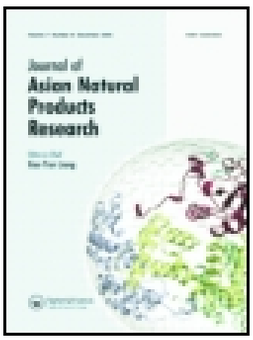

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Synthesis, antitumor activity, and molecular docking of (−)-epigallocatechin-3-gallate-4β-triazolopodophyllotoxin conjugates

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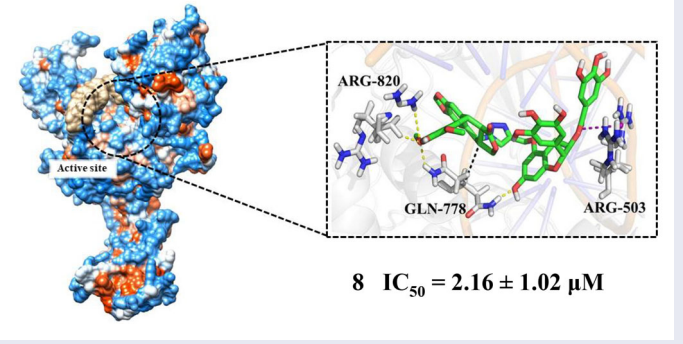
ABSTRACT

Two new (−)-epigallocatechin-3-gallate-4β-triazolopodophyllotoxin conjugates (**7** and **8**) were synthesized and evaluated for biological activity. Compound **8** showed highly potent anticancer activity against A-549 cell line with IC_{50} of 2.16 ± 1.02 μM, which displayed the highest selectivity index value (SI = 14.5) in A-549 cells. Molecular docking indicated that compound **8** could bind with the active site of Top-II. Therefore, compound **8** might be a promising candidate for further development.



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KEYWORDS
Podophyllotixin; EGCG; click reaction; antitumor activity; molecular docking

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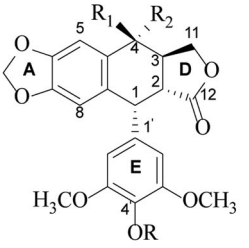
1. Introduction

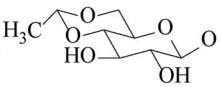
Cancer has been reported as a leading cause of death in many countries of the world in the 21st century, especially in less developed countries [1, 2]. Chemotherapy is the primary choice in cancer treatment, and development of targeted anticancer drugs, increase of bioavailability and decrease of toxicity are the key topics which are currently being studied [3]. DNA topoisomerases (Tops) enzymes relax helical supercoiling regenerated during transcription, replication, recombination and chromatin remodeling is one of the most effective and successful drug target in recent years [4, 5]. Topos enzymes are classified as topoisomerase I (Top-I) and topoisomerase II (Top-II), Top-II is a more vital nuclear enzyme involved in various DNA processed and a major target for many drugs currently used than Top-I in cancer chemotherapy [6, 7]. Thus, many researchers in medicinal chemistry recently focused on developing Top-II-selective inhibitors as anticancer agents.

The natural lignan podophyllotoxin (**PPT**, **1**, Figure 1) is extracted from the roots of *Podophyllotoxin peltatum* and shows cytotoxic activity against a various cancer cells [8, 9]. However, lack tumor selectivity and high toxicity of **PPT** has limited its application as a drug in cancer chemotherapy [10]. Many derivatives of **PPT** that are potent inhibitors have been synthesized and tested as antitumor agents. Interestingly, etoposide (**VP-16**, **2**, Figure 1) is reported as a clinical Top-II inhibitor which indicates that the substitution at C-4 position on the C-ring of **PPT** may be responsible for the Top-II inhibition [11, 12], and the active site of Top-II protein and etoposide was shown in Figure S1 (Supplementary material). Although **VP-16** is currently in use, many side effects like low blood pressure, hair loss, diarrhea, etc were accompanied with this drug [13]. Hence, development of new drugs with fewer side effects is highly desirable.

