European Journal of Medicinal Chemistry 68 (2013) 463–472

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Original article

Discovery, synthesis and biological evaluation of cycloprotoberberine derivatives as potential antitumor agents

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ARTICLE INFO

Article history:
Received 22 January 2013
Received in revised form
1 July 2013
Accepted 6 July 2013
Available online 11 August 2013

Keywords:
Cycloprotoberberine
Topoisomerase
Structure–activity relationship
Antiproliferative
Drug resistance

ABSTRACT

A series of new 1,13-cycloprotoberberine derivatives defined through variations at the 9-position were designed, synthesized and evaluated for their cytotoxicities in human HepG2 (hepatoma), HT1080 (fibrosarcoma) and HCT116 (colon cancer) cells. The preliminary structure–activity relationship (SAR) revealed that the replacement of 9-methoxyl with an ester moiety might significantly enhance the antiproliferative activity *in vitro*. Notably, compound **7f** demonstrated equipotent cytotoxicity activity against breast cancer MCF-7 (parent) and doxorubicin (DOX)-resistant MCF-7 (MCF-7/ADrR) cells, indicating a mode of action different from that of DOX. Further mechanism study showed that **7f** significantly inhibited activity of DNA topoisomerase I (Top I) and Top II. G2/M phase arrest and tumor cell growth reduction was observed thereafter. Thus, we consider cycloprotoberberine analogues to be a new family of promising antitumor agents with an advantage of inhibiting drug-resistant cancer cells.

1. Introduction

Berberine (BBR, **1**, Fig. 1), which is an isoquinoline natural product extracted from Chinese herbs *Coptis chinensis*, has been extensively used as a nonprescription drug to treat diarrhea in China for decades [1–4]. BBR has been extensively investigated since last century and was found to have a variety of pharmacological and biological activities, such as antifungal [5], anti-inflammatory [6], anti-oxidative [7], antineoplastic [8–11] and glucose-lowering [12] effects. Recently, our work has identified BBR as a novel cholesterol-lowering agent that up-regulates low-density-lipoprotein receptor (LDLR) gene expression through stabilization of LDLR mRNA; the molecular mechanism is different from that of the marketed statins [13].

In continuation of our ongoing efforts to search for derivatives of **1** as a novel class of LDLR up-regulators [14], compound 1,13-cycloprotoberberine chloride (**2**, Fig. 1) was obtained accidently and identified structurally. The modification in compound **2** completely abolished the up-regulatory effect on LDLR mRNA expression (data not shown). Screened in the models constructed in our laboratory, we found that compound **2** exerted a moderate antiproliferative activity (Tables 1 and 2) against three human tumor cell lines of HepG2 (hepatoma), HT1080 (fibrosarcoma) and HCT116 (colon cancer) with inhibition rate between 33% and 53%, greater than that of **1** in less than 5% inhibition (data not shown). The mechanism of **1** for anticancer activity is to bind to and stabilize the topoisomerase I (Top I)-mediated DNA cleavable complex by forming a drug–Top I–DNA ternary complex, just as hydroxycamptothecin (HCPT) or camptothecin does [15]. We deduced that the factor that contributes to the increased antiproliferative activity in **2** is presumably the minor change of planar structure (Fig. 1), which might enhance the potency of the compound to intercalate into free DNA, and subsequently prevent DNA cleavage by Top I.

The unique chemical scaffold and promising antiproliferative activity of **2** provoked our strong interest to further explore the structure–activity relationship (SAR) of this group of compounds. Then, the SAR investigation for the cytotoxicity was carried out with **2** as the lead in attempt to discover new chemical entity (NCE) with anticancer activity. As the methylenedioxy group plays a key role in keeping good anticancer activity [16], the present SAR

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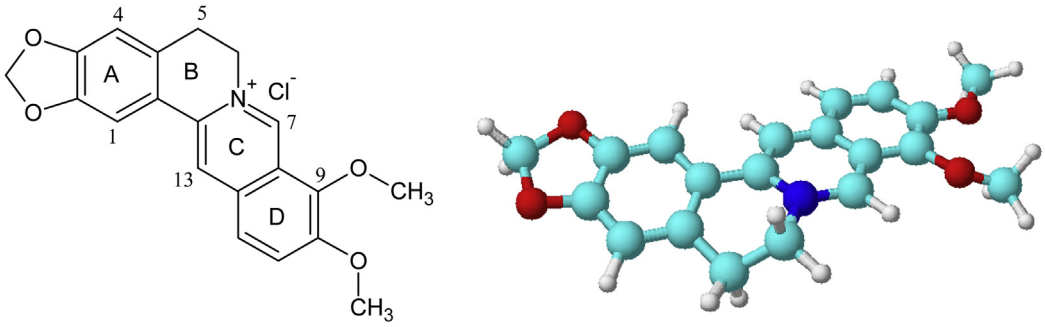
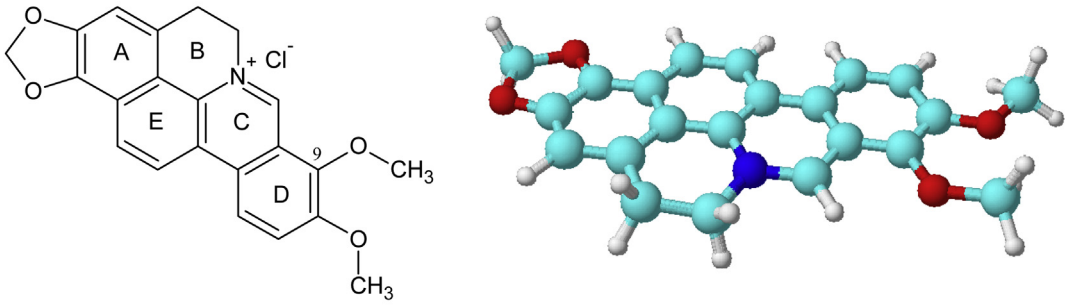
http://dx.doi.org/10.1016/j.ejmech.2013.07.026



Compound **1**



Compound **2**

Fig. 1. Modeled skeleton structures of compounds **1** and **2**. Both steric structures were constructed with ACDLAB software 12.0 version (3D Viewer).

study was mainly focused on the influence of the substituents at the 9-position. On the basis of this strategy, a series of novel 9-substituted cycloprotoberberine derivatives was designed, synthesized and evaluated. Meanwhile, with the hit **2** selected, we developed the concise synthetic routes for new cycloprotoberberine analogues (Scheme 1), which allows an extensive SAR analysis. Herein, we report the synthesis of cycloprotoberberine derivatives and antiproliferative activity in HepG2 (hepatoma), HT1080 (fibrosarcoma) and HCT116 (colon cancer) cell lines. Among these newly synthesized analogues, compound **7f** was selected as a representative agent for further antiproliferative test in multidrug resistance (MDR) cells, as well as for the primary mechanism of action.

2. Chemistry

The synthetic route used for the preparation of the hit **2** and its analogues is described in Scheme 1 with **1** as the starting material. Compound **1** was selectively reduced to the dihydroberberine (**3**) with $NaBH_4$ as a reductive agent in the presence of 5% $NaOH/K_2CO_3$ in 62% yield [17]. Compound **3** was treated with 40% glyoxal in refluxing $HOAc/CH_3CN$ to provide 13-acetaldehyde berberine (**4**) [17,18], which was easily converted into its isomer **5** in the acid condition. Cyclization of the intermediate **5** in 2% HCl at room temperature afforded **2** in 38% yield. Then, compound **2** was heated at 195–210 °C under vacuum (20–30 mmHg) and acidified in concentrated HCl/ethanol (5/95) to get the key intermediate **6** in 92% yield with the previous procedures [19]. Compound **6** was etherified or acylated to provide the final products **7a**–**s** with yields of 15–67% [19,20]. The desired products were purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent.

3. Results and discussion

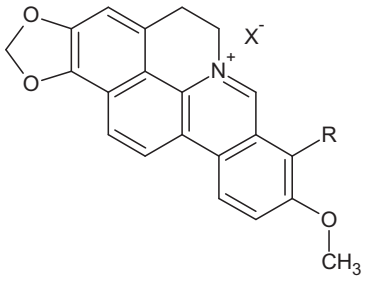
3.1. SAR analysis for the antiproliferative activity in vitro

All of the newly synthesized compounds were initially examined for their antiproliferative activity against human HepG2 hepatoma cells using the sulforhodamine B (SRB) assay with HCPT as a reference drug. Structures of the 20 cycloberberine analogues and their cytotoxicity at the concentration of 0.6 μg/mL were summarized in Table 1.

