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Combination of porphyrins and DNA-alkylation agents: Synthesis and tumor cell apoptosis induction

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Abstract—A series of porphyrin–DNA cross-linking conjugates were synthesized. Their cytotoxicities to tumor cells were tested using MTT assays first. Then, HeLa cell apoptosis induced by these cationic porphyrins under the light was examined by laser confocal microscopy, flow cytometric analysis, and further confirmed by observing the morphological changes and DNA fragmentation mainly.

1. Introduction

Porphyrins have found a wide range of medicinal applications.[1–5] It has been reported that elaboration on the meso- and the peripheral positions of the porphyrin ring will dramatically change its physical, chemical, and biological properties.[6] Introducing bioactivity groups onto the meso of porphyrins has a profound effect on the anti-cancer activity of porphyrins.[2,5] For example, the porphyrins with sugar moieties[8,10,11] were synthesized, and medicinal studies indicated that such porphyrins were very promising candidates for PDT in cancer treatment. Meanwhile, a series of porphyrin family are cationic porphyrin derivatives, which have been found to have good abilities to kill tumor cells and have been widely investigated as anti-cancer drugs in photodynamic therapy (PDT).[1,2] Mechanism studies suggested that porphyrin as a photosensitizer can localize on tumor cells and be phototriggered to produce singlet oxygen to cleave DNA and damage the tumor cells.[1a,1b,1d]

Apoptosis, or programmed cell death, is the normal pathway for clearance of defective or aged cells in the body and is the key pathway in animal development and in tissue homeostasis.[7] It offers us a new way to clean the possibly dangerous materials without causing toxicity to the body.[8] At the same time, apoptosis, in response to PDT using photosensitizers, has also been found.[4b,9] The strategy to combine many factors (such as drugs)[10] was designed to either increase or decrease the incidence of apoptosis for therapeutic cases. Apparently, any modifications of drugs could enhance the apoptosis to target tissue. As per many reports, a lot of anticancer agents are known to kill tumor cells through the mechanism of induction of apoptosis[4b,7b,11] and the techniques to identify the process of apoptosis have been reported such as morphological changes and DNA fragmentation.[7b]

Previously, we have reported that a kind of phenol quaternary ammonium could potently induce DNA cross-links by photoactivation which involved in *o*-QMs (*o*-quinone methide) intermediate.[12] Further application of this pharmacophore conjugating with porphyrins was studied and reported firstly by our previous paper.[12] In this paper, we shall continue to report our new observation on apoptosis induced by such kind of cationic porphyrins under the light.

Keywords: Porphyrin; Apoptosis; Morphological change.
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2. Results and discussion

2.1. Synthesis of the cationic porphyrin

Porphyrins **8**, **9**, **10**, and **11** have been synthesized according to our early reported method[12] and have been fully characterized by NMR, UV, and HRMS (Scheme 1, Fig. 1). In order to increase water solubility, number of charges, and *o*-quinone methide precursors of porphyrin, we designed and synthesized porphyrin **13**. Synthesis of tetra-cationic porphyrin **13** was started from 4-hydroxy-3-[(dimethylamino)methyl]benzaldehyde **4** and pyrrole (Scheme 2). The mixture was stirred in propionic acid in 120 °C for 1 h and the pure porphyrin **12** was obtained after chromatography. Finally, porphyrin **13** was formed by mixing porphyrin **12** with methyl iodide in acetone at room temperature with a fairly good yield.

2.2. Cell survival assay

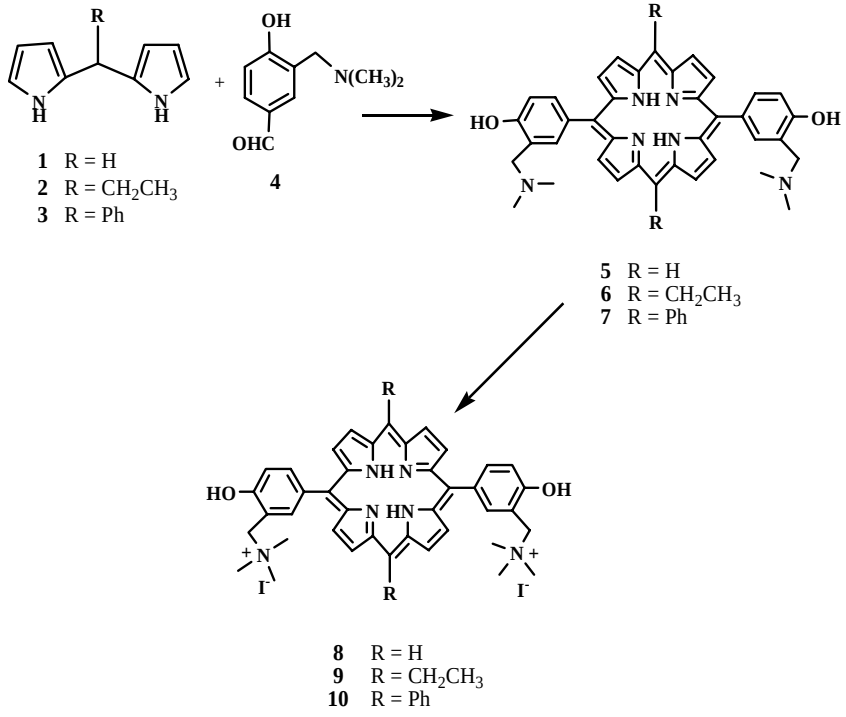
Whether synthetically porphyrins have anticancer activity to tumor cells is very significant for anticancer drugs. Therefore, an MTT assay was performed to determine the abilities to kill tumor cells of the porphyrins in vitro against HeLa and HepG2 cells.[13] Cytotoxic data were expressed as IC_{50} values (the concentration of the test agent inducing 50% reduction in cell numbers compared with control cultures), and their IC_{50} values of **8**, **9**, **10**, **11**, and **13** are shown in Table 1. According to our early reports, porphyrins **8**, **9**, **10**, and **11** could produce both singlet oxygen and *o*-QMs under photoactivation,[12] which could damage tumor cells efficiently. Furthermore, compared with these data, we found that the porphyrins **8**, **9**, **10**, and **13**, which could form *o*-quinone methide intermediates, had higher cytotoxicity compared to porphyrin **11**, which could not form *o*-quinone methide intermediate at all.

