Synthesis and biological evaluation of prodrugs for nitroreductase based 4-β-amino-4′-Demethylepipodophyllotoxin as potential anticancer agents

Zheng-Rong Wu · Wei Deng · Dian He

Received: 10 December 2021 / Accepted: 7 January 2022 / Published online: 24 January 2022
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Abstract
A series of prodrugs for nitroreductase (NTR) based 4-β-amino-4′-Demethylepipodophyllotoxin as potential anticancer agents were synthesized, and their antiproliferative activities in vitro showed compounds 2b (IC50 = 0.77, 0.83 and 1.19 μM) and 2d (IC50 = 0.98, 0.91 and 1.58 μM) were greatly selectively toxic to tumor cells A-549, HeLa and HepG2, respectively, and lower damage to normal WI-38 cells in comparison with positive agent Etoposide and Demethylepipodophyllotoxin, and induced cell cycle arrest in the G2/M phase with a concomitant decrease in the population of G1 phase in HeLa cells, which were accompanied by apoptosis. Furthermore, Molecular docking model showed that compounds 2b and 2d appeared to form stable bonds with NTR 1DS7. Taken together, these conjugates have the potential to be developed as antitumor drugs.

Graphical abstract

| Compound | A-549 (μM) | HeLa (μM) | Hep-G2 (μM) | WI-38 (μM) |
|----------|-----------|-----------|-------------|------------|
| 2b       | 1.19±0.67 | 0.77±0.76 | 0.83±0.37   | 686.54±76.58 |
| 2d       | 1.58±0.81 | 0.96±0.74 | 0.91±0.45   | 477.96±32.84 |

Keywords Podophyllotoxin · O-nitrophenoxycetyl acid · Cell cycle arrest · Apoptosis · Structure–Activity Relationship

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00044-022-02847-5.
Introduction

With the rapid growth of cancer cases across the globe, a great number of approaches are being investigated to develop an effective cure for this deadly disease [1]. Among various approaches, naturally occurring substances account for the abundant sources to identify novel hits and leads [2]. However, a number of antitumor naturally occurring substances do not show target specificity, which may even show adverse toxicity to non-carcinoma cells. In order to reduce the cytotoxicity of the natural compounds, appropriate structural modification is the vital way and has been adopted to develop new drug [3].

Podophyllotoxin is a naturally occurring aryltetralin lignin with pronounced cytotoxic activity. However, its clinical application for cancer treatment has been blocked due to its poor water solubility and selectivity [4]. For reducing cytotoxicity and increasing water solubility of the bioactive compound, a great number of structural modification have been adopted to address these limitations and obtain better therapeutic agents [5–11], and these efforts have led to the structure–activity relationships (SAR) unambiguously demonstrate that C4 is the major molecular area tolerable to significant structural diversification [12]. Therefore, most of the current derivatization on Podophyllotoxin focuses on C-4 position. Recently, A series of 4β-N substituted 4′-O-demethyl-4-deoxypodophyllotoxins have been synthesized, and exhibited significant anticancer activity [13–17]. Among these, the substituent of p-nitrobenzylpiperazine markedly affected the activity profiles of this compound class, and may serve as a potential anticancer drug [18].

NTR are a family of bacterial enzymes used in gene-directed enzyme prodrug therapy (GDEPT) that selectively activate prodrugs containing aromatic nitro groups to exert cytotoxic effects [19]. It can catalyze nitro-substituted aromatic compounds into aromatic amines and undergoes internal cyclization to a non-toxic derivative, which reduces serious side effects on healthy cells and form toxic metabolites after selective reduction by enzymes in tumor tissues [20]. In recent years, Efforts for optimization of NTR-based GDEPT have focused on the key aspects of the technology, namely the delivery system, the NTR-enzyme and the prodrug. The improved prodrug is expected to boost the clinical application of NTR-based GDEPT in future [21]. Prodrug strategies based on NTR have been extensively studied and remarkable efforts employing NTR and a library of different nitro-containing scaffolds has led to promising in vitro prodrg candidates [22–26]. O-nitrophenoxycetyl acid which containing aromatic nitro group has known as potential inhibitor of vascular endothelial growth factor in cancer chemotherapy [27–29]. NTR can catalyze nitro substituent into aromatic amines and undergoes internal cyclization to a non-toxic derivative, which reduces serious side effects on healthy cells and increases concentration of drugs at cancer cells is remarkable [21]. Furthermore, identification of structurally diverse prodrug will undoubtedly prove beneficial to design and synthesis of novel nitroaromatic prodrugs [22].

