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ORIGINAL PAPER

Synthetic and antitumor comparison of 9-*O*-alkylated and carbohydrate-modified berberine derivatives

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Abstract

Berberine is a naturally occurring isoquinoline alkaloid with moderate antitumor effect. It has been proved that berberine can affect the microenvironment of cancer cells, which is critical for cancer therapy and improving human immunity. Therefore, structural modification of berberine to obtain derivatives with high antitumor effect attracts the great attention of scientists. This paper describes the synthesis and in vitro antitumor investigation of some 9-*O*-alkylated and carbohydrate-modified berberine derivatives using high efficient strategy. The cytotoxicity investigation was accomplished on different cancer cell lines by the well-optimized MTT assay, and the results indicated that 9-*O*-hydrophobic modified berberine derivative **9** with long lipid chain showed promising in vitro antitumor activity with the IC_{50} value as low as 4.87 ± 0.24 μM. Percentage of apoptosis was measured by a Hoechst 33342/PI staining strategy on HeLa cells, which revealed that compound **9** could induce 84% of cell apoptosis at very low concentration (5 μM). Flow cytometry analysis indicated that compound **9** might inhibit HeLa cell proliferation by G2 phase arrest. The expression of various cell apoptosis proteins was measured by western blot analysis, and the results indicated that compound **9** could induce over-expression of Caspase-3, Parp, and Bax and down-regulate the expression of P53 and PCNA. All these data indicated the high potential of compound **9** for developing novel anticancer drugs.

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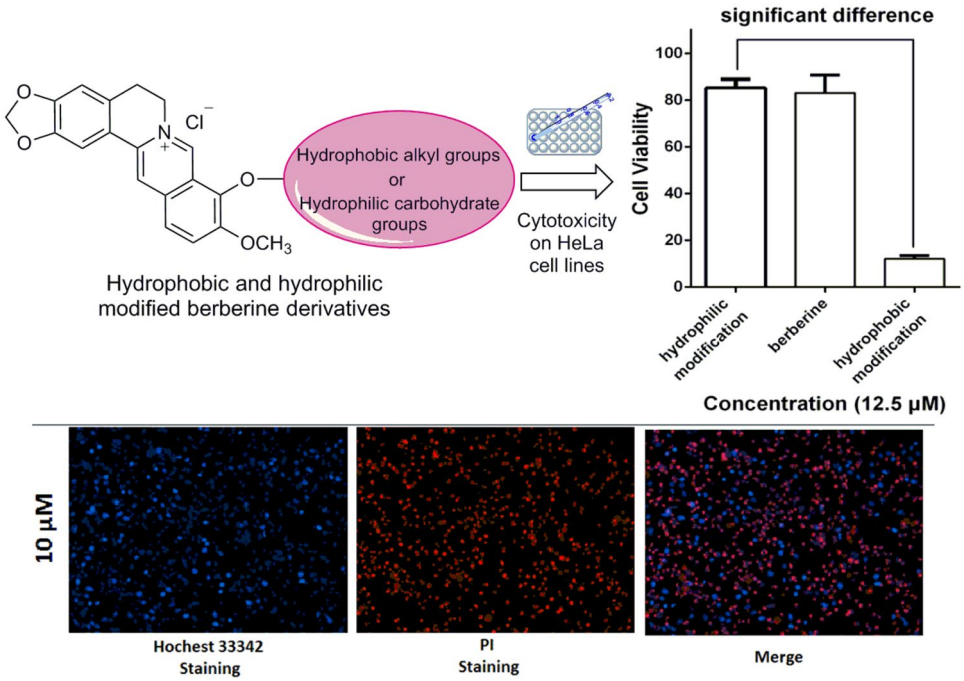
Graphic Abstract



Keywords Berberine · HeLa cell · Antitumor · Alkylated · Carbohydrate

Introduction

Berberine is a naturally occurred isoquinoline alkaloid isolated from various Berberis plants [1, 2] and has been extensively used in China for the treatment of gastroenteritis diseases and bacterial infection for hundreds of years [3, 4]. Recently, many research groups have revealed that berberine exhibited moderate antitumor effects [5, 6], such as inhibiting the proliferation of many cancer cell lines, inducing the apoptosis and differentiation of many species of tumor cells, and restraining the invasion and metastasis of tumor cells. It has been well proved that berberine can bind with the G-quadruplexes, an important structure unit in human DNA and RNA for transcriptional modulations and telomerase inhibition, which results in cancer cell senescence and apoptosis [7]. Berberine can also regulate the expression of some Bcl-2 proteins (Bax, Bim, Bcl-2, and Bcl-xL) and affect the mitochondrial membrane potential, which are critical for tumor cell apoptosis [8, 9]. Moreover, the high anti-inflammatory and antioxidant effects of berberine can improve the microenvironment of cancer cells, thereby leading to the cell cycle arrest and inhibition of cancer cell proliferation [10]. Therefore, berberine is an attracting lead molecule utilized for developing novel antitumor agents.

During the past several years, many research groups have focused on the structural modification of berberine (Fig. 1).

