Lead Structures for Applications in Photodynamic Therapy. 5. Synthesis and Biological Evaluation of Water Soluble Phosphorus(V) 5,10,15,20-Tetraalkylporphyrins for PDT

Aoife A. Ryan a, Mothi M. Ebrahim a, Rosine Petidemange a, Gisela M. Vaz b, Edyta Paszko b, Natalia N. Sergeeva b, Mathias O. Senge Dr. rer. nat., FTCD a,b,*

a School of Chemistry, SFI Tetrapyrrole Laboratory, Trinity Biomedical Sciences Institute, 152-160 Pearse Street, Trinity College Dublin, The University of Dublin, Dublin 2, Ireland
b Medicinal Chemistry, Institute of Molecular Medicine, Trinity Centre for Health Sciences, Trinity College Dublin, St James’s Hospital, Dublin 8, Ireland

* Corresponding author at: School of Chemistry, SFI Tetrapyrrole Laboratory, Trinity College Dublin, Dublin 2, Ireland
E-mail address: sengem@tcd.ie (M.O. Senge).

KEYWORDS
Photodynamic therapy; Photosensitizers; Phosphorous; Porphyrins; Cancer; Esophageal cancer

Summary
Improved photosensitizers for use in photomedicine must possess good water-solubility and optimal photophysical properties. Phosphorus(V) porphyrins fulfill these criteria and are a class of porphyrins with significant potential applications in phototherapy. Five phosphorus(V) porphyrins bearing alkyl substituents have been synthesized. Reasonable to good yields were obtained for all P(V) insertions and all compounds underwent biological evaluation for their PDT activity on two esophageal cancer cell lines, OE33 and SKGT-4. Their cellular uptake was investigated using a high content screening method. Notably, three compounds displayed good uptake and using the MTS cell proliferation assay, two were shown to have photocytotoxicity comparable to mTHPC (Temoporfin®) with IC50 values of 6.5 and 5.5 μM.
Introduction

Photodynamic therapy (PDT) is the most promising clinical application of porphyrins for cancer therapy and indication [1,2,3]. The method relies on the selective accumulation of a photosensitizer in target tissue where it can be activated with light to produce toxic singlet oxygen resulting in, e.g., tumor necrosis [4,5]. However, present PDT drugs have significant draw backs such as slow tissue clearance, low solubility, inefficient targeting, patient photosensitivity, pain, and high costs [6]. Thus, current research targets new photosensitizers with optimized photophysical and pharmacological properties [7]. This often requires significant chemical manipulation [6] (more synthetic steps) or complex formulation strategies [9,10] (liposomes, nanomaterials) whereby the biocompatibility is somewhat unclear. Current bioconjugate research developments [11,12,13,14] (antibodies, sugars, steroids, etc.) involve expensive and complicated methodology, whilst state-of-the-art drugs such as Temoporfin are undergoing further developments [15,16], mostly in terms of nanoformulations to increase their efficacy and application potential or in terms of new uses, e.g., antimicrobial action [17]. Thus, PDT development is becoming ever more specialized.

More practical advances in this area require simple approaches that can be applied to a wide variety of cases and applications. Here, we present the use of P(V) porphyrin complexes which offer such a possibility, as they allow not only the preparation of new drug candidates but simple improvements of existing drugs through P(V) complexation, i.e., water solubility [18], better uptake and potentially less side effects. P(V) porphyrin complexes are known but their exploitation in terms of PDT has been limited thus far [18,19,20,21,22,23,24,25]. We found that through P(V) complexation of simple meso tetrasubstituted porphyrins both the water solubility and the in vitro cytotoxicity of the photosensitizer can be drastically improved with respect to the non-complexed form.

