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A rational design strategy of the novel topoisomerase II inhibitors for the synthesis of the 4-*O*-(2-pyrazinecarboxylic)-4′-demethylepipodophyllotoxin with antitumor activity by diminishing the relaxation reaction of topoisomerase II-DNA decatenation

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ABSTRACT

A rational design strategy of the novel podophyllum topoisomerase II (Topo II) inhibitors for the synthesis of the esterification and amidation substituted 4′-demethylepipodophyllotoxin (DMEP) derivates was developed in order to discover the potential antitumor prodrug. Firstly, according to the structure–activity relationship, drug combination principle and bioisosterism, the –COO– and the –NH– bond substituents at the 4 position of cycloparaffin would be a great modification direction to improve antitumor activity of 4′-demethylepipodophyllotoxin (DMEP). Secondly, from the prodrug principle view, the esterification and amidation at the C-4 position of DMEP would be two useful structure modifications for improve solubility. Thirdly, from the activity pocket in Topo II-DNA cleavage complex point of view, a series of heterocyclic with pharmacological activity were chosen as module for improving antitumor activity by binding with Topo II. Finally, nine novel esterification and amidation DMEP derivates were designed and synthesized for the potential Topo II inhibitors with the superior biological activity. All the novel compounds exhibited promising in vitro antitumor activity, especially 4-*O*-(2-pyrazinecarboxylic)-4′-demethylepipodophyllotoxin (compound **1**). The antitumor activity of compound **1** against tumor cell line HeLa (i.e., the IC_{50} value of 0.60 ± 0.20 μM), A549 (i.e., the IC_{50} value of 3.83 ± 0.08 μM), HepG2 (i.e., the IC_{50} value of 1.21 ± 0.05 μM), and BGC-823 (i.e., the IC_{50} value of 4.15 ± 1.13 μM) was significantly improved by 66, 16, 12, and 6 times than that of the clinically important podophyllum anticancer drug etoposide (i.e., the IC_{50} values of 15.32 ± 0.10, 59.38 ± 0.77, 67.25 ± 7.05, and 30.74 ± 5.13 μM), respectively. Compound **1** could arrest HeLa cell cycle G_2/M and induce apoptosis by strongly diminishing the relaxation reaction of Topo II-DNA decatenation. The correctness of rational drug design was strictly demonstrated by the bioactivity test.

1. Introduction

4′-Demethylepipophyllotoxin (DMEP) was a well known naturally occurring cyclolignans with antitumor activities,[1] which can bind at the pocket between the link site of DNA and topoisomerase II (Topo II) and then diminishing the relaxation reaction of Topo II-DNA decatenation. The clinical success of etoposide and teniposide has triggered the search for compounds with a similar mechanism of action but without their inconveniences.[2] This has resulted in the discovery of many compounds with very different chemical structures. Despite most of currently numerous studies focus on the structure modification of DMEP in order to generate derivatives with superior pharmacological profiles and broader therapeutic scope,[3–5] those have only begun to appreciate the full potential of the prodrug approach in modern drug development, and many novel prodrug innovations await discovery.

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To discover a series of the novel 4′-demethylepipophyllum Topo II inhibitors, a rational prodrug design strategy should be developed to the structure modification of DMEP with the superior inhibition on Topo II, the higher antitumor activity, the lower cytotoxicity on the human normal cells and the better solubility. Firstly, the steric clash and electrostatic contour plots of the comparative molecular field analysis models[6] shows that the carbon atom at the 4 position of cycloparaffin (C ring) of DMEP is more reactive by comparing other carbon atoms in the tetranap skeleton of DMEP, and is easier nucleophillic attacked by the molecule with the electro negativity. Thus, from the structure–activity relationship point of view, the structure modifications at the 4 position of C ring of DMEP maybe a very effective pathway for improving the antitumor activity. Secondly, according to drug combination principle,[7,8] the combination of two kinds of drugs through the ester bond can gain some new compounds with better bioactivity. Many famous drugs in clinic use such as sultamicillin and benorilate were discovered through this way. So, DMEP could be combined with other compounds through ester bond for the better bioactivity and attenuated toxicity. Thirdly, according to bioisosterism,[9,10] bioisosteres are substituents with similar physical or chemical properties which produce broadly similar biological properties to a chemical compound for the activity improvement and the toxicity reduction of the lead compound. Both of the ester bond (–COO–) and the –NH– bond are the bioisosteres with the same numbers of the outermost electrons. So, the –NH– bond at the C-4 position of DMEP may be also a potential modification direction for improving the antitumor activity of DMEP. Fourthly, the prodrug approach is a very versatile strategy to increase the utility of pharmacologically active compounds.[11] Thus, according to the prodrug principle,[12] both of esters (–COOR) or amides (–NHCOR) are commonly used ionizable groups, which can be introduced into the hydroxyl, thiol, amine, or carboxylic acid functionalities of the parent drug molecule to compensate for poor aqueous solubility. A variety of esterases, amidases, and/or peptidases in plasma or in other tissues can bioconvert these prodrugs to their active counterparts. Quite often, esters and amides can also be used to enhance absorption and consequently oral drug delivery of parent drugs, because the brush-border membrane of intestinal epithelium possesses a considerable number of transporters for amino acids and peptides.[13,14] Take these into consideration, many famous drugs in clinic were discovered by using esterification and amidation such as sultamicillin and benorilate. So, esterification and amidation of DMEP at the C-4 position were permitted for two useful DMEP structure modifications. Finally, from an antitumour mechanistic point of view, substituents with the high electron density at the C-4 position of DMEP derivatives (i.e., etoposide) has been demonstrated to be bound via the hydrogen bonding interactions with the tyrosine residue of Topo II and the bases of DNA breakage.[15,16] In order to design the novel topoisomerase II inhibitors with better affinity for the active site of DNA Topo II as anti-tumor agents, a series of heterocyclic with pharmacological activity and the high electron density were chosen as module at the C-4 position of DMEP for improving the stabilization and inhibition of the complex of Topo II-DNA cleavage complex.

Based on the above analysis, this work provided an effective method in the development of the novel podophyllum Topo II inhibitors for discovery potential antitumor prodrug. Most of compounds exhibit higher antitumor activities than etoposide, which is a clinically important Topo II inhibitory anticancer drug. These results provided the determinants of DNA Top II binding affinity for this important class of anti-tumor agents and pave the way for further rational structural modification.

2. Materials and methods

2.1. Chemistry

Standard 4′-demethylepipodophyllotoxin (DMEP) (98%) was purchased from Shanxi Huisheng Medicament Technology Company, Ltd (Shanxi, China). *N*,*N*-dicyclohexylcarbodii mide (DCC) (98%), 4-dimethylami-nopyridine (DMAP) (98%), 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) (98%), 1-hydroxybenzotriazole hydrate (HOBT) (98%), 2-Pyrazinecarboxylic acid (98%), 3,4,5-Trihydroxybenzoic acid (98%), 2-Quinoline carboxylic acid (98%), Fluoroquinolonic acid (98%), 9-Anthracenecarboxylic acid (98%) and 4-Methyl-1-naphthoic acid (98%) were purchased from Shanghai Jingchun Medicament Technology Company, Ltd (Shanghai, China). Dichloromethane (DCW) was distilled from CaH_2. Precoated silica gel G plates for TLC were purchased from Merck Inc. (Darmstadt, Germany). Column chromatography was performed with Sephadex LH-20 gel (20–150 μm, Pharmacia & Upjohn Co., Switzerland). Flash column chromatography (FC) silica gel (SiO_2; 200–300 meshes) was purchased from Qing Dao Haiyang Chemical Group Co. (Shandong, China). Methanol and acetonitrile were of high-performance liquid chromatography (HPLC) grade, and all other chemicals used for extraction and isolation were of analysis grade and commercially available. Deionized water was used throughout the study.

