European Journal of Medicinal Chemistry 46 (2011) 4056–4061

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and biological evaluation of derivatives of 4-deoxypodophyllotoxin as antitumor agents

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ARTICLE INFO

Article history:
Received 16 March 2011
Received in revised form
31 May 2011
Accepted 1 June 2011
Available online 14 June 2011

Keywords:
Podophyllotoxin
4-Deoxypodophyllotoxin
Piperazine
Antitumor activity

ABSTRACT

In an attempt to generate compounds with superior bioactivity and reduced toxicity, a series of derivatives of deoxypodophyllotoxin were synthesized by reacting 4′-demethyl-4-deoxypodophyllotoxin with substituted piperazines or their amino acid amides. The cytotoxic activity of these compounds against three human cancer cell lines was evaluated. We found that *p*-nitrophenylpiperazine substitution (Compound **8b**) led to an increase in the potency of the compound. Compound **8b** exhibited the most potent cytotoxicity against A-549, HeLa and SiHa cells (IC_{50} values were 0.102, 0.180 and 0.0195 μM, respectively). In addition, flow cytometric analysis showed that **8b** induced cell cycle arrest in the G1 phase accompanied by apoptosis in A-549 cells.

1. Introduction

Podophyllotoxin (PT, **1**) and its many related derivatives have been used for a variety of therapeutic purposes including cathartic, antirheumatic and anti-viral treatments. Because of its inhibitory activity on cell growth, it is often used as a lead compound for drug design in the search for improved anti-proliferative agents [1]. Hundreds of derivatives have been prepared, together with some semi-synthetic analogues such as etoposide (VP-16), teniposide, and more recently etopophos, a more soluble prodrug of etoposide, that is currently used in chemotherapy for various types of cancer, including small cell lung cancer, testicular carcinoma, and Kaposi's sarcoma [2–5]. Surprisingly, these semi-synthetic derivatives exert their actions through different mechanisms than the parent compound, podophyllotoxin [2]. Podophyllotoxin inhibits the assembly of tubulin into microtubules through interaction with the protein at the colchicine binding site, preventing the formation of the spindle. However, etoposide and congeners induce a premitotic blockade in the late S stage of the cell cycle through the inhibition of DNA topoisomerase II (Topo II), achieved by binding to and stabilizing the DNA-protein complex, thus preventing re-ligation of the double-stranded breaks [4].

Deoxypodophyllotoxin (DPT, **2**), an analogue of PT, has anti-proliferative and antitumor activity in diverse cell types [6–10], as well as anti-inflammatory [11] and anti-viral [12] activity. Recently, it has been reported that DPT inhibits tubulin polymerization and induces cell cycle arrest at G2/M in HeLa cells, followed by apoptosis through multiple cellular processes, involving the activation of ATM, upregulation of p53 and Bax, activation of caspase-3 and -7, and accumulation of PTEN resulting in the inhibition of the Akt pathway [13–15]. Furthermore, Ahn and co-workers have described a number of DPT derivatives, such as alkyl and carboxyl alkyl esters of 4′-demethyl-4-deoxypodophyllotoxin (DDPT, **3**) (Fig. 1) and prodrugs of DPT including carbamate- and carbonate-associated forms. The antitumor activities of these compounds showed that most derivatives enhanced cytotoxic or antitumor activity compared with that of the parent compound, DPT [16,17].

In our continuing efforts to find new compounds with potent activities and low toxicity from natural products [18–24], we here present the synthesis of nine new derivatives (**8a**–**i**) of DPT and our measurements of their cytotoxic activity against A-549, HeLa, SiHa cell lines. Furthermore, the effect of **8b** on A-549 cells was also investigated by flow cytometry studies to reveal its actions on the cell cycle.

2. Chemistry

The synthetic route to the target compounds first involved generation of the intermediate DDPT (Scheme 1), which was

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doi:10.1016/j.ejmech.2011.06.004

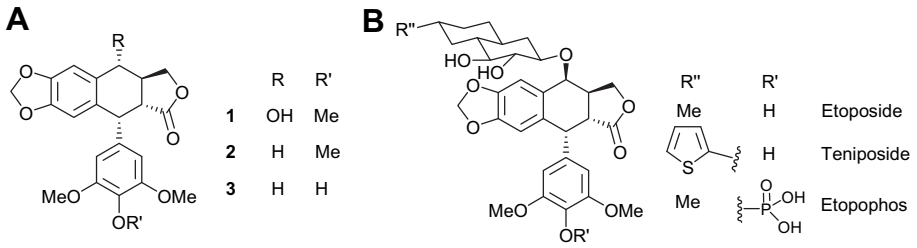


Fig. 1. Structures of podophyllotoxin (**1**) and related compounds.

prepared from **1** isolated from the Chinese medicinal herb *Podophyllum emodi* Wall. var. Chinesis Sprague. Usually, the intermediate **3** is prepared from **1** through successive 4-deoxylation by hydrogenolysis [25] and 4′-demethylation with methanesulfonic acid/sodium iodide [26]. However, this procedure gives only low yield and purity, so we modified the procedure to prepare **3** from **1** through 4′-demethylation with HBr gas in CH_2Cl_2 with subsequent 4-deoxylation by hydrogenolysis with 10% Pd/C in HAc. A previous report has also demonstrated that treatment of **3** with 4-nitrophenyl chloroformate (=4-nitrophenyl carbonochloridate) in the presence of pyridine provides the intermediate **7** in high yield [27].

