Article

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# Photodynamic Therapy Leads to Complete Remission of Tongue Tumors and Inhibits Metastases to Regional Lymph Nodes

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In patients diagnosed with oral cancer, the most important prognostic indicator for patient survival after primary treatment is metastasis to the cervical lymph nodes or distal sites. Therefore, we evaluated the utility of photodynamic therapy (PDT) mediated by aluminum-chloride-phthalocyanine entrapped in liposomes for the prevention of metastasis to regional cervical lymph nodes in the Erhlich tongue cancer model. The PDT protocol led to complete remission of tongue tumours and prevented the occurrence of regional metastasis. The prevention of regional metastasis was confirmed by histopathological and immunohistochemical analyses. In addition, PDT treatment increased the overall survival and reduced weight loss relative to control tumour-bearing mice. Thus, PDT should be clinically evaluated for use in the prevention of cervical lymph node metastasis in patients with oral cancer.

**KEYWORDS:** *Photodynamic Therapy, Oral Cancer, Metastasis, Liposome, Lymph Node.*

## INTRODUCTION

Oral cancer is the most prevalent among the cancers of the head and neck; it represents one third of all head and neck tumours and remains an important public health issue.[1] Despite efforts in the evolution of traditional surgery, radiation, and chemotherapy, the 5-year survival rate after oral cancer therapy has not improved significantly over the past decades.[2] Regional lymph node metastasis contributes significantly to the poor prognosis of patients diagnosed with oral cancer.[3] In fact, the detection of cervical lymph node or distant metastatic tissues is the most important indicator of treatment failure for oral cancer therapies.[1] Therefore, there is a need to study more effective methods for oral cancer treatment, as well as ways to manage and prevent their numerous side effects and squeal.

In the last two decades, photodynamic therapy (PDT), a non-surgical and minimally invasive procedure, has been used in the treatment of superficial epithelial tumours such as oral squamous carcinomas and skin cancer.[4,5] PDT involves the use of photochemical reactions mediated through the interaction of photosensitiser (PS) drugs, light, and oxygen. The combination of these three components leads to the destruction of cells and tissues that have selectively taken up the photosensitiser and been locally exposed to light.[4,6]

Thus, the efficiency of PDT depends upon the ability of PS to selectively accumulate in tumour tissues relative to normal tissues. One suitable strategy to preferentially

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target tumour tissues is the association of PS drugs with specific drug delivery systems, such as liposomal vehicles.[7] In addition to enhancing the tumour absorption properties of PS, liposomes are useful for the prevention of molecular aggregation of some PS, such as the phthalocyanine derivates, in physiological aqueous environments.[8] Liposomal vesicles are nano or microstructures that are 50–1000 nm in length and composed of a phospholipid bilayer surrounding an aqueous core.[7, 8] Previous studies have shown that these lipid drug carriers are selectively absorbed by surrounding blood and lymphatic tumour vessels.[9, 10]

Considering that liposomal vehicles can prevent drug aggregation and enhance the delivery of PS to tumour cells, as well as the surrounding blood and lymphatic tumour vessels,[10] it is reasonable to assume that PDT mediated by aluminium chloro phthalocyanine (AlClPc) entrapped in nanostructured liposomes could eliminate both tumour tissue and tumour-associated lymphatic vessels. Thus, the aim of the current study was to evaluate the efficacy of PDT *in vivo*, using Aluminium-Chloro-Phthalocyanine (AlClPc) entrapped in nanostructured liposomes for the treatment of lingual tumours.

## MATERIALS AND METHODS

### Liposome Preparation and Characterization

AlClPc was obtained from Aldrich Chemical Company (Saint Louis, USA). The incorporation of AlClPc into the phospholipid bilayers was performed according to the modified injection method described by Simioni et al.[11] Briefly, 360 $\mu$L dipalmitoylphosphatidycholine (DPPC) ethanolic solution (0.7 mM) and 50 $\mu$L of AlClPc (stock solution at 1.0 mmol/L) was injected with a syringe into 5 mL of PBS, pH 7.4. The injection was performed at 56 °C under magnetic stirring and at flow rate of 1 $\mu$L/s. All the experiments were performed up to 10 days after the preparation of liposomes.

