

The Cytotoxic Action of New Ag-Porphyrin as a Potential Chemotherapeutic Agent

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Abstract – Earlier we have described new water-soluble Ag- and Zn-derivatives of tetrachloride meso-tetra (4-N-oxiethylpyridyl) porphyrin (TOEtPyP) as potential anticancer drugs. In this work the effect of one of these metal porphyrins, TOEtPyP Ag, on the cell population kinetics was studied *in vitro* using morphological and biochemical techniques. The results suggested that TOEtPyP Ag action consisted in the simultaneous suppression of the cell growth and activation of the cell death. About 40% of the cells were shown to die via apoptotic pathway. So, the porphyrin studied may be attributed to inducers of both necrotic and apoptotic processes. The results obtained support our previous assertion that TOEtPyP Ag may be considered as a potential chemotherapeutic agent.

Key words : Ag-Porphyrin, Cytotoxicity *in vitro*, Chemotherapeutics

INTRODUCTION

Natural and synthetic porphyrins are organic pigments containing the porphyrin ring. Porphyrin chemistry deals with various analogues and derivatives of porphyrins including their metal complexes (Kadish *et al.* 2000).

The known ability of cationic porphyrins to bind nucleic acids has driven studies of their medical and biological activities. Recent applications of porphyrins have been based on their antiviral (Ding *et al.* 1992; Vzorov *et al.* 2002) and anticancer (Ding *et al.* 1990; Dougherty *et al.* 1998; Ohse *et al.* 2001; Berg *et al.* 2005) activities. A variety of (metallo)porphyrins has been successfully used to control harmful microorganisms (Stojiljkovic *et al.* 2001; Lambrechts *et al.* 2005; Jori 2006). Porphyrins are known photosensitizers used at the photodynamic therapy (PDT) of tumors (Doug-

herty *et al.* 1998). They have been shown to effectively accumulate and be retained for long time in tumor tissues (Vicente 2001). Generally speaking, porphyrin derivatives are believed to be promising candidates for drug discovery efforts.

Earlier a new tetrachloride meso-tetra (4-N-oxiethylpyridyl) porphyrin (TOEtPyP) and its metal derivatives were synthesized (Ghazaryan *et al.* 2006a). The toxicity of two derivatives, TOEtPyP Ag and TOEtPyP Zn, was tested *in vitro*. These metalloporphyrins were demonstrated to meet at least two important demands to potential anticancer agents as they combine high cytotoxicity with low genotoxicity (Gasparyan *et al.* 2007). At the same time these compounds' influence on the cell population kinetics and mode of induced cell death (apoptosis or necrosis) were not investigated.

In the present work the effect of TOEtPyP Ag on the cell proliferation and death rate was studied in details *in vitro* using morphological and biochemical techniques.

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MATERIALS AND METHODS

1. The porphyrin tested

The compound studied was tetranitrate meso-tetra (4-N-oxiethylpyridyl) porphyrinato Ag (II) (TOEtPyP Ag), a water-soluble metal derivative of TOEtPyP synthesized earlier by alkylation of meso-tetra (4-N-pyridyl) porphyrine at the excess of ethylenchlorhydrine in dimethylformamide. TOEtPyP Ag was synthesized by treatment of TOEtPyP by silver nitrate in dimethylformamide (Ghazaryan *et al.* 2006b). Its molecular mass is 1,152 Da.

2. Cell line and cell culture

The cell line KCL22 (suspension cell line of human chronic myeloid leukemia in blast crisis) was used. It was kindly provided by Dr. T. Liehr (Institute of Human Genetics and Anthropology, Germany). Cells were routinely maintained in the growth medium RPMI-1640 supplemented with 10% fetal bovine serum (Sigma-Aldrich and Biochrom AG, Germany) and 50 $\mu\text{g mL}^{-1}$ gentamycin (Belmedpreparaty, Belarus) at 37°C. Cells were seeded into 15 mL glass vials at the concentration $0.5 \times 10^6 \text{ cell mL}^{-1}$ (2 mL of cell suspension per vial) and incubated for 24 h. TOEtPyP Ag solved in water was added to the cell cultures at the final concentration 20 $\mu\text{M mL}^{-1}$ (this concentration was earlier determined to be equal to IC50 for an other suspension cell line of human chronic myeloid leukemia, K562, see Gasparyan *et al.* 2007. IC50 is a dose inducing 50% inhibition of cell viability). Cells were collected with 3 h-time intervals during 24 h and the cell viability, morphological changes, and DNA fragmentation were studied.