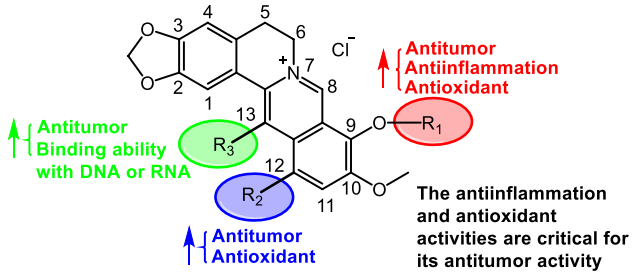
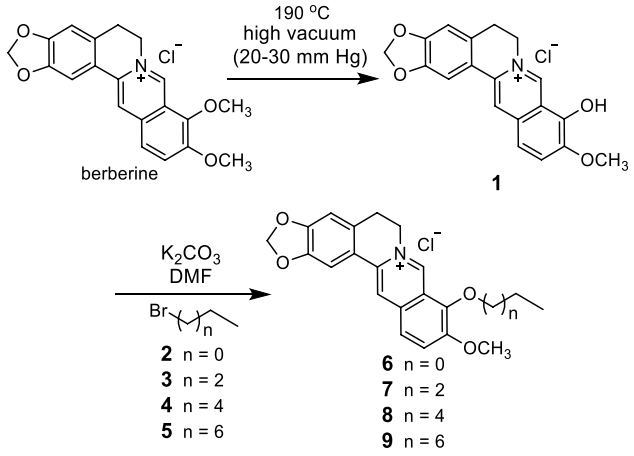


Fig. 1 Structural modification of berberine on different positions

For example, modification of berberine on its *C*-13 position with hydrophobic aromatic and lipid groups can enhance its binding ability with DNA or RNA, thus resulting in obvious improvement on its antitumor activity [9, 11]. The *C*-12 modification of berberine with Mannich bases can give berberine derivatives with both anticancer and antioxidant activities [1, 12]. However, the structural modification of berberine on its *C*-13 and *C*-12 positions is quite limited, due to the low reactivity of the hydrogen atom in these two positions. In contrast, the 9-*O*-modification of berberine is easily achievable to provide derivatives with antitumor, anti-inflammation, and antioxidant activities [11, 13, 14]. More importantly, the key



Scheme 1 Synthesis of 9-*O*-alkylated berberine derivatives **6–9**

intermediate 9-OH pseudoberberine **1** (Scheme 1) can be easily obtained through pyrolysis of berberine with high yield and selectivity [15, 16] and then will be conveniently used for introducing various functional groups on its 9-*O*-position. Consequently, many 9-*O*-modified berberine derivatives had been synthesized in the past several years, and some of them showed high antitumor activity [17]. However, the antitumor activity of these synthesized berberine derivatives is still not strong enough for developing novel antitumor agents, despite the remarkable advances made in this area. Therefore, the structural modification and functionalization of berberine still remains necessary and is of great importance.

Previous research revealed that the introduction of lipophilic groups on leading molecules could facilitate the penetration of antitumor drugs into cell membrane and enhance their cytotoxicity against many cancer cell lines [18, 19], which indicated that lipidation of berberine should be a promising modification strategy. On the other hand, the hydrophilic carbohydrate groups have also been proved critical for enhancing the antitumor activity of lead compounds through improving their bioavailability and/or receptor-binding affinity [20–25]. For example, some rhamnose-modified saponins showed high cytotoxicity against human cancer cell lines, which might be caused by the rhamnose-binding lectin expressed by cancer cells that could promote the transportation of saponins [26]. Hence, the carbohydrate modification might have some positive effect on the antitumor activity of berberine, while there is no work to compare the antitumor activity of the lipid and carbohydrate-modified berberine derivatives till now. This paper synthesized some 9-*O*-alkylated berberine derivatives with different lengths of lipid chain, and their cytotoxicity was measured in vitro on HeLa cell lines. As a comparison, the cytotoxicity of some carbohydrate-modified berberine derivatives was also measured on the same cell lines.

Experimental

General procedures

Berberine was commercially available from Tokyo Chemical Industry (TCI). Other solvents and reagents were bought as analytical grade and used without any further purification. ^{1}H NMR spectra were measured on a Bruker AV 400 spectrometer, and high-resolution mass spectra (HRMS) were measured on an Agilent QTOF 6520 mass spectrometer. HeLa cell lines were purchased from the American Type Culture Collection and cultured in DMEM medium (Invitrogen) containing 10% fetal bovine serum, 10 units/mL penicillin-G, and 10 mg/mL streptomycin. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was bought from Sigma. Hoechst 33342/PI double staining kit was obtained from Genview (China). Anti-PCNA, anti-Caspase-3, anti-Parp, anti-Bax, anti-BCL 2, anti-P53, and anti-GAPDH antibodies were bought from Cell Signaling Technology.

General procedure for synthesis of 9-O-alkylated berberines 6–9

Berberine (10 g, 27 mmol) was heated at 190 °C under reduced pressure for 30 min to give a crude solid, which was subjected to column chromatography using dichloromethane/methanol (5/1) as the eluent to afford compound **1** (7.6 g, yield = 79%) as a purple solid.

A solution of compound **1** (500 mg, 1.4 mmol), bromohydrocarbon (4.2 mmol) and K_2CO_3 (580 mg, 4.2 mmol) in 5 mL of DMF was stirred at 80 °C for 3 h. After TLC (dichloromethane/methanol 7/1) showed the conversion of the starting materials, the mixture was diluted with 50 mL of dichloromethane and washed with water (20 mL × 3). The organic layer was dried with absolute sodium sulfate and evaporated under reduced pressure. The resultant residue was purified by column chromatography using dichloromethane/methanol (10/1 → 5/1) as the eluent to provide 9-*O*-alkylated berberines **6–9**.

