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Design and synthesis of novel 4'-demethyl-4-deoxypodophyllotoxin derivatives as potential anticancer agents

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ABSTRACT

A group of podophyllotoxin (PPT) derivatives (**7a**–**j**) were synthesized by conjugating aryloxyacetanilide moieties to the 4′-hydroxyl of 4′-demethyl-4-deoxypodophyllotoxin (DDPT), and their anticancer activity was evaluated. It was found that the most potent compound **7d** inhibited the proliferation of three cancer cell lines with sub to low micromolar IC_{50} values. Furthermore, it was demonstrated that **7d** induced cell cycle arrest in G2/M phase in MGC-803 cells, and regulated the expression of cell cycle check point proteins, such as cyclin A, cyclin B, CDK1, cdc25c, and p21. Finally, 4 mg/kg of **7d** reduced the weights and volumes of HepG2 xenografts in mice. Our findings suggest that **7d** might be a potential anticancer agent.

Podophyllotoxin (PPT, 1, Figure 1) is a non-alkaloid toxin lignin extracted from the roots and rhizomes of Podophyllum species. Since the first elucidation of its structure in the 1930s, PPT has been extensively studied as a cytotoxic agent, showing strong anticancer activity toward numerous cancer cell lines [1]. Mechanism studies unravel that PPT exerts its cytotoxicity through the inhibition of both tubulin polymerization and DNA topoisomerase-II [2, 3]. However, the systemic toxicity of PPT significantly limits its clinical application as a medicinal drug [4]. This highlights the importance of further structure modifications on PPT. Over the last 30 years, a large number of PPT derivatives have been reported, leading to the discovery of several clinically valuable anticancer drugs, such as etoposide (VP-16), etoposide phosphate and teniposide (VM-26).

Structure activity relationship (SAR) revealed that ring C and E are the two most preferable portions of PPT for structure modification while the integrity of ring A, B and D is essential for retaining anticancer activity. Therefore, most of the current derivatization on PPT focuses on C-4 and C-4′ positions. 4-Deoxypodophyllotoxin (DPT, 2, Figure 1), first isolated from the root of Anthriscus sylvestris, has received substantial interest from researchers due to its diverse biological functions including potent anticancer activity [5]. The anticancer mechanisms include inhibition of tubulin polymerization, induction of cell cycle arrest, activation of caspase-3 and caspase-7, etc [6, 7]

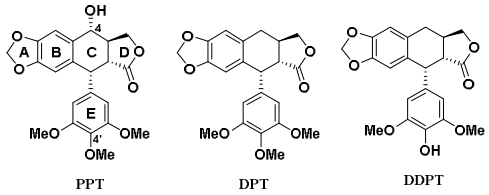

Figure 1. Chemical structures of podophyllotoxin (PPT), 4-deoxypodophyllotoxin (DPT), and 4′-demethyl-4-deoxypodophyllotoxin (DDPT).

Moreover, it was described that 4′-demethyl-4-deoxypodophyllotoxin (DDPT, **3**, Figure 1) exerted a comparable *in vitro* potency with DPT, and derivation on 4′-hydroxyl further increased *in vivo* activity. Based on this, a number of DDPT derivatives have been synthesized by coupling additional chemical moieties or drugs, such as amino acids [8], substituted piperazines [9], and 5-FU [10], to the 4′-hydroxyl of DDPT. Importantly, some of the conjugates showed superior bioactivity and reduced toxicity compared with DPT.

Inspired by the above background, herein, we present the design, synthesis, and biological evaluation of a novel series of

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DDPT 4′-derivatives, which bear an aryloxyacetanilide moiety in their structures (Figure 2). Aryloxyacetanilide represents an important skeleton in medicinal chemistry. There has been substantial evidence supporting that numerous compounds containing this moiety showed moderate to high anticancer activities *in vitro* toward various cancer cell lines [11-15], and were able to inhibit tumor growth *in vivo* [16-18]. More importantly, some of the compounds were proved to effectively inhibit microtubule assembly at both molecular and cellular levels [14]. Therefore, it is highly expected that conjugating aryloxyacetanilide moieties to the 4′-hydroxyl of DDPT would result in novel molecules with improved anticancer activity due to the synergetic effects of the two components.

