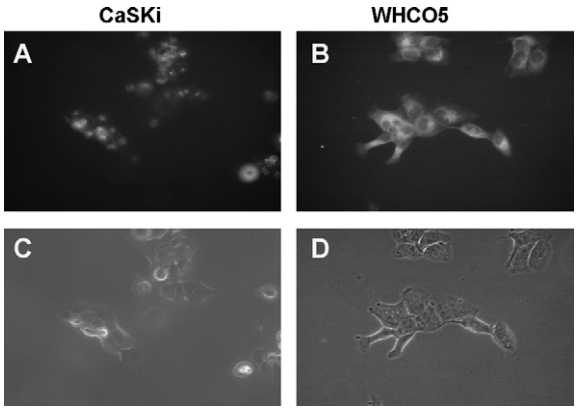

Fig. 1. Visualisation of Photolon™ in cancer cells by fluorescent microscopy. Photolon™ was added to cancer cell lines at a concentration of 10 μg/ml and cells were visualised 2 h later using a Zeiss Axiovert 200M fluorescence microscope. Fluorescence (A and B) and corresponding phase contrast (C and D) images at 40× magnification are shown for CaSki cervical cancer (A and C) and WHCO5 oesophageal cancer (B and D) cell lines. Similar observations were made with Ms751 and ME180 cervical cancer cells.

violet staining. Similar results were obtained for all of the cell lines tested and results for WHCO5 cells are shown (Fig. 2A). At the concentrations used, Photolon™ treatment had little effect on cell viability when cells were kept in the dark, implying that unactivated Photolon™ has little cytotoxicity. Exposure of Photolon™-treated cells to light, however, resulted in a significant decrease in cell survival, as observed by crystal violet staining of WHCO5 cells remaining after light treatment (Fig. 2A). Quantification of the crystal violet stained cells showed that the reduction in cell survival was dependent on the concentration of the drug. One microgram per millilitre Photolon™ had a marginal killing effect on cells exposed to light, which increased with increasing concentrations of Photolon™ (Fig. 2B). Ten micrograms per millilitre of Photolon™ was found to be the minimum concentration required for a highly significant cell killing effect ($p < 0.001$).

To compare the cytotoxic effect of Photolon™ across different cancer cell lines IC_{50}'s were calculated using