Physicochemical characterization of liposomes was performed by measuring their mean size and polydispersity index (PdI). The mean size and PdI of colloidal dispersions were performed at 25 °C by laser light scattering using a Zetasizer SZ (model 3000 HSA, Malvern PCS Instruments, England). Samples were analyzed following appropriate dilution with filtered ultrapure water. Data are the mean (±SD) of three different batches. The content of AlClPc in the formulations was determined by validated spectrophotometric method at 672 nm (Optical densities $(OD) = 0.3829 \times [\text{AlClPc}, \mu g \cdot mL^{-1}] + 0.0126$; $r = 0.9992$, the method was linear in the range of 0.50–3.00 $\mu g \cdot mL^{-1}$).[12]

### Light Source

The liposomal solution containing AlClPc was excited using a continuous low power (80 mW) diode laser (BWF light source—Tech in) adapted to fibre optics and operating at 670 nm (the wavelength of maximum optical absorption).

### Absorption and Fluorescence Spectrum of 5 $\mu$M AlClPc-Liposomal Solution

Absorbance and fluorescence spectra were recorded with a SpectraMax2 spectrophotofluorometer. For experiments, 700 $\mu$L of the 5 $\mu$M AlClPc-liposomal solution were placed in a quartz cuvette and the absorbance spectra were recorded in the 350–750 nm range, at 5-nm spectrum intervals. To determine the fluorescence emission spectra, the samples were excited at a wavelength of 350 nm, and the emission was recorded in a range of 355–750 nm, with a 5-nm interval between each point.

### Singlet Oxygen Detection

Singlet oxygen ($^1O_2$) was detected by an indirect method using the $^1O_2$-probe, 1,3-diphenylisobenzofuran (DPBF).[13] DPBF reacts irreversible with $^1O_2$, and causes a decrease in the DPBF-specific absorption band at 410 nm. For $^1O_2$ detection, 700 $\mu$L of the AlClPc-liposomal solution was placed in a quartz cuvette and the 410-nm absorbance was recorded. This first absorbance data point was considered the blank value for the reaction. In order to determine the total absorbance without irradiation, 5 $\mu$L of an alcoholic DPBF solution (1.5 mg/mL) was added to the PS solution and the absorbance was again recorded. Finally, 10–100 J/cm$^2$ was applied to the solution and the absorbance was recorded at each 10-J/cm$^2$ interval to measure the irradiation effect. The decrease in absorbance was converted to a percentage, which was indicative of the $^1O_2$ generation.

### Ehrlich Tumour

In order to test the efficacy of PDT mediated by AlClPc in a liposomal formulation in a lingual tumour model, we used the ascitic-derived Ehrlich cells. Ehrlich tumours were maintained in the ascitic form by passaging in the peritoneal cavity of mice every 3 days.

### Animals and Experimental Design

All animal handling and procedures were carried out according to the international practices for animal use and care, and approved by the Animal Ethics Committee of the University of Brasilia. Male Balb-C mice (15 weeks old) weighing 20–25 g were obtained from the Central Animal Facility of the Institute of Chemical/IQUECO (Goiânia, Brazil). A total of 36 animals were acclimatised to laboratory conditions for one week prior to starting the study; they were provided with Purina mice chow and filtered water *ad libitum*. Tumours were induced by injecting

10 $\mu$L of a suspension containing $5 \times 10^6$ ascitic-derived Ehrlich cells in the left lateral border of the tongue. This lingual tumour model was previously described for anti-tumour experiments.[14]

At 48 hours after the implantation of tumour cells, the animals were randomly divided into five groups as follows: mice without tumours, mice with tumours that were not treated, mice with tumours that were irradiated with lasers, mice with tumours that were treated with liposomal-AlClPc (5 $\mu$M) and mice with tumours that were treated with liposomal-AlClPc and 15 minutes later irradiated with laser. The energy fluence (100 J/cm$^2$) used in this study was previously tested in the same lingual tumour model.[14] To reach this energy density, the irradiation tumour fields were standardised as 10-mm circular diameters, the potency was uniform and constant at 80 mW, and the time of laser irradiation was 16 minutes. The treatments were performed every three days at three time points (for a total of six days of treatment) and the animals were evaluated on days seven or twenty-one post-treatment. The time-points of this protocol were established after a pilot study (data not shown).

Animals without tumours were considered negative controls. The experimental design is outlined in Table I. During the course of the experiment, the tongues were clinically examined and photographed in order to detect clinical alterations. In addition, animals were monitored for changes in body weight.

The animals (only 50% of the animals in the G5 group) were euthanised by cervical dislocation 24 h after the last treatment, or on days 7 or 21 post-treatment. Body weights were recorded and the tongues and cervical lymph nodes were completely excised and fixed with paraformaldehyde (4%) for histopathological and immunohistochemistry analyses.