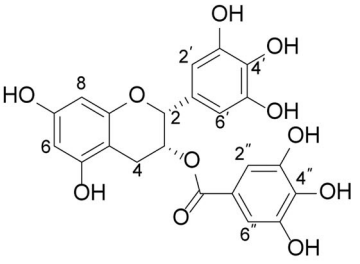
(−)-Epigallocatechin-3-gallate (**EGCG**, **3**, Figure 1) is the most abundant catechin (accounting for approximately 50% of total catechins) and has been reported to have stronger pharmacological activities [14, 15]. Interestingly, it was found that EGCG was the most potent to inhibit dose-dependently the Top-II catalytic activity [16]. However, the use of **EGCG** is often hindered by problems such as easy oxidation, ready degradation in aqueous solutions and the poor intestinal absorbance. In our



Podophyllotoxin (1) $R = CH_3$, $R_1 = H$, $R_2 = OH$

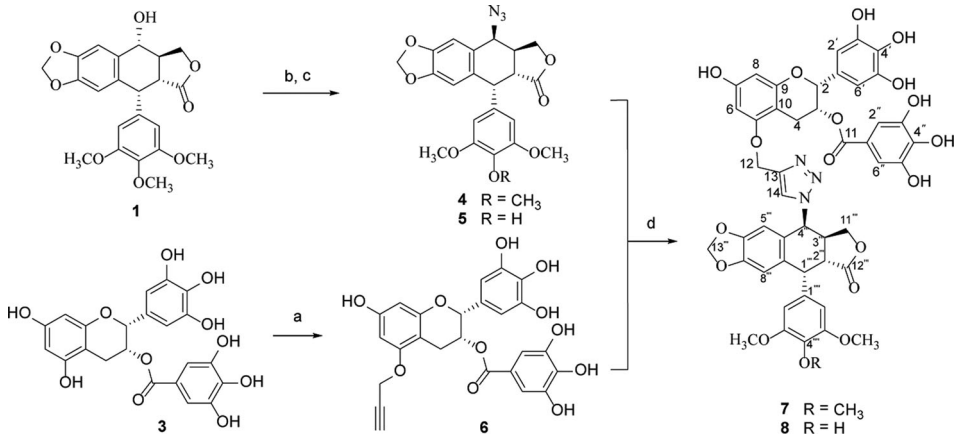


Etoposide (2) $R = H$, $R_1 =$, $R_2 = H$



(-)-Epigallocatechin-3-gallate (3)

Figure 1. Chemical structures of podophyllotixin (**PPT**, **1**), etoposide (**VP-16**, **2**) and (-)-epigallocatechin-3-gallate (**EGCG**, **3**).



Scheme 1. Synthesis of (−)-epigallocatechin-3-gallate-4β-triazolopodophyllotoxin conjugates **7** and **8**. Reagents and reaction conditions: (a) NaH, DMF, 0 °C, propargyl bromide, then, reflux, overnight, 30%; (b) $MeSO_3H$, NaI, CH_2Cl_2, then, H_2O-Acetone, $BaCO_3$, rt. 90–92%; (c) NaN_3-TFA, $CHCl_3$, 55–60%; (d) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, THF, ^{t}BuOH: H_2O (1: 1), 4 h, rt, 80–82%.

previous studies, we found that 1,2,3-trizole-**EGCG** derivatives showed high anti-cancer activities and improved the stability of the **EGCG** scaffold [17, 18].

Dual target inhibitors would likely show high cytotoxicity and more selectivity than the one. The aim of this research was interested in the development of compounds that can act on the Top-II by conjugating derivatives of the podophyllotoxin with an analogue of **EGCG**. In this paper, we have synthesized two (−)-epigallocatechin-3-gallate-4β-triazolopodophyllotoxin conjugates by click reaction, and evaluated for their cytotoxic activity against a panel of five human cancer cell lines HL-60 (leukemia), SMMC-7721 (hepatoma), A-549 (lung cancer), MCF-7 (breast cancer) and SW480 (colon cancer) *in vitro* by MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Further studies including selectivity of active compounds against cancer cells and the normal human cell line (BEAS-2B), and molecular docking were performed.