2.2. Analytical method

The chemical reaction solution was alternately washed in deionized water and saturated $NaHCO_3$. After removing the deionized water layer, drying the AcOEt layer by $NaSO_4$, and removing AcOEt by rotary evaporation, the residue was ground by aether to obtain a white powder product. The powder product (2 mg) dissolved in 1 mL methanol/water (50:50 v/v) as pre-separated sample. Pre-separated sample was filtered (0.45-μm-micropore filter) and transferred into a sampling vial for HPLC analysis. The samples were filtered with a 0.45 μm micropore filter and transferred to a sampling vial for HPLC analysis. HPLC analysis was carried out on a Waters 600 Series HPLC system, equipped with 2487 UV detector. An Akasil C18 column (5 μm, 4.6 mm × 150 mm) was used. Mobile phase was methanol/water (50:50 v/v) for the detection of alkane amination DMEP derivatives. Mobile phase was methanol/water (40:60 v/v) for the detection of esterification DMEP derivatives. The pH of mobile phase was adjusted to 3.00 with formic acid. The HPLC oven temperature was maintained at 45 °C, and the detection wavelength was 230 nm or 219 nm. The flow rate was 0.8 mL/min. All ^1H and ^{13}C NMR spectra were recorded using a mercury-300BB spectrometer (Varian, USA). Chemical shifts (δ) are reported in ppm relative to the TMS internal standard. Abbreviations are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet) and m (multiple). The synthesized compounds are at least 95% pure according to HPLC analysis. ESI-MS spectra were obtained with an agility MSD trap mass spectrometer. The separation was carried on a reversed-phase column with dimensions 150 × 4.6 mm and a particle size of 5 μm. Thin layer chromatography (TLC) analysis was carried out with a precoated silica gel G plate, and a small spot of solution containing the samples was applied onto the plate, approximately 1.5 cm from the bottom edge. The plate was then placed in a chamber with a solvent system consisting of chloroform/acetone (10:1 to 1:1, v/v). TLC spots were observed under an ultraviolet light (UV_{254}) and then observed after spraying with H_2SO_4/MeOH (10:1 v/v) and heating to 110 °C.

2.3. General procedure for the synthesis of compounds

2.3.1. Synthesis of esterification 4′-demethylepipodophyllotoxin derivatives

DMEP (400 mg, 1 mmol), carboxylic acid (3 mmol), *N*,*N*′-dicyclohexylcarbodiimide (6 mmol) and 4-dimethylaminopyridine (0.05 mmol) were mixed in 5 mL dichloromethane. All reactions were stirred under nitrogen at room temperature for 24 h and monitored by TLC (20 cm × 20 cm glass plates coated with a 0.5 mm layer of Merck silica gel GF 254). The reaction mixture was diluted with dichloromethane (100 mL), washed with saturated aqueous sodium bicarbonate (40 mL × 2), and water (40 mL × 2). The organic layer was dried over anhydrous Na_2SO_4, and then the liquid was removed by rotary evaporation in crude solid. The product was purified by silica gel column chromatography to give as a white solid.

2.3.2. Compound (1) 4-*O*-(2-pyrazinecarboxylic)-4′-demethylepipodophyllotoxin

^{1}H NMR (300 MHz, DMSO, 25 °C, TMS) and ^{13}C NMR (75 MHz, DMSO, 25 °C, TMS). ^{1}H NMR δ = 9.37 (s, 1H; CH), 9.07 (s, 1H; CH), 8.86 (s, 1H; CH), 7.05 (s, 1H; Ar-H), 6.65 (s, 1H; Ar-H), 6.38 (s, 2H; Ar-H), 6.07 (s, 1H; OCH_2O), 6.02 (s, 1H; OCH_2O), 5.73 (s, 1H; OH), 4.79 (d, ^2J (4, 4) = 4.8 Hz, 1H; CH), 4.47 (t, 1H; CH), 4.05 (t, 1H; CH), 3.85 (s, 6H; OCH_3), 3.49 (d, ^2J (1, 1) = 4.8 Hz, 1H; CH), 3.47 (d, ^2J (2, 2) = 4.8 Hz, 1H; CH), 3.26–3.18 (m, 1H; CH). ^{13}C NMR: δ = 174.42 ($COOCH_2$), 164.05 (COO), 149.54 (*C*CH), 148.48 (*C*CH), 147.80 ($COCH_3$), 146.87 ($COCH_3$), 146.68 (*C*CH3), 145.04 (CHN), 143.06 (CHN), 134.45 (CCH), 133.87 (CCH), 130.17 (CCH), 127.15 (COH), 110.69 (CH), 110.05 (CH), 107.98 (CH), 102.07 (OCH_2O), 70.65 (CH), 67.66 (CH_2), 56.74 (OCH_3), 43.96 (CH), 41.99 (CH), 37.12 ppm (CH). MS (ESI): *m*/*z*: 507 $[M+H]^+$, 524 $[M+NH_4]^+$; $C_{26}H_{22}O_9N_2$.

2.3.3. Compound (2) 4-*O*-(5-methylpyrazine-2-carboxylic)-4′-demethylepipodophyllotoxin

^{1}H NMR (300 MHz, DMSO, 25 °C, TMS) and ^{13}C NMR (75 MHz, DMSO, 25 °C, TMS). ^{1}H NMR: δ = 9.16 (s, 1H; CH), 8.60 (s, 1H; CH), 6.95 (s, 1H; Ar-H), 6.67 (s, 1H; Ar-H), 6.47 (s, 1H; Ar-H), 6.30 (s, 1H; Ar-H), 5.99 (d, 2H; OCH_2O), 5.94 (s, 1H; OH), 4.71 (d, 1H; CH), 4.38 (t, 1H; CH_2), 4.45 (t, 1H; CH_2), 3.77 (s, 6H; OCH_3), 3.42 (d, ^2J (1, 1) = 5.1 Hz, 1H; CH), 3.39 (d, ^2J (2, 2) = 4.8 Hz, 1H; CH), 3.13–3.09 (m, 1H; CH). 2.67 (s, 3H; CH_3). ^{13}C NMR: δ = 174.44 ($COOCH_2$), 163.46 (COO), 158.83 (CCH_3), 149.43 (CCH), 147.72 (CCH_3), 146.75 ($COCH_3$), 145.69 (CHN), 144.83 (CHN), 134.34 (CH), 133.74 (CH), 130.14 (CH), 127.19 (CCH), 110.58 (CH), 109.96 (CH), 107.90 (CH), 101.96 (OCH_2O), 70.30 (CH), 67.62 (CH_2), 56.66 (OCH_3), 43.90 (CH), 41.92 (CH), 37.05 ppm (CH). MS (ESI): *m*/*z*: 521 $[M+H]^+$; $C_{27}H_{24}O_9N_2$.

2.3.4. Compound (3) 4-*O*-(theophylline-7-acetic)-4′-demethylepipodophyllotoxin

^{1}H NMR (300 MHz, DMSO, 25 °C, TMS) and ^{13}C NMR (75 MHz, DMSO, 25 °C, TMS). ^{1}H NMR: δ = 8.49 (s, 1H; CH), 6.63 (s, 1H; Ar-H), 6.25 (s, 1H; Ar-H), 6.07 (s, 2H; Ar-H), 5.72 (d, ^2J (13, 13) = 3.6 Hz, 2H; OCH_2O), 5.19(s, 1H; OH), 4.92 (s, H; CH), 4.60 (s, 1H; CH), 4.10 (t, 1H; CH), 3.45 (d, 3H; CH_3); 3.35 (s, 6H; OCH3), 3.12 (m, 1H; CH), 2.64 (s, 1H; CH), 2.54 (s, 1H; CH), 1.34 (s, 3H; CH_3). ^{13}C NMR: δ = 175.32 ($COOCH_2$), 165.32 (COO), 155.38 (CO), 151.92 (CCH), 151.35 (*C*CH), 148.68 ($COCH_3$), 147.72 (CH), 142.32 (CH), 138.93 (CN), 132.24 (CH), 131.54 (CN), 127.21 (C), 110.57 (CH), 109.41 (CH), 107.73 (CH), 101.86 (OCH_2O), 67.94 (CH), 66.72 (CH), 56.43 (OCH_3), 47.10 (CH_2), 44.17 (CH), 40.62 (CH), 38.55 (CH), 30.09 (CH_3), 28.17 (CH_3), ppm (CH). MS (ESI): *m*/*z*: 621 $[M+H]^+$; $C_{30}H_{28}O_{11}N_4$.