**Table I. Experimental design.**

| Group (*n*) | Tumour | Time (days)* | Treatment | |
|---|---|---|---|---|
| | | | Laser** | AlClPc-liposome*** |
| Animals without tumours (6) | – | – | – | – |
| Animals with tumours (12) | + | 07 and 21 | – | – |
| Animals with tumours irradiated with laser (6) | + | 07 | + | – |
| Animals with tumours irradiated with AlClPc-liposome (6) | + | 07 | – | + |
| Animals with tumours treated with PDT-(12) | + | 07 and 21 | + | + |

*Notes*: *Period of observation after treatment; **Peritumoural injection of 20 $\mu$L liposomal-AlClPc (5 $\mu$M); and ***Irradiation with laser (670 nm), with a total energy of 100 J/cm$^2$.

## Histopathological and Immunohistochemistry Analysis

After fixation for 4 h, tongue samples were dehydrated and then embedded in paraffin. The samples were sectioned (5 $\mu$m) and stained with hematoxylin and eosin for histopathological analyses. Silanised slides containing paraformaldehyde-fixed and paraffin-embedded sections of tongue and lymph node samples were used for immunohistochemistry. For immunohistochemical staining, the samples were immersed in 3 mM citrate buffer (pH 6.0) for 10 min at 120 °C for antigen retrieval. Subsequently, the sections were incubated in 3% normal serum and then with the primary antibody (1:100). The sections were then incubated with biotinylated antibodies for 30 min and followed by avidin-biotin complex (LSAB-HRP Kit, Dako). Negative controls were obtained by the omission of primary antibodies. All reactions were developed using a diaminobenzidine chromogen solution (Dab substrate chromogen systemK3466, DAKO Corporation) and counterstaining was performed with Harris haematoxylin. Breast cancer histological sections were used as controls. Ehrlich tumour cells are known to express the biological marker progesterone, therefore, the tumour cells were identified using the progesterone monoclonal antibody (Clone 0098, DAKO Glostrup, Denmark); cells that stained brown were considered positive.

## Statistical Analysis

Statistical comparisons were made by one-way analysis of variance (ANOVA) using Prisma Software for PC™ and Tukey's post-hoc test was used to determine the differences. Results are presented as mean ± S.E.M. The Kaplan-Meier analyses for overall survival were compared using the log rank test (Mantel-Cox test). All statistical analyses were considered significant when $p < 0.05$.

# RESULTS

The size of the liposome vesicles are a very important parameter, because it is one of the factors controlling the kinetics of drug release as well as being able to improve the uptake by cells and tissues, due to the nanoscale size. The results of the particle size analysis indicate that the mean sizes of the system were in the nanometric range ($220 \pm 29$ nm), and the PdI values also demonstrated a narrow and unimodal vesicle size distribution ($\text{PdI} < 0.25 \pm 0.07$), i.e., the homogeneous character of colloidal formulations. The spectrophotometric method used to determine the AlClPc content in the liposomal formulation showed that, with the used method, entrapment efficiency was superior to 80% of the initial material, and these vesicles remained stable for 2–4 weeks before interaction with the biological target tissues. The stability of the nanovesicles was achieved by measuring the size and

the polydispersity index of AlClPc liposomes as described above. For the following photodynamic therapy experiments a 5 $\mu$M AlClPC liposome formulation was prepared and used.

The photophysical studies of AlClPc-liposomes was evaluated based on absorption and fluorescence spectra. As shown in Figure 1(A), the AlClPc-liposomes have the same spectral profile of light absorption as the AlClPc in monomeric form, i.e., between 650 and 700 nm with maximum absorption at 670 nm. The fluorescence emission spectra of the AlClPc-liposomes, after excitation with 350 nm, showed a maximum fluorescence emission at 680 nm (Fig. 1(B)).

In addition, we measured the generation of singlet oxygen after the exposure of the AlClPc-liposome to different red laser (670 nm) energies. Figure 1(C) shows a decrease in the typical absorbance of benzofurans at 410 nm. The same figure shows a decrease in absorbance as the supply of energy to the photosensitiser increases. This result shows the oxi-degradation of benzofurans after the generation of singlet oxygen.

PDT mediated by AlClPc entrapped in nanostructured liposomes, AlClPc-liposomes, effectively resulted in complete remission of tumours induced in the tongues of Balb-c mice with ascitic-derived Ehrlich cells (Figs. 2 and 3) and inhibited cervical lymph node metastasis (Fig. 4(B)).

