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Synthesis and biological evaluation of 4β-(thiazol-2-yl)amino-4′-*O*-demethyl-4-deoxypodophyllotoxins as topoisomerase-II inhibitors

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ABSTRACT

A series of 4β-(thiazol-2-yl)amino-4′-*O*-demethyl-4-deoxypodophyllotoxins were synthesized, and their cytotoxicities were evaluated against four human cancer cell lines (A549, HepG2, HeLa, and LOVO cells) and normal human diploid fibroblast line WI-38. Some of the compounds exhibited promising antitumor activity and less toxicity than the anticancer drug etoposide. Among them, compounds **15** and **17** were found to be the most potent synthetic derivatives as topo-II inhibitors, and induced DNA double-strand breaks via the p73/ATM pathway as well as the H2AX phosphorylation in A549 cells. These compounds also arrested A549 cells cycle in G2/M phase by regulating cyclinB1/cdc2(p34). Taken together, these results show that a series of compounds are potential anticancer agents.

Cancer has become one of the leading causes of death worldwide, according to information from the World Health Organization (WHO), it is estimated that there will be 12 million deaths from cancer in 2030. Among them, it is estimated that more than 1 million people die of lung cancer annually and approximately 1.4 million individuals are diagnosed per year, 12% of whom are new cases.[1] Thus, there is an unmet need for novel therapies to improve the prognosis of patients with lung cancer. Plant-derived compounds are known to have curative potential.

DNA is the most vulnerable material in the cell, and DNA damage induces a prominent route of cell death known as apoptosis.[2] In clinical treatment, other than surgery, the mainstay of cancer treatment to date has involved the use of DNA-damaging agents in the form of radiation and systemic chemotherapy. Radiation is responsible for approximately 40% of all cures achieved in cancer patients.[3] Commonly used DNA-damage-inducing chemotherapies include platinum salts (carboplatin, cisplatin, and oxaliplatin) that generate covalent cross-links between DNA bases,[4] and topoisomerase-II (topo-II) inhibitors (etoposide and doxorubicin) that generate topo–DNA adducts and DNA strand breaks.[5] Topo-I inhibitors induce DNA single-strand breaks, while topo-II inhibitors induce DNA double-strand breaks (DSBs). H2AX, an evolutionarily conserved variant of histone H2A, is a key histone that undergoes various posttranslational modifications in response to DSBs.[6] By virtue of phosphorylation, H2AX marks the damaged DNA double helix to facilitate local recruitment and retention of DNA repair and chromatin remodeling factors and thus restore genomic integrity.

Podophyllotoxin (PPT, **1**), derived from the roots and rhizomes of *Podophyllum* species, has cathartic, antirheumatic, and antiviral properties, and pesticidal and antimitotic activity.[7] Etoposide (VP-16, **2**) and teniposide (VM-26, **3**, Fig. 1) are semisynthetic glucosidic cyclic acetals of PPT currently used in chemotherapy for various types of cancer, including small-cell lung cancer, testicular carcinoma, lymphoma, and Kaposi's sarcoma.[8] Both of these compounds block the catalytic activity of DNA topo-II by stabilizing a cleavable enzyme–DNA-complex in which the DNA is cleaved and covalently linked to enzyme.[9] Although they are widely used in the clinic, several problems hinder their clinical efficacy such as drug resistance and poor water solubility. Therefore, there remains a need for new PPT derivatives with anticancer activity and improved water solubility. Extensive efforts have been made researchers to address these limitations.[10] Structure–activity relationship (SAR) experiments have unambiguously demonstrated that C4 is the major molecular site tolerant to significant structural diversification.[11] Furthermore, the comparative molecular field analysis ('CoMFA') models generated by Lee and coworkers

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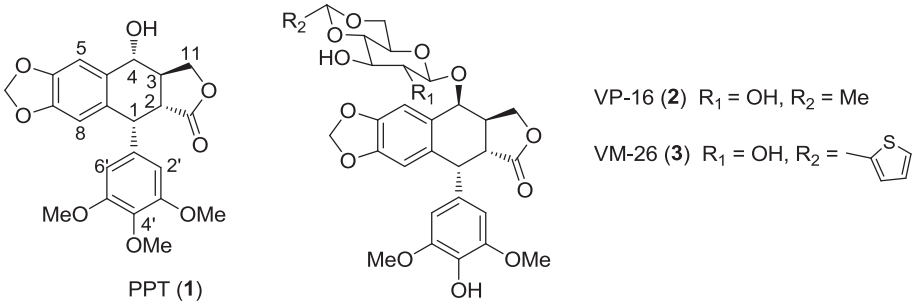


