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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, cytotoxicity, and DNA-binding property of berberine derivatives

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Abstract 9-Substituted berberine derivatives (**4a–4f**) with polyethylene glycol side chain and terminal group were synthesized and characterized by elemental (C, H, and N) and spectral analysis (NMR, HRMS and FTIR). These compounds were tested for their in vitro cytotoxic activity against four human tumor cell lines: granulocyte leukemia (HL-60), gastrocarcinoma (BGC-823), carcinoma (Bel-7402), and nasopharyngeal carcinoma (KB) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The DNA-binding properties were investigated by UV–Vis absorption, fluorescence, CD spectroscopies, and thermal denaturation measurements. The results indicated that **4a–4f** exerted cytotoxic effects with selectivity against tested cell lines. **4a** exhibited higher cytotoxicity than cisplatin, berberine, and berberrubine against HL-60 and BGC-823 cell lines. The length of side chains and nature of terminal groups played an important role in the cytotoxicity. Berberine derivatives binded to CT-DNA in an intercalating mode. The binding affinities decreased with the increasing length of side chains. Compounds **4a–4c** and **4e** could change the DNA conformation from B to A-like form.

Keywords 9-Substituted berberine derivatives · Cytotoxicity · DNA binder

Introduction

Many diseases, including cancer, are often treated with drugs that bind to DNA and/or interfere with its biological functions. Unfortunately, toxic side effects always accompany treatment using DNA-binding drugs owing to their poor selectivity towards disease-affected cells. Modification of existing drugs or natural compounds has been proven to be a feasible way for developing more promising DNA-binding candidate compounds (Paterson and Anderson, 2005).

Berberine (Fig. 1), originating from Chinese herb Huanglian and many other plants, is an isoquinoline alkaloid which has been used over many generations as an antibiotic and disinfectant. Berberine displays a wide range of biochemical and pharmacological actions such as antibiotic, immunostimulant, antitumor, and antimotility properties, and is relatively nontoxic to humans (Lau *et al.,* 2001; Jantová *et al.,* 2003). In addition, berberine is proven to be a DNA-binding agent and has the ability to induce apoptosis in HL-60 cells (Kuo *et al.,* 1995; Chen *et al.,* 2005). X-ray data certified that the berberine bond to DNA by intercalation mode (Ferraroni *et al.,* 2011). The computer-aided modeling study on the berberine–DNA complex suggested that berberine binds to DNA from its C^5–C^6–N^+–C^8 side (Mazzini *et al.,* 2003). Berberine derivatives with substitutions at 9-position were prepared as chemical modified compounds to enhance the pharmacological

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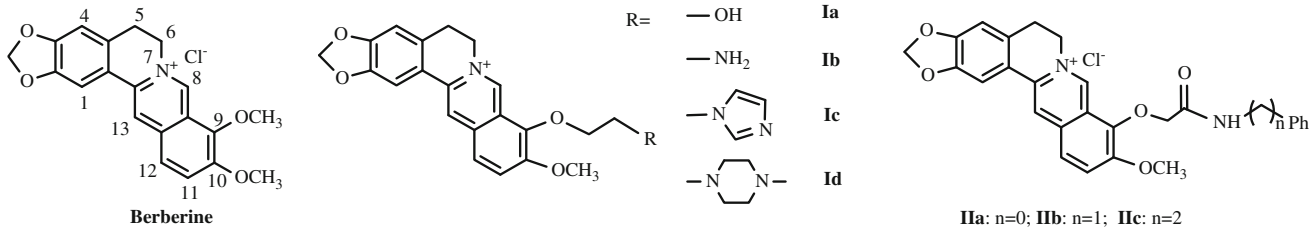


Fig. 1 Berberine and its 9-substituted derivatives

activities, as well as DNA-binding affinities. Compounds **Ia–Id** (Fig. 1) showed strong binding to CT-DNA than berberine (Pang *et al.,* 2005). Introduction of different spacers of alkyl ether chains and amino groups on the 9-position of berberine may enhance the DNA-binding affinity via hydrogen bonding and/or electrostatic interaction (Pang *et al.,* 2005). Basu *et al.* synthesized three 9-substituted berberine derivatives (**IIa–IIc**, Fig. 1), which displayed the DNA-binding affinity by more than six times compared with berberine (Basu *et al.,* 2012). All of the above prompted us to design and synthesize new 9-substituted berberine derivatives with different length of polyethylene glycol side chains and terminal groups (such as piperidine, morpholine), and investigate the DNA-binding affinity as well as their cytotoxicity.

Experimental

Materials and methods

All chemicals and reagents were of analytical grade. All reactions were carried out under N_2 using general grade solvents. Purification and handling of all compounds were carried out under air with a minimum exposure to light. Berberine was obtained from Si Chuan Hong Yi Sheng Wu Gong Cheng. LTD (purity: 97 %). CT-DNA was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Four different human carcinoma cell lines: HL-60 (granulocyte leukemia), BGC-823 (gastrocarcinoma), Bel-7402 (liver carcinoma), and KB (nasopharyngeal carcinoma) were obtained from American Type Culture Collection. Melting points were determined on a XT-4 microscopic melting point spectrometer and were uncorrected. Elemental analyses were determined on an Elementar Vario EL III elemental analyzer. The IR spectra were recorded using KBr pellets and a Perkin-Elmer Model-683 spectrophotometer. NMR spectra were recorded on a Bruker AVIII 600 NMR spectrometer using TMS as an internal standard in DMSO-d_6 or CD_3OD. The HRMS were recorded using a Bruker CVII high-resolution mass spectrometer. The optical density (OD) was measured on a microplate spectrophotometer (Bio-Rad Model 680, USA). The UV spectra and thermal denaturation experiment were measured in 50 mM Tris–HCl buffer (pH 6.35) using a UV-3600 UV–Vis spectrophotometer at room temperature. Fluorometric measurements were run on a Hitachi F-7000 PC spectrofluorophotometer. The CD spectra were recorded on a MOS-450/SFM300 spectropolarimeter.

