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Synthesis and biological evaluation of novel 4β-(1,3,4-oxadiazole-2-amino)-podophyllotoxin derivatives

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ABSTRACT

A series of new 4β-(1,3,4-oxadiazole-2-amino)-podophyllotoxin derivatives were designed and synthesized. Their cytotoxicity in vitro against six tumor cell lines (DU-145, SGC-7901, A549, SH-SY5Y, HepG2 and HeLa) were evaluated by standard MTT assay. The pharmacological results showed that most of the newly synthesized podophyllotoxin derivatives displayed potent cytotoxicity against at least one of the tested tumor cells; and among the new derivatives, **11b** was more potent than podophyllotoxin against HepG2 and Hela cell lines. Furthermore, **11b** exhibited much better selectivity toward the normal cell lines L929 and Vero than etoposide, 5-Fu and podophyllotoxin. The possible antitumor mechanism of **11b** is to inhibit the activity of DNA topoisomerase II, result in the S-phase arrest, and then cause apoptotic cell death.

Podophyllotoxin **1**, a naturally occurring cyclolignan which is the main component of podophyllum resin, shows strong cytotoxic activity against various cancer cell lines.[1] Although the therapeutic application of **1** is limited to topical use due to its high toxicity,[2] the unique cyclolignan scaffold of **1** has drawn a lot of attention for the discovery and development of new anticancer agents. Extensive structural modifications, particularly at the C4 position of podophyllotoxin have been reported in the literature.[3–5] For example, two semisynthetic derivatives of **1**, etoposide **2** and teniposide **3** (Fig. 1), are currently used in the chemotherapy for a variety of malignancies, including germ-cell malignancies, small-cell lung cancer, non-Hodgkin's lymphoma, leukemia, Kaposi's sarcoma, neuroblastoma, and soft tissue sarcoma due to their ability to inhibit the enzyme DNA topoisomerase II.[6–9] Despite of their wide clinic application, there are several limitations for these semisynthetic podophyllotoxin derivatives, which has inspired to further search for new effective antitumor agents based on this scaffold.[10]

Previous structure–activity relationship (SAR) results indicate that bulky groups are well-tolerated at C4, and the resulting derivatives based on C4 modification could significantly affect the

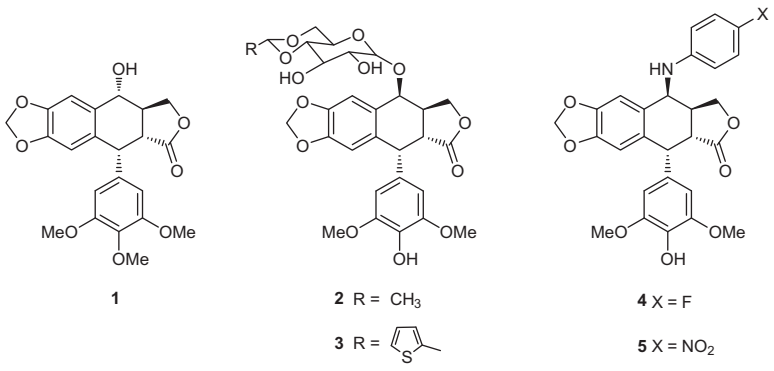

Figure 1. The structure of Podophyllotoxin **1**, Etoposide **2**, Teniposide **3**, NPF **4** and GL-331 **5**.

