Academy of Romanian Scientists *Volume 7, No. 1, 2018, pp. 37 - 44*
Annals Series on Biological Sciences ISSN 2285 – 4177

Porphyrinic Derivatives Influence on HS27 and HaCaT Cell Lines

Brândușa G. DUMITRIU[1], Laura OLARIU[2, 3], Raluca PAPACOCEA[4], Gina MANDA[5]

[1]PhD, Senior Researcher III, R&D Department, SC Biotehnos SA, 3-5 Gorunului Street, 075100-Otopeni, Ilfov, Romania (dbrandusa@biotehnos.com).
[2]PhD, Senior Researcher I, R&D Department, SC Biotehnos SA, 3-5 Gorunului Street, 075100-Otopeni, Ilfov, Romania (lolariu@biotehnos.com).
[3]Academy of Romanian Scientists, 54 Splaiul Independentei 050094, Bucharest, Romania. (ilolariu@yahoo.com).
[4] PhD, Assistant Professor, UMF Carol Davila Bucharest
[5] PhD, Senior Researcher I, INCD Victor Babes Bucharest

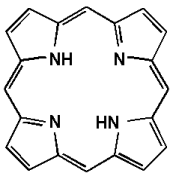
Abstract. *The porphyrin derivatives had a great development in terms of extending their applicability from initially known biological pigments to modern photodynamic or sonodynamic therapies in cancer. A first impact of these derivatives is on the skin, where minimal cytotoxic potential on normal cells should be considered. The paper presents studies on the influence of porphyrin derivatives of natural origin on HS27 and HaCaT cell lines at the level of metabolic and cytotoxic status, but also under the conditions of UV radiation. Cytotoxicity limits for Dermo-CC6 / 7b derivatives of fibroblasts and keratinocytes were determined and effects of reduction of inflammation and oxidative stress in UV-A and UV-B irradiation were revealed.*

Keywords: porphyrinic derivatives, cytotoxicity, oxidative stress, inflammation

Introduction

Porphyrin, a class of water-soluble, nitrogenous biological pigments (biochromes), derivatives of which include the (porphyrins combined with metals and protein). One of the hemoproteins is the green, photosynthetic chlorophylls of higher plants.

Due to the green colour of chlorophyll, it has many uses as dyes and pigments. It is used in colouring soaps, oils, waxes and confectionary. Chlorophyll's most important use in nature, is however, in photosynthesis. It is capable of channelling the energy of sunlight into chemical energy through photosynthesis. The chemical energy stored by photosynthesis in carbohydrates drives biochemical reactions in nearly all living organisms (1).



Porphyrin core

More recent studies show that chlorophyll protects against various chemical carcinogens. There is little known about the bioavailability of chlorophyll and how it is metabolized. What is known is that it is effective in aiding in cancer treatment and it has potent antioxidant properties. Scientific literature sustain also the use in photodynamic therapy of chlorophyll-derived photosensitizers (2).

The porphyrin derivatives had a great development in terms of extending their applicability from initially known biological pigments to modern photodynamic or sonodynamic therapies in cancer.

A first impact of these derivatives is on the skin, where minimal cytotoxic potential on normal cells should be considered.

We focused our studies on the influence of porphyrin derivatives of natural origin **(chlorophill from Salvia officinalis, name Dermo-CC6/7**) on HS27 and HaCaT cell lines at the level of metabolic and cytotoxic status, but also under the conditions of UV radiation.

Excessive production of reactive oxygen species and / or depletion of cellular antioxidants is manifested in many pathological conditions. When the relationship between pro-oxidant and anti-oxidant systems is destabilized, so structural and functional changes occur at the cellular and tissue levels, the phenomenon of "oxidative stress" is considered a catabolic event. [1, 2,18] This is based on the degradation of cellular constituents, the first targets of attack of reactive oxygen species. Although extremely difficult to quantify and elucidate in the clinical study, the role of SRO is of increasing interest in toxicology and pathogenesis, the inflammatory process being considered one of the most important mediators of tissue lesions [2, 3, 5].