3. Estimation of cell viability

The viable cell number was counted by the vital dye (trypan blue, Sigma, USA) exclusion test. Cell viability was expressed as a percentage of the controls.

Here and below at least quadruplicate cultures were scored. The Student's one-tail t-test was applied for the statistical treatment of the results that were expressed as the mean \pm SE. *p* values less than 0.05 were regarded as denoting the statistical significance.

4. Cell morphology investigation

1) Acridine orange (AO) staining

Cells were stained with 0.5 μM AO solution (Serva, Germany) in PBS for 30 min, washed twice in PBS, layered on coverslips, air dried in the dark and observed using fluorescent microscope with green light excitation (540 nm) and long pass $> 640 \text{ nm}$ barrier filter setup. Cells with nuclei colored green and red cytoplasm and nucleoli were considered to be viable. Dead cells were identified by irregular shape, red nuclei or the presence of apoptotic bodies (Darzynkiewicz *et al.* 1990; Foglieni *et al.* 2001; In Vitro Diagnostic Medical Device 1.15931 2004).

2) Giemsa staining

Cells were fixed by ethanol: acetic acid (3 : 1, v : v), layered on coverslips and air dried. Then the cells were stained with Giemsa (Sigma-Aldrich or Biochrom AG, Germany) for 20 min, rinsed in distilled water and air dried. Cells of round shape having clearly visible nuclei, structured chromatin and nucleoli were surely identified as living ones. On the contrary, cells of irregular shape with homogenous staining of chromatin and nucleus were referred as dead ones (Hayhome 1981).

The apoptotic cell death and cell growth rate were estimated both in AO and Giemsa stained cell cultures by counting of the number of cells with apoptotic bodies and mitotic cells, respectively.

5. DNA gel-phoresis

4, 9, 15, 21, and 24 h after adding of TOEtPyP Ag the cells were washed in ice-cold PBS and lysed in a buffer containing 10 mM Tris-HCl (pH 7.5), 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 100 $\mu\text{g mL}^{-1}$ proteinase K (Roche, Germany), 20 $\mu\text{g mL}^{-1}$ Ribonuclease A (Sigma, Germany) (the final concentrations) and incubated for 16 h at 50°C. DNA after extraction with an equal volume of chloroform: isoamylalcohol (24 : 1, v : v) was precipitated by adding of the mixture of 7.5 M sodium acetate and 100% ethanol, pH 5.3 (0.1 of the total volume). Precipitated DNA was collected by centrifugation at 13,000 rpm for 20 min at 4°C, washed with ice-cold 70% ethanol and air dried. DNA was then resuspended in TE buffer, pH 8.0. DNA samples were fractionated by electrophoresis in 0.6% agarose gel buffered with TBE buffer, pH 8.0, and visualized by ethidium

bromide staining using an UV transilluminator as the sole light source. The electrophoretic patterns were analyzed to reveal the existence of so called ladder distribution of DNA fragments suggesting the apoptotic cell death (Shin *et al.* 2002). The size of DNA fragments was estimated by comparing it with a standard High-Throughput I DNA ladder contained 5 DNA bands: 2,000; 1,000; 500; 250; 100 bp (Bioline, Germany).

RESULTS

1. Cell viability (by the vital dye exclusion test)

The results of the determination of the KCL22 cell line sensitivity to TOEtPyP Ag are presented in Fig. 1. The number of living cells was shown to monotonously decrease after 9 h-latent period. The TOEtPyP Ag concentration applied induced the death of approximately 3/4 of cell population over 24 h-incubation period.

