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ARTICLE

Water-soluble cationic porphyrins with enhanced phototoxicity to cancer cell lines for G4-targeting photodynamic therapy

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Porphyrins are well-known photosensitizers (PSs), a few of which are clinically approved drugs for use in photodynamic therapy (PDT). Porphyrin derivatives include tetra-cationic porphyrins, *e.g.* **TMPyP4,** are also well-studied binders for G-quadruplex (G4) DNA. Since G4 DNAs are known to play a role in malignant transformation of cells, a variety of G4 binders have been used in cancer therapy by regulating the function of G4 DNA. In this study, two water-soluble porphyrins (**1** and **2**), with four terminal cationic moieties connected with alkyl linkers were synthesized as bifunctional molecules for simultaneous G4 binding and PDT-PS. Photoinduced singlet oxygen ($^1O_2$) generation and DNA cleavage were tested under visible light (527 or 630 nm) irradiation revealing the efficient generation of $^1O_2$ in line with photoinduced DNA cleavages. Studies in a cancer cell line (HeLa) and a normal fibroblast cell line (NHDF), revealed significantly stronger photocytotoxicities of these porphyrins (**1** and **2**) in comparison to **TMPyP4,** presumably due to better cellular internalization – as observed by flow cytometry. Interestingly, enhanced photocytotoxicity of **1** and **2** was observed in HeLa in comparison to NHDF. This may be related to the fact that more G4 DNAs are present in the the nuclei of cancer cell lines, to allow binding of porphyrins **1** and **2**, as observed by fluorescence microscopy images. The interactions of porphyrin **1** or **2** with a G4-forming teloDNA were evaluated by a FRET assay and spectroscpic methods (fluorescence, UV vis, and CD) and showed selective binding to G4 DNA. The results show the potential of porphyrins **1** and **2** as PDT-PSs targeting cancer cells with higher G4-forming domains.

Introduction

Photodynamic therapy (PDT) is a non-surgical treatment used for various types of cancers by the function of reactive oxygen species (ROS) generated by photosensitizers (PSs) under photoirradiation. Most PDT-PS drugs approved or in clinical trials are porphyrin derivatives.[1, 2] In addition to their excellent ability to generate ROSs under visible light irradiation, several *in vivo* and *in vitro* studies report that porphyrins localize more in cancer cells compared to healthy cells.[3-7] This indicates their potential as PDT-PSs, enhancing damage to cancer cells while reducing unwanted damage to healthy cells.[8] However, many porphyrins suffer from low solubility in biological media, often requiring the addition of solubilizing groups or polar substituents. Furthermore, to acquire better cellular uptake,[9] amphiphilic types of porphyrins would be advantageous due to their sufficient water-solubility and lipophilicity.

Porphyrin derivatives are also known binders for guanine-quadruplex (G4), one of the higher-order structures of DNA often found in guanine-rich domains. Typically, in the presence of cations such as K^+ and Na^+, four guanine moieties form a tetrad structure *via* the Hoogsteen-type hydrogen bonds, to further form assemblies by stacking. G4 binders have attracted attention in relation to cancers and other biological functions.[10-15] For instance, promoters of oncogenes, often (>40%) containing at least one G4 motif[16] and can be stabilized by G4 binders to downregulate corresponding oncogenes.[17] G4 motifs are also found in human telomeric repeat (TTAGGG), where G4 binders stabilize their 3-D structures to disrupt the capping function of telomerases,[18, 19] which are expressed more in cancer cells.[20, 21] For these reasons, many researchers have worked on developing stronger and more selective G4-binding and/or G4-stabilizing molecules.[11] These molecules will help not only to understand the fundamental biological function of G4, but will also be useful as therapeutic drugs for selective cancer treatments. [22, 23]

Among many G4-binding small molecules,[24] a tetra-cationic porphyrin, **TMPyP4** (Fig. 1), having a planar core with four cationic moieties at its edge, has been known as a standard molecule that interacts with four negatively charged phosphate backbone in G4 structures.[25] Considering the aforementioned photoinduced ROS generation by porphyrins, **TMPyP4** and related compounds were reported as a potential core for G4-targeted PSs for photodynamic therapy (PDT).[26-33] However, despite of its binding ability to G4 DNAs, **TMPyP4** suffers from relatively low binding selectivity to G4 DNA over double strand DNA (dsDNA)[34, 35] and limited cellular accumulation.[36]

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In this study, we synthesized two water-soluble cationic porphyrins **1** and **2** with extended distance of cations from the porphyrin centre (Fig. 1). Distinct from **TMPyP4**, compounds **1** and **2** possess cationic moieties through short anchors at the edges of porphyrin core, to enhance distance from the centre, as indicated in the electrostatic surface potential map (lower row of Fig. 1 and Fig. S1 in the ESI). We expected that the flexible cation location in **1** and **2** could potentially allow a better alignment of the ligand with respect to the negatively charged phosphate backbone of DNA.[37] These cationic moieties, guanidinium[38-40] and 1-methyl imidazolium,[41] were reported to interact with phosphate in G4 DNA. Furthermore, porphyrins **1** and **2** with higher amphiphilicities than **TMPyP4** may reveal better cellular uptake. Based on these assumptions above, porphyrins **1** and **2** were designed and synthesized.[42, 43] Photoinduced singlet oxygen ($^1O_2$) generation, DNA-cleaving activity, and cytotoxicity were studied to evaluate them as PDT-PS molecules, together with cellular uptake, G4-stabilizing and binding abilities.

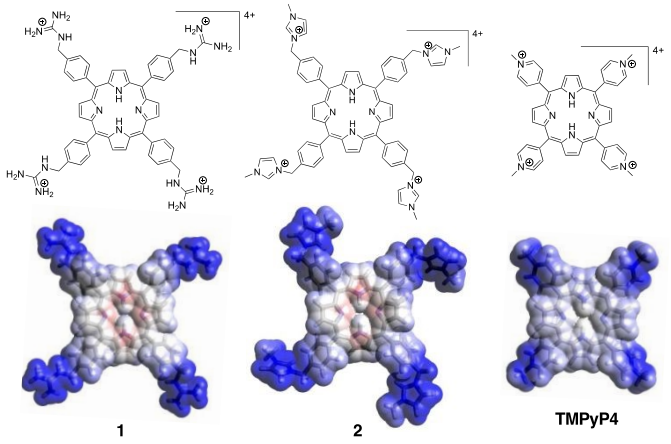

Fig. 1 Chemical structures and electrostatic surface potential maps for porphyrin **1**, porphyrin **2**, and **TMPyP4**. Conformation optimization and electrostatic surface potential calculation were performed using universal force field operated by Avogadro 1.2.0. Blue: lower electron density; red: higher electron density.