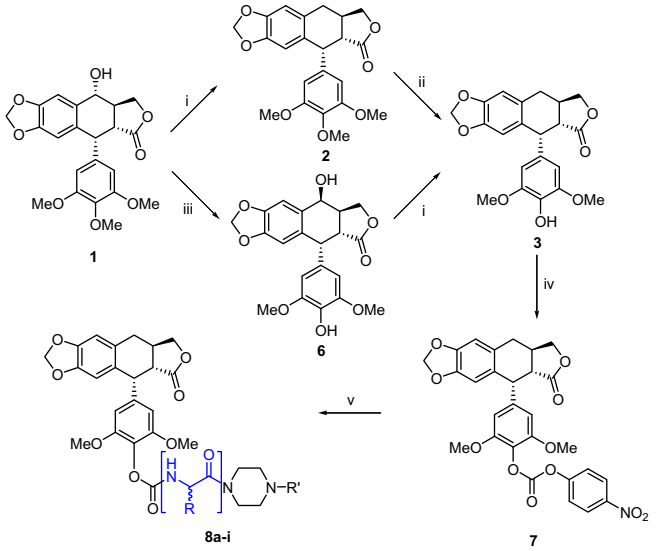
Having synthesized intermediate **7**, compounds **8a**–**e** were prepared by reacting **7** with the appropriate substituted piperazines (1-methylpiperazine, 1-*p*-nitrophenylpiperazine, 1-*p*-fluoro phenylpiperazine, 1-*p*-hydroxylphenylpiperazine, 1-cyclopentylpiperazine, respectively) in the presence of triethylamine (Et_3N) with high or moderate yields.

Amino acids are often used as carrier vehicles for some drugs because of their good water solubility when actively transplanted into mammalian tissue. As a continuation of our previous work [21,23], we also designed and synthesized compounds **8f**–**i**. 1-*p*-Nitrophenylpiperazine was condensed with the appropriate Boc-protected amino acids **4a**–**d** in the presence of benzo triazole-1-yl-oxy-tris- (dimethylamino)-phosphonium hexafluorophosphate (BOP) and *N*,*N*-diisopropylethylamine (DIEA), and subsequent deprotected with trifluoroacetic acid (Scheme 2) to provide **5a**–**d**. Compounds **8f**–**i** were generated by reacting **7** with the appropriate amino acid amides **5a**–**d** in the presence of Et_3N. The structures of compounds **8a**–**i** were established using IR, ^1H and ^{13}C NMR spectroscopy, and high-resolution mass spectrometry.

3. Biological results and discussion

The biological activities of the DPT derivative compounds **8a**–**i** were evaluated by an *in vitro* cytotoxicity test carried out with a panel of three human tumor cell lines (A-549 (lung carcinoma), HeLa (cervical carcinoma), and SiHa (cervical squamous cell carcinoma)). The results of these experiments are summarized in Table 1.

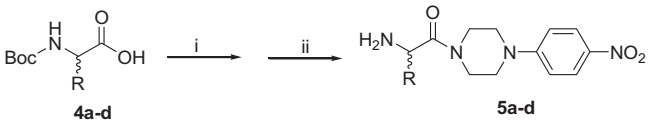
As illustrated in Table 1, the DPT derivatives, **8a**–**i**, were generally more potent than DPT, DDPT and VP-16 in their cytotoxicity to these three cell lines. As a group, these compounds were most effective in SiHa cells, and had lowest potency in HeLa cells, although the order of potency varied in each cell line. In Table 1, we also show that different piperazine substitutions in compounds **8a**–**e** have markedly different effects on the activity of the resultant compound. Notably, compound **8b** (a 1-*p*-nitrophenylpiperazinyl



Compds	8a	8b	8c	8d	8e	8f	8g	8h	8i
R	--	--	--	--	--	*L*-CH_2Ph	*D*-CH_2Ph	*L*-CH_2CH_2SMe	*D*-CH_2CH_2SMe
R′	Me	*p*-NO_2-Ph	*p*-F-Ph	*p*-OH-Ph	*p*-*cyc*-Pen	*p*-NO_2-Ph			

Scheme 1. Syntheses of compounds **8a-i**.

Reagents and conditions: i) BOP, DIEA, amine, CH_2Cl_2; ii) Trifluoroacetic acid, CH_2Cl_2



Scheme 2. Synthesis of compounds **5a**–**d**

substituted derivative of DPT) was approximately 10 times more potent than **8a**, **8c**, **8d** and **8e**.

