Chemotherapy: Open Access
ISSN: 2167-7700

Velloso et al., Chemotherapy 2012, 1:5
DOI: 10.4172/2167-7700.1000107

Research Article Open Access

Aluminum-Phthalocyanine Chloride-Based Photodynamic Therapy Inhibits PI3K/Akt/Mtor pathway in Oral Squamous Cell Carcinoma Cells *In Vitro*

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Abstract

The present study investigated the *in vitro* effects of liposomal aluminum-phthalocyanine chloride (AlPc)-based Photodynamic Therapy (PDT) on PI3K/Akt/mTOR signaling pathway, which has been reported to be constantly activated in squamous cell carcinoma. Cultured cells derived from Oral Squamous Cell Carcinoma (OSCC-3 lineage) were used. PDT was applied *in vitro* by exposing cultured cells to liposomal AlPc (0.5 or 2.5 μM for 30 minutes) and then irradiating them with laser (670 nm wavelengths, 24 J/cm^2 energy density). Cell viability was assessed by tripan blue staining and MTT assay. DNA fragmentation and mitochondrial permeability, signs of apoptosis, were also evaluated. Inhibition of PI3K/AKT/mTOR was accessed by immunofluorescence assay for detection of pS6, a downstream phosphorylated protein of this signaling pathway, in confocal microscopy. Morphological analysis was performed by FACS scan and phase contrast microscopy. Different mechanisms of cell death were induced in OSCC-3 cells, mainly apoptosis. Moreover, PI3K/Akt/mTOR signaling pathway was inhibited. The results show that cell death induced by AlPc-based PDT involves, at least partially, inhibition of the PI3K/Akt/mTOR pathway.

Keywords: Photodynamic therapy; Oral cancer; Cell death; mTOR, PI3K/Akt/mTOR pathway

Introduction

Human Oral Squamous Cell Carcinoma (OSCC) is a highly incident disease associated to several risk factors. Its treatment is usually associated with the risk of serious cosmetic or functional morbidity, mainly when surgery or radiotherapy is the mainline treatment [1,2]. Therefore, there is a need for therapeutic alternatives that preserve the normal architecture and function of the treated area and achieve tumor control comparable to or better than that obtained with classical therapies [3]. In this context, Photodynamic Therapy (PDT) has been shown to be a good candidate for treating OSCC. It involves reactions induced by light activation of a photosensitizer, namely type I and type II photoreactions (see figure 1) [4]. Both photoreactions generate oxidant species that affect cell structure and function, causing alterations in the cell itself, in its microenvironment and even in the whole organism. Type II photoreactions are generally more pronounced in PDT, since the light-activated photosensitizer catalyzes the triplet-to-singlet conversion of thousands of oxygen molecules; singlet oxygen has a markedly higher oxidant potential in comparison to triplet oxygen [5].

Some researchers have found that some specific molecular targets and organelles can be affected by PDT, profoundly influencing the fate of a PDT-treated cell and the effectiveness of the anticancer PDT itself [6]. The result depends mainly on the photosensitizer, oxygen concentration, light dose and wavelength, and the cell genotype [6]. PDT can kill cancer cells directly by efficiently inducing apoptosis as well as by activating non-apoptotic cell death pathways [6]. It can also damage the tumor-associated blood vessels, thus contributing to tumor regression [7], and activate or boost tumor-specific immune responses [8]. On one hand, for example, certain photosensitizers, particularly the hydrophobic ones, have been shown to accumulated in the mitochondria, where they can induce apoptosis when activated by light [9]; on the other hand, photosensitizers that accumulate in the plasma membrane or in lysosomes can either delay or block apoptosis and predispose cells to death by necrosis [6]. In this context, PDT can affect molecules in certain signaling pathways involved in cell death, an event that could determine what its outcomes would be [6].

The mammalian target of rapamycin (mTOR), a 289 kDa serine/threonine kinase, located downstream of the PI3K-Akt pathway, has been shown to be a major regulator of cell growth, proliferation, migration, differentiation, and survival [10]. In many human cancers, upstream (PI3K-Akt) and downstream (4E-BP1 and S6 kinase) signaling pathways of mTOR are deregulated and hence mTOR has been suggested to be a target for suppression of tumor growth [11]. In OSCC, activation of Phosphatidylinositol 3-Kinase (PI3K) is a frequent event [6] and mTOR is thus expected to be involved in the pathophysiology of this cancer. Previous work has demonstrated that the mTOR pathway is a target for PDT when an endolysosomal localizing phthalocyanine derivative, $AlPcS_2$, was used [11]. As liposomal AlPc-based PDT was shown to be effective against 4-Nitroquinoline-1-Oxide (4-NQO)-induced murine oral cancer *in vivo,* the present study evaluated the effects of this PDT protocol on the PI3K/Akt/mTOR pathway, *in vitro*. The results show that AlPc-based PDT inhibits PI3K/Akt/mTOR pathway in cultured human OSCC cells.

