# Zinc Trisulphonated Phthalocyanine Used in Photodynamic Therapy of Dysplastic Oral Keratinocytes

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*Photodynamic therapy is an alternative antitumoral procedure that requires the use of a photosensitiser and a light source emitting radiation with an adequate wavelength, suitable for activating the photosensitiser; the most common photosensitisers are porphyrins and porphyrin-related substances. Phthalocyanines are macrocyclic chemical compounds structurally related to the porphyrins, as they both feature four linked pyrrole-like subunits, therefore possessing the potential of acting as good photosensitisers; metallo-substituted sulphonated phthalocyanines are novel chemical compounds that revealed promising results when used in PDT. Activated photosensitisers can lead to the destruction of the tumoral cells through production of reactive oxygen species and the induction of apoptosis. The present study aims to assess, via protein microarray analysis, the main molecular pathways involved in the apoptosis of dysplastic oral keratinocytes following photodynamic therapy using zinc tri-sulphonated phthalocyanine.*

*Keywords: apoptosis, Zinc-trisulphonated phthalocyanine, photodynamic therapy*

Photodynamic therapy (PDT) is an alternative antineoplastic procedure directed to destroying tumoral cells; it has recently gained an increasing use in a broad range of clinical applications in various medical specialties such as gastroenterology, pneumology, urology, ophtalmology and gynecology, as well as in the field of oncologic dermatology (1). PDT requires the use of a chemical compound able to selectively accumulate in the neoplastic cells and subsequently absorb luminous radiation, acting as a *photosensitizer*, and the use of a light source of a particular wavelength, suitable for its activation (2).

Upon irradiation, the photosensitiser molecule located in the tumoral cell captures an energy quantum and follows an electronic transition from the fundamental ground *singlet* state to an excited *singlet* state, an energetic form with low stability and short lifetime ($10^{-9}$ to $10^{-6}$s) that will subsequently result either in de-excitation by releasing the energy or in an intersystem crossing into an excited *triplet* state. Being more stable and having a longer lifetime ($10^{-3}$-1s), the excited triplet state allows the deployment of photochemical reactions that will lead, through transfer of either electrons or energy between the photosensitiser molecule and the oxygen available in the reaction medium (type I and type II photodynamic reactions, respectively) to the appearance and accumulation of reactive oxygen species (ROS) such as hydroxyl radical (OH•), superoxide anion ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$), the last being more agressive than the former two (3-5) The interaction between ROS and various subcellular structures contributes, synergistically with the induction of apoptosis -the programmed cell death to the destruction of the tumoral cells (6).

Apoptosis is an active process involving ATP consumption; it can be initiated by cell exposure to a wide range of external and internal stimuli, such as nutrient or growth factors deprivation, chemotherapic agents, DNA alteration and the production of ROS (7).

Apoptosis is a multi-step program consisting in an *initiation phase* consisting in receiving and integration of the apoptotic stimuli, a *decision phase* that will determine whether the cell will or will not undergo apoptosis, an *effector phase* in which various intracellular enzymes (mainly caspases) are activated in a cascade manner and result in the cleavage of a large set of protein substrates that will lead to critical morphological alterations such as cell shrinkage, nuclear lamina lysis, DNA fragmentation and the formation of apoptotic bodies and an *elimination phase*, in which the apoptotic bodies are engulfed by the surrounding cells after recongition through various mechanism such as the externalisation of phosphatidyl-serine from the inner side to the outer surface of the cell membrane. The biochemical changes involved in apoptosis are clasically devided into *extrinsic* and *intrinsic* pathways; the extrinsic pathway involves several proteins embracing the role of membrane receptors, such as Fas (CD95) or tumoral necrosis factor receptors 1 and 2 (TNFR1, TNFR2). Upon binding of the Fas ligand (FasL) or TNF, Fas receptor and TNFR recruit specific cytosolic proteins such as FADD (Fas-associated death domain protein) and TRADD (Tumor necrosis factor receptor type 1-associated death domain protein) that will eventually activate caspase 2 and 8; the intrinsic pathway is based on the permeabilisation of the mitochondrial membrane, a central event that leads to releasing of cytochrome c from the mitochondrial intermembrane space into the cytosol, where it will associate with Apaf-1 (Apoptotic protease activating factor 1) forming a structure that can accomodate and activate procaspase 9; mitochondrial membrane permeability is achieved through pore formation by the proapoptotic Bcl proteins Bax (Bcl-2–associated X protein) and Bak (Bcl-2 homologous antagonist/killer). In