Inspired by the above background, herein, we present the design, synthesis, of a novel series of 4β-N-(4-Nitrophenyl piperazinyl)-4′-O-demethyl-4-deoxypodophyllotoxin and four different O-nitrophenoxycetyl acids(substituent of electron-donating groups, electron-withdrawing groups, steric groups, comprehensive groups, respectively) hybrids and evaluation of their cytotoxic activity against several cancer cell lines.

Scheme 1 General Synthesis of the target compounds 2a-2t.
Reagents and conditions: a halogenated acid ester, CH3CN, K2CO3. b: NaOH, HCl. ii: SOCl2. c: TMSCI/KI, CH3CN. d: CH3CN, Et3N, 1-(4-nitrophenyl) piperazine. e: SOCl2

![Scheme 1](image-url)
Results and discussion

Synthesis

The synthetic route to the target compounds is shown in Scheme 1. The O-nitrophenoxyacetyl acids were prepared according to our previous report [27]. 4β-N-(4-Nitrophenyl piperazinyl)-4′-O-demethyl-4-deoxypodophyllotoxin was according to the procedure described previous [18]. The chemical structures of the final compounds were fully characterized by $^1$H NMR, $^{13}$C NMR and HRMS spectroscopic techniques.

Cytotoxicity

The in vitro cytotoxicities of conjugates 2a-2t were evaluated against a panel of three human cancer cell lines (lung carcinoma A-549, cervical carcinoma HeLa, hepatocellular carcinoma HepG2, and human embryonic lung fibroblasts WI-38 cells), with Etoposide and Demethylepipodophyllotoxin as reference compounds. In addition, their toxicity evaluation was tested on normal embryonic lung fibroblast WI-38 cells. The screening procedure was based on the standard MTT growth inhibition assay, and the results are summarized in Table 1. As shown, most of the compounds were more potent against three tumor cell, these compounds were most effective in A549 cells and HeLa cells, and had lowest potency in HepG2 cells. The IC$_{50}$ value of compounds 2b (0.77, 0.83 and 1.19 μM) and 2d (0.98, 0.91 and 1.58 μM), and which were comparable to positive agent Etoposide and more potent than the positive agent Demethylepipodophyllotoxin (9.64, 15.29 and 12.73 μM) for HeLa, HepG2 and A549 cells, respectively. In comparison, compounds 2b (IC$_{50}$ = 688.54 μM) and 2d (IC$_{50}$ = 477.96 μM) showed greater selectivity, and were relatively less active toward normal WI-38 cells in comparison with positive agent Etoposide (IC$_{50}$ < 100 μM) and Demethylepipodophyllotoxin (IC$_{50}$ < 100 μM). Based on these results, it was possible to deduce some preliminary SAR. First of all, O-nitrophenoxyacetyl acids with electron-donating group substitution (2a-2e) appeared to be more potent than those with electron-withdrawing (2f-2k), steric (2l-2o), and comprehensive (2p-2t) groups.

Cell cycle arrest and apoptotic assay

The data above revealed that compounds 2b and 2d the most potent and selective were further investigate the effects on cell cycle progression by means of fluorescence-activated cell sorting analysis of HCT-116 cells stained with propidium iodide.