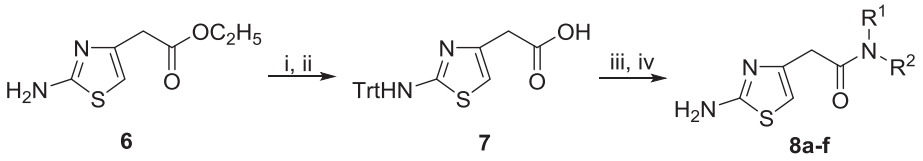
Fig. 1. The structures of podophyllotoxin (**1**), etoposide (**2**), and teniposide (**3**).

demonstrated that bulky substituent at C4 of PPT might favor DNA topo-II inhibition.[12]

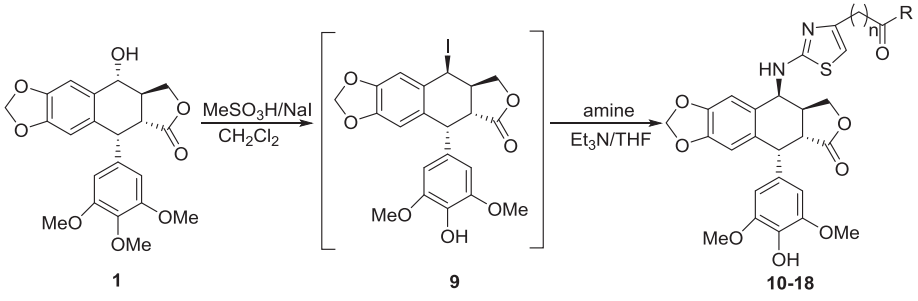
Recently, kinds of 4β-*N*-substituted 4′-*O*-demethyl-4-deoxypodophyllotoxins were generated and have been shown to exhibit more potent anticancer activity and better binding ability to DNA topo-II compared with etoposide.[13–16] As part of our ongoing efforts to develop new podophyllotoxin derivatives with potent biological activities,[17–23] herein we report the synthesis and cytotoxicities of a series of 4β-(thiazol-2-yl)amino-4′-*O*-demethyl-4-deoxypodophyllotoxins. Compound **15** and **17** were further evaluated for its effect on topo-II enzymes, H2AX phosphorylation as well as cell cycle progression.

The intermediates 2-(2-aminothiazol-4-yl)acetic carbamate **8a–f** were synthesized from ethyl 2-(2-aminothiazol-4-yl)acetate (compound **6**) using previously published methods[24] as outlined in Scheme 1. Briefly, the amino group of compound **6** was protected with trityl chloride (TrtCl) and then saponified to produce compound **7**, followed by reaction with different amines under 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDCI) in the presence of *N*-hydroxybenzotriazole (HOBT) in dichloromethane. Last, the protecting group was removed in the presence of acetic acid to provide the intermediates **8a–f**.

The target compounds **10–18** were synthesized from PPT according to previously published methods (Scheme 2).[22] Briefly,



Scheme 1. Synthesis of compounds **8a–f**. Reagents and conditions: (i) TrtCl, Et_3N, r.t (ii) NaOH, MeOH, reflux; (iii) amines, EDCI, HOBT, CH_2Cl_2, r.t; (iv) CH_3COOH, 60 °C.



Compounds	n	R	Compounds	n	R
10	1	HN-cyclopropyl	**11**	1	HN-cyclopentyl
12	1	HN-cyclohexyl	**13**	1	morpholin-4-yl
14	1	4-methylpiperazin-1-yl	**15**	1	4-cyclopentylpiperazin-1-yl
16	1	OCH_2CH_3	**17**	0	OCH_2CH_3
18	1	OH			

Scheme 2. Synthesis of target compounds **10–18**.

methanesulfonic acid/sodium iodide was used for both 4′-*O*-demethylation and C-4 epimerization of the podophyllotoxin ring system to provide the required key iodo intermediate (compound **9**). Compound **9** then underwent further one-pot reactions with the appropriate amine **8a**–**f** in the presence of triethylamine in refluxing tetrahydrofuran (THF) to produce the corresponding compounds **10**–**15**. Furthermore, to study the effects of carbamate substituted with different esters of 2-aminothiazol, we also prepared 4′-demethy-4-epipodophyllotoxin (DMEP) derivatives of ethyl 2-aminothiazol-4-yl esters **16** and **17**, as well as of 2-aminothiazol-4-yl acid **18**.

