Photodynamic Therapy

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Visible-Light-Induced Annihilation of Tumor Cells with Platinum–Porphyrin Conjugates**

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Abstract: *Despite the extensive use of porphyrins in photodynamic therapy (PDT), tetraplatinated porphyrins have so far not been studied for their anticancer properties. Herein, we report the synthesis of such novel platinum–porphyrin conjugates as well as their photophysical characterization and in vitro light-induced anticancer properties. These conjugates showed only minor cytotoxicity in the dark, but IC_{50} values down to 19 nM upon irradiation with light at 420 nm.These values correspond to an excellent phototoxic index ($PI = IC_{50}$ in the dark/IC_{50} in light), which reached 5000 in a cisplatin-resistant cell line. After incubation with HeLa cells, nuclear Pt concentrations were 30 times higher than with cisplatin. All of these favorable characteristics imply that tetraplatinated porphyrin complexes are worthy of exploration as novel PDT anticancer agents in vivo.*

Photodynamic therapy (PDT) is an approved medical technique used for the treatment of malignant tissues. In contrast to conventional anticancer treatments, PDT has several advantages, such as spatial and temporal control as well as the possibility of repeated doses.[1] Singlet oxygen ($^1O_2$) is the main cytotoxic agent in PDT for killing cancer cells through a type II mechanism.[2,3] The photosensitized generation of $^1O_2$ in PDT requires oxygen, low-energy light, and a photosensitizer (PS) that efficiently utilizes the given energy to excite triplet oxygen ($^3O_2$) into its singlet state. Ready accessibility, strong absorbance at long wavelengths, and high $^1O_2$ quantum yields are highly desirable for an ideal PS. Different porphyrins and porphyrinoids are currently FDA-approved as PSs because of their excellent photophysical properties and their selectivity for malignant tissues.

On the other hand, platinum compounds have emerged as powerful anticancer agents in the last 40 years.[4] Cisplatin and its successor carboplatin are among the most widely used anticancer drugs, active against bladder, ovarian, head and neck, testicular, and lung cancers. Oxaliplatin was able to overcome some of the resistance associated with cisplatin.[5] Despite their predominant use, all these drugs have severe side effects, such as nephrotoxicity, ototoxicity, hepatotoxicity, gastrointestinal dysfunction, and cardiotoxocity. Hence, there is a growing need for new and effective drugs. Along these lines, platinum compounds have been coupled with PSs to achieve synergistic treatment effects.[6,7] A number of platinum complexes that can be photoactivated by blue light have been developed.[8,9]

Tetraplatinated porphyrins have long been used as supramolecular guests that interact with cationic, anionic, or neutral micelles[10] and have been studied as mimics of natural peroxidase.[11] Their aggregates, which form supramolecular[12] and mesoscopic[13] assemblies, have been well characterized. Despite the use of porphyrin complexes in PDT,[3] tetraplatinated porphyrins have so far not been studied for their anticancer properties,[14] although ruthenated tetrapyridyl porphyrin complexes have been synthesized[15] and tested for their PDT ability in animal models.[16]

With the intention of improving the uptake and influencing the biodistribution of porphyrins, we decided to conjugate porphyrins to peripheral platinum centers. Herein, we report the synthesis of tetraplatinated porphyrins **2**–**4** from commercially available 5,10,15,20-tetra(4-pyridyl)porphyrin (**1**) and differently substituted platinum complexes (Scheme 1), as well as their photophysical characterization and in vitro light-induced anticancer properties.

Porphyrins **2**–**4** were characterized by IR, ^{1}H NMR, and ^{195}Pt NMR spectroscopy, ESI-MS, elemental analysis, and UV/Vis spectroscopy (see the Supporting Information). All complexes exhibited excellent quantum yields of $^1O_2$ production (Φ), thus showing that they all have potential as PDT agents. The quantum yield of $^1O_2$ was determined by using a 9,10-dimethylanthracene (DMA) based photobleaching assay[17] in *N*,*N*-dimethylformamide (DMF), as monitored by UV/Vis spectroscopy at 401 nm (see the Supporting Information). The value of $\Phi = 0.54$ obtained for porphyrin **4** in DMF is slightly greater than that of porphyrin **2** ($\Phi =$ 0.42), porphyrin **3** ($\Phi = 0.50$), and porphyrin **1** ($\Phi = 0.41$). To evaluate the potential of our porphyrins as PDT agents, we investigated the (photo)cytotoxic behavior of **2**–**4** in the dark and after irradiation with light.