As shown in Fig. 1, treatment with compounds 2b and 2d led to a dose-dependent accumulation of cells in the G2/M phase with a concomitant decrease in the population of G0/G1 phase cells respectively, and showed significantly difference compared with untreated control group ($P < 0.05$ and $P < 0.01$). Similar effects also occurred with the Annexin V/PI apoptosis detection. It was illustrated from Fig. 2 that, following the treatment, the apoptotic cell percentage was as low as 0.14% of that with untreated control group (< 0.05 and < 0.01). As suggested by the above findings, compounds 2b and 2d could efficiently induce HCT-116 cell apoptosis (Fig. 2).

These results demonstrated that compounds 2b and 2d were interfering with cell proliferation by arresting the cell cycle and induced G2/M arrest accompanied by apoptosis in HeLa cells.

### Table 1 Cytotoxicity of target compounds to different cells following 24 h of exposure

| Compds | A-549 (μM) | Hela (μM) | HepG2 (μM) | WI-38 (μM) |
|--------|------------|-----------|------------|------------|
| 2a     | 5.7 ± 0.73 | 22.92 ± 2.37 | 13.25 ± 2.53 | 583.78 ± 61.57 |
| 2b     | 1.19 ± 0.67 | 0.77 ± 0.76 | 0.83 ± 0.37 | 688.54 ± 70.58 |
| 2c     | 21.99 ± 2.7 | 12.93 ± 1.53 | 19.31 ± 0.84 | >1000 |
| 2d     | 1.58 ± 0.81 | 0.98 ± 0.74 | 0.91 ± 0.45 | 477.96 ± 32.84 |
| 2e     | 13.81 ± 3.93 | 18.55 ± 0.71 | 15.96 ± 2.23 | >1000 |
| 2f     | 16.87 ± 1.18 | 18.94 ± 1.72 | 20.67 ± 3.62 | >1000 |
| 2g     | 17.64 ± 2.15 | 16.82 ± 0.36 | 28.63 ± 2.81 | >1000 |
| 2h     | 27.44 ± 1.55 | 9.75 ± 2.42 | 23.42 ± 4.07 | >1000 |
| 2i     | 8.29 ± 0.84 | 10.44 ± 1.38 | 18.91 ± 3.09 | 744.26 ± 89.42 |
| 2j     | 16.54 ± 1.38 | 19.38 ± 2.14 | 22.55 ± 1.85 | >1000 |
| 2k     | 8.81 ± 1.15 | 5.49 ± 0.67 | 16.12 ± 2.60 | 656.67 ± 85.32 |
| 2l     | 7.08 ± 0.97 | 4.8 ± 0.36 | 19.87 ± 1.90 | 508.54 ± 42.67 |
| 2m     | 6.32 ± 1.16 | 10.02 ± 0.62 | 10.21 ± 0.95 | 727.23 ± 67.76 |
| 2n     | 7.26 ± 0.25 | 8.84 ± 0.34 | 15.19 ± 3.10 | 675.45 ± 37.87 |
| 2o     | 18.72 ± 1.89 | 10.14 ± 1.00 | 30.68 ± 2.19 | >1000 |
| 2p     | 19.82 ± 1.03 | 23.21 ± 1.33 | 19.68 ± 1.45 | >1000 |
| 2q     | 30.46 ± 4.87 | 20.31 ± 0.27 | 14.35 ± 1.41 | >1000 |
| 2r     | 28.29 ± 2.03 | 17.99 ± 1.38 | 14.64 ± 1.12 | 421.42 ± 2.10 |
| 2s     | 18.32 ± 1.55 | 15.44 ± 1.36 | 20.37 ± 2.02 | 575.11 ± 36.26 |
| 2t     | 22.21 ± 2.43 | 17.28 ± 1.72 | 18.88 ± 1.14 | >1000 |
| VP-16  | 1.42 ± 0.11 | 2.64 ± 0.45 | 1.64 ± 0.32 | <100 |
| DEPPT  | 12.73 ± 1.19 | 9.64 ± 0.58 | 15.29 ± 0.17 | <100 |