The structure of all target compounds was confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS (Supporting Information). The assignment of the configuration at C-4 position for compounds **10**–**18** was based on coupling constants $J_{3,4}$. The C-4β substituted compounds have $J_{3,4} \approx 4.0$ Hz due to a *cis* relationship between H-3 and H-4 (*eg*: $J_{3,4} = 4.2$ Hz for compound **10**), the C-4α substituted compounds, however, have $J_{3,4} \geq 10.0$ Hz because of H-3 is *trans* to H-4.[25,26] The relative stereochemistry at C-4 position was also demonstrated by the observation of 1D NOE between H-3 and H-4. While H-3 was irradiated in ^{1}H NMR spectrum for compound **10**–**18**, the H-4 was enhanced 3–5% (*eg*: 3.9% for compound **10**), however, H-2 was not enhanced. Thus we ensured that H-4 is *cis* to H-3 and H-2 is *trans* to H-3.

The in vitro anti-proliferative activities of compounds **10**–**18** were evaluated against a panel of four human cancer cell lines (lung carcinoma A549, hepatocellular carcinoma HepG2, cervical carcinoma HeLa, colon adenocarcinoma LOVO) together with the human lung fibroblast cell line WI-38, etoposide was used as a positive reference compound. The screening procedure was based on

Table 1
The in vitro anti-proliferative activities (IC_{50}, μM)[a,b] of compounds **10**–**18**.

Compds	Tumor cell				Normal cell
	A549	HepG2	HeLa	LOVO	WI-38
10	11.1 ± 0.7	10.2 ± 0.6	17.2 ± 1.0	19.1 ± 1.2	56.0 ± 2.4
11	9.1 ± 0.9	19.1 ± 1.2	14.1 ± 0.7	21.0 ± 0.8	29.0 ± 1.2
12	9.4 ± 0.5	16.3 ± 0.9	10.7 ± 0.9	19.1 ± 0.8	77.8 ± 4.6
13	18.7 ± 0.8	15.1 ± 0.8	14.8 ± 1.1	37.2 ± 1.3	33.5 ± 3.9
14	22.3 ± 1.3	18.7 ± 1.2	16.5 ± 1.0	27.4 ± 1.3	28.1 ± 2.2
15	1.3 ± 0. 9	8.5 ± 0.9	4.4 ± 0.9	9.2 ± 0.5	66.7 ± 1.2
16	9.3 ± 0.7	0.12 ± 0.03	12.5 ± 0.7	7.0 ± 0.9	44.4 ± 1.4
17	0.16 ± 0.06	0.13 ± 0.05	5.6 ± 0.7	5.0 ± 0.5	55.3 ± 2.5
18	8.3 ± 1.1	9.3 ± 0.9	8.7 ± 0.7	10.3 ± 1.0	42.5 ± 4.0
VP-16	9.1 ± 0.7	9.2 ± 0.9	11.2 ± 1.0	18.7 ± 0.9	25.7 ± 1.9

[a] Data are the mean of three independent experiments.
[b] MTT method.

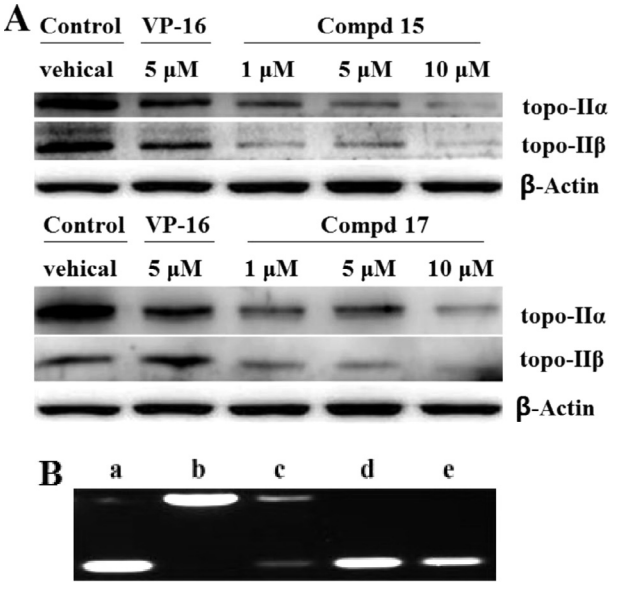


Fig. 2. The effect of compounds **15** and **17** on topo-II. (A) The effect of compounds **15** and **17** on topo-IIα and topo-IIβ protein expression in A549 cells. (B) Effect of compounds **15** and **17** on topo-IIα mediated DNA relaxation. Lane a, supercoiled pBR322 DNA. Lane b, pBR322 DNA + topo-IIα; lane c, pBR322 DNA + topo-IIα + 100 μM etoposide; lane d, pBR322 DNA + topo-IIα + 100 μM compound **17**; lane e, pBR322 DNA + topo-IIα + 100 μM compound **15**.