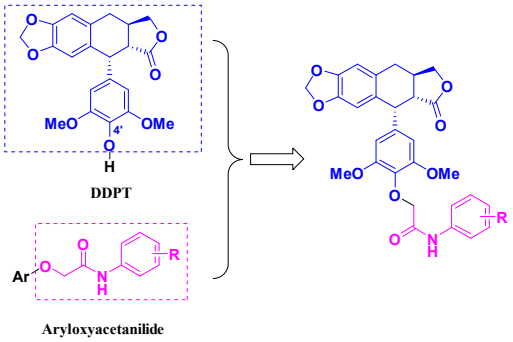

Figure 2. Design of 4′-demethyl-4-deoxypodophyllotoxin 4′-hydroxy derivatives containing an aryloxyacetanilide moiety.

The synthetic route to the target compounds is shown in Figure 3. DDPT was generated from the starting material PPT using the reported method. First, the 4-hydroxyl group of PPT was removed by reduction in the presence of sodium borohydride and trifluoroacetic acid in anhydrous THF [19]. Next, the demethylation on the C-4′ position was achieved using methylsulfonic acid and sodium iodide in anhydrous dichlormethane [20]. In order to construct the aryloxyacetanilide moiety, a series of ten phenyl amines (**4a–j**) with various substitutes on the benzene ring were employed as building blocks. The amino groups of **4a–j** were reacted with 2-chloroacetyl chloride in the presence of sodium acetate and acetic acid to introduce the chloroacetamide linker, yielding intermediates **5a–j**, respectively. Chlorine atoms in **5a–j** were then converted to iodines by refluxing in acetone with potassium iodide, giving iodoacetanilide derivatives **6a–j**. Finally, idodines atoms in **6a–j** were conjugated with the 4-hydroxy group in DDPT by refluxing in acetone using potassium carbonate as the base, resulting in final products **7a–j**. It is noteworthy that a separate step for the conversion of **5a–j** to **6a–j** by Finkelstein reaction is necessary, since direct conjugation of **5a–j** with **3** either with or without potassium iodide resulted in incomplete reaction.

The antiproliferative effects of target compounds **7a–j** were tested on three human cancer cell lines (colon carcinoma HCT-116, hepatocellular carcinoma HepG2, and gastric cancer MGC-803). Etoposide, a PPT-derived and clinically used anticancer drug was selected as positive control. The IC_{50} values for all the tested compounds are shown in Table 1. It was found that the introduction of a fluorine atom as R^1 on the aniline ring resulted in the most potent compound **7d**, which owned comparable IC_{50} value (2.20 μM) with etoposide (1.70 μM) on HCT-116 cells, and exhibited much lower IC_{50} values than etoposide on HepG2 and MGC-803 cells. Replacement of the fluorine atom in **7d** with methyl (**7b**), chloro (**7e**), or nitro (**7f**) groups led to diminished cytotoxicity, indicating that a fluoro group might be the optimal R^1. However, as for R^2, the best activity was observed when a chloro group was selected (**7h**), instead of the fluoro counterpart **7g**. The dose-response curves of **7d**, the most cytotoxic compound among this panel of molecules, on the three tested cancer cell lines are shown in Figure 4.

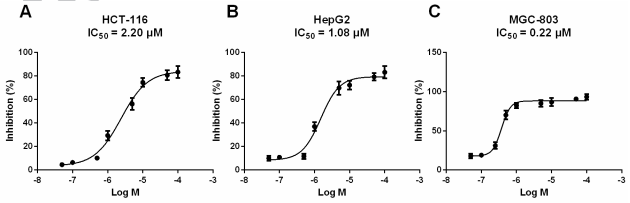

Figure 4. Dose-response curves showing the anti-proliferative effects of **7d** on (A) HCT-116, (B) HepG2, and (C) MGC-803 cell lines after an incubation of 48 h. Data are means ± SD of the inhibition (%) from three independent MTT assays.

