Bioorganic & Medicinal Chemistry 20 (2012) 2054–2066

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

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

Synthesis and biological evaluation of 4β-sulphonamido and 4β-[(4′-sulphonamido)benzamide]podophyllotoxins as DNA topoisomerase-IIα and apoptosis inducing agents

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ARTICLE INFO

Article history:
Received 28 November 2011
Revised 23 January 2012
Accepted 24 January 2012
Available online 9 February 2012

Keywords:
Etoposide
Cell cycle
Anticancer activity
Comet assay
Topo-IIα enzyme

ABSTRACT

A series of new 4β-sulphonamido and 4β-[(4′-sulphonamido)benzamide] conjugates of podophyllotoxin (**11a–j** and **15a–g**) were synthesized and evaluated for anticancer activity against six human cancer cell lines and found to be more potent than etoposide. Some of the compounds **11b**, **11d** and **11e** that showed significant antiproliferative activity in Colo-205 cells, were superior to etoposide. The flow cytometric analysis indicates that these compounds (**11b**, **11d** and **11e**) showed G2/M cell cycle arrest and among them **11e** is the most effective. It is observed that this compound (**11e**) caused both single-strand DNA breaks as observed by comet assay as well as double-strand DNA breaks as indicated by γ-H2AX. Further **11e** showed inhibition of topo-IIα as observed from Western blot analysis and related studies. Compounds caused activation of ATM as well as Chk1 protein indicating that the compound caused effective DNA damage. Moreover activation of caspase-3, p21, p16, NF-kB and down regulation of Bcl-2 protein suggests that this compound (**11e**) has apoptotic cell death inducing ability, apart from acting as a topo-IIα inhibitor.

1. Introduction

Chemotherapy is one of the main method to treat cancer. However during the course of cancer chemotherapy, it has been found that prolonged treatment of carcinoma patients with certain anticancer medicines can result in an acquired resistance toward multiple drugs, that is MDR.[1] The problem of increasingly severe MDR of tumor is a significant obstacle in providing effective chemotherapy to many patients. Development of novel antitumor agents with cytotoxicity against MDR cancer cell lines has currently become the focus of oncology.

Podophyllotoxin (**1**), a well-known naturally occurring aryltetralin lignan, is extracted as the main component from the roots and rhizomes of *Podophyllum* species such as *Podophyllum hexandrum* and *Podophyllum peltatum*.[2] The biological activity of podophyllotoxin has led to several structural modifications resulting in many useful compounds such as etoposide (**2**), teniposide (**3**) and the water-soluble prodrug, etoposide phosphate (**4**). In addition, some newly developed derivatives (e.g., NPF (**5**) and GL-331 (**6**)) display a better pharmacological profile and are currently in clinical trials. Etoposide is widely used in clinic, alone or in association with other chemotherapeutics for the treatment of testicular carcinomas and small cell lung cancer.[3] However its current therapeutic use is somewhat limited by myelosuppression, particularly neutropenia. Unlike podophyllotoxin, etoposide and teniposide do not inhibit tubulin polymerization but induce dose-dependent DNA-strand breakage associated with inhibition of DNA-topoisomerase II.[4,5]

In an ongoing effort to develop new and more potent anticancer agents, we have been involved in the last few years towards the development of new molecules based on podophyllotoxins.[6,7] In the present investigation, we report the synthesis and cytotoxic evaluation of 4β-sulphonamido and 4β-[(4′-sulphonamido)benzamide] derivatives of 4-desoxypodophyllotoxin. The choice of the sulphonamide group was dictated by the fact that it constitutes an important class of drugs, with several types of pharmacological agents possessing antitumor, anti-carbonic anhydrase, or protease inhibitory activity.[8] A host of structurally novel sulphonamide derivatives have recently been reported to show substantial antitumor activity. Some classical clinical anticancer agents that comprise of sulphonamide groups are acetazolamide (**7**), methazolamide (**8**) and ethoxzolamide (**9**)[8a] as shown in Figure 1.

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doi:10.1016/j.bmc.2012.01.039

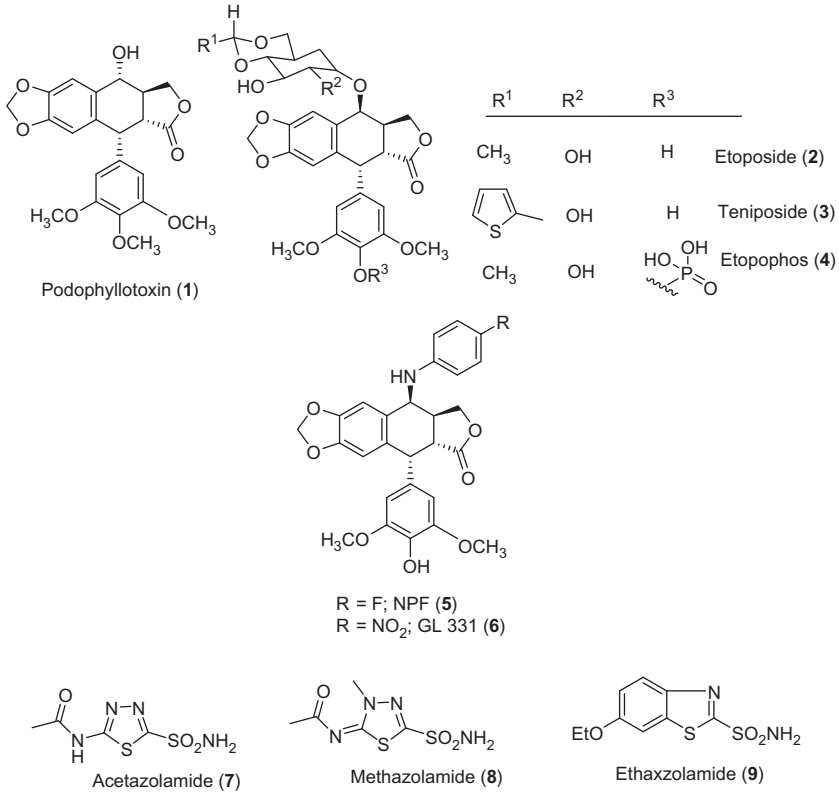


Figure 1. Structures of podophyllotoxin derivatives.

2. Chemistry

The synthesis of new 4β-sulphonamido and 4β-[(4′-sulphonamido)benzamide] congeners of 4-desoxypodophyllotoxin (**11a–j** and **15a–g**) have been carried out from podophyllotoxin as shown in Scheme 1. The key intermediates for the preparation of these podophyllotoxin derived compounds are 4β-amino podophyllotoxin (**10**)[6b,7a] and 4β-[(4′-amino)benzamide]podophyllotoxin (**14**). 4β-Aminopodophyllotoxin is coupled with 4-nitrobenzoyl chloride (**12**) in presence of Et_3N in CH_2Cl_2 to afford **13**, which upon reduction with Pd/C affords the intermediate **14**. These intermediates, 4β-aminopodophyllotoxin (**10**) and 4β-[(4′-amino)benzamide]podophyllotoxin (**14**) are then coupled with different heteroaromatic sulphonylchlorides in presence of Et_3N in CH_2Cl_2 to provide the desired sulphonamide podophyllotoxins (**11a–j** and **15a–g**). All the synthesized compounds were characterized by ^1H NMR, ^{13}C NMR, and mass spectral data.

3. Biological evaluation

3.1. Invitro cytotoxicity assay

The synthesized compounds **11a–j** and **15a–g** were evaluated for their anticancer activity in selected human cancer cell lines of breast, oral, colon, lung and ovarian origin by using sulforhodamine B (SRB) method.[9] The compounds that exhibit $GI_{50} \leqslant 10^{-5}$ M are considered to be active on the respective cell lines and the results are illustrated in Table 1. All the compounds (**11a–j** and **15a–g**) exhibit significant anticancer activity with GI_{50} values ranging from 0.04 to 2.90 μM, while the positive controls, etoposide and adriamycin demonstrated the GI_{50} in the range of 0.13–3.08 μM and 0.10–7.25 μM, respectively. The significant anticancer activity showed by the promising compounds **11b**, **11d** and **11e** prompted us to evaluate their cell viability in the Colo-205 cells, with a view to study their detailed biological effects in this cell line. Colo-205 cells were treated with compounds **11b, 11d, 11e** and etoposide (**Eto**) at 2, 4 and 8 μM concentration for 24 h and it was observed that at 4 μM concentration (Fig. 2) the cell death was reasonably effective. All these compounds showed significant cell death, with compound **11e** being the most effective.

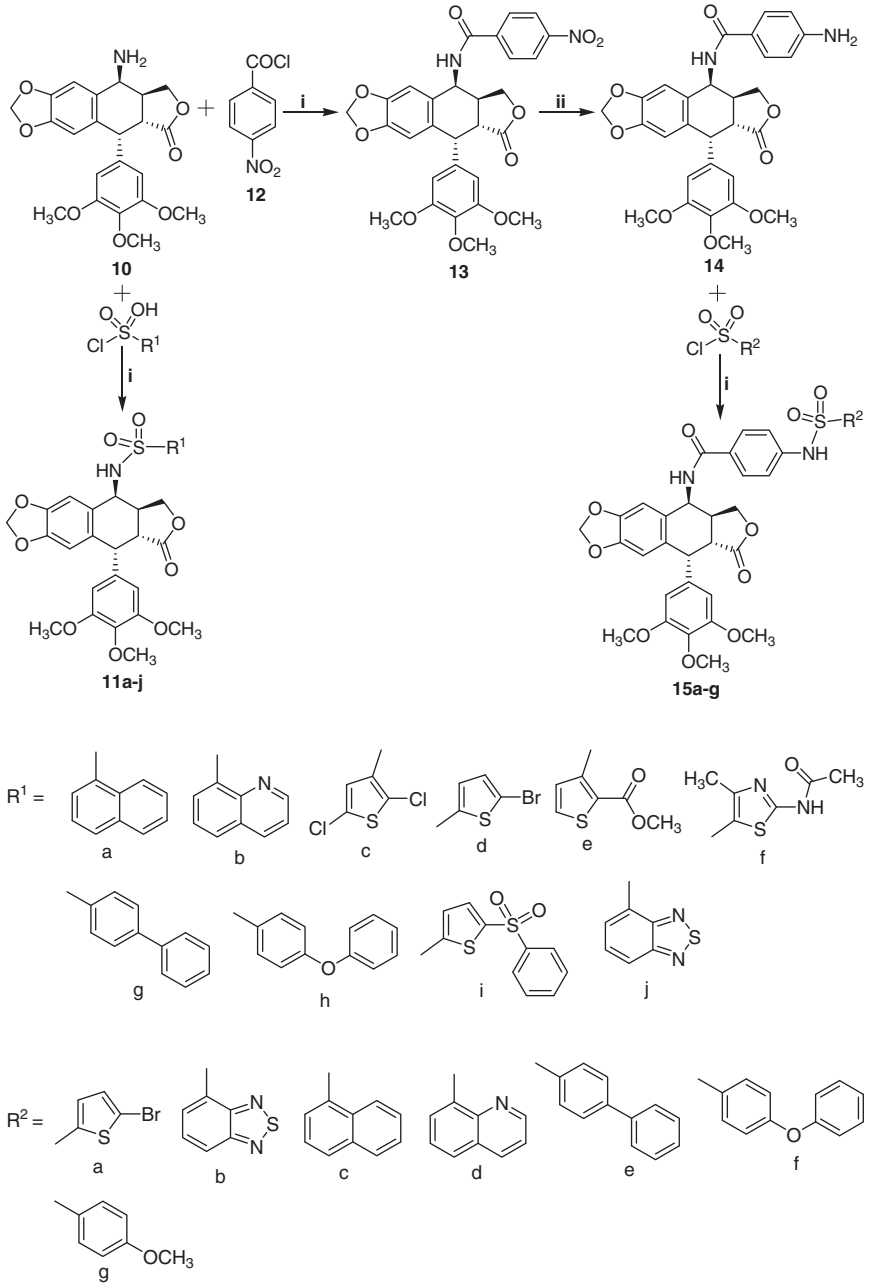
3.2. Effect of podophyllotoxin conjugates on the cell cycle