Results and discussion

Syntheses of compounds 1 and 2

Porphyrins **1** and **2** were synthesized *via* the Lindsey method[44] from the corresponding aldehydes and pyrrole (Schemes S1 and S2 in the ESI). For **1**, cleavage of the phthalimide groups of **S3** gave a porphyrin amine derivative **S4**, which was subjected to the guanidinylation to provide **1**. For **2**, a bromo substituted porphyrin **S5** was converted to porphyrin **2** by the reaction with 1-methylimidazole (Scheme S2).[43] Both compounds **1** and **2** were purified by reverse phase HPLC (Figs. S10 and S22) and structures were confirmed by ^{1}H and ^{13}C NMR and HRMS (Figs. S11-16 and S23-28).

Fig. 2a shows UV-vis spectra of porphyrins **1** and **2** and the control G4 binder **TMPyP4**. The characteristic spectra for metal-free porphyrins with a Soret band at around 420 nm and four Q bands at ca. 500-650 nm were observed in all porphyrins. Fluorescence spectra (Fig. 2b) were acquired using excitation wavelength of 420 nm and revealed that porphyrins **1** and **2** were highly fluorescent in comparison to **TMPyP4**. There was no aggregation observed in porphyrins **1**, **2** and **TMPyP4** in pH 7.4 HEPES buffer at least 10 μM as indicated by linear correlation of absorption intensity *versus* concentration at Soret band (Figs. S18 and S30). The higher fluorescence intensity of porphyrins **1** and **2** may be related to their larger hydrophobic core in comparison to **TMPyP4** leading to less quenching by water molecules.[45] There was no detectable aggregation of the molecules by DLS measurements at least up to 1 mM.

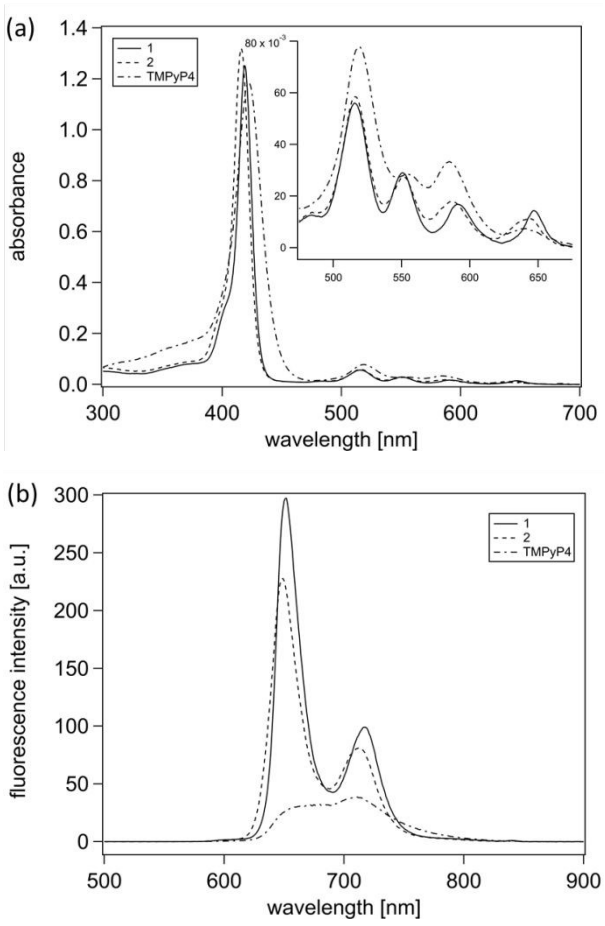

Fig. 2 UV-vis (a) and fluorescence (b) spectra of porphyrins **1**, **2**, and **TMPyP4** (5 μM in 10 mM HEPES buffer (pH 7.4)). Fluorescence spectra were recorded with an excitation wavelength at 420 nm using a slit of 5 nm.

Photoinduced singlet oxygen generation and dsDNA cleavage

Photosensitivities of **1**, **2**, and **TMPyP4** were evaluated by the singlet oxygen ($^1O_2$) generation under visible light irradiation. An ESR spin trapping method was employed for the detection of $^1O_2$ using 4-oxo-TEMP as a spin-trapping agent (scheme in Fig. 3).[46] Upon irradiation by green LED (539-541 nm), specific peaks corresponding to 4-oxo-TEMPO ($^1O_2$ adduct of 4-oxo-TEMP) were observed in the solution of each porphyrin (Fig. 3) in an irradiation-time-dependent manner (Figs. S32-37) confirming *type II* energy transfer pathway was occurring by porphyrins **1** and **2**. The relative amount of generated $^1O_2$ by each porphyrin

was evaluated by double integration value of ESR spectra (Fig. S38 in the ESI). By taking into account the relative absorption intensity of each porphyrin at 540 nm, ability of $^1O_2$ generation by porphyrins **1** and **2** under photoirradiation (540 nm) for 2 or 10 min were respectively ca. 1.4-2.0 and 1.1-1.4 times higher, that was enhanced more under 621 nm, which is advantageous in the PDT application due to the better tissue penetration of the light.

Alternatively, we also tried to observe *type I* ROS (O_2•$^-$) generated by electron transfer mechanism. Under visible light irradiation, generation of O_2•$^-$ was clearly observed as an adduct of a spin-trapping agent, DEPMPO (Fig. S39 in the ESI). However, the signals corresponding to DEPMPO•OOH were significantly suppressed in the presence of L-histidine (a $^1O_2$ quencher), suggesting that observed O_2•$^-$ was generated not by *type I* but by the reduction of $^1O_2$ once generated via *type II* pathway.

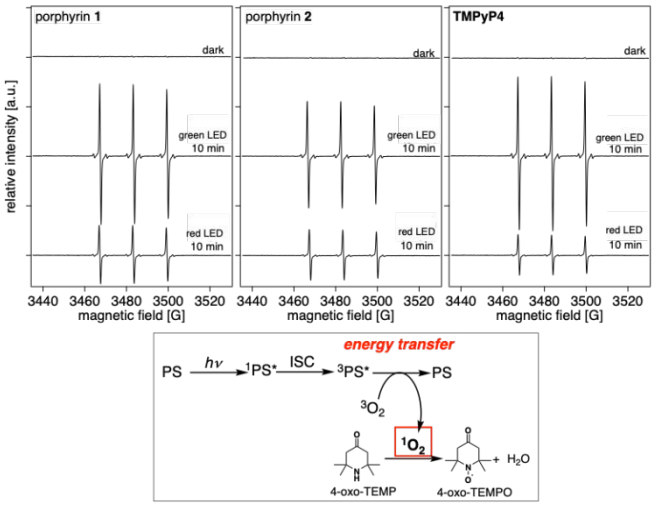

Fig. 3. X band ESR spectra of $^1O_2$ adduct of 4-oxo-TEMP observed under irradiation of visible light (green LED: 539-541 nm, 90±34% lm•W^{-1}; red LED: 616-626 nm, 30±37% lm•W^{-1}) for 10 min. Conditions: porphyrin: 50 μM; 4-oxo-TEMP: 80 mM in pH 7.4 PBS(–).