Date are the mean of three independent experiments and recorded as the mean of standard deviation (x ± SD, n = 3)

VP-16 is Etoposide and DEPPT is Etoposide Demethylepipodophyllotoxin
Fig. 1 Effects of compound 2b and 2d on HeLa cells cycle progression (48 h). A Control; B treated with 2.5 μmol/L VP-16; C treated with 50 μmol/L VP-16; D treated with 2.5 μmol/L 2b; E treated with 50 μmol/L 2b; F treated with 50 μmol/L 2d; G treated with 50 μmol/L 2d. *P < 0.05 vs control, **P < 0.01 vs control.

|        | Control | VP-16 (2.5) | VP-16 (50) | 2b(2.5) | 2b(50) | 2d(2.5) | 2d(50) |
|--------|---------|-------------|------------|---------|--------|---------|--------|
| G0/G1  | 87.76%  | 75.92%      | 67.99%*    | 59.91%* | 48.21%*| 47.29%* | 48.33%*|
| S      | 8.99%   | 18.97%      | 27.52%*    | 30.61%* | 37.21%*| 46.18%* | 42.21%*|
| G2/M   | 1.99%   | 4.01%*      | 5.46%*     | 9.27%*  | 13.92%*| 6.57%*  | 9.33%* |

Fig. 2 Apoptosis in HeLa cells were treated with compound 2b and 2d for 48 h, as detected using flow cytometry with the annexin V/PI apoptosis detection kit. A Control; B treated with 2.5 μmol/L VP-16; C treated with 50 μmol/L VP-16; D treated with 2.5 μmol/L 2b; E treated with 50 μmol/L 2b; F treated with 50 μmol/L 2d; G treated with 50 μmol/L 2d. *P < 0.05 vs control, **P < 0.01 vs control.

|        | Control | VP-16 (2.5) | VP-16 (50) | 2b (2.5) | 2b (50) | 2d (2.5) | 2d (50) |
|--------|---------|-------------|------------|---------|--------|---------|--------|
| Apoptosis | 0.14%   | 8.42%*      | 24.33%*    | 38.83%* | 70.23%*| 37.81%* | 74.55%**|
Molecular docking assay

To prove the anticancer mechanism of the action, a molecular docking investigation was undertaken. As shown in Fig. 3, which revealed that compounds 2b and 2d were well inserted into the two active pockets of NTR 1DS7. Hydrogen bonds were shown with yellow dash lines and dark green spheres. The hydrophobic interactions which are van der Waals were represented by light green spheres, these results reveal that compounds 2b and 2d having hydrophobic property, good membrane permeability and specifically targeting NTR. The binding forms are demonstrated that the para nitro and carbonyl groups in phenyl ring were able to affect the interaction on NTR significantly. These data provide certain theoretical support for experimental results.

Conclusions

In conclusion, we have designed and synthesized a number of prodrugs for nitroreductase based 4-β-amino-4′-Demethylepipodophyllotoxin as potential anticancer agents were synthesized, and their antiproliferative activities in vitro showed compounds 2b and 2d were greatly selectively toxic to tumor cells A-549, HeLa and HepG2, respectively, and lower damage to normal WI-38 cells in comparison with positive agent Etoposide and Demethylepipodophylotoxin, and induced cell cycle arrest in the G2/M phase with a concomitant decrease in the population of G1 phase in HeLa cells, which were accompanied by apoptosis. Furthermore, Molecular docking model showed that compounds 2b and 2d appeared to form stable bonds with NTR 1DS7. Taken together, these conjugates have the potential...
to be developed as antitumor drugs. Further studies on the relevant action mechanisms and structural modification of identified hits are on-going.

