Photodynamic therapy (PDT) of cancer has been known for over twenty years and is based on the dye-sensitized photooxidation of different biological targets in the tumoral tissue, yielding to a photochemically induced cell’s death via mainly apoptotic pathways. The effectiveness of this therapy depends on the photophysical and photochemical properties of the photosensitizer-drug. The purpose of this paper is to evaluate a series of aluminium phthalocyanine sensitizers compounds, in terms of photodynamic activity correlation with their structure using standard human dysplastic oral keratinocytes cell line (DOK). The photodynamic action of some aluminium sulphonated-phthalocyanines, with different sulphonation degree (AlS$_2$Pc, AlS$_4$Pc), is discussed in this matter. The in vitro photodynamic treatment (PDT) induced a drastic lethal effect on DOK cells attributed to the active singlet oxygen photochemically generated by aluminium sulphonated-phthalocyanines. The tested photosensitizers induce early membrane damage and upon irradiation AlS$_2$Pc compound is the most efficient in actively killing the tumoral cells.

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Keywords: Phthalocyanine, Photodynamic therapy, Aluminium phthalocyanines, Human dysplastic oral keratinocytes

1. Introduction

Photodynamic therapy (PDT) used in oncology is a treatment method applied in several solid tumours combining the action of oxygen, light and sensitizers [1]. PDT has a long standing history, over twenty years based on the dye-sensitized photooxidation of particular sub-cellular targets hosted by the tumor tissue [2, 3]. Since then it was approved as a curative or palliative therapy for cancer and other non-malignant conditions, being approved as an alternative to surgery for basal cell skin cancer, Bowen’s disease and actinic keratosis. While knowing the frame of the process in which, a sensitizer, light, and oxygen leads to photochemically induced cell death, the information on the intimate mechanisms that are controlling the response of malignant lesions to this phototherapeutic modality are still largely unknown. Intensive investigations are on-going in order to improve the efficiency while diminishing its side effects [4]. PDT by its own has a logical flow of processes in which the photosensitizers are applied to the tumor in the first step, an incubation period takes place in dark for an efficient up-take, followed by the illumination step when these photosensitizers usually produce singlet oxygen that acts efficiently on the cells that up-took the dye. The uptake and the retention time of the dyes should be different when registered in normal cells compared to the highly proliferative ones, like cancerous cells. The efficacy of a future PDT depends on the development of new drugs and the ability of these drugs to accumulate

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selectively in tumor tissues in comparison with normal tissues. Several new classes of photosensitizers for PDT have reached the stage of clinical trials during the past few years [5]. Phthalocyanines are among the more promising second-generation photosensitizers. There has been considerable interest in phthalocyanines for use in PDT, mainly because of their high absorbance coefficient in the far red region of the light spectrum (670-700 nm), with optimal tissue penetration by light [3]. The photophysical properties of phthalocyanines are strongly dependent on the central metal ion. The phthalocyanines photoproperties can be changed by adding a central metal to the macrocycle. Diamagnetic metals such aluminium, zinc, or tin produce long triplet states and an enhanced singlet oxygen production. Since the singlet oxygen is thought to be essential for PDT, among the metal phthalocyanines, Zn$^{II}$ and Al$^{III}$ complexes (zinc phthalocyanine, ZnPC, and chloroaluminum phthalocyanine, AlClPc) present the most favourable photophysical properties for PDT application [4], namely relatively long-lived excited singlet states (ca. 3-8 ns) and long-lived triplet states, produced in high quantum yields [6]. Also, the substitution at the macrocycle, leads to less dramatic changes in the photophysical properties, affecting the cellular uptake and solubility [7].

Taking into account the gathered data we have synthesized a series of metallo-phthalocyanines [sulphonated (n=2-4) aluminium phthalocyanines (AlS$_n$Pc)] and the relation between the structure/metal substitution and their photophysical and photodynamic properties studied and reported in this paper [8]. Particularly, the formation of singlet oxygen was determined and discussed in the context of the excited-state photophysical properties. For a future clinical application of the compounds the toxicological profile were investigated on standard DOK cell cultures and the phototoxicity induced by experimental PDT.