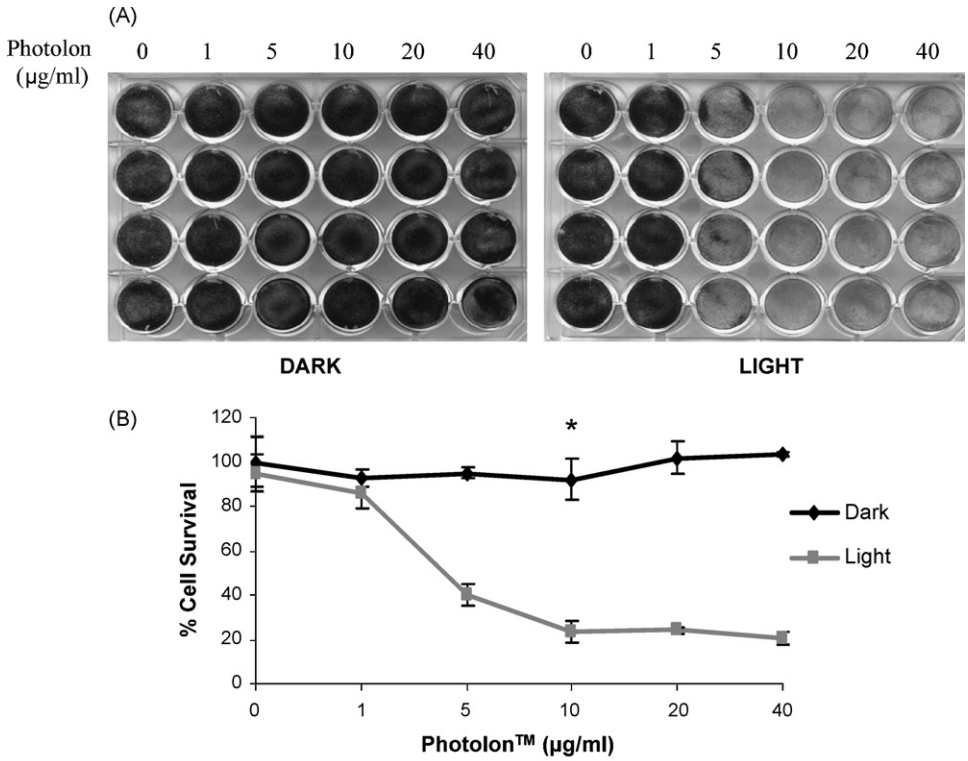

Fig. 2. Increasing concentrations of light-activated Photolon™ reduces cell survival. Cell survival after treatment with Photolon™ was determined by crystal violet staining of cells remaining on culture plates with and without exposure to light. (A) WHCO5 cells were plated in 24-well tissue culture dishes, incubated with Photolon™ for 2 h, and either kept in the dark or exposed to light. After incubation for 24 h, surviving cells remaining on the culture plates were washed and stained with crystal violet (as described in Section 2). Experiments were performed in quadruplicate and repeated at least two times. (B) Quantification of cells incubated with Photolon™ and grown in the dark or treated with light. Results shown are the mean ± S.D. of quadruplicate assay points (*$p < 0.001$) and represent light-dependent killing as Photolon™ had no effect on cells maintained in the dark. These experiments were also performed for CaSki, Ms751 and ME180 cells and similar results were obtained (see Fig. 3 for IC_{50} curves).