Comparing compounds **9** and **11**, which have similar structures but protected or unprotected by methyl groups on the phenols, a big difference to kill tumor cells. We suggested that compound **9** might form an *o*-quinone methide intermediate under illumination, compound **11** did not. Therefore, compound **9** had more potent ability to kill tumor cells than compound **11**.[12]

On the other hand, we also found that tetra-cationic porphyrin **13** had a potent ability to kill HepG2. It was probably due to the fact that more cations on the porphyrin favored it to interact with nucleic acids in the cell. Furthermore, porphyrin **13** might form more *o*-quinone methide intermediates induced by the light and have more possibility to alkylate DNA, to potently kill tumor HepG2.

2.3. Apoptosis of HeLa cells

We selected HeLa cells to carry out an apoptotic observation of the cationic porphyrins, which was carried out by using different kinds of methods and techniques. Apoptosis determination was conducted by the flow cytometry assay staining with propidium iodide. The results suggested that at a concentration of 1 μM, all porphyrins were able to recruit the cells in the apoptotic sub-G0-G1 peak (Fig. 2). The podiploid sub-G0-G1 peak (AP) represents cells undergoing apoptosis. Among these porphyrins, porphyrins **8**, **9**, and **13** which could form *o*-quinone methide intermediates, had higher abilities to induce HeLa cell apoptosis than that of



Scheme 1. Synthesis of phenol quaternary ammonium porphyrins.

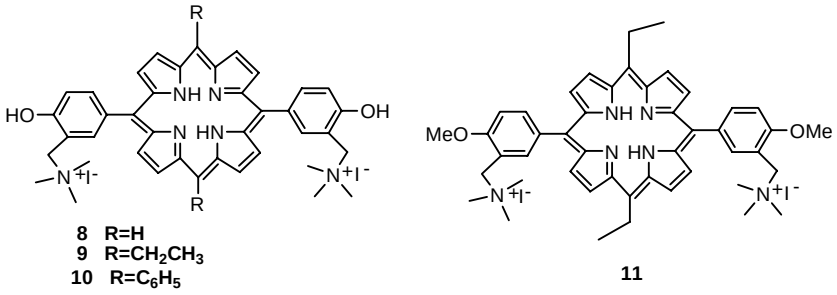
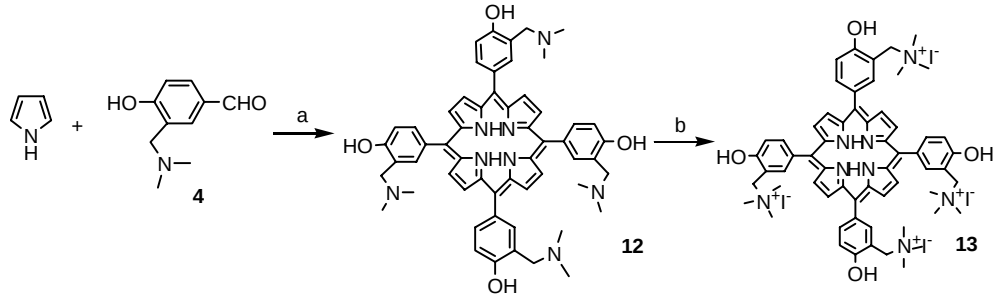


Figure 1. Structures of synthesized porphyrins.



Scheme 2. Synthesis of 5,10,15,20-tetra-{4-hydroxy-3-(trimethyl ammonium)methyl}phenylporphyrin (**13**). Reagents and conditions: (a) Propionic acid, 120 °C, 70 min, yield: 3.9%; (b) CH_3I, acetone, rt, 12 h, yield: 72%.

Table 1. IC_{50} of porphyrins **8**–**11** and **13**

IC_{50} (μM)	Porphyrin				
	8	**9**	**10**	**11**	**13**
HeLa cell	0.182	1.673	1.471	6.893	0.293
HepG2	3.038	6.893	15.813	0.293	0.404

porphyrin **11**. The exception compound **10** had close ability to induce apoptosis compared to compound **11**. We suggested that the phenyl groups on *meso*-positions of porphyrin **10** might decrease its ability to induce apoptosis either fro solubility or electronic and steric effects. Among these porphyrins, porphyrins **8** and **13** had higher abilities to induce HeLa cell apoptosis. In addition, we also performed a negative control experiment and found that untreated HeLa celld showed more than 96% survival, while cells untreated with porphyrins but irradiated for 30 min by a 50 W high pressure mercury lamp showed more than 95% survival. The cells treated with porphyrins without being irradiated by a 50 W high pressure mercury lamp also showed very low apoptosis rates (less than 6%). Therefore, the appearance of sub-G1 peak was characteristic of apoptotic cell behavior, which was not so the case of necrosis.[14] In fact, we also carried out assays of other concentrations; the results showed that both necrosis and apoptosis were observed when using a higher porphyrin conjugate. Drain also discussed varying PDT responses depending on the drug concentration.[4b]

To confirm the existence of apoptosis in the tumor cells, morphology of apoptosis was investigated by confocal microscopy staining with Hoechst 33258, a cell nucleus