Synthesis

*General procedures for the preparation of compounds **4a–4f***

Partial demethylation of berberine (**1**) at 190 °C under vacuum for 20 min, according to the reported procedure (Ouchi *et al.,* 1990; Matías *et al.,* 2007), afforded berberrubine (**2**) in 60 % yield. Treatment of **2** with oligoethyleneglycol ditosylate in DMF (Iwasa *et al.,* 1996) generated **3a**, **3b,** and **3c** in 41, 68, and 46 % yields, respectively.

To a stirred solution of **3a–3c** (0.2 mmol) in dry acetonitrile (5 mL), secondary amine (piperidine or morpholine, 2.0 mmol) and anhydrous K_2CO_3 (1.0 mmol) were added. The mixture was stirred at 80 °C for 4–6 h. Completion of the reaction was monitored by TLC. The mixture was then cooled to room temperature and filtered. The crude product was chromatographed on an Al_2O_3 column, eluted with CH_2Cl_2/MeOH to afford the proposed compounds **4a–4f** as yellow powders.

*9-O-2-(4-Morpholinyl)ethylberberine chloride (**4a**)*

Light yellow solid, yield 36 %; m.p. 194–195 °C; IR (KBr, cm^{-1}): 3,035 (C–H, Ar), 1,607–1,575 (C=C, Ar), 1,115–1,015 (C–O); ^{1}H-NMR (600 MHz, CD_3OD) δ 9.90 (s, 1H, H-8), 8.73 (s, 1H, H-13), 8.14 (d, 1H, $J = 9.0$ Hz, H-11), 8.04 (d, 1H, $J = 9.0$ Hz, H-12), 7.69 (s, 1H, H-1), 6.99 (s, 1H, H-4), 6.13 (s, 2H, OCH_2O), 4.97 (t, 2H, $J = 6.0$ Hz, H-6), 4.56 (t, 2H, $J = 6.0$ Hz, CH_2), 4.13 (s, 3H, OCH_3), 3.69 (t, 4H, $J = 4.8$ Hz, $2CH_2$), 3.29 (t, 2H, $J = 6.0$ Hz, H-5), 2.92 (t, 2H, $J = 6.0$ Hz, CH_2), 2.63 (t, 4H, $J = 4.8$ Hz, $2CH_2$); ^{13}C-NMR (150 MHz, CD_3OD) δ 150.81 (1C, C-10), 150.73 (1C, C-3), 148.57 (1C, C-2), 145.30 (1C, C-8), 143.26 (1C, C-9), 138.27 (1C, C-13a), 133.72 (1C, C-12a), 130.45 (1C, C-4a), 128.35 (1C, C-11),

125.55 (1C, C-12), 122.39 (1C, C-8a), 120.49 (1C, C-13b), 120.12 (1C, C-13), 108.00 (1C, C-4), 105.17 (1C, C-1), 102.28 (1C, O$\underline{C}H_2O$), 70.01 (1C, O$\underline{C}H_2$–CH_2–N), 66.37 (2C, $\underline{C}H_2$-O-$\underline{C}H_2$, morpholin), 58.02 (1C, O$\underline{C}H_3$), 56.21 (1C, C-6), 55.95 (1C, OCH$_2$–$\underline{C}H_2$–N), 53.38 (2C, $\underline{C}H_2$–N–$\underline{C}H_2$, morpholin), 26.84 (1C, C-5); HRMS (+)-ESI *m/z* [M-Cl]$^+$ calcd for $[C_{25}H_{27}N_2O_5]^+$: 435.19145; found: 435.19129; anal. calcd for $C_{25}H_{27}ClN_2O_5$: C, 63.76; H, 5.78; N, 5.95. Found: C, 63.71; H, 5.81; N, 5.92.

*9-O-2-(1-Piperidinyl)ethylberberine chloride (**4b**)*

Light yellow solid, yield 25 %; m.p. 194–196 °C; IR (KBr, cm^{-1}): 3,039 (C–H, Ar), 1,607–1,575 (C=C, Ar), 1,120–1,085 (C–O–C); ^{1}H-NMR (600 MHz, CD_3OD) δ 10.09 (s, 1H, H-8), 8.73 (s, 1H, H-13), 8.15 (d, 1H, $J = 9.0$ Hz, H-11), 8.05 (d, 1H, $J = 9.0$ Hz, H-12), 7.69 (s, 1H, H-1), 6.99 (s, 1H, H-4), 6.13 (s, 2H, OCH$_2$O), 4.99 (t, 2H, $J = 6.0$ Hz, H-6), 4.60 (t, 2H, $J = 6.0$ Hz, CH$_2$), 4.14 (s, 3H, OCH$_3$), 3.29 (t, 2H, $J = 6.0$ Hz, H-5), 3.13 (t, 2H, $J = 6.0$ Hz, CH$_2$), 2.83 (m, 4H, 2CH$_2$), 1.72 (m, 4H, 2CH$_2$), 1.58 (m, 2H, 2CH$_2$); ^{13}C-NMR (150 MHz, CD_3OD) δ 150.84 (1C, C-10), 150.58 (1C, C-3), 148.53 (1C, C-2), 145.24 (1C, C-8), 141.98 (1C, C-9), 138.47 (1C, C-13a), 133.75 (1C, C-12a), 130.61 (1C, C-4a), 126.00 (1C, C-11), 124.24 (1C, C-12), 121.79 (1C, C-8a), 120.40 (1C, C-13b), 120.12 (1C, C-13), 108.02 (1C, C-4), 105.19 (1C, C-1), 102.28 (1C, O$\underline{C}H_2O$), 67.70 (1C, O$\underline{C}H_2$-CH_2–N), 56.63 (1C, O$\underline{C}H_3$), 56.32 (1C, OCH$_2$-$\underline{C}H_2$–N), 55.77 (1C, C-6), 53.52 (2C, N($\underline{C}H_2CH_2$)$_2$, piperidinyl), 26.77 (1C, C-5), 22.94 (2C, N(CH$_2\underline{C}H_2$)$_2$, piperidinyl), 21.58 (1C, CH$_2$, piperidinyl); HRMS (+)-ESI *m/z* [M-Cl]$^+$ calcd for $[C_{26}H_{29}N_2O_4]^+$: 433.21218; found: 433.21196; anal. calcd for $C_{26}H_{29}ClN_2O_4$: C, 66.59; H, 6.23; N, 5.97. Found: C, 66.49; H, 6.18; N, 6.02.