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Received October 26, 2012; **Accepted** November 13, 2012; **Published** November 16, 2012

Citation: Velloso NV, Muehlmann LA, Longo JPF, da Silva JR, Zancanela DC, et al. (2012) Aluminum-Phthalocyanine Chloride-Based Photodynamic Therapy Inhibits PI3K/Akt/Mtorpathway in Oral Squamous Cell Carcinoma Cells *In Vitro*. Chemotherapy 1:107. doi:10.4172/2167-7700.1000107

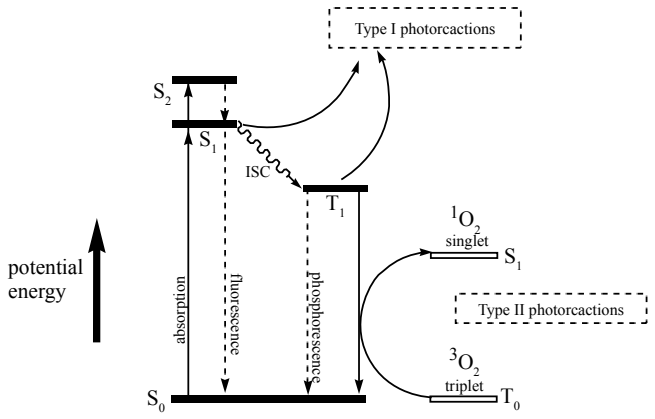

Figure 1: Jablonski diagram representing the excitation and relaxation of a photosensitizer, and type I and type II photoreactions. Horizontal bars represent energy levels of the different excitation states of a photosensitizer (full bars) or of molecular oxygen (empty bars). S_0=ground singlet state; S_1=first excited singlet state; S_2=second excited singlet state; T_0=ground triplet state; T_1=first excited triplet state; ISC=intersystem crossing. Adapted from [4].

Methods

Photosensitizer and light source

The photosensitizer AlPc was provided by Sigma-Aldrich Co. (Brazil). A diode laser (670 nm, BWF light source, Tech in) was used as the continuous light source.

Liposomal AlPc formulation

This formulation was prepared as described elsewhere [12].

Cell culture

The human oral squamous cell carcinoma cell lineage OSCC-3 was provided by the University of Michigan (USA) and maintained in 75 cm^3 culture flasks in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% (w:v) fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were incubated under 80% humidity, 5% of CO_2 and 37°C.

Treatments design

Cells were seeded in a 6-well plate, at a density of 1×10^5 cells/well, and cultured for 24 h. Then, treatments were performed as follows: control group (C) cells were incubated with PBS for 30 minutes; liposomal AlPc group (L-AlPc) cells were incubated for 30 minutes with liposomal AlPcat 0.5 µM or 2.5 µM; photodynamic therapy-treated group (PDT) cells were incubated for 30 minutes with AlPc at 0.5 µM or 2.5 µM, washed with and resuspended in PBS, and then irradiated with laser (670 nm wavelength, 24 J/cm^2 energy density). After the treatment, cells of all groups were maintained in culture conditions for 24h, as described before, and then assayed accordingly to the following protocols.

Trypan blue assay

The membrane integrity and cell proliferation were evaluated by the trypan blue (Sigma-Aldrich, USA) staining assay. Twenty four hours after each treatment, cells were detached from the plates with 0.25% (w:v) trypsin (Gibco, USA), centrifuged at 390×*g* and resuspended in 1mL DMEM. Ten microliters of this suspension was added to 40 µL 0.4% (w:v) trypan blue and cells were then counted and differentiated with a Neubauer chamber by light microscopy (Axiophot Zeiss).

MTT assay

The viability of the cells was assayed by the MTT (dimethylthiazolyl diphenyltetrazolium bromide) (Invitrogen, EUA) test. This is a colorimetric assay that relies on the ability of mitochondria from viable cells to convert MTT to an insoluble formazan, causing a yellow-to-purple color change. Briefly, twenty-four hours after treatment, medium was renewed, 20 µL 10% (w:v) MTT solution were added to each well and the cells were then incubated for 3h at culture conditions. Then, medium was removed, formazan was extracted from cells with 150 µL of dimethyl sulfoxide (Sigma, St Louis, MO) and absorbance at 595 nm was measured with a spectrophotometer (Spectra MAx M2–Molecular Devices).