**Materials and methods**

**Chemistry**

All reactions were performed with commercially available reagents and solvents without further purification. All reactions were monitored by thin-layer chromatography. $^1$H and $^{13}$C NMR spectra were recorded on Bruker AVANCE-III HD 400 MHz or NEO 500 MHz (Bruker Daltonics Inc., Germany) spectrometers using TMS as a reference. Mass spectra were recorded on a Bruker APEXI49e spectrometer (Bruker Daltonics Inc., Germany) with ESI source as ionization.

**Data for Compound 2-(3-methyl-2-nitrophenoxo)acetic acid-4β-N-(4-Nitrophenyl piperazinyl)-4'-O-demethyl-4-deoxypodophyllotoxin (2a).** $^1$H NMR (400 MHz, DMSO-$d_6$, TMS, ppm): δ: 8.06 (d, J = 9.2 Hz, 2H), 7.72 (s, 1H), 7.51 (d, J = 8.4 Hz, 1H), 7.42 (s, 1H), 7.20 (d, J = 8.4 Hz, 1H), 7.04 (d, J = 9.2 Hz, 2H), 6.99 (d, J = 8.0 Hz, 1H), 6.57 (s, 1H), 6.39 (s, 2H), 6.01 (s, 2H), 5.33 (s, 2H), 4.59 (d, J = 7.2 Hz, 1H), 4.14 (dd, J1 = 16.0 Hz, J2 = 12.0 Hz, 3H), 3.65 (s, 6H), 3.51–3.36 (m, 4H), 3.18 (dd, J1 = 12.0 Hz, J2 = 4.0 Hz, 1H), 2.63–2.51 (m, 5H), 2.37 (s, 3H), $^{13}$C NMR (400 MHz, DMSO-$d_6$, TMS, ppm): δ: 176.26, 171.0, 158.7, 153.86, 153.6, 147.3, 140.4, 139.7, 138.5, 135.1, 132.8, 131.9, 129.7, 129.4, 126.5, 125.6, 115.7, 112.6, 110.5, 110.0, 108.1, 101.7, 70.4, 69.2, 65.3, 56.8, 52.0, 50.5, 45.7, 43.7, 40.5, 19.3; MS(ESI): 783.2472 for [M + H]$^+$ (calculated 783.7597 for C$_{40}$H$_{38}$N$_4$O$_{13}$).