9-*O*-ethyl berberine chloride (**6**). Compound **1** was reacted with bromoethane **2** by the general procedure described above to give product **6** as a yellow solid (458 mg, yield = 85%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.82 (s, 1 H, C*H* = N), 8.95 (s, 1 H, Ar*H*), 8.20 (d, *J* = 8.8 Hz, 1 H, Ar*H*), 8.00 (d, *J* = 9.2 Hz, 1 H, Ar*H*), 7.80 (s, 1 H, Ar*H*), 7.10 (s, 1 H, Ar*H*), 6.18 (s, 2 H, –OCH_2O–), 4.96 (t, *J* = 6.0 Hz, 2 H, CH_2), 4.36 (9, *J* = 6.8 Hz, 2 H, OCH_2CH$_3$), 4.06 (s, 3 H, OCH_3), 3.21 (t, *J* = 6.4 Hz, 2 H, CH_2), 1.46 (t, *J* = 6.8 Hz, 3 H, CH$_2$CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 151.00, 150.29, 148.16, 145.90,

143.07, 137.94, 133.48, 131.18, 127.13, 123.81, 122.36, 120.94, 120.67, 108.89, 105.90, 102.56, 70.41, 57.52, 55.72, 26.82, 15.84. ESI-TOF HRMS (*m/z*): calcd for $C_{21}H_{20}NO_4^+$, $[M–Cl]^+$, 350.1387; found, 350.1390.

9-*O*-butyl berberine chloride (**7**). Compound **1** was reacted with bromobutane **3** by the general procedure described above to give product **7** as a yellow solid (434 mg, yield = 75%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.75 (s, 1 H, C*H* = N), 8.95 (s, 1 H, Ar*H*), 8.19 (d, *J* = 9.2 Hz, 1 H, Ar*H*), 8.00 (d, *J* = 9.2 Hz, 1 H, Ar*H*), 7.80 (s, 1 H, Ar*H*), 7.10 (s, 1 H, Ar*H*), 6.18 (s, 2 H, –OCH_2O–), 4.96 (t, *J* = 6.0 Hz, 2 H, CH_2), 4.30 (t, *J* = 6.8 Hz, 2 H, OCH_2CH$_2$CH$_2$CH$_3$), 4.06 (s, 3 H, OCH_3), 3.21 (t, *J* = 6.0 Hz, 2 H, CH_2), 1.90-1.83 (m, 2 H, OCH$_2$CH_2CH$_2$CH$_3$), 1.57-1.47 (m, 2 H, OCH$_2$CH$_2$CH_2CH$_3$), 0.99 (t, *J* = 7.6 Hz, 3 H, OCH$_2$CH$_2$CH$_2$CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 150.87, 150.30, 148.16, 145.75, 143.36, 137.94, 133.51, 131.18, 127.20, 123.74, 122.14, 120.93, 120.70, 108.89, 105.91, 102.56, 74.43, 57.54, 55.80, 32.02, 26.82, 19.03, 14.24. ESI-TOF HRMS (*m/z*): calcd for $C_{23}H_{24}NO_4^+$, $[M–Cl]^+$, 378.1700; found, 378.1703.

9-*O*-hexanyl berberine chloride (**8**). Compound **1** was reacted with bromohexane **4** by the general procedure described above to give product **8** as a yellow solid (549 mg, yield = 89%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.76 (s, 1 H, C*H* = N), 8.95 (s, 1 H, Ar*H*), 8.19 (d, *J* = 8.8 Hz, 1 H, Ar*H*), 7.98 (d, *J* = 8.8 Hz, 1 H, Ar*H*), 7.80 (s, 1 H, Ar*H*), 7.10 (s, 1 H, Ar*H*), 6.18 (s, 2 H, –OCH_2O–), 4.96 (t, *J* = 6.0 Hz, 2 H, CH_2), 4.28 (t, *J* = 6.8 Hz, 2 H, OCH_2CH$_2$CH$_2$CH$_2$CH$_3$), 4.05 (s, 3 H, OCH_3), 3.21 (t, *J* = 5.6 Hz, 2 H, CH_2), 1.92-1.84 (m, 2 H, OCH$_2$CH_2CH$_2$CH_2CH$_2$CH$_3$), 1.52-1.45 (m, 2 H, CH_2), 1.39-1.29 (m, 4 H, 2 × CH_2), 0.91 (t, *J* = 6.8 Hz, 3 H, OCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 150.89, 150.30, 148.16, 145.77, 143.37, 137.93, 133.51, 131.17, 127.19, 123.76, 122.15, 120.94, 120.70, 108.89, 105.91, 102.56, 74.74, 57.53, 55.79, 31.53, 29.92, 26.82, 25.41, 22.56, 14.42. ESI-TOF HRMS (*m/z*): calcd for $C_{25}H_{28}NO_4^+$, $[M–Cl]^+$, 406.2013; found, 406.2015.