*9-O-5-(4-Morpholinyl) diethylene glycol berberine chloride (**4c**)*

Light yellow solid, yield 43 %; m.p. 153–154 °C; IR (KBr, cm^{-1}): 3,035 (C–H, Ar), 1,605–1,573 (C=C, Ar), 1,113–1,014 (C–O–C); ^{1}H-NMR (600 MHz, CD_3OD) δ 9.83 (s, 1H, H-8), 8.75 (s, 1H, H-13), 8.14 (d, 1H, $J = 9.0$ Hz, H-11), 8.04 (d, 1H, $J = 9.0$ Hz, H-12), 7.69 (s, 1H, H-1), 6.99 (s, 1H, H-4), 6.13 (s, 2H, OCH$_2$O), 4.98 (t, 2H, $J = 6.0$ Hz, H-6), 4.58 (t, 2H, $J = 4.2$ Hz, CH$_2$), 4.13 (s, 3H, OCH$_3$), 3.91 (t, 2H, $J = 4.2$ Hz, CH$_2$), 3.71 (t, 2H, $J = 5.4$ Hz, CH$_2$), 3.66 (t, 4H, $J = 4.8$ Hz, 2CH$_2$), 3.30 (t, 2H, $J = 6.0$ Hz, H-5), 2.60 (t, 2H, $J = 5.4$ Hz, CH$_2$), 2.52 (t, 4H, $J = 4.8$ Hz, 2CH$_2$); ^{13}C-NMR (150 MHz, CD_3OD) δ 150.83 (1C, C-10), 150.74 (1C, C-3), 148.57 (1C, C-2), 145.11 (1C, C-8), 143.27 (1C, C-9), 138.30 (1C, C-13a), 133.78 (1C, C-12a), 130.46 (1C, C-4a), 126.56 (1C, C-11), 123.32 (1C, C-12), 122.38 (1C, C-8a), 120.45 (1C, C-13b), 120.15 (1C, C-13), 108.02 (1C, C-4), 105.16 (1C, C-1), 102.28 (1C, O$\underline{C}H_2O$), 72.95 (1C, Ar–O–CH$_2\underline{C}H_2O$), 69.92 (1C, Ar–O–$\underline{C}H_2CH_2O$), 67.73 (1C, O$\underline{C}H_2$–CH_2–N), 66.06 (2C, $\underline{C}H_2$–O–$\underline{C}H_2$, morpholin), 57.79 (1C, O$\underline{C}H_3$), 56.29 (1C, C-6), 56.01 (1C, OCH$_2$–$\underline{C}H_2$–N), 53.61 (2C, $\underline{C}H_2$–N–$\underline{C}H_2$, morpholin), 26.85 (1C, C-5); HRMS (+)-ESI *m/z* [M-Cl]$^+$ calcd for $[C_{27}H_{31}N_2O_6]^+$: 479.21766; found: 479.21742; anal. calcd for $C_{27}H_{31}ClN_2O_6$: C, 62.97; H, 6.07; N, 5.44. Found: C, 62.86; H, 6.14; N, 5.37.

*9-O-5-(4-Piperidine)diethylene glycol berberine chloride (**4d**)*

Light yellow solid, yield 29 %; m.p. 127–129 °C; IR (KBr, cm^{-1}): 3,035 (C–H, Ar), 1,610–1,575 (C=C, Ar), 1,117–1,088 (C–O–C); ^{1}H-NMR (600 MHz, CD_3OD) δ 9.82 (s, 1H, H-8), 8.74 (s, 1H, H-13), 8.14 (d, 1H, $J = 9.0$ Hz, H-11), 8.05 (d, 1H, $J = 9.0$ Hz, H-12), 7.68 (s, 1H, H-1), 6.98 (s, 1H, H-4), 6.12 (s, 2H, OCH$_2$O), 5.02 (t, 2H, $J = 6.0$ Hz, H-6), 4.59 (t, 2H, $J = 4.8$ Hz, CH$_2$), 4.12 (s, 3H, OCH$_3$), 4.00 (t, 2H, $J = 4.8$ Hz, CH$_2$), 3.93 (t, 2H, $J = 4.8$ Hz, CH$_2$), 3.29 (t, 2H, $J = 6.0$ Hz, H-5), 3.21–3.15 (m, 2H, 2CH$_2$), 3.11 (s, 2H, CH$_2$), 1.83–1.78 (m, 4H, 2CH$_2$), 1.62 (s, 2H, CH$_2$), 1.35–1.26 (m, 2H, CH$_2$); ^{13}C-NMR (150 MHz, CD_3OD) δ 150.82 (1C, C-10), 150.66 (1C, C-3), 148.54 (1C, C-2), 145.03 (1C, C-8), 143.15 (1C, C-9), 138.39 (1C, C-13a), 133.82 (1C, C-12a), 130.55 (1C, C-4a), 126.43 (1C, C-11), 123.45 (1C, C-12), 122.24 (1C, C-8a), 120.48 (1C, C-13b), 120.19 (1C, C-13), 108.00 (1C, C-4), 105.19 (1C, C-1), 102.26 (1C, O$\underline{C}H_2O$), 72.95 (1C, Ar–O–$\underline{C}H_2CH_2O$), 70.06 (1C, Ar–O–CH$_2\underline{C}H_2O$), 65.37 (1C, O$\underline{C}H_2$–CH_2–N), 56.65 (1C, O$\underline{C}H_3$), 56.30 (1C, OCH$_2$–$\underline{C}H_2$–N), 56.01 (1C, C-6), 53.65 (2C, N($\underline{C}H_2CH_2$)$_2$, piperidinyl), 26.86 (1C, C-5), 23.20 (2C, N(CH$_2\underline{C}H_2$)$_2$, piperidinyl), 21.81 (1C, CH$_2$, piperidinyl); HRMS (+)-ESI *m/z* [M-Cl]$^+$ calcd for $[C_{28}H_{33}N_2O_5]^+$: 477.23840; found: 477.23792; anal. calcd for $C_{28}H_{33}ClN_2O_5$: C, 65.55; H, 6.48; N, 5.46. Found: C, 65.43; H, 6.53; N, 6.40.