Table 1 summarizes the (photo)cytoxicity of **1**–**4** against the MRC-5 human fibroblast cell line, the HeLa human cervical cancer cell line, the A2780 human ovarian carcinoma cell line, and the CP70 cisplatin-resistant cell line. The reported IC_{50} values were obtained after incubation for 4 h and a washing step, followed by irradiation (6.95 J cm^{-2}) for 15 min at 420 nm. Interestingly, the combination of cisplatin

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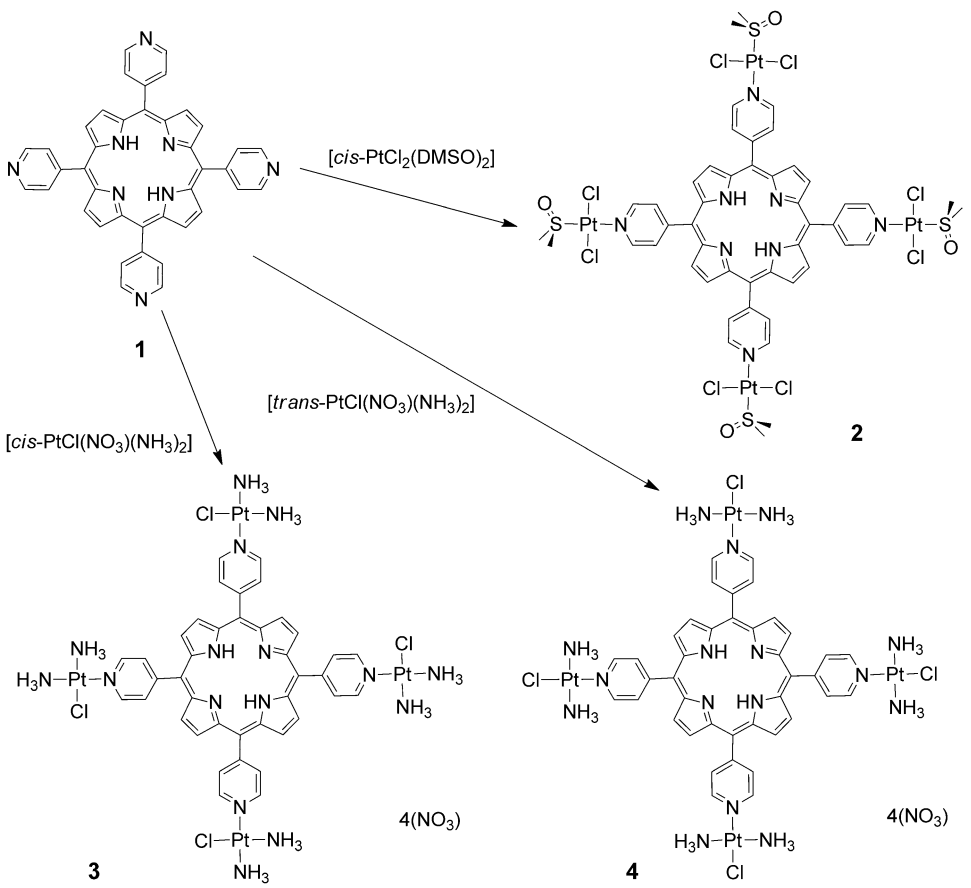

Scheme 1. Synthesis of tetraplatinated porphyrins **2**–**4**. DMSO = dimethyl sulfoxide.

and porphyrin **1** did not show a noticeable improvement in toxicity upon irradiation whereas porphyrin **1** alone had an IC_{50} value of 5.8 μM against HeLa cells when irradiated with light. Tetraplatinated porphyrins **2**–**4** formed by the conjugation of porphyrin **1** with Pt^{II} complexes showed negligible toxicity in the dark towards both noncancerous MRC-5 cells and HeLa cancer cells; however, the photocytotoxicity of these porphyrin conjugates was 600–1200 higher than their toxicity in the dark against HeLa cells. The most prominent results were obtained for porphyrins **3** and **4**, the IC_{50} values of which were 54 and 37 nM, respectively, upon irradiation with light. These low IC_{50} values were obtained with a very low irradiation dose as compared to previous studies.[6] Irradiation at a higher wavelength (575 nm, 15 min, 6.23 J cm^{-2}) also led to desirable photocytotoxicity, with IC_{50} values of 0.95 ± 0.4 μM, 1.51 ± 0.2 μM, and 0.80 ± 0.02 μM for porphyrins **2**, **3**, and **4**, respectively, against the HeLa cell line.

With these promising results in hand, we also assessed the cytotoxicity of porphyrins **2**–**4** against the cisplatin-sensitive A2780 and cisplatin-resistant CP70 human ovarian cancer cell lines. These porphyrins once again showed exceptional toxicity upon irradiation with light at 420 nm (Table 1). Upon irradiation, the toxicity towards the A2780 cell line was enhanced by a factor of 700 for porphyrin **2**, and the toxicity towards the CP70 cell line was enhanced by a factor of 1900 for porphyrin **3**. The most prominent results were observed with porphyrin **4**, which showed an over 5000-fold enhancement to yield an IC_{50} value of 19 nM upon irradiation of CP70 cells with light.