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normal conditions, Bax and Bak are sequestred through dimer formation by antiapoptotic proteins such as Bcl-2 and Bcl-XL. BH3-only proteins are a class of molecules able to be activated by various apoptotic stimuli and to release Bax and Bak from the dimers formed with Bcl-2 and Bcl-XL, consequently empowering them to form permeability pores in the mitochondrial membrane. After activation of the caspases 2, 8 and 9 respectively, both intrinsic and extrinsic pathways share a common sequence of events consisting in activation of effector caspases 3, 6 and 7 that will result in definitivation of apoptosis and extinction of the cell (8-10).

Most photosensitizers that are currently in clinical phases of investigation or already approved are based on the tetrapyrrole structure of porphyrins, chlorines and other related molecules (11). Although most of them in advanced ivestigation stages, the molecular mechanisms that underlie their anti-tumoral effect is still a matter of intense research. Phthalocyanines are macrocyclic chemical compounds related to the porphyrins, as they both feature four linked pyrrole-like subunits, therefore possessing the potential of acting as good photosensitisers (12). The addition of a central diamagnetic metal ion such as aluminium or zinc to the core structure of phthalocyanines changes their photophysical properties by enhancing singlet oxygen production and longer triplet states, while sulphonation increases the solubility of the molecules, increases their antitumoral properties and decreases the tendency of the compounds to aggregate, consequently improving their photosensitising efficacy (13). Metallo-substituted phthalocyanines yielded promising results when used as photosensitisers in PDT; Huang et al. showed in an article published in 2011 that ZnPcH1 (a mono-α-substituted zinc(II) phthalocyanine) efficiently induces apoptosis in Jurkat acute lymphoid leukemia cells and HEL acute erythroleukemia cell line, with upregulation of Bax and p53 and downregulation of HSP70, Akt and Bcl-2 (14).

As the induction of apoptosis in tumoral cells will result in destruction of the cells, this mechanism has a paramount importance in antineoplastic therapy. PDT is able to induce apoptosis in tumoral cells through various mechanisms that imply activation of multiple intracellular pathways. We have previously demonstrated that alminium di- and tetrasulphonated phtalocyanines are efficient in destroying dysplastic oral keratinocytes (13). Searching for the pathways that could be triggered in this process we have assessed an array of molecules known to be involved in apoptotic mechanisms. To the best of our knowledge there are no studies of the apoptotic pathways induced by PDT with zinc-trisulphonated phthalocyanine (ZnS3Pc) in dysplastic oral keratinocytes and moreso the usage of proteomic technology, like protein microarray to asses these pathways.

Although many studies attempted to assess the pathways employed in tumoral destruction by PDT with various photosensitisers in several cell lines, to the best of our knowledge this is the first study aimed to investigate the apoptotic mechanisms activated by PDT using a novel photosensitiser, zinc-trisulphonated phthalocyanine (ZnS3Pc), in dysplastic oral keratinocytes.

## Materials and methods

*Chemicals*

The photosensitising compound that we have investigated, zinc-trisulphonated phthalocyanine (ZnS3Pc) was prepared in the laboratory; the compound was used in a concentration of 2 μg/mL cell culture media. Prior to the PDT experimental treatment toxicity studies were performed (results not shown) from which the dark-toxicity non-toxic concentration was established. The compound's chemical structure is depicted in figure 1.