*9-O-8-(4-Morpholinyl)triethylene glycol berberine chloride (**4e**)*

Light yellow solid, yield 16 %; m.p. 152–154 °C; IR (KBr, cm^{-1}): 3,035 (C–H, Ar), 1,604–1,572 (C=C, Ar), 1,117–1,040 (C–O–C); ^{1}H-NMR (600 MHz, CD_3OD) δ 9.87 (s, 1H, H-8), 8.75 (s, 1H, H-13), 8.15 (d, 1H, $J = 9.0$ Hz, H-11), 8.05 (d, 1H, $J = 9.0$ Hz, H-12), 7.69 (s, 1H, H-1), 7.00 (s, 1H, H-4), 6.13 (s, 2H, OCH$_2$O), 4.99 (t, 2H, $J = 6.6$ Hz, H-6), 4.55 (t, 2H, $J = 4.2$ Hz, CH$_2$), 4.13 (s, 3H, OCH$_3$), 3.90 (t, 2H, $J = 4.8$ Hz, CH$_2$), 3.64 $\sim$ 3.61 (m, 6H, 3CH$_2$), 3.59 (t, 2H, $J = 6.0$ Hz, CH$_2$), 3.30 (t, 2H, $J = 6.6$ Hz, H-5); ^{13}C-NMR (150 MHz, CD_3OD) δ 150.96 (1C, C-10), 150.81 (1C, C-3),

148.57 (1C, C-2), 145.43 (1C, C-8), 143.20 (1C, C-9), 138.19 (1C, C-13a), 133.67 (1C, C-12a), 130.47 (1C, C-4a), 126.35 (1C, C-11), 123.47 (1C, C-12), 122.48 (1C, C-8a), 120.47 (1C, C-13b), 120.10 (1C, C-13), 108.04 (1C, C-4), 105.17 (1C, C-1), 102.30 (1C, O$\underline{C}H_2O$), 73.10 (1C, Ar–O–$\underline{C}H_2CH_2O$), 69.90 (1C, Ar–O–$CH_2\underline{C}H_2O$), 69.77 (1C, Ar–O–CH_2CH_2O–$\underline{C}H_2$–CH_2O), 69.71 (1C, Ar–O–CH_2CH_2O–CH_2–$\underline{C}H_2O$), 67.51 (1C, O$\underline{C}H_2$–CH_2–N), 66.01 (2C, $\underline{C}H_2$–O–$\underline{C}H_2$, morpholin), 57.57 (1C, O$\underline{C}H_3$), 56.23 (1C, C-6), 55.97 (1C, OCH_2–$\underline{C}H_2$–N), 53.56 (2C, $\underline{C}H_2$–N–$\underline{C}H_2$, morpholin), 26.85 (1C, 5-C); HRMS (+)-ESI *m/z* $[M\text{-}Cl]^+$ calcd for $[C_{29}H_{35}N_2O_7]^+$: 523.24388; found: 523.24352; anal. calcd for $C_{29}H_{35}ClN_2O_7$: C, 62.30; H, 6.31; N, 5.01. Found: C, 62.18; H, 6.38; N, 4.97.

*9-O-8-(4-Piperidine)triethylene glycol berberine chloride (**4f**)*

Light yellow solid, yield 18 %; m.p. 151–152 °C; IR (KBr, cm^{-1}): 3,039 (C–H, Ar), 1,601–1,569 (C=C, Ar), 1,117–1,046 (C–O–C); ^{1}H-NMR (600 MHz, CD_3OD) δ 9.82 (s, 1H, H-8), 8.75 (s, 1H, H-13), 8.15 (d, 1H, $J = 9.0$ Hz, H-11), 8.06 (d, 1H, $J = 9.0$ Hz, H-12), 7.69 (s, 1H, H-1), 6.99 (s, 1H, H-4), 6.13 (s, 2H, OCH_2O), 4.99 (t, 2H, $J = 6.0$ Hz, H-6), 4.56 (t, 2H, $J = 4.8$ Hz, CH_2), 4.13 (s, 3H, OCH_3), 3.98 (t, 2H, $J = 4.2$ Hz, CH_2), 3.88 (t, 2H, $J = 4.8$ Hz, CH_2), 3.80 (t, 2H, $J = 5.4$ Hz, CH_2), 3.74 (t, 2H, $J = 5.4$ Hz, CH_2), 3.62–3.58 (m, 2H, CH_2), 3.31 (t, 2H, $J = 6.0$ Hz, H-5), 3.04–2.97 (m, 2H, CH_2), 1.94–1.78 (m, 6H, $3CH_2$), 1.38 (t, 2H, $J = 6.0$ Hz, CH_2); ^{13}C-NMR (150 MHz, CD_3OD) δ 150.87 (1C, C-10), 150.85 (1C, C-3), 148.59 (1C, C-2), 145.00 (1C, C-8), 143.25 (1C, C-9), 138.42 (1C, C-13a), 133.90 (1C, C-12a), 130.52 (1C, C-4a), 126.64 (1C, C-11), 123.52 (1C, C-12), 122.39 (1C, C-8a), 120.46 (1C, C-13b), 120.22 (1C, C-13), 108.03 (1C, C-4), 105.20 (1C, C-1), 102.27 (1C, O$\underline{C}H_2O$), 73.12 (1C, Ar–O–$\underline{C}H_2CH_2O$), 70.03 (1C, Ar–O–$CH_2\underline{C}H_2O$), 69.96 (1C, Ar–O–CH_2CH_2O–$\underline{C}H_2$-CH_2O), 69.83 (1C, Ar–O–CH_2CH_2O–CH_2–$\underline{C}H_2O$), 64.33 (1C, O$\underline{C}H_2$-CH_2–N), 56.39 (1C, O$\underline{C}H_3$), 56.12 (1C, OCH_2–$\underline{C}H_2$-N), 56.10 (1C, C-6), 53.37 (2C, N($\underline{C}H_2CH_2)_2$, piperidinyl), 26.89 (1C, 5-C), 22.60 (2C, N($CH_2\underline{C}H_2)_2$, piperidinyl), 21.19 (1C, CH_2, piperidinyl); HRMS (+)-ESI *m/z* $[M\text{-}Cl]^+$ calcd for $[C_{30}H_{37}N_2O_6]^+$: 521.26461; found: 521.26414; anal. calcd for $C_{30}H_{37}ClN_2O_6$: C, 64.68; H, 6.69; N, 5.03. Found: C, 64.53; H, 6.74; N, 5.05.

