In Vitro Testing of Cyto- and Genotoxicity of New Porphyrin Water-Soluble Metal Derivatives

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Porphyrins and porphyrin derivatives have an outstanding potential for discovery of novel pharmacological agents due to their ability for numerous chemical modifications and a variety of mechanisms of biological effects. New water-soluble Ag and Zn derivatives of tetrachloride meso-tetra (4-N-oxiethylpyridyl) porphyne were synthesized. Cyto- and genotoxicity of these substances were tested in vitro by the vital dye (trypan blue) exclusion and the micronucleus tests, respectively. Both metalloporphyrins were shown to be cytotoxic for Cos-7 (fibroblast-like African green monkey kidney cells transformed by simian virus 40 [SV40]), DU 145 (epithelial-like cells of human prostate carcinoma), and K-562 (human chronic myeloid leukemia cells) cell lines. At the same time they did not cause chromosome fragmentation in K-562 cell line at as high concentrations as IC50 (20 µmol/L for Ag and 70 µmol/L for Zn derivative). Thus, the metalloporphyrins tested meet at least two important demands to potential anticancer drugs as they combine the cytotoxicity with low genotoxicity. The three in vitro tumor models used are relevant to further in vitro and in vivo preclinical investigation of the studied metalloporphyrins as potential chemotherapeutics.

Keywords  
Cytotoxicity, Genotoxicity, In Vitro Testing, Porphyrin Metal Derivatives

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Porphyrins are organic pigments of both natural and synthetic origin, containing the porphyn ring as a base of their structure. The ability for numerous chemical modifications and a variety of mechanisms of biological effects place porphyrins and porphyrin derivatives into a group of compounds with an outstanding potential for discovery of novel pharmacological agents.

Metalloporphyrins were earlier shown to express antimicrobial (Stojiljkovic and Churchward 2000; Stojiljkovic et al. 2001; Lambrechts et al. 2005; Jori 2006) and antiviral including anti-HIV (human immunodeficiency virus) (Vzorov et al. 2002) activities. Many of them are photosensitizers and this property provides the basis for their use in the photodynamic therapy of tumors (Dougherty et al. 1998; Berg et al. 2005). A water-soluble derivative of Zn-protoporphyrin was revealed to have an antitumor effect in vivo significantly reducing tumor growth in a rat model. In vitro this porphyrin induced oxidative stress, and consequently apoptotic death of cultured human cancer cell lines (Yang et al. 2001; Sahoo et al. 2002; Fang et al. 2003).

Earlier we have synthesized a new tetrachloride meso-tetra (4-N-oxiethylpyridyl) porphyn (TOEtPyP) and developed various metal derivatives of TOEtPyP. Some of them were shown to have antifungal activity (Ghazaryan, Sahakyan, and Tovmasyan 2006a). Interaction of metalloporphyrins synthesized with DNA in vitro was analyzed and their reversible binding with purified DNA was demonstrated (Dalyan et al. 2001). The dependence of the genotoxicity of porphyrins’ Mn derivatives on their chemical structure was shown (Hovhannisyan et al. 2005).

In the present work the cyto- and genotoxicity of Ag and Zn derivatives of TOEtPyP as potential chemotherapeutics were tested in vitro. Only dark toxicity of these metalloporphyrins
was studied as it was shown that these compounds are weak photosensitizers (unpublished data of Dr. G. Gyulkhandanyan, 2006).

MATERIALS AND METHODS

The Porphyrins Tested

The compounds studied were tetranitrate meso-tetra (4-N-oxyethylpyridyl) porphynato Ag (II) (TOEtPyP Ag) and tetrachloride meso-tetra (4-N-oxyethylpyridyl) porphynato Zn (II) (TOEtPyP Zn). Chemical structures of these compounds are shown in Figure 1. Both agents are water-soluble metal derivatives of TOEt-PyP synthesized by alkylation of meso-tetra (4-N-pyridyl) porphyn at the excess of ethylenchlorhydrine in dimethylformamide (Ghazaryan, Sahakyan, and Tovmasyan 2006b). TOEtPyP Ag and TOEtPyP Zn were obtained by treatment of TOEtPyP by silver nitrate in dimethylformamide and zinc chloride in ethanol, respectively (Ghazaryan, Sahakyan, and Tovmasyan 2006c). The molecular mass of TOEtPyP Ag and TOEtPyP Zn is 1152 and 1003 Da, respectively.