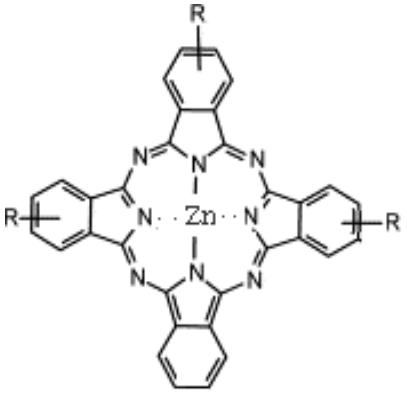


Fig. 1-Zinc trisulphonated phthalocyanine chemical structure; R= $SO_3^-$

*Cell line*

We have used cells from standard cell culture line DOK (ECACC No. 94122104), established from the dysplastic oral keratinocytes surrounding a squamous cell carcinoma of the tongue of a 57 years-old male. Cells were cultured in 5% $CO_2$ atmosphere at 37°C in Dulbecco's modified Eagle's medium (DMEDM, Sigma-Aldrich, UK) supplemented with 2mM Glutamine, 10% Fetal Calf Serum (Sigma-Aldrich, UK), 5μg/mL Hydrocortisone (Sigma-Aldrich, UK) and 50 U/mlLof Penicillin-Streptomycin (Sigma-Aldrich, UK). Cells were used for experimental PDT in their logarithmic phase of growth.

*Protein microarray analysis*

Protein microarray technology was used, *Apoptosis Phospho-Specific Array*™ kit (Abnova) detects simultaneously and highly specific 247 phosphorylated proteins; We have assessed the level of the main proteins involved in the regulation of apoptosis induced in dysplastic oral keratinocytes by PDT - ZnS3Pc. The proteomic method detects a wide range of phosphorylated or de-phosphoryated forms of various intracellular proteins. We have used arrays that allow protein expression profiling for measuring changes in phosphorylation status at specific sites and to compare the normal profile of cells with the samples subject to experimental PDT. The chosen arrays are coated with high affinity antibodies imprinted on 3-D polymer based glass substrate. Briefly the technology is presented in figure 2. The protein arrays were analyzed using GenePix® Array List (GAL) files that were provided with the kit, and the obtained data was analyzed with GenePix® Software.

*Experimental PDT*

$1x10^6$ cells/mL culture medium were incubated for 24 h with ZnS3Pc at 2 μg/mL concentration (denominated as *treated cells*). Control cells were represented by a corresponding concentration of cells not subjected to PDT. Cells were subsequently harvested and irradiated in suspension with a He-Ne laser (λ=632.8 nm, 30 mW) for 30 minutes, in saturated atmosphere of oxygen. Cells were kept on ice at 4°C, in order to avoid adherence. Aflter irradiation, according to the kit manufacturer recommandations, the cells were lysed, spinned down three times at 4°C, and treated with 100 μL protein extraction buffer. Proteins were biotinylated with N,N-Dimethylformamide and subject to protein microarray slide. The slides contain 247 highly specific antibodies fixed on a 76 x 25 x 1 mm glass slide coated. After the coupling

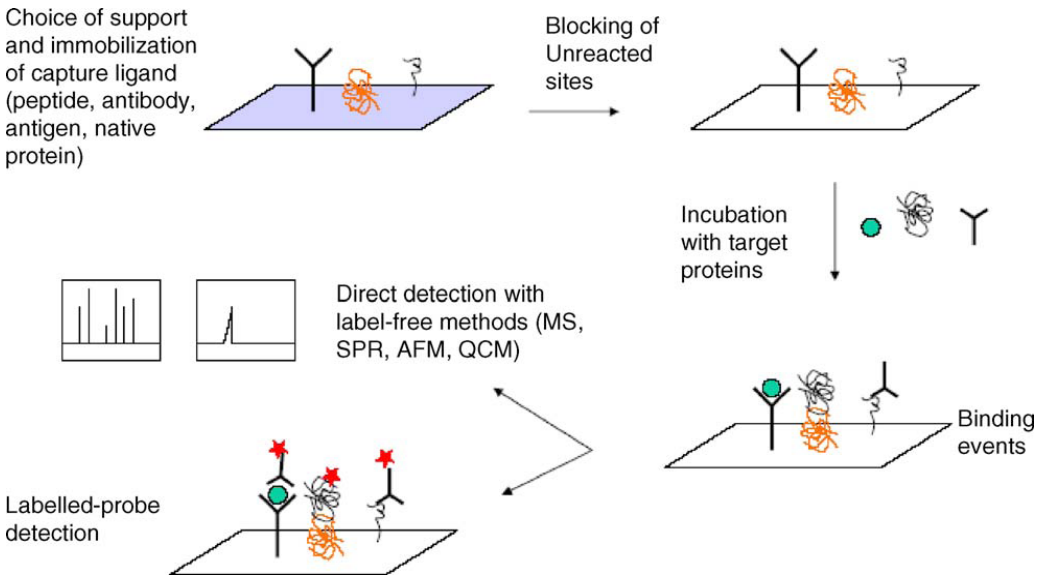


Fig. 2. Protein microarray development phases. (Cretich M, Damin F, Pirri G et al. Protein and peptide arrays: recent trends and new directions, Biomol Eng. 23(2-3),77-88 (2006) - copyright permission) (15)