Determination of in vitro cytotoxic activity

Cell culture

Four different human carcinoma cell lines: Bel-7402, KB, HL-60, and BGC-823 were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum, 100 units/mL of penicillin and 100 mg/mL of streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 in air.

Solutions

The compounds were dissolved in DMSO at a concentration of 5 mM as stock solution, and diluted in culture medium at concentrations of 0.1, 1.0, 10, and 100 mM as working solution. To avoid DMSO toxicity, the concentration of DMSO was less than 0.1 % (v/v) in all experiments.

Cytotoxicity analysis

The cells harvested from exponential phase were seeded equivalently into a 96-well plates, and then the compounds were added to the wells to achieve final concentrations. Control wells were prepared by addition of culture medium. Wells containing culture medium without cells were used as blanks. All experiments were performed in quintuplicate. The MTT assay was performed as described by Mosmann (1983). Upon completion of the incubation for 48 h, stock MTT dye solution (20 μL, 5 mg/mL) was added to each well. After 4-h incubation, DMSO (100 μL) was added to solubilize the MTT formazan. The optical density (OD) of each well was measured on a microplate spectrophotometer at wavelength of 570 nm. The IC_{50} value was determined from plot of percentage viability against dose of compounds added.

DNA-binding study

UV–Vis

Absorption spectra were conducted using a quartz cell of 1-cm optical path length. The DNA and compound-DNA samples were dissolved in Tris–HCl buffer (50 mM, pH 6.35) and incubated at room temperature for 2 h. The concentrations of compounds were fixed at 20 μM, and the concentrations of DNA varied from 0 to 400 μM. The absorbance of samples was detected on UV-3600 spectrophotometer during the wavelength range of 300–510 nm.

Fluorescence

Fluorescence spectra were conducted using a quartz cell of 1-cm optical path length. The DNA and compound-DNA samples were dissolved in Tris–HCl buffer (50 mM, pH 6.35) and incubated at room temperature for 2 h. The concentrations of compounds were fixed at 20 μM, and the concentrations of DNA varied from 0 to

400 μM. The fluorescence intensity of samples was detected on F-7000 spectrophotometer during the wavelength range of 425–690 nm (λ_{ex} = 355 nm).

Circular dichroism

CD spectra were recorded using a quartz cell of 1-cm optical path length and an instrument scanning speed of 100 nm/min with a response time of 1 s, using 0.5 points/s from 220 to 510 nm and 1 nm bandwidth. The CD spectra were obtained by taking the average of two scans made from 220 to 510 nm. The DNA and compound-DNA samples were dissolved in Tris–HCl buffer (50 mM, pH 6.35) and incubated at room temperature for 2 h. The compounds were dissolved by Tris–HCl buffer (50 mM, pH 6.35) and titrated into the DNA samples at 0.5 mol equiv up to 20 mol equiv.

Thermal melting

Melting curves of DNA and berberine derivative-DNA complexes were measured in Tris–HCl buffer (50 mM, pH 6.35). The CT-DNA and compound-DNA complexes solutions were added into a quartz cell of 1-cm optical path length. The temperature was ramped from 35 to 45 °C at a scan rate of 2 °C/s and 45–97 °C at a scan rate of 1 °C/s monitoring the absorbance changes of DNA at 260 nm.

Results and discussion

Chemistry

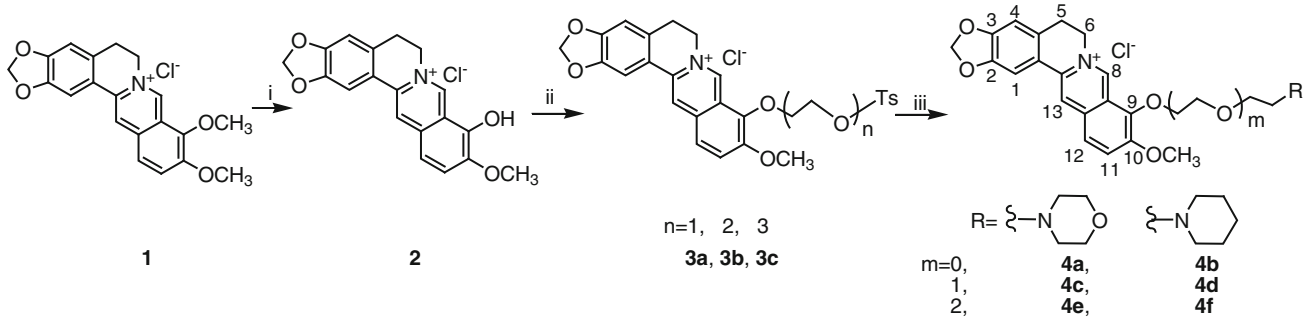
The synthesis of 9-substituted berberine derivatives (**4a**–**4f**) with different polyethylene glycol side chains and terminal groups (piperidine or morpholine) involved three steps (Scheme 1). Partial demethylation of **1** was carried out at about 190 °C under vacuum for 20 min to generate **2**. Then, treatment of **2** with various oligoethyleneglycol ditosylates in DMF afforded intermediates **3a**–**3c**, respectively. Oligoethyleneglycol ditosylates were synthesized according to the reported procedure (Garcia *et al.,* 1999). These desired compounds **4a**–**4f** were prepared by substitution reactions of **3a**–**3c** with commercially available secondary amines (Huang *et al.,* 2010).