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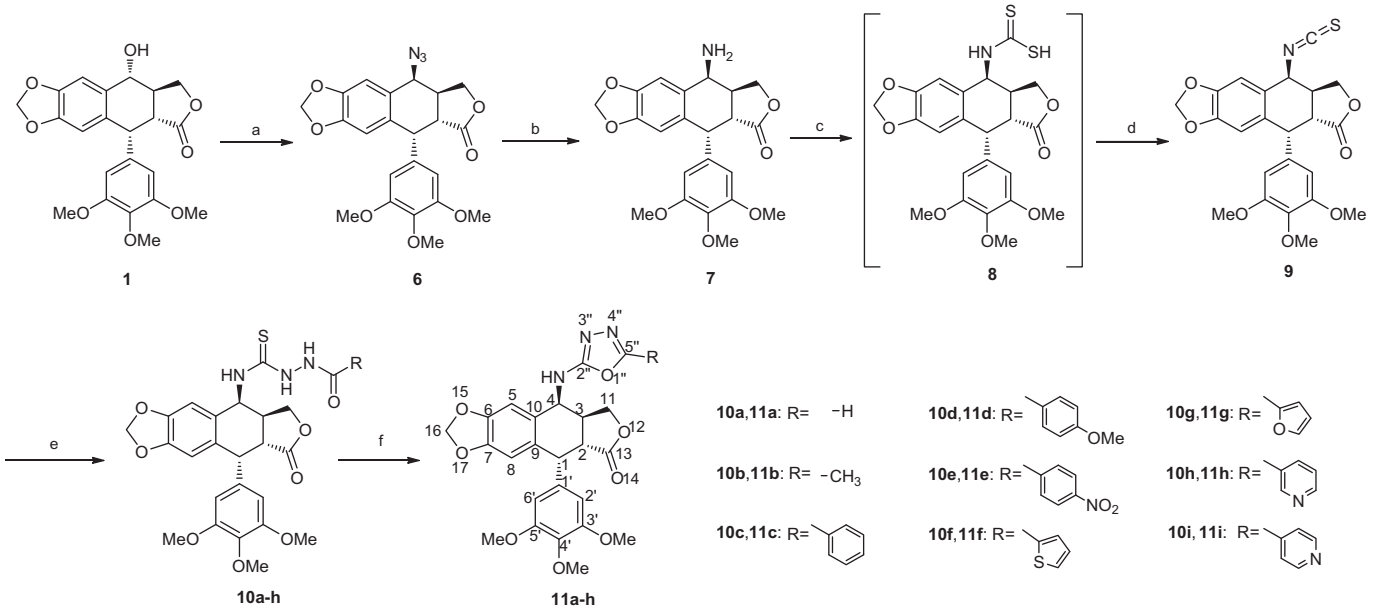


Scheme 1. Reagents and conditions: (a) NaN_3, CF_3COOH, $CHCl_3$, rt, 6 h; 90%; (b) H_2, Pd/C, MeOH, rt, 12 h; 75%; (c) Et_3N, CS_2, THF, rt, 1 h; (d) MsCl, THF, rt, 0.5 h; 81%; (e) $RCONHNH_2$, THF, rt, 2–6 h; 85%–90%; (f) pyridine, TsCl, THF, reflux,12–24 h; 64–77%.

Table 1
The cytotoxicity of compounds **11a–11i** and **12** against DU-145, SGC-7901, A549, SH-SY5Y, HepG2, HeLa, L929 and Vero cell lines

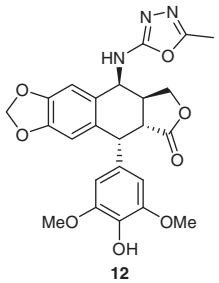
Compd	Cytotoxicity (IC_{50}, μmol L^{-1})[a]							
	DU-145	SGC-7901	A549	SH-SY5Y	HepG2	HeLa	L929	Vero
11a	11.34	>100	>100	25.81	11.85	>100	43.29	15.37
11b	>100	38.11	>100	14.66	1.51	2.68	19.62	5.49
11c	>100	>100	ND	26.88	74.20	85.34	>100	>100
11d	76.12	>100	53.66	7.39	27.95	17.66	18.15	7.43
11e	>100	>100	ND	16.58	>100	>100	>100	ND
11f	>100	56.58	>100	20.02	38.82	42.53	70.85	>100
11g	35.42	ND	69.22	29.48	38.81	58.85	>100	>100
11h	>100	>100	>100	22.47	72.77	>100	54.56	>100
11i	>100	>100	>100	41.53	96.56	>100	20.47	>100
12	60.65	9.76	>100	4.61	17.94	18.82	4.82	46.09
Podophyllotoxin	>100	2.95	13.62	2.54	2.27	7.58	1.37	1.78
Etoposide	64.26	12.09	10.12	5.37	5.48	96.26	1.00	31.14
5-Fu	12.32	14.49	8.83	1.09	32.22	15.99	1.38	>100