Organisms are protected from the aggression of reactive oxygen species in several ways: cell compartmentalization, protection provided by antioxidant compounds and enzyme systems, ability of organisms to develop inducible adaptive responses under oxidative stress conditions. In addition, repair and turnover processes help minimize lesions resulting from the attack of reactive oxygen species [6, 7, 8].

Inflammation is the protective response of tissues against cellular lesions, irritation and pathogenic invasions. Chronic inflammation is considered to be the primary mediator in the development of chronic diseases such as cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, arthritis, autoimmune and pulmonary diseases. Intensive production and secretion of cytokines and proinflammatory chemokines, once started, may form concentration gradients in the affected tissues, which may lead to the amplification of the initial inflammatory response. IL-6 is a cytokine involved not only in inflammation and response to infections, but also regulating metabolic and regenerative processes [12]. Tumor promoters, proinflammatory cytokines, endotoxins and synthetic protein inhibitors can modulate cell cycle kinetics of different cell types, stimulate the production of reactive oxygen species and induce keratinocytes to produce interleukin-8 (IL-8), a strong chemotactic for polymorphonuclear neutrophils Lymphocytes T [13]. IL-8 is involved in neutrophil activation and is released from several types of cells in response to inflammation, including monocytes, macrophages, neutrophils and intestinal, kidney, placenta and bone marrow cells [14].

After the cytotoxic profile evaluation on human normal keratinocytes and fibroblasts, we investigate the antioxidant and anti-inflammatory potential on UV irradiated keratinocytes.

The anti-inflammatory activity was studied on proinflammatory cytokines (IL-6, IL-8) release and the antioxidant effect was highlighted on intracellular reactive oxygen species H_2O_2 and O^{2-}.

Materials and Methodes

Materials

Natural compound Dermo CC: Dermo-CC is an original compound extracted from Salvia off., having a chlorophyll core.

Standardized cell lines: Fibroblast (normal cell line HS27) - cells with high proliferative capacity, cell cultures reach confluency relatively quickly (80% confluence in 3 days). Cells were cultured in monolayer in DMEM (Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 Ham, code: D8437, Sigma-Aldrich) supplemented with 10% fetal bovine serum (code: F7524, Sigma-Aldrich) used in tests between passages 20-40. Keratinocit (HaCaT) - epithelial squamous cell with a remarkable regenerative potential, which undergoes a differentiation process during its migration from the level of the germinative layer to the cortical layer, descuamative. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (code: 30-2002, ATCC®) supplemented with 10% fetal bovine serum and used in experiments at passages 40-50.

Chemical Substances:

- N-acetyl-cysteine (code: SC-202232, Santa Cruz Biotechnology) as antioxidant
- Dexamethasone (code: D4902, Sigma-Aldrich) as anti-inflammatory agent.
- CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) kits - for the determination of metabolic activity (MTS) and
- CytoTox 96® Non-Radioactive Cytotoxicity Assay from Promega - for the release of lactate dehydrogenase (LDH) in extracellular medium.
- BD Cytometric Bead Array (CBA) - Human Inflammatory Cytokines kit (BD Pharmingen) kit.

Methods

a) EVALUATION OF THE CYTOTOXIC EFFECT on a cell culture.[21]

The cytotoxic effect was evaluated by the correlation between decreased cell viability (MTS test) and enhancing lactate dehydrogenase activity in the culture medium as a toxicity marker (LDH assay). To assess the cytotoxicity of the samples studied, the cells were exposed to increasing concentrations of the test products over a period of time, depending on the cellular metabolic characteristics.[21]

Reduction of MTS, cell viability marker: Treatment of MTS cells, tetrazolium 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium tetrazolium salt allows evaluation of oxidative metabolism and response of a cell population to external factors that may have a positive or negative effect on cell life in culture. [4].

Conversion of MTS to water-soluble formazan occurs under the action of enzymes (dehydrogenases) found in metabolically active cells. The amount of formazan produced, measured as absorbance at 490 nm, is directly proportional to the number of live cells in the culture [5,6].