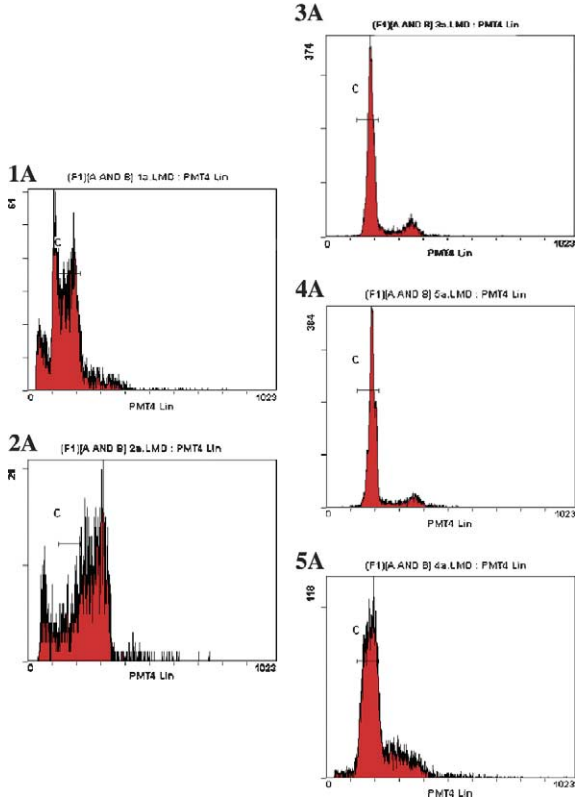


Figure 2. The histogram drug-induced cell cycle arrest and apoptosis of porphyrin (1 μM) in HeLa cells as measured by flow cytometric analysis. The *x*-axis is the DNA content and the *y*-axis the number of cells with that fluorescence intensity. 1A, 2A, 3A, 4A, and 5A represent cells that were treated with 1 μM porphyrins **8**, **9**, **10**, **11**, and **13**, respectively, for 24 h and irradiated by light for 30 min, respectively. The rate of apoptosis: 22.1%, 14%, 1%, 0.9%, and 11.5%, respectively.

present blue fluorescent DNA dye. The visualization of nuclear condensation and fragmentation are typical characteristics of apoptosis. It was found that the nucleus treated with by porphyrin became convoluted and budded off into several fragments (Fig. 3).[15] Herein, we only showed the morphology of porphyrins **8** and **13** at 5 μM. In addition, control experiments indicated that no obvious nuclear condensation and fragmentation of HeLa cells treated only with the porphyrins or the light were observed (Fig. 4).

Moreover, based on the above test results that porphyrins **8**, **13** had the best ability to induce HeLa cells to apoptosis, so they were selected to perform dual fluorescence from annexin V FITC–PI and the ordered DNA ladder detection. Further experiments were used to confirm if the tumor cell death induced by porphyrins **8** and **13** was caused by apoptosis or necrosis. In the annexin V FITC–PI test, when conjugated to a fluorescent probe, annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS) which detects the externalization of phosphatidylserine on the outer leaflet of the plasma membrane, an early feature of apoptotic cells.[16,17] Consequently, we could distinguish necrotic cells from apoptotic and living cells.[14]

In the assay, HeLa cells were treated with different concentrations of porphyrins **8** (200, 500, and 800 nM) and **13** (500 nM, 800 nM), respectively, for 24 h, then stained with annexin V and PI, and analyzed by flow cytometry. As shown in Figure 5, treatment of cells with the porphyrins indicated different apoptosis ratios in both apoptotic and necrotic cells. The total percentage of apoptotic cells was considered as the total number of early apoptotic cells and late apoptotic cells. Although negative control cells can induce little apoptosis (about 10%), the percentages of apoptotic cells treated with porphyrin were greater (generally about 18–25%) than those of control cells (the data are shown in Table 2).

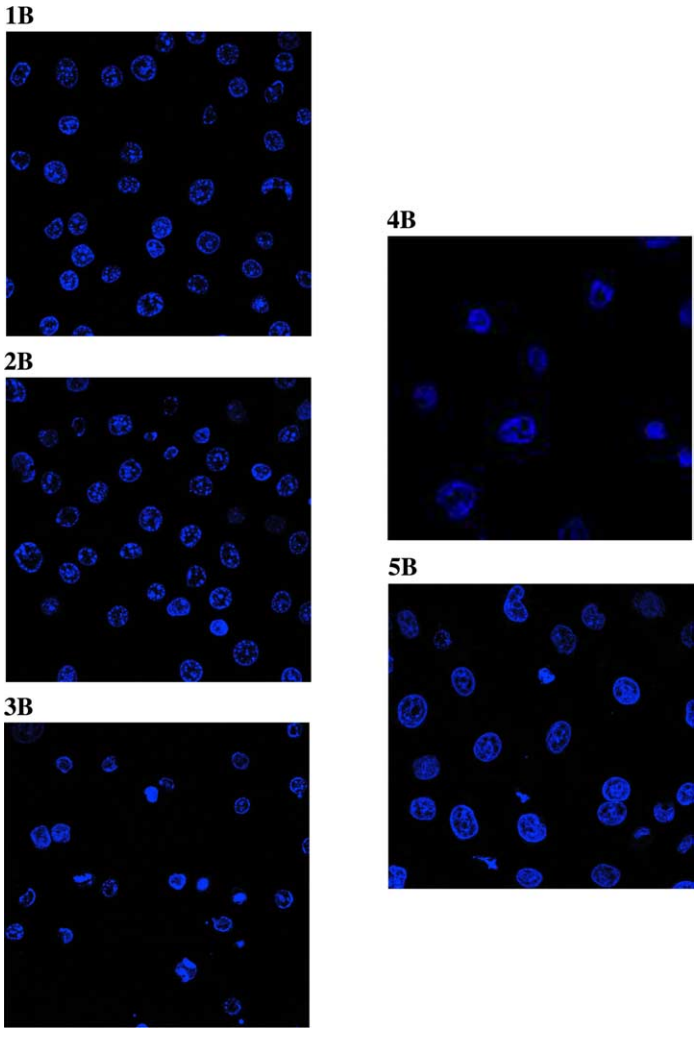


Figure 3. Confocal microphotographs of HeLa cells after treatment with the porphyrins, followed by staining with Hoechst dye 33258. HeLa cells were treated for 24 h with the porphyrins (1A, supplementary figure) 1 μM porphyrin **8**, (1B) 5 μM porphyrin **8**, (2A, supplementary figure) 1 μM porphyrin **9**, (2B) 5 μM porphyrin **9**, (3A, supplementary figure) 1 μM porphyrin **10**, (3B) 5 μM porphyrin **10**, (4A, supplementary figure) 1 μM porphyrin **13**, (4B) 5 μM porphyrin **13**, (5A, supplementary figure) 1 μM porphyrin **11**, and (5B) 5 μM porphyrin **11**, and were irradiated for 30 min by light, then stained with Hoechst 33258, fixed with 4% paraformaldehyde, and examined using a Leica Microsystems Heidelberg GmbH confocal spectral microscope.

