NEW PHOTOSENSITIZERS VERSUS AMINOLEVULINIC ACID (ALA) IN EXPERIMENTAL PHOTODYNAMIC THERAPY OF ACTINIC KERATOSIS – A CASE REPORT

DANIEL BODA[1*], MONICA NEAGU[2], CAROLINA CONSTANTIN[2], ADRIANA DIACONEASA[3], SIMONA IANOSI[4], CORNELIA AMALINEI[5], BOGDAN STANOIU[6], EDUARD CRAUCIUC[5], OVIDIU TOMA[9], RODICA-MARIANA ION[7,8]

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Abstract: Photodynamic therapy (PDT) is increasingly being recognized as an attractive, alternative treatment modality for superficial cancer, being an emerging method for local destruction of tissue by generating toxic oxygen species using light absorbed by an administered or an endogenously generated photosensitizer (porphyrins, phthalocyanines, khellin, hypericin, riboflavin). A considerable number of PDT research over the past ten years has been devoted to the development of new sensitizers. The development of new photosensitizers for localization and treatment of tumors is a research area or current interest. The data show that, when properly used, PDT is an effective alternative treatment option in oncology. This paper will present a case study of actinic keratosis treated with aminolevulinic acid (ALA) and 5, 10, 15, 20 - tetra (4-sulfophenyl) porphyrin (TSPP) in a photodynamic therapy approach. The clinical data proved the efficacy and safety of the method and the new drug. From skin biopsies we have obtained lower absolute number of keratinocytes compared to control skin. The in vitro tests showed cells having lower viability, lower proliferation capacity, and high apoptosis/necrosis percentages; therefore after applying PDT we have obtained an active destruction of the cells.

INTRODUCTION

General Principles
Photodynamic therapy (PDT) can be considered a very promising approach for anticancer research. It consists of the selectively uptake of a photosensitizing dye, often a porphyrin, by a tumor tissue and subsequent irradiation of the tumor with a light flux of an appropriate wavelength matched to the absorption spectrum of the photosensitizing dye. Topical PDT is currently mainly used to treat **actinic keratosis (AK)** and **superficial non-melanoma skin cancers** in an increasing number of countries, with many recent publications providing new evidence in this field (Dougherty et al., 1998).

In PDT a chemical reaction activated by light is used to selectively destroy tissue. The reaction requires three basic elements: *photosensitizing compound* (porphyrins, porphyrin precursors, phthalocyanines, chlorines and others); *light*; *oxygen* ($^1O_2$) and other *free radicals* (Goldman et al., 2008)**.** Depending on the power density, light and laser light in particular causes different effects in biological tissues like photochemical reactions, coagulation, photo- and thermal ablation, plasma formation and photodisruption. The photochemical interaction occurs at very small power densities (0.01 - 50 W/cm²) and plays significant role during the PDT. To perform this therapy, spectrally adapted chromophores are injected into the body, the most used being porphyrins and related compounds. Irradiation with a suitable wavelength may trigger selective photochemical reactions, resulting in desirable biological transformations (Ion et al., 2006).

Mechanism
PDT produces cytotoxic effects through photodamage of cellular organelles and biomolecules. It is known that PDT mediates tumor destruction by three mechanisms: direct cell killing, tumor vasculature damage and immune response activation. The combination of the three mechanisms is required to obtain long-term tumor control (Huang, 2005).

Direct cell killing. Experimental data show that in optimal conditions (optimum concentration of photosensitizer and light), PDT destroys tumor cells by necrosis (Neagu et al., 2007). In time, photosensitizer's concentration decreases due to „photobleaching", vascular stasis, and oedema. In these conditions, as well as in the profound regions of the tissue efficiency of photodynamic therapy lowers, some cells will enter apoptosis and some will survive by defense mechanisms or lesion repair, situation which will lead to relapse (if not destroyed by ischemia or immune response).