9-*O*-octanyl berberine chloride (**9**). Compound **1** was reacted with 1-bromooctane **5** by the general procedure described above to give product **9** as a yellow solid (480 mg, yield = 73%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.75 (s, 1 H, C*H* = N), 8.95 (s, 1 H, Ar*H*), 8.19 (d, *J* = 9.2 Hz, 1 H, Ar*H*), 8.00 (d, *J* = 9.2 Hz, 1 H, Ar*H*), 7.80 (s, 1 H, Ar*H*), 7.10 (s, 1 H, Ar*H*), 6.18 (s, 2 H, –OCH_2O–), 4.95 (t, *J* = 5.6 Hz, 2 H, CH_2), 4.28 (t, *J* = 6.8 Hz, 2 H, OCH_2CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 4.05 (s, 3 H, OCH_3), 3.21 (t, *J* = 6.0 Hz, 2 H, CH_2), 1.91-1.84 (m, 2 H, OCH$_2$CH_2CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 1.52-1.44 (m, 2 H, CH_2), 1.40-1.23 (m, 8 H, 4 × CH_2), 0.87 (t, *J* = 6.8 Hz, 3 H, OCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 150.89, 150.30, 148.17, 145.78, 143.37, 137.94, 133.51, 131.17, 127.20, 123.75, 122.15, 120.94, 120.70, 108.90, 105.90, 102.56, 74.73, 57.53, 55.79, 31.73, 29.95, 29.27, 29.16, 26.82, 25.75, 22.56, 14.44. ESI-TOF HRMS (*m/z*): calcd for $C_{27}H_{32}NO_4^+$, $[M–Cl]^+$, 434.2326; found, 434.2330.

General procedure for synthesis of carbohydrate-modified berberines 17–22

The solution of alkynylated berberine derivative **10** (100 mg, 0.25 mmol), azido sugars **11**–**16** (0.25 mmol), $CuSO_4 \cdot 5H_2O$ (33 mg, 0.13 mmol), sodium ascorbate (26 mg, 0.13 mmol) and methanol (3 mL) in 3 mL of DMF was heated to 50 °C and stirred at ambient temperature for 3 h. After TLC showed the conversion of the starting materials (dichloromethane/methanol/H_2O, 10/3/1), the solvent was evaporated and the resultant residue was subjected to column chromatography to give carbohydrate-modified berberine derivatives 17–22. The H and C NMR spectra are shown in supporting information.

Cell viability assay

HeLa cells (2×10^6/well) were cultured for 24 h at 37 °C and 5% CO_2 atmosphere in 96-well plates, using DMEM medium containing 10% fetal bovine serum (FBS), 10 units/mL penicillin-G, and 10 mg/mL streptomycin, 2 mM glutamine, and 2% sodium pyruvate. The medium was then replaced with DMEM medium containing 0.5% DMSO and different concentrations of berberine derivatives (3.125, 6.25, 12.5, 25, 50, and 100 μM). The DMEM medium containing 0.5% DMSO was employed as control well. After 24 h of incubation, 10 μL of MTT (5 mg/mL) was added to each well and the cells were cultured for another 4 h. Thereafter, the supernatant in each well was removed and 200 μL of DMSO was added. After 10 min of swirling, the optical density (OD) values of the plates were measured on a MR spectroscopy at 570 nm. The relative viability of treated cells was expressed as percentage of control untreated cells. Three replicates were run for each concentration, and all tests were repeated for 5 wells.

Hoechst 33342/PI staining for cell apoptosis

Hoechst 33342/PI double staining was carried out to determine apoptosis in HeLa cells. The cells in 96-well plates containing about 30,000 cells were incubated in binding buffer containing Hoechst 33342 and PI for 15 min at 37 °C in the dark. Fluorescence microscopic studies to detect pro-apoptotic effects were carried out per well and using a

BioTek Cytation 3 high content imager with a 20× objective. The apoptosis cells were identified based on positive staining with both propidium iodide (PI) and Hoechst 33342 and absence of nuclear fragmentation. Each plate contained 3 replicates per condition, and two independent experiments were performed. Three replicates were examined for each concentration, and all tests were repeated in 3 wells.

Cell cycle analysis

Cells were treated with different concentrations of compound **9** (2.5, 5, and 10 μM) and cultured for 24 h. Then, cells were harvested and washed with PBS, which was followed by being fixed and permeabilized at − 20 °C overnight using ice-cold 75% ethanol. After that, cells were washed with ice-cold PBS and treated with 100 μg/mL of RNase at 37 °C for half an hour. Finally, cells were stained with 1 mg/mL PI at 4 °C for half an hour and subjected to cell cycle analysis with a FACS Calibur analyzer (BD) and analyzed with FlowJo software version 7.6.1 (Ashland, OR, USA). Cells treated with solvent DMSO were used as the control groups. Three replicates were examined for each concentration. Three replicates were examined for each concentration.

Western blot

HeLa cells were cultured in 6-well plates and treated with compound **9** (0.5, 2.5, 5, 10 μM) for 24 h. Then, HeLa cells were collected and lysed by RIPA buffer. Protein concentration was determined by the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Equivalent amounts of proteins of each group were subjected to SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad, Philadelphia, PA, USA). After being blocked with 5% non-fat milk in Tris-buffered saline buffer, the membranes were incubated with primary antibodies (anti-PCNA, anti-Caspase-3, anti-Parp, anti-Bax, anti-BCL 2, anti-P53 and anti-GAPDH), (1:1000) at 4 °C overnight, followed by incubation with the corresponding secondary antibodies (1:5000). Protein bands were visualized with an ECL advanced Western blotting detection kit.

Results and discussion

Synthesis

The synthesis of lipidated berberine derivatives is depicted in Scheme 1. Pyrolysis of berberine at high temperature (190 °C) under reduced pressure (20-30 mm Hg) selectively afforded 9-*O*-demethylated berberine **1** [27], which was reacted with bromohydrocarbons **2**–**5** under the promotion of potassium carbonate in DMF to provide the desired berberine derivatives **6**–**9**. The hydrophilic carbohydrate-modified berberine derivatives **17**–**22** (Scheme 2) were prepared by a similar procedure described previously [28] from azido sugars **11**–**16** and alkynylated berberine derivative **10**, which could be obtained by reaction of berberrubine with propargyl bromide under mild conditions [28]. Previous work had been revealed that the 9-*O*-glucosyl-berberine could improve the bioavailability of berberine, but showed poor stability under both acidic physiological conditions and high temperature [29]. To solve this problem, we use a triazole linker to finish the conjugation of berberine with carbohydrate, which can provide some N-glycosides with high stability. Furthermore, the triazole linker has been proved to play important role for enhancing the antitumor activity of drugs, for example, improving the water solubility and the receptor-binding affinity [30].