Compounds **8f**–**i** generally showed comparable or weaker activity in comparison with **8b**. Different substituents at the α-carbon of the amino acid in this class of compounds (**8f** *vs* **8h**, **8g** *vs* **8i**) did not appear to have significant effects on their anti-proliferative activity in the *in vitro* assay. However, the compounds with *l*-amino acids incorporated appear to be more potent than those with *d*-amino acid substituents (**8f** *vs.* **8g**, **8h** *vs.* **8i**).

DPT is reported to induce apoptosis and cell cycle arrest in the G2/M phase in HeLa cells [14,15]. To determine whether derivatives of DPT have similar effects on other tumor cells, the effects of **8b** and DPT on cell cycle progression were also determined by FACS analysis in propidium iodide-stained A-549 cells. As shown in Fig. 2, treatment with 0.1 μM **8b** and 0.5 μM DPT led to accumulation of cells in the G1 phase; 76.8% and 85.5% of the cells were in G1 phase after 48 h treatment with **8b** and DPT, respectively, compared with 59.9% in untreated cultures. Furthermore, a population of sub-G1 phase cells, a characteristic of apoptotic cells, was observed after 48 h treatment with **8b**, DPT and VP-16 treatment. In control A-549 cells (Fig. 2A), only ~0.2% of the cells were in sub-G1 phase, whereas 21.7%, 13.1% and 4.1% of cells were in the sub-G1 phase after 48 h treatment with 0.1 μM **8b** (Fig. 2B), 0.5 μM DPT (Fig. 2) and 0.1 μM VP-16 (Fig. 2D), respectively. These data demonstrate that DPT and **8b** induce G1 arrest and apoptosis in A-549 cells. In addition, **8b** has more potent pro-apoptotic activity than DPT and VP-16. These results demonstrate that DPT and its derivatives interfere with cell proliferation by arresting the cell cycle, and that the cycle is arrested in different phases than those identified in previous studies [14,15].

Compound **8b** induces apoptosis, as verified by the morphological changes seen in A-549 cells treated with 0.1 μM **8b** as compared with control cells. As shown in Fig. 3, negative control (untreated) cells exhibited typical non-adherent, round morphology with vacuolization after 48 h incubation (Fig. 3A). Loss of cell membrane asymmetry and attachment, cell shrinkage and chromatin condensation (all of which are characteristics of apoptosis) were observed after treatment with **8b** (Fig. 3B). In addition, other changes were observed including blebbing, nuclear fragmentation and the formation of apoptotic bodies (Fig. 3C).

Table 1
Cytotoxicity of compounds **8a**–**i**, assessed by MTT method with 48 h drug exposure.

Compounds	Cytotoxicity (IC_{50}, μM)[a]		
	A-549	HeLa	SiHa
8a	3.96	0.703	4.97
8b	0.102	0.180	0.0195
8c	1.62	5.28	1.23
8d	2.39	7.01	4.76
8e	1.72	3.07	0.135
8f	0.0802	0.433	0.160
8g	0.300	0.802	0.211
8h	0.183	0.191	0.160
8i	0.208	0.203	0.0839
VP-16	14.8	50.6	30.7
DPT	0.470	36.0	10.2
DDPT	3.75	7.43	2.07

[a] Data are the mean of three independent experiments.

4. Conclusion

In summary, the podophyllotoxin class of compounds is a promising group of putative antitumor chemotherapeutics. In this present work, nine novel derivatives of DPT were successfully synthesized, and biological evaluation showed that most of these compounds exhibited improved anticancer activity as compared with etoposide and DPT. Compound **8b** possessed the most potent cytotoxicity against A-549, HeLa and SiHa cells. In addition, the synthesized derivative **8b** could induce cell cycle arrest in the G1 phase and caused apoptosis in A-549 cells. Further biological evaluation is in progress to clarify the mechanism of **8b** action and to define its effects on tubulin polymerization.

5. Experimental

Melting points were determined in Kofler apparatus and were uncorrected. Optical rotations were measured on a Perkin Elmer 341 polarimeter in a 1 dm cell at 23 °C. IR spectra were measured on a Nicolet 5DX-FT-IR spectrometer on neat samples placed between KBr plates. NMR spectra were recorded on a Bruker AM 400 at 400 MHz for ^1H and 100 MHz for ^{13}C in deuteriochloroform with TMS as an internal standard. Chemical shift values are expressed in ppm followed by multiplicity and coupling constants (J) in Hz. High resolution mass spectra were run in a Bruker Daltonics APEXII49e spectrometer with ESI source as ionization. LRMS were run in a VG-TS-250 spectrometer working at 70 eV. All reactions were monitored by thin layer chromatograph (TLC) on silica gel GF_{254} (0.25 mm thick). CC was performed on Silica Gel 60 (230–400 mesh, Qingdao Ocean Chemical Ltd., China). Podophyllotoxin was isolated from a Chinese medicinal herb *P. emodi* Wall var Chinesis Sprague, other starting materials and regents were purchased commercially and used without further purified, unless otherwise stated.