We started our SAR investigation with replacing the methoxyl at the 9-position of **2** by an ether or ester moiety. Firstly, with an introduction of a hydroxyl, ethoxyl, butyoxy, benzyloxy or 2,4-difluorobenzyloxy at position 9, respectively, five new analogues (**6**, **7a**–**d**) were generated and followed by biological test. All of the 5 compounds exhibited a moderated cytotoxicity against HepG2 with inhibitory rate of 42–63%, similar to that of the lead **2** (53%). It seemed that an ether moiety at the 9-position could retain the antiproliferative activity on HepG2, regardless of their size of the side-chains.

Next, with an introduction of an ester moiety into the C-9 of **2**, eleven new analogues (**7e**–**o**) were prepared and tested. 9-Acetyloxy compound **7e** exerted an increased cytotoxicity on HepG2 with inhibition rate by 88%, in comparison with the lead **2**. 9-Substituted benzoyloxy compounds (**7f**–**m**) including electron-withdrawing or electron-donating groups on the phenyl ring afforded a potential cytotoxicity inhibition rate ranging from 62% to 94%, much greater than that of the lead **2**. In addition, as the

Table 1
Cytotoxicity of cycloprotoberberines against human HepG2 cells with R variations (%).[a]



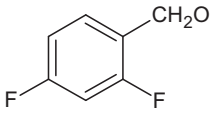
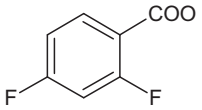
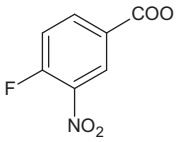
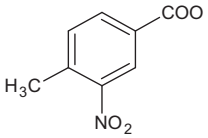
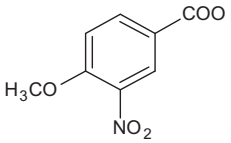
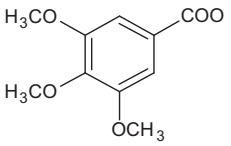
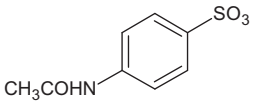
Compd	R	X	HepG2
2	OCH_3	Cl	53.4 ± 3.2
6	OH	Cl	50.0 ± 3.1
7a	OCH_2CH_3	Br	41.9 ± 3.3
7b	$OCH_2CH_2CH_2CH_3$	I	62.6 ± 1.8
7c	OCH_2Ph	Br	53.3 ± 11.5
7d		Br	50.7 ± 11.7
7e	$OCOCH_3$	Cl	88.7 ± 1.1
7f	OCOPh	Cl	86.6 ± 2.4
7g	OCOPhF-*p*	Cl	70.3 ± 2.6
7h	OCOPhF-*o*	Cl	93.7 ± 16.1
7i		Cl	92.5 ± 11.4
7j	OCOPhCl-*o*	Cl	62.3 ± 2.0
7k	$OCOPhCH_3$-*p*	Cl	63.7 ± 2.0
7l	$OCOPhOCH_3$-*p*	Cl	84.5 ± 1.9
7m		Cl	69.6 ± 2.5
7n		Cl	50.9 ± 4.3
7o		Cl	53.6 ± 2.2
7p		Cl	86.5 ± 10.6
7q	piperonyloyloxy	Cl	88.6 ± 10.8
7r	OSO_2Ph	Cl	56.2 ± 2.0

Table 1 (*continued*)

Compd	R	X	HepG2
7s		Cl	8.9 ± 5.1
HCPT			85.4 ± 3.8

[a] % of inhibition. Cells were untreated (control) or treated with the target compounds (0.6 μg/mL, 1.04 μM–1.57 μM) or HCPT (0.6 μg/mL, 1.65 μM) for 48 h, respectively.

trimethoxyphenyl and methylenedioxyphenyl might play a key role for the antiproliferative activity of anticancer drugs [16,21], they were used to replace the 9-OCH_3, from which two analogues (**7p**–**q**) were then created. The results showed that compounds **7p** and **7q** exhibited an increased activity in 87% and 89% inhibition, respectively. In another variation, benzenesulfonyl derivative **7r** retained a cancericidal activity similar to that of lead **2**, while benzenesulfonyl derivative **7s** lost the cytotoxicity completely. The results suggested that introduction of an ester moiety at C-9 of **2** might significantly enhance the antiproliferative activity on HepG2.

3.2. Antiproliferative activities of the part compounds in human tumor cell lines

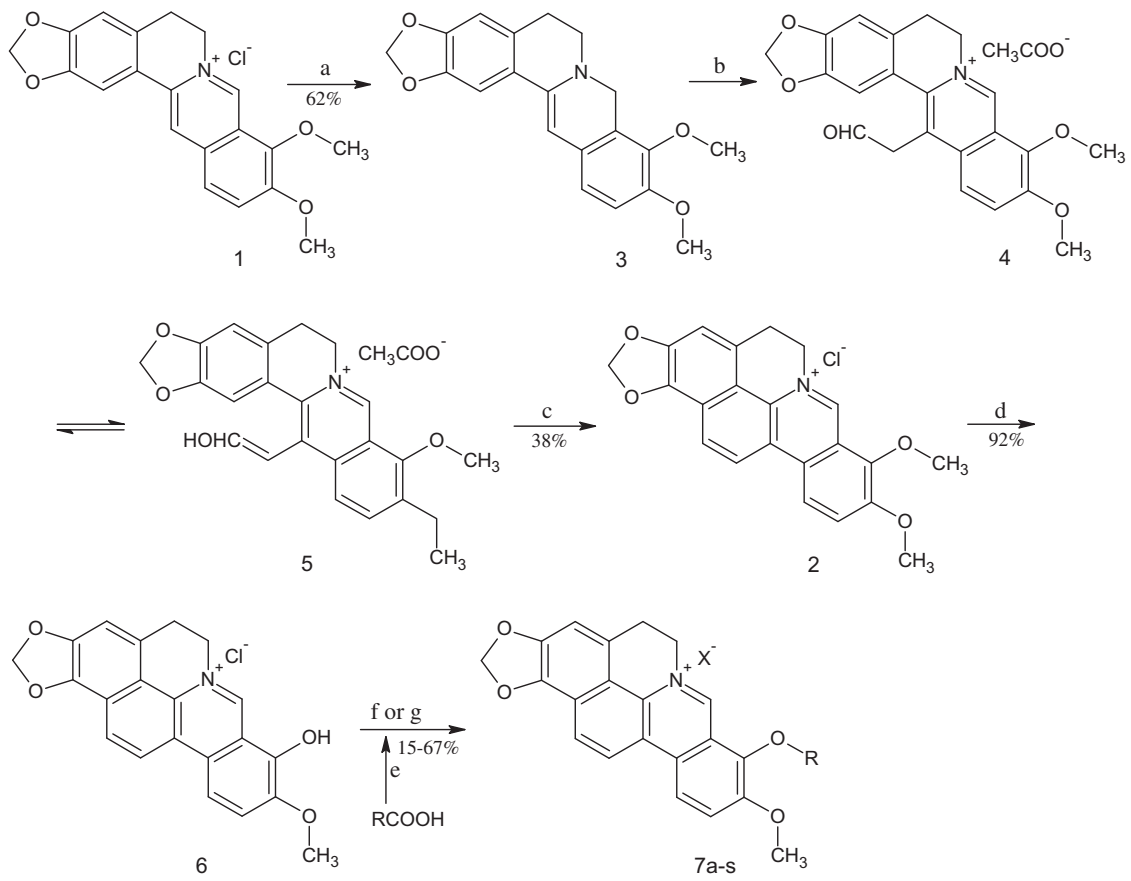
Out of the 20 newly synthesized compounds, seven analogues (**7e**–**f**, **7h**–**i**, **7l**, **7p**–**q**) demonstrated a potential cancericidal effect against HepG2 cells with above 85% growth inhibition. All of these active derivatives were chosen to measure their IC_{50} values on HepG2 and HCT116 cells for 24 and 48 h, respectively. As indicated in Table 2, most of them showed significant antiproliferative activities on HepG2 and HCT116 cells. Compound **7f** with no substituents on the phenyl ring showed the best activity on HepG2 cells, and then was selected for the further investigation.