Cell viability study

The effect of the synthesized berberine derivatives on HeLa cell lines was measured by MTT assay. Cells were treated with different concentrations (0, 3.125, 6.25, 12.5, 25, 50, and 100 μM) of berberine derivatives for 24 h, which was followed by the standard MTT analysis on a microplate reader to give the results shown in Fig. 2. Clearly, the cytotoxicity of berberine exhibited a dose-dependent manner, and the cell viability was about 51% at a concentration of 100 μM (Fig. 2a). Berberine derivative **6** with an ethyl group on its 9-*O*-position showed similar cytotoxicity as berberine both at low and high concentrations (Fig. 2b). In contrast, the cell viability of derivative **7** showed a slightly increase at low concentrations (≤ 6.25 μM), followed by a dose-dependent decrease (≥ 12.5 μM, Fig. 2c). Berberine derivative **8** showed higher cytotoxicity than berberine at low concentrations (≤ 50 μM, Fig. 2d), while its cell inhibition ability dramatically increased when the concentration reached 100 μM, which proved its potential antitumor activity at high concentrations. Interestingly, 9-*O*-octanyl berberine derivative **9** showed high cell inhibition ability both at low and high concentrations (Fig. 2e). When the concentration was 12.5 μM, the cell inhibition ability of **9** was remarkably higher than berberine, indicating its potential antitumor activity. Compared with the lipidated berberine derivatives **6**–**9**, all hydrophilic carbohydrate-modified berberine derivatives exhibited low or similar cytotoxicity against HeLa cell lines (Fig. 2f–k). This is because hydrophobic modification of berberine might exhibit high permeability into cell membrane, due to the presence of lipid bilayer in

cell surface. In contrast, the hydrophilic modified berberines, despite having good water solubility, showed poor permeability into the hydrophobic cell membrane, which resulted in low ability on suppressing cancer cell growth.

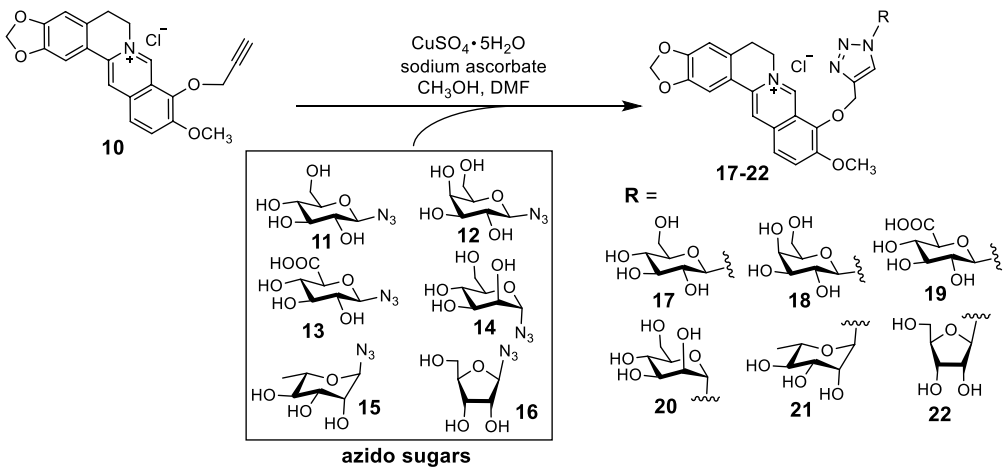
Then, compounds **8** and **9** were subjected to a series of cytotoxicity analysis against different cancer cell lines, including human lung cancer (A549), human liver cancer (HepG2), breast (MCF7), human cervical carcinoma (HeLa) and rat pheochromocytoma (PC12). The results indicated that all these two compounds displayed high cytotoxicity with IC_{50} values in the range of 4 to 10 μM (Table 1), while berberine and cisplatin showed low and moderate cytotoxicity against the cancer cell lines described above. Compound **9** showed higher high cytotoxicity than **8**, which proved that berberine derivative with long lipid chain might exhibit high antitumor activity. In view of the above results, berberine derivative **9** was chosen as the leading compound for further biological investigations.

Fluorescence photomicrographs and percentage of apoptosis

Fluorescence photomicrographs of HeLa cells treated with different concentrations of berberine derivative **9** (0.5, 2.5, 5, and 10 μM) were measured by a Hoechst 33342/PI staining strategy (Fig. 3). Hoechst 33342 is a cell membrane penetrating dye which can bind with DNA in normal cells and gives blue fluorescence [31]. In contrast, the cell-impermeable propidium iodide (PI) can bind with DNA in apoptosis cells with broken cell membrane, therefore producing red fluorescence [31]. Hence, dual staining of these two dyes can be applied to distinguish apoptosis and normal cells on a fluorescence microscope [32]. As described in Fig. 3, the non-treated normal cells (Con) with intact cell wall showed strong blue fluorescence which was generated by the cell wall penetrating dye Hoechst 33342, and the slightly red fluorescence was produced by the cell-impermeable PI dye in small amount of death cells with broken cell wall. After treating with different concentrations (0.5, 2.5, 5, and 10 μM) of derivative **9**, there was an obvious red fluorescence enhancement accompanied with a blue fluorescence decrease, due to the apoptosis of normal HeLa cells. The percentage of cell apoptosis was 46% when the concentration of **9** was 2.5 μM and reached 95% with the concentration of 10 μM (Fig. 4), which revealed the high cytotoxicity effect of **9** on HeLa cell lines. Based on the fact that apoptosis has been proved to play important role in the depression of tumor cells and keeping tissue homeostasis [33], drugs that can effectively induce tumor cell apoptosis are urgently desirable owing to their low toxic side-effects during cancer treatment [34]. Consequently, compound **9** might be a promising anticancer agent which can induce cell apoptosis at low concentrations.