In order to understand the role of these compounds (**11b**, **11d**, **11e** and etoposide) in cell cycle, FACS analysis was conducted in colon cancer cells (Colo-205).[10] The cells were treated for 24 h with compounds **11b**, **11d**, **11e** and etoposide (**Eto**) at 4 μM concentration. It was observed that Colo-205 cells showed 74%, 28%, 37% and 40% of G2/M cell cycle arrest by etoposide (**Eto**), **11b**, **11d** and **11e** respectively, whereas control (untreated cells) exhibited 26% (Fig. 3a).

Another independent experiment was carried out to observe the influence of HDAC inhibitor like trichostatin A (TSA) on cell cycle regulation caused by **11e** and etoposide (**Eto**). TSA produces dramatic change in cell cycle arrest from G2/M phase to G0 phase as indicated by profound cell death. The cell death observed was 57% and 56% in case of **11e** and etoposide (**Eto**), respectively (Fig. 3b), whereas control cells cause 4% of cell death. This observation was supported by the fact that HDAC inhibitors stabilize the DNA-topo-II complexes and cause cell death.[11,12]

3.3. Effect of 11e on DNA damage

Recent studies have indicated that many podophyllotoxin based compounds act as DNA topoisomerase-II (topo-II) inhibitors which stabilize the DNA-topo-II complexes causing the DNA double-



Scheme 1. Reagents and conditions: (i) Et_3N, CH_2Cl_2, rt, 3 h; (ii) Pd/C, H_2, EtOAc, rt, 12 h.

strand breaks.[13] Muslimovic and co-workers have shown that etoposide mainly cause single-strand DNA breaks up to 90%.[14] Broomberg and co-workers have shown that etoposide induces single-strand breaks (SSB) apart from double-strand breaks (DSB) depending upon the binding to monomer.[15] Thus we attempted to examine the effect of these podophyllotoxin conjugates for SSB as well as DSB. Hence Colo-205 cells were treated with the most effective compound (**11e**) of the series and compared with etoposide (**Eto**) at 4 μM concentration for 24 h. We observed up to 60% of SSB as indicated by the comet assay and DSB as indicated by γ-H2AX foci formation in the compound **11e** treated cells. Compound **11e** caused SSB almost similar to etoposide in Colo-205 cancer cells. However the efficiency of causing DSB was only 50%. The decreased DSB gave a thought that **11e** may function as topo-IIα inhibitor as the formation of DSB (i.e., γ-H2AX foci) needs the endogenous expression of topo-IIα, but not the topo-IIβ (Figs. 4a and 4b).

3.4. Effect of 11e on topoisomerase-IIα

DNA topoisomerase-II protein exists as two isoforms. It is well established that throughout the cell cycle of rapidly proliferating cells, topo-IIα is expressed relatively higher than topo-IIβ.[16,17] To further confirm the nature of topo-II inhibition of compound **11e**, Colo-205 cells were treated with **11e** and etoposide (**Eto**) at 4 μM concentration for 24 h. The topo-IIα down regulated in case of **11e** whereas topo-IIβ was down regulated in case of etoposide (**Eto**) treated Colo-205 cells (Fig. 5a). Our observations are in conjunction with the previous studies wherein etoposide is known to inhibit topo-IIβ (100%) and to a lesser extent of topo-IIα inhibition as observed by Western blot studies.[13] Attempts were made to observe the effect of TSA on topo-IIα inhibition caused by **11e**, etoposide (i.e., topo-II inhibitor) and camptothecin (i.e., topo-I inhibitor) compounds. The expression of topo-IIα was increased in the combinational treatments of TSA with **11e**/or etoposide (**Eto**). But not

Table 1
Cytotoxic activity (GI_{50} μM[a]) of compounds **11a–j** and **15a–g**

Compd	Zr-75-1[b]	MCF7[b]	KB[c]	DWD[c]	Colo 205[d]	A549[e]	Hop62[e]	A2780[f]
11a	2.10	0.09	2.30	2.00	2.10	0.16	2.40	1.91
11b	0.08	0.12	0.06	0.17	0.04	0.04	0.06	0.08
11c	2.50	0.11	2.10	0.15	0.15	0.14	2.00	0.16
11d	0.18	0.13	0.04	0.05	0.04	0.12	0.04	0.06
11e	0.06	0.18	0.04	0.04	0.04	0.15	0.04	0.05
11f	0.17	0.15	0.17	0.14	0.11	0.12	0.16	0.15
11g	2.30	2.61	nd	2.21	2.71	2.00	2.01	2.11
11h	2.31	2.21	2.31	2.91	nd	2.11	nd	2.40
11i	2.11	0.14	2.11	2.61	nd	nd	nd	2.30
11j	2.51	2.21	nd	nd	nd	2.71	2.21	2.311
15a	2.90	2.71	nd	2.50	nd	nd	2.01	2.11
15b	2.23	0.17	nd	2.80	nd	nd	2.91	2.11
15c	2.81	2.32	2.00	nd	2.12	2.60	2.22	2.00
15d	nd	2.90	nd	0.14	2.31	2.51	2.61	2.42
15e	2.11	2.30	2.50	2.88	2.80	nd	nd	2.51
15f	2.90	nd	2.01	2.10	nd	2.70	2.00	nd
15g	2.12	0.16	nd	2.02	2.51	nd	2.71	nd
Eto[g]	0.20	2.11	0.31	0.62	0.13	3.08	0.80	1.31
ADR[h]	1.79	0.17	0.17	0.10	0.14	7.25	0.14	0.16

[a] 50% Growth inhibition and the values are mean of three determinations.
[b] Breast cancer.
[c] Oral cancer.
[d] Colon cancer.
[e] Lung cancer.
[f] Ovarian cancer.
[g] Eto (etoposide).
[h] ADR (adriamycin), nd = not determined.

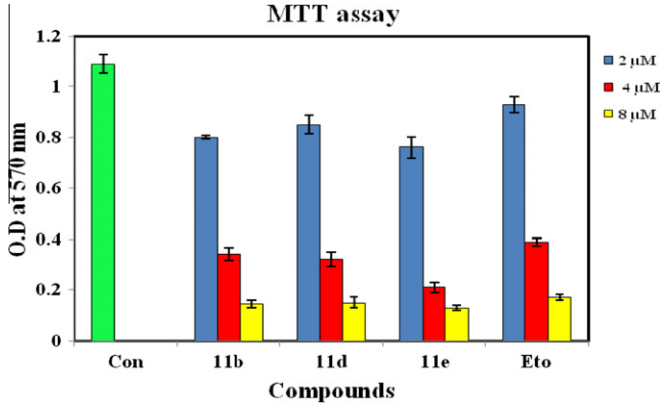


Figure 2. Colo-205 cells were treated with compounds **11b**, **11d**, **11e** and etoposide (**Eto**) at 2, 4 and 8 μM concentration for 24 h. The MTT cell viability assay was conducted and the optical density (O.D) was observed at 570 nm. Control indicates the untreated cells.

in cells treated with TSA and camptothecin (**CPT**) combination, this confirms the previous observations that HDAC inhibitors stabilize topo-II protein expression[18] and HDAC proteins interact with topo-II but not topo-I.[19] Thus we conclude that the cytotoxic event caused by compound **11e** and TSA is an additive effect on apoptosis (Figs. 5b and 5c). To further confirm the effectiveness of **11e** towards topo-IIα inhibition we carried out the topo-IIα inhibition assay using topoisomerase-IIα enzyme and PBR322 DNA.[20] The compounds (**11e** and etoposide) have shown inhibitory effect on topoisomerase-IIα mediated DNA digestion at 50 μM concentration (Fig. 5d).

3.5. Effect of 11e on ATM protein

Ataxia telengiectasia mutated (ATM) protein plays a key role as signal transducer in response to induction of DSB.[21] It was considered of interest to examine the activated ATM protein in compounds **11e** and etoposide (**Eto**) treated Colo-205 cells at 4 μM concentration. Interestingly the activation of ATM was observed in both **11e** and etoposide (**Eto**) treated cells (Fig. 6).

3.6. Effect of 11e on Chk1 activity

Check point protein (Chk1) is a G2/M specific serine threonine kinase and is activated by phosphorylation on ser 345.[22,23] To further investigate the G2/M arrest and DNA damage caused by **11e** and etoposide (**Eto**) Colo-205 cells were analyzed for Chk1-phosphorylation 24 h after the compounds (**11e** and **Eto**) treatment. Increase in Chk1 phosphorylation in both **11e** as well as etoposide (**Eto**) treated cells was observed (Fig. 7). A similar kind of observation was made by Guo and co-workers,[24] wherein benzophenanthridine (NK-314) caused the Chk1 activation during DNA damage.