Morphological analysis

Morphological analysis was based on two methods: FACS scan and phase contrast microscopy. For FACS, 24 h after the treatment, the floating and adherent cells were harvested, centrifuged, washed with 500 µL PBS and resuspended in 100 µL PBS. Next, cell granulosity and volume were both measured by FACSscan (FACS-Calibur, Becton Dickinson) flow cytometryand analyzed by CellQuest software. For phase contrast microscopy, 24 h after treatment, cells were analyzed using an inverted phase contrast microscope (Unico, EUA).

Measurement of mitochondrial membrane potential

Cytofluorometry was used to detect cellular cytotoxic effects, as described elsewhere [13]. For this assay, it was used rhodamine 123, a cationic fluorescent dye that penetrates into the cell and accumulates in mitochondria. Rhodamine effluxes from mitochondria when the membrane potential of this organelle is reduced, lowering the fluorescence of the cell. Briefly, 24 h after treatments, the floating and adherent cells were harvested, centrifuged, washed with 500 µL PBS and resuspended in 100 µL PBS. Next, cells were incubated with 0.5 µL solution of rhodamine 123 (5 mg/mL in ethanol) (Invitrogen, USA). After 15 minutes of incubation at room temperature, protected from light, cells were washed twice with 500 µL PBS. After each wash the samples were centrifuged (750×*g* for 3 minutes) and the supernatants were discarded. Next, cells were incubated for 30 minutes in 300 µl of PBS at room temperature, protected from light. Then, samples were evaluated using a FACSscan flow cytometery (FACS-Calibur, Becton Dickinson) at FL1-H (515-530 nm) and analyzed by the CellQuest software.

Detection of DNA fragmentation using FACS

This protocol is based on the fact that Propidium Iodide (PI) binds DNA allowing for measuring cell DNA content [14]. Briefly, 24h after treatment, the floating and adherent cells were harvested, centrifuged and resuspended in 100 µL PBS. For DNA fragmentation analysis, 200 µL PI-containing buffer (0.1% (w:v) sodium citrate, 0.1% (w:v) Triton X-100 and 20 µg/mL PI) (Invitrogen, USA) were added and the mixture was then incubated for 30 minutes in the dark, at room temperature. Next, samples were evaluated using a FACSscan flow cytometery (FACS-Calibur, Becton Dickinson) at FL2-H (560-580 nm) and analyzed by the CellQuest software.

Immunofluorescence detection of pS6

For immunofluorescence, a laser scanning confocal fluorescence microscope was used as previously reported [15]. This experiment was performed with groups control, L-AlPc 2.5 µM and PDT 2.5 µM. cells were cultured on polylysine coated coverslip. Twenty four hours after the treatments, cells were washed, fixed with 2% (w:v) paraformaldehyde

(Vetec Quimica Fina, Brazil) at room temperature for 10 minutes, washed with PBS and permeabilized with 0.2% (w:v) Triton X-100 for 10 minutes. Next, cells were washed with PBS, incubated with a blocking solution (2% (w:v) BSA and 5% (w:v) goat serum) for 30 minutes and incubated with the first primary antibody (ribosomal pS6, ser 240/244, Cell Signaling, USA) diluted 1:20 in blocking solution overnight at 4° C. After washing, slides were sequentially incubated for 2 h with the secondary antibody (rabbit anti-IgG FITC-Cell Signaling, USA) diluted 1:300, followed by washes with PBS. The slides were then mounted with 4`,6-diamidino-2-phenylindole (Sigma-Aldrich Co., USA) and images were captured with a confocal microscope (Leica TCS SP5, USA).

Statically analyses

Data were analyzed by ANOVA one way followed by Tukey multiple comparison tests (α=0.05). Data analysis was performed with GraphPad Prism version 5.00 for Windows.

Results

Liposomal AlPc-based PDT reduces OSCC-3 cells viability *in vitro*

The first step in this work was to investigate if liposomal AlPc-based PDT reduces the viability of OSCC-3 cells *in vitro*. Figure 2a shows the results obtained for cell viability by MTT assay. At 0.5 and 2.5 μM AlPc, in the dark, a decrease of c.a. 30% in the viability of OSCC-3 cell occurred. When cells exposed to liposomal AlPc were irradiated, a further statistically significant, AlPc dose-dependent decrease in cell viability was observed (c.a. 50% for 0.5 μM and 75% for 2.5 μM). The number of viable cells, as assessed by trypan blue staining assay (figure 2b), significantly decreased only in cells treated with PDT at 2.5 μM AlPc (c.a. 45% lower vs control).