Fig. 3. A typical array of protein microarray slide obtained after subjecting DOK cells to experimental PDT

reaction, in order to develop fluorescence, the slide was treated with Cy3-Streptavidin (0.5 mg/mL) in 60 mL detection buffer supplied by the manufacturer. The slide was scanned with a microplate reader ArrayIT (USA).

*Statistics*

The fluorescence of each spot on the slide was measured and the values reported in arbitrary units. The fluorescence was normalised, the background intensity was subtracted and the final values were used for statistical analysis after mediation. All values were measured in hexaplicates; statistical analysis was accomplished using IBM SPSS and Microsoft Excel. Results distributions were analysed using Kolmogorov-Smirnov and Shapiro-Wllk statistical tests of normality, according to the results of which we have assessed the differrences between the values for ZnS3Pc-treated cells and control cells either by using t-test after analysing the equality of variance by Levene's test or by using Mann-Whitney and Wilcoxon sum-rank statistics. P-values of less than .05 were considered as significant.

## Results and discussions

This study aimed to assess the overall apoptosis pathways evaluating the levels of non-phosphorilated/ phosphorilated proteins involved in regulation of these apoptotic trails using as an experimental model tumour keratinocytes subject to PDT. DOK cells treated with ZnS3Pc activated upon laser irradiation were compared to DOK cells not treated with the photosensitiser. The array of proteins was evaluated using a recently introduced method of analysis, namely protein microarray. The method is based upon the detection of a high number of protein in one single sample with the help of specific antibodies directed to these proteins, followed by the biotinylation and fluorecent marking of the antibody-protein couples; the results reflect the levels of the analysed proteins and are read by a microarray scanner and reported as arbitrary fluorescence units (AFU). The array chip displays fluorescence spots as shown in figure 3.

The results of the protein microarray analysis are listed in the following table (table 1); out of the 247 investigated proteins, we are presenting only the ones that have passed the statistics stringency.

Out of the 247 protein types analysed, we present 24 proteins that proved to have statistical meaning in our PDT experiment. We have observed through protein microarray analysis an important decrease in the level of antiapoptotic Bcl proteins, namely a decrease of 37.9% in Bcl-2 level and 27.47% in Bcl-XL level ($p < 0.001$) (fig. 4); we can consider this result as a marker for a permissive proapoptotic effect, considering the physiologic role of these proteins of normally blocking the apoptosis (10). The decrease in the level of the antiapoptotic proteins may be interpreted in conjunction with the significant increase in the level of pro-apoptotic proteins Bax and Bak (59.6% and 37.86% respectively, $p < 0.001$) (fig. 5). According to the data in the literature on this topic, Bax and Bak proteins form pores in the mitochondrial membrane initiating apoptotic events; therefore, we may consider these results as an proapoptotic effect of PDT using ZnS3Pc in DOK cells (16,17).

Whilst Bak protein in basal, unstimulated conditions is already anchored in the mitochondrial membrane, Bax neeeds a conformational alteration for becoming able to express the carboxi-terminal region that permits its anchoring to the mitochondria; this structural shift is favored and accelerated by the protein Bid (BH3 interacting-domain death agonist) attached to the mitochondrial membrane (17,18). Our experiment revealed an increase by 17.1% of the level of this protein, an effect that may facilitate the apoptotic process.