Our compounds are lipophilic cations. This feature may enable them to undergo passive diffusion and to profit from the electrochemical potential present between the inner and outer layer of the cellular membranes; this electrochemical potential favors the diffusion of cations, thus enhancing their cellular uptake. It was therefore of interest to study the cellular accumulation of porphyrins **2**–**4**. Fluorescence confocal laser microscopy experiments already confirmed a relevant uptake for the platinum porphyrins in HeLa cells after treatment for 2 h (Figure 1). Moreover, colocalization studies with 4′-6-diamidino-2-phenylindole (DAPI) show a pronounced selective accumulation of our compounds in the nucleus. These results are in agreement with those of previously reported studies, which showed the specific

Table 1: (Photo)cytotoxicity (IC_{50} [μM]) towards MRC-5, HeLa, A2780, and CP70 cell lines.

Compound	MRC-5 dark[a]	HeLa dark[a]	light[b]	PI[c] (HeLa) dark/light	A2780 dark[a]	light[b]	CP70 dark[a]	light[b]	PI[c] (CP70) dark/light
cisplatin	7.9 ± 1.2	12 ± 3	22 ± 6	0.51	8 ± 2	10.8 ± 1.2	32 ± 6	79 ± 15	0.41
cisplatin + **1**	10.3 ± 0.1	11 ± 3	13 ± 4	0.87	nd	nd	nd	nd	nd
1	>100	>100	5.8 ± 1.9	17.3	nd	nd	nd	nd	nd
2	>100	>100	0.15 ± 0.02	$>$ **680**	>100	0.16 ± 0.06	>100	0.09 ± 0.01	**1110**
3	50.2 ± 0.6	35 ± 4	0.05 ± 0.01	**655**	33 ± 12	0.047 ± 0.018	95 ± 4	0.049 ± 0.004	**1930**
4	93 ± 7	45 ± 8	0.04 ± 0.02	**1210**	98.3 ± 0.5	0.021 ± 0.008	>100	0.019 ± 0.004	$>$ **5260**

[a] Cells were incubated with the indicated compounds for 48 h. [b] Cells were incubated with the indicated compounds for 4 h and then irradiated with light at 420 nm (6.95 J cm^{-2}). [c] PI: phototoxic index.

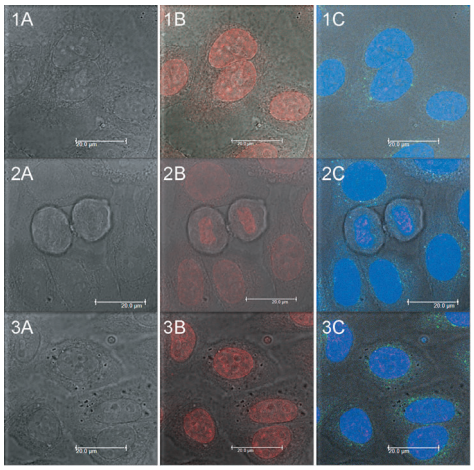

Figure 1. Cellular colocalization of 1) **2**, 2) **3**, and 3) **4** (each 1.0 μM) in HeLa cells. A) Cells visualized in DIC (differential interference contrast) mode. B) Luminescence images of **2**–**4**. C) Merged luminescence (of **2**–**4**, red), Mitotracker (green), DIC, and DAPI (blue) images. Scale bars: 20 μm. See Figure S2 in the Supporting Information for all images.

accumulation of tetracationic porphyrins in the nucleus.[18] However, eightfold positively charged complexes formed by the coordination of a tetrapyridylporphyrin by four ruthenium complexes were not found in the nucleus of human breast-cancer cells.[19]

Since porphyrin **4** showed the best anticancer profile and also demonstrated cellular and nuclear accumulation, this compound was chosen for more detailed biological and mechanistic investigations. Cellular internalization of porphyrin **4** was evident from fluorescence imaging. To confirm these results, we also evaluated the cellular uptake of platinum by ICP-MS and normalized the uptake versus protein content. For this purpose, HeLa cells were treated for 4 h with either porphyrin **4** or cisplatin (10 μM). After incubation for 4 h, the results revealed an uptake of porphyrin **4** to a concentration of about 100 ng of Pt/mg of protein as based on ICP-MS measurements. Strikingly, the use of complex **4** increased the nuclear platinum content by more than 30-fold as compared to that observed with cisplatin (Table 2). The increased nuclear platinum content is clearly only partially a consequence of the four platinum centers in porphyrin **4**. The main reason is that the conjugate is a more efficient importer of platinum into the cell nucleus than cisplatin. The highly selective accumulation of platinum in the nucleus is even more pronounced than the distribution of mitaplatin in MRC-5 cells.[20]

Table 2: Platinum content per milligram of protein in the nucleus and cytoplasm of HeLa cells after treatment for 4 h with either porphyrin **4** or cisplatin (10 μM).