2. Results and discussion

The synthesis of the novel (−)-epigallocatechin-3-gallate-4β-triazolopodophyllotoxin conjugates **7** and **8** was performed according to the reaction pathways illustrated in Scheme 1. **PPT** and **EGCG** were used as starting materials. 4β-Azido-4-deoxypodophyllotoxin (**4**) and 4β-azido-4-deoxy-4′-demethypodophyllotoxin (**5**) were synthesized according to the literature procedures [19, 20]. (−)-Epigallocatechin-3-gallate-1-propyne (**3**) was prepared by the treatment of **EGCG** with sodium hydride (NaH) and propargyl bromide to afford the terminal alkyne (**6**) in 30% yield. Then, 4β-azido-podophyllotoxins **4** and **5** were allowed to react with the above terminal alkyne (**6**) in the presence of $CuSO_4{\cdot}5H_2O$, sodium ascorbate in THF and ^{t}BtOH-H_2O (1:1) at room temperature for 4 h to give (−)-epigallocatechin-3-gallate-4β-triazolopodophyllotoxin conjugates **7** and **8** in 80–82% yields. All of synthesized compounds were characterized by ^{1}H-NMR, ^{13}C-NMR, ESI-MS and HRESI-MS analytical data.

Table 1. *In vitro* anticancer activity (IC_{50}, μM) of (−)-epigallocatechin-3-gallate-4β-triazolopodophyllotoxin conjugates **7** and **8**[a].

Compd.	IC_{50} (μM)					
	HL-60	A-549	MCF-7	SW480	SMMC-7721	BEAS-2B
7	17.16 ± 0.29	7.31 ± 1.83	13.37 ± 1.15	11.84 ± 1.92	20.67 ± 1.00	26.25 ± 0.16
8	22.76 ± 1.95	2.16 ± 1.02	12.84 ± 1.28	8.96 ± 1.11	14.63 ± 0.06	30.87 ± 0.28
PPT (**1**)	<0.064	<0.064	<0.064	<0.064	<0.064	NT[b]
EGCG (**2**)	>40	>40	>40	>40	>40	NT[b]
DDP	0.766 ± 0.03	4.72 ± 0.35	14.20 ± 0.93	10.20 ± 1.20	1.93 ± 0.43	12.86 ± 0.25

[a]Value are means of three independent experiments;
[b]NT = not tested.

Table 2. Selectivity of the cytotoxicity of (−)-epigallocatechin-3-gallate -4β-triazolopodophyllotoxin conjugates **7** and **8**.

	Selectivity index (SI[a])				
Compd.	HL-60	A-549	MCF-7	SW480	SMMC-7721
7	1.5	3.6	2.0	2.2	1.3
8	1.4	14.5	2.4	3.9	2.1
DDP	16.8	2.7	0.9	1.3	6.7

[a]Selectivity index (SI) = IC_{50} of the compound in BEAS-2B cell line/IC_{50} of the compound in cancer cell line.

The anti-proliferative activity of (−)-epigallocatechin-3-gallate-4*β*-triazolopodophyllotoxin conjugates against HL-60, SMMC-7721, A-549, MCF-7 and SW480 cell lines was screened *in vitro* by MTT assay. **PPT**, **EGCG** and cisplatin were used as positive controls. The compounds were tested in concentration range of 2.16–30.87 μM and the calculated IC_{50} values (concentration of drug inhibiting 50% cell growth) were reported in Table 1. As shown in Table 1, compounds **7** and **8** showed better growth inhibition in five human cancer cells, and **8** exhibited the most potent anti-proliferative activity against A-549 cells with IC_{50} value of 2.16 ± 1.02 μM.