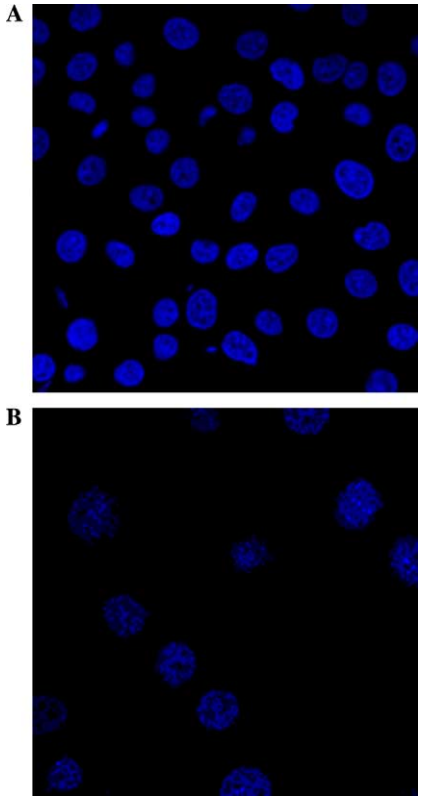


Figure 4. Control experiments for confocal microphotographs of HeLa cells after treatment without porphyrins, followed by staining with Hoechst 33258. (A) HeLa cells were irradiated for 30 min by light and cultured for 24 h; (B) Cells untreated with light and stained with Hoechst 33258. After fixing with 4% paraformaldehyde, they were examined using a Leica confocal spectral microscope.

Finally, it is known that the ordered DNA fragment pattern observed in gel electrophoresis is the biochemical hallmark of apoptosis.[7c] Then, we had examined the DNA fragment patterns and DNA ladder formation with an ordered pattern of multiple bands in agarose gels. Gel electrophoresis indicated the formation of the DNA ladder, which was induced by porphyrins **8** and **13** with 10 μM of the concentration (shown in Fig. 6). Here, clear apoptotic DNA ladders of 200 bp DNA ladder were used as control.

As we know, the only one testing method would be insufficient to confirm the induction of apoptosis, so morphologic and biochemical properties need to be studied by different techniques. The only difference between di-cationic porphyrins **9** and **11** is that the hydroxyl is protected in the peripheral substitute of the porphyrin. According to the results of MTT and apoptosis tests in Figure 2, the porphyrin **9** has higher cytotoxicity and better apoptosis than porphyrin **11**. So, we suggested that the quinone methide intermediates might be favorable for biological activities. Due to these results including appearance of the apoptotic sub-G_0-G_1 peak, visualization of nuclear condensation, and fragmentation, the result of dual fluorescence from annexin V FITC–PI and the DNA ladder formation, we believed that the cationic porphyrins **8** and **13** could induce apoptosis of HeLa cell assuredly.

3. Conclusion

In conclusion, a series of conjugation of porphyrins and DNA-alkylation has been prepared. We found that tetra-cationic porphyrins **8** and **13** had a better ability to kill tumor cells tested by MTT assays. It might be that there is more quinone methide formation after illumination and then cross-linked DNA. Finally, it could stop tumor cells from their further processing. Induced tumor cell apoptosis was the most important observation for these cationic porphyrins. All experiment data supported the fact that such quaternary ammonium porphyrins had good abilities to induce tumor cell apoptosis after photoactivation and might be potential applications for anticancer drug discovery.

4. Experiments

4.1. Materials and apparatus

The following compounds and reagents were commercially available: pyrrole (Aldrich), methyl iodide (Aldrich), TFA (Aldrich), *p*-chloranil (China Medicine Shanghai Chemical Reagent Corporation), MTT (Sigma), Hoechst 33258 (China Medicine ShangHai Chemmical Reagent Corporation), propidium iodide (Sigma), fetal calf serum (Gibco), RPMI-1640 (Gibco), Annexin V/FITC kit (Sigma), and 200-bp DNA marker (tsinghua). Pyrrole and TFA were distilled freshly before use. CH_2Cl_2 was distilled from CaH_2. $CHCl_3$ was distilled from K_2CO_3. Other reagents were analytic reagents (A.R.) and were used as obtained. 4-Hydroxy-3-[(dimethylamino)methyl]-benzaldehyde (**4**), dipyrro methane (**1**), *meso*-ethyl-2,2′-dipyrrylmethane (**2**), *meso*-phenyl-2,2′-dipyrrylmethane (**3**), and porphyrins **8**, **9**, and **10** were prepared according to a previous process.[12] Column chromatography was performed on silica (China Qingdao Chemical Corporation, 200–300 mesh). NMR spectra were recorded on a Varian mercury VX-300 MHz spectrometer. Mass spectra were obtained on an APEX II FT-ICR. UV–vis spectra were recorded on a Lambda 35 UV/vis spectrometer. Flow cytometer: EPICS ALTRA II Backman U.S.A.; Confocal Fluorescence microscopy: Leica TCA-SP2-AOBS-MP. UV–vis spectra: Lambda 35 UV/vis spectrometer. NMR: Varian Mercury VX-300 MHz spectrometer.

