Bioorganic & Medicinal Chemistry 22 (2014) 6183–6192

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

SAR analysis and biological studies of synthesized podophyllum derivates obtained by N linkage modification at C-4 position

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ARTICLE INFO

Article history:
Received 3 June 2014
Revised 21 August 2014
Accepted 22 August 2014
Available online 7 September 2014

Keywords:
Podophyllum derivates
N linkage
SAR
Microtubule
Topoisomerase II

ABSTRACT

A series of C4-N-substituted podophyllum derivatives were synthesized and tested for cytotoxicity in HeLa, BGC-823, A549, Huh7 and MCF-7 cells by MTT assay. Pharmacologically, most derivatives displayed potent cytotoxicity against at least one of the tested tumor cell lines. Structure activity relationship (SAR) analysis suggests that compounds with imidogen exposed on the pyridine, rather than pyrimidine, exhibited significantly elevated potency. Moreover, the presence of a chlorine atom in the heterocyclic ring enhanced cytotoxicity, with the order 3-position > 4-position > 5-position > 6-position. Specifically, two compounds, **3g** and **3h**, with 2-amino-3-chloropyridine substituted into the podophyllotoxin (PPT) and 4′-O-demethylepipodophyllotoxin (DMEP) scaffolds were shown to have the most potent HeLa cells cytotoxicity compared to other synthesized derivatives or reference compounds PPT, DMEP and etoposide (VP-16). The compound **3g** was shown to inhibit microtubule polymerization and compound **3h** affected topoisomerase II catalytic activity. Both compounds resulted in G_2/M phase arrest and apoptosis, purportedly by increasing the expression of P53, followed by Bax up-regulation, Bcl-2 down-regulation, and caspase-3 activation. As a result of this work, we conclude that compounds **3g** and **3h** are more potent anticancer agents than VP-16, and that they work by different antitumor mechanisms.

1. Introduction

Podophyllum peltatum L., also known as the American Mayapple, is the source of podophyllum compounds, including podophyllotoxin (PPT, **a**) (Fig. 1a) and 4′-O-demethylepipodophyllotoxin (DMEP, **b**) (Fig. 1b).[1–4] Studies show that structural modification of the podophyllum scaffold results in essential changes to the anti-microtubule activity of PPT and the anti-topoisomerase II activity of structurally modified DMEP.[5,6] Anti-microtubule agents promote cell death by interfering with the function of the mitotic spindle and induce cell apoptosis by promoting mitotic arrest, which leads to arrest of the cell cycle in metaphase.[7,8] Topoisomerase II inhibitors interfere with DNA-topoisomerase II by stabilizing the covalent DNA-topoisomerase II cleavable complex, which eventually leads to cell death due to the generation of single and double stranded DNA breaks.[9]

To date, hundreds of derivatives have been designed and synthesized using podophyllum compounds, such as etoposide (VP-16) and teniposide (VM-26) (Fig. 1c), both of which are more potent antitumor agents than DMEP.[10,11] In fact, VP-16 and VM-26 were officially approved in 1983 and 1992, respectively, for clinical use against various types of cancers, including: breast cancer, testicular cancer, small-cell lung cancer, lymphoma, Kaposi's sarcoma and childhood leukemin.[12,13] Studies have shown that the C-4 position of podophyllum analogs is a variable region, according to the composite pharmacophore model proposed by MacDonald et al.[14] However, their clinical use has been limited by unintended side effects, such as myelosuppression, neutropenia, and nausea, as well as by drug resistance and poor bioavailability. These limitations have inspired further search for new, effective antitumor agents based on the scaffolds of PPT and DMEP.[15–17] Structural modifications of the C-4 position on these compounds can be achieved through O-, S- or N-linkage. In general, the O-linked (ethers, esters) and S-linked (thioethers) compounds are less active compared to the N-linked congeners.[18–20] Recently, new synthetic N-linked congeners, such as GL-331 and NPF (Fig. 1d), were shown to exhibit improved cytotoxicity and topoisomerase II inhibitory activity.[21–25] GL-331 is currently in Phase II clinical trials for gastric carcinoma, colon cancer, non-small cell carcinoma, and etoposide resistant malignancies.[26,27] NPF, a promising new antitumor agent was found to be a 10-fold more potent inhibitor of topoisomerase II and 100-fold more cytotoxic against

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http://dx.doi.org/10.1016/j.bmc.2014.08.025
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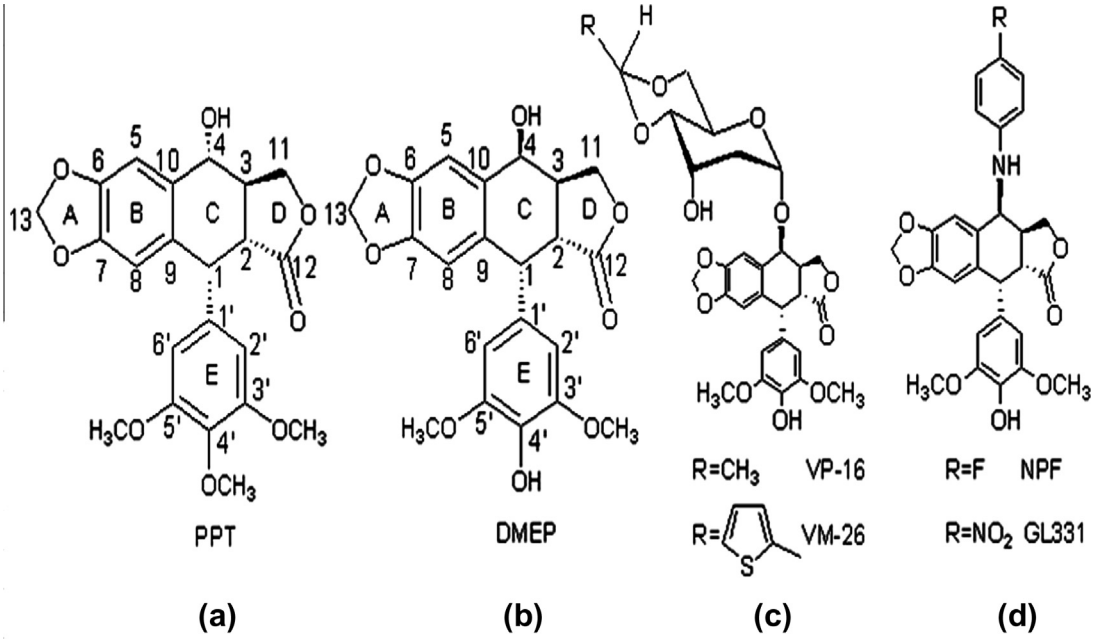


Figure 1. Structures of PPT, DMEP and related compounds.

various human tumor cells than VP-16,[28,29] but the current status of this agent is unclear.

In the past several years, multiple C4-N-substituted podophyllum derivatives were synthesized and explored,[30,31] but C4-N-amidopodophyllotoxin derivatives have received less attention. The main objective of the current work was to discover potent and selective antitumor agents using N linkage podophyllum derivatives based on the scaffolds of PPT and DMEP. The compounds 2-aminopyrimidine and 2-aminopyridine are broadly used as intermediates during derivative synthesis. In the current study, we hypothesized that these two compounds could generate more potent derivatives when substituted in the C-4 position by N linkage. Thus, we aimed to successfully synthesize four C4-N-arylamino substituted podophyllum derivatives. The resulting derivatives were measured for their cytotoxicity against the HeLa, BGC-823, A549, Huh7, and MCF-7 cell lines. The resulting data were analyzed by SAR, and the outcomes suggested that derivatives made using 2-aminopyrimidine or 2-aminopyridine precursors had improved activity. Hence we designed and synthesized a series of podophyllum derivatives and introduced a chlorine atom into various positions of the N-heterocycle, as this is purported to direct the molecular recognition process effectively and reliably in terms of overall protein–ligand binding affinity.[32] SAR analysis was once again performed to facilitate the selection of synthesized derivatives for further biological evaluation of potency to induce cell cycle arrest and apoptosis; activate apoptosis-related proteins, such as P53, members of the Bcl-2 family and caspase-3; inhibit microtubule polymerization and topoisomerase II activity. The information obtained this way maybe useful in the future for similar structural optimization as well as in easier understanding of mechanisms of action of podophyllum derivatives. This work could probably provide an essential framework of discovery and development of effective antitumor agents from natural parent compounds.