Effect of berberine derivative 9 on cell cycle

Some anticancer agents inhibit cell proliferation by interfering the cell cycle processes [35], while others result in cell apoptosis [36]. To confirm whether the decrease in cell proliferation was influenced by the cell cycle arrest, we measured the DNA content of nuclei in HeLa cells by flow cytometry (Fig. 5). Treatment of HeLa cells with different concentrations of **9** (5 and 10 μM) resulted in significant increase in cell populations (41.59% and 37.02%) at S phase, but the cell populations at G1 (54.44% and 60.51%) and G2 (3.97% and 2.47%) phases showed no obvious changes.



Scheme 2 Synthesis of carbohydrate-modified berberine derivatives **17**–**22**

These results indicated that berberine derivative **9** might inhibit cell proliferation by G2 phase arrest.

Expression of cell apoptosis proteins

PCNA is a well-kwon proliferating cell nuclear antigen which is associated with the DNA replication in the S phase of cell cycle [37], and over-expression of PCNA can lead to the proliferation of many human cancer cells [38]. Caspase-3 has been proved an executioner of cell apoptosis, which can result in cell morphological changes and apoptosis in the presence of many substrates [39]. One of the most important substrates is Parp, which is also a widely used diagnostic agent for confirming tumor cell apoptosis [40]. Furthermore, P53 tumor suppressor gene is an important regulated transcription factor which can induce the expression of Bax and reduce the production of BCL-2 and then cause tumor cell apoptosis [38]. In our experiments, western blot analysis was utilized to investigate the expression of the above proteins related to tumor cell proliferation and apoptosis. As depicted in Fig. 6, addition of increasing concentrations of berberine derivative **9** significantly stimulated the expression of Caspase-3, Parp, and Bax and reduced the production of P53, thus proving the apoptosis of tumor cells. Furthermore, the over-expression of PCNA was slightly inhibited after treatment of HeLa cells with **9**, which indicated that derivative **9** might have some effect on the S phase of cell cycle. Interestingly, there was no effect on the expression levels of BCL-2.

Conclusions

In summary, this paper synthesized some hydrophobic 9-*O*-alkylated berberine derivatives with different length of lipid chain for investigating their antitumor activities. As a comparison, some hydrophilic carbohydrate-modified berberine derivatives were also synthesized by a published procedure [28]. All these berberine derivatives were subjected to antitumor investigation on various cancer cell lines, and the results indicated that hydrophobic modification of berberine on its 9-*O*-position with long lipid chain could give derivatives with high antitumor activity. Berberine derivative **9** with an octanyl group on its 9-*O*-position showed