3.7. Effect of 11e on apoptosis

Previous studies established that the inhibition of DNA topo-II enzyme caused the replicating cells to undergo apoptosis.[25] That is upon receiving an apoptotic signal the precursor caspase undergoes proteolytic cleavage and generates an active subunit.[26] Thus as a part of understanding apoptosis, we examined the role of caspase-3, the effector caspase in compound **11e** as well as etoposide (**Eto**) treated cells. It was observed that there was an increase of caspase-3 activation in both ELISA and Western blot analysis (Fig. 8). In previous studies a similar observation was reported for deoxypodophyllotoxins wherein G2/M cell cycle arrest and apoptosis are caspase-dependent.[10]

Further we were interested to see the involvement of mitochondria as central key organelle in the apoptotic process. Bcl-2 is the oncoprotein that play a major role in apoptosis.[27] Thus an ELISA based assay against Bcl2 protein was conducted by treating Colo-205 cells with **11e**, etoposide (**Eto**) and camptothecin (**CPT**). We have observed strong down regulation of Bcl2 in compound treated cells confirming the role of mitochondria in this apoptotic event (Fig. 9).

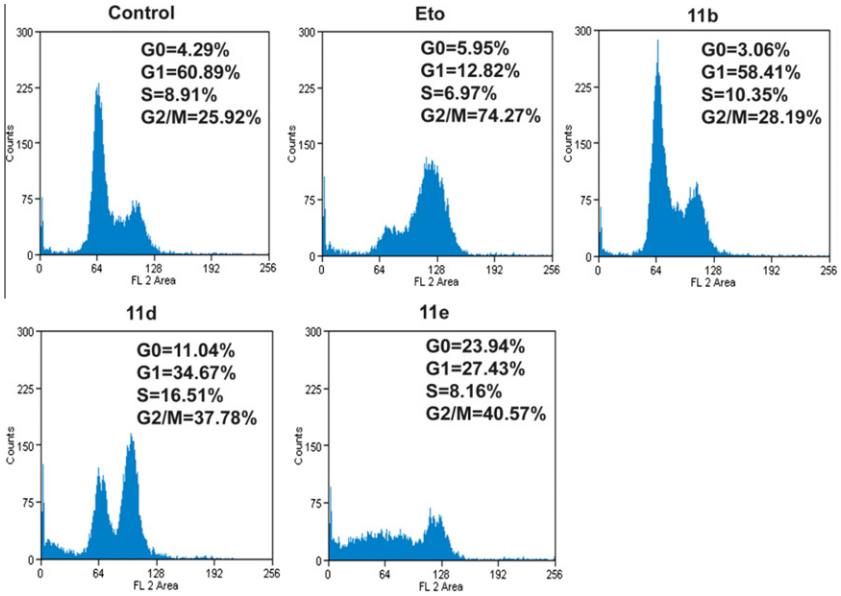


Figure 3a. Colo-205 cells were treated with compounds **11b**, **11d**, **11e** and etoposide (**Eto**) at 4 μM concentration for 24 h. The cells were further processed for FACS analysis. Compounds have shown G2/M cell cycle arrest. Control indicates the untreated cells.

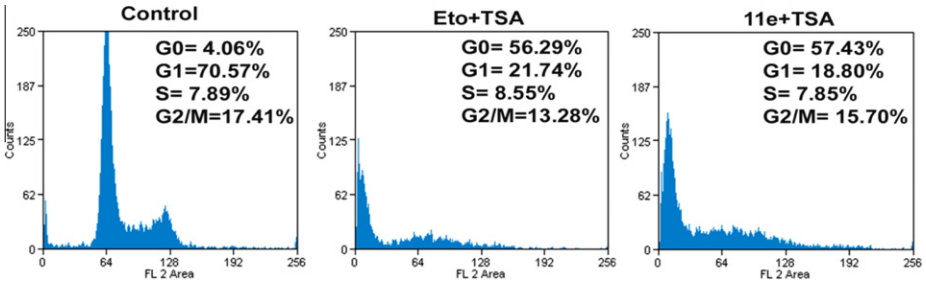


Figure 3b. Effect of trichostatin A (TSA), the known HDAC inhibitor on the cell cycle arrest caused by **11e** and etoposide (**Eto**) in Colo-205 cells at 4 μM concentration for 24 h. Addition of TSA to the cells shifted cells from G2/M arrest towards G0 phase (cell death). Control indicates the untreated cells.

3.8. Effect of 11e on p21, p16, NF-kB and caspase-3 proteins

It is known that tumor suppressor proteins such as p21 blocks the formation of tumor,[28] whereas p16 is another protein that causes senescence. Further it is well established that NF-kB plays an important role in topoisomerase-II inhibition by inducing the Fas ligand. Thus in view of the above aspects Colo-205 cells were treated with **11e** as well as etoposide (**Eto**) and the levels of p21, p16 and NF-kB proteins were analyzed. To our surprise we observed the upregulation of p21, p16 and NF-kB proteins,[29] thus suggesting the role of podophyllotoxin conjugate such as **11e** as on effective DNA topo-IIα inhibitor that possesses effective apoptotic inducing ability (Fig. 10).

4. Conclusion

In conclusion, a series of new 4β-sulphonamido and 4β-[(4′-sulphonamido)benzamide] conjugates of podophyllotoxin (**11a–j** and **15a–g**) were synthesized. These conjugates (**11a–j** and **15a–g**) were tested for anticancer activity against six human cancer cell lines and were found to be more potent than etoposide. The significant anticancer activity showed by some of the promising compounds like **11b**, **11d** and **11e** prompted us to evaluate the cell viability in the Colo-205 cells, with a view to study their detailed biological effects in this cell line. The treatment of Colo-205 cells with these conjugates (**11b, 11d, 11e**) and etoposide (**Eto**) at 2, 4 and 8 μM concentration for 24 h showed that the optimum concentration for effective cell death was 4 μM. FACS studies indicated the occurrence of G2/M cell cycle arrest in all the compounds studied with **11e** being the most effective. These compounds caused both single-strand DNA breaks (SSB) as studied by comet assay as well as double strand DNA breaks (DSB) as indicated by γ-H2AX in Colo-205 cells. Surprisingly there was reduction in the number of cells that express double-stranded breaks in **11e** compared to etoposide, as indicated by number of cells with γ-H2AX staining. This compound showed inhibitory effect on topoisomerase-IIα as observed from both Western blot analysis as well as topoisomerase-IIα enzyme inhibition studies. Similarly **11e** caused DNA damage with the activation of key signaling molecules such as ATM and Chk1. The data generated also indicate concrete evidence for the fact that rapidly replicating cells undergo apoptosis with the inhibition of topoisomerase-IIα with the activation of caspase-3 and down regulation of Bcl-2 proteins. Thus this study provides the development of a new class of podophyllotoxin conjugates as effective topoisomerase-IIα inhibitors with apoptotic death inducing ability. Moreover these findings provide an insight for future direction in the design and development of such conjugates of podophyllotoxin.

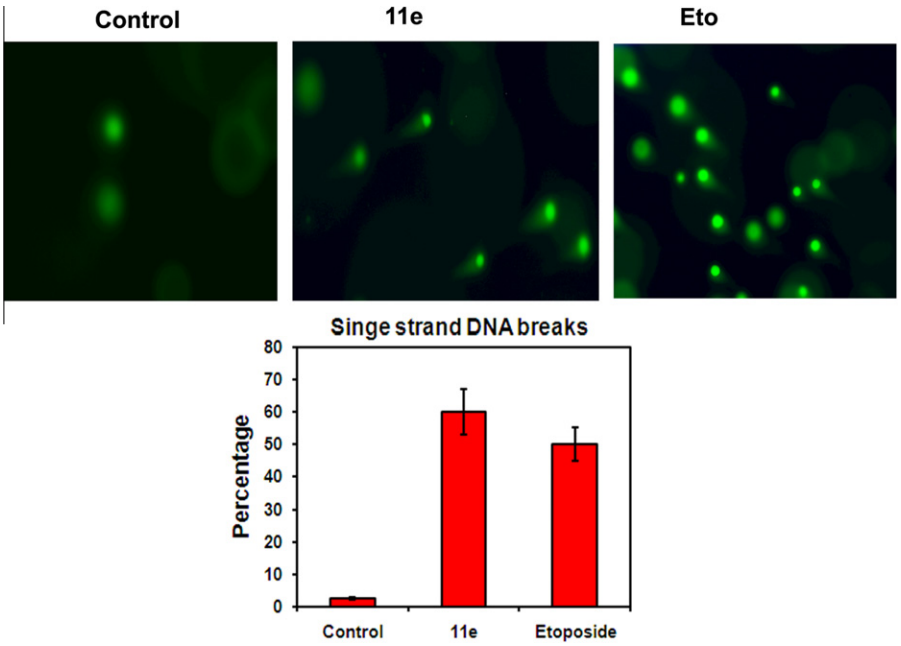


Figure 4a. The comet assay was conducted to observe the induction of single-strand DNA breaks due to compound **11e** and etoposide (**Eto**) at 4 μM concentration for 24 h. SYBR green-I dye was used to stain the DNA in both control and compound treated cells (**11e**, **Eto**). Etoposide has been employed as the positive control. Single-strand DNA breaks were identified due to the bright head and tail formation up on compound treatment. **11e** and etoposide (**Eto**) has shown single-strand DNA breaks. Whereas control cells have very negligible level of single-strand breaks as shown in this comet assay.

