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Synthesis and in vitro photodynamic activities of water-soluble fluorinated tetrapyridylporphyrins as tumor photosensitizers

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Abstract—A series of water-soluble fluorinated cationic porphyrins were designed, synthesized, and characterized. In vitro photocytotoxicity of these compounds was evaluated by MTT assay on HeLa cells. Their photocytotoxicity was dependent on the positions of the cations and the fluorines in the pyridine ring, and 5,10,15,20-tetrakis-(*N*-methyl-2-fluoro-pyridin-3-yl)-porphyrin (**8**) showed the most potent photo-induced cytotoxicity without photobleaching. PDT-induced ROS inside HeLa cells was measured with flow cytometry using ROS-sensitive fluorometric probe, 2,7-dichlorofluororescin (DCF), which revealed high correlations of ROS with cellular cytotoxicity. FACS analysis shows that PDT with porphyrin **8** induced apoptosis in HeLa cells. In summary, efficient generation of ROS, biological effectiveness, and good photostability of porphyrin **8** indicate its potential application in photodynamic therapy (PDT) in the near future.

Photodynamic therapy (PDT) is a cancer treatment leading to the selective destruction of malignancies by visible light in the presence of a photosensitizer and oxygen.[1] Upon the irradiation of visible light with appropriate wavelength, the photosensitizer can drive molecular oxygen into excited triplet state, transferring energy into ground state molecular oxygen to produce singlet molecular oxygen.[2] Activated singlet oxygen, or reactive oxygen species (ROS) in general, plays an important role in cytotoxic effects on tumor tissues. PDT can be applied as an effective cancer treatment due to enhanced permeability and retention (EPR) effect in tumors in comparison with normal tissues and is easily controlled by limiting the area of light irradiation.[3] Therefore, divergence in selective distribution to tumors and efficiency in visible light energy transfer to an intermediate are important properties of photosensitizers.

Porphyrin is a heterocyclic natural ligand of heme groups in hemoglobin or electron carriers such as cytochromes. Porphyrin-based macrocycles are one of the most frequently used photosensitizers and have been in the center of interest for the development of more sensitive analogues,[4] due to its excellent properties, especially selective association with membranes of malignant cells in vitro or in vivo studies. They also have low toxicity since they are easily cleared from tissues and blood fluids.[5] Furthermore, porphyrin-based compounds are efficient generators of reactive oxygen species (ROS) by the absorption of photons in visible region.[6a] In addition, porphyrin-based compounds showed diverse patterns of intracellular localization, predominantly at lysosomes and mitochondria, based on their structures, lipophilicity, and charges.[6b] However, a number of challenges are ahead of us to develop efficient porphyrin-based photosensitizers; longer retention time in tumor tissue, better water-solubility, and high quantum yield of reactive oxygen species. To address this issues, cationic porphyrins gained attentions in scientific community due to their advantages as a photosensitizer, such as improved water-solubility and selectivity to malignant tumor cells.[7] They also intercalate with DNA and inhibit the production of telomerase associated with the proliferation and immortality of cancerous cells.[8] In this report,

Keywords: Fluorinated tetrapyridylporphyrins; Photodynamic therapy (PDT); Photosensitizer; Reactive oxygen species (ROS); Photocytotoxicity.
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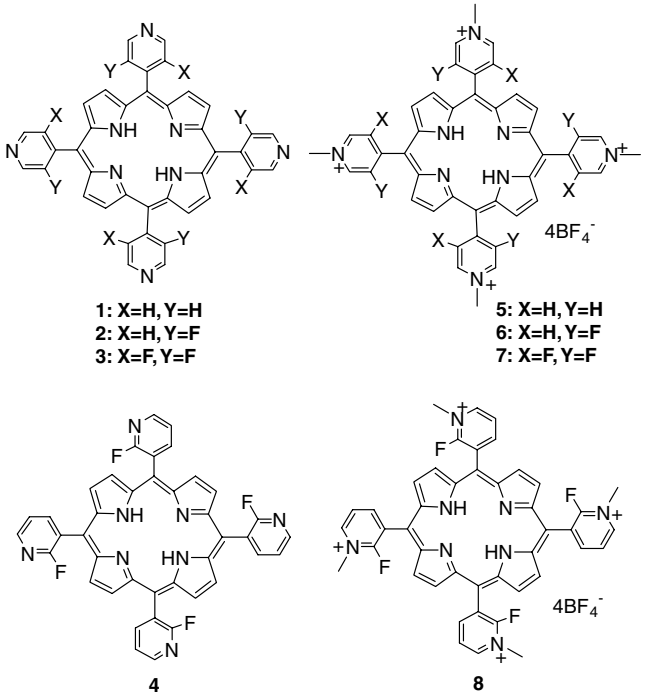