The ESI-HRMS of **4a**–**4f** exhibit the molecular ion peaks ($[M\text{-}Cl]^+$) which correspond to their molecular weight. The elemental analysis data of **4a**–**4f** are in good agreement with the calculated values. The ^{1}H-NMR spectroscopic data of these compounds showing the signals at 6.5–8.5 and 8.5–10 ppm suggest the presence of aromatic and *N*-heteroaromatic protons, which are accorded with the skeletal structure of berberine derivatives. In addition, the signals at 3–6 ppm are attached to the protons of methylene ($-CH_2-$). ^{13}C-NMR and IR also further confirmed the structure of the target products.

Cytotoxicity

The cytotoxicity of compounds **4a**–**4f**, berberine, berberrubine, and cisplatin against HL-60, BGC-823, Bel-7402, and KB cell lines was investigated. As listed in Table 1, compounds **4a**–**4f** displayed cytotoxicity with lower values of IC_{50} (<50 μM) against tested cell lines. Compound **4a** exhibited higher cytotoxicity than cisplatin against HL-60 and BGC-823 cell lines.

Accordingly, for 9-substituted berberine derivatives **4a**–**4f**, the structure–activity relationships are summarized as follows: (1) The polyethylene glycol side chain and terminal group on the 9-position of berberine enhance the cytotoxicity against Bel-7402 cell line. For example, compounds **4a**, **4c** and **4e** showed higher cytotoxicity than **1** against Bel-7402 cell line; compounds **4a**–**4f** exhibited higher cytotoxicity than **2** against Bel-7402 cell line; compound **4a** exhibited higher cytotoxicity than **1** and **2** against HL-60, BGC-823, Bel-7402 and KB cell lines. (2) The length of the side chains on the 9-position of berberine has an important effect on the cytotoxicity. For example,



1 2

Scheme 1 Synthesis of berberine derivatives. (*i*) 190 °C, 20–30 mmHg, 20 min; (*ii*) CH_3CN, K_2CO_3, $TsO(CH_2CH_2O)_nTs$ (n = 1, 2, 3), 60 °C, 3 h; (*iii*)morpholine (or piperidine), CH_3CN, K_2CO_3, 80 °C, 2–3 h

Table 1 Cytotoxicity for compounds against tumor cell lines (HL-60, BGC-823, Bel-7402, and KB)

Compound	IC_{50} (μM)			
	HL-60	BGC-823	Bel-7402	KB
1	5.83 ± 0.26	7.04 ± 0.29	16.28 ± 0.68	6.03 ± 0.24
2	11.36 ± 0.42	7.66 ± 0.34	34.31 ± 0.53	6.92 ± 0.36
4a	2.71 ± 0.23	2.82 ± 0.31	11.58 ± 0.65	5.46 ± 0.48
4b	6.51 ± 0.47	10.17 ± 0.62	27.53 ± 0.86	17.12 ± 0.63
4c	16.39 ± 0.68	11.89 ± 0.56	9.44 ± 0.38	9.93 ± 0.43
4d	46.80 ± 1.02	22.18 ± 1.17	17.86 ± 0.76	19.68 ± 0.85
4e	14.82 ± 0.53	10.84 ± 0.64	9.66 ± 0.58	10.03 ± 0.72
4f	45.76 ± 1.32	20.59 ± 1.13	33.43 ± 0.89	33.94 ± 0.76
Cisplatin	2.9 ± 0.3	6.5 ± 0.8	8.12 ± 1	2.6 ± 0.3

the cytotoxicity against HL-60, BGC-823 and KB cell lines decreases in the sequences: **4a** > **4c** > **4d**; the cytotoxicity against HL-60 and BGC-823 cell lines decreases in the sequences: **4b** ≥ **4e** > **4f**. This suggests that compounds with longer side chain may exhibit lower cytotoxicity. (3) The nature of terminal groups in the side chains affect on the cytotoxicity. For example, compound **4a** linked with morpholine displayed higher cytotoxicity than compound **4b** linked with piperidine against HL-60, BGC-823, Bel-7402, and KB cells lines. In summary, 9-substituted berberine derivatives with different lengths of polyethylene glycol side chains and terminal groups display selective cytotoxicity against tested cells lines, both the length of the polyethylene glycol side chains and the nature of the terminal groups have important effects on the cytotoxicity.

DNA-binding study

DNA is an important cellular receptor; many compounds exert their antitumor effects by binding to DNA thereby changing the replication of DNA and inhibiting the growth of the tumor cells, which is the basis of designing new and efficient antitumor drugs (Barton *et al.,* 1986; Pyle *et al.,* 1990; Friedman *et al.,* 1991; Kozurkova *et al.,* 2007). Therefore, a clear understanding of the interaction of small molecules with DNA is important in the development of DNA molecular probes and design of new and more efficient drugs targeted to DNA (Erkkila *et al.,* 1999; Maheswari and Palaniandavar, 2004). The CT-DNA-binding affinity of 9-substituted berberine derivatives **4a**–**4f** was investigated using UV–Vis, fluorescence, CD spectroscopies, and thermal denaturation experiment.