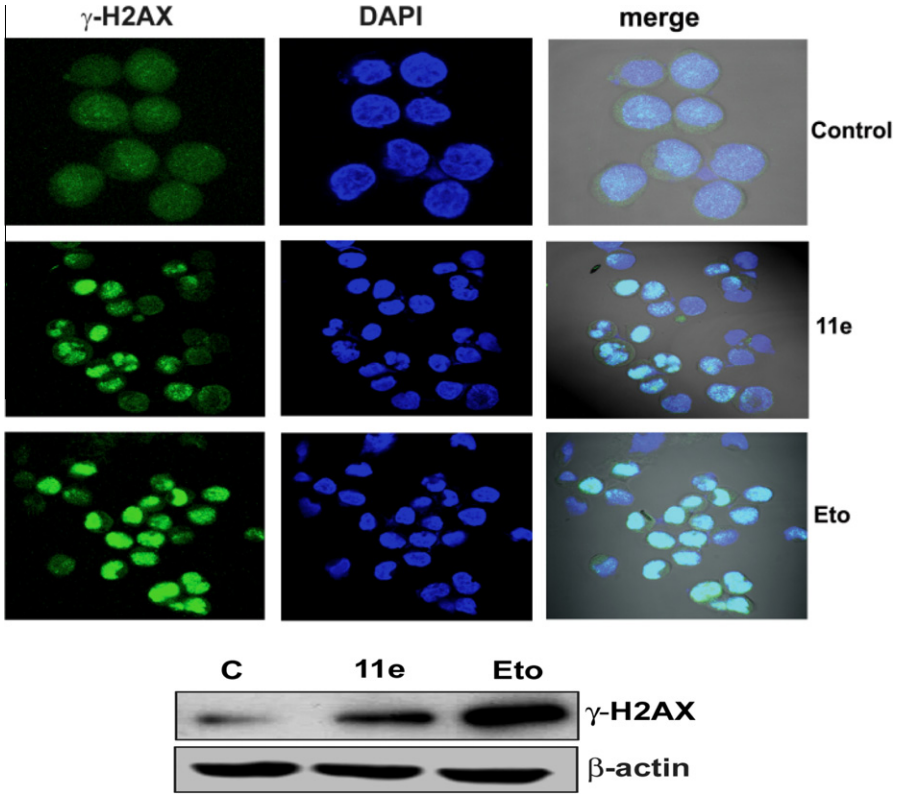


Figure 4b. γ-H2AX staining and Western blot analysis was carried out to observe the induction of double-stranded DNA breaks due to compound **11e** and etoposide (**Eto**) using antibody against ser139 residue of H2AX protein, the indicator of double stranded DNA breaks at 4 μM concentration for 24 h.

5. Experimental section

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) and were used without further purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 F-254, and visualization on TLC was achieved by UV light or iodine indicator. ^{1}H and ^{13}C NMR spectra

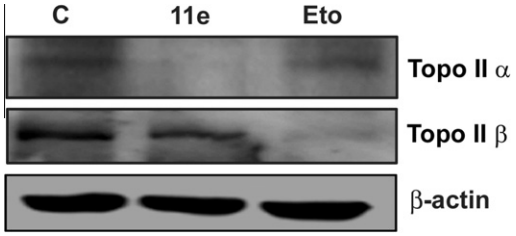


Figure 5a. The effect of compounds **11e** and etoposide (**Eto**) in Colo-205 cells on topo-IIα and β protein expression. Cells were treated with compounds **11e** and etoposide (**Eto**) at 4 μM for 24 h. Topo-IIα and β protein levels were examined in Western blot analysis. β-Actin was used as loading control.

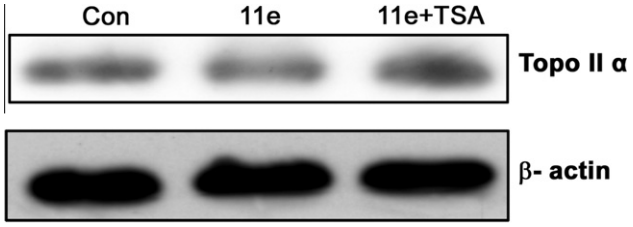


Figure 5b. The effect of compounds TSA on topo-IIα protein expression. Colon cancer cells (colo-205) were treated with compounds **11e** and (**11e** + **TSA**) combination at 4 μM for 24 h. Topo-IIα protein level was examined by conducting Western blot analysis. β-Actin was used as loading control. TSA with combination of **11e** induced expression of topo-IIα in this study.

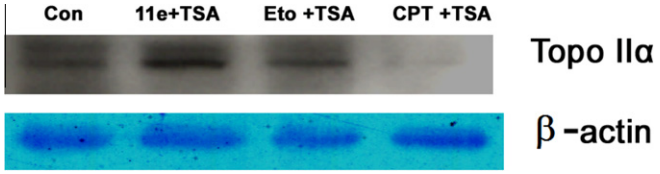


Figure 5c. The effect of combination of TSA and various DNA damaging agents on topo-IIα protein expression. Colon cancer cells (colo-205) were treated with compounds **11e** + **TSA**, **Eto** + **TSA** and **CPT** + **TSA** at 4 μM of each drug for 24 h. Topo-IIα protein level was examined by conducting Western blot analysis. β-Actin was used as loading control. TSA induced the expression of topo-IIα in the combination (**11e** & **TSA**; **Eto** + **TSA**) drugs used in this study, but not in the topo-I inhibitor (**CPT**) case. This shows the specificity of TSA towards topo-IIα induction.

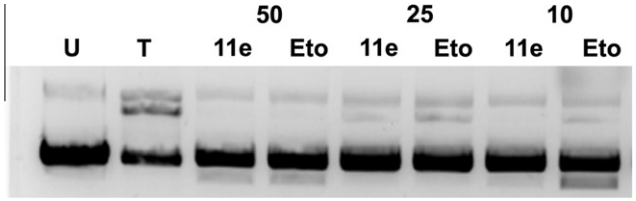


Figure 5d. The effect of compounds **11e** and etoposide (**Eto**) on topoisomerase-IIα enzyme inhibition. PBR322 plasmid DNA and compounds **11e** and etoposide (**Eto**) were added at 50, 25 and 10 μM final concentration along in which topo-IIα enzyme (4U/μL sigma company). The buffer used for enzyme activity was prepared fresh in which ATP, KCL, $MgCl_2$, DTT, Tris pH-8.0 components. The reaction was performed at 37 °C for 30 min. Here T represents the topo-II digestion of DNA with out any compound. U, represents the PBR322 DNA alone. Both **11e** and etoposide (**Eto**) inhibit the topo-IIα inhibition at 50 μM concentration.

were recorded on INOVA (400 MHz) or Gemini Varian-VXR-unity (200 MHz) or Bruker UXNMR/XWIN-NMR (300 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from internal TMS standard. ESI spectra were recorded on Micro mass, Quattro LC using ESI^+ software with capillary voltage 3.98 kV and ESI mode positive ion trap detector. Melting points were determined with an Electrothermal melting point apparatus, and are uncorrected.

5.1. 4β-[Naphthalene-1-sulphonamido]podophyllotoxin (11a)

The compound 4β-aminopodophyllotoxin (**10**) (200 mg, 0.483 mmol) was dissolved in 10 mL of dried dichloromethane, followed by addition of naphthalene-1-sulphonylchloride (131 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.966 mmol). The reaction mixture was stirred at room temperature for 3 h, till the completion of the reaction as monitored by TLC. The reaction mixture was washed with water and extracted with dichloromethane, dried over anhydrous Na_2SO_4 and the crude product was purified by column chromatography with ethyl acetate/hexane (1:1) to obtain pure compound **11a** in 260 mg, 93% yield. Mp: 139–142 °C, $[\alpha]_D^{25} = -30.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (200 MHz, $CDCl_3$): δ 2.71–2.88 (m, 1H), 2.91–3.00 (dd, 1H, $J = 4.9$, 4.6 Hz), 3.69 (s, 6H), 3.97 (s, 3H), 4.20–4.27 (m, 3H), 4.45–4.51 (m, 1H), 4.88 (d, 1H, $J = 6.6$ Hz), 5.14 (s, 1H), 5.75 (d, 2H, $J = 5.8$ Hz), 6.16 (s, 2H), 6.36 (s, 1H), 7.63–7.70 (m, 3H), 8.06 (t, 1H, $J = 6.6$, 3.3 Hz), 8.24 (d, 1H, $J = 7.4$ Hz), 8.41 (d, 1H, $J = 7.4$ Hz), 8.58 (t, 1H, $J = 3.3$, 5.8 Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.3, 43.0, 48.7, 51.0, 55.4, 59.8, 67.9, 100.7, 107.5, 107.9, 108.8, 123.9, 124.3, 126.5, 127.2, 127.8, 128.7, 128.9, 132.1, 133.6, 133.9, 134.8, 135.5, 136.1, 146.4, 147.2, 151.7; MS (ESI): 621 $[M+Na]^+$.

5.2. 4β-[Quinoline-8-sulphonamido]podophyllotoxin (11b)

This compound **11b** was prepared following the method described for the preparation of the compound **11a**, employing **10** (200 mg, 0.483 mmol) with quinoline-8-sulphonylchloride (131 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.966 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (4:6) to afford pure compound **11b** in 250 mg, 85% yield. Mp: 170–174 °C, $[\alpha]_D^{25} = +56.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 2.68–2.83 (m, 1H), 3.02–3.11 (dd, 1H, $J = 5.2$, 5.2 Hz), 3.61 (s, 6H), 3.68 (s, 3H), 4.35 (t, 1H, $J = 7.5$, 9.0 Hz), 4.44–4.50 (m, 4H), 4.56 (t, 1H, $J = 3.7$, 4.5 Hz), 4.95 (s, 1H), 5.62 (d, 2H, $J = 6.7$ Hz), 6.06 (s, 2H), 6.29 (s, 1H), 6.36 (d, 1H, $J = 4.5$ Hz), 7.44–7.49 (dd, 1H, $J = 4.5$, 4.5 Hz), 7.70 (t, 1H, $J = 7.5$, 7.5 Hz), 8.09 (d, 1H, $J = 8.3$ Hz), 8.25 (d, 1H, $J = 8.3$ Hz), 8.45 (d, 1H, $J = 7.5$ Hz), 8.77 (d, 1H, $J = 3.0$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.8, 42.9, 51.7, 55.7, 59.8, 68.3, 101.0, 109.2, 122.4, 125.7, 128.7, 129.4, 130.7, 131.8, 133.9, 135.7, 136.2, 137.1, 137.5, 142.6, 145.9, 146.9, 151.4, 151.9, 174.4; MS (ESI): 627 $[M+Na]^+$.

5.3. 4β-[2,5-Dichlorothiophene-3-sulphonamido] podophyllotoxin (11c)

This compound **11c** was prepared following the method described for the preparation of the compound **11a**, employing **10** (200 mg, 0.483 mmol) with 2,5-dichlorothiophene-3-sulphonylchloride (0.145 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.996 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (1:9) to afford pure compound **11c** in 290 mg, 95% yield. Mp: 140–143 °C, $[\alpha]_D^{25} = -38.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 2.92–3.03 (m, 2H), 3.75 (s, 6H), 3.81 (s, 3H), 4.36–4.40 (m, 2H), 4.58 (d, 1H, $J = 4.5$ Hz), 4.65–4.69 (m, 2H), 5.07 (d, 1H, $J = 6.7$ Hz), 5.98 (s, 2H), 6.05 (s, 1H), 6.24 (s, 2H), 6.51 (s, 1H), 7.28 (s, 1H), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.1, 41.0, 43.4, 52.4, 56.1, 60.6, 68.5, 101.6, 107.7, 108.0, 110.1, 126.5, 127.7, 128.1, 129.9, 132.3, 134.6, 136.8, 137.0, 147.6, 148.4, 152.4, 174.1; MS (ESI): 650 $[M+Na]^+$.