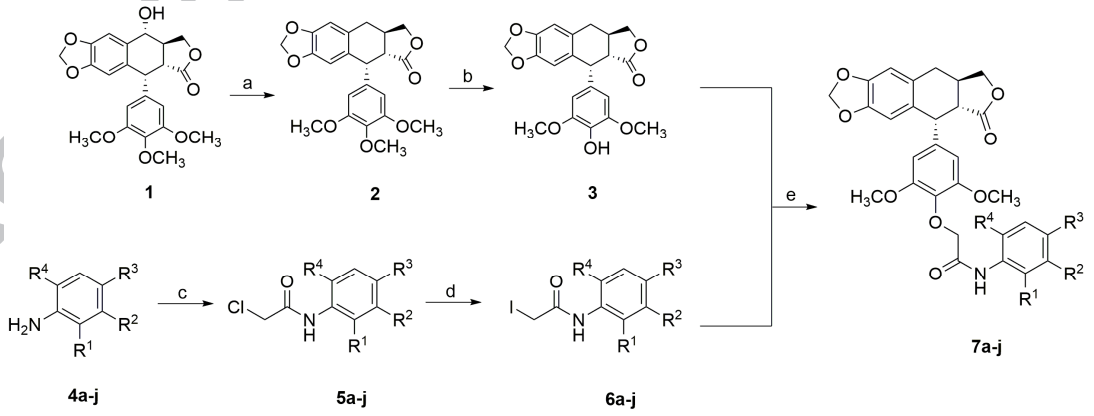

Figure 3. Synthetic route for target compounds **7a–j**. Reagents and conditions: (a) $NaBH_4$, CF_3COOH, anhydrous THF, 40 °C, 2 h. (b) NaI, CH_3SO_3H, anhydrous CH_2Cl_2, 25 °C, 4 h. (c) $ClCOCH_2Cl$, CH_3COOH, CH_3COONa, 25 °C 2 h. (d) KI, acetone, 60 °C, 2 h. (e) K_2CO_3, acetone, 60 °C, 2 h.

Table 1. Structures of **7a–j** and IC_{50} values on human cancer cell lines

Compounds	R^1	R^2	R^3	R^4	IC_{50} (μM)[a]		
					HCT-116	HepG2	MGC-803
7a	H	H	H	H	> 10	> 10	2.54 ± 0.32
7b	CH_3	H	H	H	> 10	7.80 ± 0.52	4.05 ± 0.33
7c	CH_3	H	H	Cl	> 10	> 10	6.16 ± 0.51
7d	F	H	H	H	2.20 ± 0.17	1.08 ± 0.08	0.22 ± 0.02
7e	Cl	H	H	H	> 10	> 10	> 10
7f	NO_2	H	H	H	> 10	> 10	> 10
7g	H	F	H	H	> 10	> 10	> 10
7h	H	Cl	H	H	2.68 ± 0.21	2.97 ± 0.29	1.24 ± 0.20
7i	H	NO_2	H	H	3.20 ± 0.43	> 10	2.82 ± 0.11
7j	H	H	OCH_3	H	> 10	> 10	> 10
Etoposide					1.70 ± 0.15	5.62 ± 0.61	> 10

[a]Determined using MTT assay after incubation with indicated compound for 48 h. Data are shown as mean ± SD from three separate assays.

Inhibition of tubulin polymerization has been well recognized as one of the most important mechanisms of action underlying the cytotoxicity of PPT and its derivatives. Meanwhile, all inhibition of tubulin polymerization has been implicated in G2/M cell cycle arrest in various cancer cell lines [21]. In this regard, we next investigated the effects of **7d** on cell cycle distribution in MGC-803 cells. To this end, MGC-803 cells were treated with 10, 20, and 40 μM of **7d** for 24 h, and the percentages of each cell cycle were evaluated by flow cytometry analysis. As shown in Figure 5, treatment of cancer cells with **7d** led to a concentration-dependent accumulation of cells in the G2/M phase with a concomitant decrease in the population of G1 phase cells. Cell cycle arrest in G2/M phase was initially detectable at a concentration of 10 μM (7.29%), and showed significantly difference at 20 μM (72.30%) compared with untreated control group (1.89%). These data demonstrate that **7d** induced G2/M arrest in MGC-803 cell.