2. Results and discussion

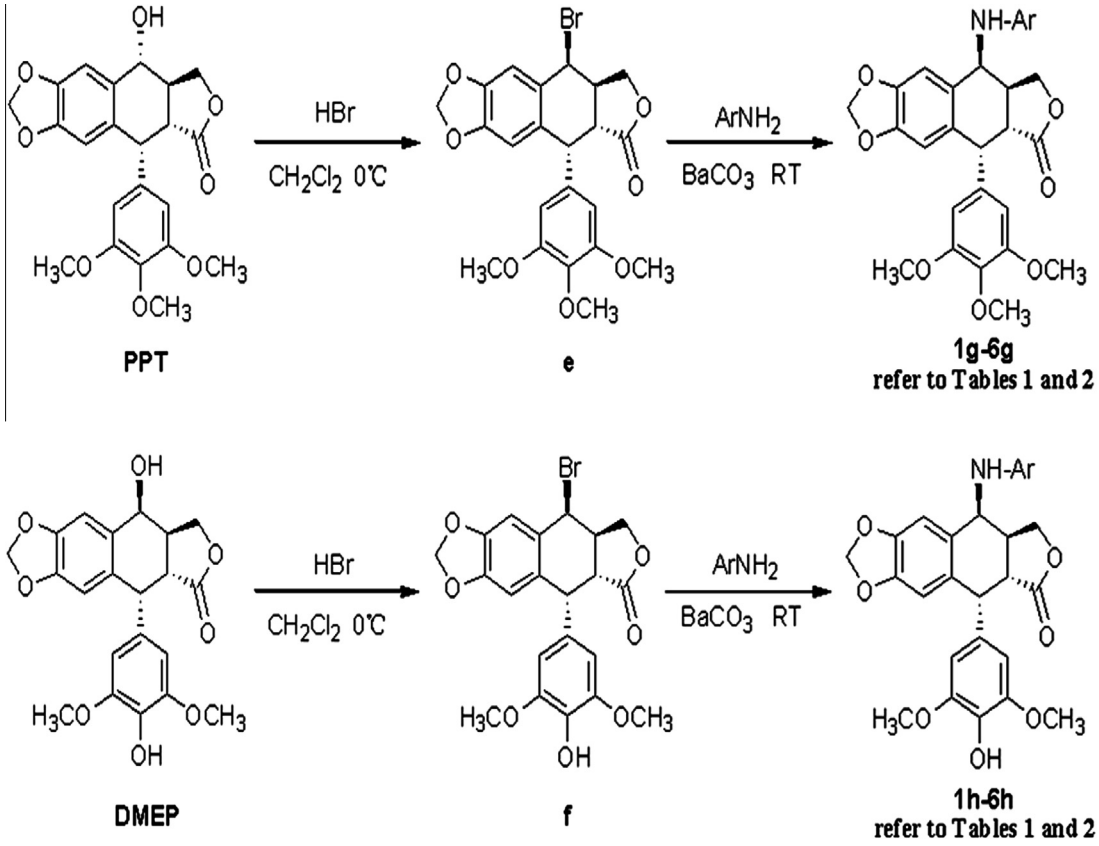
2.1. Chemistry

Podophyllum derivatives were prepared from (**a**) and (**b**). The synthetic route used to obtain title compounds is outlined in Scheme 1. The intermediates (**e**) and (**f**) were synthesized from (**a**) and (**b**), respectively, by a modified version of Kuhn's method,[33] in which a solution of (**a**) or (**b**) in dry dichloromethane was bubbled by dry hydrogen bromide gas at 0 °C. Next, the target compounds were synthesized by reduction of (**e**) or (**f**) using anhydrous barium carbonate as a catalyst in dry dichloromethane at room temperature, according to a previously published method.[34] Lastly, a series of N linkage podophyllum derivates were obtained in yields of 24–51%. The structures of the final products were confirmed by their ^{1}H NMR, ^{13}C NMR, ESI-MS spectral properties and elemental analyses, and purity estimation was performed with HPLC. Only those products with 95% purity were evaluated for biological activity.

2.2. Cytotoxicity of compounds against five tumor cell lines

The cytotoxicity of four N linkage podophyllum derivates (compounds **1g**–**2g** and compounds **1h**–**2h**) was evaluated in vitro by a MTT growth inhibition assay, which was carried out with a panel of five tumor cell lines: HeLa, BGC-823, A549, Huh7 and MCF-7. PPT, DMEP and VP-16 were used as positive controls. The screening procedure was based on the standard MTT method with calculated IC_{50} values indicating 50% inhibition of cell growth, as summarized in Table 1. According to the IC_{50} values derived from in vitro screening studies, four of the compounds demonstrated inhibition inferior to the positive controls. However, compared to compounds **1g** and **1h,** compounds **2g** and **2h** were more potent against HeLa cells (IC_{50} = 183.77 μM and 170.00 μM vs 20.44 μM and 13.16 μM), which implied that 2-aminopyridine was a better substituent in the C-4 positions of PPT and DMEP by N linkage.

On the basis of these SAR analysis results, we subsequently aimed to explore the influence of a chlorine substituent in the heterocyclic ring of 2-aminopyridine. Therefore, eight N linkage podophyllum derivatives containing a chlorine atom were synthesized by the synthetic route presented in Scheme 1. The results of in vitro cytotoxicity using the same five human tumor cell lines are presented in Table 2. All eight compounds exhibited cytotoxicity. IC_{50} values were all below micromolar levels, with some falling within the nanomolar range, which approximated or surpassed those of the reference controls PPT, DMEP and VP-16. Several



Scheme 1. Synthesis of N linkage podophyllum derivates compounds **1g–6g** and compounds **1h–6h**.

Table 1
Cytotoxicity against five tumor cell lines of compounds **1g–2g** and **1h–2h**

Compd	Ar	PPT/DMEP	IC_{50}^{a}(μM)				
			HeLa[b]	BGC-823[b]	MCF-7[b]	A549[b]	Huh7[b]
1g		PPT	183.77 ± 7.11	183.25 ± 9.69	185.47 ± 11.83	193.38 ± 13.11	288.17 ± 13.21
1h		DMEP	170.00 ± 8.75	169.45 ± 9.56	172.33 ± 12.22	184.22 ± 11.14	262.80 ± 14.34
2g		PPT	20.44 ± 1.04	19.81 ± 1.34	18.40 ± 2.52	51.86 ± 2.03	84.48 ± 3.96
2h		DMEP	13.16 ± 1.42	20.33 ± 1.42	18.39 ± 2.41	41.33 ± 1.56	63.25 ± 2.74
PPT	/	/	20.52 ± 1.38	33.35 ± 1.41	36.77 ± 2.47	67.45 ± 2.24	175.96 ± 2.56
DMEP	/	/	15.96 ± 1.22	21.26 ± 2.42	20.36 ± 1.26	52.86 ± 3.85	166.78 ± 2.29
VP-16	/	/	59.38 ± 0.77	30.74 ± 5.13	28.29 ± 1.96	67.25 ± 7.05	87.54 ± 2.14

[a] Data are the mean of three independent experiments.
[b] MTT methods, drug exposure was for 48 h.

important SARs could be deduced from these results. Firstly, we observed that the introduction of a chlorine atom into the nitrogen heterocycle caused a considerable improvement in activity. In general, the chlorine substituent podophyllum derivatives (compounds **3g–6g** and compounds **3h–6h**) showed greater cytotoxicity than their corresponding parent analogues (compound **2g** and compound **2h**). This is in contrast to the natural lignans PPT and DMEP, which had IC_{50} of 20.52 μM and 15.96 μM, respectively, against HeLa cells. Secondly, we found that changing the position of the chlorine atom in the nitrogen heterocycle caused a major alteration (compounds **3g–6g** and compounds **3h–6h**), with the order 3-position > 4-position > 5-position > 6-position. Thirdly, we observed that the trend in cell potency was maintained regardless of whether PPT or DMEP was substituted.

2.3. Induction of cell cycle arrest and apoptosis in HeLa cells by compounds 3g and 3h

Previous studies[35,36] have reported that podophyllum derivates, including VP-16, are known to cause G_2/M cell cycle arrest, followed by cell apoptosis. Accordingly, further biological evaluations focused on the highest potency compounds, **3g** and **3h,** substituted by 2-amino-3-chloropyridine in PPT and DMEP, to determine whether antitumor mechanisms were related to cell cycle arrest and apoptosis.

To define the cell cycle arrest, the most sensitive HeLa cells were treated with compound **3g** (0.25, 0.5 and 1 μM) and compound **3h** (0.125, 0.25 and 0.5 μM) for 24 h, and flow cytometric analysis was performed. A concentration dependent change was

Table 2
Further exploration of IC_{50} values for compounds **3g–6g** and **3h–6h** containing chlorine substituent

Compd	Ar	PPT/DMEP	IC_{50}^{a} (μM)				
			HeLa[b]	BGC-823[b]	MCF-7[b]	A549[b]	Huh7[b]
2g		PPT	20.44 ± 1.04	19.81 ± 1.34	18.40 ± 2.52	51.86 ± 2.03	84.48 ± 3.96
2h		DMEP	13.16 ± 1.42	20.33 ± 1.42	18.39 ± 2.41	41.33 ± 1.56	63.25 ± 2.74
3g		PPT	0.46 ± 0.03	4.21 ± 1.18	12.79 ± 2.44	18.59 ± 2.33	30.72 ± 2.93
3h		DMEP	0.24 ± 0.06	2.29 ± 0.71	9.36 ± 1.71	10.07 ± 1.25	25.32 ± 2.18
4g		PPT	3.25 ± 0.92	13.24 ± 1.41	15.41 ± 2.93	21.32 ± 2.91	59.12 ± 4.46
4h		DMEP	0.98 ± 0.09	8.47 ± 1.66	8.90 ± 1.96	11.04 ± 2.73	37.25 ± 4.08
5g		PPT	7.33 ± 0.81	20.46 ± 2.71	21.54 ± 2.45	25.44 ± 2.74	63.78 ± 4.24
5h		DMEP	5.54 ± 1.42	11.36 ± 2.35	15.57 ± 2.12	18.74 ± 2.56	40.71 ± 3.56
6g		PPT	20.33 ± 2.92	23.24 ± 1.54	24.56 ± 0.74	32.41 ± 2.11	70.47 ± 2.52
6h		DMEP	9.15 ± 1.73	13.12 ± 1.42	20.71 ± 1.29	30.41 ± 1.17	64.59 ± 2.33
PPT	/	/	20.52 ± 1.38	33.35 ± 1.41	36.77 ± 2.47	67.45 ± 2.24	175.96 ± 2.56
DMEP	/	/	15.96 ± 1.22	21.26 ± 2.42	20.36 ± 1.26	52.86 ± 3.85	166.78 ± 2.29
VP-16	/	/	59.38 ± 0.77	30.74 ± 5.13	28.29 ± 1.96	67.25 ± 7.05	87.54 ± 2.14

[a] Data are the mean of three independent experiments.
[b] MTT methods, drug exposure was for 48 h.