As we observed sufficient $^1O_2$ generation by these porphyrins under visible light (green and red), we moved to the test on the photoinduced damage to biomolecules (*e.g.* DNA). Such photoinduced DNA cleavage tests are often used as an initial assay to evaluate photosensitivity of the molecule for potential as PDT-PS drugs. Using the pBR322 supercoiled DNA as a substrate double-strand DNA (dsDNA), DNA photo-cleavage tests were carried out by co-incubation with each porphyrin at varied concentrations under visible light irradiation (527 nm green LED, 90±34% lm•W^{-1} or 630 nm red LED, 30±37% lm•W^{-1}) and subsequent gel electrophoresis analyses.

As shown in Fig. 4a, under light irradiation, DNA cleavage was observed by all porphyrins in a dose-dependent manner. Under green LED irradiation, DNA cleavage was observed in a similar range of concentrations for all porphyrins (**2** = **TMPyP4** > **1**, Fig. 4b), while both **1** and **2** showed enhanced DNA cleavage activity than **TMPyP4** under red light (**2** > **1** >> **TMPyP4**, Fig. 4c). In the presence of histidine, DNA cleavages by all three porphyrins were strongly reduced indicating that $^1O_2$ plays an important role in the DNA cleavage by these porphyrins (Fig. S41). This result was in line with the parallel data for higher $^1O_2$ generation and stronger DNA cleavage observed in porphyrins **1** and **2** in comparison to **TMPyP4**, confirming the essential role of $^1O_2$ in the photoinduced DNA cleavage by these porphyrins.

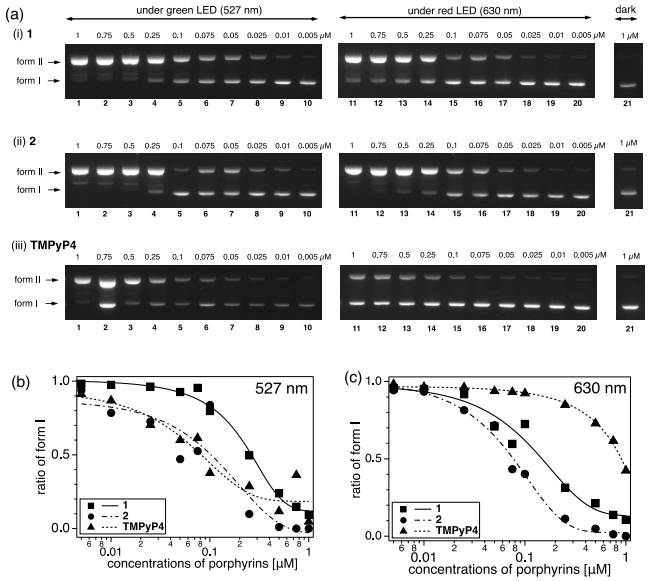

Fig. 4 (a) Photoinduced DNA cleavage of pBR322 DNA by **1**, **2** and **TMPyP4** under irradiation by LED light with a maximum at 527 nm (green, lanes 1-10) or at 630 nm (red, lanes 11-20) for 10 min. DNA: 12.5 μg•mL^{-1} in Tris-HCl-EDTA buffer (pH 8.0). (b, c) Ratio of form I intact DNA after photoirradiation (b: 527 nm, 230 mW•cm^{-2}; c: 630 nm, 255 mW•cm^{-2}) in the presence of varied concentration of **1**, **2**, or **TMPyP4**, quantified by ImageJ.

Photocytotoxicity

Following the significant $^1O_2$ generation and photoinduced DNA cleavage above, the porphyrins **1** and **2** were evaluated by photocytotoxicity tests. A cancer cell line (HeLa) and a normal cell line (NHDF cells) were used for the assays. Based on the standard methods,[47] cells were co-incubated with porphyrins at varied concentrations for 24 h then washed with PBS(–). There was no significant aggregation observed in the porphyrin solutions in medium after 24 h (Fig. S42 in the ESI). In the preliminary test, cellular uptake of the porphyrins was saturated at least after incubating for 24 h (Fig. S43 in the ESI). Subsequently, the cells were exposed to light irradiation by green (527 nm, 230 mW•cm^{-2}) or red (630 nm, 255 mW•cm^{-2}) LED and subjected to an MTT assay for viability after 3 h of incubation.

Under the dark condition, all porphyrins (**1**, **2**, and **TMPyP4**) showed no specific cytotoxicity up to at least 10 μM (Fig. 5) on either cell line. In contrast, under photoirradiation conditions, cell viability was significantly decreased in the presence of the porphyrins dose-dependently on both cell lines. Interestingly, porphyrins **1** and **2** showed much higher photocytotoxicity in comparison to control **TMPyP4**, under the irradiation of both green and red lights (Fig. 5), despite their similar $^1O_2$ generation (Fig. 3) and DNA cleaving activity (Fig. 4).

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Importantly, porphyrins **1** and **2** showed significantly higher photocytotoxicity on a cancer cell line (HeLa) than on a normal cell line (NHDF) especially under the irradiation of red light (Fig. 5a, b in the right column and Table 1). This interesting phenomenon of porphyrins **1** and **2** may be related to their properties (1) to internalize into cells better than **TMPyP4** or (2) to bind to G4 DNA, which are present more abundantly in the genome of cancer cells than in normal cells.