2.3.5. Compound (4) 4-*O*-(fluoroquinolonic)-4′-demethylepipodophyllotoxin

^{1}H NMR (300 MHz, DMSO, 25 °C, TMS) and ^{13}C NMR (75 MHz, DMSO, 25 °C, TMS). ^{1}H NMR: δ = 8.73 (s, 1H; CH), 8.09 (d, 1H; Ar-H), 7.88 (d, 1H; Ar-H), 6.82 (s, 1H; Ar-H), 6.36 (s, 1H; Ar-H), 6.28 (s, 2H; Ar-H), 5.85 (s, 2H, OCH_2O), 5.22 (s, 1H; OH), 4.77 (s, 1H; CH), 4.25 (s, 1H; CH), 4.00 (s, 1H; CH), 3.59 (d, 3H; OCH_3), 3.45 (s, 6H; CH), 3.29 (d, 1H; CH), 3.24 (s, 1H; CH), 1.41 (s, 2H; CH_2), 1.08 (s, 2H; CH_2). ^{13}C NMR: δ = 175.92 ($COOCH_2$), 161.86 (COO), 154.42 (CO), 151.81 (CCH), 149.95 (*C*CH), 148.26 ($COCH_3$), 147.46 (CH), 138.84 (CN), 137.38 (CH), 132.86 (CN), 131.48 (C), 128.62 (C), 127.44 (C), 119.46 (C), 114.01 (C),110.26 (CH), 109.95 (CH), 109.29 (CH), 107.80 (OCH_2O), 101.67 (OCH_2O), 68.37 (CH), 66.23 (CH), 56.40 (OCH_3), 49.45 (CH2), 44.24 (CH), 40.77 (CH), 38.88 (CH), 35.39 (CH_3), 28.17 (CH3), ppm (CH). MS (ESI): *m*/*z*: 664 $[M+H]^+$; $C_{34}H_{27}ClFO_{10}N$.

2.3.6. Compound (5) 4-*O*-(2-quinoline carboxylic)-4′-demethylepipodophyllotoxin

^{1}H NMR (300 MHz, DMSO, 25 °C, TMS) and ^{13}C NMR (75 MHz, DMSO, 25 °C, TMS). ^{1}H NMR δ = 8.87 (s, 1H; CH), 8.52 (s, 1H; CH), 8.26 (s, 2H; Ar-H), 7.75 (s, 1H; Ar-H), 7.34 (s, 1H; Ar-H), 7.05 (s, 1H; Ar-H), 6.65 (s, 1H; Ar-H), 6.38 (s, 2H; Ar-H), 6.02 (d, 2H; OCH_2-O), 5.73 (s, 1H; OH), 4.79 (d, 1H; CH), 4.47 (t, 1H; CH), 4.05 (t, 1H; CH), 3.85 (s, 6H; OCH_3), 3.49 (d, 1H; CH), 3.47 (d, 1H; CH), 3.22 (m, 1H; CH). ^{13}C NMR: δ = 174.42 ($COOCH_2$), 164.05 (COO), 149.54 (CCH), 148.48 (CCH), 147.80 ($COCH_3$), 146.87 ($COCH_3$), 146.68 (CCH_3), 145.04 (CHN), 143.06 (CHN), 134.45 (CCH), 133.87 (CCH), 130.17 (CCH), 127.15 (COH), 110.69 (CH), 110.05 (CH), 107.98 (CH), 102.07 (OCH_2O), 70.65 (CH), 67.66 (CH_2), 56.74 (OCH_3), 43.96 (CH), 41.99 (CH), 37.12 ppm (CH). MS (ESI): *m*/*z*: 556 $[M+H]^+$; $C_{31}H_{25}O_9N$.

2.3.7. Synthesis of amination 4′-demethylepipodophyllotoxin derivatives

DMEP (400 mg, 5 mmol), triethylamine (1.68 mL), 4-toluenesulfonylchloride (0.96 mL) were mixed in 40 mL dichloromethane. All reactions were stirred under nitrogen at room temperature for 3 h and monitored by TLC (20 cm × 20 cm glass plates coated with a 0.5 mm layer of Merck silica gel GF 254). The reaction mixture was washed with saturated aqueous sodium bicarbonate (40 mL × 2), and deionized water (40 mL × 2). The organic layer was dried over anhydrous Na_2SO_4, and then the liquid was removed by rotary evaporation in crude solid. Then, the crude solid (1 mmol), module (3 mmol) were mixed in 10 mL acetonitrile. All reactions were stirred under nitrogen at 80 °C for 48 h and monitored by TLC and then the liquid was removed by rotary evaporation. The product was purified by silica gel column chromatography to give as a white solid.

2.3.8. Compound (6) 4-*N*-(2-pyrazinecarboxylic) amino-4′-demethylepipodophyllotoxin

^{1}H NMR (300 MHz, DMSO, 25 °C, TMS) and ^{13}C NMR (75 MHz, DMSO, 25 °C, TMS). ^{1}H NMR δ = 9.37 (s, 1H; CH), 9.07 (s, 1H; CH), 8.86 (s, 1H; CH), 7.05 (s, 1H; Ar-H), 6.65 (s, 1H; Ar-H), 6.38 (s, 2H; Ar-H), 6.02 (d, 2H; OCH_2O), 5.73 (s, 1H; OH), 4.79 (d, 1H; CH), 4.47 (t, 1H; CH), 4.05 (t, 1H; CH), 3.85 (s, 6H; OCH_3), 3.49 (d, 1H; CH), 3.47 (d, 1H; CH), 3.22 (m, 1H; CH). ^{13}C NMR: δ = 174.42 ($COOCH_2$), 164.05 (COO), 149.54 (CCH), 148.48 (CCH), 147.80 ($COCH_3$), 146.87 (COCH3), 146.68 (CCH3), 145.04 (CHN), 143.06 (CHN), 134.45 (CCH), 133.87 (CCH), 130.17 (CCH), 127.15 (COH), 110.69 (CH), 110.05 (CH), 107.98 (CH), 102.07 (OCH_2O), 70.65 (CH), 67.66 (CH_2), 56.74 (OCH_3), 43.96 (CH), 41.99 (CH), 37.12 ppm (CH). ESI MS: *m*/*z*: 507 $[M+H]^+$; $C_{26}H_{22}O_9N_2$.

2.3.9. Compound (7) 4-*N*-(5-methylpyrazine-2-carboxylic) amino-4′-demethylepipodophyllotoxin

^{1}H NMR (300 MHz, DMSO, 25 °C, TMS) and ^{13}C NMR (75 MHz, DMSO, 25 °C, TMS). ^{1}H NMR: δ = 8.97 (s, 1H; CH), 8.55 (s, 1H; CH), 8.33 (s, 1H; NH), 6.79 (s, 1H; Ar-H), 6.53 (s, 1H; Ar-H), 6.24 (s, 1H; Ar-H), 5.94 (d, ^2J (13, 13) = 9.3 Hz, 2H; OCH_2O), 5.41 (t, 1H; OH), 4.47 (d, ^2J (4, 4) = 4.8 Hz, 1H; CH), 4.33 (t, 1H; CH), 3.80 (t, 6H; OCH_3), 3.47 (d, ^2J (1,1) = 5.4 Hz, 1H; CH), 3.39 (d, ^2J (2, 2) = 6.9 Hz, 1H; CH), 3.02 (s, 1H; CH), 3.25 (s, 1H; CH_3), 2.60 (s, 3H; CH_3). ^{13}C NMR: δ = 175.43 ($COOCH_2$), 164.22 (NHCO), 157.75 (CCH_3), 147.96 (CCH), 147.80 (CCH), 147.16 ($COCH_3$), 143.46 (CHN), 142.57 (CHN), 135.21 (CH), 133.52 (CH), 130.96 (CH), 130.29 (CCH), 110.17 (CH), 109.57 (CH), 108.99 (CH), 101.90 (OCH_2O), 68.97 (CH_2), 56.65 (OCH_3), 47.95 (CH), 43.63 (CH), 41.27 (CH), 37.29 ppm (CH). MS (ESI): *m*/*z*: 521 $[M+H]^+$; $C_{27}H_{24}O_9N_2$.

2.3.10. Compound (8) 4-*N*-(theophylline-7-acetic) amino-4′-demethylepipodophyllotoxin

^{1}H NMR (300 MHz, DMSO, 25 °C, TMS) and ^{13}C NMR (75 MHz, DMSO, 25 °C, TMS). ^{1}H NMR: δ = 8.71 (d, ^2J = 8.1 Hz, 1H; CH), 6.81 (s, 1H; Ar-H), 6.55 (s, 1H; Ar-H), 6.28 (d, ^2J (2', 6') = 11.7 Hz, 2H; Ar-H), 6.02 (t, 2H; OCH_2O), 5.18(m, 1H; OH), 4.99 (t, 2H; CH_2), 4.53 (d, ^2J (4, 4) = 5.1 Hz, 1H; CH), 4.23 (t, 1H; CH), 3.94 (t, 1H; NH); 3.63 (s, 6H; OCH_3), 3.44 (s, 3H; CH_3), 3.32 (s, 1H; CH), 3.21 (s, 3H; CH_3), 3.15 (d, ^2J (2 ,2) = 5.1 Hz, 1H; CH), 2.96(d, ^2J (3, 3) = 9.6 Hz, 1H; CH). ^{13}C NMR: δ = 175.18 ($COOCH_2$), 166.84 (NHCO), 151.72 (CO), 147.83 (CCH), 147.26 (CCH), 144.31 ($COCH_3$), 135.40 (CH), 133.03 (CH), 130.77 (CH), 110.12 (CH), 109.15 (CH), 107.23 (CH), 101.96 (OCH_2O), 68.95 (CH_2), 56.69 (OCH_3), 48.96 (CH), 47.87 (CH2), 43.58 (CH), 41.60 (CH), 37.28 ppm (CH). MS (ESI): *m*/*z*: 621 $[M+H]^+$; $C_{30}H_{28}O_{11}N_4$.