**Data for Compound 2-(4-methoxy-2-nitrophenoxo)acetic acid-4β-N-(4-Nitrophenyl piperazinyl)-4'-O-demethyl-4-deoxypodophyllotoxin (2d).** $^1$H NMR (400 MHz, DMSO-$d_6$, TMS, ppm): δ: 8.06 (d, J = 9.2 Hz, 2H), 7.47 (s, 1H), 7.42 (s, 1H), 7.28 (d, J = 8.0 Hz, 2H), 7.04 (d, J = 8.0 Hz, 2H), 6.57 (s, 1H), 6.38 (s, 2H), 6.01 (s, 2H), 5.25 (s, 2H), 4.59 (d, J = 4.0 Hz, 1H), 4.14 (dd, J1 = 16.0 Hz, J2 = 12.0 Hz, 3H), 3.79 (s, 3H), 3.64 (s, 6H), 3.51–3.35 (m, 4H), 3.18 (dd, J1 = 12.0 Hz, J2 = 4.0 Hz, 1H), 2.63–2.51 (m, 5H), 2.31 (s, 3H), $^{13}$C NMR (400 MHz, DMSO-$d_6$, TMS, ppm): δ: 176.26, 171.0, 158.7, 153.86, 153.6, 147.3, 140.4, 139.7, 138.5, 135.1, 132.8, 131.9, 129.7, 129.4, 126.5, 125.6, 115.7, 112.6, 110.5, 110.0, 108.1, 101.7, 70.4, 69.2, 65.3, 56.8, 52.0, 50.5, 45.7, 43.7, 40.5, 19.3; MS(ESI): 783.2472 for [M + H]$^+$ (calculated 783.7597 for C$_{40}$H$_{38}$N$_4$O$_{13}$).
Data for Compound 2-(3-fluoro-2-nitrophenoxo)acetic acid-4-β-N-(4-Nitrophenyl piperazinyl)-4′-O-demethyl-4-deoxypodophyllotoxin (2f). 1H NMR (400 MHz, DMSO-d6, TMS, ppm): δ: 8.04 (d, J = 9.2 Hz, 2H), 7.89 (dd, J1 = 8.0 Hz, J2 = 4.0 Hz, 1H), 7.66–7.62 (m, 1H), 7.41 (s, 1H), 7.36 (dd, J1 = 12.0 Hz, J2 = 4.0 Hz, 1H), 7.02 (d, J1 = 9.2 Hz, 2H), 6.55 (s, 1H), 6.37 (s, 2H), 5.99 (s, 2H), 5.32 (s, 2H), 4.57 (d, J = 4.4 Hz, 1H), 4.12 (dd, J1 = 16.0 Hz, J2 = 4.0 Hz, 1H), 3.63 (s, 6H), 3.43–3.35 (m, 4H), 3.16 (s, 6H), 3.09 (s, 6H), 3.00 (s, 6H); 13C NMR (400 MHz, DMSO-d6, TMS, ppm): δ: 176.26, 171.0, 158.7, 153.86, 153.6, 147.3, 140.4, 139.7, 138.5, 135.1, 132.8, 131.9, 129.7, 129.4, 126.5, 125.6, 115.7, 112.6, 110.5, 110.0, 108.1, 101.7, 70.4, 69.2, 65.3, 56.8, 52.0, 50.5, 45.7, 43.7, 40.5, 19.3; MS(ESI): 799.2469 for [M + H]+ (calculated 799.7582 for C40H35FN4O13).