Measurement of Spectral Properties of Porphyrins

The structure and purity of the compounds synthesized were determined by the techniques of NMR and electron absorption spectroscopy. NMR spectra were recorded on a Mercury-300 Varian NMR spectrometer (solvent: deuterated dimethyl sulfoxide). The electron absorption spectra were recorded on a Perkin-Elmer Lambda 800 UV/Vis spectrometer in the wavelength range 350 to 800 nm (solvent: distilled water). The absorption coefficients of the bands were calculated by the Beer-Lambert law using $10^{-4}$ to $10^{-6}$ mol/L porphyn solutions.

Cell Lines and Cell Culture

The cell lines used were Cos-7 (fibroblast-like African green monkey kidney cells transformed by simian virus [SV40]), DU 145 (epithelial-like human prostate carcinoma cell line; generously provided by Prof. J. Masters, Institute of Urology and Nephrology, University College London, UK), and K-562 (suspension cell line of human chronic myeloid leukemia in blast crisis; kindly provided by Dr. T. Liehr, Institute of Human Genetics and Anthropology, Germany).

Cells were routinely maintained in the growth media DMEM (Dulbecco’s modified Eagle’s medium; cell line Cos-7) or RPMI-1640 (cell lines DU 145 and K-562) supplemented with 10% fetal bovine serum (media and serum were purchased from Sigma-Aldrich and Biochrom AG, Germany) and 50 $\mu$g/ml gentamycin (Belmedpreparat, Belarus) at 37°C.

Estimation of Cell Damage

To determine the basal cytotoxicity of metalloporphyrins the cells were seeded into 15-ml glass vials (1 to 2 ml of cell suspension per vial), incubated for 24 h, and then the test compounds to be dissolved in the growth medium were added. After further 24 h of incubation, the viable cell number was counted by the vital dye (trypan blue; Sigma, USA) exclusion test. Attached cells (cell lines Cos-7 and DU 145) were previously suspended with trypsin-EDTA (Sigma-Aldrich). Cell viability was expressed as a percentage of intact controls. Doses inducing 50% inhibition of cell viability (i.e., the IC50 value) during the 24-h incubation period were determined for both metalloporphyrins in all the cell lines investigated. At least quadruplicate cultures were scored for an experimental point.

To predict starting doses for in vivo acute LD50 values (single dose, oral administration) in rodents (rats) the following prediction model (Liebsch and Spielmann 1995) was applied:

$$\log(\text{LD}_{50}[\text{mmol/kg}]) = 0.435 \times \log(\text{IC}_{50}[\text{mmol/L}]) + 0.625.$$
dissolved in RPMI-1640 medium. Four hours later cytochalasin B (Sigma-Aldrich) dissolved in ethanol was added (not more than 10 µl ethanol per 1 ml of the medium so as not to affect the cell viability and growth) to the final concentration 3 µg/ml. Cell cultures incubated with cytochalasin B without metalloporphyrins were used as negative controls. After 20 h incubation the cells were fixed with ethanol:acetic acid (3:1), spread on slides, air dried, and stained with Giemsa (Sigma-Aldrich). One thousand cells per triplicate or quadruplicate cell cultures were scored to assess the frequency of cells with one, two, or more nuclei. The cytokinesis-block proliferation index (CBPI) as a measure of cell cycle delay was expressed as

\[ \text{CBPI} = \frac{\text{number of binucleate cells} + 2\times \text{number of multinucleate cells}}{\text{total number of cells}}. \]

The number of binucleate cells with MN was counted in 1000 binucleate cells in the same cultures. Only micronuclei not exceeding 1/3 of the main nucleus diameter, not overlapping with the main nucleus, and with distinct borders were included in the scoring (Heddle 1973). The results were statistically treated with the Student’s one-tail t test.

**TABLE 1**

<table>
<thead>
<tr>
<th>Name</th>
<th>Solvent</th>
<th>NH</th>
<th>β</th>
<th>m-Py</th>
<th>CH₂N⁺</th>
<th>CH₂</th>
<th>OH</th>
<th>C₁³</th>
</tr>
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<tbody>
<tr>
<td>TOEtPyP</td>
<td>DMSO</td>
<td>−3.06</td>
<td>9.28</td>
<td>9.03</td>
<td>9.51</td>
<td>5.04</td>
<td>4.21</td>
<td>5.85</td>
</tr>
<tr>
<td>TOEtPyP Zn</td>
<td>DMSO</td>
<td>—</td>
<td>9.09</td>
<td>8.94</td>
<td>9.46</td>
<td>5.04</td>
<td>4.22</td>
<td>5.71</td>
</tr>
<tr>
<td>R</td>
<td>CH₂CH₂OH</td>
<td>CH₂CH₂OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>Cl⁻</td>
<td>Cl⁻</td>
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</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Soret</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOEtPyP</td>
<td>642(2.3) 585 (6.1) 558 (6.7) 519 (13.8) 424 (209)</td>
</tr>
<tr>
<td>TOEtPyP Ag</td>
<td>545(8.3)</td>
</tr>
<tr>
<td>TOEtPyP Zn</td>
<td>561 (14.5) 428 (215)</td>
</tr>
</tbody>
</table>

**RESULTS**

**Spectral Characteristics of Metalloporphyrins**

The NMR results registered (Table 1) were in a good agreement with the proposed structure of the metalloporphyrins tested. Their absorption spectra (Table 2) exhibited the typical Soret and Q bands specific for free-base porphyrins and their metal complexes.