The growth inhibition test in drug-resistant cells of compound **7f** was performed on human MCF-7 breast cancer cells, which have developed resistance to doxorubicin (DOX), a known Top II inhibitor used widely in clinic. In this experiment, we tested compound **7f** for its activity against parent MCF-7 cell line and DOX-resistant cells (MCF-7/ADrR), with DOX as a reference drug. As shown in Fig. 2, DOX afforded a potent activity against parent MCF-7 with an IC_{50} of 0.18 μg/mL, much greater than that in the MCF-7/ADrR cells with an IC_{50} of 24.4 μg/mL. Notably, compound **7f** demonstrated antiproliferative effect in MCF-7 cells with an IC_{50} of 2.5 μg/mL, almost equipotent to that in the MCF-7/ADrR cells (IC_{50}, 3.66 μg/mL). It suggests that cross drug resistance between cycloprotoberberines and the known Top II inhibitor might not exist.

Table 2
Antiproliferative activities of the active compounds in human tumor cell lines IC_{50}[a] (μM).

Compd	HepG2		HCT116	
	24 h	48 h	24 h	48 h
2	1.20 ± 0.08	0.71 ± 0.07	0.56 ± 0.02	1.71 ± 0.12
7e	1.01 ± 0.16	0.19 ± 0.02	0.09 ± 0.01	0.84 ± 0.17
7f	0.47 ± 0.10	0.14 ± 0.02	5.61 ± 1.54	0.41 ± 0.02
7h	0.93 ± 0.53	0.15 ± 0.02	0.42 ± 0.03	0.28 ± 0.07
7i	0.62 ± 0.20	0.13 ± 0.01	3.11 ± 0.15	0.39 ± 0.01
7l	0.44 ± 0.18	0.41 ± 0.08	0.39 ± 0.17	0.43 ± 0.01
7p	0.38 ± 0.07	0.32 ± 0.01	0.92 ± 0.08	0.37 ± 0.03
7q	0.42 ± 0.32	0.22 ± 0.02	3.06 ± 0.73	0.45 ± 0.02

[a] IC_{50}: a drug concentration required to inhibit human tumor cells proliferation by 50% after 24 and 48 h treatment, respectively.



Scheme 1. Reagents and conditions: (a) $NaBH_4$, 5% $NaOH/K_2CO_3$, CH_3OH, rt, 2 h; (b) 40% glyoxal, $HOAc/CH_3CN$, reflux, 5 h; (c) 2% HCl, rt, 5 d; (d) 20–30 mmHg, 195–210 °C; (e) $SOCl_2$, reflux, 2 h; (f) RCOCl, pyridine, CH_3CN, reflux, 6 h; (g) RX, KOH, DMF, 60 °C, 12 h.

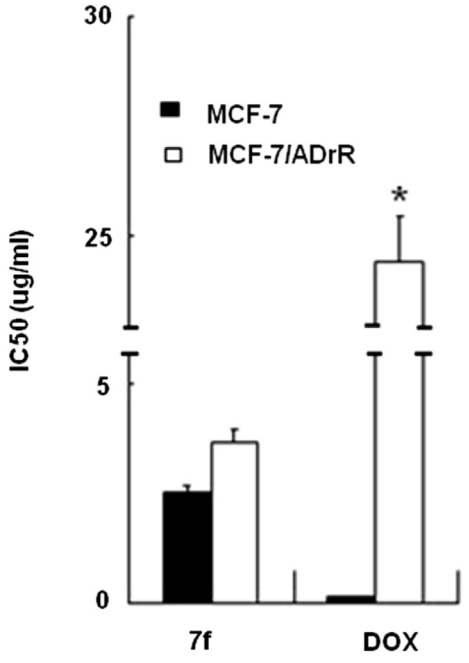


Fig. 2. IC_{50} values of **7f** and DOX in MCF-7 (parent) and DOX-resistant (MCF-7/ADrR) cell lines.

3.3. Primary mechanism

After the activity evaluation, we extended our work to the primary mechanism investigation of compound **7f**. Flow cytometric analysis of the DNA profile in the HCT116 cells (Fig. 3) showed that **7f** treatment (0.5 μg/mL for 24 h) caused a major shift of the cell population from G0/G1 to G2/M phase, revealing a significant accumulation of cells in the G2/M phase. Next, we accessed the inhibitory activity of **7f** on DNA Top I, with HCPT as a reference drug. As shown in Fig. 4 (top), compound **7f** significantly inhibited the activity of Top I at the concentration of 15 μg/mL, which was consistent with that of HCPT. Meanwhile, the inhibitory activity of **7f** on the DNA Top II was also carried out with Etoposide (VP16) as a reference, a known Top II inhibitor. As shown in Fig. 4 (bottom), compound **7f** at 15 μg/mL also significantly inhibited Top II activity, demonstrating potency greater than that of VP16 (100 μg/mL). The results indicated that compound **7f** had a potent inhibitory activity on either DNA Top I or DNA Top II.

In order to further understand the binding situation between **7f** and its targets, the docking analysis of **7f** with Top I was carried out with eHiTS software. As illustrated in Fig. 5 (top), the results indicated an intercalation of **7f** into DNA molecules. As shown in Fig. 5 (bottom), the described binding model of the **7f**–Top I–DNA showed an extended region of π–π stacking interactions between the core of **7f** and the DNA base pairs G 11 and A 113, as well as a number of hydrogen bonds formed between the oxygen atoms in **7f** and Top I–DNA residues. Besides, the benzoyloxy group at the 9-positions of **7f** might insert into the hydrophobic pocket formed by Ala 351, Met 428, Pro 431 and Lys 436.

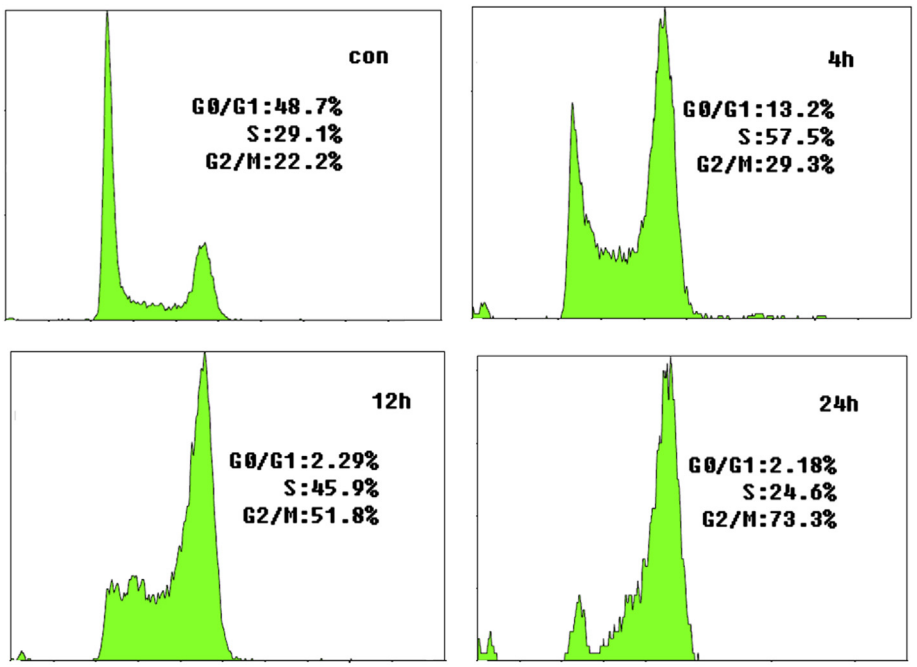


Fig. 3. Cell cycle analysis of **7f**. HCT116 cells were incubated without (control) or with **7f** (0.5 μg/mL) for 4, 12, or 24 h, respectively. Cells were then analyzed for their cell cycle distribution using flow cytometry.