The subcellular localization of the photosensitizer (PS) is extremely important. Several studies showed that PS localized in mitochondria produce cell death via apoptosis, while those localized in lysosomes and plasma membranes induce cell death predominantly by necrosis (Manda et al., 2009). Apoptosis is known in clinical practice to cause less tissue reaction compared to cell death via necrosis (after release of lysosomal enzymes and other cytotoxic agents) (Triesscheijn et al., 2006). High light doses cause rapid necrosis within a few minutes, due to massive cellular damage. The potential intracellular targets of PDT are: mitochondria, lysosomes, plasmatic membrane and nuclei of the tumor cells, as well as

tumor vasculature. PS that is not uptaken by cells is inefficient, even though it can generate high amounts of singlet oxygen. PS do not accumulate in the nucleus so they have a low potential to generate DNA lesions, mutations or carcinogenesis which is another advantage of this treatment method.

Vascular damage. Targeting tumor vasculature is one promising approach to cancer treatment. Vascular shutdown (vasoconstriction, thrombus formation) after PDT leads to the limitation of oxygen supply to the tumor and consecutive inhibition of tumor growth. Some studies showed on the other hand that vascular endothelial growth factor (VEGF) and cyclooxygenase (COX-2)- both potent angiogenic factors were upregulated during PDT, presumably due to reactive oxygen species (ROS) formation and hypoxia induced by photodynamic therapy (Taub, 1995; Hasan et al., 2003).

Immune response. The curative effect of PDT arises from the death of cancer cells that initially escape from the direct cytotoxic effect, by the intervention of the antitumor activity of inflammatory cells and tumor –sensitized immune reaction. Differences between the nature and intensity of the inflammatory reaction in normal versus cancer tissues could enhance the selectivity of PDT-induced tissue damage. The inflammatory cytokines interleukin IL-6 and IL-1 (Kelty et al., 2002) but not tumor necrosis factor-α (TNF-α) have been found upregulated after PDT. Also neutrophils were found increased in number after photodynamic therapy, slowing the tumor growth. By combining PDT with immunoadjuvants, an enhancement of the host immune reaction could be obtained, and many studies are conducted recently regarding this aspect (Oleinick et al., 2002).

Light sources

Light source in PDT (typically visible or infrared) emits light at wavelengths within the absorption spectrum of the photosensitizer. Three basic events occur when light is exposed to the skin*: reflection* from the surface of the skin (used for diagnostic purposes); *scattering* by the skin after penetrating it; *absorption* by structures within the skin which conduces to the clinical effect (Goldman et al., 2008). The choice of light source for PDT can be dictated by the location of the tumor, by the light dose delivered and by the choice of PSs. Lasers and lamps have both been employed to perform PDT and activation is obtained by illuminating the lesion site with a proper wavelength light (usually between 600-900 nm) which is absorbed by the PS. This initiates a sequence of photochemical and photobiologicall processes which lead to irreversible damages in target tissues (Nowis et al., 20050. Light penetration is a very important issue in photodynamic therapy, depending on the characteristics of the tissue (pigment-rich tissues are resistant to PDT) as well as on the wavelength of the light: longer wavelength penetrate tissues better than shorter ones. Typically, the depth of penetration is from 3 to 8 mm for wavelengths of 630-800 nm. Despite this fact, PDT can eradicate tumors with up to 1 cm depth, which can be explained by the concomitant activation of local immune response.