Data for Compound 2-(4-bromo-2-nitrophenoxo)acetic acid-4-β-N-(4-Nitrophenyl piperazinyl)-4′-O-demethyl-4-deoxypodophyllotoxin (2i). 1H NMR (400 MHz, DMSO-d6, TMS, ppm): δ: 8.14 (s, 1H), 8.05 (d, J = 8.0 Hz, 2H), 7.91 (d, J = 8.0 Hz, 1H), 7.41 (s, 1H), 7.28 (d, J = 12.0 Hz, 1H), 7.03 (d, J = 12.0 Hz, 2H), 6.55 (s, 1H), 6.37 (s, 2H), 6.00 (s, 2H), 5.36 (s, 2H), 4.57 (d, J = 4.0 Hz, 1H), 4.12 (dd, J1 = 16.0 Hz, J2 = 4.0 Hz, 1H), 2.62–2.49 (m, 5H); 13C NMR (400 MHz, DMSO-d6, TMS, ppm): 6176.26, 171.0, 158.7, 153.86, 153.6, 147.3, 140.4, 139.7, 138.5, 135.1, 132.8, 131.9, 129.7, 129.4, 126.5, 125.6, 115.7, 112.6, 110.5, 110.0, 108.1, 101.7, 70.4, 69.2, 65.3, 56.8, 52.0, 50.5, 45.7, 43.7, 40.5, 19.3; MS(ESI): 847.1464 for [M + H]+ (calculated 847.1383 for C40H35BrN4O13).
Data for Compound 2-(2-nitrophenoxo)propanoic acid-4β-N-(4-Nitrophenyl piperazinyl)-4’-O-demethyl-4-deoxypodophyllotoxin (2m). 1H NMR (400 MHz, DMSO-d6, TMS, ppm): δ: 8.04 (d, J = 4.0 Hz, 2H), 7.85 (d, J = 8.0 Hz, 1H), 7.66 (t, J = 4.0 Hz, 1H), 7.38 (s, 1H), 7.24 (d, J = 8.0 Hz, 1H), 7.14 (t, J = 4.0 Hz, 1H), 7.01 (d, J = 4.0 Hz, 2H), 6.53 (s, 1H), 6.39 (s, 2H), 6.29 (s, 2H), 5.96 (s, 2H), 5.46 (dd, J1 = 12.0 Hz, J2 = 4.0 Hz, 1H), 4.54 (d, J = 4.0 Hz, 1H), 3.31 (m, 4H), 3.13 (dd, J1 = 4.0 Hz, 1H), 2.62–2.46 (m, 5H), 1.63 (d, J = 8.0 Hz, 3H); 13C NMR (400 MHz, DMSO-d6, TMS, ppm): δ: 8.06 (d, J = 8.0 Hz, 2H), 7.98 (d, J = 8.0 Hz, 1H), 7.66 (t, J = 4.0 Hz, 1H), 7.42 (s, 1H), 7.24 (d, J = 8.0 Hz, 1H), 7.18 (t, J = 4.0 Hz, 1H), 7.01 (d, J = 4.0 Hz, 2H), 6.57 (s, 1H), 6.39 (s, 2H), 6.01 (s, 2H), 4.58 (d, J = 4.0 Hz, 1H), 4.12 (dd, J1 = 8.0 Hz, J2 = 4.0 Hz, 3H), 3.64 (s, 6H), 3.51–3.34 (m, 4H), 3.18 (dd, J1 = 12.0 Hz, J2 = 4.0 Hz, 1H), 2.62–2.49 (m, 1H), 1.98 (s, 6H); 13C NMR (400 MHz, DMSO-d6, TMS, ppm): 8176.26, 717.0, 158.7, 153.86, 153.6, 147.3, 140.4, 139.7, 138.5, 135.1, 132.8, 131.9, 129.7, 129.6, 126.5, 115.7, 112.6, 110.0, 108.1, 101.7, 70.4, 69.2, 65.3, 56.8, 52.0, 50.5, 45.7, 43.7, 40.5, 19.3; MS(ESI):797.2677 for [M + H]+ (calculated 797.7865 for C41H32B4N8O13).
Data for Compound 2-(4-methyl-2-nitrop-henoxy) propanoic acid-4β-N- (4-Nitrophenyl piperazinyl) 4'-O-demethyl-4-deoxypodophyllotoxin (2f).

\[ ^1H \text{ NMR (400 MHz, DMSO-}d_6, \text{TMS, ppm)} \delta: 8.06 (d, J = 8.0 \text{ Hz, 2H}), 7.49 (s, 1H), 7.43 (s, 1H), 7.25 (d, J = 8.0 \text{ Hz, 2H}), 7.06 (d, J = 8.0 \text{ Hz, 2H}), 6.58 (s, 1H), 6.38 (s, 2H), 6.01 (s, 2H), 5.42 (dd, J1 = 12.0 \text{ Hz, J2} = 4.0 \text{ Hz, 1H}), 4.61 (d, J = 4.0 \text{ Hz, 1H}), 4.14 (dd, J1 = 16.0 \text{ Hz, J2} = 8.0 \text{ Hz, 2H}), 3.79 (s, 3H), 3.64 (s, 6H), 3.51–3.39 (m, 4H), 3.18 (dd, J1 = 12.0 \text{ Hz, J2} = 4.0 \text{ Hz, 1H}), 2.63–2.51 (m, 5H), 1.67 (d, J = 8.0 \text{ Hz, 3H}); ^{13}C \text{ NMR (400 MHz, DMSO-}d_6, \text{TMS, ppm)}: 6176.26, 171.0, 158.7, 153.86, 153.6, 140.4, 139.7, 138.5, 135.1, 132.8, 131.9, 129.7, 129.4, 126.5, 125.6, 115.7, 112.6, 110.5, 110.0, 108.1, 101.7, 70.4, 69.2, 65.3, 56.8, 52.0, 50.5, 45.7, 43.7, 40.5, 19.3; MS(ESI): 813.3543 for [M + H]^+ (calculated 813.7857 for C_{41}H_{40}N_4O_{14}).