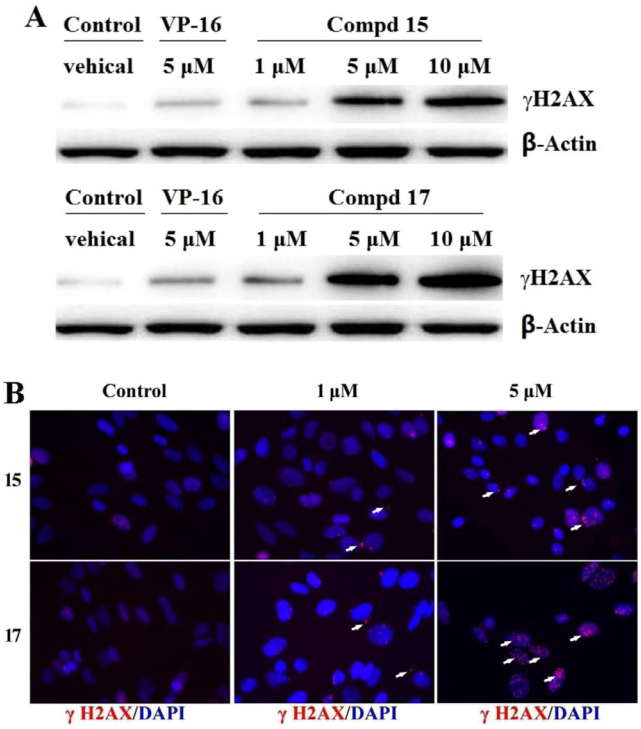


Fig. 3. Effects of **15** and **17** on H2AX phosphorylation. (A) Western blotting analysis of H2AX phosphorylation in A549 cells treated with compound **15** and **17** (vehicle, 1 μM, 5 μM and 10 μM) for 12 h; (B) A549 cells were immunostained with anti-γH2AX antibodies and counterstained with DAPI after treatment with compound **15** and **17** (vehical, 1 μM and 5 μM) for 12 h.

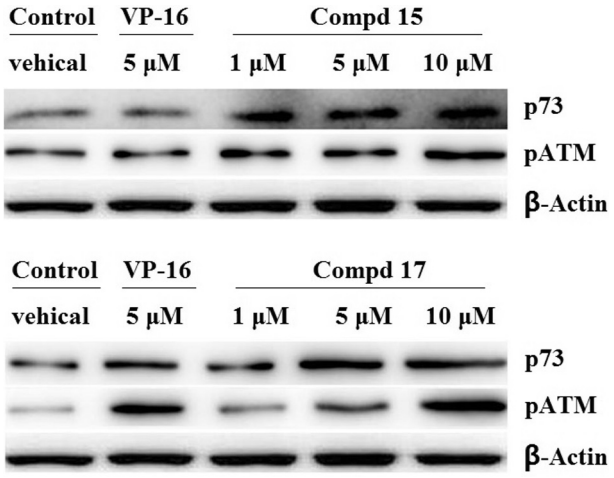


Fig. 4. Western blotting analysis p73 and pATM levels in A549 cells treated with vehical, VP16 (5 μM), **15** (1 μM, 5 μM and 10 μM) and **17** (1 μM, 5 μM and 10 μM) for 12 h.

the standard MTT method,[23] and the results are summarized in Table 1.

The compounds **10**–**18** generally showed greater or equal antiproliferation toward these four cancer cell lines and less toxicity to the normal human lung fibroblast cell WI-38 compared with VP-16 (Table 1). Based on these results, it was possible to deduce some preliminary SARs. First, the large range of activities observed for compounds **10**–**18** indicated that the substituent at C-4 of the 2-aminothiazol markedly affects the activity profile of this compound class, with 2-aminothiazol-4-yl esters appearing to be more potent compared with those of amides (compounds **16**, **17** *vs* compounds **10**–**15**). Second, the 2-(2-aminothiazol-4-yl)acetate substituted derivative of DMEP appear to be less potent than the 2-aminothiazole-4-carboxylate derivative (compound **16** *vs* compound **17**). Last, the 2-(2-aminothiazol-4-yl)acetic acid substituted compound **18** also showed moderate potent antiproliferation. These data suggested that compounds **15** and **17** might serve as potential anticancer agents. We decided to investigate the mechanism responsible for the cytotoxic effects of these compounds in A549 cells.