5.1. General procedure of synthesis of *5a–d*

To a stirred solution of the appropriate Boc-protected amino acid (2 mmol) in CH_2Cl_2 was added the 1-*p*-nitrophenylpiperazine (2.2 mmol, 1.1 equiv), BOP reagent (1.1 mmol) and DIEA (1.1 mmol) at room temperature. After stirred overnight, the reaction mixture was concentrated in vacuo, the residue was added 15 mL ethyl acetate, washed with 5% citric acid solution (6 mL × 3), 5% $NaHCO_3$ solution (6 mL × 3), then washed with saturated brine, dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was purified by flash column chromatograph to afford the appropriate Boc-amino acid amides. Boc-amino acid amides was treated with trifluoroacetic acid in CH_2Cl_2 and used directly for next reaction without further purified.

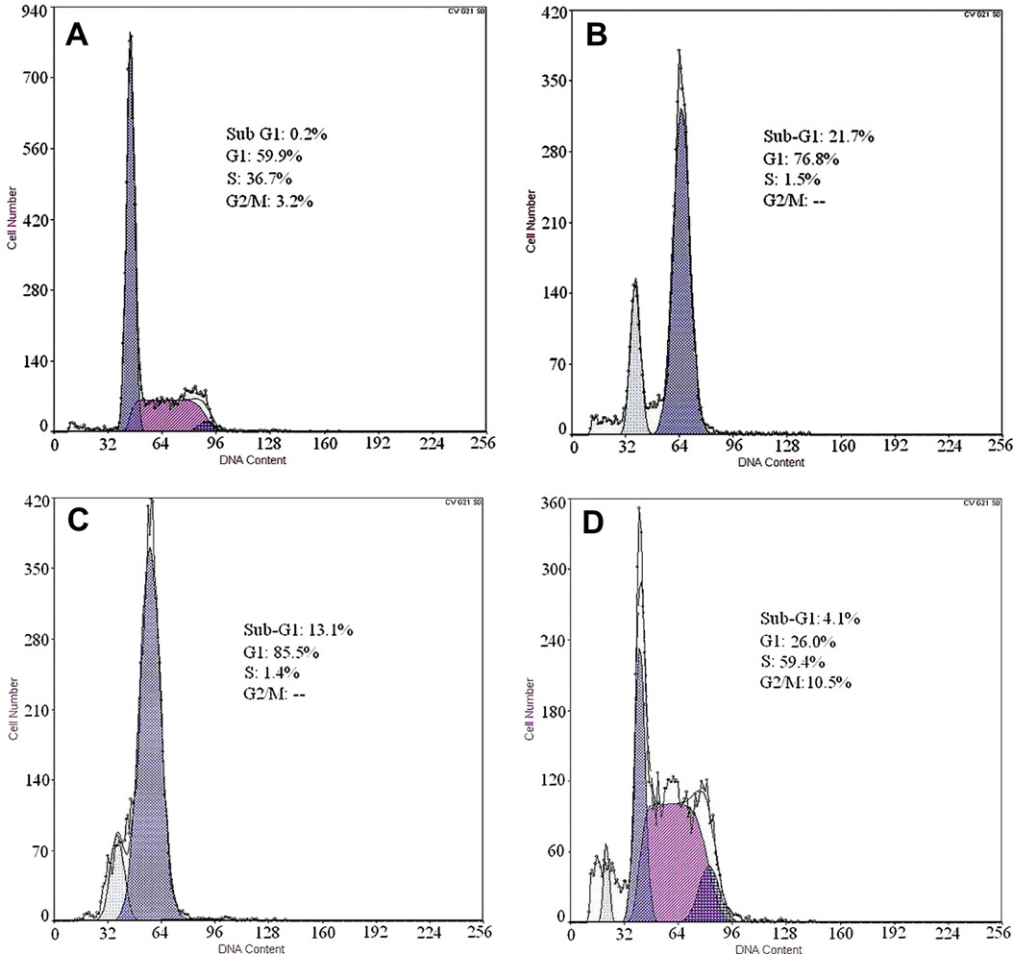


Fig. 2. Effects of **8b**, DPT and VP-16 on cell cycle progression.

5.2. Synthesis of 4′-demethyl-4-deoxylpodophyllotoxin (**3**)

A mixture of 10% Pd/C (6.0 g) in acetic acid (150 mL) was stirred under hydrogen until no further hydrogen was absorbed. Following 4′-demethyl- epipodophyllotoxin (8.0 g), which was obtained from podophyllotoxin under HBr, was added and the reaction mixture was stirred for another 5 h at 95 °C under 2 atm of hydrogen. The mixture was filtered and the filtrate was evaporated. The residue was purified by column chromatography on silica gel yielding DPT (5.6 g, yield 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.66 (s, 1 H), 6.52 (s, 1 H), 6.35 (s, 2 H), 5.94 (d, J = 9.2 Hz, 2 H), 5.41 (s, 1 H), 4.60 (s, 1 H), 4.45–4.42 (m, 1 H), 3.93–3.90 (m, 1 H), 3.78 (s, 6 H), 3.08–3.05 (m, 1 H), 2.77–2.72 (m, 3 H).