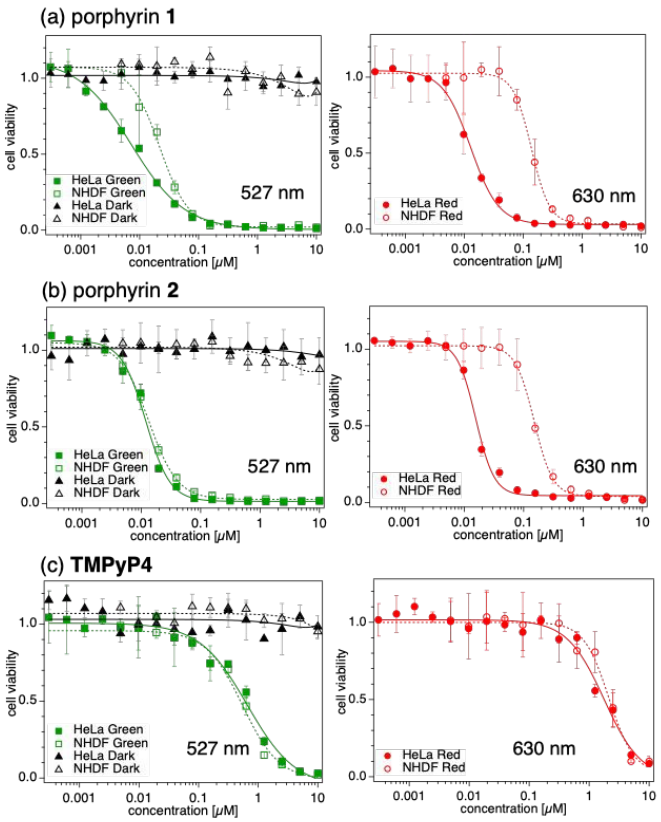

Fig. 5 Photocytotoxicities of porphyrins **1** (a), **2** (b) and **TMPyP4** (c) under the irradiation of green LED (max: 527 nm max, 230 mW•cm^{-2} for 15 min, left column) and red LED (max: 630 nm, 255 mW•cm^{-2} for 15 min, right columm) on HeLa and NHDF cells measured by MTT assay.

Table 1. IC_{50} values of photoinduced cytotoxicity of **1**, **2**, and **TMPyP4**. Values are obtained from Hill equation fitting of the data points shown in Fig. 5 by Igor Pro 9 software.

compounds	IC_{50} (SE) [nM]			
	HeLa		NHDF	
	Green	Red	Green	Red
1	7.2 (0.6)	12.8 (0.7)	21.8 (2)	138 (5.5)
2	11.9 (0.7)	15.6 (0.7)	13.4(0.9)	150 (4.4)
TMPyP4	657 (126)	1773 (376)	543 (67)	2130 (289)

Cellular uptake by flow cytometry and fluorescence microscopy

By taking advantage of the observation that porphyrins **1**, **2**, and **TMPyP4** are fluorescent compounds (Fig. 2), the cellular uptakes of these molecules to HeLa and NHDF were estimated by flow cytometry. The measurements were conducted with a laser excitation of 405 nm and detected with a filter of 678 - 706 nm based on the fluorescence spectroscopy with excitation wavelength at 405 nm, by confirming that the fluorescence intensities of compounds **1**, **2**, and **TMPyP4**, are similar in this detection wavelength range (Fig. S44). As a preliminary experiment, time-dependent cellular uptake of porphyrins was tested to confirm that the cellular uptake was saturated at 24 h of co-incubation.

Cells were incubated in the presence of each porphyrin (10 μM) for 24 h before being subjected to flow cytometry analyses. As shown in Fig. 6, cellular uptakes of all porphyrins were clearly observed by flow cytometry. The fluorescence intensity from the cells treated with porphyrins were in the order porphyrin **1** > porphyrin **2** > **TMPyP4** in both cell lines and in line with the results from photocytotoxicity. In HeLa cells, the fluorescence intensities observed in the cells treated with **1** and **2** were, respectively, ca. 13 and 4 times higher than in the cells treated with **TMPyP4,** indicating higher cellular uptake of **1** and **2** presumably due to their larger hydrophobic cores giving more amphiphilic nature. The cells treated with compound **1** had significantly higher fluorescence intensity than **2** suggesting that guanidium arms facilitated the uptake of the molecules, possibly in similar mechanisms to that observed in the uptake of arginine rich peptides.[48] In NHDF cells treated with porphyrins **1** and **2**, fluorescence intensity was, respectively, ca. 8 and 4 times higher than the one treated with **TMPyP4**. When compared between HeLa and NHDF, the mean values of fluorescence intensity observed in NHDF cells were higher than those in HeLa cells under all conditions (including control without chemicals), which is likely due to the larger size of the NHDF cells compared to HeLa cells, as can be seen on the forward scatter analysis of the flow cytometry data (Figs. S45-52).

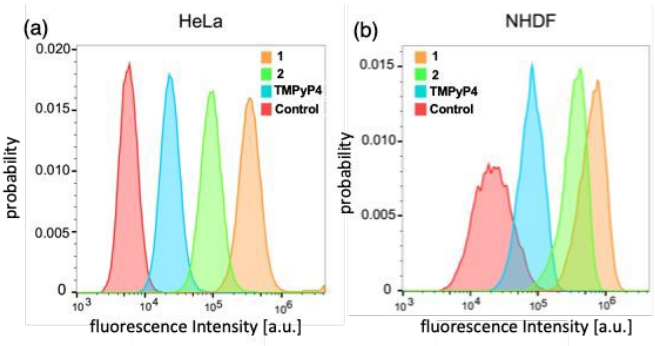

Fig. 6 Flow cytometry analyses of fluorescence emission after exposure to porphyrins 1, 2 and TMPyP4 (10 μM) in HeLa (a) and NHDF (b) cell lines. Cells were incubated with porphyrins for 24 hours and analysed with excitation wavelength of 405 nm and detection of emission 692 ± 14 nm. Mean values are (a) 399,000 (porphyrin **1**), 99,500 (porphyrin **2**), 26,000 (**TMPyP4**), 6180 (control), (b) 670,000 (porphyrin **1**), 359,000 (porphyrin **2**), 85,200 (**TMPyP4**), 25,700 (control).

Cellular uptake of the porphyrins was further confirmed by fluorescence microscopy of the HeLa and NHDF cells, incubated in the presence of each porphyrin (10 μM) for 24 h. The cells were fixed and subjected to the imaging using excitation (390/18 nm) and detection using a fluorescence filter of 700/75 nm. HeLa and NHDF cells treated with either compound **1** or compound **2** exhibited bright fluorescence, confirming porphyrin uptake (Figs. S53-S58).

Localization of porphyrin in permeabilized cells

It has been reported that cancer cells have more G4-forming domains in comparison to normal cells.[22, 23] To explain, at least in part, the higher photocytotoxicity by porphyrins **1** and **2** observed on HeLa cells in comparison to the NHDF cells, we tried to visualize the possible binding of porphyrins **1** and **2** to G4 domains using the cells that were fixed ahead of the exposure to porphyrins. Both HeLa and NHDF cells were subjected to permeabilization with 0.5% Triton X-100 and fixed with paraformaldehyde. Subsequently, the cells were co-incubated with porphyrins **1** and **2** (5 μM) and subjected to confocal microscopy imaging.

As shown in Fig. 7, in HeLa cells, porphyrin **1** was detected both in the cytoplasm and nuclei, with some enrichment in nucleoli, while **1** was detected mostly in the cytoplasm in NHDF. In contrast, porphyrin **2** was specifically detected in the nuclei with strong enrichment in nucleoli of Hela, while **2** showed strong intensity in the cytoplasm of NHDF cells with weak signals in the nuclei of some cells. These observations may suggest that both porphyrins **1** and **2** interact with G4 DNA or G4 RNA, which may be more enriched in nuclei of cancer cells than in normal cells. Cytoplasmic signals may represent their interaction with the mitochondrial DNA or cytoplasmic RNAs.