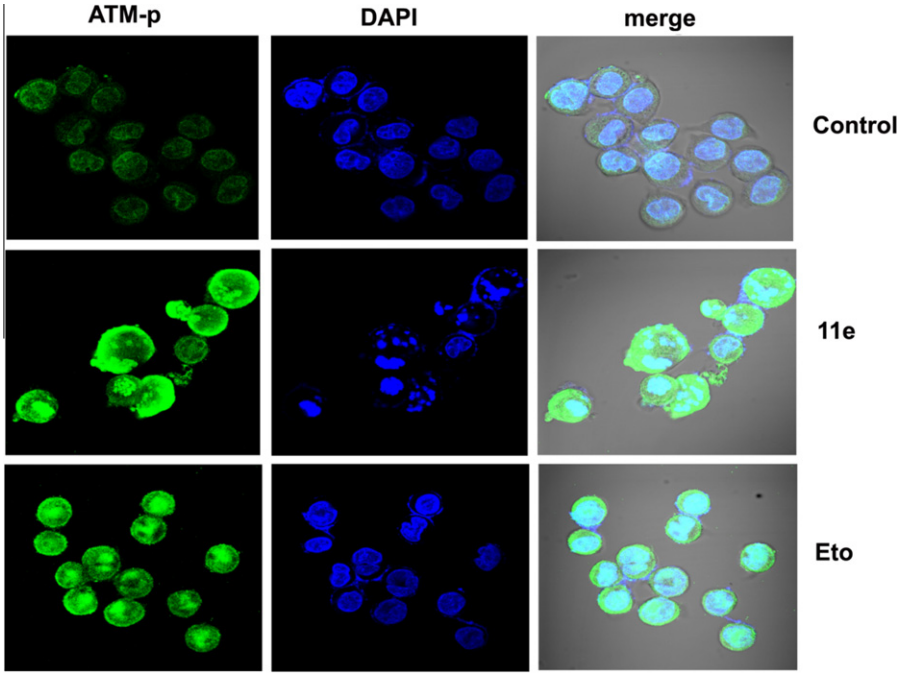


Figure 6. ATM S1981 staining was carried out to observe the ATM activation and induction of double-stranded DNA breaks due to compounds **11e** and etoposide (**Eto**) at 4 μM concentration for 24 h.

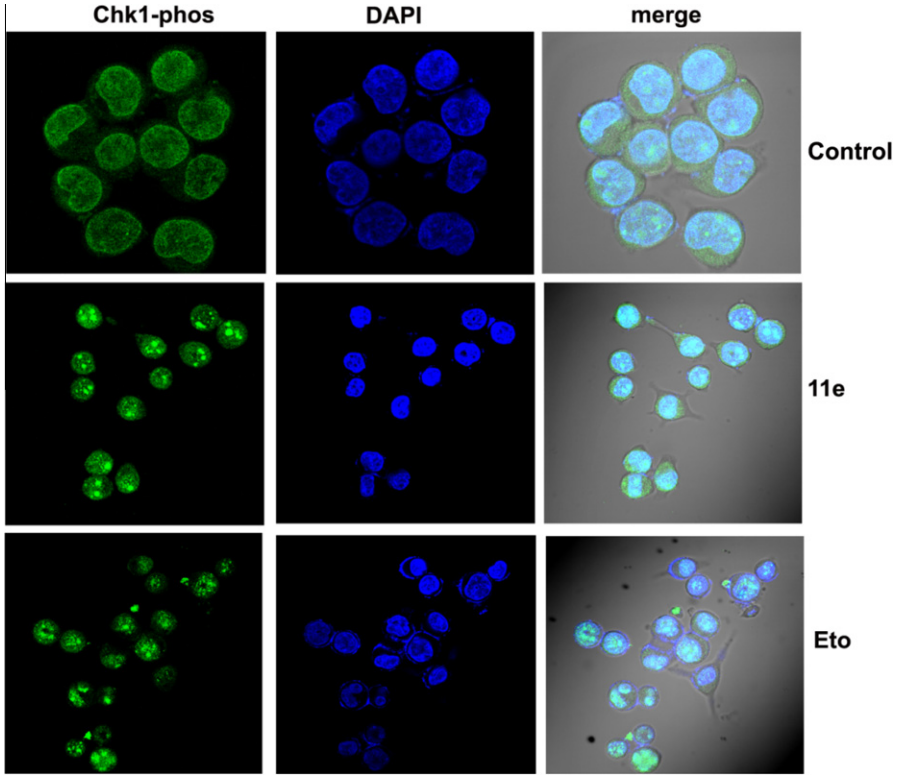


Figure 7. The Chk1 phosphorylation as a consequence of DNA damage activity in compounds **11e** and etoposide (**Eto**) treated cells.

5.4. 4β-[5-Bromothiophene-2-sulphonamido]podophyllotoxin (11d)

This compound **11d** was prepared following the method described for the preparation of the compound **11a**, employing **10** (200 mg, 0.483 mmol) with 5-bromothiophene-2-sulphonylchloride (150 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.966 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (3:7) to afford pure compound **11d** in 280 mg, 90% yield. Mp: 130–132 °C, $[\alpha]_D^{25} = -86.0$ ($c = 0.5$ in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.88–2.95 (m, 2H), 3.72 (s, 6H), 3.78 (s, 3H), 4.28–4.37 (m, 2H), 4.52 (d, 1H, $J = 3.9$ Hz), 4.62–4.64 (dd,

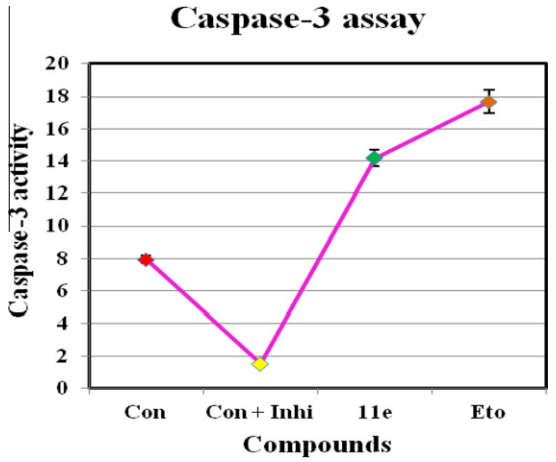


Figure 8. The effect of compounds **11e** and etoposide (**Eto**) on caspase-3 activity. The Colo-205 cells were treated with compounds **11e** and etoposide (**Eto**) at 4 μM concentration for 24 h. The cell lysates were subjected to a fluorescence based caspase-3 assay. The compounds caused increase of caspase-3 protein. Control indicates the untreated cells. Con + Inhi, indicates the caspase-3 activity in the presence of caspase-3 inhibitor.

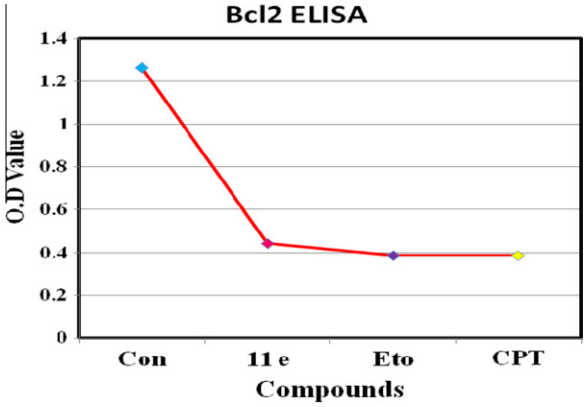


Figure 9. The Colo-205 cells were treated with compounds **11e** and etoposide (**Eto**) at 4 μM concentration for 24 h. The cell lysates were subjected to Bcl2 based ELISA assay. The compounds (**11e**, **Eto** and **CPT**) caused decrease of Bcl2 protein. Control indicates the untreated cells. Etoposide (**Eto**) and camptothecin (**CPT**) are employed as positive controls wherein etoposide serves as the positive control for topo-II inhibition and camptothecin as a positive control for the topo-I inhibition.

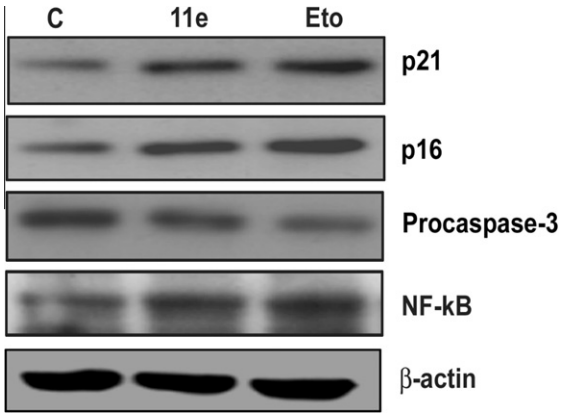


Figure 10. Treatment of Colo-205 cells with **11e** and etoposide (**Eto**) at 4 μM concentration for 24 h and subjected to western blot analysis using antibodies against p21, p16, caspase-3 full length and NF-kB proteins. The less procaspase-3 protein expression indicates more active caspase-3 formation and more apoptosis.

1H, J = 3.1, 3.9 Hz), 4.94 (d, 1H, J = 5.4 Hz), 5.86 (s, 1H), 5.93 (d, 2H, J = 3.9 Hz), 6.20 (s, 2H), 6.45 (s, 1H), 7.22 (d, 1H, J = 3.9 Hz), 7.49 (d, 1H, J = 3.9 Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.0, 41.1, 43.4, 52.4, 56.1, 60.6, 68.5, 101.5, 107.7, 108.0, 110.0, 120.5, 127.7, 130.7, 132.2, 132.4, 134.6, 136.9, 142.1, 147.5, 148.3, 152.4, 174.1; MS (ESI): 662 $[M+Na]^+$.