2. Experimental part

2.1 Chemicals

Aluminium phthalocyanines with different degrees of sulphonation, were prepared in the laboratory and reported elsewhere [9,10]. The purity of those dyes was checked by thin-layer chromatography, elementary analysis, FTIR and UV-Vis spectroscopy. Stock solutions of AlS$_{2-4}$Pc ($1.7 \times 10^{-5}$ M) were routinely prepared in PBS, and stored in the dark at 4ºC. For in vitro testing solutions were prepared in culture media and tested concentrations explained thereafter. Structural formulas of the dyes tested in this paper are shown in Table 1 and in Fig. 1.

Table 1. The molecular formula of the synthesized compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>Me</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>R$_3$</th>
<th>R$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlS$_2$Pc</td>
<td>Aluminium 8,17 di-sulphonated phthalocyanine</td>
<td>Al</td>
<td>SO$_3^-$</td>
<td>SO$_3^-$</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>AlS$_4$Pc</td>
<td>Aluminium 8,17,23,28 tetra - sulphonated phthalocyanine</td>
<td>Al</td>
<td>SO$_3^-$</td>
<td>SO$_3^-$</td>
<td>SO$_3^-$</td>
<td>SO$_3^-$</td>
</tr>
</tbody>
</table>
2.2. Apparatus

*UV-Vis spectrophotometric measurements* of the samples were made with a SPECORD M400 Carl Zeiss Jena double beam spectrophotometer, connected to microprocessor. Quartz cuvettes with 1 cm optical path lengths were used and for the in vitro PDT volumes of 3 mL containing cell suspensions with 2x10^5/ml were subjected to irradiation.

The *infrared spectra Fourier Transformed (FTIR)* spectra have been recorded directly on the sample with a Perkin Elmer Spectrum GX spectrometer, in the following conditions: range 4000 to 400 cm⁻¹, 32 scan, resolution 4 cm⁻¹, gain 1. A small quantity of matter (1 or 2 milligrams) was grounded, then mixed with KBr and placed in a DRIFT cell.

2.3. Cell culture

Standard cell culture DOK (ECACC No.94122104) is derived from dysplastic oral keratinocyte prior established from a squamous-cell carcinoma and standardized by the European Collection. The cell line is adherent and upon cultivation can prove in confluent cultures stratification properties and contains a keratin profile similar to the original dysplasia (Figure 2). It has an epithelial morphology and has in our system a duplication period at 48h.

The culture was maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, UK) enriched with 2mM Glutamine, supplemented with 10% Fetal Calf Serum (Sigma-Aldrich, UK), 50 U/ml of Penicillin-Streptomycin (Sigma-Aldrich, UK), 5µg/ml Hydrocortisone (Sigma-Aldrich, UK) at 37°C with 5% CO2 atmosphere [11]. The cells were seeded at 4x10,000 cells/cm² and when reaching sub-confluent cultures (70-80%) they were detached with 0.25% trypsin /EDTA (Sigma-Aldrich, UK) and further maintained in culture and/or subjected to further experimental procedures in 5% CO2 at 37°C.
2.4. Toxicological profiling.

For toxicological profiling, DOK were seeded at 5000 cells/well in 96 wells culture plates 24h prior to incubation with the tested compounds. For irradiation, cells incubated for 24h in compounds were washed for removal of free compounds, detached from the plates and resuspended in culture media at $2 \times 10^5$ cells/ml cell concentration. To avoid un-wanted light activation of compounds, all manipulation during compounds testing for dark-toxicity were performed in task area illumination with less than 50 lux as measured with DT-8808 Light Meter.

2.5. Laser irradiation.

DOK were irradiated with a He-Ne laser ($\lambda=632.8$ nm, 30 mW) for 30 minutes, in saturated atmosphere of oxygen. Cells were irradiated in suspension and kept on ice at 4°C for avoiding adherence.

2.5. Post-irradiation cultivation of cells.

After irradiation, the viability of cells was determined by the Trypan Blue exclusion test using an automated cell counter (Countess Invitrogen). Cells were cultivated at 5000 cells/well concentration in 96-well plates for 24h at 37°C, in 5% CO$_2$ humid atmosphere.

