Bioorganic & Medicinal Chemistry Letters 19 (2009) 1647–1649

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Porphyrins containing nitric oxide donors: Synthesis and cancer cell-oriented NO release

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ARTICLE INFO

Article history:
Received 24 October 2008
Revised 15 January 2009
Accepted 2 February 2009
Available online 7 February 2009

Keywords:
Porphyrin
Nitric oxide donors
Synthesis
Biological evaluation

ABSTRACT

Four novel porphyrins containing nitric oxide (NO) donors were synthesized, and the structures of all the products were characterized by IR, UV–vis, ^{1}H NMR, and elementary analysis. Interestingly, these new compounds not only were able to release NO, but also showed cancer cell-oriented accumulation. Higher accumulation of these new porphyrins containing NO donors in BEL-7402 liver cancer cells than in L-02 liver normal cells was corroborated by UV–vis spectroscopy. The biological activity of these porphyrins against BEL-7402 liver cancer cells was tested with a MTT assay. The studies indicated that they had more effective killing of BEL-7402 liver cancer cells than that of L-02 liver normal cells, and they had similar activity against MCF-7 breast cancer cells when compared to 5-fluorouracil in the absence of light.

Nitric oxide (NO) is a key signaling molecule involved in the regulation of many biological processes, such as regulation of blood pressure, and neurotransmission.[1,2] NO is generated either from L-arginine under the catalysis of NO synthase (NOS) or from synthetic releasing NO compounds, such as nitrate, furoxan, hydroxyguanidine, *S*-nitrosothiol, diazeniumdiolate, and others.[3,4] Recent studies have showed that NO appears to be critical for the tumoricidal activity of the immune system. High concentration of NO was cytotoxic and could induce the apoptosis of tumor cells, preventing tumors from metastasizing and assisting macrophage to kill tumor cells.[5–10] Increased scientific evidence supports that NO deficiency is implicated in many physiological and pathological processes within the mammalian body.[4] This fact provides a solid biologic basis for the application of NO replacement therapy in clinic. One of such use is to make a specific tissue-targeted delivery of NO releasing drugs to tumor cells apoptosis.[3–5] However, the development of NO donors with good tissue specificity is still very challenging.[11–13]

As porphyrin can selectively accumulate in tumor tissues,[14–17] it may serve as an ideal base for the design of new NO releasing compounds for the production of NO specifically in tumor tissues. We reasoned that porphyrins bearing moieties releasing nitric oxide could make the concentration of NO in tumor tissue higher than in surrounding normal tissue by the transport of porphyrins.[18,19] In addition, we have recently reported that some hybrid porphyrins have a better anticancer activity in the absence of light.[20,21]

In this Letter, we described the synthesis of four new porphyrins containing NO donors and their cancer cell-oriented release of NO. Furthermore, we also investigated the accumulation around the cancer cells and the preliminary anticancer activity of these new compounds.

The synthetic route to porphyrins bearing NO donors was outlined in Scheme 1. Parahydroxybenzaldehyde **1** was treated with dibromoalkanes bearing two to three carbons in K_2CO_3 and acetone at 56 °C to generate compound **2** in 63–67% yields[22], which was then reacted with $AgNO_3$ in CH_3CN to afford the corresponding nitrates **3** in good yields (70–78%).[23]

Initially, an attempt was made to synthesize porphyrins **4a–d** via the Adler–Longo method[24] by refluxing reactants in propionic acid. Different acids, such as HCOOH, CH_3COOH, and CH_3CH_2COOH were employed as refluxing solvents, and zinc acetate was used as template to stabilize the cyclic tetramer structure of intermediates. However, no product was detected. LC/MS analysis showed that the organic nitrates were decomposed in the presence of acid with high temperature. This indicates that the Adler–Longo method is not suitable for the synthesis of NO releasing porphyrins. In addition, we also found that the original reaction conditions of the Lindsey method[25] also could not afford porphyrin **4a–d**. Fortunately, the products were obtained when CF_3COOH was used as catalyst. The reaction of **3a** or **3b** with benzaldehyde or *p*-tolualdehyde with pyrrole in the ratio of 1:3:4 catalyzed by CF_3COOH smoothly afforded porphyrin **4a–d** in 6.7–8.2% yield.[26]