5.5. 4β-[Methyl-3-(sulphonamido)thiophene-2-carboxylate] podophyllotoxin (11e)

This compound **11e** was prepared following the method described for the preparation of the compound **11a**, above employing **10** (200 mg, 0.483 mmol) with 2-carbomethoxy thiophene-3-sulphonylchloride (139 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.966 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (1:9) to afford pure compound **11e** in 270 mg, 90% yield. Mp: 164–165 °C, $[\alpha]_D^{25}$ = −40.0 (c = 0.5 in $CHCl_3$), ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.86–2.98 (m, 1H), 3.08–3.14 (dd, 1H, J = 5.2, 5.2 Hz), 3.73 (s, 6H), 3.80 (s, 3H), 3.87 (s, 3H), 4.10–4.17 (dd, 1H, J = 6.7, 6.7 Hz), 4.40–4.52 (m, 2H), 4.61 (d, 1H, J = 5.2 Hz), 4.67 (t, 1H, J = 5.2, 3.7 Hz), 5.76 (s, 1H), 5.93 (s, 2H), 6.23 (s, 2H), 6.50 (s, 1H), 7.65–7.69 (m, 2H), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.62, 40.96, 43.58, 52.95, 56.21, 60.72, 60.69, 68.86, 101.59, 107.64, 108.22, 110.55, 127.60, 130.71, 131.09, 132.57, 148.43, 152.53, 174.09; MS (ESI): 640 $[M+Na]^+$.

5.6. 4β-[2-Acetamido-4-methylthiazole-5-sulphonamido] podophyllotoxin (11f)

This compound **11f** was prepared following the method described for the preparation of the compound **11a**, employing **10** (200 mg, 0.483 mmol) with 2-acetamido-4-methylthiazole-5-sulphonylchloride (147 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.966 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (1:9) to afford pure compound **11f** in 280 mg, 91% yield. Mp: 164–167 °C, $[\alpha]_D^{25}$ = −120.0 (c = 0.5 in $CHCl_3$), ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.11 (s, 3H), 2.33 (s, 3H), 3.73 (s, 6H), 3.77 (s, 3H), 2.71–2.80 (dd, 1H, J = 4.6, 4.6 Hz), 2.85–2.94 (m, 1H), 4.03–4.12 (m, 1H), 4.35–4.43 (m, 2H), 4.53 (d, 1H, J = 4.6 Hz), 5.16–5.23 (m, 1H), 5.98 (d, 2H, J = 2.7 Hz), 6.22 (s, 2H), 6.50 (s, 1H), 6.76 (s, 1H), ^{13}C NMR (75 MHz, $CDCl_3$): δ 19.12, 25.73, 42.72, 45.33, 45.45, 57.14, 62.51, 64.38, 67.71, 101.04, 107.06, 110.15, 110.85, 111.59, 130.23, 133.76, 136.00, 138.40, 144.04, 147.31, 150.73, 161.11, 170.11; MS (ESI): 632 $[M+H]^+$.

5.7. 4β-[Biphenyl-4-sulphonamido]podophyllotoxin (11g)

This compound **11g** was prepared following the method described for the preparation of the compound **11a**, employing **10** (200 mg, 0.483 mmol) with biphenyl-4-sulphonylchloride (146 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.966 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (1:1) to afford pure compound **11g** in 290 mg, 95 yield. Mp: 195–198 °C, $[\alpha]_D^{25}$ = −64.0 (c = 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.02–3.19 (m, 1H), 3.39–3.49 (dd, 1H, J = 3.7, 4.4 Hz), 3.71 (s, 3H), 3.73 (s, 6H), 3.98–4.10 (m, 2H), 4.36 (t, 1H, J = 6.6, 6.6 Hz), 4.53 (d, 1H, J = 3.7 Hz), 5.49 (t, 1H, J = 5.1, 5.1 Hz), 5.95 (d, 2H, J = 6.1 Hz), 6.28 (s, 2H), 6.49 (s, 1H), 6.84 (s, 1H), 7.11 (d, 2H, J = 8.1 Hz), 7.43–7.53 (m, 4H), 7.69 (d, 2H, J = 6.6 Hz), 7.83 (d, 2H, J = 8.1 Hz), 7.93–8.00 (m, 3H), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.2, 41.1, 43.4, 52.0, 56.1, 60.6, 68.7, 101.4, 107.8, 108.1, 110.0, 127.3, 127.6, 128.1, 128.7, 129.0, 132.2, 134.7, 137.0, 138.6, 138.9, 146.4, 147.4, 148.3, 152.4; MS (ESI): 652 $[M+Na]^+$.

5.8. 4β-[4-Phenoxybenzene-1-sulphonamido]podophyllotoxin (11h)

This compound **11h** was prepared following the method described for the preparation of the compound **11a**, employing **10** (200 mg, 0.483 mmol) with 4-phenoxybenzene-1-sulphonylchloride (155 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.966 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (4:6) to afford pure compound **11h** in 290 mg, 92% yield. Mp: 249–250 °C, $[\alpha]_D^{25} = -96.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 2.65–2.77 (m, 1H), 2.81–2.87 (dd, 1H, $J = 5.2, 5.2$ Hz), 3.63 (s, 6H), 3.65 (s, 3H), 4.16 (d, 2H, $J = 9.0$ Hz), 4.35 (d, 1H, $J = 5.2$ Hz), 4.40–4.43 (dd, 1H, $J = 3.7$ Hz), 5.10 (d, 1H, $J = 6.7$ Hz), 5.57 (s, 1H), 5.83 (s, 2H), 6.08 (s, 2H), 6.27 (s, 1H), 7.03–7.09 (m, 4H), 7.14 (t, 1H, $J = 6.7, 7.5$ Hz), 7.33 (t, 2H, $J = 7.5, 8.3$ Hz), 7.81 (d, 2H, $J = 9.0$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.2, 41.1, 43.4, 51.9, 56.1, 60.6, 68.6, 101.4, 107.7, 108.1, 110.0, 118.1, 120.1, 125.0, 128.3, 129.2, 130.1, 132.2, 133.8, 134.7, 147.4, 148.3, 152.4, 155.1, 162.0, 174.4; MS (ESI): 668 $[M+Na]^+$.

5.9. 4β-[5-(Phenylsulphonyl)thiophene-2-sulphonamido] podophyllotoxin (11i)

This compound **11i** was prepared following the method described for the preparation of the compound **11a**, employing **10** (200 mg, 0.483 mmol) with 5-phenylsulphonylthiaphene-2-sulphonylchloride (186 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.966 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (1:1) to afford pure compound **11i** in 300 mg, 88% yield. Mp: 210–213 °C, $[\alpha]_D^{25} = -62.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$): δ 2.75–2.85 (m, 2H), 3.67 (s, 6H), 3.71 (s, 3H), 4.03–4.18 (m, 2H), 4.40–4.44 (m, 1H), 4.56–4.60 (m, 1H), 5.40–5.47 (m, 1H), 5.78 (s, 1H), 5.87 (d, 2H, $J = 3.7$ Hz), 6.10 (s, 2H), 6.36 (s, 1H), 7.42–7.62 (m, 3H), 7.81 (d, 1H, $J = 1.5$ Hz), 7.91 (d, 2H, $J = 6.7$ Hz), 8.29 (d, 1H, $J = 1.5$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.0, 41.1, 43.4, 52.7, 56.1, 60.6, 68.5, 101.6, 107.6, 108.0, 110.1, 127.3, 127.6, 129.7, 129.9, 132.3, 134.0, 134.5, 136.2, 137.0, 140.1, 142.9, 145.0, 147.6, 152.5, 174.0; MS (ESI): 722 $[M+Na]^+$.

5.10. 4β-[Benzo[c][1,2,5]thiadiazole-4-sulphonamido] podophyllotoxin (11j)

This compound **11j** was prepared following the method described for the preparation of the compound **11a**, employing **10** (200 mg, 0.483 mmol) with 1,2,5-thiadiazole-4-sulphonylchloride (135 mg, 0.579 mmol), and Et_3N (1.34 mL, 0.966 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (3:7) to afford pure compound **11j** in 280 mg, 94% yield. Mp: 176–179 °C, $[\alpha]_D^{25} = +65.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (200 MHz, $CDCl_3$): δ 2.57–2.78 (m, 1H), 2.85–2.96 (dd, 1H, $J = 5.1, 5.1$ Hz), 3.54 (s, 6H), 3.60 (s, 3H), 4.15–4.26 (m, 2H), 4.33 (d, 1H, $J = 5.1$ Hz), 4.49–4.56 (m, 1H), 4.89 (s, 1H), 5.50 (d, 1H, $J = 7.3$ Hz), 5.63 (s, 2H), 5.97 (s, 2H), 6.22 (s, 1H), 7.62–7.71 (m, 1H), 8.15–8.22 (m, 2H), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.4, 40.9, 43.4, 52.9, 56.1, 60.6, 68.9, 101.4, 107.1, 108.1, 110.2, 126.9, 127.2, 128.2, 129.6, 129.9, 130.7, 131.8, 132.4, 134.5, 137.1, 146.8, 148.2, 148.8, 152.4, 155.2, 174.0; MS (ESI): 634 $[M+Na]^+$.

5.11. 4β-[(4′-Nitro)benzamide]podophyllotoxin (13)

To a solution containing 4β-aminopodophyllotoxin (**10**) (2 g, 0.820 mmol), triethylamine (2.3 mL, 1.652 mmol) in 20 mL of dichloromethane and 4-nitrobenzoyl chloride (**12**) (1.83 g, 0.991 mmol) in 10 mL of dichloromethane was added under nitrogen and stirred at room temperature for 3 h, till the completion of the reaction as monitored by TLC. The reaction mixture was washed with water and extracted with dichloromethane, dried over anhydrous Na_2SO_4 and the crude product was purified by column chromatography with ethyl acetate/hexane (3:7) to obtain pure compound **13** in 2.5 g, 92% yield. Mp: 174–177 °C; ^1H NMR (200 MHz, $CDCl_3$): δ 2.95–3.01 (m, 2H), 3.65 (s, 3H), 3.70 (s, 6H), 3.81 (t, 1H), 4.06–4.13 (dd, 1H, $J = 7.5, 6.7$ Hz), 4.37–4.46 (m, 2H), 5.43 (d, 1H, $J = 6.7$ Hz), 5.98 (d, 2H, $J = 4.5$ Hz), 6.22 (s, 2H), 6.45 (s, 1H), 6.83 (s, 1H), 7.94 (d, 2H, $J = 9.0$ Hz), 8.14 (d, 2H, $J = 9.0$ Hz); MS (ESI): 563 $[M+H]^+$.