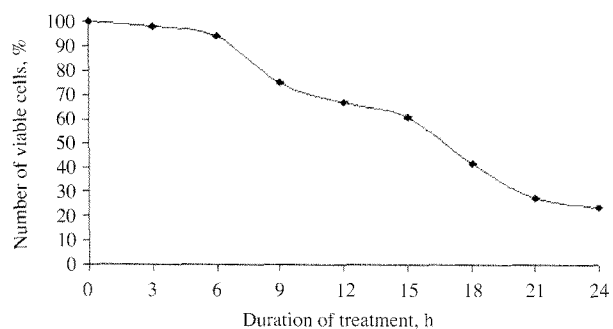


Fig. 1. Effect of TOEtPyP Ag on the viability of the KCL22 cell line (by the vital dye exclusion test).

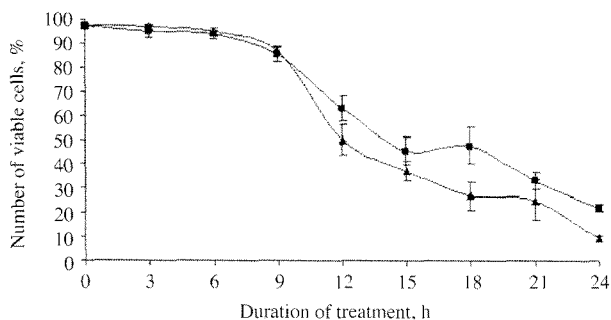


Fig. 2. Effect of TOEtPyP Ag on the viability of the KCL22 cell line (by the cell morphology, ▲-AO staining, ■-Giemsa staining).

2. Cell viability (by the cell morphology)

Results of this experiment (Fig. 2) highly agreed with the previous results (Fig. 1). The number of living cells also gradually decreased after 9 h-latent period. 78~90% of the cells dead over 24 h-incubation period.

The cell line investigated seems to be more susceptible to the cytotoxic action of TOEtPyP Ag than cell lines tested earlier (Cos-7, fibroblast-like African green monkey kidney cells transformed by SV40; DU 145, epithelial-like cells of human prostate carcinoma; K-562, human chronic myeloid leukemia cells, see Gasparyan *et al.* 2007). In fact, the porphyrin concentration applied was shown earlier to be IC50 for K562 cell line (*ibid.*); the same concentration induced the death of 75~90% KCL22 cells used in this work.

3. Rate of apoptotic cell death (by counting of the number of cells with apoptotic bodies)

The results obtained in the experiments with AO and Giemsa cell staining were similar. In both cases the number of cells with apoptotic bodies started to increase after 12 h and their maximum number (39~43% of the cell population) was observed after 21 h incubation of the culture with TOEtPyP Ag (Fig. 3). Then (24 h) the number of apoptotic cells decreased, perhaps because of so called secondary necrosis of dead cells under culture conditions (Aderem *et al.* 1999).

4. Proliferative activity of the cells (by counting of the number of mitotic cell)

TOEtPyP Ag was shown to inhibit the cell proliferation in KCL22 cell line (Fig. 4). The number of mitotic cells

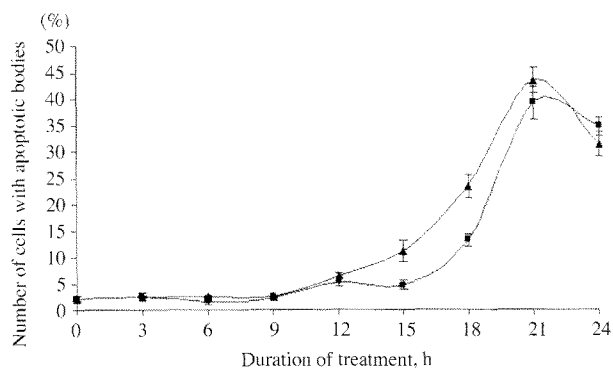


Fig. 3. Effect of TOEtPyP Ag on the apoptotic death rate of the KCL22 cell line (▲-AO staining, ■-Giemsa staining).