To evaluate the degree of selectivity of **7** and **8**, the growth inhibitory effect on a normal human bronchial epithelial cell line (BEAS-2B) was tested (Table 1), and selectivity index (SI) values of cytotoxic drugs and **DDP** were measured (Table 2). As shown in Table 2, compounds **7** and **8** have SI value ranging from 1.3 to 14.5 in all five cancer cell lines tested. Importantly, compound **8** displayed higher selectivity than **DDP** in three of the five cancer cells, and **8** showed the highest selectivity index value (SI = 14.5) in A-549 cell line. These data suggest that the highest potency compound **8** (IC_{50} = 2.16 ± 1.02 μμM) is significantly more cytotoxic to the cancer cell lines as compared with the normal cell line.

To further investigate the potential binding between Top-II and the compounds, the molecular docking was performed. In the docking results, it was observed that the vdW + Hbond + desolv energy for **7**, **8** and etoposide were −21.16 kcal/mol, −21.77 kcal/mol and −17.75 kcal/mol, respectively. It was also observed that the electrostatic energy for compound **7** is −0.03 kcal/mol, which is equal with compound **8.** The internal energy and inhibition constant were also calculated and shown in Table S1 (Supplementary material), while compounds **7** and **8** have the potential to binding Top-II. Furthermore, the inhibition constants calculated for compounds **7**, **8** and etoposide were 4190 nM, 1490 nM and 1220 nM, respectively, which indicated that compounds **7** and **8** might have considerable Top-II inhibition ability. The

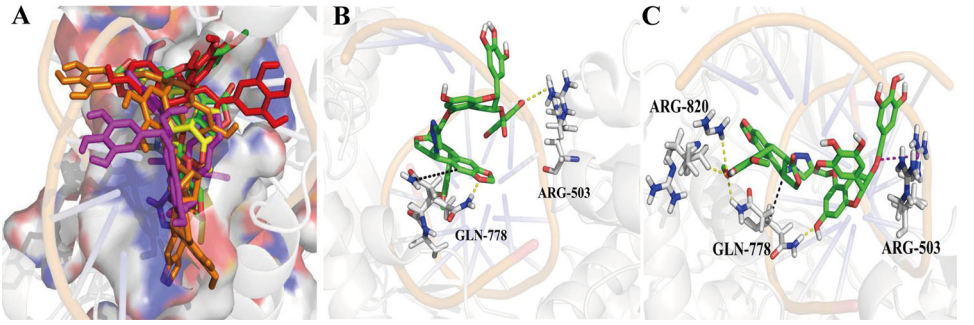


Figure 2. Binding conformations of the analogs at the active site of Top-II. A. Proposed binding modes of etoposide (in green), **PPT** (in yellow), **EGCG** (in red), **7** (in magental) and **8** (in orange) in the active site of TOP-II (PDB ID: 3QX3); B and C. Compounds **7** and **8** docked into the binding site of TOP-II: The protein is shown as cartoon; ligand and the key residues are shown as cartoon sticks (ligand color: C green, N blue, O red and polar hydrogen); hydrogen bonds are shown as yellow dotted lines; hydrophobic interactions are shown as black dotted lines; salt bridges were shown as purple dotted lines.

molecules in complex with Top-II were analyzed (Figure 2). As shown in Figure 2A, the 3 D binding modes of the compounds **PPT**, **EGCG**, **7** and **8** at the etoposide binding site of Top-II, compounds **7** and **8** could be perfectly docked in the site of Top-II. As the result presented in Figure 2B and C, it can be easily seen that two hydrogen bonds [$C^{5''}$–OH···NH_2 (ARG-503)] and [$C^{13'''}$–CH_2O···NH_2 (GLN-778)], and the E ring of PPT lead to hydrophobic interactions with the aromatic residue GLN-778 in **7**/Top-II docking complex, while four hydrogen bonds [C^{7}–OH···NH_2 (GLN-778)], [$C^{3''''}$-OCH_3···NH_2 (GLN-778)], [$C^{3''''}$–OCH_3···NH_2 (ARG-802)], and [$C^{3''''}$–OCH_3···NH (ARG-802)], the E ring of PPT leads to hydrophobic interactions with the aromatic residue GLN-778, and one salt bridge [C = O···NH_2-C(NH)=NH (ARG-503)] are found in **8**/Top-II docking complex, respectively. The presence of more hydrogen bonds and salt bridge in compound **8** seems to be the key factor for its high activity.