Materials and Methods

General procedure for cell cultures and cell proliferation assay (MTS)

Two different cell lines were used in the biological studies: the human adenocarcinoma cell line SKGT-4, and the human adenocarcinoma cell line OE33 derived from Barrett’s esophagus. The cells were routinely cultured antibiotic free in RPMI 1640 (Hyclone, USA) with 10% inactivated fetal bovine serum (Hyclone, USA) and 1 % Penicillin/Streptomycin (Hyclone, USA). These cultures were grown in sterile filtered top cell culture flasks (Nunc, Denmark). Cultures were split 1:8 when 70-80 % confluency was reached. Cell recovery was achieved using Trypsin 0.25 % with 0.1 % EDTA (Hyclone, USA) and kept at 37 °C and 5 % CO₂ in a humidified atmosphere. Culture medium was changed every 3 to 4 days until confluency was obtained. Cell lines were seeded at a concentration of 1.5×10⁶ cells/mL into sterile 96-well plates, left to attach overnight in an incubator (37 °C, 5 % CO₂) and treated. To previously prepared 96-well assay plates containing cells in 100 μL of culture medium, test
compounds, including mTHPC (Temoporfin®), at different concentrations (10-50 μM) and appropriate controls were added (untreated cells were used as control). After incubation for 24 h the medium was removed and changed for fresh one, dark controls were left in the dark for next 24 h. To assess the phototoxicity [26], the rest of the plates were illuminated for 2 min using Luxeon High Power LEDs (LXHL-BW03) as light sources emitting white light with a total fluence rate of 1.7 mW cm\(^2\) and incubated for 24 h. 20 μL of the MTS dye solution was added to each well of the dark controls and illuminated plates and these were incubated for 3 h and the absorbance was recorded at 470 nm using a 96-well plate reader and imaging technique (InCell analyzer 1000 instrument, GE Healthcare).

**Biological Evaluation**

Intracellular screening for the compounds 3a-3e was carried out in OE33 and SKGT-4 cell lines. Stock solutions of the P(V) porphyrins (0.5 mM) were prepared in ethanol:propylene glycol (60:40 v/v). Cell lines were seeded at a concentration of 3000 cells per mL into sterile 96-well plates leaving them for 24 h to attach. For imaging experiments, the cell culture medium was removed, replaced with freshly prepared solutions of the porphyrins 3a-3e of various concentrations (10–50 μM) in the medium and incubated at 37 °C under 5% CO\(_2\) for 24 h. After that the medium was removed and fixed with 4% PFA in medium and then washed with PBS. The fixed adenocarcinoma cells were then co-stained using nuclear dye Hoechst 33342 (1 μg/mL) and the bicyclic peptide DY-490 Phalloidin (1:500 in 100 μL) as cytoskeleton stain (F-actin). Fluorescent images were collected and analyzed by high content screening using InCell 1000 and in vitro images were taken at different concentrations. These images were collected using three independent channels for Hoechst, Phalloidin and the compounds 3a-3e with excitation/emission filters of 345/435 nm (blue), 475/535 nm (green) and 620/700 nm (red), respectively.

**Chemical Synthesis**

*General procedure for the synthesis of compounds 3a-e: A dry 50 mL Schlenk flask was charged with free base porphyrin (1 eq.) under Ar atmosphere. To this, dry dichloromethane 5 mL and 2,6-lutidine (115 equiv.) were added and stirred for 5 mins. A 2 M solution of PCl\(_3\) in dichloromethane (15 eq.) was added very slowly dropwise. The mixture was left to stir for 24 h (TLC control) at rt. Then the reaction was quenched by careful addition of water (2 mL) under ice cold conditions. Additional 20 mL water was added followed by extraction with dichloromethane (3 × 200 mL). The combined organic layer was washed with water and the volatiles were removed in vacuo. The crude product containing a mixture of chloro and hydroxo axial ligands was redissolved in acetone (100 mL). A 5 mL aqueous AgNO\(_3\) solution (0.75 g, 4.50 mmol, 25 eq.) was added and the mixture stirred for 2 h at rt. After complete removal of acetone, the residue was extracted with dichloromethane (3 × 200 mL), washed with water, dried over Na\(_2\)SO\(_4\) and the solvent removed. The crude product was purified by silica column chromatography. Elution with dichloromethane removed a red band containing small amount of unreacted porphyrin and other unidentified impurities. The desired product*
was then eluted as a purple band using 5-20 % acetone/dichloromethane (v/v). The sticky product obtained was triturated with hexanes to afford a purple solid.

