Photodynamic Effect of Water Soluble Piperazinium and Imidazolium Salts of HPPH on A549 Cancer Cells

Gerelt-Ireedui Sengee, Narangerel Badraa, Woo-kyoung Lee, and Young Key Shim*

*PDT Research Institute, School of Nano Engineering, Inje University, Gimhae 621-749, Korea. *E-mail: ykshim@inje.ac.kr*
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Photodynamic therapy (PDT) is a binary cancer therapy that relies on selective uptake of a photosensitizer in tumor tissues, followed by generation of singlet oxygen and other cytotoxic species upon irradiation with light of appropriate wavelength.[1,2]

Although a number of studies have documented preferential uptake of photosensitizers in tumor tissue,[3-5] exact mechanism of PDT is not fully understood. There are several possible approaches including cellular specificity such as lower pH and more low-density lipoprotein (LDL) receptors in malignant tissue than normal one.[6]

In order to find optimal photosensitizers, different compounds have been synthesized and examined including porphyrins, chlorins, phthalocyanines and purpurins.[7-10] Among them, chlorins represent the second generation of photosensitizers with promising physicochemical properties and high PDT efficiency.[11-14]

One of the challenging problems of chlorin based photosensitizers is their amphiphilic property due to a hydrophobic macrocycle of chlorins. If a photosenzitizer has too high lipophilicity, it has trouble to pass through blood vessel after intravenous injection. On the other hand, if a photosensitizer has too high hydrophilicity, it is difficult to penetrate cell membrane. Therefore it is important to compromise between hydrophilicity and lipophilicity. Introduction of hydrophilic groups imparts chlorin molecules with amphiphilic properties and, therefore, with good solubility in polar and non polar media. Such properties provide chlorin derivatives with good tumor/tissue ratio, high tumor efficacy and short clearance time.[15]

There have been several studies about protoporphyrin and chlorin e6 which are complexed with hydrophilic organic amine such as N-methyl-D-glucamine[16] and arganine[17] to improve their solubility in physiological solutions.

As far as we know, no reports have been observed in the literature on 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-a (HPPH), which is a promising photosensitizer under clinical trials, complexed with hydrophilic organic amine used as a photosensitizer for PDT.

In this study, we have designed two new chlorin derivatives in which piperazine and imidazole groups chosen as a hydrophilic organic amine are non-covalently complexed to the propionic acid residue of HPPH to improve their amphiphilicity. A comparative study of their photodynamic activities on A549 cancer cells is carried out in order to reveal influences of piperazine and imidazole moieties on biological activities of HPPH.

We have synthesized pyropheophorbide-*a* by several steps of chemical reactions including preparation of chlorophyll-*a* from *Spirulina pacifica* biomass, further conversion into HPPH, according to standard procedures.[18]

Salt-like complexes of HPPH, including complex of HPPH with piperazine (PIP) and complex of HPPH with imidazole (IMI), were obtained during HPPH treating with an organic free base-piperazine and imidazole, respectivily. Initial reagents were taken in 1:1 molar ratio in 1:3 methanol: dichloromethane. It is shown that formation of salt bond between carboxy group of parent chlorin and amino groups of piperazine and imidazole is a simple and effective method to obtain HPPH salt-like complexes (Fig. 1).

We have determined octanol/water partition coefficient (log*P*) for PIP, IMI and HPPH to evaluate amphiphilic properties. The result demontstrated that hydrophilicity of PIP (log*P* ~ 2.8) and IMI (log*P* ~ 3.1) is more than that of HPPH (log*P* ~ 5.7).

The biological activity of compound PIP and IMI complex of HPPH was evaluated in contrast to HPPH in A549 human