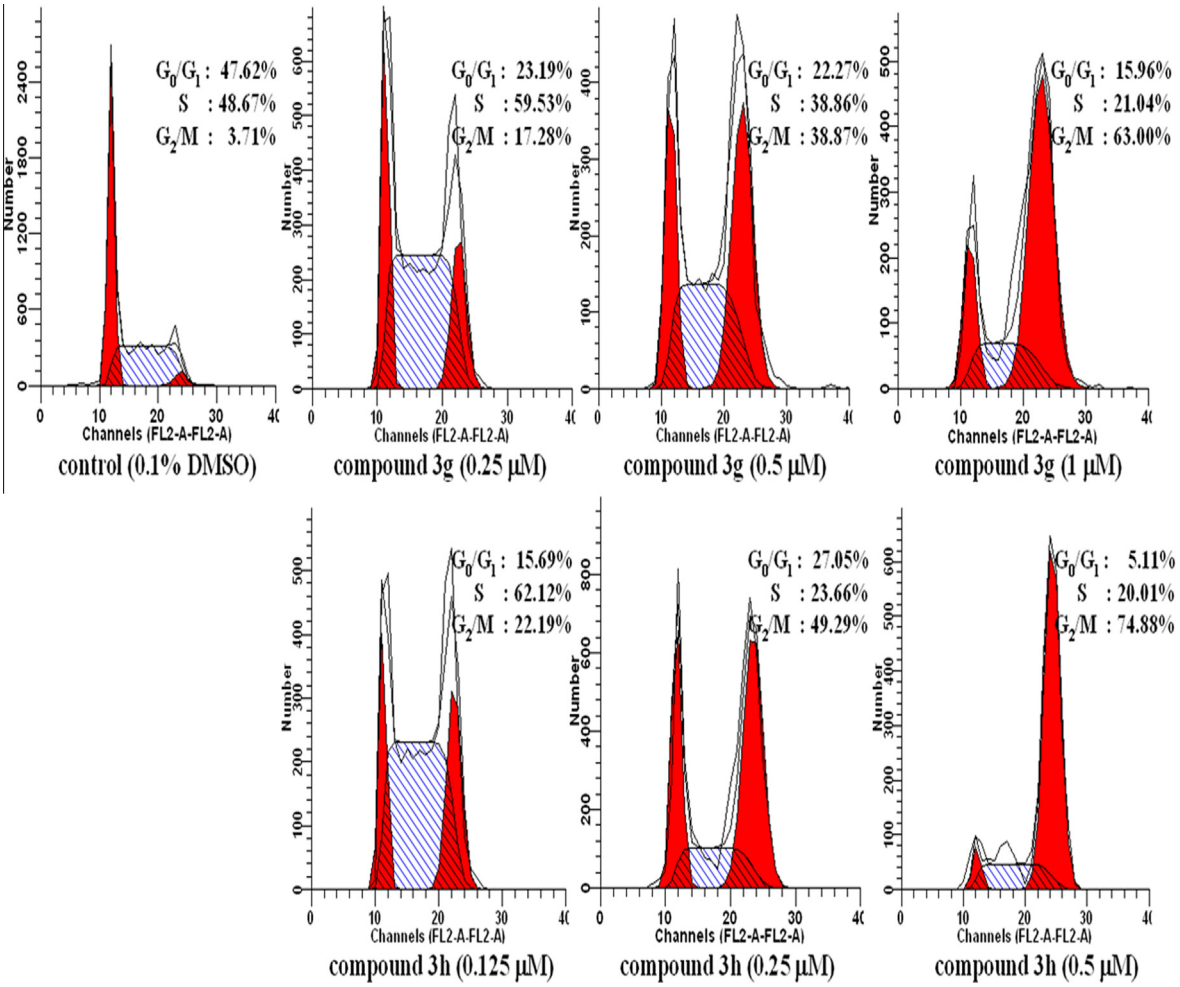


Figure 2. Effects of compounds **3g** (0.25, 0.5 and 1 μM) and **3h** (0.125, 0.25 and 0.5 μM) on cell cycle of HeLa cells. Cells were incubated for 24 h and stained with PI. Their DNA content was analyzed by fluorescence flow cytometry.

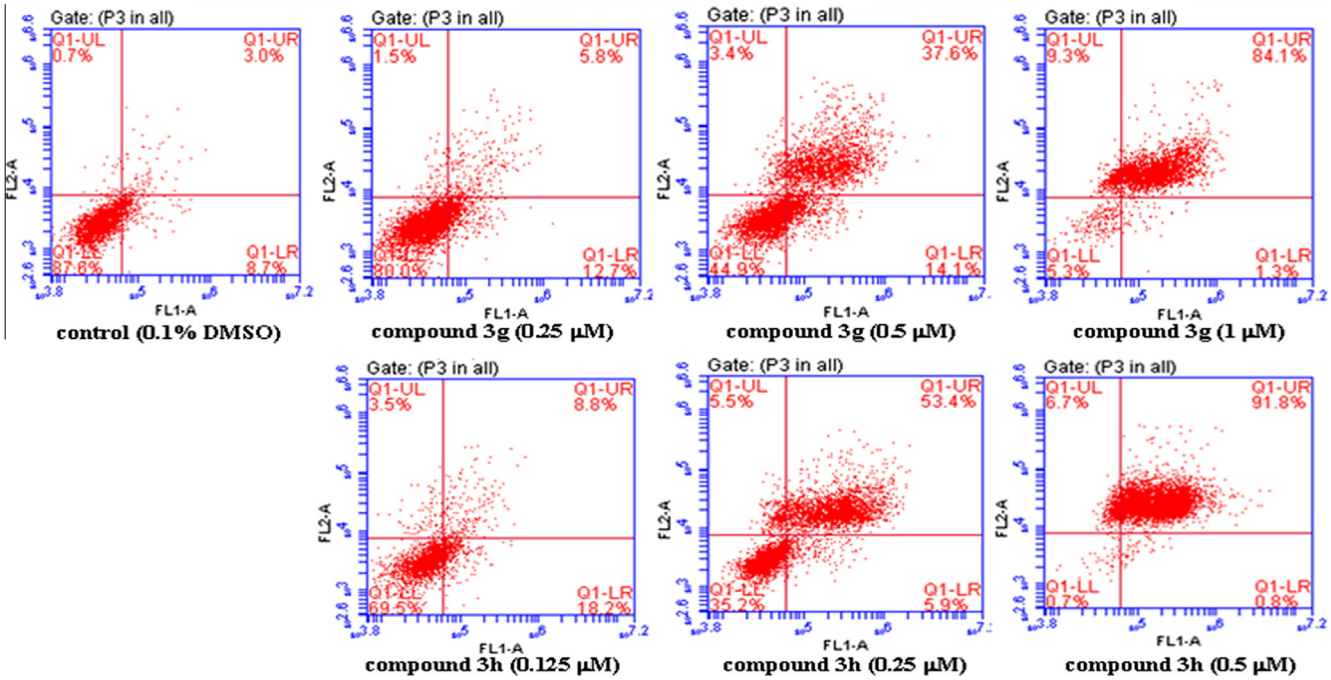


Figure 3. Effects of compounds **3g** (0.25, 0.5 and 1 μM) and **3h** (0.125, 0.25 and 0.5 μM) on cell apoptosis of HeLa cells. Cells were incubated for 48 h and induction of apoptosis was measured by Annexin-V/PI double-staining assay.

observed in the cell cycle pattern, which is shown in Figure 2. Our results demonstrate that treating HeLa cells with compound **3g** increased the percentage of cells in the G_2/M phase from 3.71% to 63.00% as the concentration increased from 0.25 to 1 μM. Likewise, after treating HeLa cells with increasing concentrations of compound **3h**, the distribution of cells in the G_2/M phase were 22.19%, 49.29% and 74.88%. The flow cytometric data clearly shows that these two compounds cause cell cycle arrest in the G_2/M phase.

In order to further explore whether the growth inhibition was related to the induction of apoptosis, we employed Annexin V/propidium iodide (PI) staining to detect the apoptosis ratio after the HeLa cells were treated with compound **3g** (0.25, 0.5 and 1 μM) and compound **3h** (0.125, 0.25 and 0.5 μM) for 48 h. As shown in Figure 3, the percentage of normal cells (left lower section of fluorocytogram) remaining after treatment with 1 μM of compound **3g** represented 5.3% of the total cells, compared to 87.6% of HeLa cells treated with 0.1% dimethyl sulfoxide (DMSO) as a control. The ratio of apoptotic cells, including early apoptosis (right lower section of fluorocytogram) and late apoptosis (right upper section of fluorocytogram), was 85.4%, whereas the control ratio was 11.7%. Furthermore, we observed that the induction of apoptosis occurred in a dose-dependent manner. There was a statistically significant difference in the degree of apoptosis induced by compound **3h** treatment at the 0.25 μM concentration compared to 0.125 μM. The apoptosis ratios for these two concentrations were 59.3% and 27.0%, respectively. The effect was maintained, even at 0.5 μM concentration, with apoptosis ratio of 92.6%, which was nine fold higher than the control ratio. Taken together, these results confirm that growth inhibition mediated by treatment with compounds **3g** and **3h** is related to the induction of apoptosis.

2.4. Effect of compounds 3g and 3h on apoptosis-related proteins

Apoptosis is mainly controlled by two major pathways, the mitochondrial pathway and the membrane death receptor pathway.[37] The majority of anti-neoplastic drugs appear to activate the mitochondrial pathway.[38] It is well known that caspases and members of the Bcl-2 family, such as Bax and Bcl-2, play important roles in mitochondrial pathway induction by various stimulations.[39–41] Bcl-2 can prevent the release of cytochrome c from mitochondria during apoptosis mediated by the mitochondrial pathway. However, Bax plays a contrary role in this pathway.[42] Among the family of caspases, caspase-3 has been reported to be the most frequently activated caspase protease in apoptotic cells, indicating its crucial role in the cell death process.[43]

To reveal the possible mechanisms responsible for the apoptotic effects of compounds **3g** and **3h**, we examined whether caspase-3, Bax and Bcl-2 were activated during apoptosis induced by varying concentrations of these two compounds. As show in Figure 4, the expression of caspase-3 and Bax was significantly increased, while Bcl-2 expression was decreased by these two compounds in a dose-dependent manner. These results suggest that the apoptotic response induced by compounds **3g** and **3h** may be associated with the mitochondrial pathway and accompanied by increased expression of Bax, decreased expression of Bcl-2, and activation of caspase-3.

Next, we tested whether the observed cell apoptosis induced by compounds **3g** and **3h** was related to the expression of P53. P53 tumor suppressor is known to integrate multiple stress signals into a series of diverse antiproliferative responses. One of the most important P53 functions is its ability to induce apoptosis, and thus, it serves as a regulator of the apoptotic process that can modulate key control points in both the extrinsic and intrinsic pathways.[44] HeLa cells P53 protein expression was increased following 48 h treatment with compounds **3g** and **3h**. This response, which was evident even at low doses, was amplified with increasing doses, implying that P53 plays an essential role in the compounds' mechanisms of action. In contrast, expression of β-actin, an internal control, was unchanged (Fig. 4). These results suggest that compounds **3g** and **3h** induce HeLa cells apoptosis by increasing P53 expression, followed by Bax up-regulation, Bcl-2 down-regulation, and caspase-3 activation.