2.3.11. Compound (9) 4-*N*-(3,4,5-trihydroxybenzoic) amino-4′-demethylepipodophyllotoxin

^{1}H NMR (300 MHz, DMSO, 25 °C, TMS) and ^{13}C NMR (75 MHz, DMSO, 25 °C, TMS). ^{1}H NMR: δ = 6.84 (s, 2H; Ar-H), 6.77 (s, 2H; Ar-H), 6.53 (s, 1H; Ar-H), 6.24 (s, 1H Ar-H), 5.97 (d, ^2J (13, 13) = 8.1 Hz 2H; OCH_2O), 5.77 (s, 3H; OH), 5.36 (s, 1H; OH), 4.47 (d, ^2J (4, 4) = 4.8 Hz, 1H; CH), 4.32 (s, 2H; CH_2), 4.32 (s, 1H; CH), 3.64 (d, ^2J (3', 5') = 16.2 Hz, 6H; OCH_3), 3.45 (d, ^2J (2, 2) = 5.1 Hz 2H, CH), 3.41 (s, 1H; CH), 3.32 (s, 1H; CH). ^{13}C NMR: δ = 175.44 ($COOCH_2$), 167.35 (CONH), 147.88 (CCH), 146.09 (CCH), 137.21 ($COCH_3$), 135.41 (CCH), 133.11 (CCH), 131.39 (COH), 130.99 (C), 124.85 (CH), 110.03 (CH), 109.58 (CH), 109.19 (CH), 107.84 (CH), 101.84 (OCH_2O), 69.14 (CH_2), 56.71 (OCH_3), 47.80 (CH), 43.68 (CH), 41.44 (CH), 37.38 ppm (CH). MS (ESI): *m*/*z*: 567 $[M+H]^+$; $C_{29}H_{26}O_{12}$.

2.4. Cytotoxicity assay

Cytotoxicity assay was performed on human lung adenocarcinoma epithelial cell line (A549), human henrietta lacks strain of cancer cells (HeLa), human gastric carcinoma cell line (BGC-823), human hepatocellular liver carcinoma cell line (HepG2), and human normal cell Hepatic immortal cell line (HL-7702). Cells (3500–13,000) in a 100 μL culture medium per well were seeded into 96-well microtest plates (Falcon, CA). A549 cell lines, HeLa cell lines and HepG2 cell lines were cultured in DMEM supplemented with 10% CS, 100 mg/L penicillin G, 100 mg/L streptomycin. BGC-823 cell lines were cultured in RPMI Medium 1640 supplemented with 10% CS, 100 mg/L penicillin G, and 100 mg/L streptomycin. The cells were incubated at 37 °C, in a humidified atmosphere with 5% CO_2 for 48 h. For all cell lines, the microculture tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; Sigma, St. Louis, MO] assay was performed to measure the cytotoxic effects. Drug stock solutions (the each drug concentration was 0.5, 1, 10, 20, 50, 100, 200, and 500 μM, respectively) were prepared in DMSO and the final solvent concentration was ⩽2% DMSO (v/v), a concentration without effect on cell replication. Initial seeding densities varied among the cell lines to ensure a final absorbance reading in control (untreated) cultures in the range 0.6–0.8 A_{492} units. Drug exposure was for 2 days, and the IC_{50} value, the drug concentration that reduced the absorbance by 50%, was interpolated from dose–response data. Each test was performed in triplicate, and absorbance readings varied no more than 5%. Values were averaged from six treated wells and values for medium alone (lane 1) were subtracted. These values were then normalized to control (unstimulated cells, lane 2) and used to calculate the effective half-maximal concentrations for each drug as well as the percentage of cell death (treated values, normalized to control × 100) induced at IC_{max}.[17]

2.5. Statistical evaluation

Data presented as means ± SD. Statistical analyses were performed by the analysis of variance (ANOVA). All statistical analyses were performed using Origin version 8.0 (GraphPad Software, OriginLab Corp., Northampton, MA, USA). Sigmoidal dose responses and non-linear regression analyses were undertaken to identify half-maximal concentrations for each of the drugs. To evaluate differences in IC_{50} concentrations, analysis of variance combined with Tukey's multiple range test was used.

2.6. Octanol–water partition coefficients

Log*P* is one criterion used in medicinal chemistry to assess the drug likeness of a given molecule, and used to calculate lipophilic efficien**cy. It was calculated with following equation: $\log P = \log[C_{in\ 1\text{-octanol}}/(C_{in\ water} * Y)]$. $C_{in\ 1\text{-octanol}}$ is the compound concentration in 1-octanol, $C_{in\ water}$ is the compound concentration in water, *Y* is a correction for index error = 2.555. The partition coefficient *P* was determined according to the standard method[18] and the reported *P* values are an average of three measurements. For a water-soluble compound, the reported log*P* values could be low.

2.7. kDNA decatenation assay

Topoisomerase II activity was determined using kit (Topogen Inc., USA, Cat No. 2000H). The substrate kDNA (134 ng) and 10 μM drug were combined in assay buffer (5 × Topo II assay buffer (A+B)) and incubated for 10 min on ice. Next, 4 U of topoisomerase II was added and the reaction was allowed to proceed for 60 min at 37 °C. The reaction was stopped by the addition of 1/10 volume of 1 mg/mL proteinase K and incubated for 15 min at 37 °C. The reaction was quenched via the addition of loading buffer (1% sarkosyl, 0.025% bromophenol blue, and 5% glycerol) and was then analyzed by electrophoresis on a 1% agarose gel in TBE buffer (89 mM Tris, 89 mM borate, and 2 mM Na-EDTA, pH 8.3) for 0.5 h at 140 V. The gel was visualized under UV illumination and photographed on an Alpha Imager.

2.8. Cell cycle arrest assay

HeLa were seeded in six multiwell plates at a density of 2×10^6 cells/plate. After 48 h of incubation with compound **1** with various concentration(0, 0.1, 0.5, 1, 5 μM) in DMEM without serum at 37 °C, cells were washed in PBS, pelleted, centrifuged, and directly stained in a propidium iodide solution (50 mg of PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) for 30 min at 4 °C in the dark. Flow cytometric analysis was performed using a FAC Scan flow cytometer (Becton Dickinson, San Jose, CA). To evaluate the

cell cycle, PI fluorescence was collected as FL2 (linear scale) by the Mod FIT software (Becton Dickinson). For the evaluation of intracellular DNA content, at least 20,000 events for each point were analyzed in at least three different experiments giving a SD of less than 5%.

2.9. Annexin V/PI apoptosis assay

An amount of 2×10^6 cells/mL was plated in 6 well plates, treated with compound **1** at a range of concentrations 0, 0.5, 1, 5, and 10 μM and incubated for 48 h. The cells were centrifuged at 1000 rpm for 10 min. The supernatant was discarded, and the cells were resuspended in 100 μL PBS. Apoptosis using a MultiSciences Biotech kit (Lot: 1200945) kit, and 10 μL annexin V and 5 μL PI was added. The samples in the labeling solution were transferred into Falcon tubes and incubated in a water bath at 37 °C for 20 min. The samples were then analyzed using a Becton Dickinson FAC scan flow cytometer with Cell Quest software. The results were tabulated as percentage of annexin V-FITC positive apoptotic cells. The experiments were performed in three replicates and repeated at least twice for each compound.