**Dihydroxo(5,10,15,20-tetra(n-butyl)porphyrinato)phosphorus(V) hydroxide, 3a:**
Compound 3a was obtained following the general procedure using 1a (0.1 g, 0.18 mmol), 2,6-lutidine (2.5 mL, 21.46 mmol) and PCl3 (2M in dichloromethane) (1.4 mL, 2.80 mmol), yield: 27 mg (0.043 mmol, 24%); Mp >300 °C; Rf (acetone, silica) 0.25; ¹H NMR (400 MHz, CDCl3): δ = 9.25 (br s, 8H, β-H), 4.33 (t, J = 8.0 Hz, 8H, CH₂), 1.91 (quintet, J = 8.0 Hz, 8H, CH₂), 1.14 (sextet, J = 8.0 Hz, 8H, CH₂), 0.78 (t, J = 8.0 Hz, 12H, CH₃) ppm; ³¹P{¹H} NMR (162 MHz, CDCl3): δ = 195.6 (s) ppm; ATR-FTIR (thin film, cm⁻¹): ν = 3093, 2951, 2929, 2865, 1651, 1626, 1516, 1439, 1373, 1311, 1231, 1159, 1102, 1083, 1069, 1032, 960 (vP-O), 892, 825, 792, 778, 747, 710, 691 cm⁻¹; UV/vis (THF): λmax (log ε) = 426 (5.27), 561 (3.84), 605 (3.48) nm; HRMS (MALDI-TOF⁺) [C₃₆H₄₆N₄O₂P₁]: calcd [M-OH]+ 597.3358, found 597.3375.

**Dihydroxo(5,10,15,20-tetrahexylporphyrinato)phosphorus(V) hydroxide, 3b:**
Compound 3b was obtained following the general procedure using 1b (0.1 g, 0.18 mmol), 2,6-lutidine (2.5 mL, 21.46 mmol) and PCl3 (2M in dichloromethane) (1.4 mL, 2.80 mmol), yield: 26 mg (0.035 mmol, 20%), Mp >300 °C; Rf (acetone, silica) 0.40; ¹H NMR (400 MHz, CDCl3): δ = 9.31 (br s, 8H, β-H), 4.37 (t, J = 8.0 Hz, 8H, CH₂), 3.71-3.64 (m, 8H, CH₂), 3.38 (dd, J = 8.0 Hz, 8H, CH₂), 3.17 (dd, J = 8.0 Hz, 8H, CH₂), 1.96-1.86 (m, 8H, CH₂), 0.75 (t, J = 4.0 Hz, 12H, CH₃) ppm; ³¹P{¹H} NMR (162 MHz, CDCl3): δ = 196.0 (s) ppm; ATR-FTIR (thin film, cm⁻¹): ν = 3098, 2951, 2865, 1651, 1626, 1516, 1439, 1373, 1311, 1231, 1159, 1102, 1083, 1069, 1032, 960 (vP-O), 892, 825, 792, 778, 747, 710, 691 cm⁻¹; UV/vis (THF): λmax (log ε) = 426 (5.27), 561 (3.86), 605 (3.52) nm; HRMS (MALDI-TOF⁺) [C₄₄H₆₂N₄O₂P₁]: calcd [M-OH]+ 709.4610, found 709.4613.