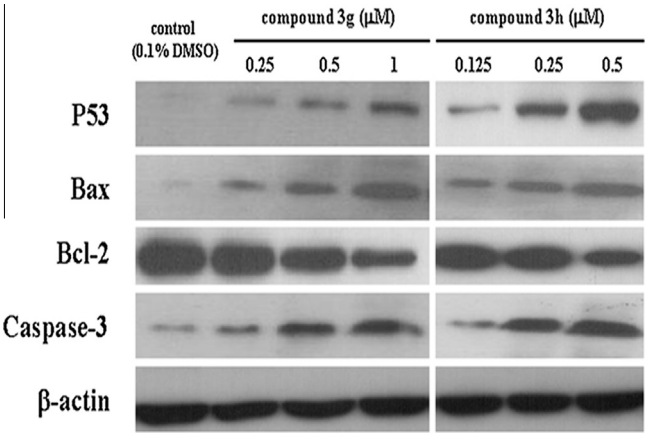


Figure 4. Effects of compounds **3g** (0.25, 0.5 and 1 μM) and **3h** (0.125, 0.25 and 0.5 μM) on expressions of apoptosis-related proteins. HeLa cells were treated with different concentrations for 48 h. Whole cell lysates were analyzed for expression levels of P53, Bcl-2, Bax, and caspase-3 by Western blotting. Control represented the HeLa cells with 0.1% DMSO treatment. β-Actin was used as a loading control.

2.5. Inhibition of microtubule polymerization by compound 3g

PPT and its analogues bind to the colchicine binding site of tubulin near the α- and β-microtubule interface and interfere with microtubule polymerization to exert antitumor effects.[45] For further insight into the biological effects of compound **3g** on cellular microtubule arrangement, we investigated microtubule structure and distribution in HeLa cells by indirect immunofluorescence (IF) using an anti α-tubulin antibody. Figure 5 shows that 0.1% DMSO treated cells (negative control) demonstrated typical interphase microtubule organization with a widespread network of long microtubules. At concentration of 0.25 μM, compound **3g** did not affect the interphase microtubule network. At the 1 μM concentration, compound **3g** completely disrupted the network of microtubules and altered the overall cellular shape, with all of the cells becoming roundish. These effects were pronounced, even compared to those seen in cells treated with the same concentration of colchicine (positive control). Overall, a significant reduction of the microtubules at the periphery of the cells was evident, the central networks were disorganized, and the cells displayed obvious apoptosis, as indicated by fragmented nuclei. Therefore, we concluded that compound **3g** definitively inhibits microtubule polymerization, and does so in a dose dependent manner.

2.6. Inhibition of human topoisomerase II kinetoplast DNA (kDNA) decatenation by compound 3h

It has been reported that the DNA-topoisomerase II complex is the molecular target of many antitumor drugs, such as the DMEP derivates, VP-16 and VM-26.[46] To further understand the antitumor mechanisms of compound **3h** substituted in the scaffold of DMEP, we evaluated the catalytic activity of topoisomerase II using a kDNA decatenation assay. As shown in Figure 6, a high level of decatenation was observed at low concentrations of **3h**. Complete inhibition of kDNA decatenation was induced by 10 μM concentration of **3h**, showing it to be more effective than the same concentration of VP-16. These results provide strong evidence that compound **3h** exhibits antiproliferative properties against human tumor cells by inhibiting the activity of topoisomerase II.

3. Conclusions

In this study, we synthesized a series of N linkage podophyllum derivates, several of which displayed improved anticancer activity compared to PPT or DMEP and VP-16. SAR analysis indicated that podophyllum derivates containing 2-aminopyridine at the C-4 position (compounds **2g** and **2h**) show optimum biological activity. Compounds containing a chlorine atom substituted into the heterocyclic ring (compounds **3g**–**6g** and compounds **3h**–**6h**) displayed enhanced activity compared to their corresponding parent analogues (compound **2g** and compound **2h**). What is more, changing the position of the chlorine substituent on the nitrogen heterocycle caused a significant improvement in the potency, with the order 3-position (compounds **3g** and **3h**) > 4-position

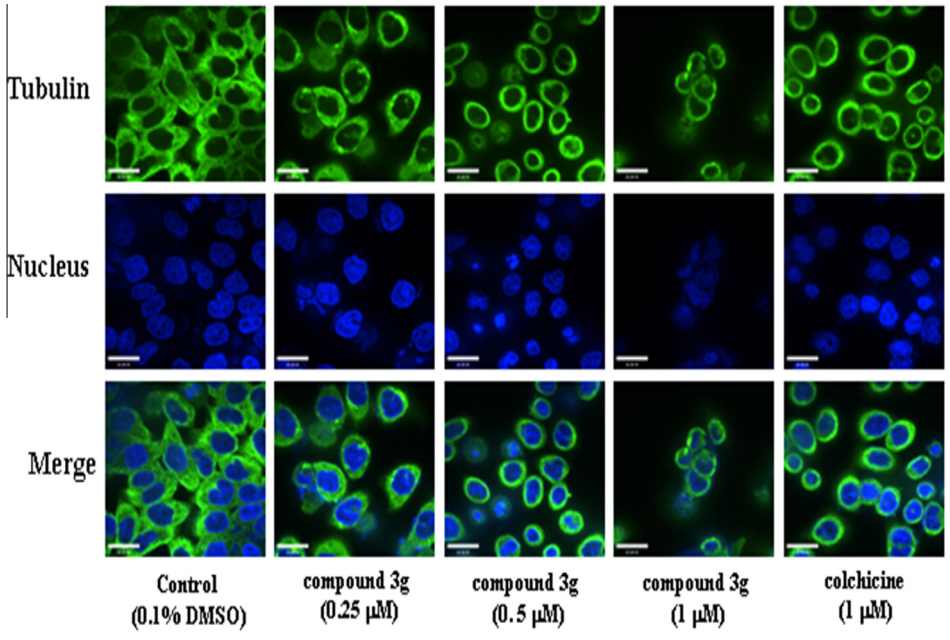


Figure 5. Effects of compound **3g** (0.25, 0.5 and 1 μM) on microtubule assembly. Microtubule organization in HeLa cells exposed for 24 h to solvent vehicle alone (0.1% DMSO, negative control), compound **3g** (0.25, 0.5 and 1 μM) and colchicine (1 μM, positive control), as revealed by immunofluorescence localization of α-tubulin (green) and nuclei staining (blue). Bar: 22 μm.

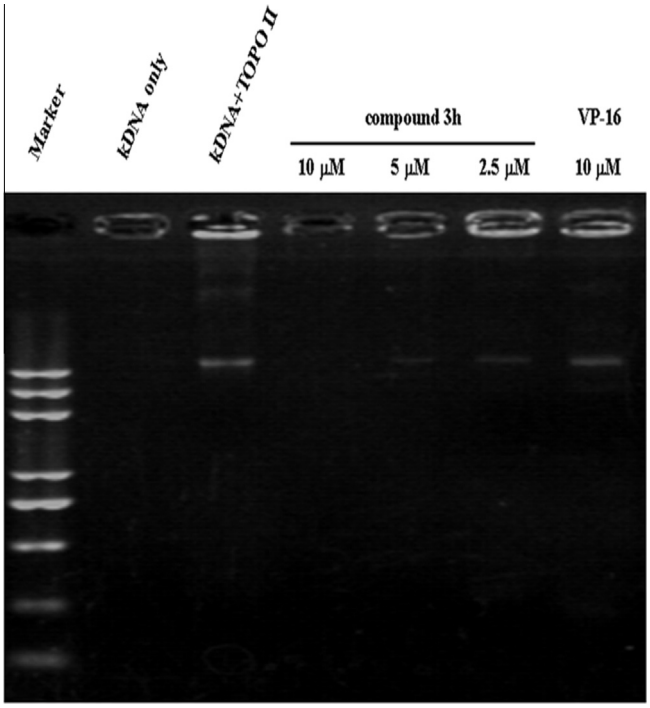


Figure 6. Effects of compound **3h** (2.5, 5 and 10 μM) on decatenation of kDNA by topoisomerase II (TOPO II). VP-16 was used as reference at 10 μM. DNA samples were separated by gel electrophoresis.

(compounds **4g** and **4h**) > 5-position (compounds **5g** and **5h**) > 6-position (compounds **6g** and **6h**). Upon further study, we found that compounds **3g** and **3h** exhibited the strongest antitumor potency, arrested HeLa cells in the G_2/M phase of the cell cycle, and caused apoptosis in HeLa cells. The latter effect may be due to increased P53 expression resulting in the up-regulation of Bax, the down-regulation of Bcl-2, and the activation of caspase-3. Mechanistic study demonstrated that compound **3g** exerted its antitumor activity through the inhibition of microtubule polymerization, while compound **3h** inhibited topoisomerase II catalytic activity. The present findings provide evidence that compounds **3g** and **3h** possess antitumor mechanisms of action. In summary, the presented data provide insights into the modification of podophyllum derivatives. This work also adds to the current body of knowledge detailing the mechanisms of action of podophyllum derivatives. In addition, it provides useful information for structural optimization studies in general, as well as for studies focused on the discovery and development of effective antitumor agents from natural parent compounds. In future research, these findings should be followed by a detailed investigation utilizing binding models to identify more derivatives and improve upon IC_{50} values. We will continue to investigate the biological activity of these compounds to contribute to a better understanding of their antitumor mechanisms of action and evaluate their potential to be potent anticancer agents.

4. Experimental

4.1. General experimental procedures

Solvents were purified in the usual way. Analytical thin-layer chromatography (TLC) was performed on precoated Merck silica Gel 60 F254 plates. Column chromatography was carried out on silica gel (60–120 mesh, Merck chemicals). ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker AC-300/400 MHZ NMR spectrometer with tetramethylsilane (TMS) as the internal standard. All chemical shifts are expressed in parts per million (ppm, for δ). Electrospray ionization mass spectrometry (ESI-MS) spectra were obtained with an agility MSD trap mass spectrometer after dissolving the compounds in methanol. Elemental analyses were done in a Perkin Elmer CHNS/O Analyzer 2400.