| Molecule | Control cells * | ZnS3Pc-treated cells * | Variation | p-value |
|---|---|---|---|---|
| Akt Phospho Thr308 | 544.33 (36.27) | 396.17 (36.30) | -27.22% | <.001 |
| Akt Phospho Ser473 | 520.17 (47.32) | 356.33 (35.04) | -31.50% | <.001 |
| Bcl-2 | 4882.17 (328.08) | 3032.00 (216.58) | -37.89% | <.001 |
| Bcl-XL | 3927.17 (257.5) | 2848.33 (170.13) | -27.47% | <.001 |
| Bax | 2137.67 (245.71) | 3412.00 (189.58) | 59.61% | <.001 |
| Bak | 2330.17 (195.78) | 3212.50 (275.27) | 37.86% | <.001 |
| Bid dephosphorylated | 891.67 (43.68) | 1044.00 (64.26) | 17.08% | <.001 |
| Bid Phospho Ser78 | 288.00 (24.79) | 304.33 (27.47) | 5.67% | 0.305 |
| Bim dephosphorylated | 1215.17 (157.21) | 1475.33 (80.20) | 21.41% | 0.041 |
| Bim Phospho Ser69 | 374.33 (31.33) | 383.50 (22.81) | 2.45% | 0.576 |
| BAD dephospho Ser112 | 577.17 (47.81) | 2698.50 (127.96) | 367.54% | <.001 |
| BAD dephospho Ser136 | 607.00 (52.78) | 2858.83 (200.26) | 370.98% | <.001 |
| BAD dephospho Ser155 | 849.50 (67.38) | 1645.83 (95.84) | 93.74% | <.001 |
| BAD Phospho Ser112 | 1835.83 (85.59) | 456.17 (32.11) | -75.15% | <.001 |
| BAD Phospho Ser136 | 2242.33 (176.54) | 614.83 (30.11) | -72.60% | <.001 |
| BAD Phospho Ser155 | 1377.67 (56.53) | 699.67 (52.64) | -49.21% | <.001 |
| Caspase 3 | 2537.17 (207.77) | 3677.83 (230.11) | 44.96% | <.001 |
| Caspase 6 | 1696.00 (87.45) | 2213.17 (192.79) | 30.50% | <.001 |
| Caspase 8 | 2721.33 (138.81) | 3232.50 (202.21) | 18.80% | <.001 |
| Caspase 9 | 1794.83 (200.93) | 3189.00 (211.27) | 77.70% | 0.002 |
| Caspase 10 | 2222.67 (125.23) | 2378.00 (96.22) | 6.99% | 0.038 |
| Caspase 9 PhosphoSer196 | 615.33 (58.38) | 682.17 (59.61) | 10.86% | 0.078 |
| Cytochrome c | 3790.33 (242.70) | 4102.17 (372.54) | 8.23% | 0.117 |
| p53 | 2894.33 (246.01) | 3336.67 (109.27) | 15.30% | 0.009 |

**Table 1**
RESULTS OF PROTEIN MICROARRAY ANALYSIS OF THE MAIN PROTEINS INVOLVED IN APOPTOSIS IN DOK CELLS FOLLOWING PDT USING ZnS3Pc; *MEAN VALUES ARE REPORTED, MEASURED IN ARBITRARY FLUORESCENT UNITS; STANDARD DEVIATIONS ARE MENTIONED IN BRACKETS