**Dihydroxo(5,10,15,20-tetrakis(2-methylpropyl)porphyrinato)phosphorus(V) hydroxide, 3c:**
Compound 3c was obtained following the general procedure using 1c (0.1 g, 0.18 mmol), 2,6-lutidine (2.5 mL, 21.46 mmol) and PCl3 (2M in dichloromethane) (1.4 mL, 2.80 mmol), yield: 53 mg (0.088 mmol, 49%); Mp >300 °C; Rf (acetone, silica) 0.45; ¹H NMR (400 MHz, CDCl3): δ = 9.30 (br s, 8H, β-H), 4.38 (d, J = 8.0 Hz, 8H, CH₂), 1.89-1.83 (m, 4H, CH₂), 0.48 (s, 12H, CH₃), 0.46 (s, 12H, CH₃) ppm; ³¹P{¹H} NMR (162 MHz, CDCl3): δ = 195.8 (s) ppm; ATR-FTIR (thin film, cm⁻¹): ν = 3101, 2955, 1646, 1365, 1321, 1228, 1167, 1120, 1078, 1036, 970, 882, 820, 781, 707 cm⁻¹; UV/vis (THF): λmax (log ε) = 426 (5.28), 561 (3.86), 605 (3.52) nm; HRMS (MALDI-TOF⁺) [C₃₆H₄₆N₄O₂P₁]: calcd [M-OH]+ 597.3358, found 597.3367.

**Dihydroxo(5,10,15,20-tetrakis(1-methylpropyl)porphyrinato)phosphorus(V) hydroxide, 3d:**
Compound 3d was obtained following the general procedure using 1d (0.1 g, 0.18 mmol), 2,6-lutidine (2.5 mL, 21.46 mmol) and PCl3 (2M in dichloromethane) (1.4 mL, 2.80 mmol), yield: 42 mg (0.070 mmol, 39%); Mp >300 °C; Rf (acetone, silica) 0.47; ¹H NMR (400 MHz, CDCl3): δ = 9.23 (br s, 8H, β-H), 4.18-4.09 (m, 4H, CH₂), 2.44-2.33 (m, 8H, CH₂), 2.29-2.25 (m, 12H, CH₃), 0.68-0.62 (m, 12H, CH₃) ppm; ³¹P{¹H} NMR (162 MHz, CDCl3): δ = 198.4 (s) ppm; ATR-FTIR (thin film, cm⁻¹): ν = 3134, 2961, 2926, 2871, 1696, 1500, 1457, 1375, 1315, 1228, 1075, 1038, 1002, 879, 824, 782, 714
Dihydroxo(5,10,15,20-tetrakis(1-ethylpropyl)porphyrinato)phosphorus(V) hydroxide, 3\textit{e}: Compound 3\textit{e} was obtained following the general procedure using 1\textit{e} (0.1 g, 0.15 mmol), 2,6-lutidine (1.8 mL, 17.25 mmol) and PCl\textsubscript{3} (2M in dichloromethane) (1.1 mL, 2.31 mmol), yield: 61 mg (0.090 mmol, 60\%); Mp >300 °C; R\textsubscript{f} (acetone, silica) 0.45; \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta = 9.27\) (br s, 8H, \(\beta\)-H), 3.93-3.86 (m, 4H, CH\textsubscript{2}), 2.59-2.48 (m, 16H, CH\textsubscript{2}), 0.77 (br, 24H, CH\textsubscript{3}) ppm; \textsuperscript{31}P\textsubscript{1}H NMR (162 MHz, CDCl\textsubscript{3}): \(\delta = 197.7\) (s) ppm; ATR-FTIR (thin film, cm\textsuperscript{-1}): \(\nu = 3223, 2962, 2931, 2871, 1695, 1500, 1458, 1376, 1312, 1226, 1084, 1039, 950, 880, 827, 785, 711, 658\) cm\textsuperscript{-1}; UV/vis (THF): \(\lambda_{\text{max}}\) (log \(\epsilon\)) = 428 (5.27), 565 (4.06), 605 (3.88) nm; HRMS (MALDI-TOF\textsuperscript{+}) \([\text{C}_{40}\text{H}_{54}\text{N}_{4}\text{O}_{2}\text{P}_{1}]\): calcd [M-OH]\textsuperscript{+} 653.3984, found 653.3978.

Results and Discussion

Synthesis

Meso aryl and \(\beta\)-octaethyl P(V) porphyrins have been prepared before, mostly for coordination studies, redox chemistry investigations, and structural analyses [19,20,21,22,23,24]. The procedure we adopted followed that developed for P(V) insertion of 2,3,7,8,12,13,17,18-octaethylporphyrin derivatives, whereby PCl\textsubscript{3} acts as a more effective reagent for insertion over POCl\textsubscript{3} due to a lower phosphorus oxidation state [23,24]. Our initial studies to prepare P(V) alkylporphyrins gave mixtures of the chloro and hydroxide complexes. Similar results were obtained with other reagents. However, optimization studies revealed that a change of the \textit{in situ} generated chloro complexes to the hydroxide complexes was possible using aqueous silver nitrate as a halide abstractor.