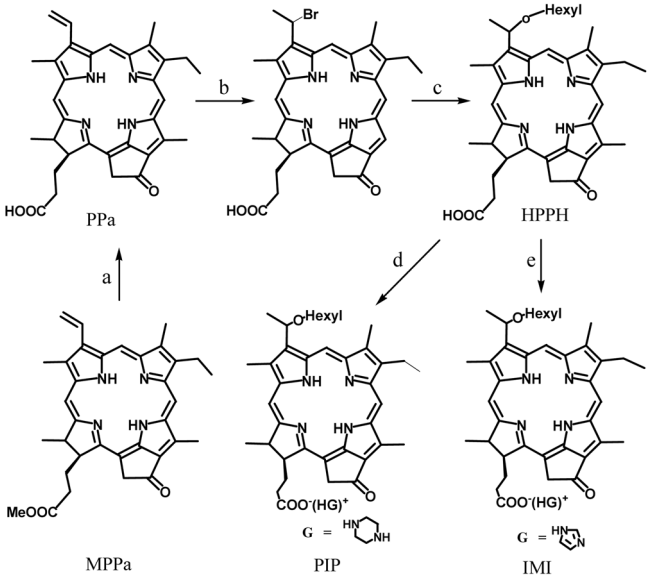

Figure 1. Synthetic pathways of complexes of HPPH with piperazine (PIP) and imidazole (IMI), Reagents: a) KOH, MeOH/THF; b) HBr/AcOH; c) Hexanol/K_2CO_3/CH_2Cl_2; d) piperazine/MeOH/MC; e) imidazole/MeOH/MC.

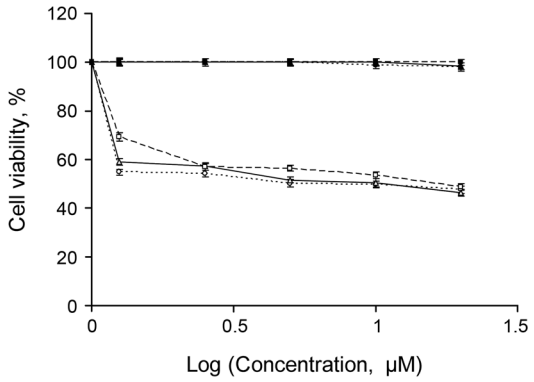

Figure 2. Dark (black figure: ▲, ●, ■) and phototoxicity (white figure: △, ○, □) of PIP (line-triangle-line), IMI (dot-circle-dot) and control starting material HPPH (dash-square-dash) toward A549 cells using 2 J cm^{-2} dose light after 24 h incubation time.

lung adenocarcinoma cells. One of the very desirable properties for new photosensitizers is high phototoxicity with minimal or no dark toxicity. Therefore, we measured the dark toxicity and phototoxicity of those compounds by means of MTT assays. The percentage of viable cells was evaluated at 24 h and 48 h incubation time after treatment with increasing concentrations of those compounds as shown in Figure 2 and Figure 3.

In comparison between dark and phototoxicity among those photosensitizing agents, each of them at various concentrations up to 20 μM exhibits a significant decrease in cell viability of samples received light irradiation (2 J cm^{-2}) compared to samples kept in dark.

For 24 h incubation time, dark toxicity of PIP is higher than that of IMI at the high concentrations when those compounds kept in dark without any irradiation. Both of newly synthesized PIP and IMI exhibit slightly higher dark toxicity than control treatment HPPH at the concentrations more than 6 μM. However, all of those compounds have relatively low dark toxicity at the all concentrations up to 20 μM.

From phototoxicity results, PIP and IMI exhibit higher phototoxicity than control treatment HPPH at all concentrations. Moreover, phototoxicity of IMI is higher than that of PIP at concentration less than 10 μM whereas PIP has more phototoxicity than that of IMI at concentrations more than 10 μM. Cell survival is not significantly decreased with increasing concentration of agent for the PIP and IMI except for the lowest concentration of 1.25μìM.

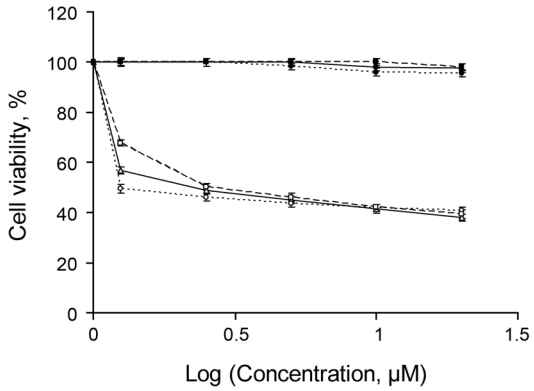

Figure 3. Dark (black figure: ▲, ●, ■) and phototoxicity (white figure: △, ○, □) of PIP (line-triangle-line), IMI (dot-circle-dot) and control starting material HPPH (dash-square-dash) toward A549 cells using 2 J cm^{-2} dose light after 48 h incubation time.