5.12. 4β-[(4′-Amino)benzamide]podophyllotoxin (14)

To a solution of 4β-[(4′-nitro)benzamide]podophyllotoxin (**13**) (1.54 g, 3.53 mmol) in 80 mL of ethyl acetate was added 300 mg, of 10% palladium on activated carbon. The mixture was stirred overnight under hydrogen, the reaction mixture was filtered and the filtrate was evaporated. The crude product was purified by column chromatography with ethyl acetate/hexane (6:4) to obtain pure compound **14** in 1.42 g, 98% yield. ^1H NMR (200 MHz, $CDCl_3$): δ 2.93–3.01 (m, 2H), 3.601 (s, 3H), 3.69 (s, 6H), 3.80 (t, 1H), 4.01–4.10 (dd, 1H, $J = 7.5, 6.7$ Hz), 4.30–4.44 (m, 2H), 5.49 (d, 1H, $J = 6.7$ Hz), 6.00 (d, 2H, $J = 4.5$ Hz), 6.23 (s, 2H), 6.43 (s, 1H), 6.80 (s, 1H), 7.90 (d, 2H, $J = 9.0$ Hz), 8.16 (d, 2H, $J = 9.0$ Hz); MS (ESI): 533 $[M+H]^+$.

5.13. 4β-[4′-(5-Bromothiophene-2-sulphonamido)benzamide] podophyllotoxin (15a)

This compound **15a** was prepared following the method described for the preparation of the compound **11a**, employing **14** (200 mg, 0.375 mmol) with 5-bromothiophene-2-sulphonylchloride (116 mg, 0.451 mmol), and Et_3N (1.03 mL, 0.751 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (1:1) to afford pure compound **15a** in 270 mg, 95% yield. Mp: 213–215 °C, $[\alpha]_D^{25} = -53.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 2.82–3.15 (m, 1H), 3.74 (s, 6H), 3.78 (s, 3H), 4.05–4.34 (m, 2H), 4.39–4.52 (m, 1H), 4.58 (d, 1H, $J = 4.4$ Hz), 5.37–5.44 (m, 1H), 5.97 (d, 2H, $J = 3.6$ Hz), 6.28 (s, 2H), 6.53 (s, 1H), 6.79 (s, 1H), 7.12 (d, 1H, $J = 4.4$ Hz), 7.17 (d, 2H, $J = 8.0$ Hz), 7.49 (d, 1H, $J = 4.4$ Hz), 7.78 (d, 2H, $J = 8.0$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.2, 41.6, 43.6, 48.7, 60.6, 68.9, 101.5, 108.0, 108.9, 110.0, 123.6, 128.3, 129.8, 130.6, 131.4, 132.4, 134.5, 135.3, 136.0, 137.0, 138.7, 147.6, 148.3, 152.5, 166.1, 174.3; MS (ESI): 780 $[M+Na]^+$.

5.14. 4β-[4′-(Benzo[c][1,2,5]thiadiazole-4-sulphonamido) benzamide]podophyllotoxin (15b)

This compound **15b** was prepared following the method described for the preparation of the compound **11a**, employing **14** (200 mg, 0.375 mmol) with 1,2,5-thiadiazole-4-sulphonylchloride (105 mg, 0.451 mmol), and Et_3N (1.03 mL, 0.751 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (1:1) to afford pure compound **15b** in 269 mg, 98% yield. Mp: 247–249 °C, $[\alpha]_D^{25} = -78.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$): δ 2.86–2.99 (m, 2H), 3.73 (s, 6H), 3.77 (s, 3H), 4.06–4.13 (dd, 1H, $J = 6.0, 5.2$ Hz), 4.24–4.38 (m, 2H), 4.46 (d, 1H, $J = 3.0$ Hz), 5.33–5.37 (m, 1H), 5.93 (d, 2H, $J = 9.8$ Hz), 6.23 (s, 2H), 6.41 (s, 1H), 6.76 (s, 1H), 7.28 (d, 1H, $J = 9.0$ Hz), 7.65 (d, 2H, $J = 8.3$ Hz), 7.73–7.79 (m, 3H), 8.34 (t, 1H, $J = 8.3, 9.0$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.2, 41.5, 43.5, 48.6, 56.0, 60.7, 61.9, 68.9, 101.4, 108.0, 108.9, 109.9, 127.8, 128.0, 128.3, 128.4, 129.5, 129.8, 132.4, 132.8, 135.0, 135.8, 147.4, 148.2, 148.7, 152.4, 155.2, 166.1, 174.3; MS (ESI): 731 $[M+H]^+$.

5.15. 4β-[4′-(Napthalene-1-sulphonamido)benzamide] podophyllotoxin (15c)

This compound **15c** was prepared following method described for the preparation of the compound **11a**, employing **14** (200 mg, 0.375 mmol) with napthalene-1-sulphonylchloride (101 mg, 0.451 mmol), and Et_3N (1.03 mL, 0.751 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (4:6) to afford pure compound **15c** in 268 mg, 98% yield. Mp: 180–183 °C, $[\alpha]_D^{25} = -66.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 2.89–3.00 (m, 2H), 3.74 (s, 6H), 3.76 (s, 3H), 4.08–4.15 (dd, 1H, $J = 6.7$, 6.7 Hz), 4.26–4.30 (m, 1H), 4.38–4.43 (m, 1H), 5.29–5.38 (m, 2H), 5.87 (d, 2H, $J = 21.9$ Hz), 6.20 (s, 2H), 6.22 (s, 1H), 6.74 (s, 1H), 6.99 (d, 1H, $J = 8.3$ Hz), 7.22 (t, 1H, $J = 7.5$, 8.3 Hz), 7.50–7.59 (m, 4H), 7.62 (d, 1H, $J = 8.3$ Hz), 7.92 (d, 1H, $J = 8.3$ Hz), 8.04 (d, 1H, $J = 8.3$ Hz), 8.13 (d, 1H, $J = 8.3$ Hz), 8.24 (d, 2H, $J = 7.5$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.2, 41.5, 43.4, 48.6, 56.0, 60.5, 68.9, 101.3, 107.9, 108.8, 109.8, 123.8, 124.0, 127.0, 127.9, 128.1, 128.2, 128.3, 129.0, 132.2, 132.3, 132.6, 133.0, 133.6, 134.6, 134.8, 136.2, 136.3, 147.3, 148.0, 152.3, 166.0, 174.3; MS (ESI): 740 $[M+NH_4]^+$.

5.16. 4β-[4′-(Quinoline-8-sulphonamido)benzamide] podophyllotoxin (15d)

This compound **15d** was prepared following the method described for the preparation of the compound **11a**, employing **14** (200 mg, 0.375 mmol) with quinoline-8-sulphonylchloride (102 mg, 0.451 mmol), and Et_3N (1.03 mL, 0.751 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (3:7) to afford pure compound **15d** in 268 mg, 98% yield. Mp: 190–193 °C, $[\alpha]_D^{25} = -160.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 2.86–3.08 (m, 2H), 3.74 (s, 6H), 3.79 (s, 3H), 4.04–4.21 (m, 2H), 4.224.44 (m, 1H), 4.57 (d, 1H, $J = 4.4$ Hz), 5.22–5.39 (m, 1H), 5.96 (d, 2H, $J = 2.9$ Hz), 6.25–6.31 (m, 4H), 6.51 (s, 1H), 6.72 (s, 1H), 7.09 (d, 2H, $J = 8.0$ Hz), 7.50–7.66 (m, 4H), 8.02 (t, 1H, $J = 8.0$, 8.8 Hz), 8.31 (t, 1H, $J = 7.3$, 7.3 Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 41.5, 43.6, 48.3, 56.0, 60.5, 61.9, 68.9, 101.3, 107.9, 108.8, 109.9, 120.3, 122.3, 125.4, 128.1, 128.5, 129.2, 129.4, 129.8, 131.8, 132.4, 133.9, 134.5, 137.1, 140.4, 142.7, 147.4, 151.2, 152.3, 174.2. MS (ESI): 724 $[M+H]^+$.

5.17. 4β-[4′-(Biphenyl-4-sulphonamido)benzamide] podophyllotoxin (15e)

This compound **15e** was prepared following the method described for the preparation of the compound **11a**, employing **14** (200 mg, 0.375 mmol) with biphenyl-4-sulphonylchloride (113 mg, 0.451 mmol), and Et_3N (1.03 mL, 0.751 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (3:7) to afford pure compound **15e** in 271 mg, 96% yield. Mp: 205–207 °C, $[\alpha]_D^{25} = -83.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (200 MHz, $CDCl_3$): δ 3.39–3.49 (m, 2H), 3.73 (br s, 9H), 3.96–4.16 (m, 2H), 4.27–4.46 (m, 1H), 4.53 (d, 1H, $J = 3.7$ Hz), 5.41–5.60 (m, 1H), 5.95 (s, 2H), 6.24 (s, 2H), 6.50 (s, 1H), 6.80 (s, 1H), 7.14 (d, 2H, $J = 7.9$ Hz), 7.41–7.51 (m, 5H), 7.62 (d, 2H, $J = 7.9$ Hz), 7.75 (d, 2H, $J = 7.9$ Hz), 7.97 (d, 2H, $J = 8.3$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.3, 41.7, 43.6, 48.7, 56.1, 60.6, 68.9, 101.5, 108.0, 108.9, 110.1, 127.3, 127.6, 128.1, 128.4, 128.8, 128.9, 129.0, 131.9, 132.5, 134.6, 134.8, 137.3, 137.4, 138.7, 147.0, 147.6, 148.4, 152.5, 166.2, 174.2; MS (ESI): 749 $[M+H]^+$.

5.18. 4β-[4′-(4-Phenoxyphenylsulphonamido)benzamide] podophyllotoxin (15f)

This compound **15f** was prepared following the method described for the preparation of the compound **11a**, employing **14** (200 mg, 0.375 mmol) with 4-phenoxybenzene-1-sulphonylchloride (120 mg, 0.451 mmol), Et_3N (1.03 mL, 0.751 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (1:1) to afford pure compound **15f** in 278 mg, 96% yield. Mp: 190–193 °C, $[\alpha]_D^{25} = -83.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$): δ 2.74–2.85 (m, 1H), 3.38–3.43 (dd, 1H, $J = 5.3$, 5.3 Hz), 3.73 (s, 6H), 3.75 (s, 3H), 3.88 (t, 2H, $J = 9.8$, 9.8 Hz), 4.39 (t, 1H, $J = 8.0$, 8.0 Hz), 4.55 (d, 1H, $J = 4.4$ Hz), 5.48–5.51 (dd, 1H, $J = 5.3$, 5.3 Hz), 5.93 (d, 2H, $J = 6.2$ Hz), 6.28 (s, 2H), 6.50 (s, 1H), 6.83 (s, 1H), 7.02–7.10 (m, 6H), 7.24 (t, 1H, $J = 7.1$, 7,1 Hz), 7.42 (t, 2H, $J = 7.1$, 7.1 Hz), 7.78 (d, 2H, $J = 8.9$ Hz), 7.93 (d, 2H, $J = 7.1$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.6, 43.1, 47.5, 55.7, 59.8, 68.4, 101.2, 108.1, 109.1, 109.3, 117.4, 120.4, 125.4, 128.9, 130.0, 130.5, 130.8, 131.1, 131.8, 132.1, 135.6, 135.8, 136.1, 136.3, 146.6, 147.2, 151.9, 154.2, 162.3, 165.5, 174.5; MS (ESI): 765 $[M+H]^+$.