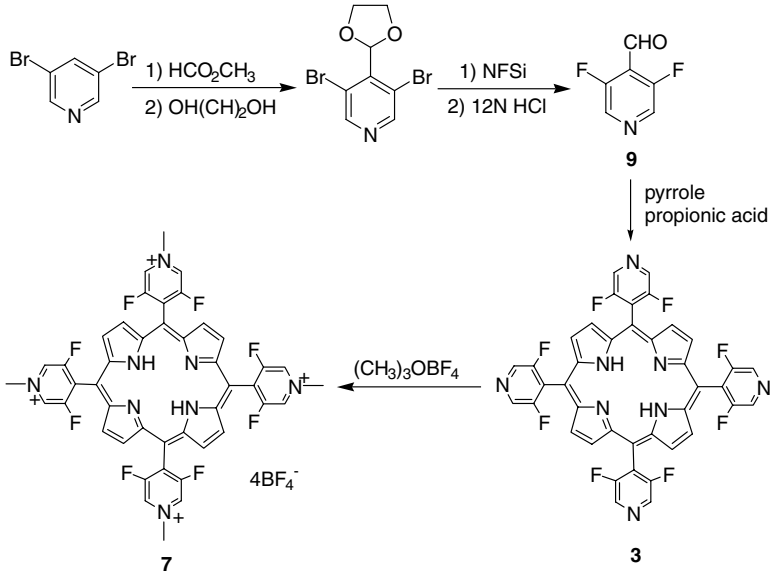
Figure 1. Porphyrin analogues.

we replaced some of the hydrogen atoms on cationic porphyrin system with fluorine to obtain high quantum yields and effective energy absorption. Generally, the fluorinated porphyrins have greater triplet quantum yields,[9] and their fluorine substitution is known to play a key role in anti-tumor activity.[10] For systematic comparison of the structure–activity relationship, non-fluorinated cationic porphyrin **5** and three fluorinated cationic porphyrin analogues (**6**–**8**) were synthesized in our laboratory. The structures of these compounds are shown in Figure 1.

In this paper, we reported the synthesis, photophysical properties, and photostability of fluorinated cationic porphyrin derivatives. The results of photocytotoxicity studies as well as cellular location and efficiency of reactive oxygen species (ROS) generation in HeLa cells in vivo are also presented.

The porphyrins were prepared through a typical reaction between equimolar amounts of fluoropyridine carbaldehyde and pyrrole in refluxing propionic acid.[11] Porphyrins **1**[12] and **2**[13] were prepared from pyridine-4-carbaldehyde and 3-fluoropyridine-4-carbaldehyde, respectively. The synthesis of **3** was achieved in five steps (Scheme 1). After formylation of 3,5-dibromopyridine at the 4-position, the formyl group was converted into a 1,3-dioxolane group via a conventional protection protocol. The NFSi-mediated difluorination and subsequent deprotection of the 1,3-dioxolane group to an aldehyde yielded compound **9**.[14] The typical reaction of aldehyde **9** with pyrrole in propionic acid generated an 8% yield of porphyrin **3** purified by silica gel flash column chromatography ($MeOH/CHCl_3$, 1:40). Porphyrin **4** was successfully obtained from highly volatile 2-fluoropyridine-3-carbaldehyde[15] at a 6% yield which was purified by silica gel flash column chromatography ($MeOH/CHCl_3$, 1:40). Methylation of porphyrins (**1**–**4**) with trimethyloxonium tetrafluoro-borate afforded an excellent yield (>90%) of the water-soluble cationic porphyrins (**5**–**8**). Porphyrins **3** and **4** and their salts, **7** and **8**, are novel compounds. These compounds were characterized by conventional spectroscopic techniques (NMR, HRMS).[16] ^{19}F NMR signals of the fluorinated porphyrins **5**–**8** showed a single peak at around δ 150 ppm in DMSO-d_6.

The fluorescence emission and UV–visible spectral data of these cationic porphyrins in water are listed in Table 1. The fluorescence maxima show two bands (~660 and 720 nm) upon the excitation by visible light



Scheme 1.