Table 1. IC_{50} values of PIP, IMI and HPPH after 24 h and 48 h incubation times against A549 cancer cells

Incubation time after irradiation	IC_{50} Value (μM)		
	PIP	IMI	HPPH
24 h	10.1 ± 3.7	5.0 ± 3.4	17.5 ± 10.5
48 h	2.3 ± 0.9	1.2 ± 0.7	2.5 ± 1.1

In case of 48 h incubation time, dark toxicity of PIP and IMI is higher than that of HPPH at the concentrations more than 3 μM and 6 μM respectively. Moreover, PIP and IMI have higher phototoxicity than HPPH at concentrations less than 10 μM. But at concentrations more than 10 μM, phototoxicity of IMI is lower than that of HPPH while PIP exhibits higher phototoxicity than HPPH.

To evaluate photodynamic activities of the agents, all of IC_{50} values of three agents are determined for light irradiated condition after 24 h and 48 h incubation time. Data of IC_{50} value for those compounds is shown in Table 1.

For light irradiated condition, the photocytotoxicity results of 24 h incubation after treatment were shifted to the those of 48 h with significant improvement which is shown determined values of IC_{50}.

The phototoxicity of those compounds was also examined by monitoring cell death at 24 h and 48 h incubation after photoirradiation under microscopic observation. No cell death was observed without the agent, whereas cell death was observed at increased concentration of PIP and IMI.

Figure 4 shows the time course for cell death at a concentration of 2.5×10^{-6} M.

As a result from phototoxic effect, compound PIP and IMI has higher phototoxicity, which is a favorable characteristic

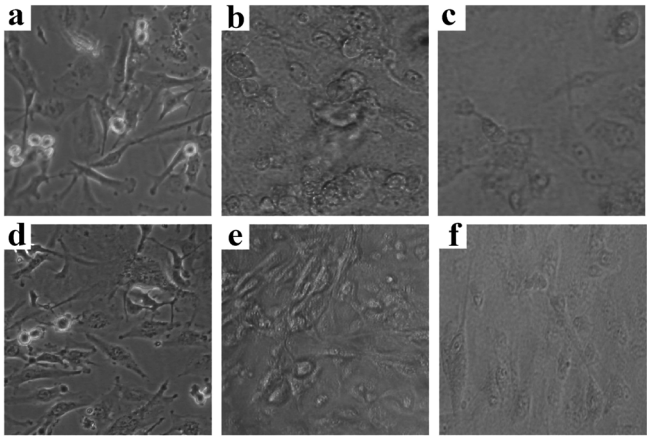

Figure 4. Optical image of morphological changes induced by PIP (b and c) and IMI (e and f) for 24 and 48 h respectively after irradiation compared with control (a and d) at 2.5×10^{-4} M concentration.

for PDT, than HPPH. According to the dark and phototoxicity study, IMI exhibits not only low dark toxicity but also high phototoxicity, whereas PIP exhibits high phototoxicity as well, but shows slightly high dark toxicity only at high concentrations. The increasements of dark and phototoxicity for IMI and PIP may depend on specific properties of piperazine and imidazole which are involved in anticancer and antibacterial medications.[19,20]

In conclusion, complexing hydrophilic organic amines such as piperazine and imidazole into the propionic acid residue of chlorin macrocycle not only improve its amphilic property but also enhances its phototoxicity without greatly increasing dark toxicities. This work demonstrates that complexes of HPPH with hydrophilic organic amine, particularly IMI, could be promising candidates for a new PDT-agent and is worth further investigations.

Experimental Section

Complex of HPPH with piperazine (PIP). To a solution of 0.1 mmole HPPH in MeOH/MC (3:1), a solution of 0.1 mmole piperazine in MeOH/water was added and organic solvents were evaporated *in vacuo*. Resulting aqueous solution was filtered through membrane and freeze dried to give complex of HPPH with piperazine.