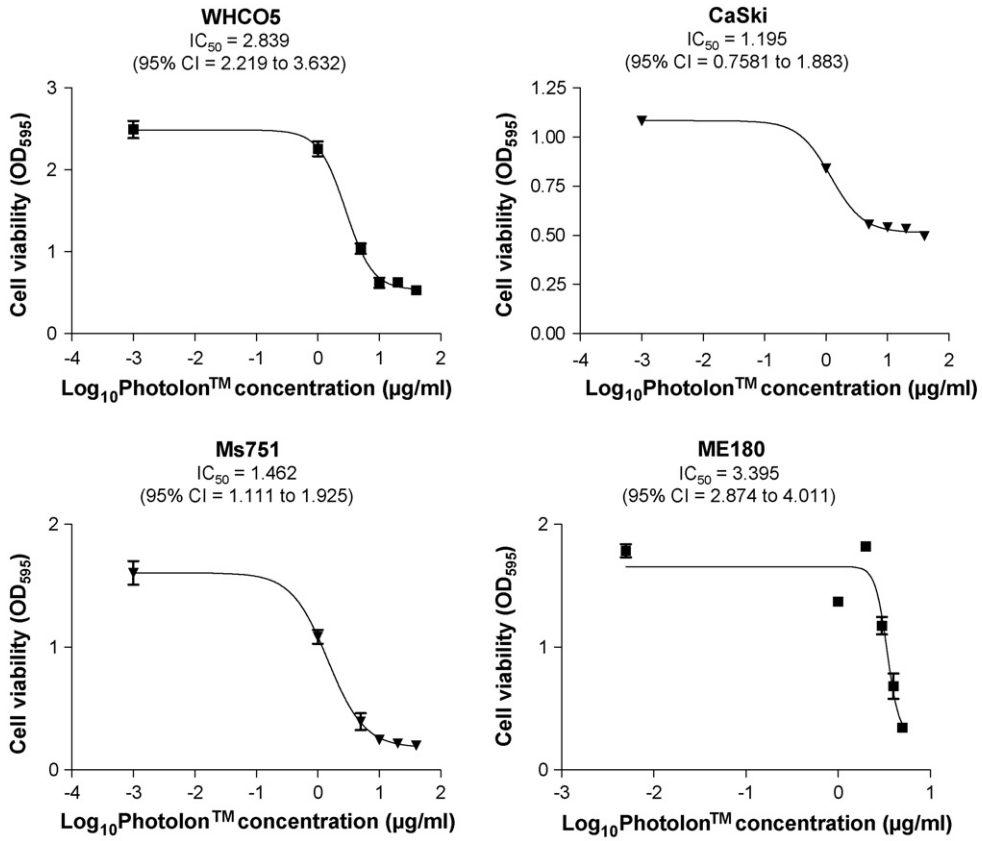

Fig. 3. Light-dependent killing of different cancer cell lines by Photolon™. WHCO5, CaSki, Ms751 and ME180 cells were treated with varying concentrations of Photolon™ (1–40 μg/ml) and exposed to light after incubation for 2 h. Viable cells after light-activation of Photolon™ were quantified the following day as described in Fig. 2. The resulting data was analysed in GraphPad Prism Version 4.00 by plotting the log of Photolon™ concentration versus the OD_{595} of surviving (viable) cells. IC_{50}'s and 95% confidence intervals were calculated and are shown for individual cell lines. Similar IC_{50}'s were obtained for WHCO5, CaSki, Ms751 and ME180. Each assay point represents the mean ± S.D. of experiments performed in quadruplicate.

GraphPad Prism for WHCO5, ME180, Ms751 and CaSki cells that were treated with Photolon™ and exposed to light. The IC_{50} values (with 95% confidence intervals) were similar between the different cell types, varying between 1 and 3 μg/ml, suggesting that Photolon™ had a similar cytotoxic effect on these cancer cells (Fig. 3).

Having determined that light-activated Photolon™-induced the most significant cell death at a concentration of 10 μg/ml (Fig. 2), this concentration was used to investigate whether Photolon™-induced cell death occurred as a result of cytotoxic or cytostatic effects. MTT assays were used to measure cell proliferation in the absence and presence of light-activated Photolon™ using two representative cell lines, CaSki and WHCO5. While both cell lines continued to proliferate when incubated with unactivated Photolon™ (i.e. cells not exposed to light treatment), light treatment of Photolon™-containing cells resulted in an immediate inhibition of growth and reduction in cell number in both CaSki and WHCO5 cells (Fig. 4A and B). These results suggest that the concentration of Photolon™ used has a cytotoxic effect rather than causing cell growth arrest followed by cell death (cytostatic). Since Photolon™-induced cell death occurred in a rapid manner we postulated that this was likely to occur via the production of reactive oxygen species (ROS).

3.2. *Photolon™-induced cell death occurs via type I ROS reactions*

The production of reactive oxygen species (ROS) stimulates cytotoxicity and has been shown to be an initiating event in PDT-induced cell death. To determine whether Photolon™-induced cell death occurred via the production of ROS, we measured its production in control and light-activated cells using DCF-DA. Our results showed a significant increase in ROS production in light-activated Photolon™ containing cells (Fig. 5A). The accumulation of ROS occurred within