UV–Vis absorption titration analysis

As shown in Fig. 2 and Fig. S1, the absorption intensity of these compounds decreases with the increasing concentration of CT-DNA. The obvious hypochromicities (17–25 %) and bathochromic shifts (5–8.5 nm) suggested that these berberine derivatives can bind to CT-DNA in intercalative mode. The values of red shifts and hypochromicities for these compounds are higher than that of berberine (with the value of hypochromicity is 5 %, and bathochromic shift is 1 nm), this indicates that the introduction of side chains and terminal groups at 9-position of berberine enhances the binding ability of berberine to CT-DNA. The binding properties are similar to those previously observed intercalative ligand-DNA complexation (Friedman *et al.,* 1991) and are indication of intermolecular interaction involving the π stacking with the base pairs of CT-DNA. Binding

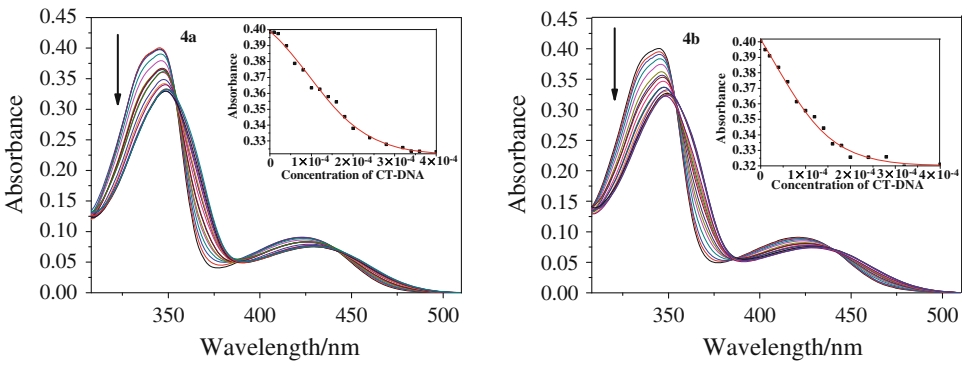


Fig. 2 Absorption spectra of **4a** and **4f** with increasing concentration of CT-DNA (0–400 μM) at the concentration of 2.0×10^{-5} M in 50 mM Tris–HCl buffer (pH 6.35) at r.t. *Arrows* show the absorbance changes upon increasing DNA concentration. *Inset* Plots of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA] for the compounds with CT-DNA

Table 2 Binding constants (K) and photo physical properties of **4a**–**4f** with CT-DNA

Compound	K (M^{-1})	Bathochromic shift[a] (nm)	Hypochromicity[a] (%)
4a	3.55×10^8	8.0 (345–353)	17
4b	1.27×10^8	8.5 (345–353.5)	21
4c	6.32×10^6	7.0 (345.5–352.5)	21
4d	1.49×10^6	5.5 (345.5–351)	19
4e	3.45×10^5	8.5 (345–353.5)	25
4f	1.86×10^5	5.0 (346–351)	21

[a] Obtained at the absorption maximum

constants (K) of these compounds binding to CT-DNA are calculated by the nonlinear least-squares curve-fitting method (Kozurkova *et al.,* 2007). As shown in Table 2, the K values of these compounds binding to CT-DNA decrease with the increasing length of the side chains. For example, the K values decrease in the sequence: **4a** > **4c** > **4d** and **4b** > **4e** > **4f**.

Fluorescence studies

To further ascertain the interaction of **4a**–**4f** with CT-DNA, the fluorescence spectra were detected in the range from 425 to 690 nm with an exciting wavelength at 355 nm at room temperature. As shown in Fig. 3 and Fig. S2, berberine derivatives have weak fluorescence properties at around 525 nm. The fluorescence intensity of compounds **4a**–**4f** increased largely with the increasing concentration of CT-DNA, which indicated that these compounds strongly interacted with CT-DNA. Furthermore, the maximum emission wavelength undergoes an obvious blue shift of up to 6 nm (from 531 to 525 nm), which suggests that the berberine backbones of these compounds intercalate into the base pairs of DNA (Barton *et al.,* 1986). The observed fluorescence intensities were quantified by plotting F/F_0 (F and F_0 are the fluorescence intensity in the presence and absence of CT-DNA) as a function of CT-DNA concentrations, and the value of F/F_0 is obtained according to Stern–Volmer analysis (Erkkila *et al.,* 1999). As shown in Table 3, the Stern–Volmer plots denote that these compounds are sensitive to the concentration of CT-DNA.

Circular dichroism studies

CD is a useful technique to investigate the conformational changes in DNA morphology during small molecule-DNA interactions. The CD spectra of compounds-DNA were recorded and shown in Fig. 4. The spectra demonstrated that the CT-DNA has the characterization of double helix B-DNA conformation, with a positive band at 277 nm and a negative band at 247 nm, which are generated by the base stacking and right-handed helicity structure, respectively. Increases in the intensity of the positive band and decrease in the intensity of the negative band for CT-DNA in the presence of compounds **4a**, **4b**, **4c**, and **4e** were observed, which indicated that the compounds could intercalate into the CT-DNA molecular, resulting in the conformation of B-DNA change into A-like form (Maheswari and Palaniandavar, 2004). On the other hand, both positive and negative bands increase in intensity when the compounds **4d** and **4f** are added to the CT-DNA solution, which is typical intercalation involving π-stacking and stabilization of the right-handed B form of CT-DNA (Xie *et al.,* 2011).