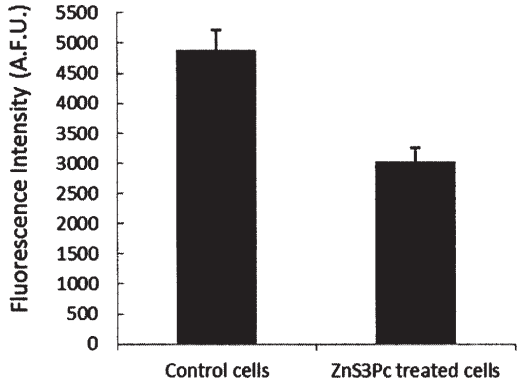


Fig. 4. Bcl-2 protein level variation following ZnS3Pc-PDT

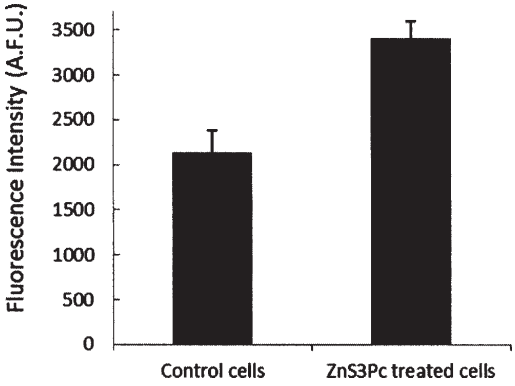


Fig. 5. Bax protein level before and after ZnS3Pc-PDT

Bid can be phosphorylated at Serine 78 residue in the aminoacid chain, an alteration that leads to antiapoptotic function consisting in Bid functional shifting toward blocking cell cycle in order to repair various DNA alterations caused by physical or chemical agents (19). Analysing our results, the statistical data regarding the level of Bid phosphorylated at Serine 78 revealed the lack of a significant change in this level (increase 5.67%, p=0.305, non-significant), ruling out the involvement of this molecular mechanism in PDT with ZnS3Pc in DOK cells.

The BH3-only protein Bim (Bcl2-interacting mediator of cell death) is a proapoptotic protein; O'Connor has proven that Bim is able to link to Bcl-2 and Bcl-XL, releasing proapoptotic proteins sequestred as heterodimers formed with these antiapoptotic proteins (20). Our PDT experiment revealed an 1.214 times increase in Bim level following therapy with ZnS3Pc (p=.041). According to Luciano et al., Bim may be phosphorylated at Serine 69 residue, a chemical alteration of structure that leads to rapid proteosomal degradation of Bim and apoptosis inhibition (21). However, aiming to assess the level of phosphorylated Bim, we have observed the lack of a significant change in the level of this form of protein Bim (p=0.576), suggestive for the lack of activation of this phosphorylation mechanism by PDT using ZnS3Pc.

Akt kinase is an enzyme involved in a large number of intracellular processes, amongst which the phosphorylation of the proapoptotic BH3-only protein BAD (Bcl-2-associated death promoter). Akt is a key protein at the cross-roads of many intracellular signaling pathwyas involved in both tumorigenesis and apoptotic proccesses (22). The complete activation of Akt kinase may be achieved only through its double phosphorylation at Threonine 308 and Serine 473 (23), therefore the level of phosphorylation paralells its activation state. As assessing the change in the level of the phosphorylated forms of Akt, we have observed a decrease of Thr308-phosphorylated Akt at 72.8% and a decrease of Ser473-phosphorylated Akt at 68.5% compared to control cells; the decrease in the activation state of Akt and the involvement of Akt in several intricate pathways may explain a sum of other protein level alteration that we have observed, as stated below.

BAD is able to interact with the antiapoptotic proteins, blocking them and therefore permitting apoptosis (8). Phosphorylation at Ser112 and/or Ser136 in its protein chain leads to BAD sequestration in cytosol by the 14-3-3 proteins, with subsequent inhibition of apoptosis, whereas phosphorylation at Ser155 blocks the interaction between BAD and Bcl-XL (24-26). In our experiment, the level of dephosphorylated BAD increased 4.67 times for dephosphoBAD-Ser112, 4.71 times for dephosphoBAD-Ser136 and 1.937 times for dephosphoBAD-Ser155 following PDT in the cells incubated with ZnS3Pc, while