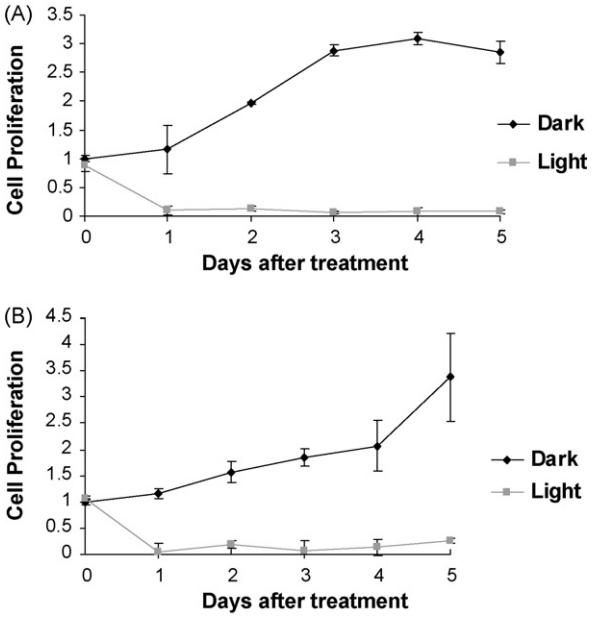

Fig. 4. Activated Photolon™ inhibits cell proliferation. Cell proliferation was assayed for (A) CaSki and (B) WHCO5 cells. Cells were grown in 96 well plates, treated with 10 μg/ml Photolon™, and maintained either in the dark or exposed to light for 15 min. Following light exposure they were incubated at 37 °C for the indicated days (described in Section 2). Cell proliferation was monitored using the MTT assay over a period of 5 days and results shown are the mean ± S.D. All experiments were performed in quadruplicate and repeated at least two times. Photolon™-induced inhibition of cell proliferation was light-specific since cells maintained in the dark continued to proliferate over the assay period.

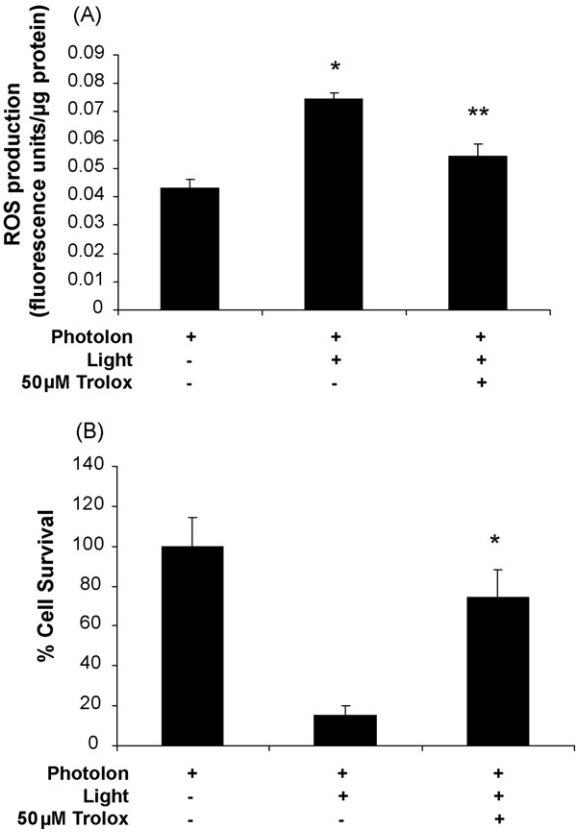

Fig. 5. Photolon™-induced ROS production and cell killing can be reversed by the ROS scavenger, Trolox. (A) Reactive oxygen species (ROS) production was measured in WHCO5 cells using DCF-DA as described in Section 2. The amount of fluorescence detected is a direct indication of ROS production. Light-activated Photolon™ resulted in a significant increase in ROS production compared to cells maintained in the dark (*$p < 0.001$). The addition of Trolox prior to incubation with Photolon™ and exposure to light significantly inhibited activated Photolon™-induced ROS production (**$p < 0.01$). (B) WHCO5 cell survival as measured by the MTT assay 24 h after Trolox and Photolon™ treatment shows that scavenging ROS production by inclusion of Trolox significantly inhibited light-activated Photolon™-induced cell death (*$p < 0.005$). Results shown are the mean ± S.D. of experiments performed in quadruplicate.

15 min of exposure to light. Incubation with Trolox, a vitamin E analogue ROS scavenger, prior to the addition of Photolon™ and light exposure, significantly decreased overall ROS production (Fig. 5A), suggesting that one of the initiating events in Photolon™-induced cell death is the production of ROS. Since Trolox scavenged excess ROS produced as a result of light-activation of Photolon™, we next tested whether incubation with Trolox would consequently prevent Photolon™-induced cell death. Our results show that the treatment of cells with Trolox prior to incubation with Photolon™ and light treatment significantly reduced the growth inhibitory effect of activated Photolon™ (Fig. 5B). These results suggest that activated Photolon™ induces ROS which is required for cell death by this PDT.