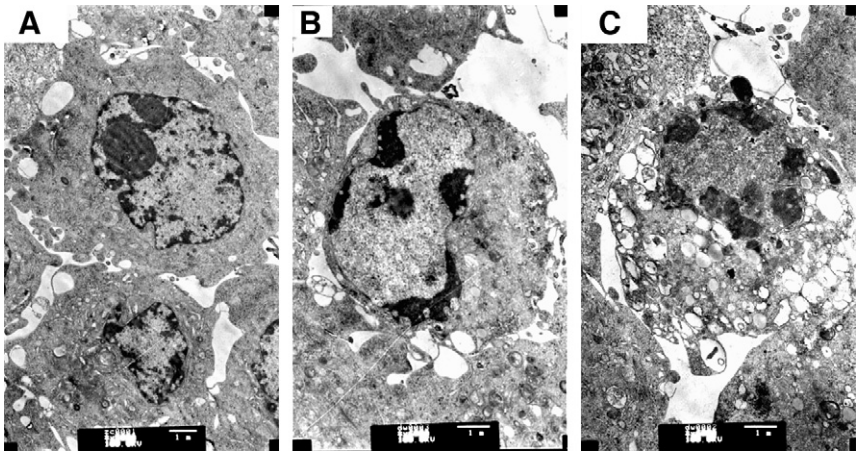


Fig. 3. Comparison of A-549 cell morphology after treatment with or without **8b**.

5.3. Synthesis of 4′-demethyl-4-deoxylpodophyllotoxin 4′-(p-nitrophenyl) carbonate (**7**)

To a solution of *p*-nitrophenyl chloroformate (1.3 g, 6.35 mmol) in anhydrous CH_2Cl_2 (5 ml) was added dry pyridine (0.6 ml). Instantaneously a white precipitate was formed. A solution of DDPT (7.2 g, 1.87 mmol) in anhydrous CH_2Cl_2 (10 ml) was added dropwise under an argon atmosphere and allowed the mixture was stirred for further 45 min at room temperature. The mixture was purified by silica gel chromatography using a mixture of dichloromethane: ethyl acetate = 8:1 as the eluent to afford compounds **8** (8.6 g, yield: 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 (m, J = 8.8 Hz, 2 H, *p*-Ph), 7.50 (m, J = 9.2 Hz, 2 H, *p*-Ph), 6.68 (s, 1 H, H-5), 6.52 (s, 1 H, H-8), 6.43 (s, 1 H, H-2′,6′), 5.96 (m, 2 H, OCH_2O), 4.66 (d, 1 H, 1−H), 4.49 (m, 1 H, H-11a), 3.96 (t, 1 H, H-11b), 3.77 (s, 6 H, $2OCH_3$), 3.11 (m, 1 H, H-2), 2.82 (m, 2 H, H-2,3,); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.8, 155.6, 151.1, 150.1, 147.1, 145.3, 140.0, 130.0, 128.3, 127.5, 125.2, 121.6, 110.4, 108.5, 107.62, 101.2, 77.32, 72.03, 56.21, 47.32, 43.27, 33.00.

5.4. General procedure of synthesis of ***8a–i***

To a stirred solution of **7** (0.1 mmol, 55 mg) in dry dichloromethane (3 ml) was added the appropriate amine (0.12 mmol, 1.2 equiv) and triethylamine (0.12 mmol) at room temperature. After stirring for overnight, the reaction mixture was purified directly by silica gel column chromatography.

5.4.1. 4′-Demethyl-4-deoxylpodophyllotoxin 4′-(4-methyl-piperazine) carbomate (***4a***)

The yield: 68%; white amorphous solid; m.p.:232−234 °C; $[\alpha]_D^{23}$ -37 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3397, 2923, 1771, 1722, 1599, 1443, 1454, 1421, 1224, 1034, 999; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (s, 1 H), 6.66 (s, 1 H), 6.52 (s, 1 H), 6.37 (s, 2 H), 5.94 (d, J = 6.4 Hz, 2 H), 4.71 (brs, 1 H), 4.63 (t, J = 6.4 Hz, 1 H), 4.47−4.42 (m, 1 H), 3.93−3.44 (m, 1 H), 3.71 (brs, 4 H), 3.57 (br, 2 H), 3.04−3.04 (m, 1 H), 2.40−273 (m, 3 H), 2.44 (t, J = 4.4 Hz, 4 H), 2.33 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.4, 153.3, 151.4 (2 C), 147.0, 146.7, 134.4, 134.7, 130.4, 124.4, 124.3, 110.5, 104.4, 107.4 (2C), 101.1, 72.0, 56.2 (2 C), 54.6 (2 C), 47.4, 46.1, 44.7, 44.1, 43.7, 33.1, 32.6; HRMS (ESI) 511.2066 for $[M + H]^+$ (calcd 511.2075 for $C_{27}H_{31}N_2O_4$).