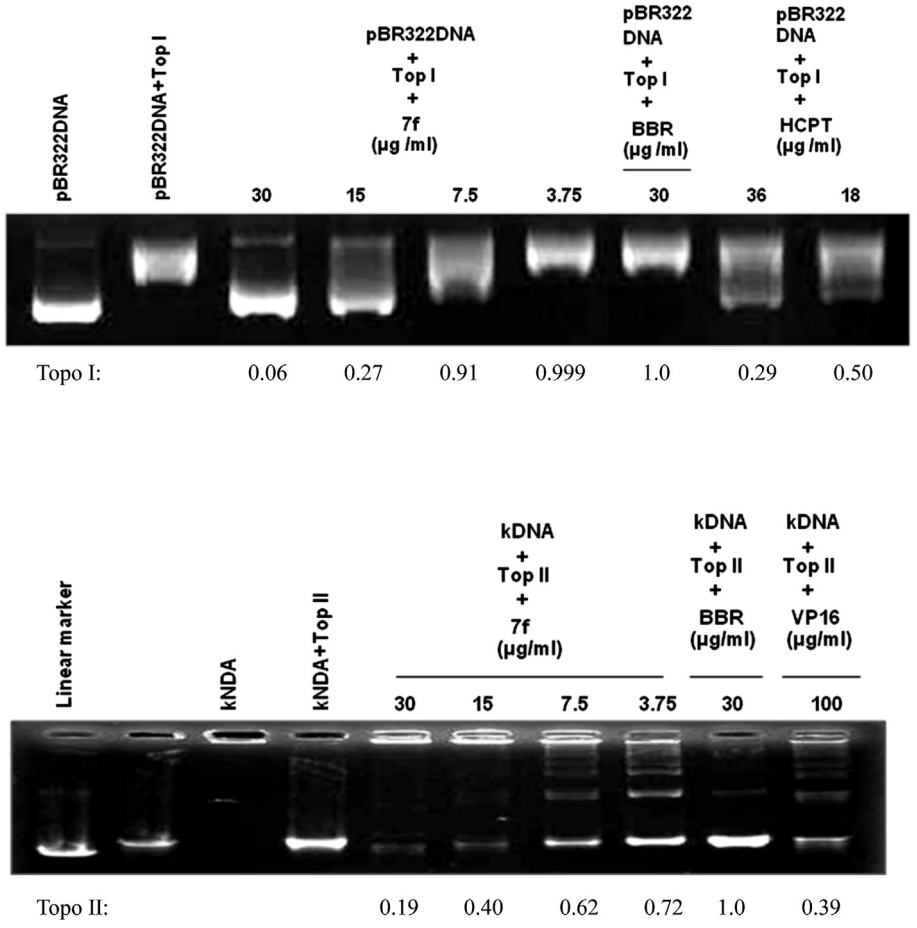


Fig. 4. DNA Top I (top) and Top II (bottom) inhibitory activity of **7f** at different concentration using HCPT and VP16 as a positive control respectively.

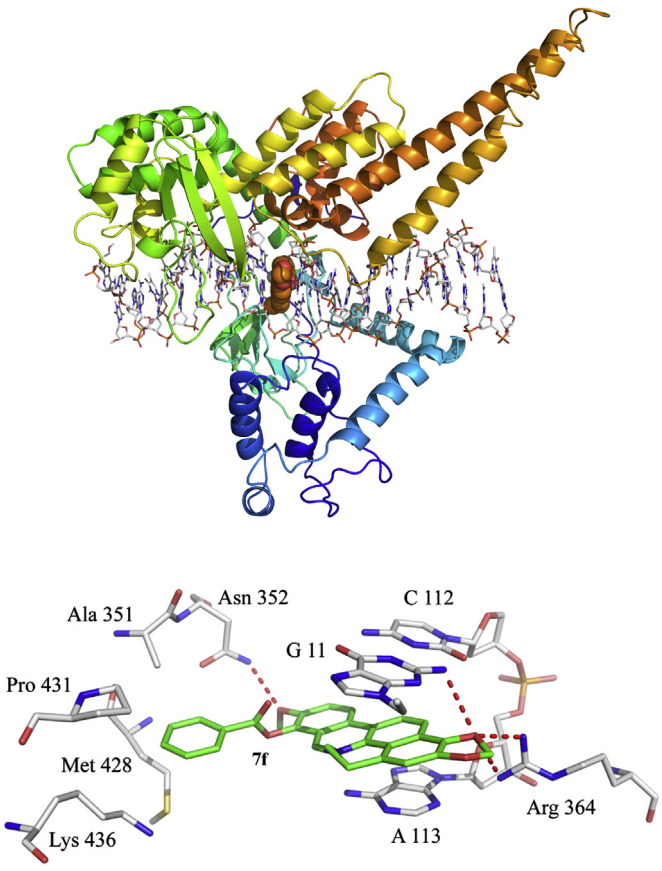


Fig. 5. Binding modes of **7f** within Top I/DNA covalent complex (top) and Top I–DNA domain (bottom). Molecular figures were generated by PyMOL.

4. Conclusions

A series of novel cycloprotoberberines defined through modifications at the 9-posotion were prepared and evaluated for their antiproliferative activities against human tumor cell lines with **2** as the lead. The SAR analysis indicated that replacement of the 9-methoxyl in **2** by an ester moiety might significantly enhance the antiproliferative activity. Notably, compound **7f** showed promising potency *in vitro* in either naive breast cancer MCF-7 or MCF-7/ADrR cells, suggesting an antiproliferative mechanism different from that of DOX. Further study showed that compound **7f** inhibited the activity of DNA Top I and Top II, and arrested the cell cycle at G2/M phase. The results suggest that these cycloprotoberberine derivatives constitute a novel family of promising antitumor agents with an advantage of inhibiting drug-resistant cancer cells and merit further investigation.

5. Experimental section

5.1. Chemistry

Unless otherwise noted, all commercial reagents and solvents were obtained from the commercial provider and used without further purification. The ^{1}H NMR spectra was performed on a Varian Inova 400 MHz spectrometer (Varian, San Francisco, CA) and ^{13}C NMR on a Bruker Avance III 400 spectrometer in CD_3OD or DMSO-d_6, with Me_4Si as the internal standard. Chemical shifts are given in ppm scale and coupling constants (J values) are expressed in Hz. ESI high-resolution mass spectra (HRMS) were recorded on an Autospec Uitima-TOF mass spectrometer. Flash column chromatography was performed on Combiflash Rf 200, particle size 0.038 mm. Melting points (mp) were obtained with CXM-300 melting point apparatus. HPLC analyses were performed on an Agilent 1200 using a 5.0 μm C18 reversed phase column. Purities of biologically important compounds were ≥95%. For purities estimated by HPLC, the major peak accounted for ≥95% of the combined total peak area when monitored by an MWD detector at 278 nm.

5.1.1. 2,3-Methylenedioxy-9,10-dimethoxy-1,13-cycloprotoberberine chloride (**2**)

To a stirred solution of **1** (7.4 g, 20 mmol) and K_2CO_3 (8.3 g, 60 mmol) in methanol (250 mL), 5% NaOH (10 mL) solution containing $NaBH_4$ (1.2 g, 32 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 2 h and the precipitated solid was filtered, washed with distilled water (100 mL) and 80% ethanol (100 mL) to give kelly solid **3** (4.2 g, 62%). Intermediate **3** (4.0 g, 12 mmol) was then reacted with 40% glyoxal (2 mL) in the stirred solvent mixture of CH_3CN (160 mL) and HOAc (40 mL), which was heated to 85–95 °C for 5 h to prepare the desired compound **4** (**5**). The solvent was evaporated under vacuum, and 2% HCl (200 mL) was added into the residue. The reaction mixture was stirred at room temperature for 1 h and filtered to remove undissolved material. The filtrate was stirred at room temperature for 5 d, then evaporated under vacuum and recrystallized from 95% ethanol to obtain orange solid **2** (1.8 g, 38%). Mp 185–187 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.62 (t, J = 6.8 Hz, 2H), 4.12 (s, 3H), 4.16 (s, 3H), 5.24 (t, J = 6.8 Hz, 2H), 6.39 (s, 2H), 7.59 (s, 1H), 8.21 (d, J = 9.2 Hz, 1H), 8.31 (d, J = 9.6 Hz, 1H), 8.85 (d, J = 9.6 Hz, 1H), 8.89 (d, J = 9.2 Hz, 1H), 10.14 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 26.07, 55.72, 57.01, 62.18, 102.89, 110.32, 115.97, 117.00, 119.46, 120.02, 120.98, 122.15, 122.86, 126.03, 126.50, 128.06, 129.35, 140.90, 145.60, 146.61, 146.96, 150.58; HRMS: calcd for $C_{22}H_{18}NO_4Cl$ $[M - Cl]^+$, 360.1226; found, 360.1216.