Morphology of OSCC-3 is altered by liposomal AlPc-based PDT

Alterations in cell shape and an increase in the number of detached cells were observed under phase contrast microscopy on PDT groups in a dose-dependent manner in comparison to control group, as shown in figure 3a. Alterations in cell volume and cytoplasm granulosity (Figure 3c) were also observed in PDT groups.

OSCC-3 cells express signs of apoptosis after treatment with PDT based on liposomal AlPc

Figure 4a shows anAlPc dose-dependent decrease in mitochondrial membrane potential ($\Delta\Psi m$) in PDT groups (15% PDT 0.5 μM and PDT 2.5 μM 23% vs control, p<0.05). Moreover, DNA fragmentation

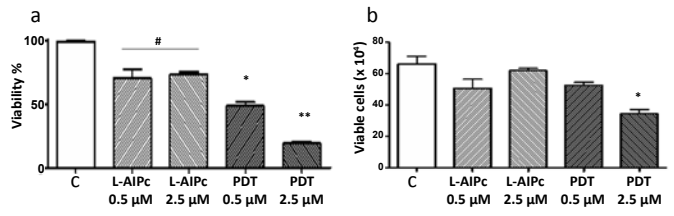

Figure 2: Viability of OSCC 3 cells after PDT treatment. a) Percentage of viable cells, measured by MTT assay; #p<0.05 vs C; *p<0.05 vs control, and L-AlPc 0.5 and 2.5 μM; **p<0.05 vs all other groups. b) Number of viable cells counted after trypan blue staining; *p<0.05 vs all other groups. Results are presented as mean ± standard deviation from 3 independent experiments.

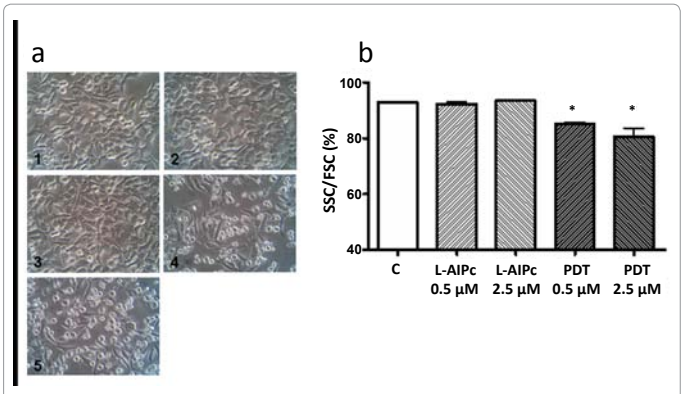

Figure 3: Morphological analysis of OSCC 3. a) phase contrast microscopy of control (1), L-AlPc 0.5 μM (2), L-AlPc 2.5 μM (3); PDT 0.5 μM (4) e PDT 2.5 μM (5); b) ratio of cell granulosity (SSC) to volume (FSC) evaluated by flow cytometry. Results are presented as mean ± standard deviation from 3 independent experiments. *p<0.05 vs C, and L-AlPc 0.5 and 2.5 μM.

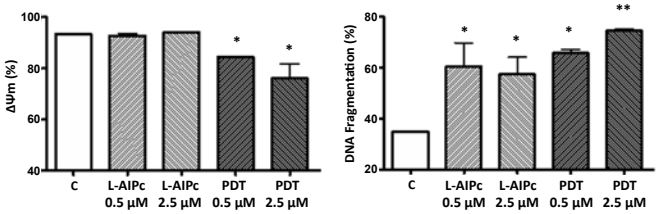

Figure 4: Mitochondrial membrane potential ($\Delta\Psi m$, left) and DNA fragmentation (right) in OSCC-3 cells. Results are presented as mean ± standard deviation from 2 experiments.*p < 0.05 vs control. **p<0.05 vs all other groups.

was intense in cells of PDT 0.5 and 2.5 μM group, although this event was also observed in cells treated with liposomal AlPc in the dark.

Liposomal AlPc-based PDT reduces the amount of pS6 per OSCC-3 cell

The analysis of pS6 protein showed that cells from control group presented intense fluorescence on the cytoplasm, evidencing the presence of pS6 (Figure 5a). After application of PDT, this fluorescence was reduced, indicating an inhibition of the PI3K/Akt/mTOR pathway (Figure 5b). There was no visible difference between cells of control and L-AlPc 2.5 μM groups for the presence of pS6.