4.2. Synthesis of compounds (e) and (f)

A solution of (**a**) (PPT, 10 g, 24 mmol) or (**b**) (DMEP, 9.6 g, 24 mmol) in 250 ml of dry dichloromethane was kept at 0 °C, and dry hydrogen bromide gas was bubbled into the solution for about 2 h until TLC showed the total disappearance of (**a**) or (**b**). Then, nitrogen was bubbled through the solution to drive off excess hydrogen bromide and the solution was evaporated in vacuum. Water, formed in the reaction was removed using benzene as an azeotropic mixture. Lastly, the desired product (**e**) (11.8 g) or (**f**) (11.5 g) was obtained and used in the next reaction step without further purification.[47]

4.3. Synthesis of N linkage podophyllum derivates

A solution containing (**e**) (716 mg, 1.5 mmol) or (**f**) (695 mg, 1.5 mmol), anhydrous $BaCO_3$ (590 mg, 3.0 mmol), and the appropriate arylamine (1.65 mmol) in 15 ml dry dichloromethane under nitrogen was stirred overnight at room temperature. The reaction mixture was filtered, diluted with ethylacetate, washed with water, dried over anhydrous $MgSO_4$, and purified by silica gel column chromatography using dichloromethane–acetone–ethylacetate (100:5:5) or toluene–ethylacetate (3:1) as eluents.[48]

4.3.1. 4-*N*-(2-Aminopyrimidine)-4-deoxidation-4′-podophyllotoxin (1g)

The yield 31%; white powder, ^{1}H NMR (300 MHz, $CDCL_3$, 25 °C, TMS): δ = 3.05 (s, 1H; H-3), 3.06 (s, 1H; H-2), 3.74 (s, 3H; OCH_3), 3.80 (s, 6H; OCH_3), 3.94 (s, 1H; H-11), 4.40 (d, J = 6 Hz, 1H; H-11), 4.60 (s, 1H; H-1), 5.29 (d, J = 15 Hz, 2H; H-4 and NH), 5.93 (d, J = 9 Hz, 2H; H-13), 6.32 (s, 3H; Ar-H), 6.51 (s, 1H; Ar-H), 6.61 (s, 1H; Ar-H), 6.82 (s, 1H; Ar-H), 8.16 ppm (s, 1H; Ar-H); ^{13}C NMR (75 MHz, $CDCL_3$, 25 °C, TMS): δ = 38.29 (C-3), 42.13 (C-2), 44.03 (C-1), 50.28 (C-4), 56.45 (OCH_3), 60.98 (OCH_3), 69.63 (C-11), 101.78 (C-13), 108.49 (Ar-C), 108.63 (Ar-C), 109.73 (Ar-C), 110.15 (Ar-C), 111.94 (Ar-C), 130.01 (Ar-C), 132.39 (Ar-C), 135.27 (Ar-C), 147.79 (Ar-C), 148.51 (Ar-C), 152.83 (Ar-C), 158.20 (Ar-C), 161.65 (Ar-C), 174.94 ppm (C-12). MS (ESI): m/z: 491.17 $[M+H]^+$; Anal. Calcd for $C_{26}H_{25}N_3O_7$: C, 63.54; H, 5.13; N, 8.55. Found: C, 63.55; H, 5.06; N, 8.44.

4.3.2. 4-*N*-(2-Aminopyrimidine)-4-deoxidation-4′-demethylepipodophyllotoxin (1h)

The yield 24%; white powder, ^{1}H NMR (300 MHz, $CDCL_3$, 25 °C, TMS): δ = 2.96 (s, 1H; H-3), 2.97 (s, 1H; H-2), 3.70 (s, 6H; OCH_3), 3.76 (s, 1H; H-11), 4.31 (s, 1H; H-11), 4.53 (s, 1H; H-1), 5.23 (s, 1 H; H-4), 5.27 (d, J = 3 Hz, 1H; exchangeable NH), 5.86 (d, J = 9 Hz, 2H; H-13), 6.26 (s, 3H; Ar-H), 6.45 (s, 1H; Ar-H), 6.55 (s, 1H; Ar-H), 6.75 (s, 1H; Ar-H), 8.10 ppm (s, 1H; Ar-H); ^{13}C NMR (75 MHz, $CDCL_3$, 25 °C, TMS): δ = 38.17 (C-3), 42.19 (C-2), 43.81 (C-1), 50.25 (C-4), 56.65 (OCH_3), 69.58 (C-11), 101.75 (C-13), 108.18 (Ar-C), 109.65 (Ar-C), 110.16 (Ar-C), 111.82 (Ar-C), 129.90 (Ar-C), 130.73 (Ar-C), 132.56 (Ar-C), 134.30 (Ar-C), 146.72 (Ar-C), 147.73 (Ar-C), 148.50 (Ar-C), 158.17 (Ar-C), 161.40 (Ar-C), 174.99 ppm (C-12). MS (ESI): m/z: 477.15 $[M+H]^+$; Anal. Calcd for $C_{25}H_{23}N_3O_7$: C, 62.89; H, 4.86; N, 8.80. Found: C, 63.16; H, 5.21; N, 8.61.

4.3.3. 4-*N*-(2-Aminopyridine)-4-deoxidation-4′-podophyllotoxin (2g)

The yield 28%; white powder, ^{1}H NMR (300 MHz, CD_3OD, 25 °C, TMS): δ = 3.04 (s, 1H; H-3), 3.05 (s, 1H; H-2), 3.71 (s, 3H; OCH_3), 3.73 (s, 6H; OCH_3), 4.62 (s, 2H; H-11), 4.63 (s, 1H; H-1), 5.08 (s, 1H; H-4), 5.98 (s, 2H; H-13), 6.28 (s, 2H; Ar-H), 6.52 (s, 1H; Ar-H), 6.81 (t, J = 6 Hz, 1H; Ar-H), 7.00 (d, J = 12 Hz, 2H; Ar-H), 7.80 ppm (d, J = 6 Hz, 2H; Ar-H); ^{13}C NMR (75 MHz, CD_3OD, 25 °C, TMS): δ = 38.30 (C-3), 42.16 (C-2), 51.71 (C-1), 52.84 (C-4), 55.37 (OCH_3), 59.87 (OCH_3), 69.13 (C-11), 101.85 (C-13), 107.02 (Ar-C), 108.27 (Ar-C), 109.58 (Ar-C), 113.09 (Ar-C), 114.59 (Ar-C), 125.62 (Ar-C), 131.29 (Ar-C), 137.03 (Ar-C), 138.81 (Ar-C), 141.95 (Ar-C), 147.72 (Ar-C), 149.08 (Ar-C), 151.12 (Ar-C), 153.06 (Ar-C), 172.49 ppm (C-12). MS (ESI): m/z: 490.17 $[M+H]^+$; Anal. Calcd for $C_{27}H_{26}N_2O_7$: C, 66.11; H, 5.34; N, 5.71. Found: C, 65.95; H, 5.27; N, 5.53.

4.3.4. 4-*N*-(2-Aminopyridine)-4-deoxidation-4′-demethylepipodophyllotoxin (2h)

The yield 30%; white powder, ^{1}H NMR (300 MHz, CD_3OD, 25 °C, TMS): δ = 3.03 (s, 1H; H-3), 3.04 (s, 1H; H-2), 3.71 (d, J = 9 Hz, 6H; OCH_3), 3.78 (s, 1 H; H-11), 4.59 (s, 1H; H-11), 4.63 (s, 1H; H-1), 5.05 (s, 1H; H-4), 5.98 (s, 2H; H-13), 6.25 (s, 2H; Ar-H), 6.52 (s, 1H; Ar-H), 6.83 (d, J = 6 Hz, 1H; Ar-H), 6.98 (d, J = 15 Hz, 2H; Ar-H), 7.79 ppm (d, J = 6 Hz, 2H; Ar-H); ^{13}C NMR (75 MHz, CD_3OD, 25 °C, TMS): δ = 40.71 (C-3), 41.83 (C-2), 50.22 (C-1), 51.32 (C-4), 53.97 (OCH_3), 59.11 (C-11), 100.24 (C-13), 105.36 (Ar-C), 106.59 (Ar-C), 108.07 (Ar-C), 111.50 (Ar-C), 113.00 (Ar-C), 123.97 (Ar-C), 128.13 (Ar-C), 130.15 (Ar-C), 133.30 (Ar-C), 137.26 (Ar-C), 140.36 (Ar-C), 146.24 (Ar-C), 147.46 (Ar-C), 149.57 (Ar-C), 171.74 ppm (C-12). MS (ESI): m/z: 476.16 $[M+H]^+$; Anal. Calcd for $C_{26}H_{24}N_2O_7$: C, 65.54; H, 5.08; N, 5.88. Found: C, 65.29; H, 5.00; N, 5.71.

4.3.5. 4-*N*-(2-Amino-3-chloropyridine)-4-deoxidation-4′-podophyllotoxin (3g)

The yield 46%; white powder, ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 3.04 (s, 1H; H-3), 3.05 (s, 1H; H-2), 3.71 (s, 6H; OCH_3), 3.73 (d, J = 4 Hz, 1 H; H-11), 3.76 (s, 3H; OCH_3), 4.33 (t, J = 8 Hz, 1 H; H-11), 4.57 (d, J = 4 Hz, 1H; H-1), 5.08 (d, J = 8 Hz, 1H; H-4), 5.38 (d, J = 4 Hz, 1H; exchangeable NH), 5.90 (d, J = 4 Hz, 2H; H-13), 6.30 (s, 2H; Ar-H), 6.47 (s, 1H; Ar-H), 6.58–6.61 (m, 1H; Ar-H), 6.75 (s, 1H; Ar-H), 7.45 (d, J = 4 Hz, 1H; Ar-H), 7.97 ppm (d, J = 4 Hz, 1H; Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS): δ = 38.14 (C-3), 41.89 (C-2), 43.76 (C-1), 50.00 (C-4), 56.14 (OCH_3), 60.69 (OCH_3), 69.74 (C-11), 101.52 (C-13), 108.16 (Ar-C), 109.20 (Ar-C), 109.89 (Ar-C), 114.32 (Ar-C), 115.17 (Ar-C), 130.08 (Ar-C), 132.34 (Ar-C), 135.14 (Ar-C), 136.51 (Ar-C), 145.77 (Ar-C), 147.60 (Ar-C), 148.26 (Ar-C), 152.51 (Ar-C), 153.15 (Ar-C), 174.82 ppm (C-12). MS (ESI): m/z: 524.14 $[M+H]^+$; Anal. Calcd for $C_{27}H_{25}ClN_2O_7$: C, 61.78; H, 4.80; N, 5.34. Found: C, 61.79; H, 4.75; N, 5.27.