	Porphyrin **4**		Cisplatin	
	ng Pt/mg protein	%	ng Pt/mg protein	%
nucleus	103 ± 46	99.5	3.26 ± 0.15	94.1
cytoplasm	0.54 ± 0.13	0.5	0.21 ± 0.02	5.9

Having established the nuclear uptake of porphyrin **4**, we hypothesized the involvement of DNA damage/interaction as a possible mode of action of the complex. Viscometry experiments (see Figure S3 in the Supporting Information) revealed a gradual increase in the relative viscosity of a solution of calf-thymus DNA (ctDNA) as the concentration of porphyrin **4** increased: a trend similar to that observed with ethidium bromide (EB), although less pronounced. EB is known as a classic intercalator and causes an increase in the viscosity of ctDNA owing to the lengthening and the stiffening of ctDNA.[21] The viscosity results show that porphyrin **4** binds to DNA by a mixed binding mode involving intercalation and platinum coordination.

The competition of porphyrin **4** with EB for binding sites on ctDNA was investigated by a fluorescence EB-displacement titration (see Figure S4). The emission intensity of the EB spectrum decreased as a result of the competitive binding of porphyrin **4** to ctDNA. From these data, an apparent binding constant $K_{app} = 7.5 \times 10^6\,\text{M}^{-1}$ was determined.[22] This binding constant suggests a strong binding affinity for ctDNA, comparable to that of EB. These results were nicely confirmed by absorption spectroscopy. A significant change in the absorption spectrum of porphyrin **4** was observed upon the addition of ctDNA (see Figure S5), with a large hypochromic effect (60%) coupled with a red shift (11 nm) for the characteristic peak, thus confirming the stacking between porphyrin **4** and the base pairs of DNA.[23] The high binding affinity for ctDNA was quantified by using the method of Bard and co-workers,[24] which gave an intrinsic binding constant (K_b) of $2.1 \times 10^6\,\text{M}^{-1}$. Circular dichroism studies gave additional insight into the binding mode between porphyrin **4** and ctDNA (see Figure S6). CD spectra of ctDNA treated with porphyrin **4** ($r = 0.05$–0.2) showed a negative peak in the Soret region of the porphyrin spectrum and thus revealed intercalative behavior.[23]

To gain further insight into the binding mode between porphyrin **4** and DNA bases, we carried out ^{1}H NMR spectroscopic studies (see Figure S7). The reaction of **4** with four equivalents of guanosine was monitored in a 1:1 [D_7]DMF–D_2O mixture. The H8 chemical shift located 0.84 ppm downfield from that of free guanosine was assigned to platinum(II)-coordinated guanosine, which is comparable to that of cisplatin-modified guanosine 5′-monophosphate.[25] On the basis of the above experiments, we can conclude that porphyrin **4** is able to bind to DNA by coordination to the nitrogen atoms of guanosine as well as by intercalation, although the platination of N7 only progressed to less than 50% within 10 days and is therefore much slower than intercalation.

All results described above suggest that DNA could be a main target for porphyrin **4** even without the involvement of light. However, the target platinum complex displayed a pronounced light-mediated cytotoxicity. In DNA photocleavage experiments with porphyrin **4** (see Figure S8), no DNA cleavage was observed upon incubation in the dark. In contrast, upon irradiation with light, the compound induced significant cleavage of the supercoiled configuration (intact DNA), and an increase in intensity of the nicked band (damaged DNA) was observed.

In summary, porphyrins **2**, **3**, and **4** were found to have a promising toxicity profile under low-energy irradiation, with high potency toward cancerous cell lines. Porphyrin **4** showed promising photocytotoxic properties, with extremely high toxicity towards human cancerous cell lines upon irradiation (HeLa: $IC_{50} = 37$ nM; A2780: $IC_{50} = 21$ nM; CP70: $IC_{50} = 19$ nM) as well as an excellent phototoxic index of up to 5000. Cellular-uptake studies disclosed the internalization of the porphyrins into the nucleus. Porphyrin **4** was found to interact with ctDNA mainly through intercalation, as demonstrated by viscosity, EB-displacement, and UV/Vis spectroscopic experiments. Porphyrin **4** was also found to induce DNA photocleavage at its IC_{50} concentration, thus confirming DNA as a probable main target of this complex. These favorable characteristics together imply that it may be worth exploring tetraplatinated porphyrin complexes as novel PDT anticancer agents in vivo.

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