Histological analysis confirmed the remission of tumours as a result of PDT. Although no tumour cells were detected in the tongues of PDT-treated animals (Figs. 5(C) and (D)), a specific tissue organisation with viable tumour cells, which were actively proliferating among the muscle fibres, was detected in untreated tumour-bearing mice (Figs. 5(A) and (B)). The tongues of PDT-treated animals showed the presence of edema, identified by increased extracellular empty space, as well as an increase in inflammatory cell infiltration (Figs. 5(C) and (D)).

Twenty-one days after initial PDT treatment, the edema tissue was replaced by fibrotic tissue characterised by the deposition of collagen fibres among the muscle fibres (Figs. 5(E) and (F)).

There was an absence of metastatic tumour cells in the cervical lymph nodes of the PDT-treated animals (Figs. 6(A) and (B)); however, metastatic tumour cells were detected in the untreated tumour-bearing animals (Figs. 6(C) and (D)). Notably, the PDT-treated animals did not have swollen lymph nodes (Fig. 4). The absence of tumour cells in the cervical lymph nodes of PDT-treated animals was confirmed by immunohistochemistry analysis; PDT-treated animals did not have positively stained tumour cells in their lymph nodes (Fig. 7).

In addition, PDT treatment prevented the significant body weight loss that was observed in untreated tumour-bearing animals (Fig. 8(A)). Moreover, PDT-treated animals also had a higher survival rate (Fig. 8(B)); two weeks

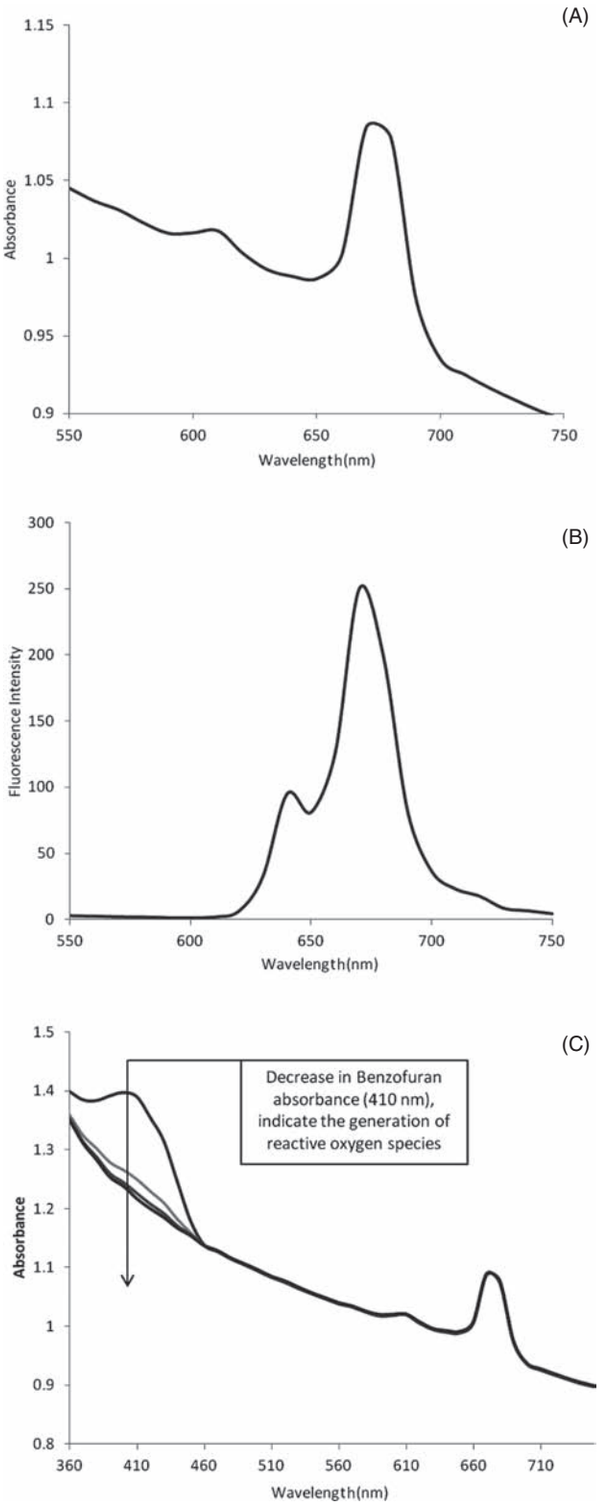


**Figure 1. Photophysical studies of AlClPc-liposomes. The absorption spectra (A) and the fluorescence emission spectra (B) of the AlClPc-liposomes. The generation of singlet oxygen from the AlClPC-liposome solution after red laser irradiation (C). Singlet oxygen was quantified by the degradation of benzofuran. Benzofuran oxi-degradation was monitored by the decrease in specific optical absorbance at 410 nm.**