**Cytotoxicity of Metalloporphyrins**

Both TOEtPyP Ag and TOEtPyP Zn were demonstrated to cause a dose-dependent cell survival reduction in the cell lines studied (Figure 2). The sensitivity of all the cell lines to both metalloporphyrins was nearly the same (IC₅₀ = 70–90 μmol/L; Table 3) with one exception: the cell line K-562 was 3.5 to 4.5 times more sensitive to TOEtPyP Ag (IC₅₀ = 20 μmol/L) than the others. This difference possibly suggests the cell line-specificity of toxic action of this porphyrin.

The extrapolation of IC₅₀ to LD₅₀ values (Table 3) suggested that TOEtPyP Ag and TOEtPyP Zn belong to the 4th toxicity class (harmful if swallowing; LD₅₀ from 300 to 2000 mg/kg) (GSH Rev.1 2005).

**Cell Cycle Effects**

Determination of the cell proliferation rate (as CBPI; see Materials and Methods) showed (Table 4) that TOEtPyP Ag at doses IC₅₀ and IC₅₀/2 caused a dose-dependent cell cycle delay in K-562 cell line (63.6% and 75% of the negative control, respectively). At the same time the difference with positive control was not significant. Thus, the cell cycle suppressive effect of TOEtPyP Ag may be estimated as weak and comparable with those of

**TABLE 3**

<table>
<thead>
<tr>
<th>Cell</th>
<th>IC₅₀ of TOEtPyP Ag</th>
<th>LD₅₀ of TOEtPyP Ag</th>
<th>IC₅₀ of TOEtPyP Zn</th>
<th>LD₅₀ of TOEtPyP Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cos-7</td>
<td>0.07</td>
<td>1.326</td>
<td>0.07</td>
<td>1.326</td>
</tr>
<tr>
<td>DU 145</td>
<td>0.09</td>
<td>1.479</td>
<td>0.07</td>
<td>1.326</td>
</tr>
<tr>
<td>K-562</td>
<td>0.02</td>
<td>0.769</td>
<td>0.07</td>
<td>1.326</td>
</tr>
</tbody>
</table>

Note. TOEtPyP Ag is a paramagnetic compound and its NMR spectrum cannot be interpreted.
FIGURE 2
The cytotoxicity of TOEtPyP Ag (a–c) and TOEtPyP Zn (d–f) in Cos-7 (a, d), DU 145 (b, e), and K-562 (c, f) cell lines.

the known chemotherapeutic cyclophosphamide. TOEtPyP Zn was shown not to change the rate of the cell cycle progression in K-562 cells.

Chromosome Damages
Both metalloporphyrins studied at concentrations from IC_{50} to IC_{50}/5 were demonstrated not to induce MN formation. The MN number in treated cultures did not exceed the control values (Table 5).

DISCUSSION
Porphyrin derivatives have been earlier demonstrated to exhibit a marked cytotoxic anticancer activity both in vitro and in vivo (Ohse et al. 2001; Grand et al. 2002; Asayama et al. 2007; Wang et al. 2007).

In the present work it was shown that newly synthesized porphyrin metal derivatives, TOEtPyP Ag and TOEtPyP Zn, were cytotoxic for various cell lines in vitro (IC_{50} values were from 20 to 90 \mu mol/L). Converting of IC_{50} values obtained in our in vitro experiments into in vivo LD_{50} values (see Table 3) demonstrated that these compounds might be classified as harmful (GSH Rev.1, 2005). All the cell lines used, in spite of their various origins (different species: monkey and human; different tissues: kidney, prostate, and blood), were almost similarly sensitive to both metalloporphyrins. A sole exception was higher sensitivity of human myeloid leukemia cell line K-562 to TOEtPyP Ag. As tumor cell lines have been shown to retain many of genotypic and phenotypic properties of their corresponding tumors (Wistuba et al. 1998, 1999; Brown and Botstein 1999; Masters 2000) and tissue genotypic features (Perou et al. 1999; Ross et al. 2000), it is reasonable to propose that the TOEtPyP Ag toxicity, in contrast to that of TOEtPyP Zn, is cancer type (and, maybe, tissue) specific.