This allowed for the synthesis of simple S\textsubscript{4}-symmetric P(V) porphyrins 3\textit{a}-3\textit{e} using a two-step method, involving the use of anhydrous dichloromethane under an inert atmosphere, in moderate to good yields of 20-60 \% (Scheme 1). Despite the presence of meso alkyl residues these compounds proved to be highly soluble in organic solvents \textit{and} water and much easier to work with than related meso aryl counterparts. Additionally, the synthetic sequence is a much simpler strategy than that of commercial photosensitizers such as \textit{mTHPC} (Temoporfin\textsuperscript{®}), whereby multiple steps, harsher conditions and lengthy chromatographies are necessary. In contrast, here, simple P(V) insertion is obtained using mild conditions in a 2-component 1-step strategy, requiring minimal chromatography.

Figure 1 shows the UV-vis absorption spectra of compound 3\textit{d} and that of the known photosensitizer \textit{mTHPC}. A distinct bathochromic shift in the Soret band absorption of 3\textit{d} is observed in comparison to \textit{mTHPC} (428 vs. 415 nm). A similar effect is observed in comparison with the uncomplexed form 2\textit{d}. Whilst \textit{mTHPC} displays a typical chlorin absorption at 651 nm (\(\epsilon \sim 22000\)), 3\textit{d} displays a stronger Soret absorption than that of \textit{mTHPC} (\(\epsilon \sim 196,000\) vs. 146,000).
<table>
<thead>
<tr>
<th>Entry</th>
<th>$R^1$</th>
<th>Compound</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$n$-butyl</td>
<td>3a</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>$n$-hexyl</td>
<td>3b</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>2-methylpropyl</td>
<td>3c</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>1-methylpropyl</td>
<td>3d</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>1-ethylpropyl</td>
<td>3e</td>
<td>60</td>
</tr>
</tbody>
</table>

**Scheme 1:** Synthesis of P(V) porphyrins. *Reagents and conditions:* i) $P(V)$ insertion: PCl$_3$, 2,6-lutidine, DCM, rt, argon; ii) Ligand exchange: aq. AgNO$_3$, acetone.

**Figure 1:** UV-vis absorption spectra of 3d (blue) versus mTHPC (green) in MeOH.
**In vitro Studies**

All compounds synthesized were screened for PDT activity. Specifically, their photodynamic potential as novel and effective PDT reagents for esophageal cancer treatment [27] was evaluated on human esophageal adenocarcinoma cell lines SKGT-4 and OE33 [28,29]. Preliminary investigations show that the water soluble P(V) porphyrin compounds, which are relatively easy to synthesize, can be used as versatile materials in PDT. Imaging analysis using high content screening (HCS) [26] showed that the P(V) porphyrins are readily taken up by SKGT-4 and OE33 cell lines after 24 h incubation and showed no visible toxicity in the dark. Images obtained by the InCell analyzer are shown in Figure 2 and show the photosensitizer uptake in red, dispersed within the cytoplasm of the cells. Table 1 shows the PDT effect of compounds 3d, 3e and 3a against OE33 and SKGT-4 cancer cell lines. Assessment of (photo)toxicity (toxicity in the dark = cytotoxicity vs. light toxicity = phototoxicity) was performed using cell proliferation assays (MTS) using white light as a light source for our preliminary *in vitro* screen. Primary results of the three non-specifically designed porphyrins showed that after a post-illumination incubation of 24 h, two compounds exhibit promising phototoxicity and a minimal dark toxicity.