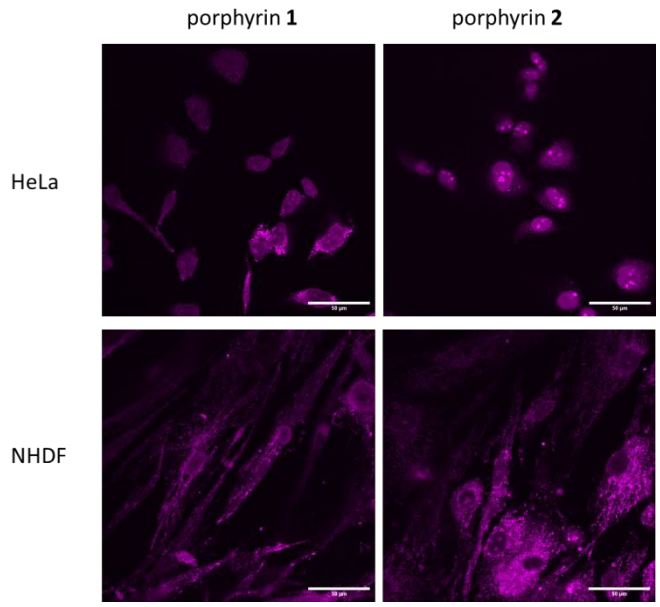

Fig. 7 Fluorescence microscopy images of HeLa cells and NHDF cells in the presence of porphyrins **1** and **2** (5 μM). Cells were premetallized and fixed prior to the addition of porphyrins.

Interaction of porphyrin 1 or 2 with telo24 DNA

To investigate more about the potential interaction of porphyrins and G4 DNA in cells, we investigated the G4-binding ability of **1** and **2** using G4 DNA in solution using **TMPyP4** as a standard. Possible interaction of **1** or **2** with G4 DNA was measured by fluorescence spectroscopy, FRET melting assay, UV-vis titration, and CD measurements. **TMPyP4**, a known G4-binder, was used as a standard, and the telo24 DNA (d(TTAGGG)$_4$), a human telomeric DNA sequence which **TMPyP4** binds, was used as a G4 DNA.[25]

Fluorescence Spectroscopy. To a solution of each porphyrin (**1**, **2**, or **TMPyP4**, 5 μM) in pH 7.4 HEPES buffer, telo24 G4 DNA was added at 0 to 15-25 μM. In the experiments with a known G4-binder, **TMPyP4** (Fig. 8c), increase in the emission at 660 nm was observed upon addition of telo24 in a good agreement with the previous reports, suggesting some change of local environment of **TMPyP4** caused by the binding to G4 DNA.[49, 50, 51] In the case of porphyrin **1**, upon addition of telo24 DNA, fluorescence intensity at 651 nm decreased dose-dependently (Fig. 8a). This could be explained by photo-induced electron transfer from the electron rich guanine to the porphyrin, similar to the previous reports on the fluorescence quenching by DNA.[52, 53] The fluorescence intensity of porphyrin **2** at 648 nm showed an initial decrease upon addition of lower concentrations of DNA and a subsequent increase at higher concentrations of DNA with a slight red shift (650 nm), with a somewhat similar tendency as **TMPyP4**, indicating similar interaction modes between **TMPyP4** and **2**.

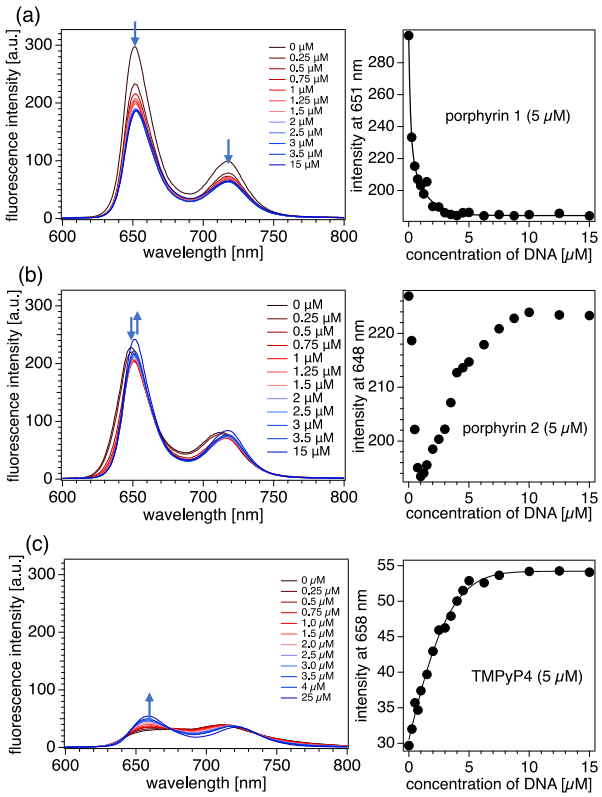

Fig. 8 Fluorescence spectra of **1** (a), **2** (b) and **TMPyP4** (c) (5 μM) in 10 mM HEPES, (pH 7.4 with 1 mM Na_2EDTA and 100 mM KCl) in the presence of telo24 G4 DNA (0-15 μM). Excitation wavelength: 423 nm for **1**, 420 nm for **2** and 432 nm for **TMPyP4**.

FRET melting assay. Based on the fluorescence measurements above indicating possible interaction of porphyrin **1** and **2** with telo24 G4 DNA (Fig. 8), the G4 DNA-stabilization abilities of the porphyrins were evaluated by a FRET melting assay. To a telo24 G4 FRET probe, functionalized

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with 6-carboxylfluorescein (FAM) at 5'-end and tetramethyl rhodamine (TAMRA) at 3'-end, each porphyrin (**1**, **2**, or **TMPyP4**) was added and subjected to fluorescence intensity measurements at 510-530 nm (with wavelength of 450-480 nm) at varied temperatures. Upon temperature increase from 25 to 100 °C, the FAM emission derived from destabilized G4 probes was increased corresponding to quenching of FRET signals (Fig. S59).

In the presence of G4 stabilizers, this increase is supressed. As shown in Fig. 9a, both porphyrins **1** and **2** showed dose-dependent stabilization effects of G4 DNA at concentrations above 1.3 and 0.16 μM, respectively. While porphyrin **1** required higher concentration to stabilize G4 DNA, G4 stabilization by **2** was more efficient than **1,** with efficiency similar to that of **TMPyP4** (Fig. 9a).