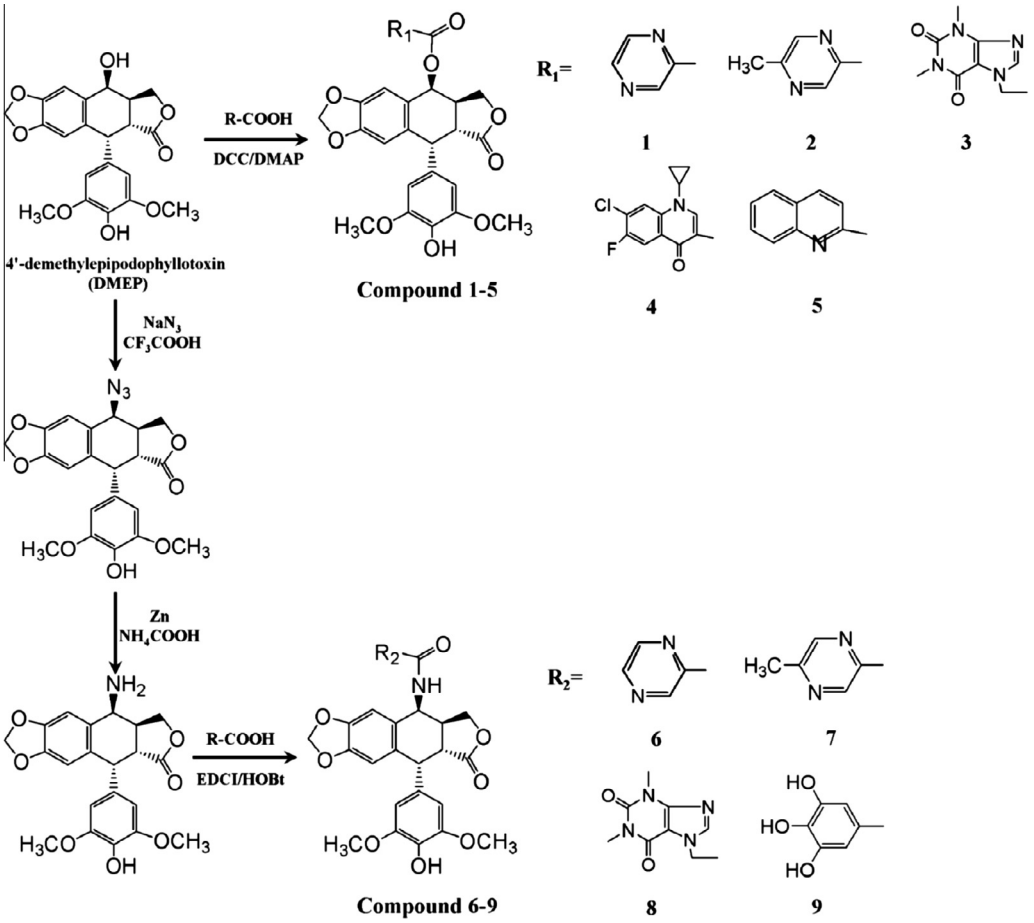
2.10. Computational docking simulations

Molecular docking of compounds into the X-ray structure of the human topoisomerase II, DNA and VP-16 complex (PDB: 3QX3) from the RCSB Protein Data Bank was carried out by using the Auto-Dock software (version 4.2) via the graphic user interface Auto-Dock Tool Kit (ADT 1.5.4).[19] The grid maps of docking studies were computed by using AutoGrid 4, which was included in the Autodock4 software. The graphical user interface ADT was employed to set up the enzymes; in that, all hydrogen atoms were added, the Gasteiger charges were calculated, and nonpolar hydrogen atoms were merged with the carbon atoms. For macromolecules, the generated pdbqt files were saved. Then, based on the Auto-Dock software, a docking procedure was applied to position the conformation of these compounds correctly with regard to their active sites. Grid maps representing the topoisomerase II protein in the actual docking process were constructed with Auto Grid. The points of the grids were thus set at $60 \times 60 \times 60$ with a grid spacing of 0.375 Å to ensure that it was sufficiently large to include not only the active site but also significant portions of the surrounding surface.

3. Results and discussion

3.1. Drug molecular design

The process of drug molecular design was performed in three steps. Firstly, numerous studies on 4′-demethylepipodophyllotoxin (DMEP) currently focus on its structure modification of the cycloparaffin (C-ring) in the tetranap skeleton, and the steric clash and electrostatic contour plots of the comparative molecular field analysis models[6] shows that the C-4 position would be a great modification position to improve antitumor activity of DMEP. Meanwhile, in our previous work, the DMEP derivate 4β-*S*-(1,2,4-triazole-3)-4-demethylepipodophyllotoxin that structurally modified at C-4 position of DMEP against tumor cell line BGC-823 (IC_{50} values of



Scheme 1. Synthesis of esterification and amidation 4′-demethylepipodophyllotoxin derivatives **1**–**9**.

0.28 μM), A549 (IC_{50} values of 0.76 μM) and HepG2 (IC_{50} values of 0.42 μM) were significantly improved by 91, 221 and 2.73 times than those of etoposide (IC_{50} values of 25.72, 167.97 and 1.15 μM), respectively.[20] Furthermore, the structure of the Topo II-DNA cleavage complex finding that electrophilic group in the C-4 position of DMEP rests in a spacious binding pocket with relatively few interactions, so it can be either modified or replaced to produce derivatives with enhanced Topo-poisoning activities, it appears that those non-C4 substitutions would cause steric conflicts and impair the drug-binding site. Thus, the C-4 position of DMEP was ensured for an effective modification site for generating new bioactive DMEP derivatives. Secondly, according to drug combination principle,[7,8] the combination of two kinds of drugs through the ester bond can gain some new compounds with better bioactivity. Many famous drugs in clinic use such as sultamicillin and benorilate were discovered through this way. So, DMEP could be combined with other compounds through ester bond for the better bioactivity and attenuated toxicity. Thirdly, according to bioisosterism[9,10], both of the ester bond (–COO–) and the –NH– bond are the bioisosteres with the same numbers of the outermost electrons but with different electronegativity. Electronegativity (electronegativity = constant*(|EA| + |IE|)/2, EA: energy liberated when an extra electron attaches to a single neutral atom, IE: energy required to remove an existing valence electrons from the neutral atom) generally increases from left to right on the periodic table and decreases from top to bottom. The most commonly used scale of electronegativity is that developed by Linus Pauling[21,22] in which the value 3.44 is assigned to oxygen, 3.04 is assigned to oxygen, and 2.10 is assigned to hydrogen. Although nitrogen has low EA, the chemical bond –NH– with the polarity has enough IE for higher Electronegativity than oxygen. Because hydrogen has an electrognegativity of 2.10 and nitrogen has an electronegativity of 3.04, they could form a polar molecule with the nitrogen being the negative side of the dipole. The –NH– with the greater electronegativity may be denser than that of the oxygen atom. So, the amidation is another effective pathway to improve the antitumor activity of DMEP. Fourthly, it is extensively reported that the cytotoxicity of the Topo II poisons etoposide stems from its ability to stabilize the DNA-Topo II complex and prevent the relegation of the double-stranded breaks, which also results in inhibition of DNA replication and apoptotic cell death.[23] Comparatively, the further studies focused on the C-4 position modification of the substituents with high electron density were much more promising. The substituents with high electron density were favorable for stabilization the complex of Topo II-DNA cleavage complex. So, a series of heterocyclic with pharmacological activity and electronegative atom (i.e., oxygen, nitrogen) were chosen as module. *N*-Benzyl heterocycles, which were frequently found in medicinal chemistry, the higher electron density of nitrogen atom making them easily attack the action of target protein and improve the antitumor activity of leading compounds.[24,25] Benzene ring contains a large π bond, which can easily form π–π accumulation with biological molecules, thus can enhance the interaction between drugs and target proteins. The quinolones[26,27] derivates were a family of anti-tumor drugs target to DNA topoisomerase II, the same as DMEP derivates. Combing quinolones with DMEP enable to enhance the targeting of the new DMEP derivates to DNA topoisomerase II. Base on the above analysis, we ensured amination and esterification for two modification directions. In this effort, nine novel esterification and alkane amination DMEP derivates were rationally designed as target compounds, the structure of target compounds were shown in Scheme 1.