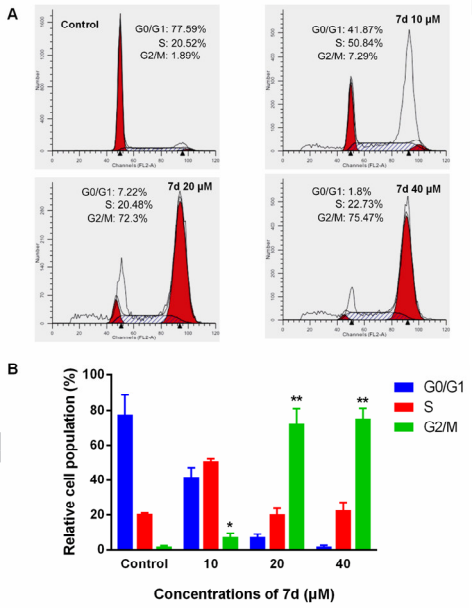

Figure 5. Effect of **7d** on cell cycle progression in MGC-803 cells. MGC-803 cells were treated with indicated concentrations (0, 10, 20, and 40 μM) of **7d** for 24 h, and the percentages of each cell cycle were analyzed by flow cytometry. Data are expressed as means ± SD from three independent experiments. *P < 0.05 vs control, **P < 0.01 vs control.

To further elucidate the roles of **7d** in cell cycle related signaling pathways, MGC-803 cells were treated with different concentrations (0, 10, 20, and 40 μM) of **7d** for 24 h, and the expression levels of cell cycle related proteins were detected using Western blot assay. The cyclin-CDK (cyclin dependent kinase) complexes play essential roles in cell cycle progression. Cyclin A associates with CDK1 from late S phase, and is replaced by cyclin B when progressing to late G2 phase [22]. Cyclin B-CDK1 is the primary complex responsible for the transition of cell cycle phase from G2 to M [23]. It was found that **7d** inhibited the expression of cyclin A, cyclin B and CDK1 in a dose-dependent manner, while the level of CDK2 was not affected (Figure 6). Furthermore, **7d** suppressed the expression of CDK activator cdc25c [24], but increased the expression of CDK inhibitor p21 [25]. Taken together, it was demonstrated that **7d** regulated the expressions of cell cycle related proteins.

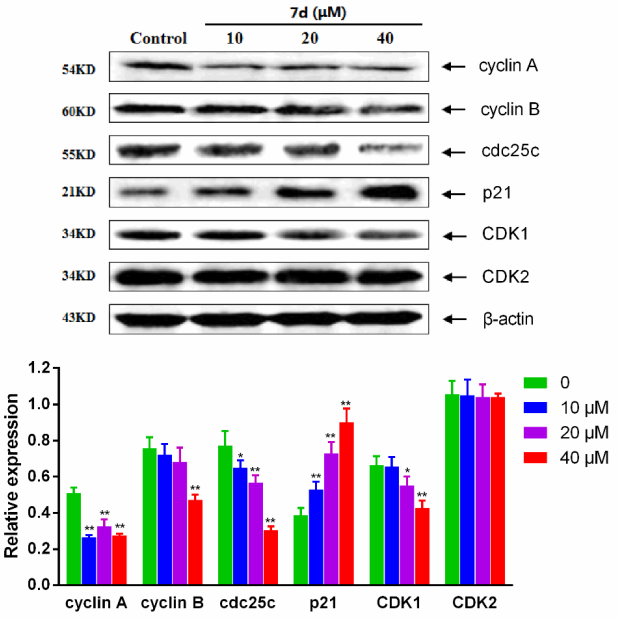

Figure 6. Effects of **7d** on the expression of cell cycle related proteins in cancer cells. MGC-803 cells were incubated with indicated concentrations of **7d** for 24 h, and the levels of the indicated protein expression were detected by a Western blot assay using specific antibodies. *P < 0.05 vs 0 μM, **P < 0.01 vs 0 μM.

Table 2. Effects of **7d** on HepG2 tumor weights and mouse body weights *in vivo*[a]

Entry	Dose	Weight (g)		Tumor weight (g)	Inhibitory ratio (%)
		Day 1	Day 14		
Control	0.4 mL/mouse	21.00 ± 0.89	30.10 ± 2.39	1.54 ± 0.22	
	1 mg/kg	20.82 ± 1.34	29.09 ± 1.56	1.45 ± 0.24	6.09
7d	2 mg/kg	20.91 ± 1.31	28.36 ± 2.80	1.13 ± 0.23[b]	26.59
	4 mg/kg	21.30 ± 0.64	27.20 ± 2.23	0.84 ± 0.24[b]	45.56

[a]Data shown are means ± SD of tumor weights and mouse body weights for each group of mice (n = 10). [b]$P < 0.01$ vs control.