ND: not determined.
[a] Data are the mean of three independent experiments.

antitumor spectra of podophyllotoxin.[11] One of the major breakthroughs in this field underlines that the sugar moiety of etoposide is not essential for topoisomerase II inhibition.[7] 4β-Non-sugar-substituted, especially amino group substituted podophyllotoxin derivatives, such as NPF **4** and GL-331 **5**, have exhibited improved cytotoxicity and DNA topoisomerase II inhibition activity.[12,13] SAR studies suggest that 4β-stereochemistry and 4-N-linkage are the essential structural features for topoisomerase II inhibitory activity. Moreover, oxadiazoles are common bioisosteres for biologically active amides, esters and ureas. In particular, 2-amino-1,3,4-oxadiazoles are present in a wide range of pharmaceutical substances, such as antimicrobial agents, antitumor agents, anti-inflammatory agents and anticonvulsant agents.[14]

Our objective was to discover more potent and selective antitumor agents based on podophyllotoxin scaffold. Therefore, we have designed and synthesized a series of novel N-substituted derivatives **11a–11i**, in which 2-amino-1,3,4-oxadiazole moieties have been introduced at the C4 position of podophyllotoxin, and the new products were evaluated for their cytotoxicity against DU-145, SGC-7901, A549, SH-SY5Y, HepG2 and HeLa cell lines. urthermore, the possible mechanism of HepG2 cell growth inhibition by **11b** was also investigated in the present study.

Nine 4β-(1,3,4-oxadiazole-2-amino)-podophyllotoxin derivatives were synthesized starting from **1** through a convenient six-step procedure as described in Scheme 1. In the first step, the 4α-OH group in **1** was efficiently converted to 4β-azide (compound



Scheme 2. The structure of C-4′ demethylated derivative **12**.

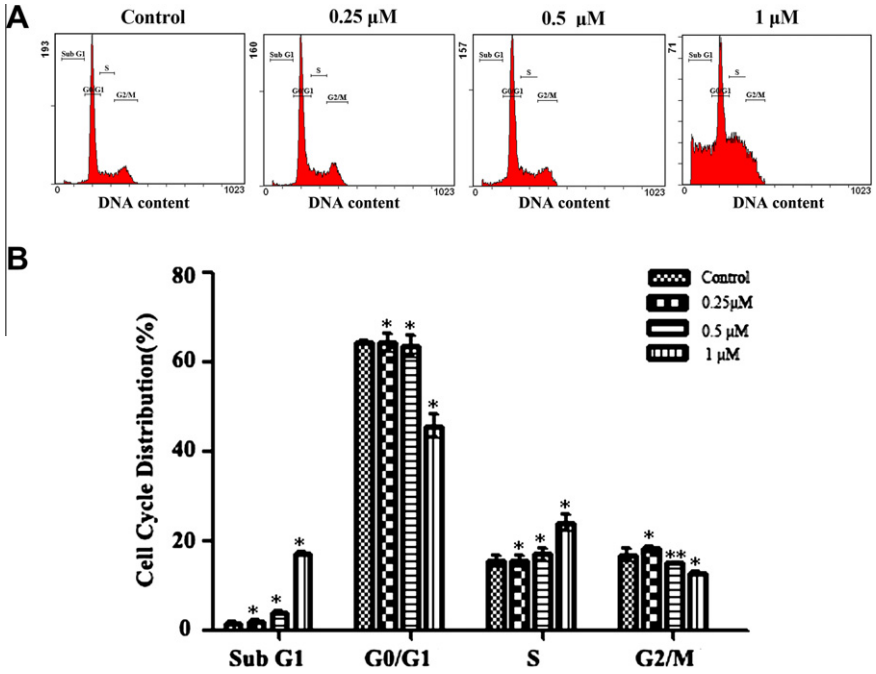


Figure 2. Effects of compound **11b** on cell cycle of HepG2 cells. Cells were treated with compound **11b** (0.25, 0.5 and 1 μM) for 48 h, and the DNA content of 10,000 events was analyzed by flow cytometry. The profiles showed the cell cycle (A) and the proportions (%) in each phase (B) of HepG2 cells treated with **11b**. $^{*}p$ <0.05, $^{**}p$ <0.01 compared to control.