3.2. Synthesis of esterification and amidation 4′-demethylepipodophyllotoxin derivatives

The synthetic route to the target compounds is depicted in Scheme 1. DMEP was condensed with the appropriate carboxylic acids in the presence of *N*,*N*-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino) pyridine (DMAP) to provide the ester linkage compounds **1**–**5** under an atmosphere of nitrogen at room temperature.[28] Compounds **6**–**9** were synthesized by three steps as described in the reported method.[29,30] Firstly, using sodium azide as catalyst, 4′-demethylepipodophyllotoxin was reducted to 4-azido-4′-demethylepipodophyllotoxin. Then, the key intermediate 4-amino-4′-demethylepipodophyllotoxin was obtained by

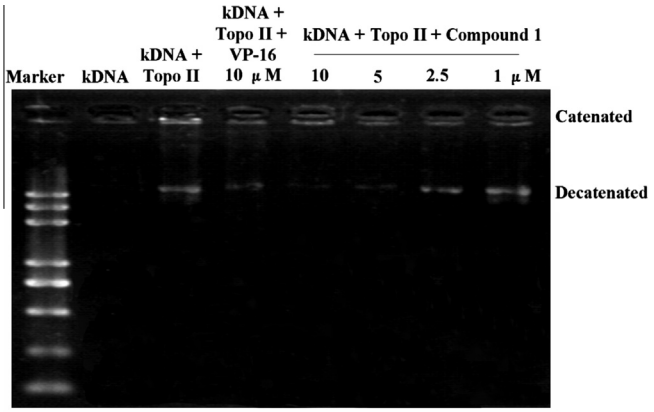


Figure 1. Inhibition of human topoisomerase II (Topo II) catalytic activity by compound **1**. All reaction samples were electrophoresed in 1% agarose gels as described in the Topo II mediated kDNA relaxation assay.

Table 1
The cytotoxic activity and the solubility of the esterification and amidation podophyllum compounds

Compound	Cytotoxic activity[a] (IC_{50}, μM)					Log*P*[a]
	HepG2[b]	HeLa[b]	A549[b]	BGC-823[b]	HL-7702[b]	
1	1.21 ± 0.05	0.88 ± 0.20	3.83 ± 0.08	4.15 ± 1.13	52.43 ± 5.32	0.33 ± 0.11
2	14.22 ± 0.68	9.56 ± 2.44	10.51 ± 2.91	1.50 ± 0.09	18.63 ± 5.23	0.38 ± 0.23
3	7.91 ± 1.67	0.65 ± 0.11	6.91 ± 1.15	2.00 ± 0.97	38.01 ± 3.42	0.43 ± 0.15
4	16.03 ± 2.54	0.60 ± 0.08	10.05 ± 2.43	17.41 ± 5.07	41.77 ± 3.91	0.69 ± 0.21
5	9.18 ± 1.21	20.53 ± 3.50	19.20 ± 4.04	28.81 ± 3.34	20.09 ± 3.11	0.80 ± 0.05
6	6.64 ± 1.42	10.84 ± 1.82	16.46 ± 1.82	14.38 ± 2.91	53.63 ± 6.92	0.43 ± 0.12
7	6.78 ± 1.08	16.05 ± 2.70	18.71 ± 2.24	3.49 ± 1.12	20.68 ± 1.05	0.59 ± 0.08
8	2.35 ± 0.99	3.18 ± 0.19	7.32 ± 0.56	5.88 ± 1.07	50.82 ± 4.53	0.56 ± 0.19
9	27.41 ± 3.98	22.27 ± 0.77	33.54 ± 4.71	24.17 ± 3.42	14.82 ± 3.40	0.29 ± 0.02
DMEP	18.74 ± 2.71	15.96 ± 1.22	52.08 ± 0.85	21.26 ± 2.42	13.04 ± 1.55	0.73 ± 0.08
Etoposide	15.32 ± 0.10	59.38 ± 0.77	67.25 ± 7.05	30.74 ± 5.13	24.61 ± 3.82	0.52 ± 0.13

[a] The value was the average of triplicates.
[b] MTT methods, drug exposure was for 48 h.

reducting 4-azido-4′-demethylepipodophyllotoxin, which was treated zinc (Zn) in ammonium formate. At last, combination 4β-amino-4′-demethylepipodophyllotoxin with various carboxylic acids in presence of 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) and 1-hydroxybenzotriazole hydrate (HOBT), the target compounds were successfully synthesized. The details were shown in Scheme 1.

3.3. Activity evaluation

3.3.1. Effect of compounds on the proliferation of human tumor cell lines

In the present study, the antitumor activities in vitro of nine novel esterification and alkane amination DMEP derivates on the several human tumor cell lines were investigated. The antineoplastic drug etoposide (VP-16) was used as reference standard. Cytotoxicity values for DMEP were included in Table 1 for comparative purposes. When these cells treated with different concentrations of DMEP derivatives cultured up to 48 h, there was an increase in the percentage of cells being shrunk observed with inverted microscope.

As shown in Table 1, with few exception, the results of inhibitory indicated that the most of nine novel esterification and alkane amination DMEP derivates potential antitumor activity on the HepG2, HeLa, A549 and BGC-823 tumor cell lines. *N*-Benzyl heterocycle-substitution DMEP derivate 4-*O*-(2-pyrazinecarboxylic)-4′-demethylepipodophyllotoxin (compound **1**). The antitumor activity of compound **1** against tumor cell line HeLa (i.e., the IC_{50} value of 0.60 ± 0.20 μM), A549 (i.e., the IC_{50} value of 3.83 ± 0.08 μM), HepG2 (i.e., the IC_{50} value of 1.21 ± 0.05 μM), and BGC-823 (i.e., the IC_{50} value of 4.15 ± 1.13 μM) was significantly improved by 66, 16, 12, and 6 times than that of the clinically important podophyllum anticancer drug etoposide (i.e., the IC_{50} values of 15.32 ± 0.10, 59.38 ± 0.77, 67.25 ± 7.05, and 30.74 ± 5.13 μM), respectively.

As shown in Table 1, although the growth of all cell lines was inhibited by any of the compounds used independently in a dose-dependent manner, the data demonstrated that different compounds display different sensitivity to the same cell. Firstly, esterification derivates (e.g., compounds **1**–**3**) displayed better antitumor activities than amidiation derivates (e.g., compounds **6**–**8**) against four cancer cell lines. Secondly, N-benzyl heterocycle-substitution derivates (e.g., compounds **1**–**3** whose 50% effective concentration between 1–10 μM) showed better antitumor activities than benzene ring-substitution derivates (e.g., compound **9** whose 50% effective concentration between 22–33 μM). At last, another important critical trend observed across the entire data set was compounds possessing an electrondonating substituent showed enhanced antitumor activity such as the presence of F, Cl atom (i.e., compound **4**) increased the antitumor activity against four cell lines. On the contrary, starting from compounds **1** and **2**, removing the methyl group resulted in a moderate drop of potency (i.e., compounds **6** and **7**) antitumor activity. Simultaneously, most of the compounds exhibited the low toxic-side effects. As shown in Table 1, the IC_{50} value of compound **1** (i.e., 52.43 ± 5.32 μM), compound **3** (i.e., 38.01 ± 3.42 μM), compound **4** (i.e., 41.77 ± 3.91 μM), compound **6** (i.e., 53.63 ± 6.92 μM), and compound **8** (i.e., 50.82 ± 4.53 μM) against on the human normal