Photosensitisers are known to induce cell killing via either type I reactions in which electron-transfer occurs between the light-excited photosensitiser and cellular constituents, and/or type II reactions that involve energy transfer between the excited photosensitiser and molecular oxygen, to produce singlet oxygen (Calzavara-Pinton et al., 2007). To assay for the involvement of type I reactions, we analysed the effect of scavengers of type I-involved species using DMTU, a strong scavenger of hydroxyl radicals (OH^-), superoxide anion radicals (O_2^-) and hydrogen peroxide (Killilea, Hester, Balczon, Babal, & Gillespie, 2000; Sparrow et al., 2002). This scavenger significantly reduced ROS production as measured using dihydrorhodamine 123 (DHR) ($p < 0.01$) (Fig. 6A). In addition, BHA, an antioxidant that inhibits the mitochondrial electron transport system (Uslu et al., 2000), also dramatically reduced ROS production ($p < 0.01$) (Fig. 6A). Our earlier finding that Trolox

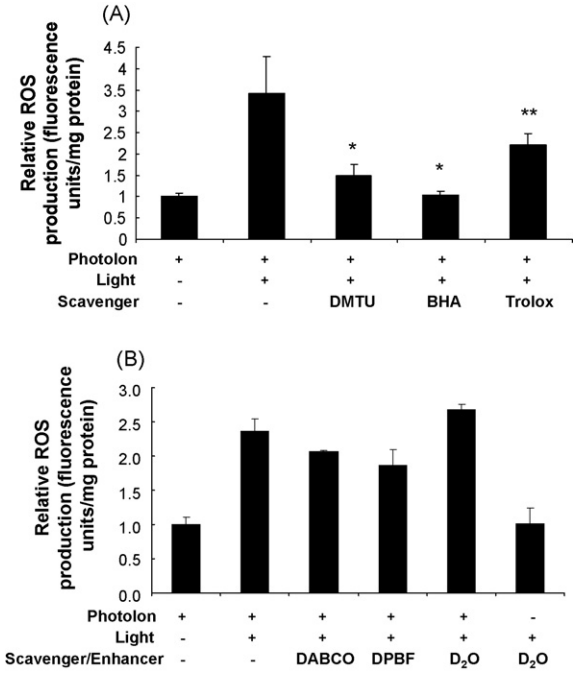

Fig. 6. Photolon™ induces ROS production via type I free radical-mediated reactions. ROS was measured using DHR, which measures both type I and type II reaction intermediates. The effect of various scavengers (DABCO, DPBF, DMTU, BHA, Trolox) and enhancers (D_2O) of oxidative damage were determined. (A) Type I free radical scavengers used at concentrations previously reported to affect ROS (10 mM DMTU, 100 μM BHA and 50 μM Trolox), significantly reduced Photolon™-induced ROS (*$p < 0.01$, **$p < 0.05$) while (B) type II singlet oxygen modulating agents, DABCO (1 mM), DPBF (50 μM) and D_2O had no effect. Data shown are the mean ± S.D. of experiments performed in quadruplicate and repeated at least two times.

could scavenge Photolon™-induced ROS as measured using DCF-DA was supported by assays based on the oxidation of DHR (Fig. 6A). To determine the possibility of type II reactions, we analysed the effect of enhancers and quenchers of singlet oxygen on ROS production after light-activated Photolon™ treatment. D_2O, has been reported to significantly prolong the lifetime of singlet oxygen and hence serves as an enhancer (Wlaschek et al., 1997). If activated Photolon™ produced singlet oxygen species; we anticipated that ROS production would be enhanced with D_2O. Our results however, showed no effect when cells were exposed to light-activated Photolon™ in the presence of D_2O (Fig. 6B). D_2O on its own also had little to no effect on ROS production in control cells. Moreover, the presence of singlet oxygen quenchers DABCO and DPBF caused no significant change in Photolon™-induced ROS production (Fig. 6B). Taken together, these results suggest that Photolon™ phototoxicity is likely to be mediated through the type I reaction rather than that of a type II reaction.