3. Experimental

3.1. General experimental procedures

(−)-Epigallocatechin-3-gallate and podophyllotoxin were purchased from Chengdu Proifa Technology Development Co., Ltd (Chengdu, China); 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dichloromethane and acetonitrile were distilled over calcium hydride. All reagents were commercially available and used without further purification unless indicated otherwise. Melting points were measured by an X-4 melting point apparatus and are uncorrected. MS data were obtained in the ESI mode on API Qstar Pulsar instrument; HRMS data were obtained in the ESI mode on LCMS-IT-TOF (Shimadzu, Kyoto, Japan); ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker AVANCE III 500 MHz, or 600 MHz (Bruker BioSpin GmbH, Rheinstetten,

Germany) instruments, using tetramethylsilane (TMS) as an internal standard: chemical shifts (δ) are given in ppm, coupling constants (J) in Hz. Column chromatography (CC) was performed over silica gel (200–300 mesh; Qingdao Makall Group CO., LTD; Qingdao; China). All reactions were monitored by thin-layer chromatography (TLC), which was visualized by ultraviolet light (254 nm) and sprayed with 5% H_2SO_4 in EtOH, followed by heating.

3.2. Synthesis of (−)-epigallocatechin-3-gallate-1-propyne (6)

(-)-Epigallocatechin-3-gallate (458 mg, 1.0 mmol) was dissolved in N,N-dimethylformamide (DMF) (5 ml), then sodium hydride (60 mg, 1.5 mmol) was added at 0 °C under nitrogen and the mixture was stirred at room temperature for 0.5 h. Propargyl bromide (0.1 ml, 1 mmol) was quickly added and the reaction was stirred at 80 °C for 12 h. After cooling, the mixture was concentrated under vacuum and the resulting residue was purified by silica gel chromatography with $CHCl_3/CH_3OH$, (9:1→4:1) to afford the product **6** (137 mg, 30%). ^{1}H-NMR (CD_3OD, 500 MHz) δ 6.90 (s, 2H, $C^{2''}$-H, $C^{6''}$-H), 6.50 (s, 2H, $C^{2'}$-H, $C^{6'}$-H), 5.96 (s, 2H, C^6-H, C^8-H), 5.53 (brs, 1H, C^3-H), 4.97 (s, 1H, C^2-H), 4.78 (d, 2H, J = 2.4 Hz, OCH_2), 3.29 (t, 1H, J = 1.6 Hz, C≡CH), 2.96 (dd, 1H, J = 4.6, 12.0 Hz, C^4-H_a), 2.85 (dd, 1H, J = 4.6, 12.0 Hz, C^4-H_b); ^{13}C-NMR (CD_3OD, 125 MHz) δ 167.0 (C = O), 157.9 (C-7), 157.8 (C-9), 157.2 (C-5), 151.9 (C-3′, C-5′), 146.7 (C-3″, C-5″), 138.4 (C-4″), 133.8 (C-4′), 130.7 (C-1′), 127.1 (C-1″), 110.1 (C-2″, C-6″), 106.8 (C-2′, C-6′), 99.3 (C-10), 96.5 (C-8), 95.9 (C-6), 80.4 (C-2), 79.5 (*C*≡CH), 78.5 (C≡*C*H), 76.7 (C-3), 60.0 (OCH_2), 26.8 (C-4); ESIMS: *m/z* 495 $[M - H]^-$.