6) via nucleophilic substitution with NaN_3 and TFA (CF_3CO_2H) at room temperature. The corresponding 4β-amino compound **7** was prepared by reducing the azide **6** with catalytic hydrogenosis.[15,16] A dithiocarbamate salt **8** was readily generated at room temperature in THF by slow addition of 1 equiv of CS_2 to a solution of amine **7** and 3.5 equiv of Et_3N. Then MsCl was used to facilitate the elimination of H_2S from the dithiocarbamate to afford the isothiocyanate **9**.[17] In the final phase of the synthesis, **9** was treated with various hydrazides at room temperature to generate key intermediates **10a–10i** in 85–90% yields. Upon treatment with tosyl chloride (TsCl) and Et_3N, thiosemicarbazides **10a–10i** were cyclized and produced the corresponding 4β-(1,3,4-oxadiazole-2-

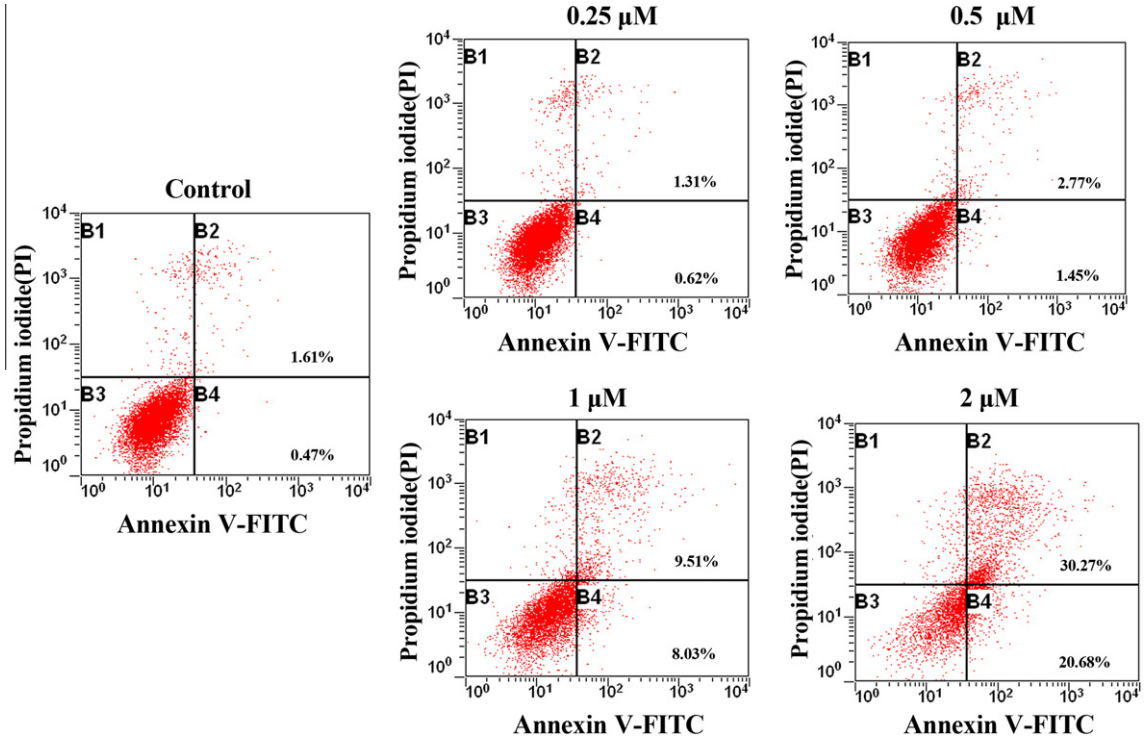


Figure 3. Effects of compound **11b** on cell apoptosis of HepG2 cells. Induction of apoptosis were measured by Annexin-V/PI double-staining assay after treatment with compound **11b** (0.25, 0.5, 1 and 2 μM) for 48 h.