Actinic keratosis

Actinic keratosis (AK) is the most common skin lesion with malignant potential, with a prevalence ranging from 11% to 25% in the Northern Hemisphere and from 40% to 50% in Australia (Brand et al., 2000; Frost et al., 1994). The main factors responsible for these lesions are UV light, ionizing radiations, radiant warm and exposure to carbon processing products. The most affected persons are those with Fitzpatrick I and II phototype, men being more susceptible than women; this are considered *in situ* squamous cell carcinoma (SCC) by the most clinicians (Lebwohl, 2003). In time, these lesions could remain unchanged, could spontaneously regress or could progress to SCC and further developing on the support of pre-existing actinic keratosis. 5 to 20% of AK will transform into SCC within 10 to 25 years, with reported annual transformation rates ranging widely, from 0.25% to 16%. (Salasche 2000; Glogau, 2000). This statistic data suggest the powerful impact of actinic keratosis in health population even this lesions are in most cases ignored or wrong diagnosed. The common therapy could be surgical (cryotherapy, removing with or without cauterization or laser therapy) or medication based on topic drugs like 5-flourouracyl, imiquimod, diclofenac gel or PDT. PDT works as a selective targeted treatment with high cure rates. The advantage of porphyrins-PDT is no general cutaneous photosensibility. The application time and dose could differ depending of cell type characteristics.

Photosensitizers

Different photosensitizers have been tested in human tumor tissues. Porphyrin has been the preferred drug used as a photosensitizing substance due to its high affinity for the tumor cell and its strong cellular effect after light irradiation. Ion et al. [Ion 2006] showed that porphyrins can form a variety of structures from linear head-to-tail or J-aggregates to fractal aggregates grown under different regimes of aggregation, and can exhibit rich photophysical properties. Photophysical properties of TSPP porphyrin, as a synthetic compound have been extensively studied both in vivo and in vitro with application for the photodynamic therapy of cancer.

The majority of molecules that result in photosensitization contain a heterocyclic ring structure similar to that of chlorophyll or haem. The most extensively studied PSs so far are the porphyrins. In the last years, more selective and potent sensitizers have been developed, being under investigation in clinical trials. The ideal PS should be a single compound, biologically stable and photochemically efficient, should be retained selectively in the target tissues, displays no dark cytotoxicity, has good cytotoxic oxygen species generation, generates a high quantum yield of triplets, and has increased absorbance in the red region of visible light, for a better light penetration. The localization of PS in the target tissues is of great importance to understand the mechanisms involved in the cytotoxicity of PDT. The cytotoxic product of the photochemical process- singlet oxygen- $^1O_2$ can migrate less than 0.02 μm from its production site; therefore the sites

of photodamage will reflect the localization of the sensitizer at the time of irradiation. Since most PSs are fluorescent, drug localization can be determined by fluorescence microscopy using a sensitive system.

This class of molecules is able of using that energy to produce reactive oxygen species (singlet oxygen, superoxide anion, hydroxyl radicals, so on). The mechanism of cytotoxicity involves generation of singlet oxygen and other free radicals when the light-excited sensitizer loses or accepts an electron.

The porphyrins have been the preferred drugs as photosensitizing substances due to their high affinity for the tumor cell and its strong effect under light irradiation. Different others photosensitizers have been tested for localization in human tumor tissues: fluorescein, cyanine dyes, phthalocyanines, eosin, tetracycline, acridine orange, Rhodamine 123, etc) (Braathen et al., 2007; Frackowiak et al., 2001, 2008; Alexandrova et al., 2004). In addition, several second-generation photosensitizers are undergoing clinical testing. These second-generation compounds are generally pure, can be activated by light in the range of 630-800 nm, and share in common a lower incidence of prolonged cutaneous photosensitivity than Photofrin. One of these is d-aminolevulic acid (ALA), a precursor of the photosensitive protoporphyrin IX in the haeme biosynthetic pathway. A limitation of Photofrin and ALA is their low extinction at their absorption peak furthest into the red region (630 nm).

An ideal PDT photosensitizer should meet several criteria:

- chemical purity and reproducible synthesis;
- no toxicity;
- triplet excitation state of the photosensitizer must have a sufficient life time to be able to generate singlet oxygen;
- preferential accumulation in tumor tissue;
- physical and chemical stability;
- short time interval between photosensitizer administration and its maximum accumulation in tumor tissue;
- optimum absorption properties in the therapeutical window;
- solubility in biological fluids of the body for a rapid reaching at tumor site;
- rapid removal from the body.