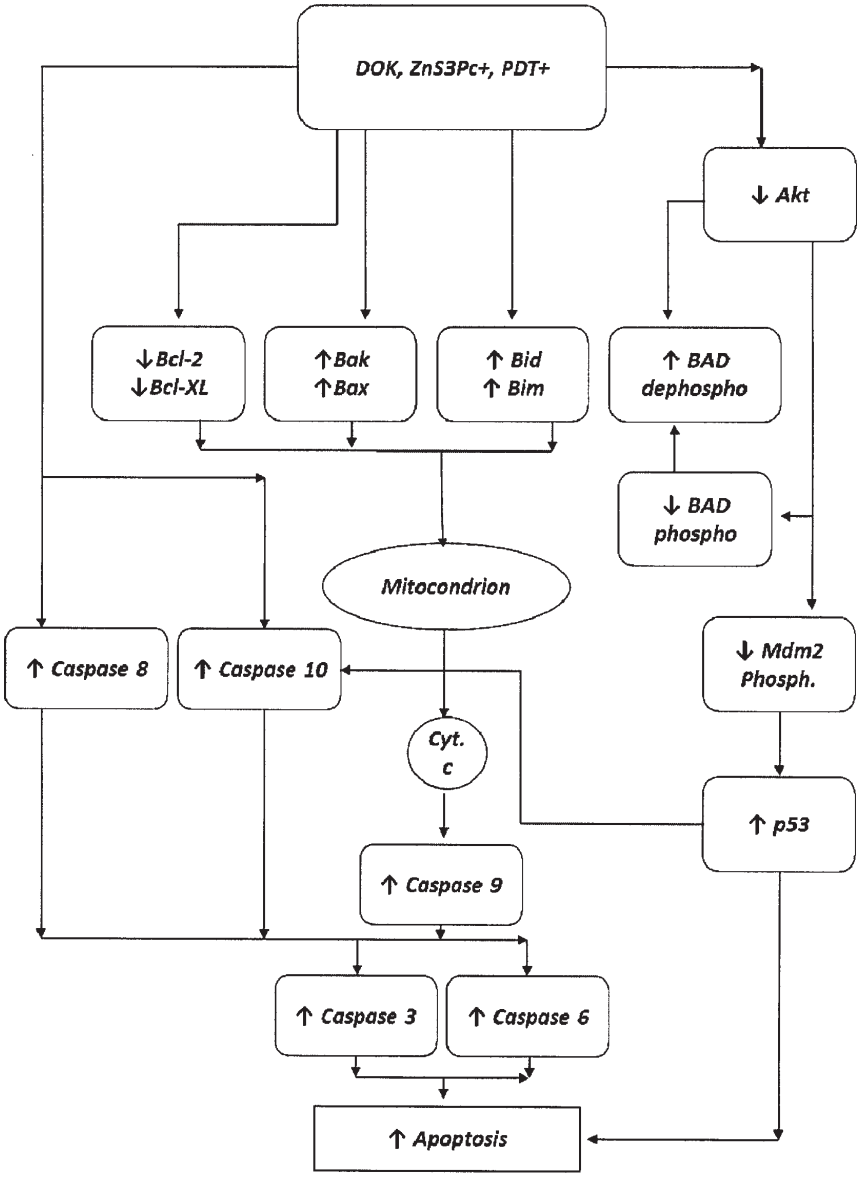


Fig.6. Schematic representation of the main apoptotic pathways activated by experimental photodynamic therapy using as photosensitizer ZnS3Pc loaded in DOK cells. Diagram resulted from the evaluation of 247 apoptotic-related proteins by means of protein-microarray technology

the levels of the corresponding phosphorylated counterparts decreased to 24.8, 27.4 and 50.8% for control cells values, respectively. These results plead for BAD involvement in DOK cells apoptosis induced by PDT. Taking into consideration the fact that Akt kinase is responsible for BAD phosphorylation at Ser136, the decrease in the level of phosphorylated BAD at this aminoacid residue may be explained by the decrease values of the level of the phosphorylated (and consequently, activated) forms of Akt, as previously asserted.

The caspases (acronym for cysteine-dependent aspartate-directed proteases) are a family of proteolytic enzymes able to cleave various subsets of proteins. After an aspartate residue in their chemical structure the caspase's family of enzymes contain a catalytic situs formed by five aminoacids (QACXG). The caspases involved in apoptosis are clasically divided into *initiator* caspases (caspases 2, 8, 9 and 10) and *effector* caspases (caspases 3, 6 and 7) (27). In the cells treated with ZnS3Pc and subject to PDT, we have observed singificant increase in the level of caspase 3 and 6 (44.96% and 30.5% respectively, $p<0.001$), accompanied by a increase of 77.7% in the level of caspase 9 ($p=0.002$). Previous results published by our group showed a similar caspase 3 – mediated apoptosis after experimental PDT, but in myelogenous leukemia K562 cell line (28). The level of caspase 8, a caspase canonically activated *via* the extrinsic apoptotic pathways, presented a smaller increase, of 18.8% ($p<0.001$), while the level of caspase 10 increased with 6.99% ($p=0.038$). Taking into account our previous resulsts as well in conjunction with these data altogether suggest that apoptosis in DOK cells treated by ZnS3Pc-PDT is mainly activated *via* the intrinsic pathway.

The phosphorylation of caspase 9 at Ser196 abolishes its apoptotic role; this structural modification is achieved by the action of Akt kinase (29). The level of phosphoSer196 form of caspase 9 does not increase significantly following ZnS3Pc-PDT ($p=0.078$), an aspect that may be as well correlated with the decrease in the level of phosphorylated Akt at Thr308 and Ser473, as above stated (23).