λ_{max} (CH_2Cl_2)/nm (rel.int.) 411.2 (2.6564); 506.3 (0.2259); 537.5 (0.2391); 605.5 (0.2216); 661.7 (1.6256); δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.73 (1H, s, H-5); 9.44 (1H, s, H-20); 8.49 (1H, s, H-10); 7.20 (2H, s, H-NH_2-pip) 5.9 (1H, q, H-3^1); 5.25 and 5.12 (2 H, dt, H-15^1); 4.47 (1H, q, H-18); 4.25 (1H, dt H-17); 3.75 (2H, q, H-8^1); 3.60 (3H, s, H-12); 3.51-3.41 (8H, m, H-CH_2-pip); 3.34 (3H, s, H-1^1); 3.24 (3H, s, H-7^1); 2.7 (2H, m, H-17^1); 2.56 (2H, m, H-17^2); 2.10 (3H, s, H-3^2); 2.0 (1H, br, NH-pip); 1.76 (3H, dt, H-18^1); 1.67 (3H, t, H-8^2, *J* 7.6, 7^2 Me); 1.3-0.75 (13H, m, H-hexyl); 0.36 (1H, br, H-NH); −1.74 (1H, br, H-NH).

Complex of HPPH with imidazole (IMI). To a solution of 0.1 mmole HPPH in MeOH/MC (3:1), a solution of 0.1 mmole imidazole in MeOH/water was added and organic solvents were evaporated *in vacuo*. Resulting aqueous solution was filtered through membrane and freeze dried to give complex of HPPH with imidazole.

λ_{max} (CH_2Cl_2)/nm (rel.int.) 410.7 (2.2274); 506.2 (0.2159); 537.5 (0.2114); 605.4 (0.1717); 661.7 (0.9359); δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.74 (1H, s, H-5); 9.48 (1H, s, H-20); 8.51 (1H, s, H-10); 7.68 (1H, s, H^2-imi) 7.08 (2H, s, H^4 and H^5-imi) 5.88 (1H, q, H-3^1); 5.25 and 5.12 (2H, dt, H-15^1); 4.47 (1H, q, H-18); 4.25 (1H, dt H-17); 3.74 (2H, q, H-8^1); 3.64 (3H, s, H-12); 3.36 (3H, s, H-1^1); 3.25 (3H, s, H-7^1); 2.72 (2H, m, H-17^1); 2.57 (2H, m, H-17^2); 2.10 (3H, s, H-3^2); 1.81 (3H, dt, H-18^1); 1.69 (3H, t, H-8^2, *J* 7.2, 7^2 Me); 1.3-0.75 (13H, m, H-hexyl); 0.46 (1H, br, H-NH); −1.74 (1H, br, H-NH).

Phototoxicity. Cell culture and photoirradiation. The cell line tested was A549 (human lung carcinoma cell). The cell line was obtained from the cell line bank at Seoul National University's Cancer Research Center (Korea) and were grown in medium RPMI-1640 (Sigma-Aldrich) with 10% fetal bovine serum, glutamine, penicillin and streptomycin at 37 °C in humidified atmosphere of 5% CO_2 in air. Phosphate buffered saline (PBS) (Sigma-Aldrich), microscope (Olympus, CK40-32 PH, Japan), ELISA-reader (BioTek, Synergy HT, USA), trypsin-EDTA solution, incubator (37 °C, 5% CO_2) were used. The PDT was carried out using a diode laser generator apparatus (BioSpec LED, Russia) equipped with a halogen lamp, a band-pass filter (< 700 nm), and a fiber optics bundle. The wavelength was set at an appropriate level depending on absorption maximum of the photosensitizer. Duration of the light irradiation, under PDT treatment, is calculated taking into account of the empirically found effective dose of light energy in J cm^{-2}.

Values are reported as the mean ± standard error of mean (SEM) of three independent experiments done in triplicate.

IC_{50} (dose affecting 50% of cells) values were obtained by nonlinear regression analysis, using the GraphPad PRISM 5.01 sofware (GraphPad Software Inc.).

Morphological changes induced by PDT. Cells of each of the cell lines were inoculated into a 96-well chamber slide at a volume of 100 mL (5×10^4 cells/well) for stationary culture. 24 h later, photosensitizer (2.5 μM) was then added at a volume of 100 μL/well. After a predetermined time, the photosensitizer solution was discarded, and the culture was again washed three times in physiologic saline and medium added a volume of 100 mL/well. The cultures were then subjected to LED irradiation at the distance of 20 cm for 10 min, followed 24, and 48 h later by optical microscopy to comparatively determine the morphologic changes induced with those in the cultures not subjected to irradiation. The time course of the changes in survival rate after irradiation was observed.

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