Two *photosensitizers* are currently approved for topical PDT in dermatology. Methyl aminolevulinate (MAL) marketed as Metvix® is used in Europe, Australia and New Zealand. 5-aminolevulinic acid (ALA) marketed as Levulan® is approved by the FDA and is used in the United States and Canada. The techniques and dosage regimen vary considerably from author to author and their discussion is beyond the scope of this article (Goldman et al., 2008). Promising clinical results have been obtained using them in a variety of superficial malignant and non-malignant lesions such as basal cell carcinoma of the skin, Bowen's disease, mycosis fungoides, psoriasis and actinic keratosis.

Our current study focuses on actinic keratosis treated by ALA-PDT versus a new photosensitizer, 5,10,15,20-tetra(4-sulfonatophenyl) porphyrin (TSPP). The novelty of the research is represented by innovative formulation of porphyrinic materials depending on biological system tested. Previous studies (Ion et al., 1998; Neagu et al., 2007; Ion et al., 2008), from synthesis to clinical application confirmed its safety profile and good clinical response. The preliminary results of the team, suggest that TSPP has a similar efficacy like ALA but with lower secondary effects. Also, every new data will confer a direct impact to clinicians by implementation of a new treatment procedure – photodynamic therapy with a novel porpyrinic photosensitizer in actinic keratosis.

MATERIAL AND METHODS

We present the case of a patient, 61 years old, male presenting with multiple actinic keratosis (AK) on the scalp and face, with no previous therapy. AKs were classified into 3 grades (according to Olsen et al., 1991): 1=mild, 2=moderate, 3=severe. Only isolated 1 and 2 grade AKs were chosen for the study in order to warrant accurate comparison. Grade 3 AKs were excluded from the study comparison because they are known to respond less to PDT treatment. Control photographs were taken immediately after the treatment, 5 days and 10 days after. The patient gave written consent to participate in the study.

All grade 1 and 2 lesions were evaluated by a blinded observer immediately after the treatment, 5 days and 10 days after. A clinical score was used for assessing erythema, hyperkeratosis and thickness of the lesion on a scale between 0 and 3 (0=absent, 1=slight, 2=moderate and 3 =severe). Complete response was defined as a sum score of 0.

On the left side of the scalp and face was applied 20% ALA and on the right side TSPP dissolved in an oil-in-water cream, both for 2 hours incubation time under occlusion. Identical irradiation protocol was used: one session of irradiation 130 J/cm2 fluence at 635 nm wavelength. Pain during treatment was quantified by the patient on a 0 to 10 analog scale.

5,10,15,20-tetra-(4-sulfonatophenyl)porphyrin (TSPP) was synthesized and purified in the laboratory after the literature methods (Ion, 2006). It was solubilized in water at 10^{-4} M concentration. All the stock solutions were stored at 4°C in the dark and used in the 14 days interval.
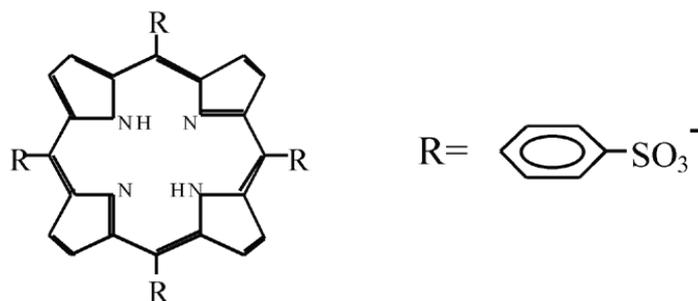