4.1.1. 10,20-{4-Hydroxy-3-(dimethylamine)methyl}phenyl porphyrin (5). Samples of 4-hydroxy-3-[(dimethyl amino)methyl]-benzenedaldehyde (197 mg, 1.1 mmol) and dipyrromethane (164 mg, 1.0 mmol) were dissolved in CH_2Cl_2/EtOH (95:5, 110 mL) in a 250 mL round-bottomed flask in N_2, and then TFA (1.96 mmol, 0.15 mL) was added slowly over 30 s. The reaction was stirred at room temperature for 20 h. Then *p*-chloranil (387 mg, 1.5 mmol) was added, and the reaction mixture was stirred at room temperature for a further 3 h. We obtained the desired porphyrin (6.2%) by chromatography purification with silica gel (a mixture solvent of $CHCl_3$ and MeOH).

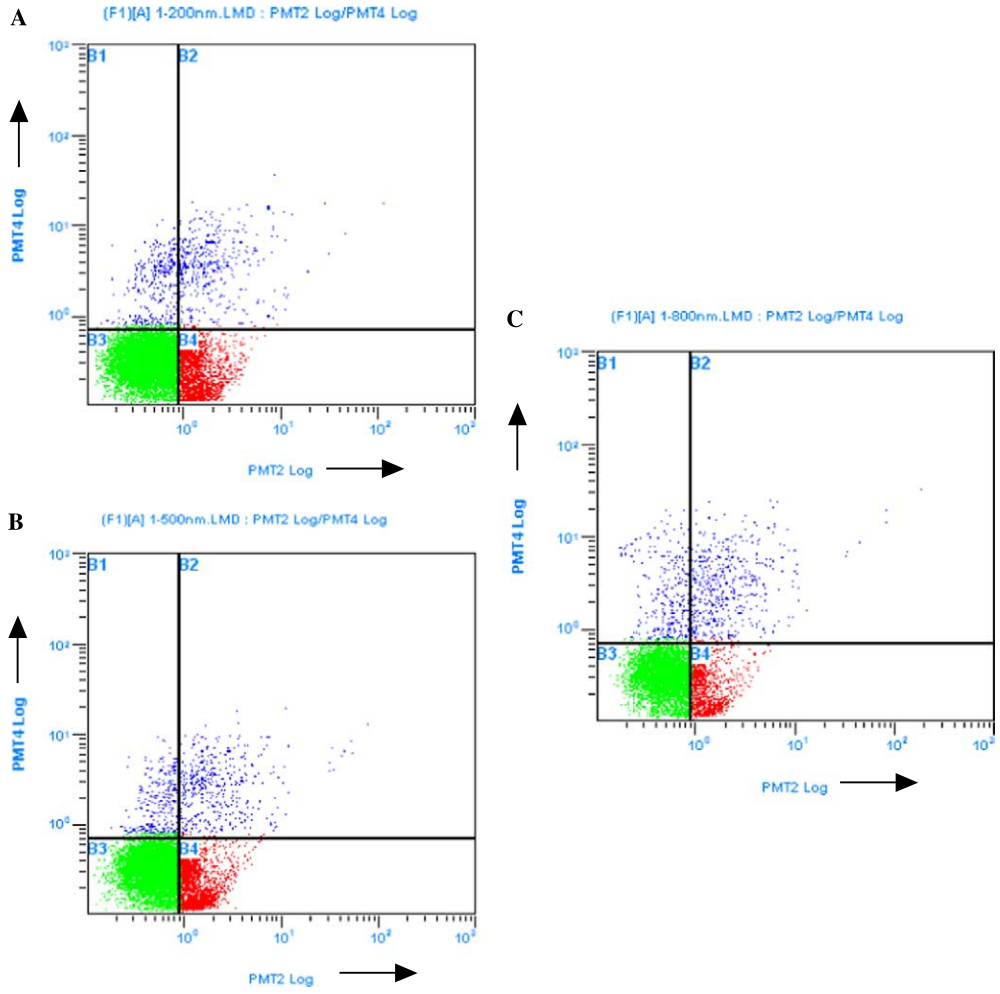


Figure 5. Apoptosis detection in HeLa cells using the Annexin V assay after 24 h. B1 (upper left corner); B3 (lower left corner); B2 (upper right corner); B4 (lower right corner). The total percentage of apoptotic cells was considered as B2 + B4. The cells irradiated for 30 min by light and treated with 200 nM (A), 500 nM (B), 800 nM (C) porphyrin **8**, 500 nM (D), 800 nM (E) porphyrin **13** for 24 h respectively; Control cells (F): Not irradiated and untreated with porphyrins; Control cell (G): irradiated for 30 min by light and no porphyrin.

^{1}H NMR (d_6-DMSO, 300 MHz): 10.26 (s, 2H), 9.36 (d, J = 2.1 Hz, 4H), 9.10 (d, J = 2.4 Hz, 4H), 8.07 (t, J = 3.4 Hz, 2H), 7.84 (d, J = 3.9 Hz, 2H), 7.28 (d, J = 3.9 Hz, 2H), 3.94 (s, 4H), 2.55 (s, 12H), −3.07 (br, 2H); ^{13}C NMR (d_6-DMSO, 75 MHz): 44.9, 63.3, 105.3, 115.0, 119.4, 120.8, 131.2, 131.6, 132.3, 135.1, 135.5, 145.2, 147.8, 158.3; UV–vis (CH_3OH) λ_{max} (nm, log ε): 408 (5.56), 506 (4.38), 543 (4.07), 578 (3.84), 636 (3.46); ESI HRMS for $C_{38}N_6O_2H_{36}$ [M^++H] calcd 609.2973. Found 609.2976.

4.1.2. 5,15-Diethyl-10,20-{4-hydroxy-3-(dimethylamine)-methyl}phenylporphyrin (6). Condensation of 4-hydroxy-3-[(dimethyl amino)methyl]benzenedaldehyde (197 mg, 1.1 mmol) and *meso*-ethyl-2,2′-dipyrrylmethane (164 mg, 1.0 mmol) in CH_2Cl_2/EtOH (95:5, 110 mL) in a 250 mL round-bottomed flask in N_2, and then TFA (1.96 mmol, 0.15 mL) was added. After purification, compound **6** was obtained and gave a purple solid (15.8%).