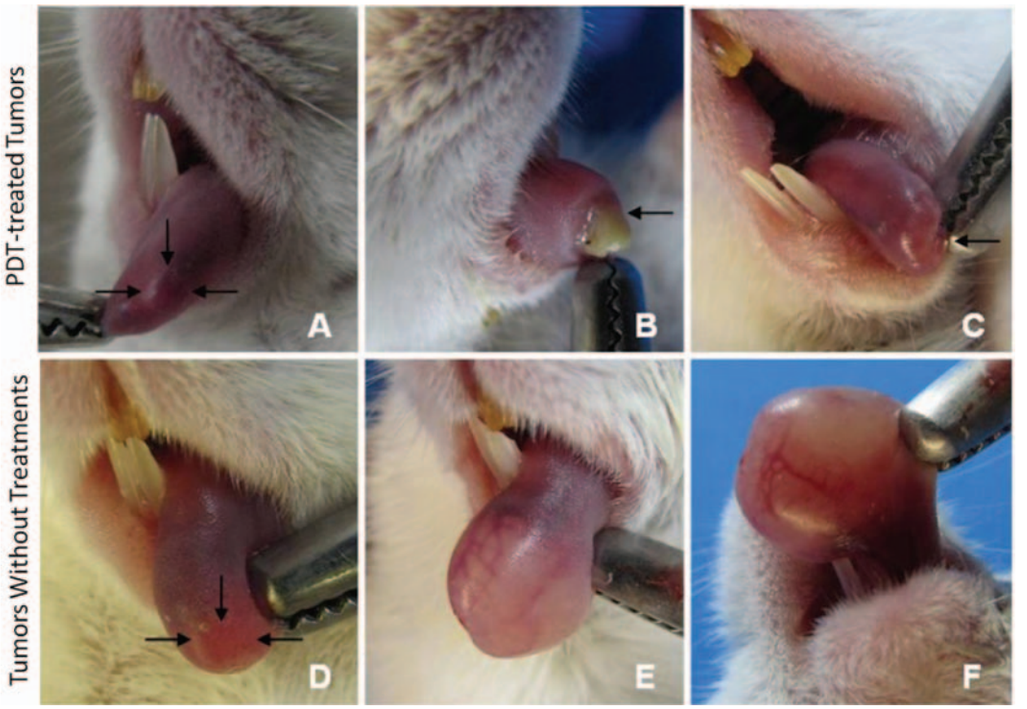


**Figure 2.** **Clinical aspects of the Erhlich tongue tumours during the first seven days of experiments. The tumours of a PDT-treated mouse prior to treatment (A), on day 3 (B) and on day 7 (C) post-treatment. There was development of necrotic tissue near the PDT treatment site and the absence of tumour mass by the end of the treatment period. The untreated Erhlich tongue tumours over the experimental timeline (D)–(F).**

after tumour induction, while 100% of the untreated animals died, the survival rate was 60% among PDT-treated animals. Furthermore, no skin phototoxicity was observed in the treated mice during the experimental protocol.

## DISCUSSION

PDT mediated by AlClPc-liposomes prevented regional cervical lymph node metastasis and induced the complete remission of Erhlich tongue tumours. These are interesting findings because the literature shows that more than 20% of patients with initial head and neck cancers could have occult metastases to the cervical lymph nodes.[1, 15] The absence of tumour cells in sentinel lymph nodes, in our study, is likely related to the ability of PDT to target lymphatic vessels, which can inhibit tumour cell migration. Tammela et al. (2011)[10] have shown that in a skin melanoma mouse model, PDT-treated animals did not present with metastases due to the selective destruction of draining lymphatic vessels; whereas animals that had their tumours excised