Figure 1. The chemical structure of TSPP

Keratinocyte isolation
Skin biopsies were taken before and after PDT therapy and human keratinocyte cultures were obtained after a protocol comprising several combined methods [Green et al, 1979; Gragnani et al, 2003; Boyce, 1999; E. Radu et al, 2002] in order to obtain the best yield of primary keratinocytes. Briefly, 3 mm^2 punch biopsies before and after PDT were incubated for 24h in antibiotic cocktail medium, after thorough removal of antibiotics, biopsies were incubated for another 24h in PBS without Ca++ and Mg++ containing 2 U/ml dispase (Sigma Chemical Co.), at 4^0C. The epidermis was mechanically removed with twisters. The epidermis was sliced in very small fragments with scissors and incubated for 15-30 min. in trypsin-EDTA (Sigma) at 4^0C. The suspension that contains keratinocytes was washed in PBS without Ca++ and Mg++ by centrifugation at 1000rpm for 10 min at 4^0C. After repeating the washing, cells were resuspended in KMK-2 medium (Sigma). For cultivating keratinocytes, KMK-2 medium was supplemented with bovine pituitary extract, insulin, hydrocortisone, transferine and EGF. Cell suspensions were counted in Trypan blue and further tested.

Cell viability
Besides Trypan blue exclusion test, cell viability was evaluated using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit [Technical Bulletin #TB163, Promega], that measures the lactat dehidrogenase (LDH) released in the culture medium by cultivated cells, being a suitable test for cell membrane integrity.

Proliferation capacity
Isolated keratinocytes were tested for the proliferation capacity using an indirect test that quantifies the intracellular oxidases, namely CellTiter 96 Aqueous One Solution Cell Proliferation kit (Promega) [Technical Bulletin #TB112], as a measure of metabolically active cells in culture.

Apoptosis
Annexin V-FITC (Ann-FITC-green fluorescence) propidium iodide (PI-red fluorescence) kit (BD Biosciences) was used for assessing the apoptosis by flow cytometry. The early feature of apoptosis characterized by morphological change in plasma membrane which involves the translocation of the membrane phospholipid phosphatidilserine from internal layer to external layer of cell membrane is identified by high affinity binding of Ann-FITC. Late apoptotic/necrotic cells in which phospholipid phosphatidilserine translocation has occurred and the cell membrane is damaged, cells are double stained with Ann-FITC and PI. Samples were immediately analyzed in a FACScalibur cytometer (Becton Dickinson) within one hour, using Cell Quest software. Results are presented as follows: percentage cells stained double negative – live cells, double positive – dead/necrotic cells and positive for Annexin, negative for propidium iodide – apoptotic cells (Nowis et al., 2005).

RESULTS AND DISCUSSIONS

TSPP is an anionic porphyrin, a very large disk-shape molecule which posses four negative charges sustained by the sulphonate groups from the four corners. In aqueous solutions, at neutral pH, the electronic absorption spectrum of TSPP is typical of free base porphyrins (D_{2h}

symmetry) and is characterized by an intense Soret band at around 420 nm and four Q bands in the 500-700 nm range (the aetio-type spectrum).

Aggregation of TSPP can be activated by decreasing pH or increasing ionic strength of the solution, and it can be monitored using different positions of absorption peaks corresponding to different species (Figure 2). Because of static Coulombic repulsion, the two central NH fragments are probably distorted out of the aromatic plane, as reported elsewhere (Salasche, 2000). The planar TSPP zwitterionic monomer subunit leads to the suggestion of a straightforward structure for both J- and H-aggregates. In acidic medium, new absorption bands (from 490, 707 nm) become dominant when the concentration of TSPP exceeds 10^{-5} M and they are attributed to the aggregated forms of TSPP, Table I. H and J-aggregates of some porphyrins are based on the intermolecular interactions of 3-5 Kcal/mol per porphyrin face. Such aggregates have the size of 5-6 nm in solution. The columnar structure formed by porphyrins has a length of 5 to 27 porphyrin unities (Ion et al., 2008).
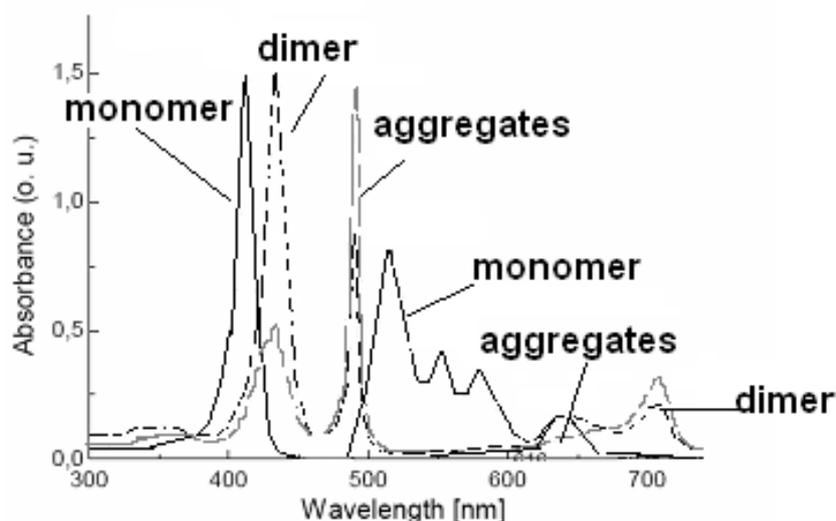