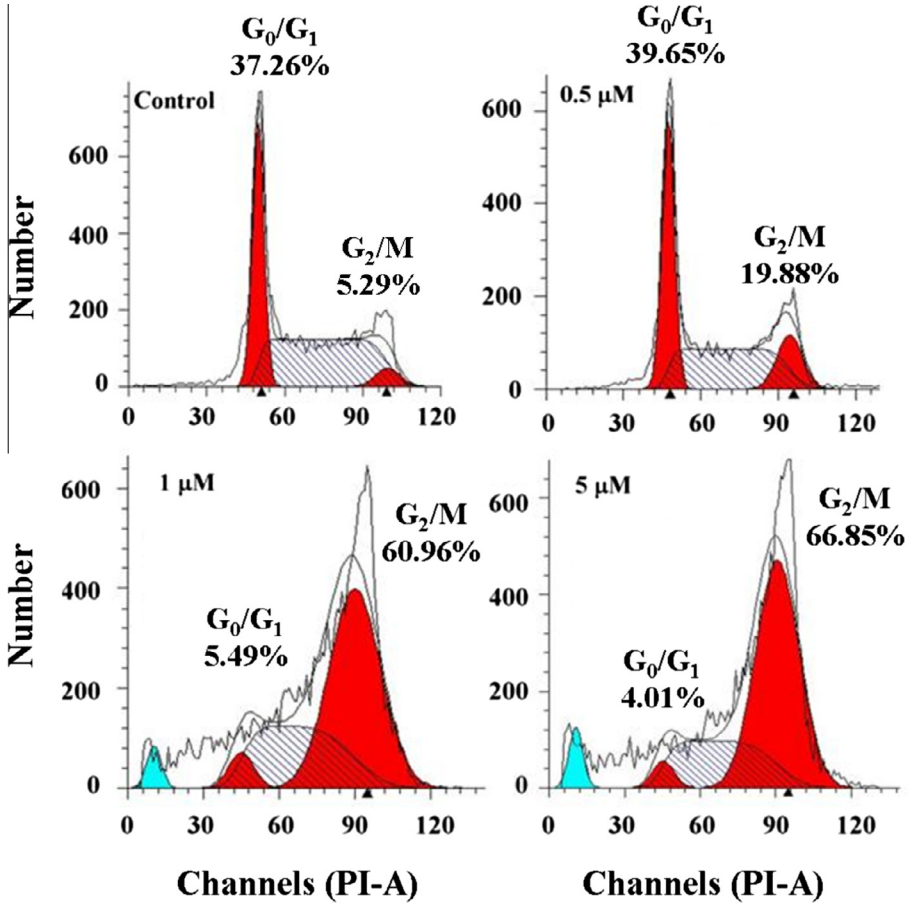


Figure 2. Effect of compound **1** on the HeLa cell cycle arrest. Cell cycle arrest detection in HeLa cells using propidium iodide (PI) double staining treatment with 0, 0.5, 1, and 5 μM compound **1** for 24 h.

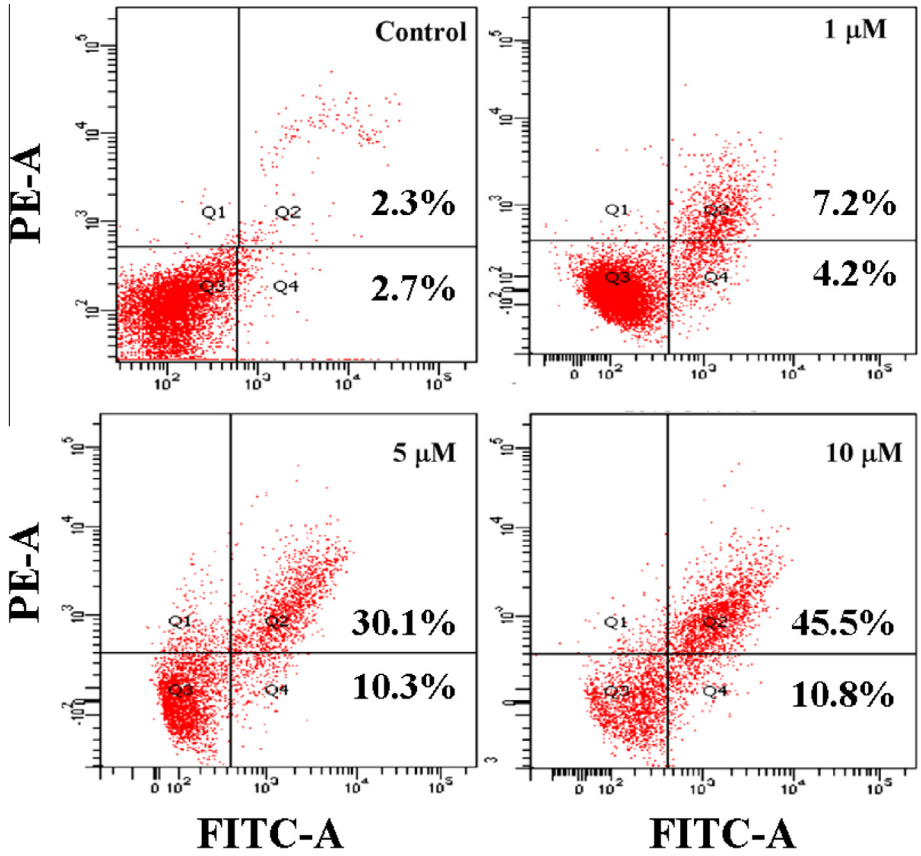


Figure 3. Compound **1** induced HeLa cell apoptosis. Apoptosis detection in HeLa cells using annexin V and propidium iodide (PI) double staining treatment with 0, 1, 5, and 10 μM compound **1** for 48 h. The cell scatter could be composed of four subgroups as follows: Q1 quadrant was the necrotic cells, Q2 quadrant was late apoptotic cells, Q3 quadrant was the living cells late, and Q4 quadrant was the early apoptotic cells.

cell HL-7702 was improved 114%, 54%, 70%, 117%, and 106% than that of etoposide (i.e., 24.61 ± 3.82 μM), respectively. The IC_{50} values indicate that most of compounds have promising anti-tumor activity and lower cytotoxic efficacy on normal human cell line HL-7702 than the standard compounds etoposide. On the other hand, As expected, the partition coefficient log*P* indicated that most of compounds exhibited the higher the water-solubility than that of etoposide, and compound **9** had the lowest the partition coefficient (i.e., log*P* = 0.29 ± 0.02) among the other compounds. Compared with etoposide (i.e., log*P* = 0.52 ± 0.13), the partition coefficient of compound **9** (i.e., log*P* = 0.32 ± 0.11) was significantly decreased 79%. This maybe the trihydroxy benzene with three hydroxyl groups at C-4 position of compound **9** resulted in the improvement of the water-solubility. Furthermore, interestingly, the water-solubility of compound **1**(i.e., log*P* = 0.33 ± 0.11) was better than that of compound **6** (i.e., log*P* = 0.43 ± 0.12), the water-solubility of compound **2** (i.e., log*P* = 0.38 ± 0.23) was better than that of compound **7** (i.e., log*P* = 0.59 ± 0.08), the water-solubility of compound **3** (i.e., log*P* = 0.43 ± 0.15) was better than that of compound **8** (i.e., log*P* = 0.56 ± 0.19). These demonstrate that the same substitution with the ester bond at the C-4 position of DMEP was greater effect than the amido bond for improving the water-solubility.

3.3.2. Effect of compounds on the Topo II DNA decatenation assay

DMEP derivates have been reported to induce cell death by inhibiting DNA Top II activity. So we investigated the efficacy of the newly synthesized compounds for human DNA Top II enzyme inhibiting ability. The kDNA decatenation assay has been utilized to test drug potential to inhibit DNA Top II activity in a cell-free system. To understand the action mechanisms of antitumor activity of these compounds, the effect on the catalytic activity of human topoisomerase II was evaluated. As shown in Figure 1, compound **1** diminished the relaxation reaction at a concentration 10, 5, 2.5, and 1 μM, respectively, and completely inhibited the catalytic activity of topoisomerase II at 10 μM of concentration, which was much better than the effect of etoposide at same concentration. These results provide strong evidence that compound **1** with pyrazine exhibit antiproliferative properties against human cancer cells by inhibiting the activity of topoisomerase II. These may be also indicated a different mode of action for that pyridine is competitive nonselective phosphodiesterase inhibitor which raises intracellular cAMP and activates apoptosis pathway correlated with antitumor activity.

3.3.3. Effect of compound 1 on the HeLa cells cycle arrest and apoptosis

On the basis of the above results, further biological evaluations have been focused on compound **1**. To gain further insight into the mode of action of compound **1**, we examined the effects of compound **1** on cell cycle by flow cytometry in HeLa cells. Interestingly, a concentration dependent change was observed in the cell cycle pattern (Fig. 2). Our results demonstrated that treatment of HeLa cells for 24 h with 0.5, 1 and 5 μM of compound **1** could cause cell cycle arrest at G_2/M phase from 15.44% to 66.85%. To probe whether compound **1** induced HeLa apoptosis, Flow cytometry was used to generate an apoptotic cell scatterplot of the control groups treated with compound **1**. As shown in Figure 3, different concentrations (i.e., 1 , 5, and 10 μM) of compound **1** were treated