Data for Compound 2-(4-methoxy-2-nitro-phenoxo)propanoic acid-4β-N- (4-Nitrophenyl piperazinyl)-4'-O-demethyl-4-deoxypodophyllotoxin (2t).

\[ ^1H \text{ NMR (400 MHz, DMSO-}d_6, \text{TMS, ppm)} \delta: 8.07 (d, J = 8.0 \text{ Hz, 2H}), 7.49 (s, 1H), 7.43 (s, 1H), 7.25 (d, J = 8.0 \text{ Hz, 2H}), 7.06 (d, J = 8.0 \text{ Hz, 2H}), 6.64 (s, 1H), 6.38 (s, 2H), 6.01 (s, 2H), 4.75 (dd, J1 = 12.0 \text{ Hz, J2} = 4.0 \text{ Hz, 1H}), 4.61 (d, J = 4.0 \text{ Hz, 1H}), 4.14 (dd, J1 = 16.0 \text{ Hz, J2} = 8.0 \text{ Hz, 2H}), 6.58 (s, 1H), 6.38 (s, 2H), 6.01 (s, 2H), 5.42 (dd, J1 = 12.0 \text{ Hz, J2} = 4.0 \text{ Hz, 1H}), 4.61 (d, J = 4.0 \text{ Hz, 1H}), 4.14 (dd, J1 = 16.0 \text{ Hz, J2} = 8.0 \text{ Hz, 2H}), 3.79 (s, 3H), 3.64 (s, 6H), 3.51–3.39 (m, 4H), 3.18 (dd, J1 = 12.0 \text{ Hz, J2} = 4.0 \text{ Hz, 1H}), 2.63–2.51 (m, 5H), 1.67 (d, J = 8.0 \text{ Hz, 3H}); ^{13}C \text{ NMR (400 MHz, DMSO-}d_6, \text{TMS, ppm)}: 6176.26, 171.0, 158.7, 153.86, 153.6, 140.4, 139.7, 138.5, 135.1, 132.8, 131.9, 129.7, 129.4, 126.5, 125.6, 125.6, 115.7, 112.6, 110.5, 110.0, 108.1, 101.7, 70.4, 69.2, 65.3, 56.8, 52.0, 50.5, 45.7, 43.7, 40.5, 19.3; MS(ESI): 813.3543 for [M + H]^+ (calculated 813.7857 for C_{41}H_{40}N_4O_{14}).

Cytotoxicity assay

The cytotoxicity of the derivatives in vitro was evaluated using the MTT assay. Cells were incubated at 37 °C in a 5% CO₂ atmosphere. The synthetic compounds 2a-2t and reference compound (Etoposide and Demethyllepodophyllotoxin) were dissolved and the cells were plated in 96-well plates determined according to the reported method [18].

Cell cycle and apoptotic assay

For cell cycle and apoptotic assay were determined according to the reported methods [14]. Detection of apoptosis using Annexin V-FITC/PI staining, after incubation for 24 h at 37 °C in a humidified atmosphere with 5% CO₂, various concentrations of compound 2b and 2d were added to the cultures, and the plate was incubated for further 72 h. After that, HCT-116 cells were collected, washed, resuspended in 200 µL binding buffer of the Annexin V-FITC kit, stained with propidium iodide and incubated for 30 min at 37 °C prior to flow cytometric analysis.

Molecular docking model assay

Based on the redocking process, molecular docking was applied between the chosen two compounds and NTR model. To clarify the binding forms of the chosen compounds, binding affinities were also predicted by using ligand–enzyme molecular docking calculations with different approaches.

Author contributions Z-RW: software, data curation, writing-original draft. WD: Supervision. DH: funding acquisition, writing—review. Supervision.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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