Figure 2. The absorption spectra of TSPP monomer, dimer and aggregates forms

Table I. The specific absorption bands of different TSPP forms

Porphyrinic forms	B band λ/ε x10^3(nm/M^{-1}.cm^{-1})	Q bands λ/ε x10^3(nm/M^{-1}.cm^{-1})
H_2TSPP^{-4}	412/355	515/130
		551/4.5
		579/1.9
		33/1.01
H_4^{+2}TSPP Dicationic form	433/357	550/140
		594/3
		644/14

H_4^{2+}TSPP J-aggregate	422	490		707
H_4^{2+}TSPP H aggregate		401		517 552 593

Ion, in 2006 and Frackowiak and co-workers, in 2001 showed that the efficiency of the incorporation of these dyes into cellular membrane changes in the same manner as the singlet oxygen generation (Ion, 2006; Frackowiak et al., 2001). The sulphonated porphyrins were better incorporated (unlike to the non-sulphonated ones). The finding can suggest that aggregated forms (J-aggregates) generated for the porphyrins in water are better penetrating the membranes and once in the cellular membrane, dyes are disaggregated by the interaction with lipids and exhibit photodynamic efficiency as the monomeric form.

In our experimental approach, we have obtained from untreated skin biopsies a mean of 2.8×10^4 keratinocytes/cm^2 skin (Figure 3) with a mean of 65% viability. After therapy from the same skin region and from the same surface isolated keratinocytes were less than half compared to the control skin, displaying as well a lower viability (Figure 3).
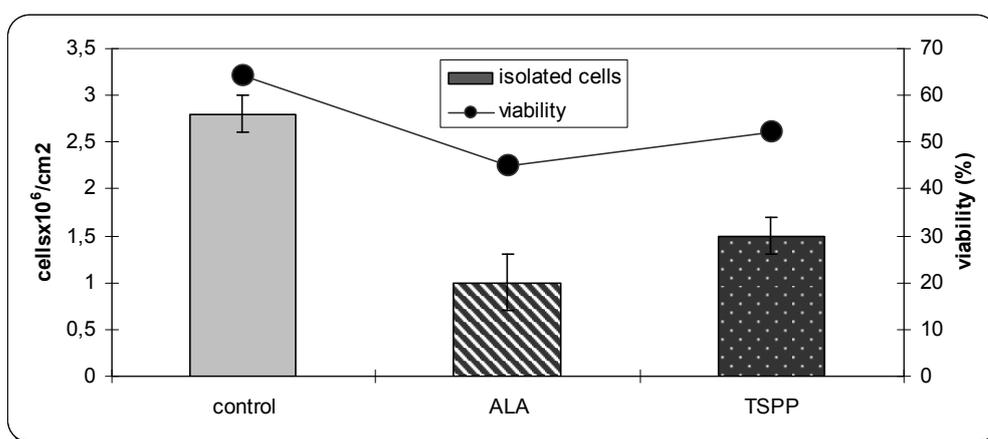