3.3. *Photolon™-induced cell death involves cellular necrosis*

Photodynamic therapies have been reported to induce cell killing via necrosis, apoptosis, or both. We speculate that the subcellular localisation of a photosensitiser has important implications for the mechanism through which cell death occurs. This is based on reports by Kessel and Luo (1998) showing that an apoptotic response occurred if the photosensitiser was localised to the mitochondria, while a necrotic mechanism of cell death occurred when the photosensitiser caused damage to the lysosomes or cell membrane. In order to determine whether Photolon™-induced cell death occurred via either of these mechanisms we assayed cells for caspase-3/7 activity as an indicator of apoptosis and LDH release as an indicator of necrosis. No increase in caspase-3/7 activity was observed but rather our results showed a decrease in Photolon™-treated cells exposed to light, while the caspase-3/7 activity of cells kept in the dark remained largely unchanged (Fig. 7A). Fluctuations in caspase-3/7 activity of cells kept in the dark were observed across the different time points; these, however, were not significantly different from controls at time 0. The decrease in caspase-3/7 activity in light-treated cells is possibly due to an increase in protein degradation during the process of cell death. Concurrent with the decrease in caspase-3/7 activity was a significant increase in LDH release in light-exposed cells (Fig. 7B). A similar change in LDH release was not observed in control cells kept in the dark, suggesting that LDH release occurred only as a result of light-activated Photolon™. LDH activity increased relatively quickly after light treatment (within 1 h), suggesting that cell death occurred via necrosis. Caspase-3/7 and LDH activity in Photolon™-treated CaSki cells were also performed and similar results were obtained (results not shown). These results suggest that light-activated Photolon™ induces necrosis rather than apoptosis, since no increase in caspase activity but an increase in LDH release was observed.

4. Discussion

Photodynamic therapy (PDT) is a powerful approach for the treatment of cancer, as it involves the local destruction of cancerous or precancerous tissue, while preserving the surrounding normal tissue. Since it has shown great potential as a therapeutic approach, newer agents have become the focus of much research. In this

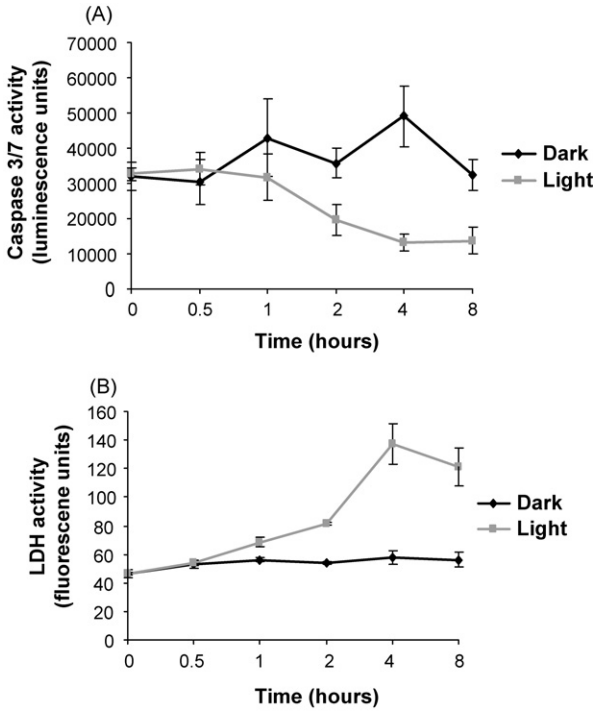

Fig. 7. Caspase-3/7 and LDH activity in Photolon™-treated cells. (A) Caspase-3/7 activity and (B) LDH activity in WHCO5 cells treated with Photolon™ were used as a measure of apoptosis and necrosis, respectively. Caspase-3/7 and LDH activity were assayed in cells treated with Photolon™ in its light-activated (light) and unactivated (dark) forms. A decrease in caspase-3/7 activity (A) and corresponding increase in LDH release (B) was observed when cells were exposed to light. Results shown are the mean ± S.D. of experiments performed in quadruplicate and repeated at least two times.

study Photolon™-induced cell death was analysed in an *in vitro* cell culture system. We show that Photolon™ exhibits no cytotoxicity in its unactivated form at concentrations that have a significant cell killing effect. Photolon™ appears to accumulate rapidly in cells and once activated by light it causes a rapid increase in cell death.