2.6. Cellular viability.

Cell viability was determined by the LDH release test using Cytotox96 Non-Radioactive Cytotoxicity Assay (Promega). LDH is released in higher amount by dying cells with disturbances of membrane integrity. After incubation, cells were centrifuged for 5 min. at 250g and 50μL of supernatant were harvested from each sample. The LDH release test was performed according to the manufacturer instructions. Briefly, supernatants were incubated with 50μL of Substrate Reaction Mix for 30 min. at room temperature protected from light. The reaction was stopped and the optical density (OD) was measured at 492 nm. A sample containing only the culture medium was considered the control. The absorbance value determined from control was subtracted from...
OD values obtained from the experimental samples. LDH tests were performed for toxicological profiling and after PDT treatment for investigating the cellular membrane damage.

2.7. Cellular proliferation.

Cell multiplication was investigated by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] reduction test using CellTiter 96 Aqueous One Solution Cell Proliferation kit (Promega). The reaction intensity depends on the number of living cells in culture. Briefly, 50 μL of cell suspension from each sample were transferred in a 96-well plate with flat bottom and 50 μL of fresh culture medium were added. 20μl of CellTiter 96® AQueous One Solution Reagent was then rapidly added to each well and cells were incubated for 3h at 37°C. After 3h of incubation, the OD at 492 nm, with a reference wavelength of 620 nm, was recorded using a 96-well plate reader (PR 3100, Bio-Rad). MTS tests were performed for toxicological profiling and after PDT treatment for investigating the capacity of cells to proliferate post-treatment.

2.8. Statistics.

All experiments were performed in triplicates, mean and standard deviation of the mean and/or proliferation indexes were represented. Cell kill following PDT were converted into percentage of cell kill using a published formulae [12].

\[% \text{Cell Kill}=100 \times (\text{OD}_{490 \text{ Control Cells}}-\text{OD}_{490 \text{ Treated Cells}}/)\text{OD}_{490 \text{ Control Cells}}.\]

3. Results and discussion

Phthalocyanines, as expected from the extensively conjugated aromatic chromophore, exhibit UV-Visible absorption spectra with intense π–π* transitions, usually referred to as Q bands in the range 660-700 nm (ε >10^5 M^-1.cm^-1) with associated higher energy vibrational components in the range 600-660 nm. The phthalocyanines strongly absorb clinically useful red light (630-800 nm) with molar absorption coefficient 2-4 x 10^5 M^-1cm^-1. Metalation, which reduces the electron density at the inner nitrogen atoms, is predicted to produce a hypsochromic shift. λ_{max} shift to shorter wavelength depends on the metal electronegativity. In contrast, electron-withdrawing groups at the periphery of the molecule are predicted to give rise to a bathochromic shift. The 18th electron inner ring system of the phthalocyanines determines the bathochromicity and the substitution at the benzene rings has only a minor effect on the λ_{max} of the phthalocyanine chromophores.

For the studied aluminium phthalocyanines, the absorption spectra are shown in Table 2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>λ_1/ε</th>
<th>λ_2/ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlS_2Pc</td>
<td>PBS</td>
<td>609/18679</td>
<td>673/27960</td>
</tr>
<tr>
<td>AlS_4Pc</td>
<td>PBS</td>
<td>612/8679</td>
<td>676/19834</td>
</tr>
</tbody>
</table>

It can be assumed that aluminium is able to change the distribution of the electron cloud. The higher reactivity of metal increases π-electrons delocalization which leads to more bathochromic shift in the absorption spectrum and the more intensive PDT effects [13a]. Not only
metal can enhance PDT effect but also the peripheral groups attached to the main molecular core, which can have photoresponse. Such a delocalization can weaken electrons bonding with the maternal molecules followed by the enhancement of their activity. The spatial spreading of the molecules after substitution might therefore be energetically favourable in term of activity. A higher number of $\pi$-electrons and their delocalization lead to the improvement of PDT effect.

*Fig. 3. The absorption spectra of AlPcS$_4$ and ZnS$_2$Pc in PBS*

The degree of sulphonation has been detected by FTIR spectroscopy, too. Figure 3 represents the FTIR allure for AlS$_2$Pc and AlS$_4$Pc, the band from 757 cm$^{-1}$ (attributed to SO$_4$ group) is higher for tetrasulphonated compound than for disulphonated one.