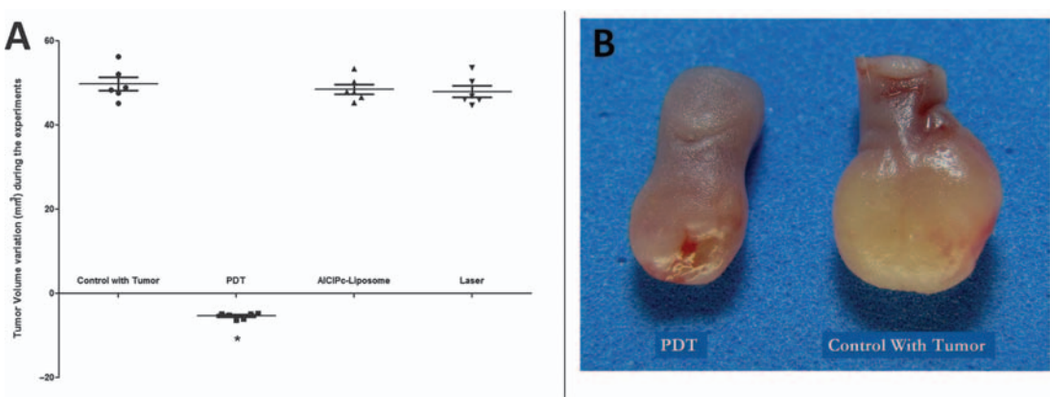


**Figure 3.** **The variation in tumour volume after the first 7 days of treatment (A). *Denotes a statistically significant difference among the experimental groups. The clinical appearance of the excised tongues on day 7 post-tumour cell implantation (B). There is an increased tongue/tumour volume in the untreated tumour-bearing mice compared to the PDT-treated tongue.**

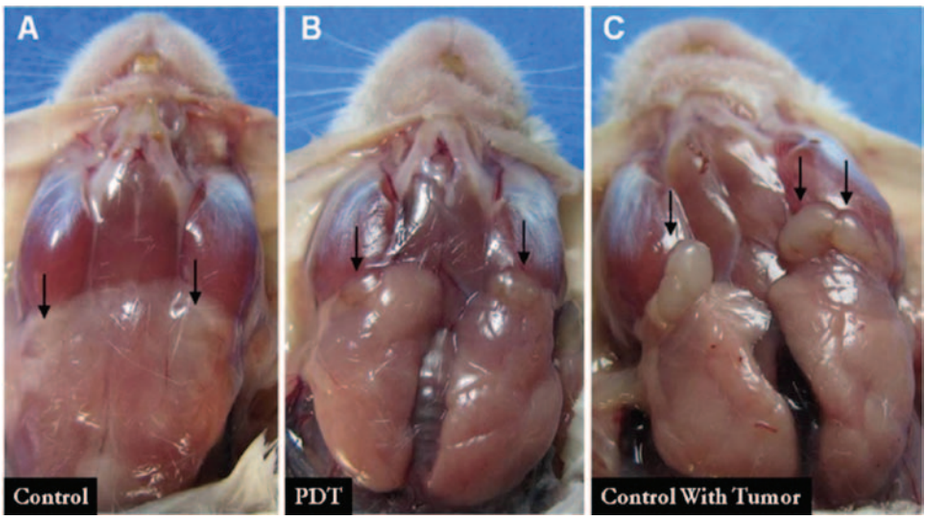


**Figure 4.** **Photographs of regional cervical lymph nodes from non tumour-bearing animals (A) and PDT-treated animals (B) and the swollen cervical lymph nodes of untreated tumour-bearing animals (C).**

developed regional metastasis. Within this context, evaluations of regional lymph node metastatic behaviours in oral cancer are essential to test new treatments and therapeutics since these clinical conditions are a decisive factor for the prognosis of patients diagnosed with oral cancer.[2]

Importantly, although head and neck cancer therapy consists of the surgical removal of the primary tumour with the excision of the associated draining lymph nodes, tumour cells in metastatic transit inside the lymphatic vessels are not removed during surgery.[16] Moreover, it is known that lymph nodes are occasionally located along some lymph vessels of the tongue and are known as lingual lymph nodes. The lingual lymph nodes cannot be removed with usual neck dissection, so metastasis to these nodes may cause recurrence of oral cancer in the neck.[17, 18] Thus, although, the frequency of metastasis to the lingual lymph nodes among oral cancer patients is relatively low,[18] we do think that PDT could be effective for the prevention of recurrence of oral cancer.