The IC$_{50}$ after illumination of 3d was 6.5 ± 0.01 μM and 7.0 ± 0.01 μM in SKGT-4 cells and OE33 cells, respectively. The IC$_{50}$ in the dark for 3d was 42.5 ± 0.02 μM in SKGT-4 cell line and 43.0 ± 0.02 μM in OE33 cell line. Similar results were observed for 3a where IC$_{50}$ values of 5.5 ± 0.01 μM and 6.0 ± 0.01 μM were observed in SKGT-4 and OE33 cell lines, respectively. However, an enhanced dark toxicity was observed for this compound, whereby IC$_{50}$ values of 24.5-28.0 μM were seen with both cell lines. Compound 3e demonstrated meaningful lower cytotoxicity, with an IC$_{50}$ of 45 μM in SKGT-4 and 48 μM in OE33 cell lines after illumination and no significant dark toxicity up to 50 μM. These results show that PDT activity of P(V) porphyrins is comparable to the commercially available mTHPC. Temoporfin was determined to have an IC$_{50}$ of approx. 5 μM in both SKGT-4 and OE33 cancer cell lines with minimal dark toxicity, via control experiments.

In conclusion, we have synthesized simple, S$_4$ symmetric P(V) alkylporphyrins which are water soluble and even the chemically simplest symmetric dyes show PDT activity in esophageal cells on par with the contemporary, clinically approved photosensitizer mTHPC (Temoporfin, Foscarn®) despite the different localization behavior of water soluble versus non water soluble photosensitizers. The novel photosensitizing agents demonstrate significant toxicity after irradiation with light and minimal dark cytotoxicity, making them potential inexpensive candidates for PDT. Although these compounds have yet to be optimized in terms of enhanced absorption for better light penetration and enhanced target tissue accumulation via the inclusion of bioconjugates, our preliminary studies prove that this “P(V) effect” methodology has the potential to enhance the PDT activity and cellular uptake of even the simplest of tetrapyrrole. It is anticipated that these finding will enable the extension of the studies to the *in vivo* stage and beyond. Future work will involve localization studies, and singlet oxygen quantum yield determination. Additionally, the antimicrobial PDT activity of compounds 3a and 3d will be investigated for their applicability to serve as surface disinfectants. In order to improve uptake and intracellular localization within
adenocarcinoma cells we will prepare a set of P(V) porphyrins with more unsymmetrically substituted porphyrins. This concept will be used to prepare amphiphilic derivatives (with polar and nonpolar side groups) to improve membrane passage. Our simple and facile method for P(V) insertion allows the conversion of any other porphyrin free base, including those that have been specifically prepared for PDT e.g. two photon absorbers, conjugated systems (both of which exhibit a red shift in absorption for use with red/near-IR light source irradiation), arrays and bioconjugates. In doing so these macrocyclic systems with already optimized substitution patterns may be further improved through the “P(V) effect”, i.e. increased water solubility, increased cytotoxicity and better absorption properties.
Figure 2: Images collected from InCell analyzer. Images present OE33 and SKGT-4 cells treated with porphyrins 3d, 3e and 3a after 24 h incubation. Blue color represents nuclei stained by Hoechst; green color shows F-actin stained by phalloidin; red color represents photosensitizer.

Table 1: PDT effect of porphyrins 3d, 3e and 3a using MTS assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>SKGT-4</th>
<th>OE33</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ light µM ± SD</td>
<td>IC₅₀ dark µM ± SD</td>
</tr>
<tr>
<td>Temoporfin® (control)</td>
<td>5 µM (±0.01)</td>
<td>&gt;50 µM</td>
</tr>
<tr>
<td>3d</td>
<td>6.5 µM (±0.011)</td>
<td>42.5 µM (±0.022)</td>
</tr>
<tr>
<td>3e</td>
<td>45 µM (±0.022)</td>
<td>&gt;50 µM</td>
</tr>
<tr>
<td>3a</td>
<td>5.5 µM (±0.01)</td>
<td>28 µM (±0.020)</td>
</tr>
</tbody>
</table>
Acknowledgments

This work was supported by grants from Science Foundation Ireland (SFI P.I. 09/IN.1/B2650 and 12/TIDA/B2381) and the Irish Research Council.

References