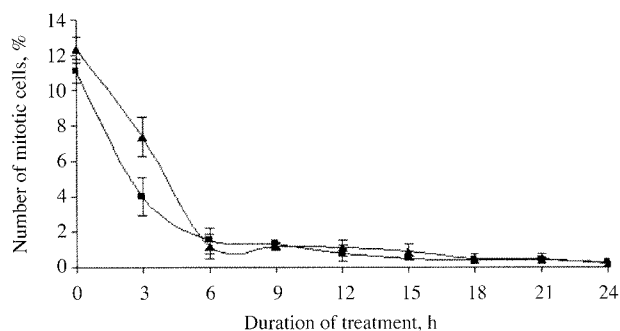


Fig. 4. Effect of TOETPyP Ag on the mitotic division rate of the KCL22 cell line (\blacktriangle -AO staining, \blacksquare -Giemsa staining).

dropped abruptly from 11~12% to 7.4~4.0% (AO and Giemsa staining, respectively) after 3 h of the cell treatment and to 1.2~1.5% after 6 h of incubation. At the end of observations (24 h) the number of dividing cells was as low as 0.2~0.3%.

5. DNA fragmentation

The apoptotic response, as judged by the appearance of a DNA ladder, was examined by the gel electrophoresis in order to determine the apoptotic effect of TOETPyP Ag. Short-term (4 h) treatment of cells with the agent seemed not to induce any DNA fragmentation (Fig. 5). The characteristic nucleosomal DNA fragmentation pattern, which is the biochemical hallmark of apoptosis, was first appeared 9 h after exposure of the cell culture to TOETPyP Ag and remained till 15 h. Later (21 and 24 h) it fully disappeared. Beginning from 9 h and till the end of observations (24 h) the smeared DNA fluorescence was also visible. The described dynamics of DNA fragmentation suggested that the apoptotic processes were induced only in a part of the cells and were followed by secondary necrotic destruction of the cells affected. These results are in agreement with results of morphological study presented above.

DISCUSSION

Now a wide-range efforts are directed to new porphyrins and metalloporphyrins discovery to control malignant cells (Luo *et al.* 1996; Szurko *et al.* 2003; Ogura *et al.* 2005; Al-Mutairi *et al.* 2006), harmful microorganisms (Tome *et al.* 2004; Lambrechts *et al.* 2005; Jori 2006), human immunodeficiency virus (Vzorov *et al.* 2002), etc. We have been

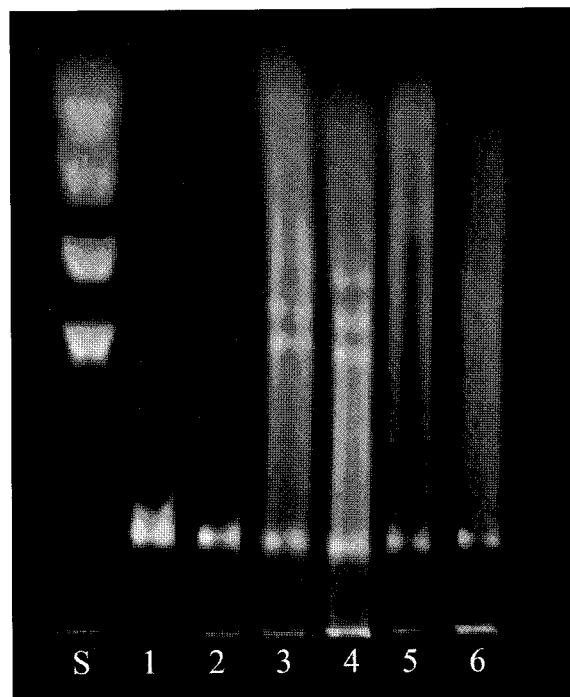


Fig. 5. Effect of TOETPyP Ag on the DNA fragmentation of the KCL22 cell line. Duration of the cell incubation with TOETPyP Ag: 1-0 h; 2-4 h; 3-9 h; 4-15 h; 5-21 h; 6-24 h.

synthesizing a variety of meso-substituted porphyrins and metalloporphyrins (Ghazaryan *et al.* 2006c). Some of them were demonstrated to possess not only above-mentioned but also catalytic (Martirosyan *et al.* 2006), bactericide, fungicide (Ghazaryan *et al.* 2006b), and plant growth stimulating (Ghazaryan *et al.* 2006a) activities.