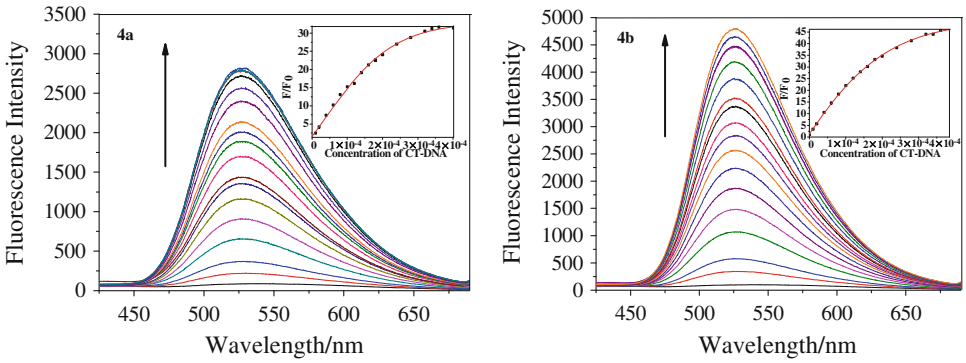


Fig. 3 Fluorescence spectra of compounds **4a** and **4f** with increasing concentration of CT-DNA (0–400 μM) at the concentration of 2.0×10^{-5} M in 50 mM Tris–HCl buffer (pH 6.35) at at r.t., $\lambda_{ex} = 355$ nm. *Arrow* shows the emission intensity increases upon increasing DNA concentration

Table 3 F/F_0 of **4a**–**4f** with CT-DNA

Compound	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**
F/F_0 (M^{-1})	1.27×10^5	7.09×10^4	1.82×10^5	8.01×10^4	1.79×10^5	1.01×10^5

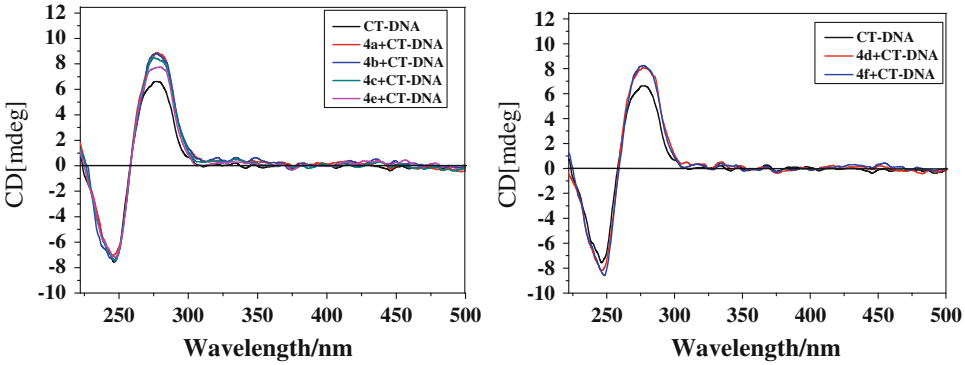


Fig. 4 CD spectra of CT-DNA and compound **4a–4f** with the fixed concentration of CT-DNA (60 μM) at the concentration of 2.0×10^{-5} M in 50 mM Tris–HCl buffer (pH 6.35) at r.t

Thermal denaturation studies

To further verify the mode of interaction of berberine derivatives with CT-DNA, thermal denaturation experiment was conducted. As well known, double helix DNA is very stable due to the presence of hydrogen bonds and base stacking interactions. The two strands of DNA will disintegrate into single when the hydrogen bonds between the base pairs are broken by heating. Small molecules, which can intercalate into the base pairs, will make the value of melting point of CT-DNA (T_m) increase obviously (Chaveerach *et al.,* 2010). As shown in Fig. 5, the T_m of CT-DNA is 75.8 °C in the absence of any compounds. The T_m of CT-DNA increases in the presence of berberine derivatives **4a–4f**, with the values of increased melting point ($\triangle T_m$) are 1.4, 1.7, 1.5, 2.2, 1.2, and 1.3 °C, respectively (as shown in Table 4). These spectral properties suggested that these 9-substituted berberine derivatives could intercalate into the base pairs of CT-DNA, and increase the stability of CT-DNA (Waning, 1965; Neyhart *et al.,* 1993).

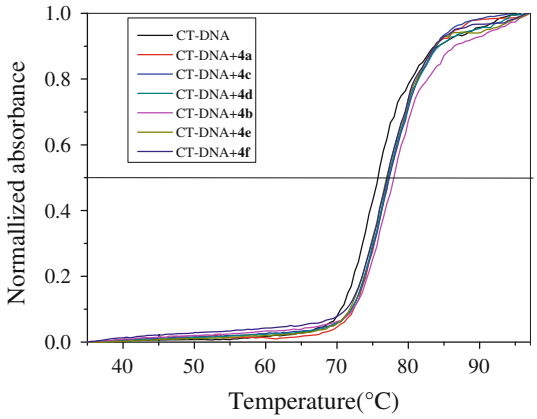


Fig. 5 The melting curves of CT-DNA (10 μM) in the absence and presence of compounds **4a–4f** (100 μM) in 50 mM Tris–HCl buffer (pH 6.35) at at r.t

Table 4 T_m and ΔT_m for CT-DNA in the absence and presence of compounds **4a–4f**

Compound	T_m (°C)	ΔT_m (°C)
CT-DNA	75.8	–
4a + CT-DNA	77.2	1.4
4b + CT-DNA	78	2.2
4c + CT-DNA	77.5	1.7
4d + CT-DNA	77.3	1.5
4e + CT-DNA	77	1.2
4f + CT-DNA	77.1	1.3

Conclusions

We have described the synthesis of a new series of 9-substituted berberine derivatives with a varying length of polyethylene glycol side chain and piperidine/morpholine as a terminal group. The cytotoxicity assay suggested that compound **4a** exhibited higher cytotoxicity than cisplatin against HL-60 and BGC-823 cell lines. Both the length of polyethylene glycol side chain and the nature of terminal group have important effects on the cytotoxicity. The cytotoxicity of these compounds decrease with the increasing length of the side chains. Berberine derivative **4a** linked with morpholine exhibited higher cytotoxicity than **4b** linked with piperidine against HL-60, BGC-823, Bel-7402, and KB cells lines. These semi-synthesized berberine derivatives could intercalate into the base pairs of CT-DNA. The DNA-binding affinities of these compounds decrease with the increasing length of the side chains. Compounds **4a–4c** and **4e** could change the DNA conformation from the B to the A-like form, and compounds **4d** and **4f** could stabilize the right-handed B form of CT-DNA. These studies may provide some guidance for the development of natural compounds as anticancer agents with potential clinical value.

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