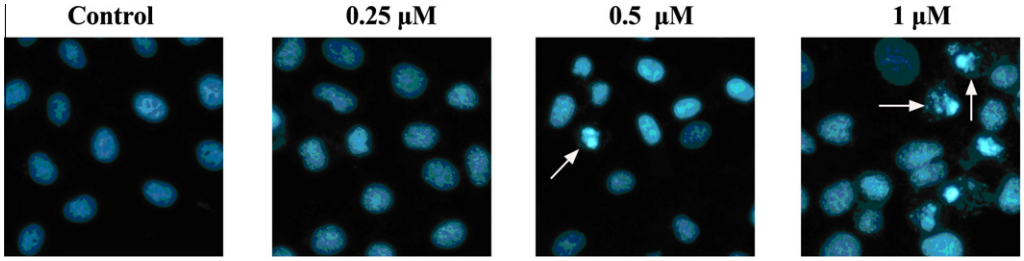


Figure 4. Effects of compound **11b** on morphological changes of HepG2 cells. HepG2 cells were incubated with various concentrations of **11b** (0.25, 0.5 and 1 μM) for 48 h and stained by Hoechst 33258 for morphology observation.

amino) podophyllotoxin derivatives **11a–11i**, respectively.[18] The structures of the intermediates and final products were confirmed by their MS, IR, ^{1}H NMR and ^{13}C NMR spectral properties (Supplementary data). Purity estimation was done with HPLC, and only those with purity around 95% were tested for biological activity.

The biological activities of the podophyllotoxin derivatives **11a–11i** were evaluated by an in vitro cytotoxicity test carried out with a panel of six human tumor cell lines (human prostate carcinoma cell line DU-145, human gastric cell line SGC-7901, human lung cancer cell line A549, human neuroblastoma cell line SH-SY5Y, human hepatoma cell line HepG2 and human cervical carcinoma cell line HeLa) and two normal cell lines (mouse fibroblast cell line L929 and Africa green monkey kidney cell line Vero) by MTT method.[19] Podophyllotoxin, etoposide and 5-fluorouracil (5-Fu) were selected as the positive controls. The results expressed as IC_{50} values are summarized in Table 1.

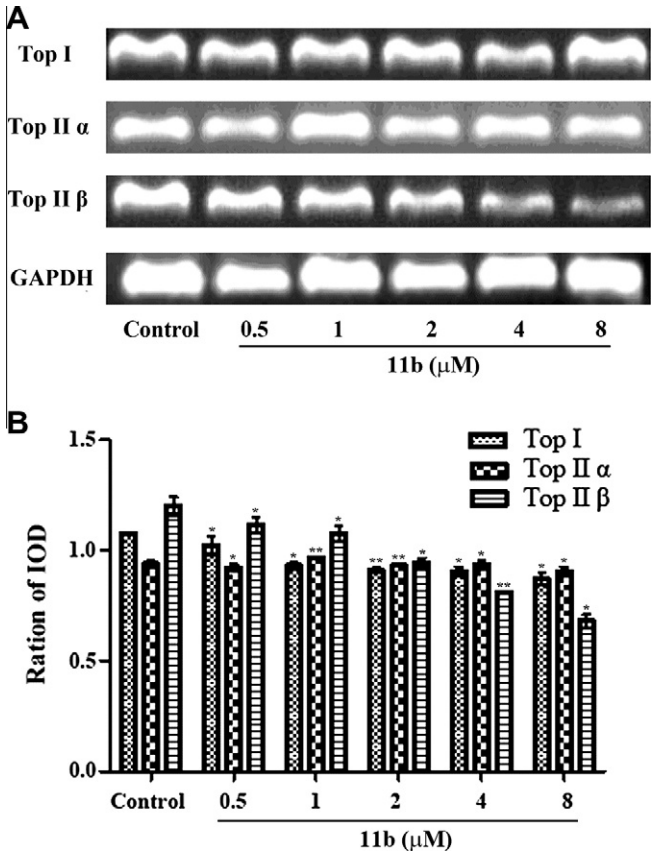


Figure 5. Effects of compound **11b** on topoisomerase I, topoisomerase IIα and topoisomerase IIβ gene expressions in HepG2 cells. HepG2 cells were treated with various concentrations of compound **11b**. Control cells were incubated with vehicle alone. (A) Topoisomerase mRNA expressions were assessed by RT-PCR in HepG2 cells exposed to compound **11b** for 6 h. (B) The amount of RNA loaded in each lane was confirmed by GAPDH mRNA. $^{*}p$ <0.05,$^{**}p$ <0.01 compared to control.