Table 1. Fluorescence emission and UV–vis data of porphyrin derivatives

Compound	λ_{em} (nm)		λ_{max} (nm) ($\varepsilon \times 10^{-4}$, M^{-1} cm^{-1})				
			Soret band	Visible bands			
5	660	720	422 (17.2)	515 (1.5)	550 (0.6)	589 (0.6)	647 (0.3)
6	658	717	424 (21.1)	513 (2.0)	563 (1.2)	582 (1.2)	—
7	665	721	420 (9.7)	513 (1.3)	—	587 (0.6)	659 (0.3)
8	662	726	424 (28.3)	516 (1.9)	552 (0.6)	593 (0.6)	648 (0.2)

with absorption maxima wavelength in Q bands. Their UV–visible spectra were consisting of the intense Soret band around 420 nm and weaker Q bands in the 510–660 nm intervals with very little difference in extinction coefficients. Based on our observation, fluorinated substituents on cationic porphyrins do not significantly influence their fluorescence emission maxima (626–630 nm) as well as UV–vis absorption maxima (Fig. 2).

To evaluate the biological functions of our novel compounds, we initiated an in vitro cytotoxicity assay with photodynamic activity of porphyrins (**5**–**8**) on HeLa cells.[16] Cells were pre-incubated with various concentrations of porphyrins (**5**–**8**) for 1 h followed by irradiation with fluorescent lamp (530–620 nm) at a fluence rate of 1.6 J/cm^2 (0.44 mW/cm^2 for 1 h) without washing. Then, HeLa cells were incubated for 48 h at 37 °C. The cell survival fraction was measured for photo-induced growth inhibition by photosensitizers using MTT assay. Figure 2 shows the concentration-dependent survival curves for porphyrins **5**–**8**. Most of the survival rates ranged up to 90% in the absence of light under the wide range of concentration, which revealed the minimal or moderate cytotoxicity in darkness (Fig. 3).

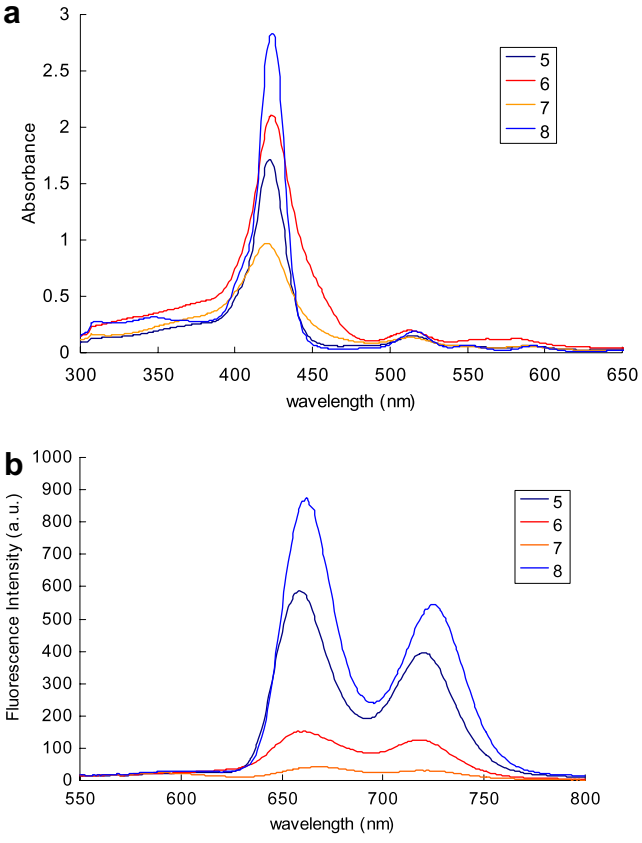


Figure 2. (a) UV–vis absorbance spectra of cationic porphyrin derivatives (**5**–**8**); (b) Emission spectra of cationic porphyrin derivatives (**5**–**8**).

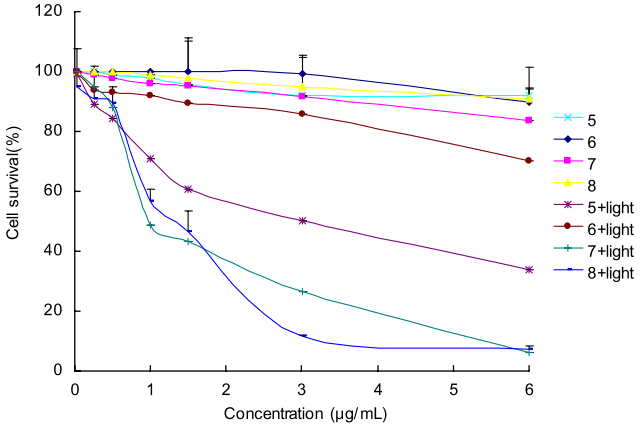


Figure 3. Photocytotoxic effect of porphyrin derivatives on HeLa cells in the absence of light and the irradiation of visible light for 1 h.