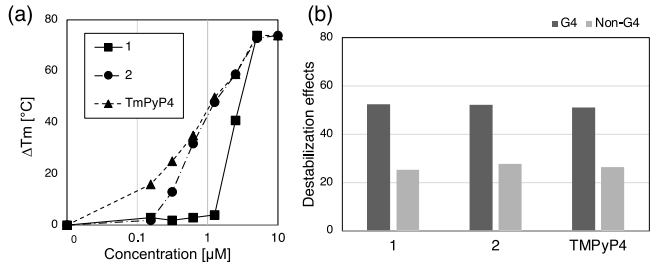

Fig. 9 (a) The stabilization of G4 DNA by porphyrins, analyzed by FRET assay. The change of Tm values (ΔTm) of telo24 G4 DNA in the presence of the porphyrin **1**, **2** or **TMPyP4** compared to those without the compounds was plotted. (b) The selectivity for G4 DNA of porphyrins analyzed by competition FRET assay. The normalized FAM emission signals of labeled G4 probe in the presence of competitor telo24 G4 DNA (G4) or telo24 mut (non-G4) were shown.

Since many G4-binders interact with not only with G4 DNA but also non-G4 DNA, the specificity of each porphyrin in stabilizing G4 over non-G4 DNA was investigated by a competitive FRET test. The non-labelled competitor, (1) telo24 G4 DNA (a G4 competitor) or (2) ssDNA with telo24 mutant sequence (a non-G4 competitor), was added to the FRET assay system and the stabilization effect by the porphyrins was evaluated. The G4 stabilization was decreased significantly by the addition of competitor G4 DNA but not by non-G4 DNA (Fig. S60). Notably, in the presence of the G4 competitor, stronger effects of G4 destabilization were observed in comparison to the case of non-G4 competitor (Figs. S60-S61). At the temperatures with highest differences in destabilization between G4 and non-G4 competitors (47, 40, and 25 ºC respectively for **1**, **2**, and **TMPyP4**) (Fig. S62), the destabilization effects by the competitors were quantified. As a result, in the presence of **1**, the destabilization effect of G4 and non-G4 competitors were 52% and 25%, respectively, indicating the stabilization selectivity of **1** with G4 over non-G4 was 2.1 times larger (Fig. 9b and Table S2). Similarly, the stabilization selectivity of **2** with G4 over non-G4 was 1.9 times larger, estimated from the destabilization effect by the G4 competitor (53%) and non-G4 competitor (28%). The results for the standard compound **TMPyP4** (1.9 times) was in line with a previous report,[54] indicating that porphyrins **1** and **2** present G4 stabilization effects with selectivity for G4 DNA at a level similar to that shown by **TMPyP4**.

UV-vis titration. To obtain more insight into the interactions of porphyrins **1** and **2** with G4 DNA, we employed a UV-vis titration assay. Measurements were performed with 5 μM porphyrin solution in pH 7.4 HEPES buffer in the presence of varied concentrations of telo24 DNA (0 to 15 μM) (Fig. 10). Upon addition of telo24 DNA, a significant red shift at the Soret band of the porphyrins was observed in all compounds, indicating that all three porphyrins had some interaction with telo24 DNA. As a result, compound **1** revealed a bathochromic shift of the Soret band (from 419 to 422 nm) with hypsochromism upon addition of telo24 DNA (Fig. 10a). Compound **2** and **TMPyP4** showed a similar pattern with changes of the Soret band (416 nm and 422 nm), which decreased in the presence of lower concentrations (0 – 2 μM) of telo24 DNA and displayed an increase of new peaks (424 nm for **2** and at 437 nm for **TMPyP4**) at higher concentration (≥ 3 μM) (Fig. 10b,c). These results suggest that there is some difference in the binding mode of porphyrin **1** *versus* porphyrin **2** and **TMPyP4** to the telo24 DNA. Both porphyrin **2** and **TMPyP4** revealed an isosbestic point respectively at 420 and 432 nm, while **TMPyP4** showed a higher red shift than porphyrin **2** in the presence of Telo24. The spectral pattern of **TMPyP4** with G4 DNA was in line with previous reports.[49, 55]

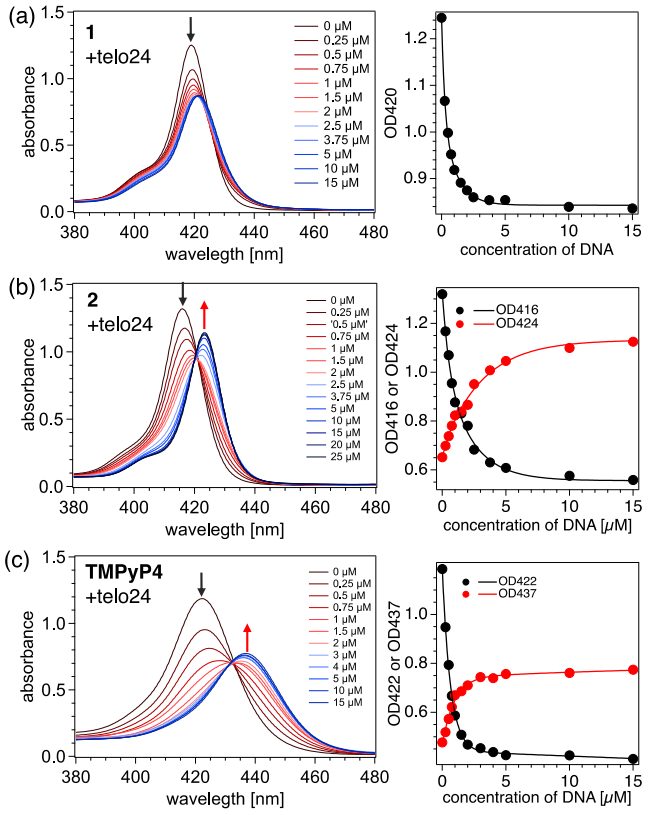

Fig. 10 UV-vis absorption spectra at around Soret band of **1** (a), **2** (b) and **TMPyP4** (c) (5 μM) in 10 mM HEPES (pH 7.4 with 1 mM Na_2EDTA and 100 mM KCl) in the presence of telo24 G4 DNA (0-15 μM).

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From the UV-vis titration data, K_d values for **1**, **2** and **TMPyP4** with Telo24 DNA were calculated (Figs. S63-64) and listed in Table 2.[56] It was observed that **2** showed relatively lower K_d value with slower dissociation kinetics, indicating stronger affinity towards the telo24 DNA, whereas **1** and **TMPyP4** had similar K_d values. Selectivity in the K_d values of these porphyrins to G4 DNA over dsDNA were evaluated by titration studies performed using calf thymus (CT) DNA under the same conditions (Figs. S65-67). Calculated K_d values were much higher with CT DNA in comparison to telo24 DNA in all porphyrins. When comparing these porphyrins, higher K_d values were observed for both **1** and **2** in comparison to **TMPyP4**, indicating that the dissociation kinetics of **1** and **2** from CT DNA was much faster than **TMPyP4,** showing better G4 selectivity of **1** and **2** (Table 2).