To examine whether the newly synthesized porphyrins containing NO donors could indeed release NO, the percentage of NO released in vitro from **4a** to **4d** upon incubation with L-cysteine for 1.5 h at 37 °C, was determined (Table 1).[27]

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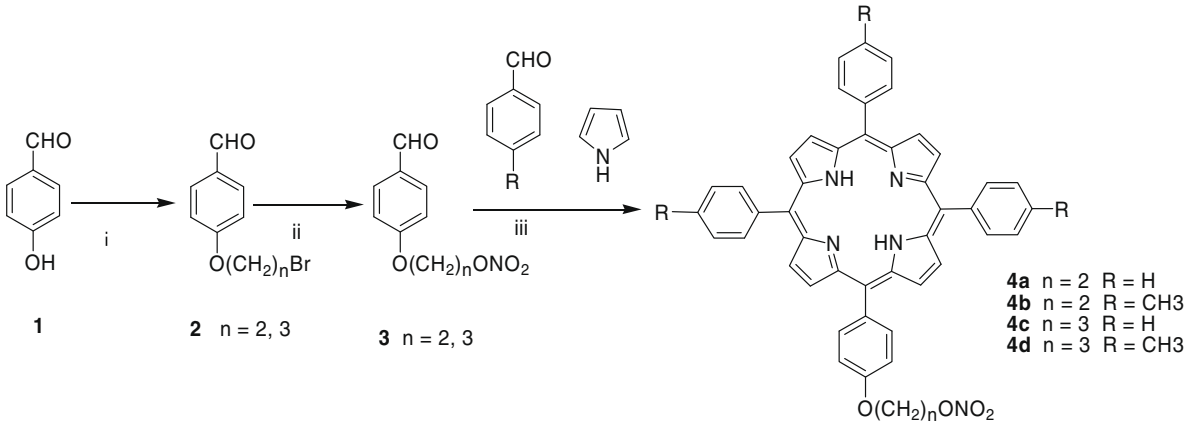
doi:10.1016/j.bmcl.2009.02.005



Scheme 1. Synthetic routes of porphyrins bearing NO donor outlined. Reagents and conditions: (i) Br $(CH_2)_n$Br, K_2CO_3, CH_3COCH_3, 56 °C, 20 h (**2a** 63%, **2b** 67%); (ii) CH_3CN, $AgNO_3$, reflux (**3a** 70%, **3b** 78%). (iii) (a) CF_3COOH, CH_2Cl_2, room temperature 12 h; (b) *p*-chloranil reflux 30 min (6.7–8.2%).

Table 1
Nitric oxide released from **4a** to **4d**[a]

Compound	% NO release in PBS[b]	% NO release in 18 μM L-cysteine[c]
4a	0.43 ± 0.01	5.67 ± 0.08
4b	0.55 ± 0.03	3.12 ± 0.04
4c	0.47 ± 0.03	3.95 ± 0.06
4d	1.10 ± 0.06	6.25 ± 0.07

[a] Percentage of nitric oxide released (mean value, $n = 3$) relative to a theoretical maximum release of 1 mol of NO/mol of test compound was determined using the Griess reaction.
[b] Incubated in phosphate buffer solution (PBS, pH 7.4) at 37 °C for 1.5 h.
[c] Incubated in the presence of 18 μM L-cysteine in phosphate buffer solution (pH 7.4) at 37 °C for 1.5 h.