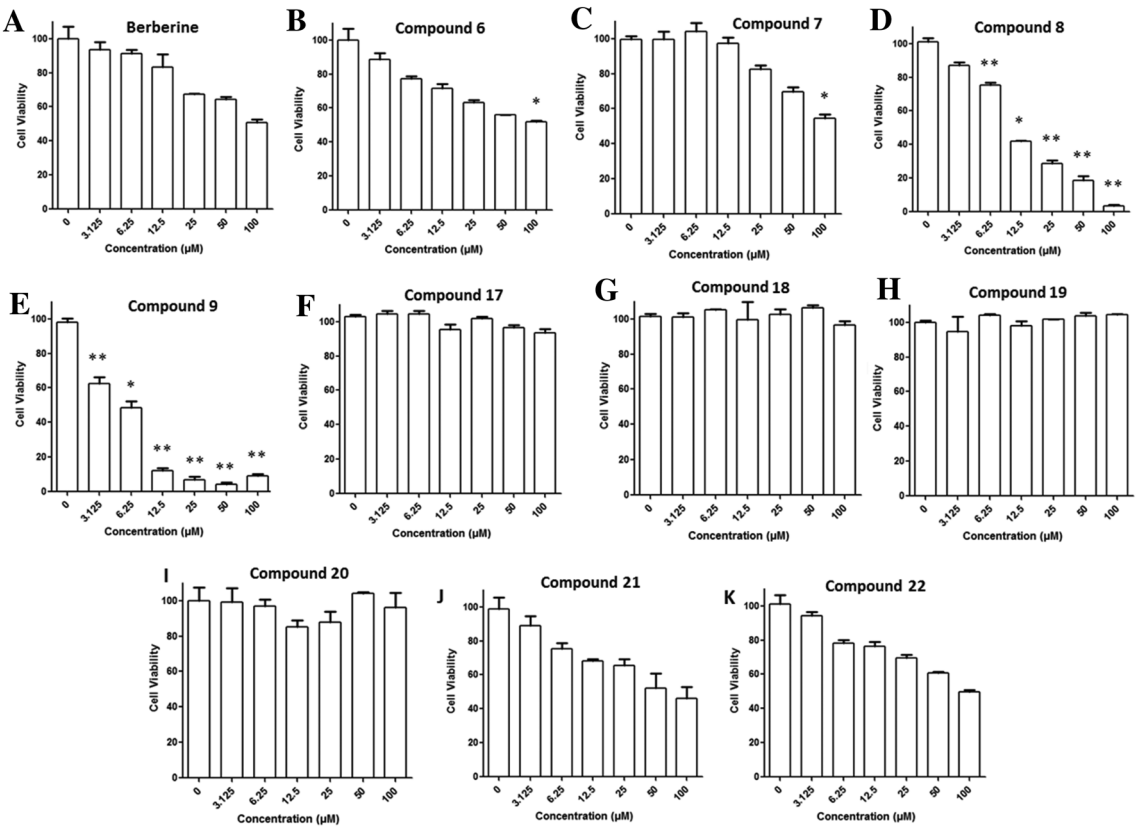


Fig. 2 Cytotoxic effects of berberine derivatives on HeLa cell lines after 24 h of incubation. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control

Table 1 IC_{50} values (μM) for berberine, cisplatin, and berberine derivatives 8 and 9. Cells were treated with different concentrations (40, 20, 10, 5, 2.5, 1.25, and 0.625 μM) of drugs for 24 h

Compound	IC_{50} values (μM)				
	A549	HepG2	HeLa	MCF7	PC12
8	8.56 ± 1.34	8.34 ± 1.45	9.58 ± 1.89	7.65 ± 2.22	8.52 ± 2.12
9	5.54 ± 0.22	5.54 ± 0.52	5.02 ± 0.12	4.87 ± 0.24	6.54 ± 1.31
Berberine	> 40	> 40	> 40	> 40	> 40
Cisplatin	25.03 ± 0.85	24.91 ± 0.41	20.32 ± 1.52	19.70 ± 1.01	21.69 ± 1.88

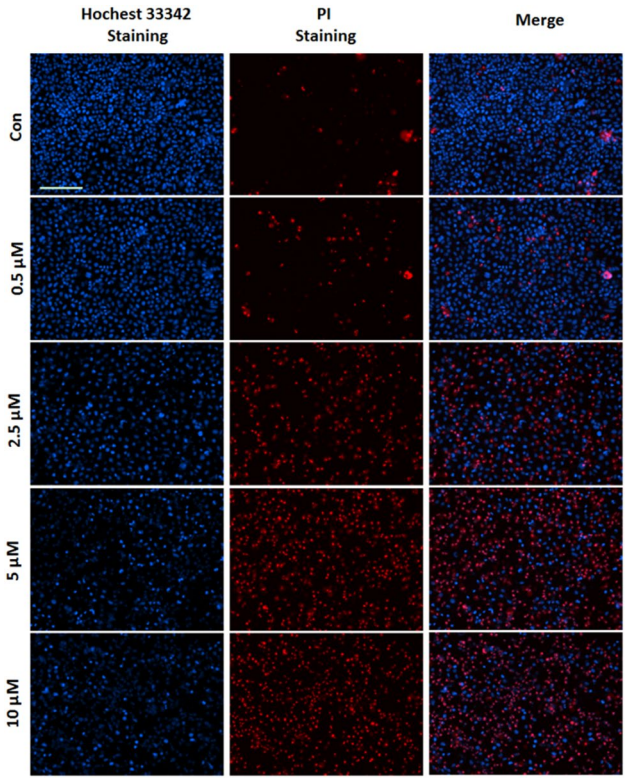


Fig. 3 The effect of different concentrations (0.5, 2.5, 5, and 10 μM) of berberine derivative 9 on fluorescence photomicrographs of HeLa cells detected by dual staining of Hoechst 33342/PI after 24 h of incubation. Con: Non-treated cells

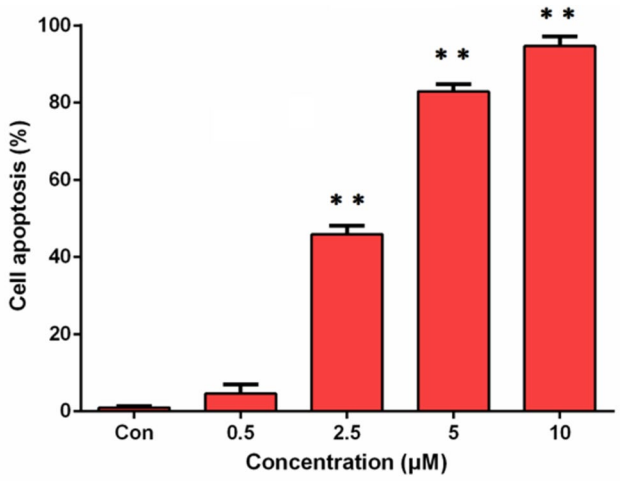


Fig. 4 The percentage of apoptosis HeLa cells after treatment with different concentrations (0.5, 2.5, 5, and 10 μM) of berberine derivative 9. Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$ versus control

excellent in vitro antitumor activity and could induce 84% of cell apoptosis at very low concentration (5 μM). More interestingly, compound **9** could result in an increase in HeLa cell populations (41.59% and 37.02%) at S phase, which indicated that compound **9** might inhibit cell proliferation by G2 phase arrest. Western blot analysis revealed that compound **9** could induce the down-regulation or up-regulation of various cell apoptosis proteins, such as Caspase-3, Parp, Bax, and P53. All these results proved that hydrophobic modification of berberine with long lipid chain can provide derivatives with high antitumor activity, for example, 9-*O*-octanylated berberine **9** should be a potential target for further investigation.