^{1}H NMR (d_6-DMSO, 300 MHz): 9.43 (d, J = 2.4 Hz, 4H), 8.91 (d, J = 2.4 Hz, 4H), 8.01 (t, J = 4.5 Hz, 2H), 7.75 (d, J = 5.7 Hz, 2H), 7.22 (d, J = 3.9 Hz, 2H), 5.00 (q, J = 3.6 Hz, J = 3.9 Hz, 4H), 2.11 (t, J_1 = 3.9 Hz, J_2 = 3.9 Hz, 6H), 3.92 (s, 4H), 2.55 (s, 12H), −2.65 (br, 2H); ^{13}C NMR (d_6-DMSO, 75 MHz): 23.7, 29.5, 45.4, 63.5, 114.5, 118.9, 120.0, 120.9, 133.3, 134.4, 134.8, 157.5; UV–vis (CH_3OH) λ_{max} (nm, log ε): 420 (5.43), 518 (4.58), 554 (4.50); ESI HRMS for $C_{42}N_6O_2H_{44}$ [M^++H] calcd 665.3599. Found 665.3607.

4.1.3. 5,15-Diphenyl-10,20-{4-hydroxy-3-(dimethyl-amine)methyl}phenylporphyrin (7). Condensation of 4-hydroxy-3-[(dimethylamino)methyl]benzenedaldehyde (197 mg, 1.1 mmol) and *meso*-phenyl-2,2′-dipyrrylmethane (164 mg, 1 mmol) in CH_2Cl_2/EtOH (95:5, 110 mL) in a round-bottomed flask in N_2, and then TFA (0.15 mL, 1.96 mmol) was added. After purification, compound **7** was formed and gave a purple solid (15%).

^{1}H NMR (d_6-DMSO, 300 MHz): 8.84 (q, J_1 = 4.8 Hz, J_2 = 12 Hz, J_3 = 4.5 Hz, 8H), 8.17–8.19 (m, 4H), 8.01–7.98 (m, 2H), 7.73–7.71 (m, 8H), 7.20–7.17 (m, 2H), 3.89 (s, 4H), 2.52 (s, 12H), −2.76 (br, 2H); ^{13}C NMR

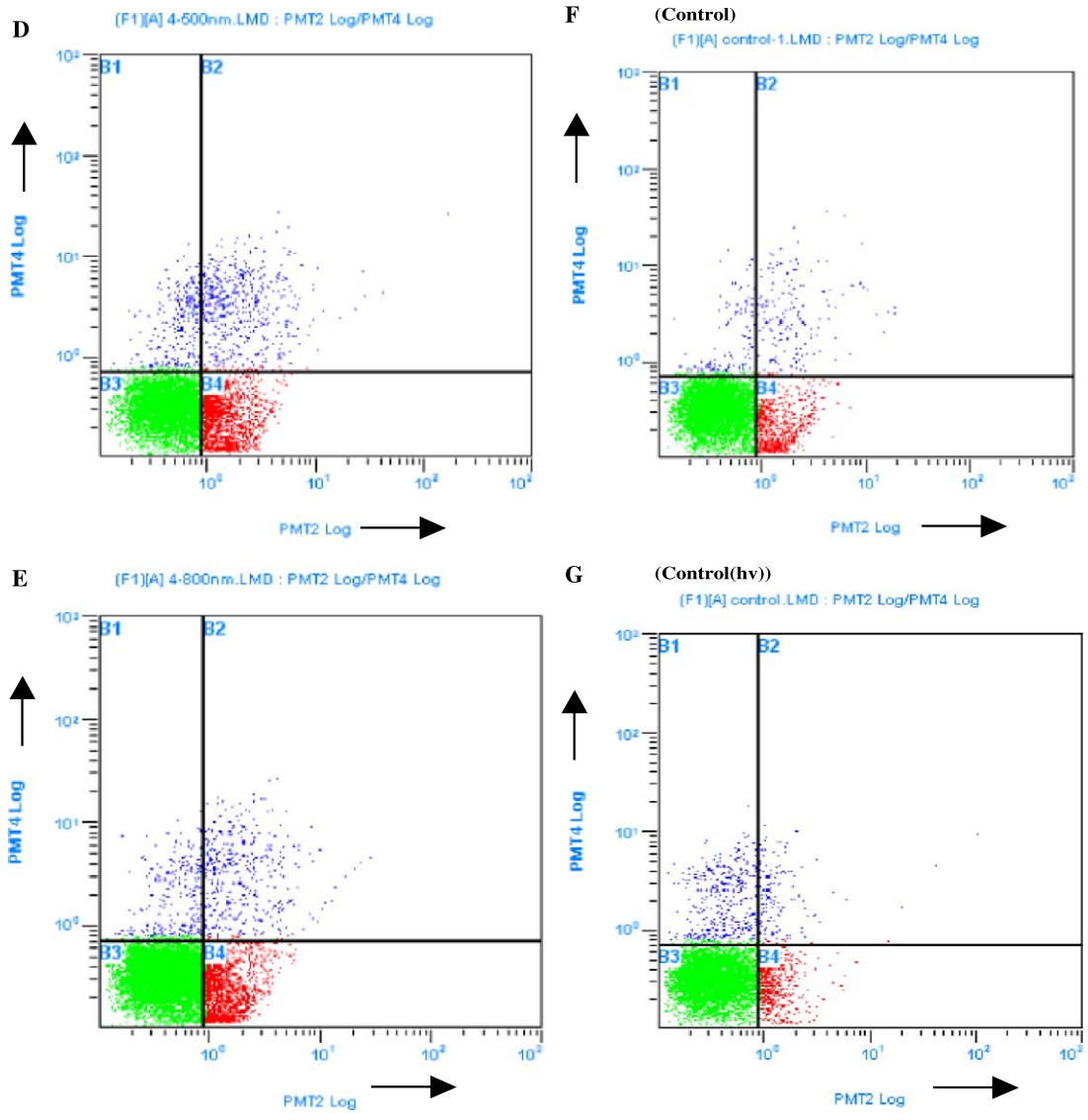


Figure 5. *(continued)*

Table 2. The percentage of apoptotic cells and negative control cells. The total percentage of apoptotic cells was considered as B2 + B4