5.1.2. 2,3-Methylenedioxy-9-hydroxy-10-methoxy-1,13-cycloprotoberberine chloride (**6**)

Compound **2** (3.6 g, 9.1 mmol) was heated at 195–210 °C in a dry oven under vacuum (20–30 mmHg) for 30 min and the crude material was acidified with concentrated HCl/ethanol (5/95) to obtain red solid **6** (3.2 g, 92%). Mp 269–271 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.56 (t, J = 6.8 Hz, 2H), 4.03 (s, 3H), 5.10 (t, J = 6.8 Hz, 2H), 6.36 (s, 2H), 7.52 (s, 1H), 8.02 (d, J = 8.8 Hz, 1H), 8.10 (d, J = 9.2 Hz, 1H), 8.26 (d, J = 8.4 Hz, 1H), 8.75 (d, J = 9.2 Hz, 1H), 9.96 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 26.17, 55.30, 56.93, 102.79, 110.21, 113.86, 115.85, 116.13, 117.00, 121.10, 121.98, 122.45, 124.44, 126.37, 127.48, 128.89, 140.80, 145.79, 145.99, 146.32, 147.15; HRMS: calcd for $C_{21}H_{16}NO_4Cl$ $[M - Cl]^+$, 346.1073; found, 346.1069.

5.1.3. General procedures for the synthesis of compounds (**7a–d**)

A mixture of **6** (198 mg, 0.52 mmol), alkyl halide (2.08 mmol) and KOH (60 mg, 1.04 mmol) in DMF (10 mL) was heated for 12 h at 60 °C. The solvent was evaporated under vacuum, and dilute HCl was added into the residue to get a suspension of pH 3. After filtration, the resulting residue was purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent to obtain the desired compound.

*5.1.3.1. 2,3-Methylenedioxy-9-ethoxy-10-methoxy-1,13-cycloprotoberberine bromide (**7a**).* The title compound was prepared from **6** and ethyl bromide (2 mL) in the same manner as described above. Yield: 22%; Red solid; mp 216–218 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 1.49 (t, J = 7.2 Hz, 3H), 3.62 (t, J = 6.8 Hz, 2H), 4.10 (s, 3H), 4.44 (q, J = 7.2 Hz, 2H), 5.26 (t, J = 6.8 Hz, 2H), 6.39

(s, 2H), 7.58 (s, 1H), 8.20 (d, J = 9.2 Hz, 1H), 8.29 (d, J = 9.2 Hz, 1H), 8.83 (d, J = 9.2 Hz, 1H), 8.88 (d, J = 9.2 Hz, 1H), 10.05 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 15.36, 26.06, 55.76, 56.96, 70.22, 102.88, 110.29, 115.95, 116.96, 119.25, 120.42, 120.93, 122.11, 122.82, 125.83, 126.49, 128.04, 129.30, 140.87, 144.53, 146.57, 146.87, 150.67; HRMS: calcd for $C_{23}H_{20}NO_4Br$ $[M - Br]^+$, 374.1391; found, 374.1397.

*5.1.3.2. 2,3-Methylenedioxy-9-butoxy-10-methoxy-1,13-cycloprotoberberine iodide (**7b**).* The title compound was prepared from **6** and 1-butyl iodide in the same manner as described above. Yield: 27%; Red solid; mp 202–204 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 1.00 (t, J = 7.2 Hz, 3H), 1.51–1.57 (m, 2H), 1.87–1.94 (m, 2H), 3.62 (t, J = 6.8 Hz, 2H), 4.10 (s, 3H), 4.37 (t, J = 6.8 Hz, 2H), 5.25 (t, J = 6.8 Hz, 2H), 6.40 (s, 2H), 7.59 (s, 1H), 8.22 (d, J = 9.2 Hz, 1H), 8.30 (d, J = 9.2 Hz, 1H), 8.84 (d, J = 9.2 Hz, 1H), 8.89 (d, J = 9.2 Hz, 1H), 9.98 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 13.76, 18.55, 26.07, 31.51, 55.85, 56.99, 74.20, 102.88, 110.29, 115.97, 117.00, 119.19, 120.23, 120.96, 122.17, 122.87, 125.94, 126.49, 128.11, 129.34, 140.90, 144.81, 146.60, 146.70, 150.56; HRMS: calcd for $C_{25}H_{24}NO_4I$ $[M - I]^+$, 402.1705; found, 402.1702.

*5.1.3.3. 2,3-Methylenedioxy-9-benzyloxy-10-methoxy-1,13-cycloprotoberberine bromide (**7c**).* The title compound was prepared from **6** and benzyl bromide in the same manner as described above. Yield: 60%; Orange solid; mp 131–133 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.61 (t, J = 6.8 Hz, 2H), 4.14 (s, 3H), 5.22 (t, J = 6.8 Hz, 2H), 5.43 (s, 2H), 6.39 (s, 2H), 7.35–7.43 (m, 3H), 7.59 (s, 1H), 7.62 (d, J = 6.8 Hz, 2H), 8.21 (d, J = 9.2 Hz, 1H), 8.32 (d, J = 9.6 Hz, 1H), 8.86 (t, J = 9.2 Hz, 2H), 9.98 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 26.09, 55.88, 57.01, 75.60, 102.90, 110.34, 115.95, 117.01, 119.72, 120.45, 120.97, 122.14, 122.98, 125.88, 126.48, 128.03, 128.38 (2), 128.46, 128.82 (2), 129.29, 136.33, 140.93, 143.94, 146.65, 146.77, 150.87; HRMS: calcd for $C_{28}H_{22}NO_4Br$ $[M - Br]^+$, 436.1547; found, 436.1562.

*5.1.3.4. 2,3-Methylenedioxy-9-(2,4-difluorobenzyloxy)-10-methoxy-1,13-cycloprotoberberine bromide (**7d**).* The title compound was prepared from **6** and 2,4-difluorobenzyl bromide in the same manner as described above. Yield: 55%; Red solid; mp 138–140 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.61 (t, J = 6.8 Hz, 2H), 4.11 (s, 3H), 5.22 (t, J = 6.8 Hz, 2H), 5.47 (s, 2H), 6.40 (s, 2H), 7.14–7.19 (m, 1H), 7.28–7.33 (m, 1H), 7.60 (s, 1H), 7.81 (q, J = 8.4 Hz, 1H), 8.22 (d, J = 9.2 Hz, 1H), 8.33 (d, J = 9.6 Hz, 1H), 8.88 (d, J = 4.4 Hz, 1H), 8.90 (d, J = 4.0 Hz, 1H), 9.93 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 600 MHz) δ: 26.46, 56.34, 57.39, 69.16, 103.30, 104.23, 104.41, 110.75, 111.95, 112.09, 116.36, 117.43, 120.43, 120.71, 121.39, 122.61, 123.45, 126.27, 126.90, 128.51, 129.80, 133.37, 133.44, 141.35, 143.94, 146.90, 147.08, 151.27; HRMS: calcd for $C_{28}H_{20}NO_4F_2Br$ $[M - Br]^+$, 472.1360; found, 472.1347.

*5.1.4. General procedures for the synthesis of compounds (**7e**–**s**)*

A mixture of **6** (99 mg, 0.26 mmol), acyl chloride (1.04 mmol) and anhydrous pyridine (84 μL, 1.04 mmol) in anhydrous CH_3CN (5 mL) was refluxed for 6 h. After cooling, the mixture was filtered and the resulting residue was purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as gradient eluent to obtain desired compounds.

*5.1.4.1. 2,3-Methylenedioxy-9-acetyloxy-10-methoxy-1,13-cycloprotoberberine chloride (**7e**).* The title compound was prepared from **6** and acetyl chloride in the same manner as described above. Yield: 64%; Red solid; mp 183–185 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 2.56 (s, 3H), 3.64 (t, J = 6.8 Hz, 2H), 4.09 (s, 3H), 5.24 (t, J = 6.8 Hz, 2H), 6.41 (s, 2H), 7.61 (s, 1H), 8.26 (d, J = 9.2 Hz, 1H), 8.40 (d, J = 9.2 Hz, 1H), 8.95 (d, J = 9.2 Hz, 1H), 9.09 (d, J = 9.2 Hz, 1H), 10.15 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 20.68, 25.91, 55.92, 57.22, 102.96, 110.37, 115.88, 117.01, 119.81, 120.87, 122.25, 122.70, 123.28, 125.30, 126.62, 128.03, 129.74, 135.44, 140.97, 145.79, 146.81, 150.43, 168.02; HRMS: calcd for $C_{23}H_{18}NO_5Cl$ $[M - Cl]^+$, 388.1181; found, 388.1188.