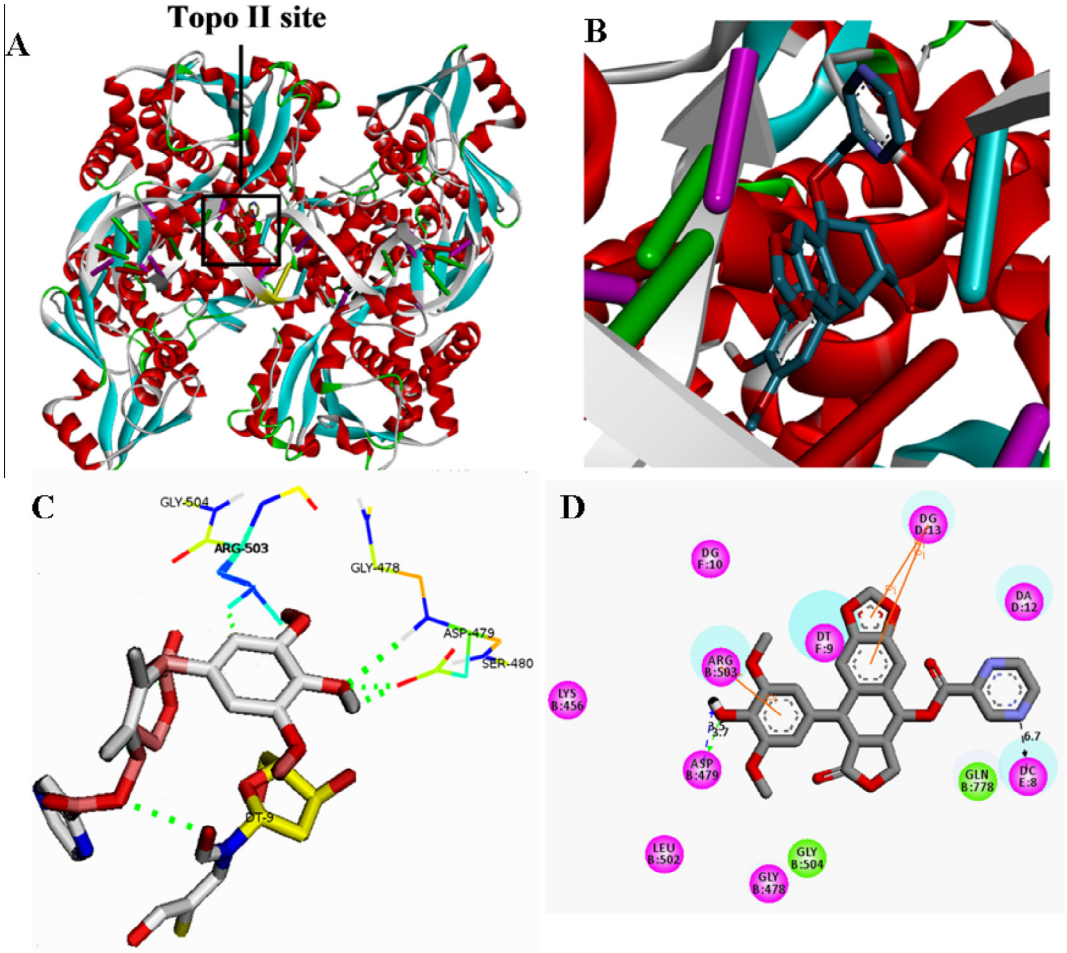


Figure 4. (A) Structure of the topoisomerase II cleavage complex stabilized by compound **1**. (B) Active pocket of topoisomerase II dimer bound with compound **1**; (C) 3D view of binding mode (the green dotted lines are H-bond); (D) 2D view of binding mode.

in HeLa for 48 h, the cells count in Q2 quadrants were considered to be late apoptotic cells and the total percentages were 19.1%, 19.6%, 39.1% and 63.8%, respectively. The results showed that the effect was observed in a dose-dependent manner and the apoptotic rates of HeLa cells increased with the increase of concentration. It demonstrated that compound **1** induced apoptosis of cells and thus as expected, caused the anticancer effects. In summary, we have prepared a series of 4′-demethylepipodophyllotoxin derivatives. Antitumor evaluation indicates most of them performed better antitumor activity than etoposide. For the most promising compound **1**, antitumor mechanism study showed that compound **1** completely arrested HeLa cell at G_2/M and eventually induce apoptosis through inhibiting the activity of Topo II.

3.3.4. Docking studies

As for the most promising compound **1**, antitumor mechanism study showed that it completely arrested HeLa cell in G_2/M with 5 μM and eventually induce apoptosis through inhibiting the activity of Topo II. So, to study the molecular basis of interaction and affinity of binding of the 4′-demethylepipodophyllotoxin analogues, compound **1**, the most active compound, was used as the representative one to investigate the binding modes of synthesized 4′-demethylepipophyllotoxin derivatives.

Compound **1** was docked into the active site of Topoisomerase-II for etoposide as reported earlier[16,31], docking results are given in Figure 4. Compound **1** was found to have a score of −12.85 kg/mol with 1 H-bonds with Arg 503 and 3 H bonds with Asp 479 (Fig. 4C). It is quite similar with the binding mode of etoposide as reported earlier. Hence, the amino acid residues with dioxolane ring and trans-lactone ring can match that of compound **1** in space to firm the conformation of the complex.

A careful inspection of the binding pocket showed (Fig. 4D) that the trimethoxyphenyl ring (E ring) of 1 was inserted in a bigger hydrophobic cavity formed by Arg 503, Lys 456, Asp 479, Leu 502, Gly 478, Gly 504. This result indicated that the hydrophobic property was one of the main forces governing the interaction between 1 and Topo II. On the other hand, the interaction was not exclusively hydrophobic. It should be noted that there were several polar residues (Arg 503, Lys 456, Asp 479, Gly 478, Gly 504, Gln 778)in the proximity to 1, which played important roles in stabilizing the Topo II and compound **1** complex via electrostatic interaction and Van der Waals' force (Fig. 4D). The hydrogen-bonding or electrostatic interaction acted as an anchor, intensely determining the 3D space position of 1 in the binding pocket and facilitating the hydrophobic interaction of the ligand 1 with the side chain of protein. In addition, it can be seen from the Figure 4D, the nitrogen heterocyclic-substituted ring of compound **1** made a π–π interaction with DC: 8, which may contribute to the binding and stabilization of the compound **1** with DNA, thereby, resulting in strengthening the stability of DNA, Topo II and compound **1** ternary complex. From the results, it was evident that the in silico findings were well related to the IC_{50} values obtained through in vitro cytotoxicity assay, furthermore, it displayed that compound **1** induced apoptosis potentially by the strong interaction of compound **1** with Topo II.

4. Conclusion

This work presented the rational design method of the novel topoisomerase II (Topo II) inhibitors for the synthesis of the esterification and amidation substituted 4′-demethylepipodophyllotoxin (DMEP) derivates exhibiting antitumor activity. The correctness of rational design was strictly demonstrated by the biological activity tests. The bioassay tests showed that the designed novel 4-*O*-(2-pyrazinecarboxylic)-4′-demethylepipodophyllotoxin (compound **1**) had superior in vitro biological activities including the stronger anti-tumor activity, the lower cytotoxic activity on normal human cells, and the higher solubility, cell cycle G_2/M arrest, and induce apoptosis. The antitumor activity of 4-*O*-(2-pyrazinecarboxylic)-4′-demethylepipodophyllotoxin (compound **1**) against tumor cell line HeLa (i.e., the IC_{50} value of 0.60 ± 0.20 μM), A549 (i.e., the IC_{50} value of 3.83 ± 0.08 μM), HepG2 (i.e., the IC_{50} value of 1.21 ± 0.05 μM), and BGC-823 (i.e., the IC_{50} value of 4.15 ± 1.13 μM) was significantly improved by 66, 16, 12, and 6 times than that of the clinically important podophyllum anticancer drug etoposide (i.e., the IC_{50} values of 15.32 ± 0.10, 59.38 ± 0.77, 67.25 ± 7.05, and 30.74 ± 5.13 μM), respectively. Especially, unlike the clinically important podophyllum anticancer drug etoposide (VP-16), compound **1** not only showed the strong anti-tumor activity, but also exerted significant the superior inhibitory on topoisomerase II (Topo II) for diminishing the relaxation reaction of topoisomerase II-DNA decatenation in vitro. Interestingly, the active pockets of Topo II and DMEP structure–function relationship suggested that alkylation moieties at the C-4 position such as tris(hydroxymethyl)metylaminomethane, cyclopentylamine, (3-aminopropyl)imidazole, and (4-pyridyl)thioglycolic acid is an effective modification. The above analyses indicated that the esterification and amination substituted at C-4 position of DMEP was crucial and contributed to improve the antitumor activity against the most tumor cell lines.

We are now in the process of further exemplification of the method and its application to the synthesis of a range of naturally occurring and biologically significant target products. Furthermore, to perfect the drug design method, the pharmacological mechanism of the active DMEP derivates needs to be studied in detail.

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