In order to evaluate the efficacy of PDT mediated by AlClPc-liposomes for the prevention of metastasis, we decided to use a tumour model based on the orthotopic transplantation of Ehrlich tumour cell suspensions. The Ehrlich tumour is an aggressive tongue tumour that induces regional lymph node metastasis in a few days. Indeed, in the present study, one hundred percent of mice developed regional cervical lymph node metastasis. Furthermore, we also decided to inject peritumoural AlClPc-liposomes to facilitate uptake by lymph capillaries. According to Torchilin et al. (2005),[9] subcutaneous administration of liposomes results in their uptake by draining lymphatic capillaries at the injection site. Recently, Tammela et al. (2011)[10] have also shown that intradermal injections of photosensitiser drugs entrapped in liposomes vesicles lead to their specific accumulation in the lymphatic vessels. In addition, PDT also causes damage to the tumour vasculature leading to indirect tumour cell death by hypoxia or starvation.[14, 19, 20] In the present study, cell death by necrosis and an increased number of inflammatory cells

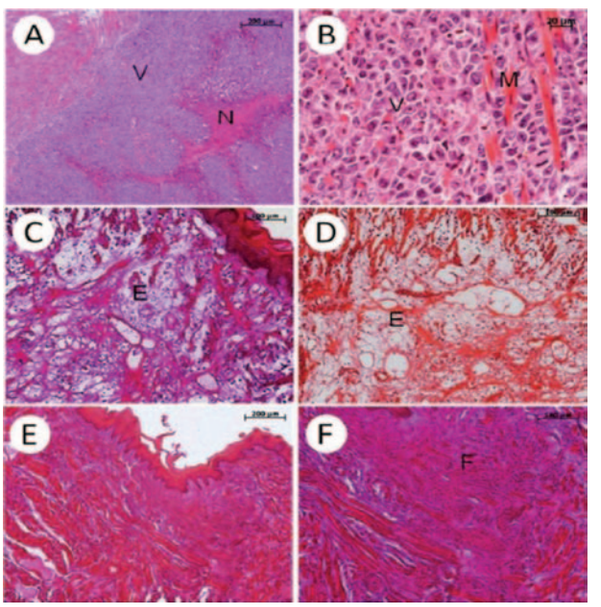


**Figure 5.** **Histopathological aspects of the Erhlich tongue tumours in the different experimental groups. The viable tumour tissue (*V*) proliferating among the muscle fibres in an untreated tumour-bearing mouse (A) and (B). Several foci of tissue necrosis (*N*) were detected within the viable tumour tissue. The absence of tumour tissue in the PDT-treated tumours (C) and (D). The presence of tissue edema (E), represented by increased interstitial space among the remaining connective and muscular cells. The presence of a fibrotic tissue (F) in the 21-post PDT-treated tumours (E) and (F). This fibrotic tissue was detected in the space that was previously occupied by the edema.**

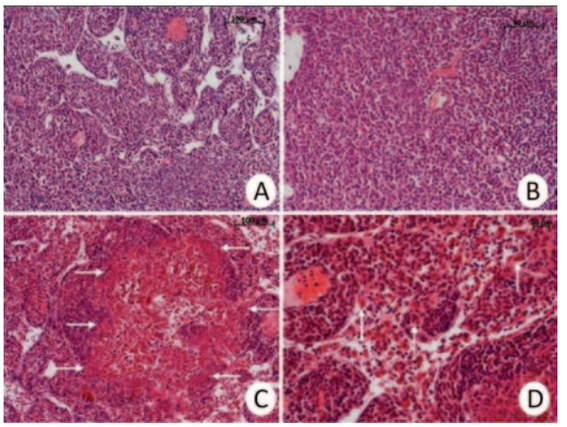


**Figure 6.** **Histopathological aspects of the cervical lymph nodes of PDT-treated mice. Normal lymph node architecture and the absence of Erhlich tumour tissue (A) and (B). The cervical lymph node of a mouse with untreated tumours shows the presence of Erhlich tumours cells (arrows) among the normal lymphoid tissue (C) and (D).**

in the tongues of PDT-treated animals were observed. We previously demonstrated that PDT mediated by AlClPc-liposomes induces necrosis and vascular damage in mouse tongue tumours.[14] In addition to that, this AlClPc-liposome formulation has been previously evaluated in different cell models.[6, 21] In both reports, the formulation did not promote cytotoxicity effects when used alone, however promoted a high level of cell death when the photosensitizer formulation was combined with red light irradiation.