TOETPyP Ag, a novel porphyrin metal derivative, was earlier demonstrated to have high cytotoxicity and low genotoxicity for cells *in vitro* (Gasparyan *et al.* 2007). This work aimed to study in details the effects of TOETPyP Ag on the cell population kinetics. The cell line KCL22 highly susceptible to the toxic action of TOETPyP Ag was used. About 75~90% of cells died during 24 h of treatment with the agent. The results of our observations suggested that TOETPyP Ag action consisted in the simultaneous suppression of the cell growth and activation of the cell death. About 40% of the cells were shown to die via apoptotic pathway. So, the porphyrin studied may be attributed to inducers of both necrotic and apoptotic processes. Taken together, our previous and presented here results supplement each other and elucidate the mechanism of TOETPyP Ag cytotoxic effect. The ability of TOETPyP Ag to kill the cells via apoptotic way seems to be important for its appli-

cation as a medicine. It will allow weakening or avoiding the inflammatory tissue reactions induced by necrotic cells.

The results of this investigation support our previous assertion (*ibid.*) that TOETPyP Ag may be considered as a potential chemotherapeutic agent. It seems to be reasonable to undertake a preclinical study of this compound both *in vitro* and *in vivo*.

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REFERENCES

- Aderem A and DM Underhill. 1999. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* 17:593-623.
- Al-Mutairi DA, JD Craik, I Batinic-Haberle and LT Benov. 2006. Photosensitizing action of isomeric Zn N-methylpyridyl porphyrins in human carcinoma cells. *Free Radical Res.* 40(5):477-483.
- Berg K, PK Selbo, A Weyergang, A Dietze, L Prasmickaite, A Bonsted, BO Engesaeter, E Angell-Petersen, T Warloe, N Frandsen and A Hogset. 2005. Porphyrin-related photosensitizers for cancer imaging and therapeutic applications. *J. Microsc.* 218(2):133-147.
- Cohen GM, X Sun, RT Snowden, D Dinsdale and DN Skilletter. 1992. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochemical J.* 286:331-334.
- Darzynkiewicz Z. 1990. Differential staining of DNA and RNA in intact cells and isolated cell nuclei with acridine orange. *Methods Cell Biol.* 33:285-98.
- Ding L, G Etemad-Moghadam and B Meunier. 1990. Oxidative cleavage of DNA mediated by hybrid metalloporphyrin-ellipticine molecules and functionalized metalloporphyrin precursors. *Biochemistry.* 29:7868-7875.
- Ding L, J Balzarini, D Schols, B Meunier and E De Clercq. 1992. Anti-human immunodeficiency virus effects of cationic metalloporphyrin-ellipticine complexes. *Biochem. Pharmacol.* 44:1675-1679.
- Dougherty TJ, CJ Gomer, BW Henderson, G Jori, D Kessel, M Korbek, J Moan and Q Peng. 1998. Photodynamic therapy. *J. Natl. Cancer Inst.* 90:889-905.
- Foglieni C, C Meoni and AM Davalli. 2001. Fluorescent dyes for cell viability: an application on prefixed conditions. *Histochem.Cell Biol.* 115:223-229.
- Gasparyan G, G Hovhannisyanyan, R Ghazaryan, L Sahakyan, A Tovmasyan, R Grigoryan, N Sarkissyan, K Margaryan, S Haroutiunian and R Arutyunyan. 2007. *In vitro* testing of cyto- and genotoxicity of new porphyrin water-soluble metal derivatives. *Int. J. Toxicol.* 26:479-502.