5.4.2. 4′-Demethyl-4-deoxylpodophyllotoxin 4′-(4-p-nitrophenyl-piperazine) carbomate (***4b***)

The yield: 72%; yellow amorphous solid; m.p.:149−150 °C; $[\alpha]_D^{23}$ -45 (c 0.3, $CHCl_3$); IR (cm^{-1}) 2916, 1773, 1719, 1597, 1505, 1445, 1423, 1324, 1224, 1127, 1034, 996; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.13 (d, J = 9.2 Hz, 2 H), 6.44 (d, J = 9.6 Hz, 2 H), 6.66 (s, 1 H), 6.51 (s, 1 H), 6.34 (s, 2 H), 5.93 (d, J = 4.0 Hz, 2 H), 4.63 (d, J = 4.0 Hz, 1 H), 4.45 (t, J = 6.4 Hz, 1 H), 3.90 (t, J = 9.2 Hz, 1 H), 3.46 (br, 2 H), 3.77−3.3.73 (m, 2 H), 3.70 (s, 6 H), 3.51 (br, 4 H), 3.06 (dd, J = 14.4, 5.6 Hz, 1 H), 2.40−270 (m, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.4, 154.5, 153.2, 151.4 (2 C), 147.1, 146.7, 134.9, 134.7, 130.3, 124.3, 124.1, 125.9 (2 C), 112.9 (2 C), 110.5, 104.4, 107.4 (2C), 101.2, 72.0, 56.2 (2 C), 47.4, 46.4 (2 C), 44.1, 43.4, 43.3, 33.1, 32.7; HRMS (ESI) 640.1915 for $[M + Na]^+$ (calcd 640.1902 for $C_{32}H_{31}N_3NaO_{10}$).

5.4.3. 4′-Demethyl-4-deoxylpodophyllotoxin 4′-(4-p-flurophenyl-piperazine) carbomate (***4c***)

Yield: 47%; white amorphous solid; m.p.:142−143 °C; $[\alpha]_D^{23}$ -72 (c 0.3, $CHCl_3$); IR (cm^{-1}) 2911, 1774, 1723, 1600, 1509, 1423, 1336, 1223, 1157, 1129, 1039, 995; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.00−6.96 (m, 2 H), 6.92−6.40 (m, 2 H), 6.66 (s, 1 H), 6.52 (s, 1 H), 6.34 (s, 2 H), 5.93 (d, J = 4.0 Hz, 2 H), 4.63 (d, J = 3.6 Hz, 1 H), 4.46 (t, J = 4.4 Hz, 1 H), 3.93 (t, J = 9.2 Hz, 1 H), 3.44 (brs, 2 H), 3.70 (brs, 4 H), 3.15−3.10 (m, 4 H), 3.06 (dd, J = 14.4, 4.4 Hz, 1 H), 2.40−2.73 (m, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.4, 153.2, 151.4 (2 C), 147.0, 146.6, 134.5, 130.3, 124.3, 124.2, 114.5, 114.4, 115.7, 115.4, 110.5, 104.4, 107.4 (2 C), 101.1, 77.3, 72.0, 56.2 (2 C), 47.4, 43.7, 33.0, 32.6; HRMS (ESI) 591.2132 for $[M + H]^+$ (calcd 591.2137 for $C_{32}H_{32}FN_2O_4$).

5.4.4. 4′-Demethyl-4-deoxylpodophyllotoxin 4′-(4-p-hydroxylphenyl-piperazine) carbomate (***4d***)

Yield: 43%; white amorphous solid; m.p.:140−142 °C; $[\alpha]_D^{23}$ -65 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3361, 2920, 1771, 1711, 1599, 1512, 1424, 1223, 1124, 1034, 995; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.46 (d, J = 4.0 Hz, 2 H), 6.77 (d, J = 4.0 Hz, 2 H), 6.66 (s, 1 H), 6.52 (s, 1 H), 6.34 (s, 2 H), 5.93 (d, J = 7.6 Hz, 2 H), 4.63 (d, J = 3.6 Hz, 1 H), 4.46−4.40 (m, 1 H), 3.91−3.79 (m, 2 H), 3.79−3.72 (m, 3 H), 3.70 (s, 6 H), 3.10−3.00 (m, 5 H), 2.40−2.70 (m, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.4, 153.4, 152.3 (2 C), 147.6, 147.2, 139.3, 130.4, 124.4 (2C), 117.0, 111.0, 104.9, 104.3 (2 C), 101.7, 77.3, 72.6, 56.7, 53.9, 47.9, 44.2, 33.6, 33.2; HRMS (ESI) 549.2173 for $[M + H]^+$ (calcd 549.2141 for $C_{32}H_{33}N_2O_9$).

5.4.5. 4′-Demethyl-4-deoxylpodophyllotoxin 4′-(4-cyclopentylpiperazine) carbomate (***4e***)