*Fig.4 FTIR spectra for AlS$_2$Pc and AlS$_4$Pc*
Similar to other tetapyrrolic macrocycles [14], phthalocyanines in water have a strong tendency for stacking (or aggregate) to form dimers and high order oligomers, induced by the tendency of the hydrophobic skeleton to avoid the contact with water. Stacking decreases photosensitizing efficacy because of energy transfer between the aggregated molecules. The sulphonated aluminium phthalocyanines are among the most used compounds in PDT [15]. Sulphonation has a double achievement: it increases the efficiency of antitumor properties [16]; and it leads to a substantial repulsion of the phthalocyanine rings, making them soluble in water as monomers [17,18]. As the formation of aggregates strongly influences the photoactivity of phthalocyanines, the dimerization was studied in detail. The absorption spectra recorded in freshly prepared solutions, exhibit temporal changes, one of the reasons being the dimerization of the compounds under study. The process manifests itself as a decrease of the intensities of the Soret band, the Q band and its vibrational satellite at 610 nm. The resulting increase of the dimer concentration gives rise to the build-up of the band at 640 nm and, possibly, of the weak wings below 590 nm and above 720 nm. In some cases a broad shoulder on the red edge of the Q band was seen, probably due to dimmers and/or higher aggregates [7].

The absorption spectra of AlS\(_2\)Pc undergo temporal changes due mainly to the dimerization of the molecules in solutions. A qualitative explanation seems obvious: the molecules are fully dissociated in alkaline solutions, and a strong electrostatic repulsion prevents aggregation of the molecules. Moreover, it should also be noted that our results seem to indicate that AlS\(_4\)Pc does not dimerize or dimerizes to a degree below our detectability level.

**Photodynamic activity**

Sulphonated phthalocyanines bearing a central aluminum ion have been extensively studied in vitro as well as in vivo. There are many literature reports confirming their photodynamic activity.

Therefore in a recently published study [12] DOK cells were tested in an erythrosine PDT model along with other oral epithelial tumor cells (H357). The study revealed that DOK cells have an increased sensitivity to their PDT model compared to the other cell line. Another study performed on human oral keratinocytes using chloroaluminum-phthalocyanine reinforced the possibility of using light-irradiated chloroaluminum-phthalocyanine with anti-tumoral effect [19]. Using phthalocyanine photosensitizers in A431 epidermoid standard cells it was reported that this type of photosensitizers can appraise the treatment of T-cell-mediated skin diseases, such as cutaneous lymphomas, dermatitis, lichenoid tissue reactions and psoriasis [20].

In spite of the fact that the literature seems abundant in sulphonated phthalocyanines PDT testing, few PDT studies are addressing DOK cells. In our paper, we are reporting for the first time the results obtained in an in vitro experimental PDT model using AlS\(_2\)Pc and AlS\(_4\)Pc as photosensitizers for inducing photodamage on oral displazic human keratinocytes.

**Toxicological profiling of AlS\(_2\)Pc and AlS\(_4\)Pc**

DOK cells were cultivated for short (2h) or longer term (24h) in the tested photosensitizers in a range of concentration of 90-0.150 microM. We have avoided the AlPcS3 version of the tested compounds due to its aggregation tendency in biological friendly media. Testing the LDH release in short term cultivation we have obtained a dose-effect response (Figure 5 A, B) that reveals a specific interaction with the cellular membrane depending on the photosensitizer structure. Therefore in the lower concentration range - 0.146 - 1.406 microM and higher concentrations range - 18.75 - 90 microM the LDH release is statistically identical (see vertical arrows Figure 5 A,B). The actual interaction of the compounds with the membrane and namely registering a completely different effect on the LDH release is in the middle range of concentrations 1.406-22.5 microM. In this domain while AlS\(_2\)Pc favours the LDH release making cell membrane more fluid, the AlS\(_2\)Pc compound induces a rigidization of the membrane, thus a low LDH release. In this “middle” domain of concentration we can notice a different pattern due to the interaction with the membrane of subtle different structures.
Fig. 5. LDH release in the culture medium after 2h of DOK incubation with the photosensitizers in the dark

When assessing 24h cultivation in the compounds (Figure 6 A, B) the pattern of LDH release in relation to the concentration is similar in the 0.146-1.406 microM range. In the middle concentration range AIS2Pc there is almost an identical dose-effect curve with Figure 5A, while in this concentration range AIS4Pc does not affect the LDH release on DOK cells.