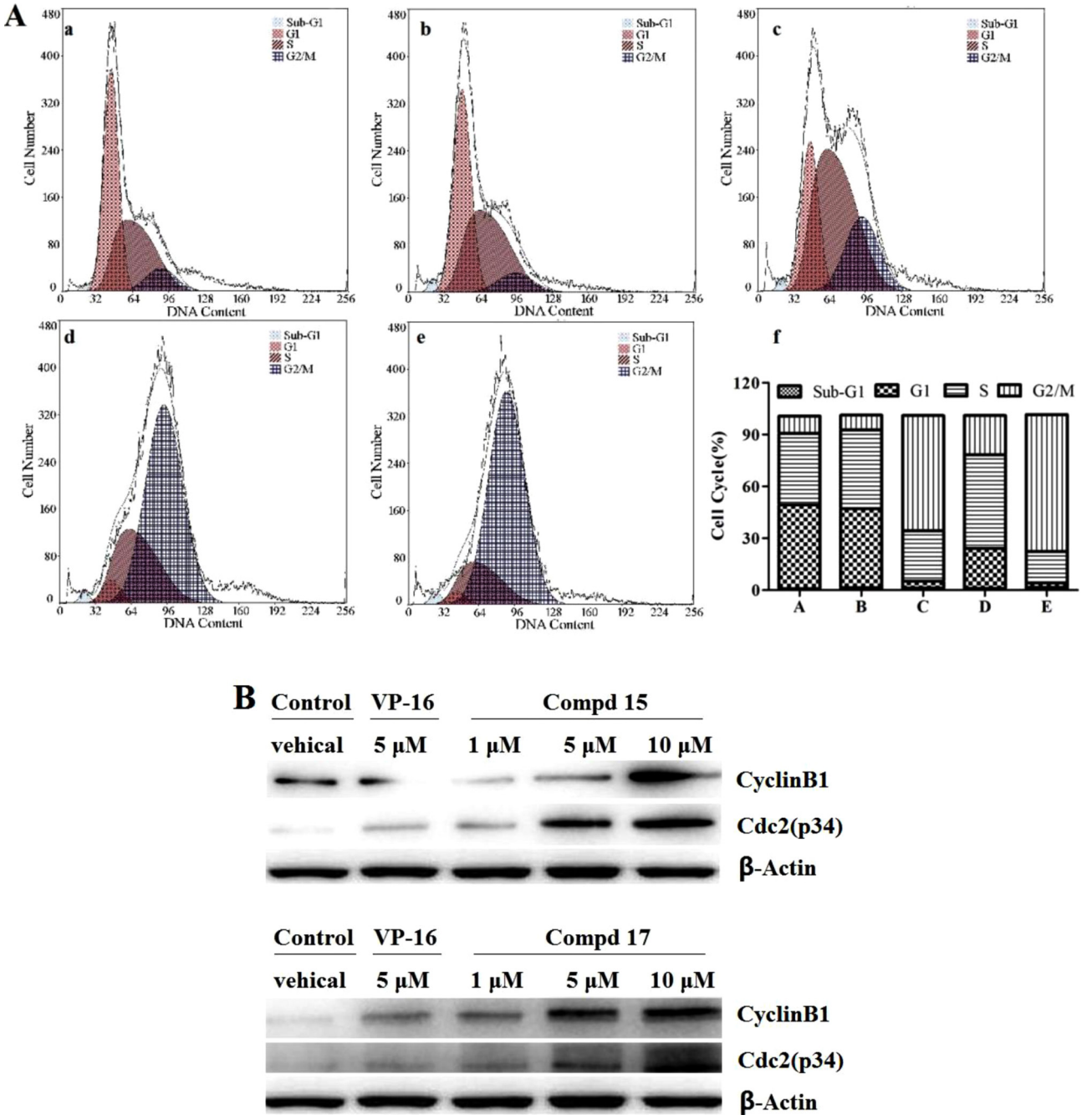


Fig. 5. Effects of **15** and **17** on cell cycle progression. A) flow cytometric analysis. a) A549 cells treated with vehical; b) A549 cells treated with 1 μM compound **15** for 12 h; c) A549 cells treated with 5 μM compound **15** for 12 h; d) A549 cells treated with 1 μM compound **17** for 12 h; e) A549 cells treated with 5 μM compound **17** for 12 h; f) Bar graphs showing compound **15** and **17** on cell cycle progression. B) Western blotting analysis G2/M cell-cycle regulators expression of cyclin B1, cdc2(p34) treated with compounds **15** and **17** various concentration in A549 cells for 12 h.