Photolon™ is a water-soluble compound; the conjugation of chlorin e6 to the hydrophilic polymer polyvinylpyrrolidone (PVP) making Photolon™ hydrophilic in nature (Chin, Heng, et al., 2006). While lipophilic photosensitisers (for example lipophilic porphyrins) are preferentially taken up through the plasma membrane of the cell, hydrophilic photosensitisers tend to be taken up by pinocytosis (Calzavara-Pinton et al., 2007), a form of endocytosis, where particles are taken in by the cell in endosomal vesicles which subsequently fuse with lysosomes (i.e. lysosomotropic delivery). Although the localisation of Photolon™ was not studied in the present study, it is likely that Photolon™ is internalised into tumour cells via endocytosis and subsequent trafficking delivers it to lysosomes. We have preliminary data that suggest it localises to lysosomes (data not shown). These findings would correlate well with a another study (Roberts & Berns, 1989), where mono-L-aspartyl chlorin e6 (Npe6), another water-soluble Ce6, enters the cell through endocytosis and localises in lysosomes.

While photosensitisers can localise to the plasma membrane and organelles such as the lysosome, mitochondria, golgi apparatus and endoplasmic reticulum (Calzavara-Pinton et al., 2007), as PDT's they are reported to induce the generation of reactive oxygen species (Dolmans et al., 2003). A recent study showed that after treatment with the photosensitiser, Pc4, ROS production was most pronounced in lysosomes, even though the photosensitiser itself was not specifically localised to lysosomes (Sakharov, Elstak, Chernyak, & Wirtz, 2005). These authors propose that lysosomes have a specific environment which makes them very susceptible targets for reaction oxygen species, and the attack of ROS on lipids in the lysosomes leads to lipid peroxidation. Lipid peroxidation induces a free-radical chain reaction that propagates, leading to lysosomal damage. The permeability of the lysosome increases, ultimately causing its rupture. Lysosomal hydrolases are released that interact with and degrade cellular membranes, thereby inducing cellular necrosis. Lactate dehydrogenase (LDH) is released from cells with damaged membranes, making it a suitable marker of necrosis.

Photosensitisers can produce ROS via type I (involving the formation of free radicals) or type II reactions (involving the generation of singlet oxygen), for example the photosensitiser Neutral Red acts via type I reactions, while the photosensitiser Rose Bengal acts via type II reactions (Fischer, Krieger-Liszkay, & Eggen, 2005). We show that light-activated Photolon™ leads to the production of reactive oxygen species through type I reactions, as type I free radical scavengers significantly reduced the production of Photolon™-induced ROS, while singlet oxygen scavengers and an enhancer of singlet oxygen production, D_2O, had no effect. We suggests that the mechanism of Photolon™-induced cell death may involve electron transfer to oxygen, and the subsequent formation of toxic photo-products that presumably cause cellular damage, resulting in the initiation of cellular necrosis. Blocking ROS production by the ROS scavenger Trolox blocks Photolon™'s phototoxic effect. Our results add to previously reported findings by (Kessel & Luo, 1996), that phototoxicity was prevented on addition of radi-

cal scavengers to cells treated with lysosomal-targeting photosensitisers.

In conclusion, our data using *in vitro* cell culture systems (1) supports clinical findings that Photolon™ is a highly effective second-generation photosensitiser and (2) suggests that its mechanism of action is via the production of type I reactive oxygen species followed by cell death through necrosis.

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