Table 2. Summary of UV-vis titration data of porphyrins **1**, **2**, and **TMPyP4** with telo24**.** Values were obtained using linear regression on the binding model developed by Wolfe et al.[56] using GraphPad Prism 8 software.

compounds	λmax [nm]	K_d [μM] (SE)	
		telo24 DNA	CT DNA
1	419	0.75 (0.08)	67.7 (5.6)
2	416	0.34 (0.07)	64.8 (6.2)
TMPyP4	422	0.93 (0.18)	48.1 (5.2)

Circular dichroism. To investigate the effect of porphyrins on the topologies of G4-forming DNA, circular dichroism (CD) measurements were employed. It has been reported that human telomeric DNA is polymorphic and observed to form several topologies.[57, 58] Among them, 3+1 types of hybrid structures are most relevant in the presence of higher K^+ concentrations,[59] which is a similar condition to the one in the cells (ca. 140 mM).[60] In this study, the effect of porphyrins **1**, **2**, and **TMPyP4** on the topologies of telo24 DNA were investigated by CD studies under three conditions, (1) in the presence of 100 mM KCl, (2) in the presence of 100 mM NaCl, and (3) in the absence of K^+ and Na^+, that were supposed to provide different 3D topologies of G4. In the presence of K^+, a CD signal with a maximum at 295 nm and a minimum around 240 nm with two shoulders around 247 and 270 nm were observed for telo24 DNA (Fig. 11).[61] To this DNA, each porphyrin was added at varied concentrations.

As shown in Fig. 11a, the CD signal at 295 nm increased in the presence of 6.25 μM (0.5 equiv) of porphyrin **1**, while no additional increase was observed by further addition of **1** (≥12.5 μM). In the case of porphyrin **2**, on the other hand, enhanced CD band of telo24 at 295 nm was observed dose-dependently up to 4-5 equivalent addition of porphyrin (Fig. 11b), with simultaneous increase of the CD shoulder bands at 270 nm and decrease of the shoulder at 247 nm. These results suggest that the 3+1 hybrid topology of telo24 DNA was more stabilized in the presence of porphyrin **2**. Under the same concentrations, the addition of control **TMPyP4** resulted only in a slight decrease of the signal at 295 nm (Fig. 11c).

CD spectra measured in the presence of 100 mM NaCl are shown in Fig. S68. Under this condition, telo24 DNA forms an antiparallel conformation, indicated by characteristic signals at 295 nm (maximum) and at 265 nm (minimum).[58] Upon addition of porphyrin **1**, CD spectra of telo24 resulted in a slight decrease of the peak at 295 nm and slight increase of the peak at 265 nm (Fig. S68a). The addition of porphyrin **2**, resulted in the signal increases at 295 nm and decrease at 265 nm (Fig. S68b), suggesting the destabilization of telo24 in an antiparallel conformation by **2**. Addition of control **TMPyP4** caused the decreases both 295 nm and 265 nm signals (Fig. S68c). In the absence of K^+ and Na^+, all three porphyrins cause an increase of the 295 nm peak, especially in the case of compound **2**, presumably due to its ability to induce formation of G4 (Fig. S69).

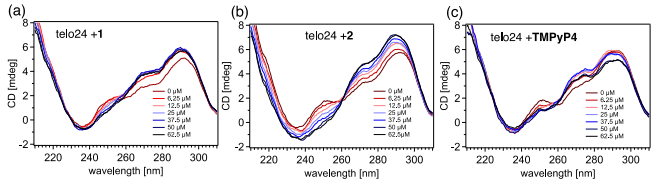

Fig. 11 CD spectra of telo24 DNA (12.5 μM) in the presence of 0 - 62.5 μM of porphyrin **1** (a), porphyrin **2** (b), and **TMPyP4** (c) in pH 7.4 Tris HCl buffer (50 mM) in the presence of 100 mM KCl and 1 mM Na_2EDTA

Experimental

Detection of ROS by ESR spin trapping reagents. ESR spectra were recorded on Bruker EMX, Continuous Wave X-Band EPR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Suprasil® ESR tube with a diameter of 4 mm, length of 250 mm and a wall thickness of 0.8 mm were used (SP Wilmad-LabGlass, NJ, USA). The 2,2,6,6-tetramethylpiperidin-4-one (4-oxo TEMP) was purchased from ABCR (Karlsruhe, Germany) and purified by sublimation prior to use. Irradiation was performed by green (539-541 nm, 90±34% lm•W^{-1}) or red LED light (616-626 nm, 30±37% lm•W^{-1}) from Lumiflex300 Pro RGB LED Stripes (LUMITRONIX LED-Technik GmbH, Hechingen, Germany), 120 LED lamps assembled in an aluminium cylindrical container with a diameter of 8.5 cm.

DNA photocleavage assay. A mixture of an aliquot (10 μL) of DNA solution (25 ng•μL^{-1} in Tris-HCl buffer) and **1**, **2** or **TMPyP4** solution in water (10 μL) with each concentration was irradiated in U-shape 96-well (round bottom) by Lumidox® II 96-well LED Array (Analytical Sales and Services, Inc., NJ, USA) equipped with either 527 nm max (230 mW•cm^{-2}) or 630 nm max LED (255 mW•cm^{-2}) for 10 min. Subsequently, Gel Loading Dye Purple (6X) (4 μL) was added to each well, and each mixture was analysed by electrophoresis (1% agarose in 0.5X TBE buffer) run at 100V for 80 min using 0.5X TBE as the running buffer. The gel was stained using GelRed® Nucleic Acid Stain for 1 hour and subjected to ChemiDoc Imaging System (Bio-Rad Laboratories, Inc., CA, USA). The images were analyzed using ImageJ software.