4.3.6. 4-*N*-(2-Amino-3-chloropyridine)-4-deoxidation-4′-demethylepipodophyllotoxin (3h)

The yield 42%; white powder, ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 3.04 (s, 1H; H-3), 3.06 (s, 1H; H-2), 3.73 (d, J = 12 Hz, H; H-11), 3.79 (s, 6H; OCH_3), 4.38 (d, J = 8 Hz, 1H; H-11), 4.60 (d, J = 4 Hz, 1H; H-1), 5.09 (d, J = 4 Hz, 1H; H-4), 5.41 (s, 1H; exchangeable NH), 5.95 (d, J = 4 Hz, 2H; H-13), 6.33 (s, 2H; Ar-H), 6.53 (s, 1H; Ar-H), 6.62–6.65 (m, 1H; Ar-H), 6.78 (s, 1H; Ar-H), 7.49 (d, J = 8 Hz, 1H; Ar-H), 8.00 ppm (t, J = 4 Hz, 1H; Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS): δ = 38.07 (C-3), 42.05 (C-2), 43.62 (C-1), 50.02 (C-4), 56.40 (OCH_3), 69.76 (C-11), 101.52 (C-13), 107.89 (Ar-C), 109.17 (Ar-C), 109.96 (Ar-C), 114.32 (Ar-C), 115.22 (Ar-C), 130.08 (Ar-C), 130.57 (Ar-C), 132.56 (Ar-C), 136.53 (Ar-C), 145.79 (Ar-C), 146.43 (Ar-C), 147.58 (Ar-C), 148.26 (Ar-C), 153.18 (Ar-C), 174.93 ppm (C-12). MS (ESI): m/z: 510.12 $[M+H]^+$; Anal. Calcd for $C_{26}H_{23}ClN_2O_7$: C, 61.12; H, 4.54; N, 5.48. Found: C, 61.27; H, 4.66; N, 5.26.

4.3.7. 4-*N*-(2-Amino-4-chloropyridine)-4-deoxidation-4′-podophyllotoxin (4g)

The yield 51%; white powder, ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 2.95 (s, 1H; H-3), 2.96 (s, 1H; H-2), 3.68 (s, 6H; OCH_3), 3.69 (s, 3H; OCH_3), 3.73 (d, J = 8 Hz, 1H; H-11), 4.31 (d, J = 8 Hz, 1H; H-11), 4.46 (d, J = 4 Hz, 1H; H-1), 5.19 (d, J = 8 Hz, 1H; H-4), 5.32 (s, 1H; exchangeable NH), 5.81 (s, 1H; H-13), 5.87 (s, 1H; H-13), 6.25 (s, 2H; Ar-H), 6.42 (s, 2H; Ar-H), 6.56 (d, J = 8 Hz, 1H; Ar-H), 6.75 (s, 1H; Ar-H), 7.90 ppm (d, J = 4 Hz, 1H; Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS): δ = 38.08 (C-3), 41.74 (C-2), 43.69 (C-1), 49.61 (C-4), 56.07 (OCH_3), 60.61 (OCH_3), 69.67 (C-11), 101.44 (C-13), 108.03 (Ar-C), 108.23 (Ar-C), 109.36 (Ar-C), 114.19 (Ar-C), 130.27 (Ar-C), 132.06 (Ar-C), 135.26 (Ar-C), 136.80 (Ar-C), 144.46 (Ar-C), 147.38 (Ar-C), 147.95 (Ar-C), 148.60 (Ar-C), 152.44 (Ar-C), 158.39 (Ar-C), 175.08 ppm (C-12). MS (ESI): m/z: 524.14 $[M+H]^+$; Anal. Calcd for $C_{27}H_{25}ClN_2O_7$: C, 61.78; H, 4.80; N, 5.34. Found: C, 61.66; H, 4.79; N, 5.25.

4.3.8. 4-*N*-(2-Amino-4-chloropyridine)-4-deoxidation-4′-demethylepipodophyllotoxin (4h)

The yield 48%; white powder, ^{1}H NMR (400 MHz, CD_3OD and $CDCl_3$, 25 °C, TMS): δ = 3.18 (s, 1H; H-3), 3.19 (s, 1H; H-2), 3.78 (s, 6H; OCH_3), 3.85 (d, J = 8 Hz, 1H; H-11), 4.40 (t, J = 8 Hz, 1H; H-11), 4.61 (d, J = 4 Hz, 1H; H-1), 4.87 (d, J = 4 Hz, 1H; H-4), 5.97 (d, J = 4 Hz, 2H; H-13), 6.34 (s, 2H; Ar-H), 6.54 (d, J = 8 Hz, 2H; Ar-H), 6.64 (s, 1H; Ar-H), 6.76 (s, 1H; Ar-H), 7.53 ppm (s, 1H; Ar-H); ^{13}C NMR (100 MHz, CD_3OD and $CDCl_3$, 25 °C, TMS): δ = 42.12 (C-3), 45.57 (C-2), 47.31 (C-1), 54.67 (C-4), 59.99 (OCH_3), 72.41 (C-11), 105.47 (C-13), 111.93 (Ar-C), 112.96 (Ar-C), 113.66 (Ar-C), 130.47 (Ar-C), 132.80 (Ar-C), 133.56 (Ar-C), 134.04 (Ar-C), 135.99 (Ar-C), 138.35 (Ar-C), 147.50 (Ar-C), 150.97 (Ar-C), 151.46 (Ar-C), 152.32 (Ar-C), 159.79 (Ar-C), 179.36 ppm (C-12). MS (ESI): m/z: 510.12 $[M+H]^+$; Anal. Calcd for $C_{26}H_{23}ClN_2O_7$: C, 61.12; H, 4.54; N, 5.48. Found: C, 61.18; H, 4.50; N, 5.28.

4.3.9. 4-*N*-(2-Amino-5-chloropyridine)-4-deoxidation-4′-podophyllotoxin (5g)

The yield 39%; white powder, ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 2.97 (s, 1H; H-3), 2.98 (s, 1H; H-2), 3.69 (s, 6H; OCH_3), 3.72 (s, 3H; OCH_3), 3.74 (d, J = 8 Hz, 1H; H-11), 4.33 (s, 1H; H-11), 4.48 (d, J = 4 Hz, 1H; H-1), 4.98 (d, J = 4 Hz, 1H; H-4), 5.28 (s, 1H; exchangeable NH), 5.85 (s, 1H; H-13), 5.89 (s, 1H; H-13), 6.26 (s, 2H; Ar-H), 6.35 (d, J = 8 Hz, 1H; Ar-H), 6.44 (s, 1H; Ar-H), 6.75 (s, 1H; Ar-H), 7.29 (d, J = 4 Hz, 1H; Ar-H), 7.97 ppm (d, J = 4 Hz, 1H; Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS): δ = 38.16 (C-3), 41.80 (C-2), 43.69 (C-1), 49.86 (C-4), 56.10 (OCH_3), 60.66 (OCH_3), 69.73 (C-11), 101.47 (C-13), 108.09 (Ar-C), 109.32 (Ar-C), 109.56 (Ar-C), 109.77 (Ar-C), 120.63 (Ar-C), 130.33 (Ar-C), 132.07 (Ar-C), 135.21 (Ar-C), 137.24 (Ar-C), 145.89 (Ar-C), 147.43 (Ar-C), 148.01 (Ar-C), 152.46 (Ar-C), 155.87 (Ar-C), 175.08 ppm (C-12). MS (ESI): m/z: 524.14 $[M+H]^+$; Anal. Calcd for $C_{27}H_{25}ClN_2O_7$: C, 61.78; H, 4.80; N, 5.34. Found: C, 61.61; H, 4.91; N, 5.08.

4.3.10. 4-*N*-(2-Amino-5-chloropyridine)-4-deoxidation-4′-demethylepipodophyllotoxin (5h)

The yield 37%; white powder, ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 2.97 (s, 1H; H-3), 2.98 (s, 1H; H-2), 3.73 (s, 6H; OCH_3), 3.75 (d, J = 12 Hz, 1H; H-11), 4.34 (s, 1H; H-11), 4.50 (s, 1H; H-1), 4.88 (d, J = 8 Hz, 1H; H-4), 5.27 (d, J = 8 Hz, 1H; exchangeable NH), 5.88 (s, 1H; H-13), 5.92 (s, 1H; H-13), 6.28 (s, 2H; Ar-H), 6.37 (d, J = 8 Hz, 1H; Ar-H), 6.47 (s, 1H; Ar-H), 6.76 (s, 1H; Ar-H), 7.32 (d, J = 12 Hz, 1H; Ar-H), 8.00 ppm (d, J = 4 Hz, 1H; Ar-H); ^{13}C

NMR (100 MHz, $CDCL_3$, 25 °C, TMS): δ = 38.11 (C-3), 41.93 (C-2), 43.52 (C-1), 49.97 (C-4), 56.35 (OCH_3), 69.72 (C-11), 101.47 (C-13), 107.84 (Ar-C), 109.26 (Ar-C), 109.51 (Ar-C), 109.84 (Ar-C), 120.73 (Ar-C), 130.27 (Ar-C), 130.61 (Ar-C), 132.32 (Ar-C), 137.30 (Ar-C), 145.96 (Ar-C), 146.41 (Ar-C), 147.40 (Ar-C), 148.05 (Ar-C), 155.86 (Ar-C), 175.11 ppm (C-12). MS (ESI): *m*/*z*: 510.12 $[M+H]^+$; Anal. Calcd for $C_{26}H_{23}ClN_2O_7$: C, 61.12; H, 4.54; N, 5.48. Found: C, 61.10; H, 4.68; N, 5.34.