Compound	B1 (%)	B2 (%)	B3 (%)	B4 (%)	B2 + B4 (%)
8					
800 nM	5.7	7.06	70.64	16.61	23.67
500 nM	3.27	3.53	73.18	20.02	23.55
200 nM	3.51	3.31	76.42	16.76	20.07
13					
800 nM	3.81	5.63	71.19	19.85	25.48
500 nM	3.20	3.61	77.83	15.35	18.96
Control cell	0.96	1.83	87.32	9.89	11.72
Control cell (hv, 30 min)	6.23	1.92	83.67	8.17	10.09

(d_6-DMSO, 75 MHz): 45.4, 63.5, 114.6, 119.9, 120.2, 120.3, 126.6, 127.7, 131.2, 132.9, 134.5, 134.7, 135.0, 142.4, 158.0; UV–vis (CH_3OH) λ_{max} (nm, log ε): 421 (5.51), 517 (4.36), 554 (4.18), 650 (3.91); ESI HRMS for $C_{50}N_6O_2H_{44}$ [M^++H] calcd 761.3599. Found 761.3601.

4.1.4. 5,10,15,20-Tetra-(4-hydroxy-3-(dimethylamine)-methyl)phenylporphyrin (12). A solution of the pyrrole (0.21 mL, 3 mmol) in 5 mL propionic acid was dropped to 15 mL propionic acid of 4-hydroxy-3-[(dimethylamino)methyl]benzenedaldehyde **4** (269 mg, 1.5 mmol). The mixture was stirred for 60 min at 120 °C, then neutralized up to pH 7 with Na_2CO_3 and washed by CH_2Cl_2 after cold. The organic layer was dried with Na_2SO_4 after being washed with water, and most of the solvent was removed under vacuum. The resulting dark red solution was purified with chromatography (silica gel, $CHCl_3$/MeOH/Et_3N = 50:1:0.8). The desired porphyrin **12** was obtained (yield: 3.9%, 13 mg).

^{1}H NMR (d_6-DMSO, 300 MHz): 8.88 (s, 8H), 8.03 (d, J = 8.1 Hz, 4H), 7.80 (s, 4H), 7.23 (m, 4H), 3.92 (s, 8H), 2.54 (s, 24H), −2.74 (br, 2H); ^{13}C NMR (d_6-DMSO, 75 MHz): 45.0, 63.3, 101.6, 114.6, 120.1, 120.4, 133.2, 134.7, 135.1, 158.1; UV–vis (CH_3OH): λ_{max} (log ε) = 427.0 nm (5.09), 520.0 nm (4.22), 558.0 nm (4.15), 577.5 nm (4.17), 652.0 nm (4.14); ESI HRMS

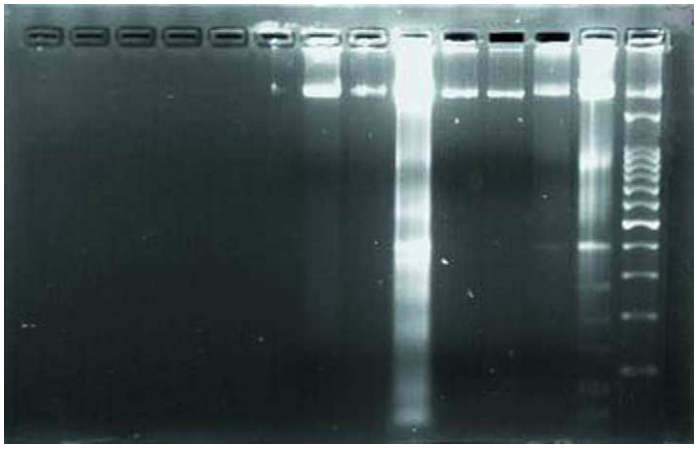


Figure 6. Agarose gel electrophoresis analysis of DNA fragmentation. HeLa cells were treated with porphyrins **8**, **13** and subsequently cultured in fresh medium for 24 h. After harvesting of the cells, nuclear DNA fragmentation was analyzed by 1.6% agarose gel electrophoresis. Lane 1, cells treated to 1 μM porphyrin **13** and irradiated for 30 min by light; lane 2, cells treated to 5 μM porphyrin **13** and irradiated for 30 min by light; lane 3, cells treated to 10 μM of porphyrin **13** and irradiated for 30 min by light; lane 4, cells irradiated for 30 min by light; lane 5, untreated cells; lane 6, cells treated to 5 μM of porphyrin **8** and irradiated for 30 min by light; lane 7, cells treated to 10 μM of porphyrin **8** and irradiated for 30 min by light; lane 8, 200-bp DNA marker.

for $C_{56}N_8O_4H_{58}$ [M^++H] calcd 907.4657. Found 907.4649.

4.1.5. 5,10,15,20-Tetra-{4-hydroxy-3-(trimethylammonium)methyl}phenylporphyrin(13). Tetra[4-hydroxy-3-[(dimethylamine)methyl]phenyl]porphyrin **12** (30 mg, 0.02 mmol) was added to a solution of methyl iodide (15 mmol, 0.9 mL) in 50 mL acetone and the mixture was stirred at room temperature for 12 h. After finishing the reaction, the solvent was removed under reduced pressure. The crude product was washed several times with CH_2Cl_2 and the precipitate was crystallized three times with ethanol and *n*-hexane co-solvents. The desired porphyrin **13** was obtained in 35 mg with the yield of 72%.