Yield: 47%; white amorphous solid; m.p.:130−132 °C; $[\alpha]_D^{23}$ s-41 (c 0.3, $CHCl_3$); IR (cm^{-1}) 2955, 2913, 2469, 1774, 1721, 1600, 1442, 1423, 1335, 1222, 1129, 1034, 997; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.66 (s, 1 H), 6.52 (s, 1 H), 6.37 (s, 2 H), 5.93 (d, J = 4.0 Hz, 2 H), 4.63 (d, J = 3.6 Hz, 1 H), 4.46 (t, J = 4.4 Hz, 1 H), 3.90 (t, J = 9.2 Hz, 1 H), 3.70 (brs, 4 H), 3.54 (brs, 2 H), 3.06 (dd, J = 12.4, 4.4 Hz, 1 H), 2.40−2.70 (m, 3 H), 2.54 (brs, 5 H), 1.47 (s, 2 H), 1.95−1.45 (m, 2 H), 1.73−1.69 (m, 2 H), 1.60−1.55 (m, 2 H), 1.47−1.42 (m, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.9, 153.4, 151.9 (2 C), 147.1, 146.7, 134.5, 130.5, 124.5, 124.4, 110.7, 104.5, 107.9 (2 C), 101.2, 72.1, 67.5, 56.4 (2 C), 52.0 (2 C), 47.6, 44.9, 44.1, 43.4, 33.2, 32.7, 30.4 (2 C), 24.1 (2 C); HRMS (ESI) 565.2553 for $[M + H]^+$ (calcd 565.2544 for $C_{31}H_{37}N_2O_4$).

5.4.6. N-(1-oxyl-4′-demethyl-4-deoxypodophyllic)-L-phenylalanine 4′-(4-p-nitrophenyl -piperazine) carbomate (***4f***)

Yield: 32%; yellow amorphous solid; m.p.:132−134 °C; $[\alpha]_D^{23}$ -31 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3307, 2920, 1771, 1734, 1710, 1645, 1597, 1502, 1446, 1324, 1224, 1124, 1035, 996; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.10 (d, J = 9.2 Hz, 2 H), 7.30−7.27 (m, 5 H), 6.70 (d, J = 9.2 Hz, 2 H), 6.67 (s, 1 H), 6.52 (s, 1 H), 6.34 (s, 2 H), 6.14 (d, J = 4.4 Hz, NH), 5.95 (d, J = 4.4 Hz, 2 H), 4.94−4.47 (m, 1 H), 4.63 (d, J = 3.6 Hz, 1 H), 4.46 (t, J = 6.4 Hz, 1 H), 4.12 (q, J = 7.2 Hz, 2 H), 3.92 (t, J = 4.4 Hz, 1 H), 3.70 (s, 6 H), 3.50−3.45 (m, 1 H), 3.39−3.34 (m, 1 H), 3.21−3.15 (m, 3 H), 3.10−3.04 (m, 3 H), 2.41−2.74 (m, 3 H), 2.69−2.61 (m, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.4, 169.4, 154.0, 153.5, 151.4 (2 C), 147.0, 146.6, 134.4, 136.0, 130.2, 129.5 (2 C), 129.4, 124.6 (2 C), 124.3, 127.6, 127.1, 125.4 (2 C), 112.7 (2 C), 110.4, 104.4, 107.7 (2 C), 101.1, 72.0, 60.3, 56.1 (2 C), 51.9, 47.3, 46.3, 44.5, 43.7, 41.2, 39.4, 33.0, 32.6; HRMS (ESI) 765.2744 for $[M + H]^+$ (calcd 765.2766 for $C_{41}H_{41}N_4O_{11}$).

5.4.7. N-(1-oxyl-4′-demethyl-4-deoxypodophyllic)-D-phenylalanine 4′-(4-p-nitrophenyl -piperazine) carbomate (***4g***)

Yield: 32%; yellow amorphous solid; m.p.:133−135 °C; $[\alpha]_D^{23}$ -53 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3274, 2914, 1737, 1594, 1500, 1449, 1331, 1230, 1115, 1035, 996; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.10 (d, J = 9.2 Hz, 2 H), 7.30−7.27 (m, 5 H), 6.69 (d, J = 9.6 Hz, 2 H), 6.66 (s, 1 H), 6.49 (s, 1 H), 6.34 (s, 2 H), 6.07 (d, J = 4.4 Hz, NH), 5.93 (d, J = 6.4 Hz, 2 H), 4.91−4.49 (m, 1 H), 4.62 (d, J = 4.0 Hz, 1 H), 4.45 (t, J = 6.4 Hz, 1 H), 4.12 (q, J = 7.2 Hz, 2 H), 3.92 (t, J = 4.4 Hz, 1 H), 3.70 (s, 6 H), 3.52−3.47 (m, 1 H), 3.39−3.34 (m, 1 H), 3.20−3.15 (m, 3 H), 3.10−3.02 (m, 3 H), 2.74−2.71 (m, 3 H), 2.66−2.63 (m, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ HRMS (ESI) 765.2762 for $[M + H]^+$ (calcd 765.2766 for $C_{41}H_{41}N_4O_{11}$).