As illustrated in Table 1, all the newly synthesized podophyllotoxin derivatives (**11a–11i**) exhibited much lower cytotoxicity toward the tested normal cell lines (L929 and Vero) than Podophyllotoxin. The results in Table 1 also shown that the derivatives with alkyl groups at C5″ position (**11a** and **11b**) had relative better activity profile than those with aryl groups (e.g., **11c–11i**). Among the tested compounds, **11b** displayed the most potent cytotoxicity against SH-SY5Y, HepG2 and HeLa, with IC_{50} values of 14.66 μM, 1.29 μM and 2.68 μM, respectively. The dose-and time-dependent curve of **11b** in HepG2 hepatoma cells was further observed (data not shown). The cytotoxicity selectivity against the tumor cells and normal cells is the most important characteristic property of the new podophyllotoxin derivatives. According to the data in Table 1, **11b** has much better cytotoxicity selectivity against the tumor cells and normal cells than etoposide, 5-Fu and podophyllotoxin itself. For example, the IC_{50} values of etoposide against tumor cell HepG2 and normal cells L929 are 5.48 μM and 1.00 μM, respectively; whereas, for **11b**, the IC_{50} values for the two cell lines are 1.51 μM and 19.62 μM. The results indicate that **11b** is less cytotoxic to the normal cells.

For some known podophyllotoxin derivatives, such as etoposide **2**, teniposide **3**, NPF **4** and GL-331 **5**, the C4′ position is a free hydroxyl group, instead of a methoxy group. In order to investigate the effect of a free hydroxyl group on the antitumor activity of **11b**, a demethylated derivative **12** (Scheme 2) at C4′ position has been synthesized (the detailed experimental procedure is described in the Supplementary data). The assay data in Table 1 shown that **12** demonstrated stronger inhibitory activity against SGC-7901 and SH-SY5Y cell lines (9.76 μM and 4.61 μM, respectively), but weaker activity against HepG2 and HeLa cell lines (17.94 μM and 18.82 μM). On the other hand, **12** did not show good selectivity toward the normal cell L929 (4.82 μM). Therefore, we might conclude that for the 4β-(1,3,4-oxadiazole-2-amino)-podophyllotoxin derivatives, a free hydroxyl group at the C4′ position does not have obvious positive effects on their antitumor activity.

Compound **11b** was chose to further evaluate its antitumor activity in human hepatoma cell line (HepG2), and study was carried out to determine whether or not its antitumor mechanism is related to DNA topoisomerase, cell cycle arrest and apoptosis.

The HepG2 cells were treated with **11b** (0.25, 0.5 and 1 μM) for 48 h, and the DNA content of 10,000 events was analyzed by flow cytometry (Fig. 2). The profiles shown the cell cycle (Fig. 5A) and the proportions (%) in each phase (Fig. 5B) of HepG2 cells treated with **11b**. Flow cytometric analysis of DNA profile in the HepG2 cells shown that **11b** treatment produced a dose-dependent shift of the cell population from G0/G1 to S-phase, revealing a significant accumulation of cells in the S-phase. At the same time, Figure 2 demonstrated a dose-dependent increase of apoptosis induction which was indicated by percentage of sub-diploid DNA content.