Finally, the anticancer activity of **7d** was evaluated in mice. HepG2 xenograft models were established on ICR mice, which were then randomized into four groups. Three groups were injected with different doses of **7d** (1, 2, 4 mg/kg, respectively) intravenously every two days, and the fourth group was treated as vehicle control. It was found that **7d** exhibited tumor-retarding effects in a concentration-dependent way as measured by tumor weight after two weeks (Table 2). Meanwhile, 4 mg/kg of **7d** significantly reduced tumor volume as compared with control (Figure 7). In addition, treatment of **7d** showed no significant effects on mouse body weights after two weeks (Table 2), indicating the safety of **7d** *in vivo*.

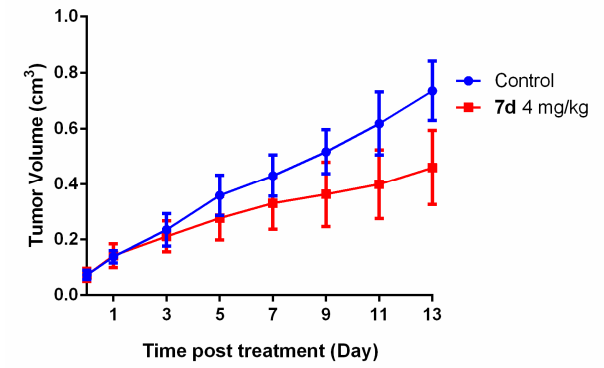

Figure 7. Inhibitory effects of **7d** on the growth of HepG2 xenografts *in vivo*. Mice inoculated with HepG2 xenografts were randomly treated with 4 mg/kg of **7d** or vehicle every two days, and the volumes of tumors were measured at the indicated time points. Data are shown as means ± SD from each group of mice (n = 10).

PPT represents an attracting lead compound for anticancer drug development. In current study, diverse aryloxyacetanilide moieties were conjugated to the 4′-hydroxy of 4′-demethyl-4-deoxypodophyllotoxin (**3**), generating a panel of novel PPT derivatives (**7a**–**j**). MTT assays led to the discovery of the most potent compound **7d**, which inhibited the proliferation of cancer cells with sub to low micromolar IC_{50} values. Further mechanism studies revealed that **7d** induced cancer cell cycle arrest in G2/M phase. Western blot assays indicated that **7d** suppressed the expression of cyclin A, cyclin B, CDK1, and cdc25c, while induced the expression of p21. Moreover, *in vivo* study demonstrated that 4 mg/kg of **7d** reduced the volumes and weights of HepG2 mice xenografts without dramatically affecting mouse body weights. In summary, our study suggested **7d** as a potential anticancer compound, and further evaluation of **7d** is on-going.

Acknowledgments

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Supplementary Material

Supplementary data (synthesis of **2**, **3**, **5a–j**, **6a–j**, **7a–j**, ^{1}H NMR and IR spectra of **7a–j**, and biological assay methods) can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.

Graphical Abstract

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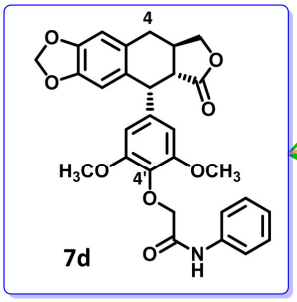

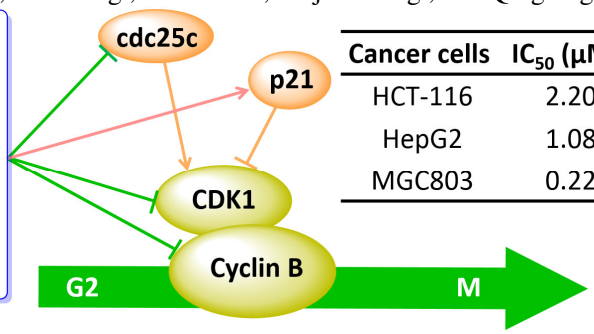

Cancer cells	IC_{50} (μM)
HCT-116	2.20
HepG2	1.08
MGC803	0.22