Etoposide is well-known drug that causes double-strand DNA breaks and is a topo-II inhibitor.[7,8] As these 4β-(thiazol-2-yl) amino-4′-*O*-demethyl- 4-deoxypodophyllotoxins are closely related molecules to etoposide, it was considered of interest to examine the effects of topo-II and DSBs. Thus A549 cells were treated with etoposide (5 μM), **15** and **17** at 1, 5 and 10 μM for 12 h.[23] Interestingly both of compounds **15** and **17** decreased the expression of either topo-IIα or topo-IIβ in A549 cells in a dose-dependent manner (Fig. 2A). Furthermore, compounds **15** and **17** induced supercoiled pBR322 DNA relaxation to form linear DNA (Fig. 2B). This was strongly supported by the fact that these derivatives of podophyllotoxin can act as topo-II inhibitor, and the percentage of cells with DSBs were greater than those of etoposide (at the same molarity).

It has been well documented that phosphorylation at Ser-139 on H2AX protein (i.e., γ-H2AX) is a direct indicator of double-strand DNA breaks in cells.[27,28] To further gain insight into the mechanisms of action of compounds **15** and **17**, phosphorylations of H2AX were analyzed by western blotting in A549 cells treated with etoposide (5 μM) and compounds **15** and **17** (1, 5, or 10 μM) for 12 h. As shown in Fig. 3A, increased γH2AX was observed in cells treated with both of compounds **15** and **17** compared with control, and this effect was in a dose dependent manner. Furthermore, formation of γH2AX nuclear foci (a marker of DNA DSBs, arrows) was observed by immunofluorescence in A549 cells treated with compounds **15** and **17** for 12 h (Fig. 3B). Collectively, these results reveal that compounds **15** and **17** induced H2AX phosphorylation as characteristic of DSBs.

Previous studies have showed that p73 plays a central role in DSBs checkpoints,[29] and ataxia-telangiectasia mutated (ATM) is the main physiological mediator of H2AX phosphorylation in response to DSBs.[30] Next we evaluated the effects of compounds **15** and **17** on induce p73 up-regulation and phosphorylation of ATM in A549 cells. As shown in Fig. 4, p73 and pATM levels increased in a dose-dependent manner after treatment with compounds **15** and **17** (1, 5, or 10 μM) compared with control, meanwhile the effects on p73 and pATM were stronger than those of etoposide (at the same molarity). These results indicated that p73 and ATM is essential for inducing DSBs and suggested a central role for the p73/ATM/γH2AX pathway in mediating the activity of these compounds.

Podophyllum derivatives including VP-16 inhibit cancer cells though inducing G2/M cell cycle arrest.[31] Thus, we investigated the effect of compounds **15** and **17** on cell cycle progression by means of fluorescence-activated cell sorting analysis of A549 cells stained with propidium iodide. Treatment with compounds **15** and **17** led to a dose-dependent accumulation of cells in the G2/M phase along with a concomitant decrease in the population of G1 phase cells (Fig. 5A). During normal cell cycle, G2/M progression is triggered by activation of the cyclin B1-cdc2 kinase.[32] Therefore, we also investigated the levels of cdc2(p34) and cyclin B1. A549 cells were exposed to compound **15** or **17** (1 μM, 5 μM and 10 μM) for 12 h and evaluated by western blotting. As shown in Fig. 5B, there was a marked turn in cyclin B1 and cdc2(p34) protein levels as compared with the control. These results showed that compounds **15** and **17** induce G2/M cell cycle arrest via cyclin B1 and cdc2(p34) in a dose-dependent manner.

In summary, a series of novel 4β-(thiazol-2-yl)amino-4-*O*-demethyl-4-deoxypodophyllotoxins show promising in vitro cytotoxicity and less toxicity. These compounds appear to induce DSBs by blocking the activity of DNA topo-II, and by activating phosphorylation of H2AX in a p73 and ATM dependent manner. Furthermore, this series of derivatives induce G2/M cell cycle arrest via the cycle regulators cyclin B1 and cdc2(p34). These results suggest that compounds **15** and **17** have potential for further development as anticancer agents.

Acknowledgements

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.12.012.

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