It has been reported that a reduced thiol group such as L-cysteine, L-cysteamine, or glutathione is required for the release of NO from certain NO donor agents such as those containing an organic nitrates moiety.[28] The data of the percentage of NO release acquired in this study are consistent with the literature since the percentage of NO released from **4a** to **4d** were higher upon incubation in the presence of L-cysteine (3.12–6.67%) compared to that determined in phosphate buffer solution (PBS) at pH 7.4 (0.43–1.10%). The superior NO release of these compounds is in good agreement with the reported results that high dose of NO showed potent cytotoxicity on tumor cells.[6,7]

MTT cytotoxicity assay[29] was widely used to estimate the bioactivity of a drug molecule. Compounds **4a–d** and the reference compounds tetraphenylporphyrin and 5-fluorouracil, were evaluated for their tumor cell cytotoxicity using a MTT cytotoxicity assay[30] (Table 2). All compounds showed higher activity against BEL-7402 liver cancer cells when compared to 5-fluorouracil in the absence of light. Compound **4a** showed similar activity against MCF-7 breast cancer cells when compared to 5-fluorouracil. In contrast, tetraphenylporphyrin was significantly less active against tested cancer cell lines in the absence of light. This result is in accordance with the fact that tetraphenylporphyrin has low dark cytotoxicity.[21] Furthermore, these results suggested the cytotoxicity activity of these new compounds might come from the part of NO donors.

Table 2
Cytotoxicity of the target compounds against BEL-7402 and MCF-7 cells in vitro

Compound	Cytotoxicity (IC_{50}, μM)[a,b]	
	BEL-7402	MCF-7
4a	0.8 ± 0.04	8.7 ± 0.6
4b	1.8 ± 0.2	55 ± 4.9
4c	3.0 ± 0.1	30 ± 3.2
4d	1.4 ± 0.06	46 ± 3.1
Tetraphenylporphyrin	350 ± 18.3	245 ± 21.8
5-Fluorouracil	4.3 ± 0.7	4.7 ± 0.4

[a] The IC_{50} values represent the concentration which results in a 50% decrease in cell growth after 72 h incubation.
[b] Values are the means of three experiments.

An enhanced accumulation of compound **4a** in BEL-7402 liver cancer cells compared to L-02 liver normal cells was detected by UV–vis spectroscopy.[31] The absorbance of the cell culture fluid was detected after incubation of **4a** (100 μmol/L) for 30 min (Fig. 1). The absorbance of the cell culture fluid in BEL-7402 liver cancer cells decreased more when compared to the absorbance of the cell culture fluid in L-02 liver normal cells. These results showed that the new porphyrins selectively release NO in tumor cells.

To determine whether NO releasing derivatives of porphyrin exhibit different toxicity between tumor and normal cells, **4a–d** and tetraphenylporphyrin were evaluated toward L-02 liver normal cells and Bel 7402 liver cancer cells using a MTT based cell viability assay. As shown in Figure 2, **4a–d** showed more effective killing of BEL-7402 liver cancer cells than that of L-02 liver normal cells at the concentration of 1.4 μmol/L after 72 h of incubation. Compared with TPP, **4a–d** showed a statistical difference in viability ($p < 0.001$). Tetraphenylporphyrin exhibited less activity against tested cell lines in the absence of light and compounds **3a** and **3b** exhibited more toxicity toward L-02 liver normal cells than toward BEL-7402 liver cancer cells. This indicates that new porphyrins release more NO in the circumstance of cancer cells than in

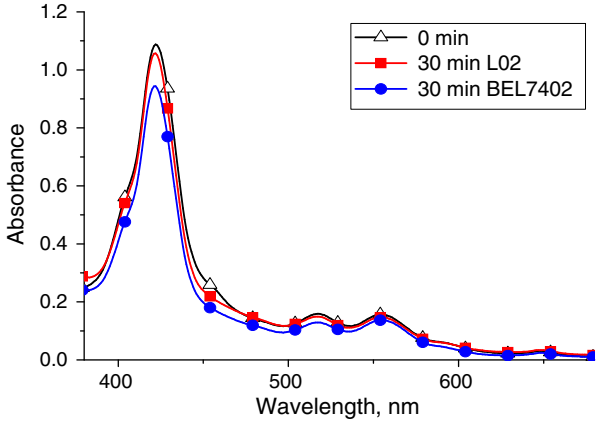


Figure 1. The absorbance of cell culture fluid after incubation of 100 μmol/L **4a** for 30 min.