According to Preise et al. (2011),[22] a rapid vascular shutdown and consequent massive ischemic death of tumour tissue, accompanied by a prompt inflammatory reaction, provide an environment for the efficient stimulation of the host immune system. From a tissue perspective, the occurrence of tissue necrosis after PDT mediated by AlClPc-liposomes is due to the release of tissue immunogenic debris that leads to an intense immunological response against the tumour tissue.[20] It is important to emphasise that after the first week of treatment, tissue edema formed as a result of the inflammation process that induced tumour cell

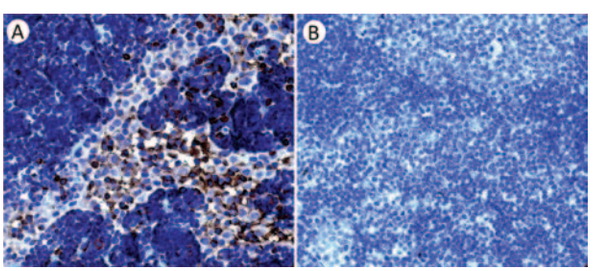


**Figure 7.** **Expression of progesterone positive tumour cells. Tumour cells were stained in brown in the lymph node sections of an untreated tumour-bearing mouse (A). The lymph node tissue from a PDT-treated mouse shows no positive staining for progesterone expression in tumour cells (B).**

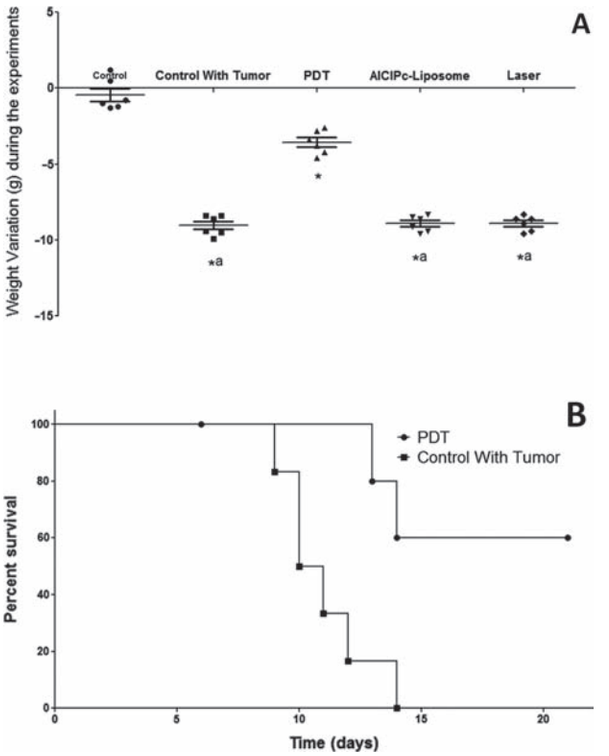


**Figure 8.** **The variation in mice weight during the first 7 days of treatments (A). *Represents a statistically significant difference among the experimental groups. The Kaplan-Meier analyses of the PDT-treated and the untreated mice with tongue tumours during the 21 days of experiments (B). A decrease in the survival of control tumour-bearing mice compared to the PDT-treated animals during the experimental period.**

death, thus producing empty tissue space that was subsequently occupied by collagen-rich tissue after three weeks of observation. This pattern is indicative of tongue tissue repair after tumour remission.[23]

Although PDT induce damage to the blood vessels and consequent tissue hypoxia, the presence of oxygenated tissues is important to produce the best photodynamic effect. Based on previous studies in which we achieved a 90% reduction of tumour burden after a single treatment,[14] we hypothesised that a three day interval should be sufficient to allow oxygenation of the treated tissue. Therefore, to improve the efficacy of PDT in the present study, we repeated three applications of PDT with a 3/3 day interval, with a total of seven treatment days. This protocol allowed the complete remission of tumours and the prevention of cervical lymph node metastasis. Another interesting result achieved using this PDT protocol was the improvement of overall survival and the significant delay in weight loss. These results show that PDT is an innovative therapy and a useful anti-tumour technique that does not promote the severe clinical side-effects that are often associated with traditional cancer treatments, such as chemotherapy and radiotherapy.[24]

In conclusion, PDT mediated by AlClPc-liposomes effectively prevented cervical lymph node metastasis, controlled Erhlich tongue tumour burden, and increased overall survival. The prevention of regional metastasis observed in our results is a fundamental feature due to the importance of this type of metastasis in oral cancer patients, and its negative impact on quality of life and overall survival. Furthermore, PDT should be clinically evaluated as a treatment modality for the prevention of cervical lymph node metastasis in oral cancer patients.

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