Photocytotoxicity Assay. Photocytotoxicity of **1**, **2** and **TMPyP4** was tested on HeLa and NHDF cell lines. HeLa and NHDF cells were purchased from ATCC (Manassas, VA, USA). Preincubated cells were harvested at log-growth-phase and cell suspension in growth medium (DMEM containing 10% FBS, 2

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mM glutamine, and 1% penicillin-streptomycin (100 µL)) was seeded to a 96-well plate (flat bottom) with a density of 1000 cells per well. After incubation for 24 h at 37 ºC with 5% CO_2 atmosphere, the medium of each well was exchanged with the porphyrin solutions in growth media and the cells were incubated for additional 24 h. Subsequently, cells were washed with PBS(–) and phenol red-free DMEM was added to each well. The cells in 96-well plates were subjected to photoirradiation using Lumidox® II 96-well LED Array by green LED (230 mW•cm^{-2}) or red LED (255 mW•cm^{-2}) for 15 min. After photoirradiation, DMEM medium in each well was exchanged with MTT solution in phenol red-free DMEM (0.5 mg•mL^{-1}, 100 µL) and cells were incubated for additional 3 h. Subsequently, the media was removed from each well and was replaced with DMSO (100 µL) to measure OD_{560} values to evaluate cell viabilities in relative to negative control (no chemical) and positive control (treated with Tween-20).

Flow cytometry. HeLa and NHDF cells at log-growth-phase were seeded in a 6-well plate with a density of 3x10^6 cells per well and incubated in growth medium for 24 h. Subsequently, the cells were incubated in the presence of 10 µM of each porphyrin for additional 24 h. Cells were treated with tripsin and centrifuged and obtained pellets were washed with PBS(–) for three times and resuspended in PBS(–) (500 µL) for measurement. Flow cytometry measurements were performed on Cytek® Aurora system (Cytek Biosciences, Fermont, CA, USA). Data were analyzed using FlowJo software with the V12 channel (excitation wavelength: 405 nm; emission wavelength: 692 nm (center) with 28 nm width).

Confocal microscopy. Both HeLa and NHDF cells were incubated in Gibco™ DMEM High Glucose containing 10% FBS and 1% penicillin-streptomycin. The cells at log-growth-phase were treated with tripsin and seeded in ibidi™ µ-Slide 8 Wells with a density of 10,000 cells in 250 µL. Subsequently, the cells were washed with PBS(–), fixed using 4% paraformaldehyde solution, and permeabilized by 0.5% Triton X-100 (1 min) and exposed to porphyrins (5 µM in PBS(–)) for 5 min. Cells were washed with PBS(–) and subjected to confocal fluorescence imaging in N_2-saturated PBS(–) on Microscope (Nikon TiE2) with Yokogawa Confocal Scanner Unit CSU-W1 (Excitation: 405 nm laser, emission filter: ET700/75).

Fluorescence spectroscopy. Fluorescence emission spectra were recorded on a Varian Cary Eclipse spectrophotometer (Agilent Technologies, Inc., Santa Clara, California, U.S.). Each solution of **1**, **2** or **TMPyP4** (5 µM) was prepared in 10 mM HEPES buffer (pH 7.4, containing 100 mM KCl and 1 mM Na_2EDTA). A solution of single strand telo24 DNA (500 µM) with the sequence of d(TTAGGGTTAGGGTTAGGGTTAGGG) was prepared in same buffer and subjected to the pre-annealing process by heating at 90 °C for 10 min and cooling back to room temperature over 3 h. To each porphyrin solution (2 mL) in a quartz cuvette (path length: 1 cm), an aliquot of the DNA solution was added and left to equilibrate for 2 min upon mixing to record fluorescence spectra.

FRET melting assay. G4 stabilization by porphyrins was tested by FRET assay using telo24 DNA labelled with 6-carboxyfluorescein (FAM) at 5”-end and tetramethyl-rhodamine (TAMRA) at 3’-end (Fasmac Co., Ltd., Kanagawa, Japan). The details are described in the ESI.

UV-Vis. UV Absorption spectra were recorded on a JASCO V-570 UV/VIS/NIR spectrophotometer (JASCO Co., Tokyo Japan). Each solution of **1**, **2** or **TMPyP4** (5 µM) was prepared in 10 mM HEPES buffer (pH 7.4, containing 100 mM KCl and 1 mM Na_2EDTA). A solution of single strand telo24 DNA (500 µM) with the sequence of d(TTAGGGTTAGGGTTAGGGTTAGGG) was prepared in same buffer and subjected to the pre-annealing process by heating at 90 °C for 10 min and cooling back to room temperature over 3 hours. To each porphyrin solution (2 mL) in a quartz UV cuvette (path length: 1 cm), an aliquot of the DNA solution was added and left to equilibrate for 2 min upon mixing to record UV-vis spectra. The titration was stopped when there was no change observed upon addition of DNA.

Circular dichroism. CD spectra were recorded on a Jasco J-1500 Circular Dichroism Spectrophotometer (JASCO Co., Tokyo Japan). High performance quartz cell with an optical path of 1 mm was used. Solutions of telo24 DNA (12.5 µM) in 10 mM Tris HCl buffer (pH 7.4) were prepared under three different conditions; containing (1) 100 mM KCl, (2) 100 mM NaCl or (3) without K^+ or Na^+. To the DNA solution in each buffer, an aliquot of compound **1**, **2** or **TMPyP4** was added to measure the CD spectrum.

Conclusions

Two types of cationic porphyrin derivatives, **1** and **2**, were designed and synthesized as bi-functional molecules with photosensitization and G4 DNA-binding activity. In comparison to a well-studied standard G4 binder **TMPyP4**, **1** and **2** exhibited similar $^1O_2$ generation and dsDNA cleavage. However, significantly enhanced photocytotoxicity was observed in **1** and **2** compared to **TMPyP4,** presumably due to better cellular internalization of the molecules. Interactions with telo24 G4 DNA were studied by spectroscopic methods, revealing similar levels of binding stability and slightly better selectivity with **1** and **2** compared to **TMPyP4**. Interestingly, stronger photocytotoxicity was observed in a cancer cell line (HeLa) in comparison to the normal cell line (NHDF) with all porphyrins (**1**, **2**, and **TMPyP4**) upon red light irradiation. This may be related to more abundant existence of G4 on the cancer cell genomes. This is in line with the localization of porphyrin molecules in cellular nuclei observed by fluorescence microscopy. Recently, we reported bifunctional Gd(III)- and Mn(III)-porphyrin molecules with photosensitization and relaxivity.[63, 64] Considering the excellent photosensitivity of porphyrins **1** and **2** in this study, with binding ability to human telomeric G4, these molecules can be considered as promising model compounds for further development as G4 targeting photosensitizers.

Author contributions

N.Y.-S., H.M., and Y.Y. designed the overall project. C.C. designed detailed structures of the molecules and contributed

to their synthesis and structural characterization in collaboration with Y.Y. N.K. performed FRET assay in collaboration with H.M. and N.Y.-S. C.C. performed fluorescence, UV vis, and CD spectroscopies. CC performed ROS generation assay by ESR, DNA cleavage tests, photocytotoxicity assay, and fluorescence microscopy analyses in collaboration with S.S.L and Y.Y. T.X performed flow cytometry assay in collaboration with C.C. and Y.Y. The manuscript was written with contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the ESI.†

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