Release of lactate-dehydrogenase in cell medium, cytotoxicity marker: Lactate dehydrogenase (LDH) is a cytosolic enzyme present in all cells which, under normal physiological conditions of the plasma membrane, remains in the cytoplasm. The in vitro release of LDH provides a precise way of measuring cell membrane integrity and, implicitly, cell viability [7]. The release of LDH in the cell culture supernatant is measured by a test in which two coupled enzymatic reactions catalyzed by LDH and diaphorase are carried out which result in the conversion of a tetrazolium salt into a red formazan compound [8].

b) DETERMINATION OF EXTRACELLULAR PRO-INFLAMMATORY CYTOKINES by flow cytometry and use of capture beads:

The assay uses a series of discrete fluorescence intensity particles for the simultaneous detection of several soluble analytes (inflammatory cytokines). Each beads of the kit has a capture surface coated with antibodies specific for IL-8, IL-6 [11]. The capture beads, the conjugated detection antibodies and the recombinant standards or assay samples are incubated together to form a sandwich complex that is visualized in APC-A / PE-A coordinates following acquisition of flow cytometry. Analysis of fluorescence histograms and interpolation of values on calibration curves is performed with FCAP Beads Array software.

c) OXIDATIVE BALANCE MODULATION - Flow cytometry for reactive oxygen species – ROS – quantification – DCFH-DA (for HYDROGEN PEROXIDE) and HE (for SUPEROXIDE ANION) staining [22]

Cellular oxidative stress through intracellular activation of superoxide anionand and hydrogen peroxide is quantified by simultaneous measurement of intracellular levels of H_2O_2 and O_2 - DCFH-DA (dichlorofluoresceine diacetat), and HE (hydroxiethidium) staining and flow cytometry analysis. DCFH-DA is embedded in lipid hydrophobic region of membrane where hydrolytic enzymes clive the diacetat residues, releasing the membrane permeant configuration which is oxidized in the cytoplasm by the intercellular hydrogen peroxide, producing FITC-A fluorescence (530nm emission). HE permeates the cell membrane and is oxidized by superoxide anion ethidium bromide which tight bond DNA and emits at 620nm (PE-A). Hydrogen peroxide and superoxide anion quantities are proportional with the variation of mean fluorescence channel: FITC–A mean – for Hydrogen peroxide and PE-A mean – for superoxide anion. Flow cytometry diagrams shows cellular subpopulation (green and blue) producing hydrogen peroxide (FITC-A positive) and superoxide anion (PE-A positive).

Results and discussions

Evaluation of the cytotoxic effect of Dermo CC 6 and 7 on keratinocytes and fibroblasts cell culture

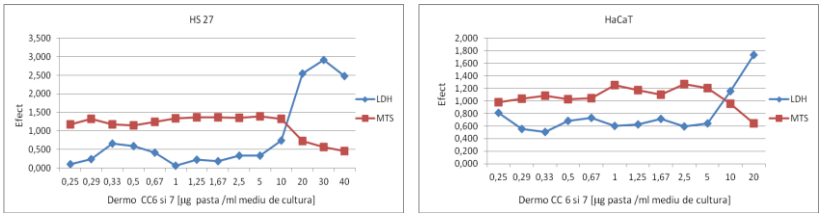


Fig. 1. Evaluation of the cytotoxic potential of chlorophylic extracts revealed by MTS and LDH assays on HaCaT - keratinocyte (a.) and HS27 - fibroblast (b.) cell lines. The cells were allowed to adhere for 24h (7000 cells / well) and treated for 48 hours with the test substance according to the protocol for the specific reagent kit (MTS / LDH). All concentrations of active principle were tested in triplicate.

Dermo CC 6 /7 maintain a low level of cytotoxicity on both cell lines, characterised by poor LDH release in culture medium and a rising metabolic activity.On the HaCaT cell line, the cytotoxicity threshold is 5µg/ml, this concentration increases the amount of lactate dehydrogenase released in the medium, while the reduction in MTS decreases, indicating a loss of cell membrane integrity and decreased cell viability. On the HS27 cell line, the chlorophilic complex has a cytotoxic effect starting at a 10% concentration. Concentrations below this limit has superior ratio of sample/ control effects, the metabolic activity evaluated by MTS technique being higher, suggesting an activation of cellular metabolism induced by extract components.