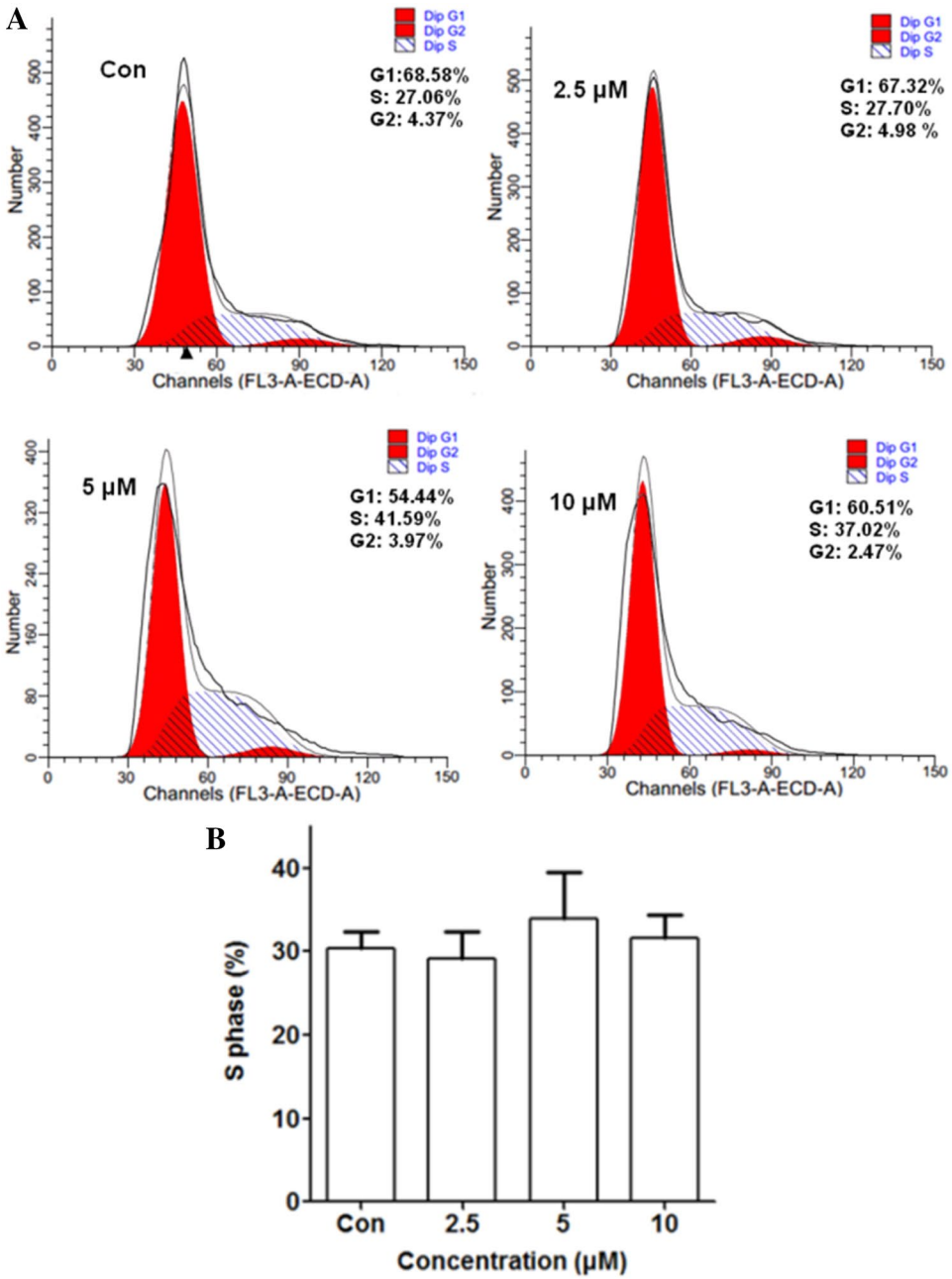


Fig. 5 Effect of berberine derivative 9 on cell cycle distribution (**a**) and S phase variation (**b**). HeLa cells were treated with different concentrations (2.5, 5, and 10 μM) of 9 for 24 h and untreated HeLa cells were used as a control

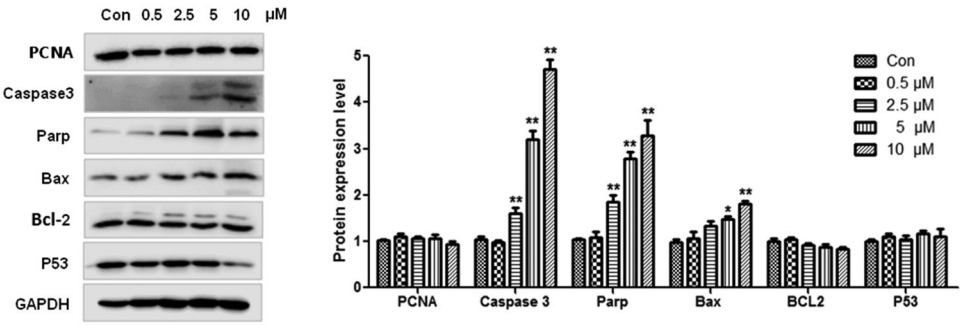


Fig. 6 Western blot analysis of the effect of berberine derivative 9 (0.5, 2.5, 5, and 10 μM) on cell apoptosis proteins of HeLa cells. GAPDH served as the internal control

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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