Figure 3. Primary keratinocytes isolated from normal human skin before and after PDT (isolated cells/cm^2 skin and viability) (mean±SD of triplicates)

Primary keratinocytes were further cultivated until the culture could not be maintained. The proliferation capacity of primary keratinocytes extracted from PDT skin biopsies was significantly lower compared to control skin (Figure 4). After 7-8 days from the isolation primary keratinocytes could not be maintained in culture. The functional testing of isolated keratinocytes was done at 72-96h from isolation.
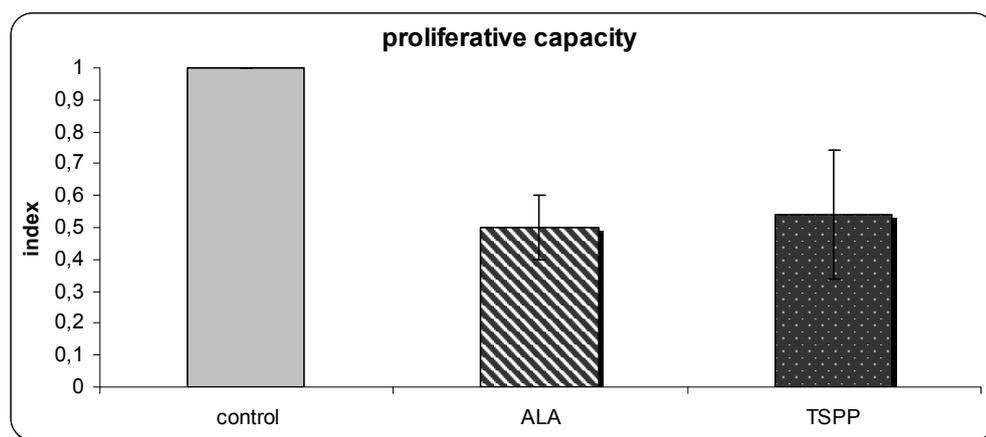
Figure 4. Proliferative capacity of isolated keratinocytes from skin subjected to ALA-PDT (mean±SD of triplicates)

The proliferation capacity matches the high apoptotic pattern of isolated cell post-PDT (Figure 5) in both compounds used in vivo.
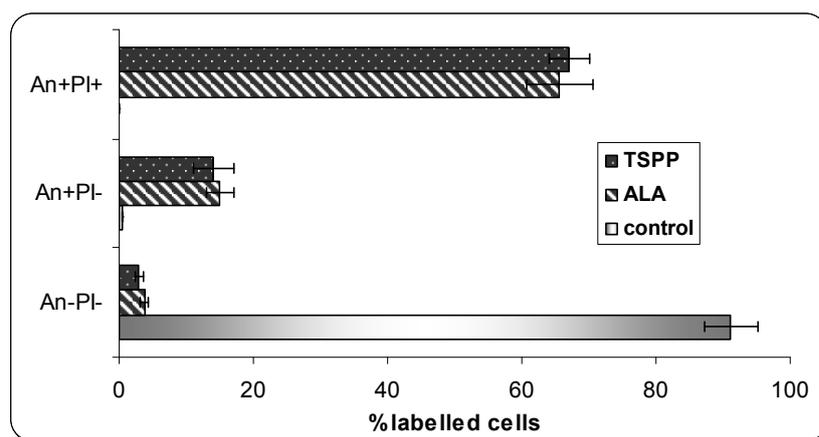

Figure. 5. Annexin-V and propidium iodide labelling of isolated keratinocytes after in vivo PDT compared to control keratinocytes (mean±SD of triplicates) – Necrosis/late apoptosis: An+PI+; Early apoptosis: An+PI-; Live cells: An-PI-.