Oxidative stress and extracellular release of pro-inflammatory cytokines (IL-6 and IL-8) by UV irradiated keratinocytes.

The cells were adhered 24h, pretreated 24h with the test substances, irradiated with specific doses and then cultivated for another 16h in the presence of Dermo-CC6/7. Irradiation was performed under reproducible conditions with controlled irradiation equipment: Bio-Sun system (Vilber Lourmet).

We settled an experimental system of 3 test series:

- Non-irradiated group
- UV-A irradiation **9J/cm2**
- UV-B irradiation **0.02J/cm2**

We studied the cellular impact of Dermo-CC6/7 treatment on oxidative stress and inflammation.

Positive controls:

- **N-acetylcysteine** were used as a potent anti-oxidant – 0.2mM
- **Dexametasone** is a well-established anti-inflammatory agent – 200ng/ml

Oxidative stress: Hydrogen peroxide and intracellularly released superoxide anion - the results are presented in the table below, as follows: The amount of oxygenated water and the intracellular superoxide anion correspond to fluorescence channel fluctuation in the two coordinates: FITC - A mean - hydrogenated water and PE-A mean - for the superoxide anion

Tested compound	Fluorescence channel FITC-A (H_2O_2)			Fluorescence channel PE-A (O_2^-)		
	Unirradiated cells	UVA	UVB	Unirradiated cells	UVA	UVB
Cellular control	6536	10688	32820	6826	9448	13184
Solvent control	6388	14971	29950	6332	11609	22406
Dermo-CC 2.5µg/ml	5982	10265	26412	5963	10256	12554
Dermo-CC 5 µg/ml	6044	11587	22447	5877	9872	11789
N Acetil Cysteine 10mM	6558	17896	18774	6522	16254	15443

Dermo-CC acts in a dose-effect manner to reduce the reactive oxygen species, being a potent antioxidant, similar to N-acetyl Cysteine. We had to mention the inhibition of hydrogen peroxide on UV-A irradiated keratinocytes and the decrease of both oxygen reactive species in the case of UV-B impact.

Inflammation: pro-inflammatory cytokines IL6 and IL8; - the effect of the tested compounds on IL6 and IL8 proinflammatory cytokines released under UV irradiation conditions is presented in the table below:

Tested compound	IL6 (pg/ml)			IL8 (pg/ml)		
	Unirradiated cells	UVA	UVB	Unirradiated cells	UVA	UVB
Cellular control	135,36	275,36	2105,6	476,5	607,99	885,8
Solvent control	126,57	279,37	2274,8	432,6	564,68	991,22
Dermo-CC 2.5µg/ml	134,2	178,18	1813,9	445,1	556,55	969,81
Dermo-CC 5 µg/ml	137,1	133,16	1599,4	461,7	486,88	728,82
N Acetil Cysteine 10mM	107,28	105.8	1208	536,03	120.8	458,77

Dermo-CC reduces the secretion of IL6 and IL8 cytokines for both types of irradiation at the higher dose tested (5 µg / ml).

Conclusions

Dermo-CC6 / 7 (Chlorophyll COMPLEX) derivatives has a good cytotoxic profile on human normal fibroblasts and keratinocytes.

It also reduce the inflammation (IL6 and IL8 signaling) and reactive oxygen species release (hydrogen peroxide and superoxide anion) in UV-A and UV-B irradiated keratinocytes.

These effects suggest its future use in photodynamic therapy as an antioxidant adjuvant or as a cellular protector.

Acknowledgment

The study was performed as part of the 55/2016 project: *"Advanced theranostic approach in cancer combining photodynamic therapy and nanoparticles"*, as part of M-Era.Net Topic 5 "Tailoring of bioactive material surfaces for health applications"

References

[1] Russian Journal of Applied Chemistry December 2003, Volume 76, Issue 12, pp 1958–1961 ,Chlorophyll and Its Derivatives, Chlorins and Porphyrins, as a Promising Class of Environmentally Friendly Dyes

[2] Joerg G. Moser, Angelika C. Rueck, Hans-Joachim Schwarzmaier, Christel Westphal-Frosch, "Photodynamic cancer therapy: fluorescence localization and light absorption spectra of chlorophyll-derived photosensitizers inside cancer cells," Opt. Eng. 31(7) (1 July 1992.