3.3. General procedure for the synthesis of (−)-epigallocatechin-3-gallate -4β-triazolopodophyllotoxin conjugates (7 and 8)

To a solution of 4β-azido-podophyllotoxins **4**/**5** (0.1 mmol) and (−)-epigallocatechin-3-gallate-1-propyne (**6**) (0.1 mmol) in THF (1.0 ml) and ^{t}BtOH-H_2O (1.0 ml, 1:1) at room temperature, copper (II) sulfate pentahydrate (0.1 mmol) and sodium ascorbate (0.05 mmol) were added and the reaction mixture was stirred for 4 h. Removal of the solvents gave a residue which was chromatographed on silica gel ($CHCl_3$:CH_3OH = 9:1) to afford the product.

3.3.1. (−)-Epigallocatechin-3-gallate-[4β-(1,2,3-triazol-1-yl-4-deoxypodophyllotoxin)] ether (7)

White amorphous powder; yield 82%; m.p. 192–194 °C; ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.47 (s, 1H, C^{14}-H), 6.86 (s, 2H, $C^{2'}$, $C^{6'}$-H), 6.59 (s, 1H, $C^{5'''}$-H), 6.57 (s, 1H, $C^{8'''}$-H), 6.50 (s, 2H, $C^{2''''}$, $C^{6''''}$-H), 6.40 (s, 2H, $C^{2'}$, $C^{6'}$-H), 6.38-6.39 (m, 2H, C^6-H, C^8-H), 6.18 (d, 1H, J = 4.9 Hz, $C^{4'''}$-H), 5.90-5.97 (m, 4H, C^3-H, C^2-H, $C^{13'''}$-CH_2), 5.22-5.24 (m, 2H, C^{12}-CH_2), 4.76 (d, 1H, J = 4.8 Hz, $C^{1'''}$-H), 4.18-4.20 (m, 2H, $C^{11'''}$-CH_2), 3.74 (s, 3H, $C^{4'''}$-OCH_3), 3.72 (s, 6H, $C^{3'}$, $C^{5'}$-OCH_3), 2.98-3.01 (m, 1H, $C^{3'''}$-H), 2.95-2.96 (m, 1H, C^4-H_a), 2.85-2.87 (m, 2H, $C^{2'''}$-H, C^4-H_b); ^{13}C-NMR (CD_3OD, 125 MHz) δ 175.8 (C-12‴), 166.9 (C-11), 164.9 (C-5), 157.9 (C-7), 157.8 (C-9), 153.9

(C-3‴′, 5‴′), 151.9 (C-3‴, 5‴), 150.6 (C-6‴), 149.3 (C-7‴), 146.7 (C-3″, 5″), 142.3 (C-13), 136.7 (C-4″), 134.7 (C-1‴′), 131.3 (C-4‴′), 130.7 (C-4′), 127.3 (C-1′), 126.6 (C-9‴), 126.5 (C-10‴), 119.4 (C-14), 115.9 (C-5‴), 115.3 (C-8‴), 111.1 (C-1″), 110.2 (C-2″, 6″), 109.5 (C-2′, 6′), 106.8 (C-2″, 6″), 103.3 (C-13‴), 100.1 (C-10), 96.5 (C-8), 95.9 (C-6), 79.9 (C-12), 78.5 (C-2), 70.4 (C-4‴), 68.9 (C-3‴), 67.5 (C-11), 61.1 (4‴′-OCH_3), 56.6 (3‴′, 5‴′-OCH_3), 44.8 (C-1‴), 42.4 (C-2‴), 38.6 (C-3‴), 29.3 (C-4); ESIMS: *m/z* 958 $[M+Na]^+$.