The tested TSPP showed an effective *in vivo* destructive effect on keratinocytes in the patient with actinic keratosis doubled by a good clinical response. The adverse events to PDT (pain, erythema) were slightly lower in the TSPP treated area (Figure 6).
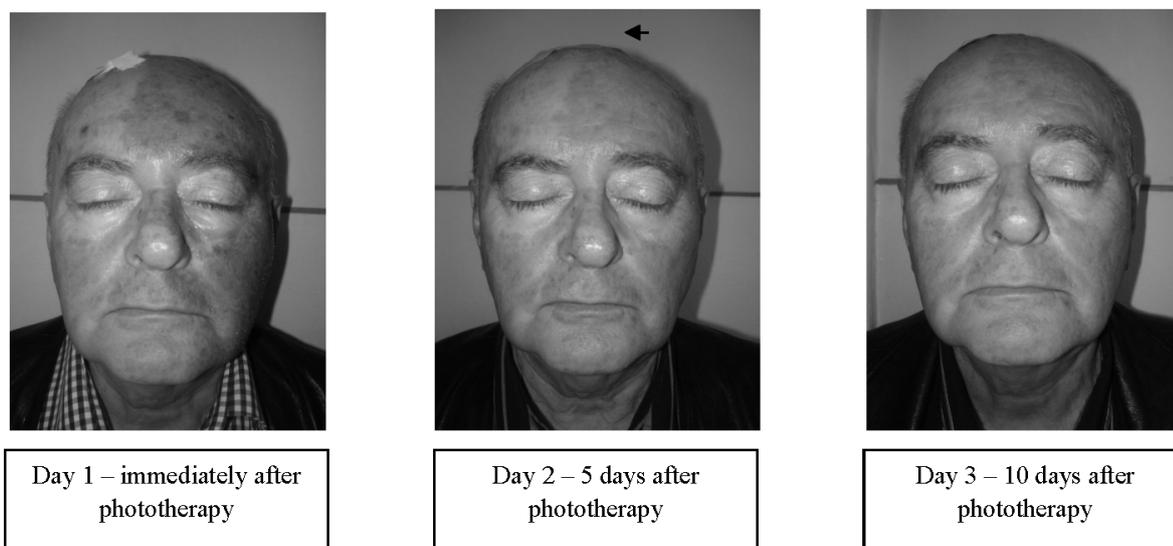
| Day 1 – immediately after phototherapy | Day 2 – 5 days after phototherapy | Day 3 – 10 days after phototherapy |

Figure 6. The volunteer patient at different stages at PDT treatment

CONCLUSIONS

We have obtained from the treated skin lower absolute number of keratinocytes compared to control skin. The cells displayed a lower viability proving a lower proliferation capacity in vitro and high apoptosis/necrosis percentages, therefore an active destruction of the cells.
Photodynamic therapy is a potentially effective treatment approach for superficial human cancers and selected benign conditions. The technique can be used as an adjuvant therapy with surgery, radiation or chemotherapy. Newer generation photosensitizers are being tested which may produce less photosensitivity.
TSPP can be a good competitor for ALA in PDT therapy considering the fact that it is cheaper and being a porphyrin by itself it doesn't induce intracellular local protoporphyrin synthesis. Further clinical studies will try to improve TSPP irradiation protocols.

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[1] „Carol Davila"University of Medicine and Pharmacy Bucharest, Dermatology

[2] "Victor Babes" National Institute of Pathology, Bucharest, Immunology Department

[3] „Grigore Alexandrescu" Hospital Bucharest, Dermatological Department

[4] University of Medicine and Pharmacy Craiova, Dermatology

[5] „Gr.T. Popa" University of Medicine and Pharmacy Iasi, Histology

[6] University of Medicine and Pharmacy Craiova, Cell and Molecular Biology

[7] National Institute of Research and Development for Chemistry and Petrochemistry – ICECHIM, Bucharest

[8] Valahia University, Targoviste, Romania

[9] "Alexandru Ioan Cuza" University of Iasi

*dan.boda@gmail.com