4.3.11. 4-*N*-(2-Amino-6-chloropyridine)-4-deoxidation-4′-podophyllotoxin (6g)

The yield 47%; white powder, ^1H NMR (400 MHz, $CDCL_3$, 25 °C, TMS): δ = 2.96 (s, 1H; H-3), 2.97 (s, 1H; H-2), 3.69 (s, 6H; OCH_3), 3.71 (s, 3H; OCH_3), 3.75 (d, *J* = 4 Hz, 1H; H-11), 4.37 (d, *J* = 4 Hz, 1H; H-11), 4.46 (d, *J* = 4 Hz, 1H; H-1), 5.08 (d, *J* = 8 Hz, 1H; H-4), 5.28 (d, *J* = 4 Hz, 1H; exchangeable NH), 5.83 (s, 1H; H-13), 5.88 (s, 1H; H-13), 6.25 (s, 2H; Ar-H), 6.28 (s, 1H; Ar-H), 6.41 (s, 1H; Ar-H), 6.57 (d, *J* = 8 Hz, 1H; Ar-H), 6.76 (s, 1H; Ar-H), 7.26 ppm (t, *J* = 8 Hz, 1H; Ar-H); ^{13}C NMR (100 MHz, $CDCL_3$, 25 °C, TMS): δ = 38.17 (C-3), 41.72 (C-2), 43.69 (C-1), 49.73 (C-4), 56.10 (OCH_3), 60.64 (OCH_3), 69.71 (C-11), 101.47 (C-13), 106.56 (Ar-C), 108.08 (Ar-C), 109.35 (Ar-C), 112.74 (Ar-C), 130.06 (Ar-C), 132.10 (Ar-C), 135.23 (Ar-C), 136.89 (Ar-C), 139.56 (Ar-C), 147.40 (Ar-C), 148.01 (Ar-C), 149.42 (Ar-C), 152.45 (Ar-C), 157.53 (Ar-C), 175.08 ppm (C-12). MS (ESI): *m*/*z*: 524.14 $[M+H]^+$; Anal. Calcd for $C_{27}H_{25}ClN_2O_7$: C, 61.78; H, 4.80; N, 5.34. Found: C, 61.68; H, 4.93; N, 5.26.

4.3.12. 4-*N*-(2-Amino-6-chloropyridine)-4-deoxidation-4′-demethylepipodophyllotoxin (6h)

The yield 36%; white powder, ^1H NMR (400 MHz, $CDCL_3$, 25 °C, TMS): δ = 2.93 (s, 1H; H-3), 2.94 (s, 1H; H-2), 3.69 (s, 6H; OCH_3), 3.71 (d, *J* = 12 Hz, 1H; H-11), 4.33 (d, *J* = 8 Hz, 1H; H-11), 4.42 (d, *J* = 4 Hz, 1H; H-1), 5.19 (d, *J* = 4 Hz, 1H; H-4), 5.28 (d, *J* = 4 Hz, 1H; exchangeable NH), 5.82 (d, *J* = 4 Hz, 1H; H-13), 5.87 (s, 1H; H-13), 6.26 (s, 2H; Ar-H), 6.28 (d, *J* = 8 Hz, 1H; Ar-H), 6.40 (s, 1H; Ar-H), 6.57 (d, *J* = 8 Hz, 1H; Ar-H), 6.75 (s, 1H; Ar-H), 7.25 ppm (d, *J* = 8 Hz, 1H; Ar-H); ^{13}C NMR (100 MHz, $CDCL_3$, 25 °C, TMS): δ = 38.13 (C-3), 41.77 (C-2), 43.48 (C-1), 49.69 (C-4), 56.33 (OCH_3), 69.72 (C-11), 101.45 (C-13), 106.59 (Ar-C), 107.85 (Ar-C), 109.33 (Ar-C), 112.66 (Ar-C), 130.03 (Ar-C), 130.68 (Ar-C), 132.29 (Ar-C), 133.86 (Ar-C), 139.56 (Ar-C), 146.38 (Ar-C), 147.28 (Ar-C), 147.96 (Ar-C), 149.39 (Ar-C), 157.60 (Ar-C), 175.25 ppm (C-12). MS (ESI): *m*/*z*: 510.12 $[M+H]^+$; Anal. Calcd for $C_{26}H_{23}ClN_2O_7$: C, 61.12; H, 4.54; N, 5.48. Found: C, 61.16; H, 4.53; N, 5.40.

4.4. Cell culture

Five human tumor cell lines, HeLa, BGC-823, A549, Huh7, MCF-7, were cultured on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 25 cm^2 culture flasks, at 37 °C in an incubator with a humidified atmosphere containing 5% CO_2.

4.5. Evaluation of cytotoxicity in vitro

Cytotoxicity was assessed by the MTT assay[49] as an indicator of metabolically active cells. Cells were harvested from the culture during the experimental growth phase and seeded in a 96-well plate at a density of 5×10^4–1×10^5 cells/ml in fresh medium. After overnight growth, cells were treated with compounds, including PPT, DMEP and VP-16, compounds **1g**–**6g** and **1h**–**6h** with eight concentrations (0.01, 0.1, 1, 10, 50, 100, 200 and 500 μM) (pre-dissolved in DMSO) and incubated for 48 h. Afterwards, the medium was discarded and replaced with 10 μl of 5 mg/ml MTT solution (diluted in sterile PBS) diluted in serum-free medium. After the plates were incubated at 37 °C for 4 h, the resulting formazan crystals were solubilized in 100 μl DMSO, followed by shaking for 15 min on an oscillator. The optical density was read at 492 nm with a microplate reader (Biotek synergy 2). IC_{50} values were determined from a log plot of percent of control versus concentration. All assays were performed in triplicate and mean ± SD values were used to estimate cell viability.

4.6. Analysis of cell cycle

The effects of compounds on cell cycle were analyzed using flow cytometry as described by Kim et al.[50] Briefly, HeLa cells were treated with different concentrations of compounds **3g** (0.25, 0.5 and 1 μM) and **3h** (0.125, 0.25 and 0.5 μM) for 24 h. After incubation, a total of 1.5×10^5 cells were harvested from the treated and normal samples. The cells were washed twice with PBS and fixed overnight in 70% ethanol at 4 °C. The cells were then centrifuged to remove the fixative, washed three times with PBS, and incubated with 100 μg/ml of Rnase A and 50 μg/ml of PI in the dark at room temperature for 30 min to stain DNA. The cell cycle distribution was detected by FACSCalibur flow cytometer (BD).

4.7. Analysis of apoptosis

Apoptosis analysis was performed according to Ren et al.[51] Briefly, HeLa cells were treated with different concentrations of compounds **3g** (0.25, 0.5 and 1 μM) and **3h** (0.125, 0.25 and 0.5 μM) for 48 h. After incubation, cells were collected and washed twice in cold PBS and re-suspended in 200 μl of binding buffer at a concentration of 1.5×10^5 cells/ml. The samples were incubated with 5 μl of Annexin V-FITC and PI in the dark for 15 min at room temperature. Samples were then analyzed by FACSCalibur flow cytometer (BD) and evaluated based on the percentage of cells positive for Annexin V.

4.8. Western blot analysis

HeLa cells treated with different concentrations of compounds **3g** (0.25, 0.5 and 1 μM) and **3h** (0.125, 0.25 and 0.5 μM) for 48 h were harvested, rinsed twice with cold PBS, and lysed with lysis buffer. Protein concentration in the resulting lysate was determined using the bicinchoninic acid (BCA) assay. Appropriate amounts of protein (20 μg–30 μg) were separated by electrophoresis in 10–12% Tris–glycine polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked and then incubated overnight with appropriate primary antibody at dilutions specified by the manufacturer. The next day, they were washed and incubated with the corresponding HRP-conjugated secondary antibody at a 1:1000 dilution in Tris-buffer Saline-Tween 20 (10 mM, Tris–Cl [PH = 7.4], 150 mM NaCl, 0.1% Tween-20). Bound secondary antibody was detected using an enhanced chemiluminescence system (Pierce Biotechnology).

4.9. Microtubule polymerization inhibition assays

Intracellular microtubule organization was revealed by indirect IF analyses. Experiments were carried out with cells plated on glass coverslips at a density of 1.5×10^4 cells/cm^2 and grown for 24 h in control medium followed by an incubation of 24 h in the presence of different concentrations of compound **3g** (0.25, 0.5 and 1 μM) or solvent vehicle alone (0.1% DMSO). Colchicine (1 μM) was used as a positive control. At the end of the treatments, cells were fixed and stained as previously described.[52] Briefly, HeLa cells were fixed and permeabilized for 10 min with methanol at −20 °C, washed with PBS and blocked in PBS and 1% bovine serum albumin (BSA) for 15 min at room temperature. To localize tubulin, the cells were

incubated with monoclonal antitubulin antibody (clone B-5-1-2, Beyotime), 1:500 in PBS for 1 h at room temperature. As secondary antibody, we used FITC-labeled Goat Anti-Mouse IgG (Beyotime), 1:1000 in PBS and 5% BSA for 1 h at room temperature. Nuclei staining were performed by incubation with DAPI (0.25 mg/ml in PBS) for 5 min at room temperature. The coverslips were examined with a Zeiss Axiovert 200 microscope equipped with a 60× Neofluor lens. Images were acquired with an Axiocam camera (Zeiss) and PC running Axiovision software (Zeiss).

4.10. Topoisomerase II-mediated kDNA decatenation assays

Based on a previous report by Hasinoff et al.,[53] a gel assay was carried out to determine if compound **3h** inhibited the catalytic decatenation activity of topoisomerase II by the ATP-dependent decatenation of kDNA. In general, 0.2 μg catenated kDNA (TOPOGEN, Ohio) was incubated at 37 °C for 1 h in the presence of compound **3h** at different concentrations (2.5, 5 and 10 μM) and 10 μM VP-16 in a final volume of 20 μl containing 50 μM Tris–Cl (pH = 8.0), 150 mM NaCl, 10 μM $MgCl_2$, 5 μM ATP, 0.5 μM DTT and 30 μg/ml BSA. The reaction was stopped by further 30 min incubation at 37 °C with 3 μl SDS containing 1 mg/ml proteinase K. Gel electrophoresis and detection were performed as described above. Subsequently, samples were separated by submarine 1% agarose gel electrophoresis (55 V, 2 h), and gels were stained with 10 μl/100 ml ethidium bromide for 20 min. The gel was visualized under UV illumination and photographed on an Alpha Imager.