*5.1.4.2. 2,3-Methylenedioxy-9-benzoyloxy-10-methoxy-1,13-cycloprotoberberine chloride (**7f**).* The title compound was prepared from **6** and benzoyl chloride in the same manner as described above. Yield: 67%; Orange solid; mp 228–230 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.61 (t, J = 6.8 Hz, 2H), 4.07 (s, 3H), 5.22 (t, J = 6.8 Hz, 2H), 6.41 (s, 2H), 7.60 (s, 1H), 7.71 (t, J = 7.6 Hz, 2H), 7.86 (t, J = 7.6 Hz, 1H), 8.26–8.31 (m, 3H), 8.46 (d, J = 9.6 Hz, 1H), 8.98 (d, J = 9.6 Hz, 1H), 9.15 (d, J = 9.2 Hz, 1H), 10.24 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 25.89, 55.86, 57.30, 102.96, 110.36, 115.87, 117.04, 119.92, 120.91, 122.27, 122.94, 123.32, 125.30, 126.65, 127.96, 128.09, 129.10 (2), 129.77, 130.45 (2), 134.63, 135.61, 140.98, 145.86, 146.82, 150.39, 163.44; HRMS: calcd for $C_{28}H_{20}NO_5Cl$ $[M - Cl]^+$, 450.1346; found, 450.1336.

*5.1.4.3. 2,3-Methylenedioxy-9-(p-fluorobenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7g**).* The title compound was prepared from **6** and 4-fluorobenzoyl chloride in the same manner as described above. Yield: 31%; Orange solid; mp 218–220 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.61 (t, J = 6.4 Hz, 2H), 4.08 (s, 3H), 5.21 (t, J = 6.4 Hz, 2H), 6.41 (s, 2H), 7.56 (t, J = 8.8 Hz, 2H), 7.60 (s, 1H), 8.28 (d, J = 9.2 Hz, 1H), 8.35–8.39 (m, 2H), 8.46 (d, J = 9.6 Hz, 1H), 8.99 (d, J = 9.2 Hz, 1H), 9.16 (d, J = 9.6 Hz, 1H), 10.24 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 25.89, 55.87, 57.29, 102.97, 110.35, 115.80, 116.23, 116.45, 116.98, 119.81, 120.81, 122.18, 122.96, 123.26, 124.61, 125.22, 126.60, 128.02, 129.69, 133.49, 133.58, 135.41, 140.94, 145.81, 146.81, 150.34, 162.50, 164.64; HRMS: calcd for $C_{28}H_{19}NO_5FCl$ $[M - Cl]^+$, 468.1248; found, 468.1256.

*5.1.4.4. 2,3-Methylenedioxy-9-(o-fluorobenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7h**).* The title compound was prepared from **6** and 2-fluorobenzoyl chloride in the same manner as described above. Yield: 27%; Red solid; mp 181–183 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.62 (t, J = 6.8 Hz, 2H), 4.10 (s, 3H), 5.22 (t, J = 6.8 Hz, 2H), 6.41 (s, 2H), 7.51–7.57 (m, 2H), 7.60 (s, 1H), 7.88–7.92 (m, 1H), 8.28 (d, J = 9.2 Hz, 1H), 8.30–8.32 (m, 1H), 8.46 (d, J = 9.2 Hz, 1H), 8.98 (d, J = 9.2 Hz, 1H), 9.16 (d, J = 9.2 Hz, 1H), 10.25 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 25.88, 55.93, 57.35, 102.97, 110.36, 115.82, 116.41, 116.99, 117.60, 119.78, 120.86, 122.22, 123.07, 123.28, 125.01, 125.28, 126.63, 128.07, 129.75, 132.95, 135.15, 136.95, 140.95, 145.80, 146.82, 150.28, 160.54, 163.22; HRMS: calcd for $C_{28}H_{19}NO_5FCl$ $[M - Cl]^+$, 468.1247; found, 468.1247.

*5.1.4.5. 2,3-Methylenedioxy-9-(2,4-difluorobenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7i**).* The title compound was prepared from **6** and 2,4-difluorobenzoyl chloride in the same manner as described above. Yield: 17%; Red solid; mp 191–193 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.62 (t, J = 6.4 Hz, 2H), 4.10 (s, 3H), 5.21 (t, J = 6.4 Hz, 2H), 6.41 (s, 2H), 7.41–7.46 (m, 1H), 7.61 (s, 1H), 7.62–7.68 (m, 1H), 8.28 (d, J = 9.2 Hz, 1H), 8.39 (q, J = 8.4 Hz, 1H), 8.46 (d, J = 9.6 Hz, 1H), 8.98 (d, J = 9.6 Hz, 1H), 9.16 (d, J = 9.6 Hz, 1H), 10.24 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 25.89, 55.96, 57.37, 102.98, 106.07, 110.39, 112.60, 112.81, 113.32, 115.87, 117.05, 119.79, 120.91, 122.28, 123.17, 123.36, 125.33, 126.66, 128.14, 129.84, 135.01, 135.10, 135.21, 141.00, 145.79, 146.86, 150.31, 159.79; HRMS: calcd for $C_{28}H_{18}NO_5F_2Cl$ $[M - Cl]^+$, 486.1153; found, 486.1151.

*5.1.4.6. 2,3-Methylenedioxy-9-(o-chlorobenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7j**).* The title compound was prepared from **6** and 2-chlorobenzoyl chloride in the same manner as described above. Yield: 23%; Red solid; mp 164–166 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.63 (t, J = 6.8 Hz, 2H), 4.11 (s, 3H), 5.24 (t, J = 6.8 Hz, 2H), 6.41 (s, 2H), 7.61 (s, 1H), 7.66–7.69 (m, 1H), 7.79–7.81 (m, 2H), 8.28 (d, J = 9.2 Hz, 1H), 8.45–8.48 (m, 2H), 8.99 (d, J = 9.6 Hz, 1H), 9.16 (d, J = 9.2 Hz, 1H), 10.24 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 25.90, 55.96, 57.39, 102.97, 110.38, 115.86, 117.05, 119.73, 120.91, 122.27, 123.09, 123.33, 125.36, 126.66, 126.86, 127.64, 128.14, 129.81, 131.64, 133.18, 133.99, 134.99, 135.29, 140.99, 145.77, 146.85, 150.23, 161.41; HRMS: calcd for $C_{28}H_{19}NO_5Cl_2$ $[M - Cl]^+$, 484.0952; found, 484.0952.

*5.1.4.7. 2,3-Methylenedioxy-9-(p-methylbenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7k**).* The title compound was prepared from **6** and 4-methylbenzoyl chloride in the same manner as described above. Yield: 41%; Red solid; mp 221–223 °C; ^{1}H NMR (CD_3OD, 400 MHz): δ 2.47 (s, 3H), 3.60 (t, J = 6.8 Hz, 2H), 4.06 (s, 3H), 5.17 (t, J = 6.8 Hz, 2H), 6.29 (s, 2H), 7.40 (s, 1H), 7.43 (d, J = 8.0 Hz, 2H), 8.19 (d, J = 8.0 Hz, 2H), 8.27 (d, J = 9.6 Hz, 1H), 8.32 (d, J = 9.6 Hz, 1H), 8.76 (d, J = 9.2 Hz, 1H), 8.96 (d, J = 9.2 Hz, 1H), 9.89 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 21.35, 25.90, 55.87, 57.29, 102.96, 110.37, 115.91, 117.07, 120.00, 120.94, 122.30, 122.86, 123.34, 125.21, 125.32, 126.67, 128.11, 129.62 (2), 129.80, 130.53 (2), 135.75, 141.00, 145.29, 145.87, 146.83, 150.45, 163.43; HRMS: calcd for $C_{29}H_{22}NO_5Cl$ $[M - Cl]^+$, 464.1499; found, 464.1489.