The proliferative capacity of DOK was represented as proliferation indexes to better emphasize the effect induced by the tested photosensitizers on the metabolic activity of the cells. In presence of the tested compounds cells, as expected, display after 2h of incubation a rather normal proliferative capacity up to 11 microM (Figure 7 A). At higher concentrations both compounds per se hinder the capacity of cells to proliferate and/or to be metabolically active in this case. We straighten that, in contrast to the cellular membrane test, no statistically notable differences were registered between the two compounds when incubating cells only for a short period of time in concentrations lower than 11 microM.
Fig. 6. LDH release in the culture medium after 24h of DOK incubation with the photosensitizers in the dark.

After 24h of incubation in the photosensitizers (Figure 7 B) the proliferative capacity of DOK cells displays a similar profile in both compounds. We note, though a slight increased toxicity of AlS4Pc in the tested range of concentrations. In this case, we have tested the actual proliferative capacity of cells as the incubation period was 24h.
Experimental PDT with AlS₂Pc and AlS₄Pc

Taking on board the obtained results for the toxicological profile on the tested compounds, dark-toxicity testing showed us that concentrations lower than 4 microM for both compounds are suitable for pursuing the actual PDT treatment, namely loading DOK cells with compounds and subjecting them to irradiation as described. Thus, we have loaded the cells 24h with 2microM AlS₂Pc or AlS₄Pc and after removing the unbound compounds we have subjected them to irradiation as described in Materials section.

Although adherent cells, DOK were irradiated in suspension due to the available equipment and kept on 4°C for avoiding adherence on the quartz cuvette. Immediately after irradiation we have counted the absolute number of cells and recorded their viability. In both cases the experimental PDT has killed a mean of 70% of DOK cells loaded with the photosensitizers. In a system prior developed by us [13b], we have cultivated in standard condition cells that survived PDT treatment were as described above and their LDH release in the culture media and proliferative capacity were registered after 24h cultivation post-PDT. The LDH release registered after 24h post-PDT (Figure 8 A) surprisingly shows that compared to non-irradiated counter-parts the LDH release is statistically similar. We can although notice that the actual means of non-irradiated cells are higher compared to the irradiated cells, meaning that cells are still metabolically active. This assertion is sustained by the results obtained in terms of proliferation index (Figure 8 B) where the irradiated cells have low proliferating capacity 24h post-treatment, capacity that remains low even 72h post-PDT. Compound AlS₂Pc when loaded at 2microM in
DOK and activated via irradiation kills compared to control 3 times more cells (Figure 8C) while AlS4Pc only twice compared to un-loaded but irradiated cells.

Fig. 8. Cellular parameters registered post-PDT. LDH release of DOK cells surviving experimental PDT (A). Proliferative capacity of viable cells surviving experimental PDT represented as index calculated to the non-irradiated cultures (B). % of cells killed by experimental PDT (C)

Overall the tested phthalocyanines have a dark-toxicity profile that recommends them to be further useful in PDT, non-toxic concentrations below 4 microM, matching physiological ranges. After performing in vitro PDT the best results were obtained with AlS2Pc. It is noticeable that AlS2Pc induces membrane related processes after very short incubation and overall has a better phototoxicity in our system.
4. Conclusions

Data regarding the use of DOK in PDT models are scarce and we report for the first time the effect of experimental PDT on this type of pre-malignant cell type. All the photodynamic experiments were in vitro performed on DOK cells as model for a future clinical application of PDT in oral cancer.

The photodynamic action of some sulfonated-phthalocyanines complexed with different metals, such as Al, and with different sulphonation degree (AlS_2Pc, AlS_3Pc), is discussed in this paper. Photodynamic treatment (PDT) method can induce a lethal effect on human displazic keratinocytes, effect attributed to the active singlet oxygen photochemically generated from the tested compounds.

In dark-toxicity testing, the subtle different structure induces different actions on DOK cell membrane in short term cultivation, while no effect on the proliferative capacity. In long term dark-toxicity testing the proliferative capacity is hindered only at higher concentrations. When subjecting cells to PDT after loading them with non-toxic concentrations of the compounds we have registered the best anti-tumoral behaviour by AlS_2Pc. The tested compounds recommend them to future development for clinical application in keratinocyte-related diseases.

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