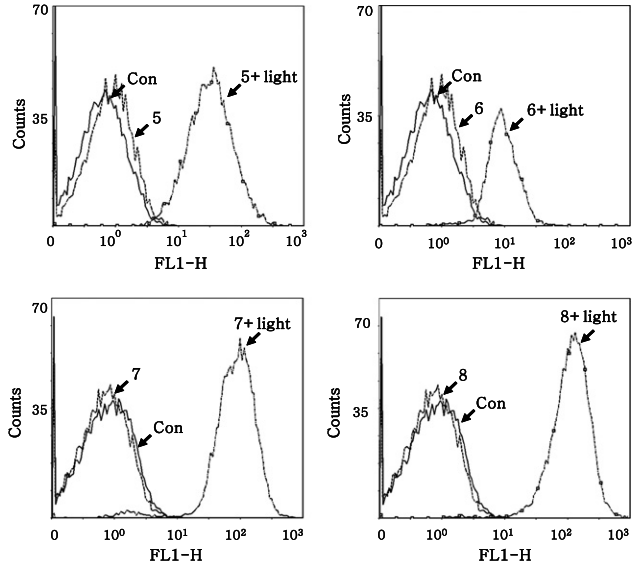


Figure 4. Porphyrins induce the generation of ROS. HeLa cells were incubated in the presence or absence of 30 μg/mL porphyrins for 2 h. DCF fluorescence was detected by flow cytometry after 2 h of porphyrin treatment.

As shown in Figure 3, porphyrins **7** and **8** exhibit significantly higher photocytotoxic effects on HeLa cells. IC_{50} values are 0.87 μM for porphyrin **7** and 1.1 μM for porphyrin **8**, while IC_{50} values for porphyrin **5** and **6** are 3 μM and 40 μM, respectively. Particularly, porphyrin **8** showed a remarkable difference in photosensitizing activity from porphyrin **6**, which led us to the conclusion that the position of positive charges on pyridyl moieties exerts a strong influence on the photosensitizing activity. Among **7** and **8**, although both porphyrins give a significantly high photocytotoxicity, porphyrin **8** turned out to be a more effective PDT photosensitizer based on the studies with HeLa cells.

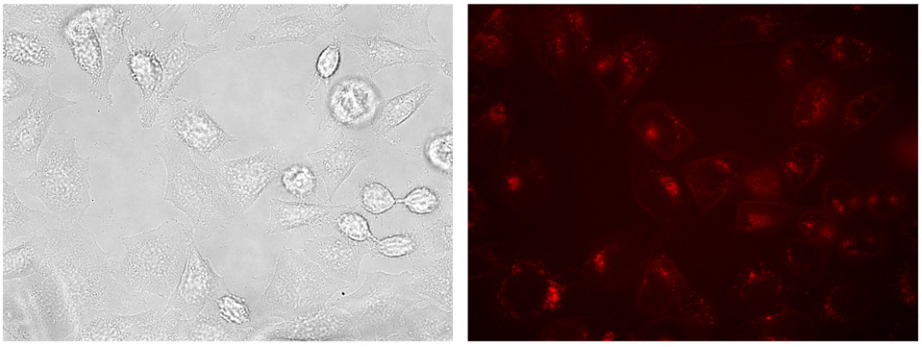


Figure 5. Intracellular localization images of porphyrin **8** captured by Axiovert200 (Zeiss, Germany). HeLa cells were cultured in the presence of 30 μM porphyrin **8** for 24 h. (Left) Phase contrast image; (Right) Fluorescence image.