Acknowledgments

We wish to thank the editor, the associate editor, and the two anonymous reviewers for their helpful comments and suggestions, which have led to an improvement of this article.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.08.025.

References and notes

1. Kamal, A.; Suresh, P.; Mallareddy, A.; Kumar, B. A.; Reddy, P. V.; Raju, P.; Tamboli, J. R.; Shaik, T. B.; Jain, N.; Kalivendi, S. V. *Bioorg. Med. Chem.* **2011**, *19*, 2349.
2. Schrecker, A. W.; Hartwell, J. L. *J. Org. Chem.* **1956**, *21*, 381.
3. Macrae, W. D.; Hudson, J. B.; Towers, G. H. N. *Planta Med.* **1993**, *55*, 531.
4. Bohrin, L.; Rosen, B. *Drug Discovery Today* **1996**, *1*, 343.
5. Pitts, S. L.; Jablonksy, M. J.; Duca, M.; Dauzonne, D.; Monneret, C.; Adrimondo, P. B.; Anklin, C.; Graves, D.; Oscheroff, E. N. *Biochemistry* **2011**, *50*, 5058.
6. Passarellla, D.; Giardini, A.; Petretto, B.; Gabriele, F.; Sacchetti, A.; Silvani, A.; Ronchi, C.; Cappelletti, G.; Caetelli, D.; Blorlak, C.; Danieli, B. *Bioorg. Med. Chem.* **2008**, *16*, 6269.
7. Snyder, J. A.; McIntosh, R. J. *Annu. Rev. Biochem.* **1976**, *45*, 699.
8. Margolis, R. L.; Wilson, L. *Cell* **1978**, *13*, 1.
9. Yamashita, A.; Tawa, R.; Imakura, Y.; Shibuya, M.; Lee, K. H. *Mol. Pharmacol.* **1994**, *47*, 1920.
10. Canela, C.; Moraesb, R. M.; Dayana, F. E.; Ferreira, D. *Phytochemistry* **2000**, *54*, 115.
11. Wang, Z. Q.; Kuo, Y. H.; Schnur, D.; Bowen, J. P.; Liu, S. Y.; Han, F. S.; Cheng, Y. C.; Lee, K. H. *J. Med. Chem.* **1990**, *33*, 2660.
12. Baldwin, E. L.; Osheroff, N. *Curr. Med. Chem. Anticancer Agents* **2005**, *5*, 363.
13. Kamal, A.; Kumar, B. A.; Arifuddin, M. *Tetrahedron Lett.* **2003**, *44*, 8457.
14. MacDonald, T. L.; Lehnert, E. K.; Loper, J. T.; Chow, K. C.; Ross, W. E. *DNA Topoisomerase in Cancer*; Oxford University Press: New York, 1991; pp 119–214.
15. Kamal, A.; Gayatri, N. L.; Reddy, D. R.; Reddy, M. M.; Arifuddin, M.; Dastidar, S. G.; Kondapi, A. K.; Rajkumar, M. *Bioorg. Med. Chem.* **2005**, *13*, 6218.
16. Kamal, A.; Kumar, B. A.; Suresh, P.; Juvekar, A.; Zingde, S. *Bioorg. Med. Chem.* **2011**, *19*, 2975.
17. Kamal, A.; Kumar, B. A.; Suresh, P.; Shankaraiah, N.; Kumar, M. S. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 350.
18. Zhou, X. M.; Wang, Z. Q.; Chang, J. Y.; Chen, H. X.; Cheng, Y. C.; Lee, K. H. *J. Med. Chem.* **1991**, *34*, 3346.
19. Xiao, Z.; Xiao, Y. D.; Golbraikh, A.; Tropsha, A.; Lee, K. H. *J. Med. Chem.* **2002**, *45*, 2294.
20. Van Vilet, D. S.; Tachibana, Y.; Bastow, K. F.; Huang, E. S.; Lee, K. H. *J. Med. Chem.* **2001**, *44*, 1422.
21. Castro, M. A.; Corral, J. M.; Gordaliza, M.; Garcia, P. A.; Zurita, M. A.; Feliciano, A. S. *J. Med. Chem.* **2004**, *47*, 1214.
22. Bhat, B. A.; Reddy, P. B.; Kumar Agrawal, S. K.; Saxena, A. K.; Sampath Kumar, H. M.; Qazi, G. N. *Eur. J. Med. Chem.* **2008**, *43*, 2067.
23. Reddy, D. M.; Srinivas, J.; Chashoo, G.; Saxena, A. K.; Sampath Kumar, H. M. *Eur. J. Med. Chem.* **2011**, *46*, 1983.
24. Lee, K. H.; Beers, S. A.; Mori, M.; Wang, Z. Q.; Kuo, Y. H.; Li, L.; Liu, S. Y.; Cheng, J. Y.; Chan, F. S.; Cheng, Y. C. *J. Med. Chem.* **1990**, *33*, 1364.
25. Kamal, A.; Kumar, A.; Arifuddin, M.; Dastidar, S. G. *Bioorg. Med. Chem.* **2003**, *11*, 5135.
26. Kamal, A.; Kumar, B. A.; Arifuddin, M. *Tetrahedron Lett.* **2003**, *44*, 8457.
27. Chang, H.; Shyu, H. G.; Lee, C. C.; Tsai, S. C.; Wang, B. W.; Lee, Y. H.; Lina, S. K. *Biochem. Biophys. Res.* **2003**, *302*, 95.
28. Kamal, A.; Lakshmi Gayatri, N. *Tetrahedron Lett.* **1996**, *37*, 3359.
29. Daley, L.; Meresse, P.; Bertounesque, E.; Monneret, C. *Tetrahedron Lett.* **1997**, *38*, 2673.
30. Zhang, Y. L.; Guo, X.; Chen, Y. C.; Lee, K. H. *J. Med. Chem.* **1994**, *37*, 446.
31. Zhang, J. Q.; Zhang, Z. W.; Hui, L.; Chen, S. W.; Tian, X. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 983.
32. Lu, Y. X.; Shi, T.; Wang, Y.; Yang, H. Y.; Yan, X. H.; Luo, X. M.; Jiang, H. L.; Zhu, W. L. *J. Med. Chem.* **2009**, *52*, 2854.
33. Beers, S. A.; Imakura, Y.; Dai, H. J.; Li, D. H.; Cheng, Y. C.; Lee, K. H. *J. Nat. Prod.* **1988**, *51*, 901.
34. Thurston, L. S.; Imakura, Y.; Haruna, M.; Li, D. H.; Liu, Z. C.; Liu, S. Y.; Cheng, Y. C.; Lee, K. H. *J. Med. Chem.* **1989**, *32*, 604.
35. Knudsen, K. E.; Booth, D.; Naderi, S.; Sever-chroneos, Z.; Fribourg, A. F.; Hunton, I. C.; Feramisco, J. R.; Wang, J. Y.; Knudsen, E. S. *Mol. Cell. Biol.* **2000**, *20*, 7751.
36. Hu, C. Q.; Xu, D. Q.; Du, W. T.; Qian, S. J.; Wang, L.; Lou, J. S.; He, Q. J.; Yang, B.; Hu, Y. Z. *Mol. BioSyst.* **2010**, *6*, 410.
37. Zhao, Y.; Hui, J.; Wang, D.; Zhu, L.; Fang, J. H.; Zhao, X. D. *Chem. Pharm. Bull.* **2010**, *58*, 1324.
38. Kaufmann, S. H.; Earnshaw, W. C. *Exp. Cell. Res.* **2000**, *256*, 42.
39. Salvesen, G. S.; Dixit, V. M. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10964.
40. Pugazhenthi, S.; Nesterova, A.; Sable, C.; Heidenreich, K. A.; Boxer, L. M.; Heasley, L. E.; Reusch, J. E. *J. Biol. Chem.* **2000**, *275*, 10761.
41. Qi, S. N.; Jing, Y. X.; Dong, G. X.; Chen, Y.; Yoshida, A.; Ueda, T. *Oncol. Rep.* **2007**, *18*, 273.
42. Liu, J.; Li, Y.; Ren, W.; Hu, W. X. *Cancer Lett.* **2006**, *242*, 133.
43. Cohen, G. M. *Biochem. J.* **1997**, *326*, 1.
44. Jordan, S. F.; Scott, W. L. *Oncogene* **2003**, *22*, 9030.
45. Md, A.; Pradeep, K. N. *J. Mol. Graph. Model* **2009**, *27*, 930.
46. Desben, S.; Giorgi-Renault, S. *Curr. Med. Chem.* **2002**, *2*, 71.
47. Zhu, X. K.; Guan, J.; Xiao, Z. Y.; Cosentino, L. M.; Lee, K. H. *Bioorg. Med. Chem.* **2004**, *12*, 4267.
48. Lee, K. H.; Beers, S. A.; Mori, M.; Wang, Z. Q.; Kuo, Y. H.; Li, L.; Liu, S. Y.; Chang, J. Y.; Han, F. S.; Cheng, Y. C. *J. Med. Chem.* **1990**, *33*, 1364.
49. Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827.
50. Kim, N. D.; Yoon, J.; Kim, J. H.; Lee, J. T.; Chen, Y. S.; Hwang, M. K.; Ha, L.; Song, W. *Bioorg. Med. Chem.* **2006**, *15*, 3772.
51. Ren, J.; Wu, L.; Xin, W. Q.; Chen, X.; Hu, K. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4778.
52. Hamel, E. *Cell Biochem. Biophys.* **2003**, *38*, 1.
53. Hasinoff, B. B.; Kazlowska, H.; Creighton, A. M.; Allan, W. P.; Thampatty, P.; Yalowich, J. C. *Mol. Pharmacol.* **1997**, *52*, 839.