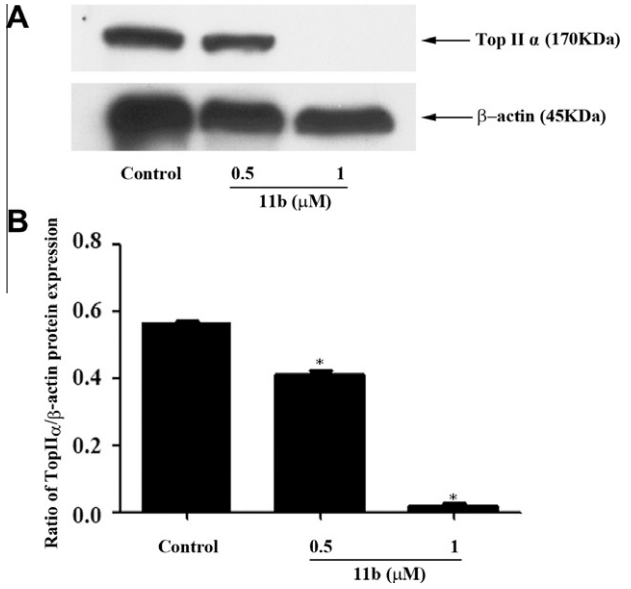


Figure 6. Effects of compound **11b** on topoisomerase IIα protein expression in HepG2 cells. HepG2 cells were treated with various concentrations of compound **11b**. Control cells were incubated with vehicle alone. (A) Topoisomerase IIα protein expression was monitored 48 h after treatment of cells with compound **11b**. (B) The relative Topoisomerase IIα protein levels were measured by scanning densitometry of the band. $^{*}p$ <0.05 compared to control.

In order to explore whether the tested compounds-mediated inhibition in cell growth is related to the induction of apoptosis, Annexin V/PI staining was used to detect the apoptotic ratio of compound **11b** treated cells. Induction of apoptosis was measured by Annexin-V/PI double-staining assay after treatment with **11b** (0.25, 0.5, 1 and 2 μM) for 48 h. As shown in Figure 3, after treatment with 2 μM of **11b** in HepG2 cells for 48 h, the early to mid-apoptotic cells (right lower section of fluorocytogram) represented 20.68% of the total cells, whereas the control had only 0.47% apoptotic cells. Meanwhile, the late apoptotic and necrotic cells (right upper section of fluorocytogram) represented 30.27% of the total cells, whereas the control had only 1.61% necrotic cells. The result confirmed the induction of apoptosis after compound **11b** treatment.

To further define the cell cycle arrest, morphology examination was also performed. HepG2 cells were incubated with various concentrations of **11b** (0.25, 0.5 and 1 μM) for 48 h, and stained by Hoechst 33258 for morphology observation. Almost all the cells in the control group were normal, however, HepG2 hepatoma cells treated with **11b** for 48 h displayed the characteristic features of apoptosis (Fig. 4); that is, the disappearance of nuclear membrane and appearance of apoptotic body.

Finally, we investigated the effects of **11b** on the DNA topoisomerase I, DNA topoisomerase IIα and DNA topoisomerase IIβ gene expression (Fig. 5). Topoisomerase I, topoisomerase IIα and topoisomerase IIβ mRNA expression were assessed by RT-PCR in cells treated with different concentrations of **11b** (0.5, 1, 2, 4 and 8 μM) for 6 h (Fig. 5A), and the relative gene expression levels were measured by scanning densitometry of the band intensities (Fig. 5B). As shown in Figure 5, **11b** significantly inhibited the mRNA expression of DNA topoisomerase IIβ. Furthermore, in order to explore whether **11b** target DNA topoisomerase IIα, we investigated the effect of **11b** on the DNA topoisomerase IIα protein expression (Fig. 6). DNA topoisomerase IIα protein expression was assessed by western blot in cells treated with different concentrations of **11b** (0.5 and 1 μM) for 48 h (Fig. 6A), and the relative protein expression levels were measured by scanning densitometry of the band intensities (Fig. 6B). As shown in Figure 6, **11b** significantly inhibited the protein expression of DNA topoisomerase IIα. The results indicated that **11b** could act as a topoisomerase II inhibitor.

In conclusion, we have designed, synthesized and evaluated for the antitumor activity of ten novel 4β-(1,3,4-oxadiazole-2-amino)-podophyllotoxin derivatives. Among these new compounds, **11b** exhibited promising antiproliferative activity in a selective HepG2 cell line. The mechanism study showed that **11b** inhibits the gene and protein expressions of DNA topoisomerase IIβ, suspends the cell cycle at S-phase, and eventually leads the tumor cells to apoptosis. Further biological evaluation is in progress to define its effects on tubulin polymerization.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05.059.

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