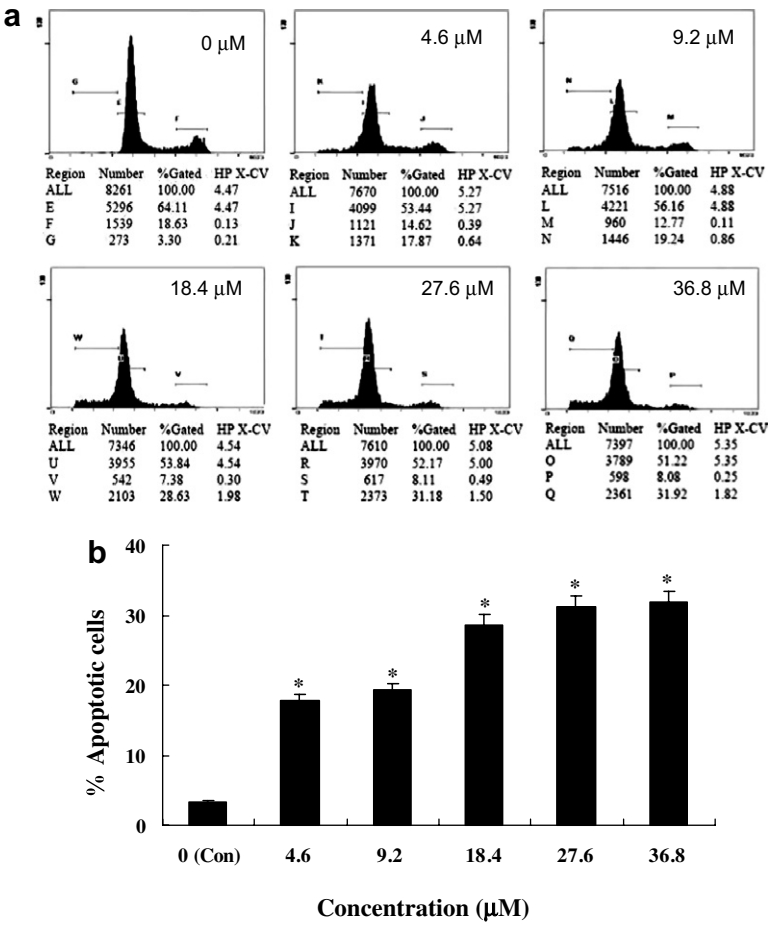


Figure 6. Sub-G1 cell population in **8**-treated HeLa cells stained with propidium iodide (PI) was determined by FACS flow cytometry. (a) data from a typical experiment; (b) histogram from FACS analysis of cells incubated under control conditions in the presence of various concentrations of **8**. The values are means ± S.D. of three independent experiments and analyzed by using Student's *t*-test. (*$p < 0.001$ when compared to the control (0 μM)).

The cytotoxicity of photosensitizer is caused by reactive oxygen species (ROS), generated upon the irradiation. ROS has been demonstrated to be an early signal mediating apoptosis.[17] To investigate whether intracellular ROS is involved in cationic porphyrin-mediated cell death, we next measured the level of ROS within the cells using a ROS-sensitive fluorometric probe, 2,7-dichlorofluororescin (DCF), that detects a wide range of ROS by flow cytometric analysis.[18] As shown in Figure 4, the basal level of DCF-sensitive ROS in HeLa cells was not readily detectable, however significant generation of ROS was observed upon the treatment of 30 μg/mL porphyrins followed by visible light irradiation. The PDT-induced ROS generation was highly correlated with the cytotoxic data of porphyrins **5**–**8** in HeLa cells. Especially, porphyrins **7** and **8** showed significant enhancement of ROS inside HeLa cells, which is consistent with the data of cytotoxicity assay. In addition, cellular uptake image of porphyrin **8** indicates that porphyrin **8** is localized to the cytoplasm (possibly to the mitochondria) as previously reported (Fig. 5).[19] Therefore, we can conclude that ROS generation in the cytoplasm by PDT-sensitizers is the key element for cellular cytotoxicity.

We also performed a photobleaching experiment with cationic porphyrins under the same irradiation conditions used for photocytotoxicity in the HeLa cells. However, we observed minimal decay of emission intensity at λ_{em} = 662 nm even with extensive irradiation (up to 500-fold light dosage of PDT cytotoxicity study) of visible light at 516 nm (see Supporting information). It is noteworthy that porphyrins **8** and **7** showed high photostability, which is the desired property of a PDT-sensitizer.

To address the cell death caused by porphyrin **8**-mediated PDT, the extent of apoptotic death was assessed using FACS flow cytometry through the determination of sub-G1 cell population by propidium iodide (PI) staining (Fig. 6). Analysis of the sub-G1 peak on DNA content frequency histograms revealed that there was a significant dose-dependent increase in the number of cells undergoing apoptotic cell death in HeLa cells (Fig. 6b). These results indicate that porphyrin **8** induced cell death mainly by apoptosis, in agreement with the suggestions of other authors.[20]

In conclusion, a series of water-soluble fluoro-substituted porphyrins has been designed, synthesized, and characterized. The potentials of fluorinated cationic porphyrin derivatives as PDT-sensitizers were evaluated by in vitro photocytotoxicity measurement using MTT assay on HeLa cells. Notably, porphyrin, **7** and **8** show significantly higher photocytotoxicity, presumably due to the efficient generation of reactive oxygen species (ROS) in the cytoplasm and their photostability. FACS analysis also showed that PDT with porphyrin **8** induced apoptosis in HeLa cells. Further developments and improvements using water-soluble fluorinated porphyrins are in progress and more extensive biological studies are on going.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.02.083.

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