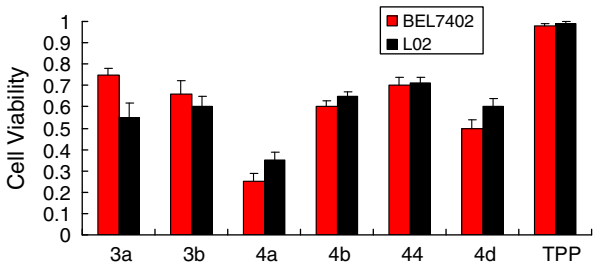


Figure 2. Cytotoxicity (data were subjected to one-way analysis of variance (ANOVA), followed by multiple comparisons with least significant differences (LSD) test. Statistical significance was considered with $P < 0.05$) of the target compounds between tumor and normal cells at the concentration of 1.4 μmol/L.

normal cells, and it is consistent with the finding that new compounds accumulate more in the circumstance of cancer cells than in normal cells.

In summary, a series of novel porphyrins releasing NO were synthesized and their biological activities were evaluated. All new compounds can release a high percentage of NO and have better accumulation toward cancer cells than toward normal cells. Consequently, they showed better activities towards cancer cells. We believe that they could be employed as promising agents in chemotherapy.

Acknowledgment

The authors gratefully thank the financial supports of National Natural Science Foundation of China (Grants CN J0830415).