- Ghazaryan RK, LA Sahakyan and AG Tovmasyan. 2006a. Patent of the Republic of Armenia No 1716 A2.
- Ghazaryan RK, LA Sahakyan and AG Tovmasyan. 2006b. Patent of the Republic of Armenia No 1715 A2.
- Ghazaryan RK, LA Sahakyan and AG Tovmasyan. 2006c. Patent of the Republic of Armenia No 1714 A2.
- Hale AJ, CA Smith, LC Sutherland, VE Stoneman, V Longthorne, AC Culhane and GT Williams. 1996. Apoptosis: molecular regulation of cell death. *Eur. J. Biochem.* 237: 884.
- Hayhome BA. 1981. Giemsa Staining to Monitor Cell Viability in *Peridinium cinctum* fa. *Ovoplanum*. *Trans. Am. Microsc. Soc.* 100:410-414.
- In Vitro Diagnostic Medical Device. 2004. Acridine orange zinc chloride double salt (C.I.46005) Certistain.
- Jori G. 2006. Photodynamic therapy of microbial infections: state of the art and perspectives. *J. Environ. Pathol. Toxicol. Oncol.* 25:505-519.
- Kadish KM. 2000. The Porphyrin Handbook (Smith KM and Guillard R Eds.). Academic Press. San Diego.
- Lambrechts SA, MC Aalders, FD Verbraak, JW Lagerberg, JB Dankert and JJ Schuitmaker. 2005. Effect of albumin on the photodynamic inactivation of microorganisms by a cationic porphyrin. *Photochem Photobiol.* 79:51-57.
- Luo Y, CK Chang and D Kessel. 1996. Rapid Initiation of Apoptosis by Photodynamic Therapy. *Photochem. Photobiol.* 63(4):528-534.
- Martirosyan A, V Hovhannesyan, S Gasparyan, S Mamayan, R Ghazaryan and V Madakyan. 2006. Metal-complex catalysis during the reduction of functional groups by borohydride in the synthesis of pyrrolidine derivatives. *Chem. Heterocyclic Compounds.* 42(2):180-183.
- Ogura Sh, T Kamachi and I Okura. 2005. Cellular localization and photodynamic effect of chlorin e6-monoclonal antibody conjugate. *J. Porphyrins Phthalocyanines.* 9:138-141.
- Ohse T, S Nagaoka, Y Arakawa, H Kawakami and K Nakamura. 2001. Cell death by reactive oxygen species generated from water-soluble cationic metalloporphyrins as superoxide dismutase mimics. *J. Inorg. Biochem.* 85(2):201-208(8).
- Shin MJ, HJ Kim, SJ Yang. 2002. Arsenic trioxide induces apoptosis in chronic myelogenous leukemia K562 cells:

- possible involvement of p38 MAP kinase. *J. Biochem. Mol. Biol.* 35:377-383.
- Stojiljkovic I, BD Evavold and V Kumar. 2001. Antimicrobial properties of porphyrins. *Expert Opin Investig Drugs.* 10:309-320.
- Szurko A, G Kramer-Marek, M Widel, A Ratuszna, J Habdas and P Kus. 2003. Photodynamic effects of two water soluble porphyrins evaluated on human malignant melanoma cells in vitro. *Acta Biochim. Pol.* 50(4):1165-1174.
- Tome JPC, MGPMS Neves, AC Tome, JAS Cavaleiro, M Soncin, M Magaraggi, S Ferro and G Jori. 2004. Synthesis and antibacterial activity of new poly-S-lysine-Porphyrin conjugates. *J. Med. Chem.* 47:6649-6652.
- Vicente MGH. 2001. Porphyrin-based sensitizers in the detection and treatment of cancer: recent progress. *Curr. Med. Chem. Anticancer Agents.* 1:175-194.
- Vzorov AN, DW Dixon, JS Trommel, LG Marzilli and RW Compans. 2002. Inactivation of human immunodeficiency virus type 1 by porphyrins. *Antimicrob Agents Ch.* 46: 3917-3925.
- Wyllie AH, JFR Kerr, AR Currie. 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68:251-306.

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