*5.1.4.8. 2,3-Methylenedioxy-9-(p-methoxybenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7l**).* The title compound was prepared from **6** and 4-methoxybenzoyl chloride in the same manner as described above. Yield: 51%; Orange solid; mp 202–204 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.61 (t, J = 6.8 Hz, 2H), 3.92 (s, 3H), 4.06 (s, 3H), 5.22 (t, J = 6.8 Hz, 2H), 6.41 (s, 2H), 7.22 (d, J = 8.8 Hz, 2H), 7.59 (s, 1H), 8.24 (d, J = 8.8 Hz, 2H), 8.27 (d, J = 9.6 Hz, 1H), 8.44 (d, J = 9.2 Hz, 1H), 8.98 (d, J = 9.6 Hz, 1H), 9.13 (d, J = 9.6 Hz, 1H), 10.20 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 500 MHz) δ: 26.02, 55.93 (2), 57.37, 103.07, 110.46, 114.52 (2), 116.03, 117.17, 120.08, 120.19, 121.06, 122.43, 122.87, 123.44, 125.42, 126.77, 128.22, 129.89, 132.86 (2), 135.98, 141.10, 145.98, 146.93, 150.62, 163.19, 164.35; HRMS: calcd for $C_{29}H_{22}NO_6Cl$ $[M - Cl]^+$, 480.1449; found, 480.1442.

*5.1.4.9. 2,3-Methylenedioxy-9-(m-nitro-p-fluorobenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7m**).* A mixture of 4-fluoro-3-nitrobenzoic acid (193 mg, 1.04 mmol) and $SOCl_2$ (5 mL) was refluxed for 2 h, then the surplus $SOCl_2$ was evaporated under vacuum to prepare acyl chloride. The title compound was prepared from **6** and 4-fluoro-3-nitrobenzoyl chloride in the same manner as described above. Yield: 25%; Red solid; mp 187–189 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.62 (t, J = 6.4 Hz, 2H), 4.09 (s, 3H), 5.20 (t, J = 6.4 Hz, 2H), 6.41 (s, 2H), 7.61 (s, 1H), 7.93–7.98 (m, 1H), 8.29 (d, J = 9.2 Hz, 1H), 8.48 (d, J = 9.2 Hz, 1H), 8.67–8.68 (m, 1H), 8.94 (dd, J = 2.0, 7.2 Hz, 1H), 8.99 (d, J = 9.2 Hz, 1H), 9.18 (d, J = 9.6 Hz, 1H), 10.29 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 25.90, 55.97, 57.39, 102.99, 110.41, 115.87, 117.07, 119.66, 119.79, 120.01, 120.92, 122.26, 123.39 (2), 125.11, 125.34, 126.65, 128.16, 128.40, 129.86, 134.88, 137.31, 138.11, 141.01, 145.87, 146.89, 150.26, 161.04; HRMS: calcd for $C_{28}H_{18}N_2O_7FCl$ $[M - Cl]^+$, 513.1096; found, 513.1083.

*5.1.4.10. 2,3-Methylenedioxy-9-(m-nitro-p-methylbenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7n**).* A mixture of 4-methyl-3-nitrobenzoic acid (188 mg, 1.04 mmol) and $SOCl_2$ (5 mL) was refluxed for 2 h, then the surplus $SOCl_2$ was evaporated under vacuum to prepare acyl chloride. The title compound was prepared from **6** and 4- methyl-3-nitrobenzoyl chloride in the same manner as described above. Yield: 25%; Dark red solid; mp 165–167 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 2.70 (s, 3H), 3.62 (t, J = 6.8 Hz, 2H), 4.08 (s, 3H), 5.20 (t, J = 6.8 Hz, 2H), 6.41 (s, 2H), 7.61 (s, 1H), 7.87 (d, J = 8.0 Hz, 1H), 8.28 (d, J = 9.2 Hz, 1H), 8.47 (d, J = 9.2 Hz, 2H), 8.79 (d, J = 1.2 Hz, 1H), 8.99 (d, J = 9.6 Hz, 1H), 9.17 (d, J = 9.2 Hz, 1H), 10.27 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 19.81, 25.89, 55.94, 57.35, 102.98, 110.38, 115.85, 117.05, 119.71, 120.88, 122.24, 123.35, 125.29, 126.02, 126.63, 127.09, 128.11, 129.81, 133.39, 133.91, 134.33, 135.06, 139.59, 140.99, 145.85, 146.86, 149.12, 150.28, 161.77; HRMS: calcd for $C_{29}H_{21}N_2O_7Cl$ $[M - Cl]^+$, 509.1348; found, 509.1343.

*5.1.4.11. 2,3-Methylenedioxy-9-(m-nitro-p-methoxybenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7o**).* A mixture of 4-methoxy-3-nitrobenzoic acid (205 mg, 1.04 mmol) and $SOCl_2$ (5 mL) was refluxed for 2 h, then the surplus $SOCl_2$ was evaporated under vacuum to prepare acyl chloride. The title compound was prepared from **6** and 4-methoxy-3-nitrobenzoyl chloride in the same manner as described above. Yield: 15%; Dark red solid; mp 158–160 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.62 (t, J = 6.4 Hz, 2H), 4.08 (s, 3H), 4.11 (s, 3H), 5.20 (t, J = 6.4 Hz, 2H), 6.41 (s, 2H), 7.60 (s, 1H), 7.68 (d, J = 8.8 Hz, 1H), 8.28 (d, J = 9.2 Hz, 1H), 8.46 (d, J = 9.6 Hz, 1H), 8.51 (dd, J = 2.4, 8.8 Hz, 1H), 8.74 (d, J = 2.4 Hz, 1H), 8.98 (d, J = 9.6 Hz, 1H), 9.16 (d, J = 9.2 Hz, 1H), 10.25 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 25.91, 55.95, 57.35, 57.63, 102.98, 110.39, 115.07, 115.91, 117.09, 119.83, 119.90, 120.94, 122.30, 123.15, 123.38, 125.34, 126.66, 127.23, 128.15, 129.85, 135.22, 136.36, 139.19, 141.02, 145.87, 146.87, 150.38, 156.40, 161.63; HRMS: calcd for $C_{29}H_{21}N_2O_8Cl$ $[M - Cl]^+$, 525.1299; found, 525.1295.

*5.1.4.12. 2,3-Methylenedioxy-9-(3,4,5-trimethoxybenzoyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7p**).* A mixture of 3,4,5-trimethoxybenzoic acid (221 mg, 1.04 mmol) and $SOCl_2$ (5 mL) was refluxed for 2 h, then the surplus $SOCl_2$ was evaporated under vacuum to prepare acyl chloride. The title compound was prepared from **6** and 3,4,5-trimethoxybenzoyl chloride in the same manner as described above. Yield: 21%; Orange solid; mp 210–212 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.62 (t, J = 6.4 Hz, 2H), 3.83 (s, 3H), 3.92 (s, 6H), 4.08 (s, 3H), 5.24 (t, J = 6.4 Hz, 2H), 6.41 (s, 2H), 7.56 (s, 2H), 7.60 (s, 1H), 8.28 (d, J = 9.2 Hz, 1H), 8.46 (d, J = 9.6 Hz, 1H), 8.99 (d, J = 9.2 Hz, 1H), 9.16 (d, J = 9.6 Hz, 1H), 10.20 (s, 1H); HRMS: calcd for $C_{31}H_{26}NO_8Cl$ $[M - Cl]^+$, 540.1658; found, 540.1685.

*5.1.4.13. 2,3-Methylenedioxy-9-piperonyloyloxy-10-methoxy-1,13-cycloprotoberberine chloride (**7q**).* A mixture of piperonylic acid (173 mg, 1.04 mmol) and $SOCl_2$ (5 mL) was refluxed for 2 h, then the surplus $SOCl_2$ was evaporated under vacuum to prepare acyl chloride. The title compound was prepared from **6** and piperonyloyl chloride in the same manner as described above. Yield: 21%; Orange solid; mp 192–194 °C; ^{1}H NMR (CD_3SOCD_3, 400 MHz): δ 3.61 (t, J = 6.4 Hz, 2H), 4.07 (s, 3H), 5.23 (t, J = 6.4 Hz, 2H), 6.25 (s, 2H), 6.41 (s, 2H), 7.22 (d, J = 8.0 Hz, 1H), 7.60 (s, 1H), 7.73 (d, J = 1.6 Hz, 1H), 7.91 (dd, J = 1.6, 8.4 Hz, 1H), 8.27 (d, J = 9.2 Hz, 1H), 8.44 (d, J = 9.6 Hz, 1H), 8.97 (d, J = 9.6 Hz, 1H), 9.13 (d, J = 9.6 Hz, 1H), 10.19 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 500 MHz) δ: 26.02, 55.99, 57.38, 102.63, 103.07, 108.74, 109.79, 110.46, 115.99, 117.14, 120.03, 121.00, 121.59, 122.36, 122.91, 123.41, 125.39, 126.75, 127.02, 128.17, 129.85, 135.80, 141.09, 145.95, 146.92, 147.99, 150.56, 152.76, 162.78; HRMS: calcd for $C_{29}H_{20}NO_7Cl$ $[M - Cl]^+$, 494.1240; found, 494.1239.