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23. *Procedure of synthesis of substituted benzaldehyde* **3**. A mixture of **2** (5 mmol), silver nitrate (10 mmol), and acetonitrile (10 ml) was stirred at refluxed temperature for 8 h, and then filtered and concentrated. The product was purified with column chromatography (silica gel, 1:1, petroleum ether/ethyl acetate). 4-(2′-Nitrooxyethoxy)-benzaldehyde **3a**. Yield 70%; MS *m*/*z*: 212 $(M+H)^+$; ^1H NMR ($CDCl_3$, 400 MHz): δ 9.90 (1H, CHO), 7.86–7.84 (d, 2H, ArH), 7.02–7.00 (d, 2H, ArH), 4.86 (t, 2H, OCH_2, J = 4.4 Hz), 4.34 (t, 2H, OCH_2, J = 4.4 Hz), ppm. Anal. Calcd for $C_9H_9NO_5$: C, 51.19; H, 4.30; N, 6.63; O, 37.88. Found: C, 51.07; H, 4.44; N, 6.35; O, 37.54. 4-(3′-Nitrooxypropoxy)-benzaldehyde **3b**. Yield 78%; MS *m*/*z*: 226 $(M+H)^+$; ^1H NMR ($CDCl_3$, 400 MHz): δ 9.88 (1H, CHO), 7.85–7.82 (d, 2H, ArH), 7.01–6.99 (d, 2H, ArH), 4.68 (t, 2H, OCH_2, J = 6.4 Hz), 4.16 (t, 2H, OCH_2, J = 5.6 Hz), 2.26 (m, 2H, $-CH_2-$, J = 6.0 Hz), ppm. Anal. Calcd for $C_{10}H_{11}NO_5$: C, 53.33; H, 4.92; N, 6.22; O, 35.53. Found: C, 53.53; H, 4.72; N, 6.05; O, 37.50.
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26. *Procedure of synthesis of porphyrin* **4a**–**d**. The reactions were performed in a 250 mL three neck round bottomed flask fitted with a gas inlet port. The flask was charged with 100 mL of distilled CH_2Cl_2, 1.0 mmol of substituted benzaldehyde **3a** or **3b**, 3.0 mmol of benzaldehyde or *p*-tolualdehyde and 4 mmol of pyrrole. The resulting solution was magnetically stirred at room temperature and purged by nitrogen gas for 20 min. After addition of 2.5 mmol of CF_3COOH into the solution, the reaction flask was shielded from light for 12 h, then 1.6 mmol of *p*-chloranil was added at once. The solution was stirred for another 2–3 min before it was refluxed for 30 min in water bath. After cooling down to room temperature, 2 mmol of Et_3N was added to neutralize the acid. The solvent (CH_2Cl_2) was removed by rotary evaporation under vacuum. The crude product was purified by column chromatography with mixture eluent of $CHCl_3$ and petroleum ether. The second band was collected. The solvent was removed under vacuum to obtain the desired products. *meso*-5-(2′-Nitrooxyethoxyphenyl)-10,15,20-triphenylporphine (**4a**). Yield 8.2%; mp > 300 °C; MS *m*/*z*: 720 $(M+H)^+$; ^1H NMR ($CDCl_3$, 400 MHz): δ 8.84 (8H, pyrrolic), 7.27–8.23 (19H, ArH), 4.99 (t, 2H, OCH_2, J = 4.4 Hz), 4.52 (t, 2H, OCH_2, J = 4.4 Hz), −2.79 (2H, NH) ppm. Anal. Calcd for $C_{46}H_{33}N_5O_4$: C, 76.76; H, 4.62; N, 9.73; O, 8.89. Found: C, 76.72; H, 4.62; N, 9.70; O, 8.92; IR (KBr, cm^{-1}): 3317 (N–H), 1607, 1506, 1473 (C=C) cm^{-1}; UV–vis [λ_{max}, nm ($\varepsilon \times 10^{-3}$ cm^{-1} mol^{-1} L)] in CH_2Cl_2: 417.5, 515, 550, 589, 647.5. *meso*-5-(2′-Nitrooxyethoxyphenyl)-10,15,20-tri-(methylphenyl)porphyrin (**4b**). Yield 6.7%; mp > 300 °C; MS *m*/*z*: 762 $(M+H)^+$; ^1H NMR ($CDCl_3$, 400 MHz): δ 8.85 (8H, pyrrolic), 7.27–8.10 (16H, ArH), 5.01 (t, 2H, OCH_2, J = 4.4 Hz), 4.54 (t, 2H, OCH_2J = 4.4 Hz), 2.70 (s, 9H, CH_3), −2.81 (2H, NH) ppm. Anal. Calcd for $C_{49}H_{39}N_5O_4$: C, 77.25; H, 5.16; N, 9.19; O, 8.40. Found: C, 77.14; H, 5.11; N, 9.16; O, 8.43; IR (KBr, cm^{-1}): 3315 (N–H) 1627, 1505, 1463 (C=C) cm^{-1}; UV–vis [λ_{max}, nm ($\varepsilon \times 10^{-3}$ cm^{-1} mol^{-1} L)] in CH_2Cl_2: 418.5, 515, 552,591.5, 647. *meso*-5-(3′-Nitrooxypropoxyphenyl)-10,15,20-triphenylporphine (**4c**). Yield 7.2%; mp > 300 °C; MS *m*/*z*: 734 $(M+H)^+$; ^1H NMR ($CDCl_3$, 400 MHz): δ 8.83 (8H, pyrrolic), 7.27–8.22 (19H, ArH), 4.86 (t, 2H, OCH_2, J = 6.4 Hz), 4.37 (t, 2H, OCH_2, J = 6.0 Hz), 2.41 (m, 2H, $-CH_2-$, J = 6.0 Hz), −2.77 (2H, NH) ppm. Anal. Calcd for $C_{47}H_{35}N_5O_4$: C, 76.93; H, 4.81; N, 9.54; O, 8.72. Found: 76.90; H, 4.83; N, 9.56; O, 8.71; IR (KBr, cm^{-1}): 3317 (N–H), 1635, 1508, 1467 (C=C) cm^{-1}; UV–vis [λ_{max}, nm ($\varepsilon \times 10^{-3}$ cm^{-1} mol^{-1} L)] in CH_2Cl_2: 417.5, 515, 550.5, 589, 646. *meso*-5-(3′-Nitrooxypropoxyphenyl)-10,15,20-tri-(methylphenyl)porphyrin (**4d**). Yield 7.8%; mp > 300 °C; MS *m*/*z*: 776 $(M+H)^+$; ^1H NMR ($CDCl_3$, 400 MHz): δ 8.85 (8H, pyrrolic), 7.27–8.13 (16H, ArH), 4.85 (t, 2H, OCH_2, J = 6.4 Hz), 4.36 (t, 2H, OCH_2, J = 5.6 Hz), 2.70 (s, 9H, CH_3), 2.40 (m, 2H, $-CH_2-$, J = 6.0 Hz), −2.77 (2H, NH) ppm. Anal. Calcd for $C_{50}H_{41}N_5O_4$: C, 77.40; H, 5.33; N, 9.03; O, 8.24. Found: C, 77.46; H, 5.28; N, 9.00; O, 8.26; IR (KBr, cm^{-1}): 3311 (N–H) 1630, 1508, 1472 (C=C) cm^{-1}; UV–vis [λ_{max}, nm ($\varepsilon \times 10^{-3}$ cm^{-1} mol^{-1} L)] in CH_2Cl_2: 419, 481, 517.5, 552, 647.
27. *In vitro nitric oxide release assays*. (1) Incubation with 18 mM L-cysteine in phosphate buffer (pH 7.4). A solution of the test compound (1 mL of a 0.2 mM solution in 0.1 M phosphate buffer, pH 7.4) was mixed thoroughly with a freshly prepared solution of L-cysteine (1 mL of a 3.6 mM solution in 0.1 M phosphate buffer, pH 7.4), and the mixture was incubated at 37 °C for 1.5 h in the absence of air. After exposure to air for 10 min at 25 °C, an aliquot of the Griess reagent (1 mL) (freshly prepared by mixing equal volumes of 1.0% sulfanilamide and 0.1% *N*-naphthylethylenediamine dihydrochloride in water) was added to an equal volume (1 mL) of each test compound's incubation solution with mixing. After 10 min had elapsed, absorbance was measured at 540 nm using a Simadzu UV 2100 UV–vis scanning spectrophotometer. Solutions of 0–100 μM sodium nitrite were used to prepare a nitrite absorbance versus concentration curve under the same experimental conditions. The percent nitric oxide released (quantitated as nitrite ion) was calculated (±SEM, n = 3) from the standard nitrite versus concentration curve. (2) Incubation with phosphate buffer (pH 7.4). This assay was performed as described under procedure 1 above except that a solution of the test compound (2 mL of a 2 mM solution in 0.1 M phosphate buffer pH 7.4) was used and no L-cysteine was added.
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30. *In vitro cell cytotoxicity test (MTT assay)*. The cytotoxic effects of the compounds on Bel-7402, MCF-7 and L-02 cells were determined by using the MTT assay. Cells were planted in 100 μL medium at a concentration of 1×10^3 cells per well in 96-well microtiter plates. Plates had been incubated for 24 h at 37 °C under an atmosphere of air containing 5% CO_2. Medium (100 μL) containing the test drugs were added to quadruplicate wells and incubated for additional 72 h. The medium was then removed from the wells and 200 μL MTT (1 μg/mL in complete medium) was added to each well, and then incubated for another 4 h. The formazan crystals were dissolved in 100 μL dimethylsulfoxide buffered with 25 μL glycine–NaCl solution (0.1 M glycine, 0.1 M NaCl, pH 10.5). The absorbance was measured in an enzyme-linked immunoabsorbent assay plate reader (Bio-Rad) at a wavelength of 570 nm. The concentration required for 50% inhibition of cell viability (IC_{50}) was determined for the various compounds tested.
31. *In vitro accumulation assays test*. BEL-7402 and L-02 cell cultures were grown in 24-well plates at 37 °C in 5% CO_2 atmosphere until at least 70% confluency. The medium was removed and replaced by a 100 μmol/L drug-containing one (500 μL). After 0 and 30 min incubation at 37 °C in 5% CO_2 atmosphere, the drug-containing medium was removed and their absorbance was measured using a Simadzu UV 2100 UV–vis scanning spectrophotometer.