The release of cytochrome c from the intramembranar mitochondrial space to the cytosol is an event in the intrisic apoptotic pathway (9,10). Our experiment analyses the total level of proteins, not the level of the proteins from specific subcellular compartments, therefore the lack of a significant increase of the level of cytochrome c observed in this study ($p=0.117$). Mitochondrial depolarization studies could shed new light and explain more throughly the involvement of cytochrome c in experimental PDT. Our previous results performed on K562 cell lines proved a mitochondrial up-take mechanisms for Zn-complexed photosensitizers (30). Therefore we can not rule out the lack of differences in cytochrome c level to be the result of different explanations, dissimilar accumulation in intra-cellular site for this photosensitizer or merely the strict time-

dependency of the phenomenon and hence our „frozen-in-time" evaluation.

P53 protein, "the guardian of the genome", is the target of various mutations in the vaste majority of human neoplasias; in the squamous cell carcinomas of the oral cavity, the frequency of these mutations in p53 may be as high as 60%; Burns et al. showed that DOK cells present a constitutive elevated level of p53 protein (31,32). Accordingly to their observations, our experiment revealed a high level of p53 protein; moreover, an increase by 15.3% in the level of this protein ($p=0.009$) could be observed in the cells treated with ZnS3Pc and subject to PDT. The augmentation of the level of p53 may also explain the increase of the level of caspase 10 (7%, $p=0.038$), according to the data in the literature, as p53 has been proven to stimulate the expression of caspase 10 (33).

Taking into consideration that Akt kinase phosphorylates Ser186 from Mdm2 protein, a protein that sequestres p53 and increases its proteosomal degradation, the level of Akt in our experiment may also be correlated with the level of p53 protein; as Mdm2 phosphorylation by Akt increases its activity and consequently decreases the level of p53 (34), the decrease of phosphorylated (and therefore active) form of Akt that we have observed may explain the 15.3% increase in the level of p53.

The ratio between the pro- and anti-apoptotic proteins, in the form of Bax to Bcl-2 ratio (Bax/Bcl-2) is usually employed by the studies in the literature on this topic. The ratio usefulness results from the fact that its decrease shows the predominance of the antiapoptotic proteins, while its increase supports the propensity for an evolution to apoptosis. Thus, the therapeutic agents and procedures that induce apoptosis determine the increase of this ratio (35).

$$\frac{Bax\ (ZnS3Pc\ treated\ cells)}{Bcl-2\ (ZnS3Pc\ treated\ cells)}:$$

$$\frac{Bcl-2\ (control\ cells)}{Bax\ (control\ cells)} = 1.596/.621 = 2.57$$

The evaluation of this parameter revealed in our study a supraunitary value, proving that this procedure efficiently induces apoptosis in DOK cells following PDT with ZnS3Pc. We can draw the virtual molecular pathways that are involved in ZnS3Pc-PDT-induced apoptosis in oral dysplastic keratinocytes, as suggested by the results of our study and depicted by figure 6.

## Conclusions

Photodynamic therapy with ZnS3Pc activates apoptosis, mainly on the intrisic pathway, by increasing the levels of the active forms of proapoptotic proteins, while decreasing the levels of the antiapoptotic proteins; levels of Bax and Bak are increasing, whereas levels of Bcl-2 and Bcl-XL vary in the opposite direction, favoring apoptosis. Several other proteic molecules are involved in the pathways activated by ZnS3Pc-PDT, such as p53, Akt kinase and BH3-only proteins Bim, Bid and BAD. The study establishes an important theoretical basis for the action of PDT using ZnS3Pc. Our study proves the usefulness of proteomic technology, such as protein microarray evaluation for complex molecular pathways and to the best of our knowledge this is the first study investigating the main apoptotic pathways activated by photodynamic therapy with trisulphonated zinc phthalocyanine in DOK cells.

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*All authors have equally contributed to this work - Conceived and designed the experiments; Performed the experiments; Analyzed the data; Wrote the paper: MT, CM RI, MN, CC. The study was partially financed by Project PN-II-ID-PCE-2011-3-0918.*

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