*5.1.4.14. 2,3-Methylenedioxy-9-benzenesulfonyloxy-10-methoxy-1,13-cycloprotoberberine chloride (**7r**).* The title compound was prepared from **6** and benzenesulfonyl chloride in the same manner as described above. Yield: 28%; Red solid; mp 223–225 °C; ^{1}H NMR

(CD_3SOCD_3, 400 MHz): δ 3.62 (t, $J = 6.8$ Hz, 2H), 3.76 (s, 3H), 5.20 (t, $J = 6.8$ Hz, 2H), 6.42 (s, 2H), 7.63 (s, 1H), 7.73 (t, $J = 8.0$ Hz, 2H), 7.91 (t, $J = 7.6$ Hz, 1H), 7.97 (d, $J = 7.2$ Hz, 2H), 8.28 (d, $J = 9.6$ Hz, 1H), 8.33 (d, $J = 9.6$ Hz, 1H), 8.95 (d, $J = 9.2$ Hz, 1H), 9.16 (d, $J = 9.6$ Hz, 1H), 9.79 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 25.98, 56.47, 56.95, 103.05, 110.51, 115.73, 117.09, 120.51, 120.86, 122.42, 123.74, 124.55, 125.68, 126.70, 128.60, 128.68 (2), 129.75 (2), 130.06, 132.86, 134.40, 135.65, 141.10, 145.17, 147.08, 151.63; HRMS: calcd for $C_{27}H_{20}NO_6SCl$ $[M - Cl]^+$, 486.1017; found, 486.1001.

*5.1.4.15. 2,3-Methylenedioxy-9-(N-acetylsulfanilyloxy)-10-methoxy-1,13-cycloprotoberberine chloride (**7s**).* The title compound was prepared from **6** and *N*-acetylsulfanilyl chloride in the same manner as described above. Yield: 15%; Red solid; mp 205–207 °C; ^1H NMR (CD_3SOCD_3, 400 MHz): δ 2.12 (s, 3H), 3.60 (t, $J = 6.4$ Hz, 2H), 3.81 (s, 3H), 5.18 (t, $J = 6.4$ Hz, 2H), 6.42 (s, 2H), 7.63 (s, 1H), 7.86 (s, 4H), 8.28 (d, $J = 9.2$ Hz, 1H), 8.34 (d, $J = 9.6$ Hz, 1H), 8.95 (d, $J = 9.2$ Hz, 1H), 9.15 (d, $J = 9.6$ Hz, 1H), 9.74 (s, 1H), 10.61 (s, 1H); ^{13}C NMR (CD_3SOCD_3, 400 MHz) δ: 24.19, 25.91, 56.48, 57.01, 103.03, 110.49, 115.71, 117.05, 118.62 (2), 120.48, 120.86, 122.40, 123.67, 124.40, 125.69, 126.62, 126.94, 128.55, 129.94, 130.28 (2), 133.04, 141.06, 145.26, 145.57, 147.03, 151.76, 169.49; HRMS: calcd for $C_{29}H_{23}N_2O_7SCl$ $[M - Cl]^+$, 543.1227; found, 543.1203.

5.2. Biological methods

5.2.1. Tumor cell lines

The human tumor cell lines HepG2, HCT116, HT1080, MCF-7 were obtained from American Type Culture Collection, Cells were cultured in the MEM-EBSS medium (Invitrogen) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. MCF-7/ADrR cells were kindly a gift from Dr Qiyang He (Institute of Medicinal Biotechnology) and grown in medium containing 10 mM DOX.

5.2.2. Cell growth inhibition assay

The effect on cell growth was determined using an SRB assay [22]. Cells were seeded in 96-well plates at a density of 4×10^3–8×10^3/mL and treated with the tested compounds (100 μl) at different concentrations of 0.039, 0.15, 0.63 and 2.5 μg/mL respectively. After 24 h and 48 h, cells were fixed with 50% trichloroacetic acid (TCA), and then 0.4% (w/v) SRB in acetic acid (1%) was added to stain cells. Unbound SRB was washed out by 1% acetic acid and SRB bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 492 nm. The growth inhibition (%) was calculated at each concentration and the IC_{50} for reducing the cell number to 50% of the control was obtained. Results were obtained from triplicate determinations and are shown as mean and SD.

5.2.3. Flow cytometric analysis

Flow cytometric analysis was performed in our previous report [22]. After treated with compound **7f** (0.5 μg/mL) for 0 h, 4 h, 12 h and 24 h, HCT116 cells were harvested and fixed with 75% ethanol at −20 °C overnight. Cells were stained with propidium iodide (25 mg/mL) and RNase A (200 mg/mL) at 37 °C for 30 min. The DNA content was analyzed with a FACScan flow cytometer (COULTER EPICS XL, Fullerton, Cal, USA).

5.2.4. Top I inhibition assay

Top I enzyme activity was assayed by measuring the decreased mobility of the relaxed isomers of supercoiled pBR322 DNA in an agarose gel after it had been treated with human Top I [23]. 1 Unit of human Top I (Beyotime, Haimen, Jiangsu, China) and the different concentrations compound were added and mixed, the reaction took place at 37 °C for 20 min, then 0.5 μg pBR322 DNA (Beyotime, Haimen, Jiangsu, China) was added and the reaction continued to take place at 37 °C for 30 min and was terminated by adding 0.5% SDS, 0.25 μg/mL bromophenol blue, and 15% glycerol.

5.2.5. Top II inhibition assay

Top II enzyme activity was assessed by measuring the decatenation of kinetoplast DNA using the Top II Assay Kit (TopoGEN, Columbus, OH) [24]. Briefly, 0.15 μg of kinetoplast DNA was incubated with 4 U of human Top II (TopoGEN) in 20 μl of the assay buffer [50 mmol/L Tris–HCl (pH 8), 120 mmol/L KCl, 10 mmol/L $MgCl_2$, 0.5 mmol/L ATP, 0.5 mmol/L dithiothreitol and 30 μg/mL bovine serum albumin] at 37 °C for 30 min in the presence or absence of **7f** or VP16. The reaction was stopped by the addition of 5 μl of gel loading dye [5%Sarkosyl, 0.0025% bromophenol blue and 25% glycerol]. The reaction products were electrophoresed in a horizontal 1% agarose gel in tris-acetate/EDTA buffer at 4 V/cm for 40 min at room temperature stained with 5 μg/mL ethidium bromide, distained in water, and photographed under UV light.

5.2.6. Molecular docking

The X-ray crystallographic structures of the Indolocarbazole (SA315F)–DNA–Top I complex (PDB: 1SEU) [25] in Protein Data Bank were selected for the docking study. The eHiTS software package was used for flexible docking. eHiTS is an exhaustive flexible docking algorithm with a scoring function which incorporates both empirical and knowledge-based features [26,27]. Active site detection of the Indolocarbazole (SA315F)–DNA–Top I complex was carried out using the "-complex" parameter. The program automatically detected the SA315F in the complex and selected the part of enzyme and DNA within a 7 Å radius around the SA315F to be the active site. The compound **7f** was then docked into the active site using the highest accuracy mode of docking (accuracy set to 6). Finally, the program gave 32 docked conformers ranking the Scores. We selected the conformer with the best score and speculated the detail binding patterns. Molecular figures were generated by PyMOL [28].

Acknowledgements

This work was supported by the "National Mega-project for Innovative Drugs" (2012ZX09301002-001-017) and the National Natural Science Fund for Young Scientists (81102464) and the IMB Research Foundation.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.07.026.

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