[3] Valko M, Leibfritz D, Moncol J, Cronin M, Mazur M, Telser J., Free radicals and antioxidants in normal physiological functions and human disease, The International Journal of Biochemistry & Cell Biology, 2007, 39:44–84

[4] Rees MD, Kennett EC, Whitelock JM, Davies MJ., Oxidative damage to extracellular matrix and its role in human pathologies, Free Radical Biology & Medicine, 2008, 44:1973–2001.

[5] Malich G., Markovic B., Winder C., The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines, Toxicology, 1997, 124(3), 179-92.

[6] Barltrop J.A. et al. 5-(3-carboxymethoxyphenyl)-2-(4,5-dimenthylthiazoly)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators, Bioorg. Med. Chem. Lett., 1991, 1, 611–4.

[7] Riss T.L., Moravec R.A., Comparison of MTT, XTT, and a novel tetrazolium compound for MTS for in vitro proliferation and chemosensitivity assays. Mol. Biol. Cell (Suppl.), 1992, 3, 184a.

[8] Fotakis G., Timbrell J.A., In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride, Toxicology Letters, 2006, 160, 171-177. 10.

[9] Dean R.T., Fu S., Stocker R., Davies M.J. (1997) Biochemistry and pathology of radical-mediated protein oxidation. Biochem.J. 324: 1-18.

[10] Allen M., Millett P., Dawes E., Rushton N., Lactate dehydrogenase activity as a rapid and sensitive test for the quantification of cell numbers in vitro, Clin Mater., 1994, 16(4):189-94.
[11] Decker T., Lohmann-Matthes M.L., A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity, J Immunol Meth, 1988, 115:61-9. 14.
[12] Stoker R. (1990) Induction of heme oxygenase as a defence against oxidative stress. Free Rad.Res.Comms. 9: 101-112.
[13] Cory A.H., Owen T.C., Barltrop J.A., Cory J.G., Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture, Cancer Commun., 1991, 3, 207-12.
[14] Cook E.B., Stahl J.L., Lowe L., Chen R., Morgan E., Wilson J., Varro R., Chan A., Graziano F.M., Barney N.P., Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics, Journal of Immunological Methods, 2001, 254, (1–2), 109-118.
[15] Stoker R., Lai A., Peterhans E., Ames B.N. (1989) Antioxidant activities of bilirubin and biliverdin. In: Medical, Biochemical, and Chemical Aspects of Free Radicals, Elsevier, Amsterdam, pp.465-468
[16] Scheller J., Chalaris A., Schmidt-Arras D., Rose-John S., The pro- and anti-inflammatory properties of the cytokine interleukin-6, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2011, 1813, 5, 878-888.
[17] Wilmer J.L., Luster M.I., Chemical induction of interleukin-8, a proinflammatory chemokine, in human epidermal keratinocyte cultures and its relation to cytogenetic toxicity, Cell Biology and Toxicology, 1995; 11, 37-50.
[18] Carini M., Aldini G., Piccone M., FACINO R.M., Fluorescent probes as markers of oxidative stress in keratinocyte cell lines following UVB exposure, Il Farmaco; 55(8),526–534, (2000).
[19] Robinson J.P. , Current Protocols in Cytometry. vol 2, Unit 9.7, 9.7.1 – 9.7.14, Ed. John Wiley &Sons., (2009).
[20] Bickel M., The role of interleukin-8 in inflammation and mechanisms of regulation, J Periodontol., 1993, 64, 456-60.
[21] Crăciun, Dumitriu, Ene, A.Adil, Olariu, Rosoiu, *Valorification of grape marc by obtaining bioactive complexes tested through in vitro experimental models,* Analele AOSR, vol6, nr.2/2017, 5-14.
[22] Olariu, Dumitriu, Ene, A.Adil, , Rosoiu, Alflutop modulates în vitro"relevant mechanisms of osteoarthritic pathologies, Analele AOSR, vol6, nr.1/2017, 5-14, 82-99