Being cytotoxic, the metalloporphyrins studied only slightly delayed the cell progression through the cell cycle (TOEtPyP Ag) or had no effect on the cell growth rate (TOEtPyP Zn). At the same time both TOEtPyP Ag and TOEtPyP Zn were demonstrated to not generate significant levels of MN in mammalian cells, i.e., they had no propensity to induce chromosome breakage and formation of chromosome fragments (Curry et al. 1966) and seem not to present a potential genotoxic hazard.
These known features of porphyrin pharmacokinetics for long period of time (El-Far and Pimstone 1984; Vicente 2001). Low levels of genotoxicity and cell cycle side effects are also very desirable features of chemotherapeutics in order to meet at least two important criteria of potential anticancer drugs. So they suggest that their application as chemotherapeutics can enhance local tumor control and minimize drug toxic load on normal tissues. These features are believed to be applicable to the metalloporphyrins tested in this work.

Further research in vitro and in vivo is needed to reveal either these compounds meet other requirements as potential anticancer drugs (e.g., low level of tissue- and organ-specific toxicity and adverse effects, etc.). The three in vitro tumor models used (monkey SV40-transformed kidney cells, human prostate carcinoma, and human leukemia) are relevant to these further preclinical investigations of the metalloporphyrins studied.

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>Effect of metalloporphyrins on the cell proliferation rate in K-562 cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>Versus the negative control</td>
</tr>
<tr>
<td>Negative control</td>
<td>22.0 ± .86</td>
</tr>
<tr>
<td>Positive control</td>
<td>15.35 ± 2.72</td>
</tr>
<tr>
<td>TOEtPyP Ag, IC50</td>
<td>14.0 ± .97</td>
</tr>
<tr>
<td>TOEtPyP Ag, IC50/2</td>
<td>16.5 ± 1.65</td>
</tr>
<tr>
<td>TOEtPyP Ag, IC50/5</td>
<td>19.6 ± 1.17</td>
</tr>
<tr>
<td>Negative control</td>
<td>16.5 ± .52</td>
</tr>
<tr>
<td>Positive control</td>
<td>13.6 ± 1.22</td>
</tr>
<tr>
<td>TOEtPyP Zn, IC50</td>
<td>16.5 ± 3.57</td>
</tr>
<tr>
<td>TOEtPyP Zn, IC50/2</td>
<td>15.3 ± .87</td>
</tr>
<tr>
<td>TOEtPyP Zn, IC50/5</td>
<td>14.9 ± .82</td>
</tr>
</tbody>
</table>

Note. Averaged results of three experiments

It is a common idea that the cytotoxicity of a drug applied in cancer therapy may be of decisive importance (Colombo et al. 2001). Low levels of genotoxicity and cell cycle side effects are also very desirable features of chemotherapeutics in order to not provoke secondary mutations and dangerous chromosome damage in the process of therapy (Vogel et al. 1991).

The porphyrin metal derivatives tested were demonstrated to possess cytotoxicity together with low genotoxicity. So they meet at least two important criteria of potential anticancer drugs. Moreover, porphyrins and related compounds have been shown to effectively accumulate within tumor cells and to be retained for long period of time (El-Far and Pimstone 1984; Vicente 2001). These known features of porphyrin pharmacokinetics

<table>
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<tr>
<th>TABLE 5</th>
<th>Effect of metalloporphyrins on the MN frequency in K-562 cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>MN number (%)</td>
</tr>
<tr>
<td>Negative control</td>
<td>1.3 ± 0.26</td>
</tr>
<tr>
<td>Positive control</td>
<td>4.7 ± 0.58</td>
</tr>
<tr>
<td>TOEtPyP Ag, IC50</td>
<td>0.7 ± 0.19</td>
</tr>
<tr>
<td>TOEtPyP Ag, IC50/2</td>
<td>1.9 ± 0.38</td>
</tr>
<tr>
<td>TOEtPyP Ag, IC50/5</td>
<td>1.1 ± 0.44</td>
</tr>
<tr>
<td>Negative control</td>
<td>1.9 ± 0.44</td>
</tr>
<tr>
<td>Positive control</td>
<td>3.2 ± 0.20</td>
</tr>
<tr>
<td>TOEtPyP Zn, IC50</td>
<td>1.4 ± 0.36</td>
</tr>
<tr>
<td>TOEtPyP Zn, IC50/2</td>
<td>0.6 ± 0.31</td>
</tr>
<tr>
<td>TOEtPyP Zn, IC50/5</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>