3.3.2. (−)-Epigallocatechin-3-gallate-[4β-(1,2,3-triazol-1-yl)-4-deoxy-4′-demethylpodophyllotoxin] ether (8)

White amorphous powder; yield 80%; m.p. 200–202 °C; ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.47 (s, 1H, C^{14}-H), 6.96 (s, 1H, $C^{5'''}$-H), 6.86 (s, 2H, $C^{2'}$, $C^{6'}$-H), 6.74-6.78 (s, 1H, $C^{8'''}$-H), 6.58 (s, 2H, $C^{2'''}$, $C^{6'''}$-H), 6.50 (s, 2H, $C^{2'}$, $C^{6'}$-H), 6.37 (s, 2H, C^6-H, C^8-H), 6.18 (d, 1H, $J=4.9$ Hz, $C^{4'''}$-H) , 5.90-5.97 (m, 2H, C^3-H, C^2-H, $C^{13'''}$-CH_2), 5.22-5.24 (m, 2H, C^{12}-CH_2), 4.76 (d, 1H, $J=4.8$ Hz, $C^{1'''}$-H), 4.17-4.20 (m, 4H, $C^{11'''}$-CH_2), 3.72 (s, 6H, $C^{3'}$, $C^{5'}$-OCH_3), 2.98-3.00 (m, 1H, $C^{3'''}$-H), 2.96-2.98 (m, 1H, C^4-H_a), 2.84-2.86 (m, 1H, $C^{2'''}$-H), 2.85 (dd, 1H, $J=4.6$, 12.0 Hz, C^4-H_b); ^{13}C-NMR (CD_3OD, 125 MHz) δ 176.1 (C-12‴), 165.0 (C-11), 162.1 (C-5), 157.9 (C-7), 157.4 (C-9), 151.9 (C-3‴, 5‴), 148.7 (C-3′, 5′), 146.7 (C-3″, 5″), 146.1 (C-6‴, C-7‴), 144.5 (C-13), 142.0 (C-4″), 132.3 (C-1‴′), 131.3 (C-4‴′), 130.8 (C-4′), 127.3 (C-1′), 126.6 (C-9‴), 126.5 (C-10‴), 119.4 (C-14), 115.9 (C-5‴), 115.3 (C-8‴), 111.1 (C-1″), 110.2 (C-2″, 6″), 109.4 (C-2′, 6′), 106.8 (C-2‴′, 6‴′), 103.2 (C-13‴), 100.1 (C-10), 96.4 (C-8), 95.9 (C-6), 79.9 (C-12), 78.5 (C-2), 70.4 (C-4‴), 68.9 (C-3), 67.5 (C-11‴), 56.8 (3‴′, 5‴′-OCH_3), 44.7 (C-1‴), 42.6 (C-2‴), 38.6 (C-3‴), 29.3 (C-4); ESIMS: *m/z* 944 $[M+Na]^+$.

3.4. Cytotoxicity assay

The cytotoxicity of all compounds against HL-60, A-549, MCF-7, SW480, and SMMC-7721 cell lines was measured using MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [21]. Adherent cells (100 μl) were seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition, both with an initial density of 1×10^5 cells/ml in 100 μl of medium. Each tumor cell line was exposed to the test compound at various concentrations in triplicate for 48 h. After the incubation, MTT (100 μg) was added to each well, and the incubation continued for 4 h at 37 °C. The cells were lysed with SDS (200 μl) after removal of 100 μl of medium. The optical density of lysate was measured at 595 nm in a 96-well microtiter plate reader (Bio-Rad 680). IC_{50} values were calculated by Reed and Muench's method.

3.5. Molecular modeling

The crystal structure of Top-II (code ID: 3QX3) [22] was obtained in Protein Data Bank. PyMol 2.3 software was used to the preparation of ligand and receptor.

Autodock Tools v1.56 was used for grid and docking according to the literature [23]. Docking parameters were set as the defaults values, except "The Number of GA Runs" was set to 50 and maximum number of evals (medium) was set to 5,000,000 on AutoGrid v4.2.6 and AutoDock v4.2.6. Docking conformations were classified into different clusters by binding energy, and the cluster with the lowest binding energy was selected. In the selected cluster, conformations with the lowest binding energy and RMSD (<2.0 Å) were finally chosen to analyze the receptor-ligand interaction. Etoposide was chosen as control ligand.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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