5.19. 4β-[4′-(4-Methoxyphenylsulphonamido)benzamide] podophyllotoxin (15g)

This compound **15g** was prepared following the method described for the preparation of the compound **11a**, employing **14** (200 mg, 0.375 mmol) with 4-methoxybenzene-1-sulphonylchloride (93 mg, 0.451 mmol), Et_3N (1.03 mL, 0.751 mmol) and the crude product was purified by column chromatography with ethyl acetate/hexane (3:7) to afford pure compound **15g** in 259 mg, 98% yield. Mp: 147–150 °C, $[\alpha]_D^{25} = -20.0$ ($c = 0.5$ in $CHCl_3$), ^1H NMR (200 MHz, $CDCl_3$): δ 2.88–3.09 (m, 2H), 3.75 (s, 3H), 3.80 (s, 3H), 3.91 (s, 6H), 4.12–4.30 (m, 2H), 4.47 (t, 1H, $J = 6.6$, 6.6 Hz), 4.59 (d, 1H, $J = 2.9$ Hz), 5.32–5.44 (m, 1H), 5.96 (d, 2H, $J = 5.1$ Hz), 6.30 (s, 2H), 6.52 (s, 1H), 6.79 (s, 1H), 7.02 (d, 2H, $J = 8.8$ Hz), 7.11 (d, 2H, $J = 8.0$ Hz), 7.76 (d, 2H, $J = 8.0$ Hz), 7.79 (d, 2H, $J = 8.8$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 41.5, 43.5, 48.6, 55.6, 56.0, 60.5, 61.9, 68.7, 101.4, 107.9, 108.9, 109.9, 114.1, 128.0, 128.4, 129.5, 129.8, 130.2, 130.6, 131.7, 132.3, 134.5, 134.7, 137.5, 147.4, 148.2, 152.4, 163.9, 166.3, 174.3; MS (ESI): 703 $[M+H]^+$.

6. Procedure of the SRB-assay

The synthesized compounds (**11a–j** and **15a–g**) have been evaluated for their in vitro cytotoxicity in human cancer cell lines. A protocol of 48 h continuous drug exposure has been used and a sulforhodamine B (SRB) protein assay has been used to estimate cell viability or growth. The cell lines were grown in DMEM medium containing 10% fetal bovine serum and 2 mM *L*-glutamine and were inoculated into 96 well microtiter plates in 90 microliter at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. Aliquots of 10 μL of the drug dilutions were added to the appropriate microtiter wells already containing 90 μL of cells, resulting in the required final drug concentrations. For each compound four concentrations (0.1, 1, 10 and 100 μM) were evaluated and done in triplicate wells. Plates were incubated further for 48 h and assay was terminated by the addition of 50 μL of cold trichloro acetic acid (TCA) (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 mL) at 0.4% (w/v) in 1% acetic acid was added to each of the cells, and plates were incubated for 20 min at room temperature. The

residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm with 690 nm reference wavelengths. Percent growth was calculated on a plate by plate basis for test wells relative to control wells. The above determinations were repeated three times. Percentage growth was expressed as the (ratio of average absorbance of the test well to the average absorbance of the control wells) × 100. Growth inhibition of 50% (GI_{50}) was calculated from [(Ti-Tz)/(C-Tz)] × 100 ¼ 50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. Where, Tz ¼ Optical density at time zero, OD of control ¼ C, and OD of test growth in the presence of drug ¼ Ti.

7. Cell culture

Human colon cancer cell line Colo-205 cells were purchased from American Type Culture Collection that were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 2 mM glutamax (Invitrogen), 10% fetal calf serum and 100U/ml Pencillin and 100 μg/mL streptomycin sulfate (Sigma). The cell line was maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in the incubator.

8. In vitro evaluation of cytotoxicity (MTT assay)

Cell viability was assessed by the MTT assay, a mitochondrial function assay. It is based on the ability of viable cells to reduce the MTT to insoluble formazan crystals by mitochondrial dehydrogenase. The Colo-205 cells were seeded in a 96-well plate at a density of 10,000 cells/well. After overnight incubation, cells were treated with compounds **11e** and etoposide (**Eto**) at 2, 4 and 8 μM concentration and incubated for 24 h. Medium was then discarded and replaced with 10 μL MTT dye. Plates were incubated at 37 °C for 2 h. The resulting formazan crystals were solubilized in 100 μL extraction buffer. The optical density (O.D) was read at 570 nm with micro plate reader (Multi-mode Varioskan instrument-Themo Scientific).

9. Cell cycle analysis

5×10^{-5} Colo-205 cells were seeded in 60 mm dish. Cells were treated with **11b**, **11d**, **11e** and etoposide (**Eto**) at 4 μM for 24 h. Trichostatin A (TSA) at 4 μM concentration for 12 h was used in this study. Immediately after incubation period the cells were incubated for an additional 24 h. Cells were harvested with Trypsin-EDTA, fixed with ice-cold 70% ethanol at 4 °C for 30 min, washed with PBS and incubated with 1 mg/mL RNase solution (Sigma) at 37 °C for 30 min. Cells were collected by centrifugation at 2000 rpm for 5 min and further stained with 250 μL of DNA staining solution [10 mg of Propidium Iodide (PI), 0.1 mg of trisodium citrate, and 0.03 mL of Triton X-100 were dissolved in 100 mL of sterile MilliQ water at room temperature for 30 min in the dark]. The DNA contents of 20,000 events were measured by flow cytometer (DAKO CYTOMATION, Beckman Coulter, Brea, CA). Histograms were analyzed using Summit Software.

10. Comet assay (single cell gel electrophoresis)

The Colo-205 cells were seeded in 60 mm dishes and were treated with etoposide (**Eto**) and podophyllotoxin conjugate **11e** at a concentration for 4 μM concentration for 24 h. Briefly, 20,000 cells were embedded in agarose and deposited in microscope slides. The slides were incubated 2 h in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 10% DMSO, 1% Triton X-100) followed by 3 washes in neutralizing buffer (0.4 M Tris–HCl, pH 7.5). Electrophoresis was carried out for 20 min at 40 V in 0.5% TBE buffer (pH 8). During electrophoresis the damaged DNA migrates away from the nucleus towards anode. Slides were then stained with a 20 mg/mL Syber green solution for 30 min. Images from were taken in fluorescent microscope at 20×.

11. Immunofluorescence

Human colon cancer cells (Colo-205) cells were seeded on cover slips and treated with podophyllotoxin conjugate, the effective conjugate **11e** and etoposide (**Eto**) at concentration of 4 μM for 24 h. After treatment, cover slips were fixed with a paraformaldehyde solution (4% in 1X PBS) for 20 min at room temperature. Cell permeabilization was achieved by administration of a Triton X-100 solution (0.2% in 1X PBS) for 5 min. Further the cover slips were kept overnight in 100% methanol at 4 °C. Subsequently, cover slips were blocked with a 1% BSA solution for 60 min and then incubated with anti-γ H2AX, phos-ATM, phos-CHK1 (1:100) antibody at room temperature for 2 h. The slides were washed three times each of 5 min with PBST. Then cover slips were incubated with a FITC-conjugated anti-rabbit secondary antibody (Jackson Immuno Research Laboratories Inc., Pennsylvania, USA) for one hour and cover slips were washed three times with PBST solution and mounted with DAPI/PI solution. Finally, cells were observed under confocal microscope (Olympus FV1000). Images taken were processed with the support of the flow view version 1.7c software program.

12. Protein extraction and Western blot analysis

Total cell lysates from cultured Colo-205 cells treated with **11e** and etoposide (**Eto**) were obtained by lysing the cells in ice-cold RIPA buffer (1XPBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) and containing 100 μg/mL PMSF, 5 μg/mL Aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin and 100 μg/mL NaF. After centrifugation at 12,000 rpm for 10 min, the protein in supernatant was quantified by Bradford method (BIO-RAD) using Multimode Varioskan instrument (Thermo-Fischer Scientifics). Fifty micrograms of protein per lane was applied in 12% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to polyvinylidine difluoride (PVDF) membrane (GE Biosciences). The membrane was blocked at room temperature for 2 h in TBS + 0.1% Tween20 (TBST) containing 5% blocking powder (Santacruz). The membrane was washed with TBST for 5 min, primary antibody was added and incubated at 4 °C overnight (O/N). Topo-IIα, and β were purchased From LS biotech and γ-H2AX, p21, p16, pro-caspase-3, NF-kB (p65) antibodies were from Cell Signalling, USA. β-Actin antibody was purchased from Imgenex company. The membrane was incubated with corresponding horseradish peroxidase-labeled secondary antibody (1:2000) (Santa Cruz) at room temperature for 1 h. Membranes were washed with TBST three times for 15 min and the blots were visualized with chemiluminescence reagent (Thermo Fischer Scientifics Ltd). The X-ray films were developed with developer and fixed with fixer solution.

13. Caspase-3 assay

The caspase-3 fluorescence assay kit (Clonetech, CA) was applied to evaluate the caspase-3 activity, using the procedures provided by the manufacturer Colo-205 cells were treated with compounds **11e** and etoposide (**Eto**) at 4 μM concentration as obtained from FACS analysis. Cell lysates were added to the 2X reac-

tion buffer containing DTT and caspase substrate was added and incubation was carried out at 37 °C for 1 h. Readings were taken at excitation wavelength 400 nm and emission wavelength 505 nm.

14. Bcl2 ELISA

Bcl2 ELISA was conducted with Alexis Biochemical's Bcl2 ELISA kit. Colo-205 cells were treated with compounds **11e**, etoposide (**Eto**) and camptothecin (**CPT**) at 4 μM concentration for 24 h. The cell lysates were isolated and added to the micro wells which contains Bcl2 antibody. After the addition of lysates 100 μL of biotin conjugated anti-human Bcl2 monoclonal antibody was added. After the incubation period and washing steps the bound Bcl2 was detected by using 150 μL Streptavidin-HRP secondary antibody. The colored product obtained was detected by O.D at 450 nm. O.D is directly proportional to the amount of Bcl2 protein in the sample.

Acknowledgments

The author P.S. is thankful to UGC, India and, A.M.R. is thankful to CSIR, India for the award of research fellowships.

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