*5.4.8. N-(1-oxyl-4′-demethyl-4-deoxypodophyllic)-L-methine 4′-(4-p-nitrophenyl- piperazine) carbomate (**4h**)*

Yield: 32%; yellow amorphous solid; 32%. m.p.:134–139 °C; $[\alpha]_D^{23}$ -41 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3406, 2973, 2925, 1690, 1596, 1501, 1452, 1395, 1326, 1235, 1167, 1129, 1027, 994; ^1H NMR (400 MHz, $CDCl_3$) δ 4.14 (d, $J = 4.4$ Hz, 2 H), 6.45 (d, $J = 4.4$ Hz, 2 H), 6.66 (s, 1 H), 6.52 (s, 1 H), 6.34 (s, 2 H), 6.30 (d, $J = 4.4$ Hz, NH), 5.94 (d, $J = 4.4$ Hz, 2 H), 4.63 (d, $J = 3.6$ Hz, 1 H), 4.45 (t, $J = 7.2$ Hz, 1 H), 3.92 (t, $J = 9.6$ Hz, 1 H), 3.90–3.46 (m, 2 H), 3.74–3.72 (m, 2 H), 3.70 (s, 6 H), 3.50 (brs, 4 H), 3.09–3.04 (m, 1 H), 2.40–2.66 (m, 3 H), 2.63–2.59 (m, 2 H), 2.09 (s, 3 H), 2.04–2.02 (m, 2 H); HRMS (ESI) 749.2464 for $[M + H]^+$ (calcd 749.2447 for $C_{37}H_{41}N_4O_{11}S$).

*5.4.9. N-(1-oxyl-4′-demethyl-4-deoxypodophyllic)-D-methine 4′-(4-p-nitrophenyl- piperazine) carbomate (**4i**)*

Yellow amorphous solid, Yield:; m.p.:134–139 °C; $[\alpha]_D^{23}$ -50 (c 0.3, $CHCl_3$); IR (cm^{-1}) 3343, 2962, 2916, 1771, 1734, 1710, 1645, 1597, 1503, 1445, 1460, 1326, 1229, 1127, 1035, 997; ^1H NMR (400 MHz, $CDCl_3$) δ 4.14 (d, $J = 9.2$ Hz, 2 H), 6.43 (d, $J = 9.2$ Hz, 2 H), 6.66 (s, 1 H), 6.50 (s, 1 H), 6.37 (s, 2 H), 5.96 (d, $J = 4.4$ Hz, NH), 5.95 (d, $J = 4.4$ Hz, 2 H), 4.94–4.44 (m, 1 H), 4.62 (d, $J = 3.6$ Hz, 1 H), 4.45 (t, $J = 6.4$ Hz, 1 H), 3.94–3.44 (m, 3 H), 3.40–3.72 (m, 2 H), 3.64 (s, 6 H), 3.50–3.39 (m, 4 H), 3.10–3.04 (m, 1 H), 2.40–2.74 (m, 3 H), 2.65–2.59 (m, 2 H), 2.12 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.4, 169.4, 154.2, 153.4, 151.4, 147.0, 146.7, 139.1, 134.9, 130.2, 124.3, 125.9, 113.0, 110.5, 104.5, 107.7, 101.2, 72.0, 56.1, 53.7, 49.6, 47.4, 47.2, 46.7, 44.7, 43.7, 41.5, 33.0, 32.9, 32.6, 29.9, 29.2, 15.6; HRMS (ESI) 749.2499 for $[M + H]^+$ (calcd 749.2447 for $C_{37}H_{41}N_4O_{11}S$).

5.5. Cytotoxicity assays

Cells were incubated at 37 °C in a 5% CO_2 atmosphere. The MTT assay was used to determine growth inhibition [28]. The derivatives were dissolved in saline for five concentrations (0.005–50 μM). Cells were plated in 96-well plates and allowed to attach for 24 h, then exposed in quadplex well for 48 h. The media were aspirated, and 10 μL of 5 mg/mL MTT solution (dilute in sterile PBS) diluted in serum-free media was added to each well. After 4 h of incubation, the solution was centrifuged for 10 min under 2000 rpm. The supernatant was mixed with 150 μL DMSO, then was shaken on a oscillator. The absorbance at λ_{570} was determined on a microplate reader (BIO-RAD m680, USA). IC_{50} values were determined from a log plot of percent of control versus concentration.

5.6. Analysis of cell cycle by flow cytometry

For cell cycle analysis, we used the human lung carcinoma A-549 cell line grown in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 24 μg/mL gentamicin and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Untreated and drug-treated cells ($(3–5) \times 10^5$) were harvested and fixed overnight in 70% ethanol at 4 °C. Cells were then washed three times with PBS, incubated for 1 h with 1 mg/mL RNase A and 20 μg/mL propidium iodide at room temperature, and analyzed with a flow cytometer (COULTER EPICS XL, USA) as described previously [29]. Quantification of apoptotic cells was calculated as the percentage of cells in the sub-G1 phase (hypodiploidy) in the cell cycle.

5.7. Analysis of morphological changes

A-549 cells treated with 0.1 μM **8b** or vehicle were harvested after 48 h, fixed with glutaraldehyde and osmium tetroxide, dehydrated with acetone, then embedded, sectioned, stained and examined for morphological changes using a transmission electron microscope (JEOL JEM-1230, Japan) [30].

Acknowledgements

This work was financially supported by the Doctoral Found of Qingdao University of Science and Technology (QUST 0022380), Open Funds of Key Lab of Preclinical Study for New Drugs of Gansu Province (GSKFKT-00801) and the Fundamental Research Funds for the Central Universities, Lanzhou University (lzujbky-2010-136).

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