Discussion

The results obtained in this work show that the treatment of OSCC 3 cells with liposomal AlPc-based PDT induced a significant, dose-dependent reduction of cell viability and the number of cells 24h after its application, particularly in cells exposed to 2.5 μM AlPc. This result confirms that this liposomal formulation is effective against cancer cells, as previously demonstrated *in vivo* [16] and *in vitro* [3]. The reduced viability and number of cells after PDT treatment was paralleled by alterations on cell morphology. Under phase contrast microscopy, OSCC-3 cells treated with PDT, at both 0,5 μM and 2.5 μM AlPc, presented lower adhesion to the culture plate when compared to control cells. The analysis using flow cytometry showed reduction in volume and in quantity of cell granules in OSCC-3 cells of the PDT 2.5 μM group. These evidences suggested that cells could be undergoing an apoptotic process induced by PDT [3].

In fact, evidences indicate that PDT can directly kill the cancer cells by the induction of apoptosis, necrosis or the autophagy [6]. The next

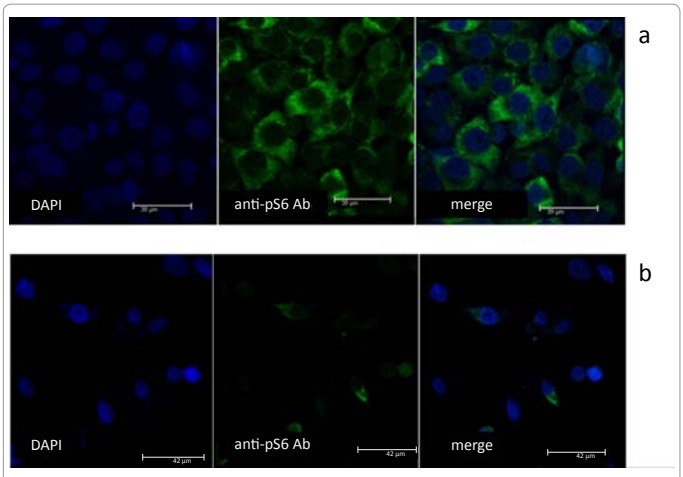

Figure 5: Immunofluorescence for detection of pS6, a downstream phosphorylated protein in the PI3K/Akt/mTOR pathway. Cells were stained with DAPI and FITC-anti pS6.In (a), OSCC-3 cells from control group. In (b), OSCC-3 cells from PDT 2.5 μM group (OSCC 3).

step in this work was to investigate if PDT induced apoptosis in OSCC-3 cells. In this context, mitochondria are increasingly recognized as an important target organelle during photo damage to tumor cells [17]. Many hydrophobic photosensitizers, such as AlPc itself, localize in mitochondria membranes where they can trigger apoptosis. This event is typically observed just after PDT application and is followed by the apoptosome-mediated caspase activation cascade [18]. As previously observed, during PDT-mediated apoptosis, mitochondrial membrane potential progressively collapses due to a lack of membrane selective permeability [18]. Reduced mitochondrial membrane potential was, in fact, observed in this study with OSCC-3 cells submitted to PDT with both AlPc concentrations tested. This result suggests that liposomal AlPc-based PDT induces apoptosis in OSCC-3 cells. DNA fragmentation, another sign of apoptosis [19], was also induced by PDT, particularly at 2.5 μM AlPc. The liposomal formulation itself, in the dark, induced significant DNA fragmentation, but it was lower in comparison to PDT groups.

PDT is known to induce oxidative stress and induce apoptosis both *in vitro* and *in vivo* [5]. Multiple signaling pathways are involved in PDT-induced cell death. In mammals, one of the main pathways involved in apoptosis control is the PI3K/Akt/mTOR pathway [1]. In the present work the inhibition of the PI3K/Akt/mTOR pathway was evaluated through immunofluorescence for the detection of the phosphorilated protein, downstream of mTOR, pS6 [1]. The present study shows that PDT with 2.5 μM AlPc reduced the amount of pS6 per cell. This result can support further investigations on how PDT affects cell signaling pathways and how it influences the fate of a treated cell.

Concluding, the results presented here suggest that the treatment with PDT using liposomal AlPc triggers apoptosis in OSCC-3 cells, an event that may be related to an inhibition of PI3K/Akt/mTOR signaling pathway. Further studies on how PDT can affect this and other pathways affecting cell death may be useful for improving PDT protocols. Photosensitizer molecules and formulations may be designed to affect specific molecules or organelles in a target cell to enhance PDT effectiveness. This may be the future of modern PDT.

Acknowledgements

Financial support from the Brazilian agencies MCT/CNPq, FINEP, CAPES, FAP-DF, and FINATEC is gratefully acknowledged.

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