^{1}H NMR (d_6-DMSO, 300 MHz): 10.95 (s, 2H), 8.97 (s, 8H), 8.21 (s, 8H), 7.46 (d, J = 8.1 Hz, 4H), 4.75 (s, 8H), 3.26 (s, 36H), −2.92 (br, 2H); ^{13}C NMR (d_6-DMSO, 75 MHz): 52.7, 63.4, 113.6, 114.9, 119.3, 132.2, 157.5. UV–vis (CH_3OH): λ_{max} (log ε) = 428 nm (5.13), 521 nm (4.24), 556 nm (4.09), 579.5 nm (4.01), 650 nm (4.17); ESI HRMS for $C_{60}N_8O_4H_{70}$ ([(M^+−4I)/4]) calcd 241.6375. Found 241.6375.

4.2. Cell survival assay

HeLa living cells were grown according to media component mixtures, designated by American Type Culture Collection in RPMI-1640 + 10% FCS (fetal calf serum) in a 5% CO_2 humidity incubator at 37 °C. Cells were harvested using trypsin and washed with PBS. The cells, about 4×10^4 were added to well of a 96-well plate and incubated for 12 h at 37 °C in a 5% CO_2 incubator. Then the various concentration porphyrins were added, and the cells were irradiated for 30 min by a 50 W high pressure mercury lamp in the distance of 13 cm after incubation for 4 h at 37 °C in a 5% CO_2 incubator. Continuously, the cells were incubated for a further 20 h. Ten microliters of MTT (5 mg/mL) was added to each well and incubated for another 4 h. The cells were centrifuged at 2000 rpm for 10 min and then the medium was removed. Adding 100 μL DMSO to each well, the values of OD were detected at 570 nm. Cytotoxicity data were expressed as IC_{50} values.

4.3. Confocal fluorescence microscopy for morphology

Morphology of apoptosis was investigated by labeling the cells with the nuclear stain Hoechst 33258 and visualized under confocal spectral microscope (fluorescence microscopy). HeLa cells (1×10^6) were preplated in a 6-well plate and treated with the different concentrations of porphyrins. Cells were grown and harvested as described above. Then, the HeLa cells (1×10^6) were treated with 1 and 5 μM porphyrins, respectively, for 20 h. After that, untreated or porphyrin-treated cells were collected by centrifugation and suspended in PBS (phosphate-buffered saline). Finally, they were fixed in 4% paraformaldehyde followed by staining of the nucleus Hoechst 33258. Apoptotic cells were distinguished from control cells by the presence of a fragmented or highly condensed nucleus. So the cells were observed and confirmed under a confocal fluorescent (Leica Microsystems Heidelberg GmbH) microscope for chromosomal condensation and nuclear fragmentation.

4.4. Flow cytometric determination of apoptosis

Cells were grown and harvested as described above. Cell cycle progression and apoptosis were analyzed by quantitative DNA content after staining with propidium iodide (PI). Untreated or porphyrin-treated cells were collected by centrifugation and suspended in PBS. Finally, they were fixed in an ice-cold 70% ethanol solution at −20 °C overnight. The cell–ethanol solution was stored at −20 °C until being further treated for the analysis. To prepare the sample well for the flow cytometry analysis, the cell–ethanol solu-

tion was centrifuged and the cells were treated using RNAse (1 mg/mL) for 30 min at 37 °C. The mixture was suspended in 1 mL solution of PBS containing propidium iodide PI (100 μg/mL) in the polypropylene tubes. The cells were incubated for 1 h at room temperature. Then, the tubes were kept at 4 °C in the dark until further used for flow cytometry analysis by a flow cytometer (Beckmen Coulter Eltra flow cytometer). The linear DNA content data were processed by MultiCycle DNA Content and Cell Analysis software for cell cycle analysis. Apoptotic cells were evaluated as a sub-G0-G1 hypodiploid peak and the FITC intensity was used for apoptosis analysis.

4.5. Annexin V FITC-PI assay

Early stages of apoptosis were characterized by perturbations in the cellular membrane, which led to a redistribution of phosphatidylserine to the external side of the cell membrane. This caused a flux of calcium and it was required by the fluorescence-labeled annexin V to selectively bind to phosphatidylserine in order to identify cells undergoing apoptosis. Furthermore, cells were also stained with propidium iodide to distinguish early and late apoptotic cells from necrotic cells. The cells about 1×10^6 were added to the well of a six-well plate and incubated for 12 h at 37 °C in a 5% CO_2 incubator. Then the various concentration porphyrins were added, and the cells were irradiated for 30 min by a 50 W high pressure mercury lamp in the distance of 13 cm after incubation for 4 h at 37 °C in a 5% CO_2 incubator and replaced with fresh RPMI-1640 + 10% FCS. The cells were incubated for a further 20 h. The cells were resuspended in 195 μL binding buffer and incubated in the dark with both annexin V and propidium iodide for 40 min. Then, a sample of 400 μL of binding buffer was added and 10^4 cells were analyzed by the flow cytometer (Beckman Coulter, Expo32 MultiCOMP software). The percentage of live, dead, and apoptotic cells was determined. In the corresponding figure that illustrates the apoptosis, the viable cells (B3) were located in the lower left corner (negative in both annexin V-FITC and propidium iodide). Early apoptotic cells (B4) were in the lower right corner (annexin V-FITC positive). Late apoptotic cells (B2) caused cellular membrane and nuclear damage were in the upper right corner (positive in both annexin V-FITC and propidium iodide). The total percentage of apoptotic cells was combined with B2 + B4.

4.6. DNA gel electrophoresis

Cells were grown and harvested as described above. Cells were collected and washed with PBS twice. Then they were lysed in 500 μL lysis buffer (50 mM Tris–Cl, pH 8.0; 10 mM EDTA; 0.5% sodium lauroyl sarcosinate; and 1 mg/mL proteinase K) for 3 h at 56 °C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) before loading. Samples were mixed with loading buffer and loaded onto a 1.6% agarose gel containing 0.1 mg/mL ethidium bromide. The agarose gels were run at 50 V for 2 h in TAE buffer. And the gels were detected and photographed under UV light.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmc.2005.09.041.

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