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One-pot synthesis of podophyllotoxin–thiourea congeners by employing NH_2SO_3H/NaI: Anticancer activity, DNA topoisomerase-II inhibition, and apoptosis inducing agents

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ABSTRACT

A facile one-pot method for the synthesis of novel podophyllotoxin–thiourea congeners has been developed by using NH_2SO_3H/NaI system. Interestingly, 4β-azido podophyllotoxin reduction with concomitant aryl isothiocyanates coupling under mild reaction conditions has been achieved. These compounds have been investigated for their in vitro cytotoxicity against A549, MDA MB-231, DU-145, LNCaP, and HGC-27 cancer cell lines. Some of the representative compounds have selectively exhibited cytotoxicity on DU-145 (human prostate cancer) cells and the most potent compound was **4a** (IC_{50} of 0.50 ± 0.03 μM) with optimal safety therapeutic window (81.7 fold) on normal human prostate cell line (RWPE-1, IC_{50} of 40.85 ± 0.78). The flow-cytometric analysis of the compound **4a** in prostate cancer cells indicated a strong G2/M-phase arrest and significant topoisomerase II inhibition activity. Furthermore, these compounds induce apoptosis as observed by Acridine Orange and Ethidium Bromide (AO/EB) staining and Annexin V binding assay. Molecular docking results of the title compounds with topoisomerase-IIα were presented as theoretical support for the experimental data.

Carbamoylation/thiocarbamoylation of nucleophilic compounds plays a significant role for the synthesis of various biologically important scaffolds.[1] A variety of methods for the conversion of azides into amines have been reported by using radical initiators,[2] metal-catalyzed hydrogenations,[3] lithium aluminum hydride,[4] borohydrides,[5] triphenylphosphine,[6] etc. Recently, Azhayev and co-workers have reported a direct method for the conversion of azides to urea derivatives by employing trialkylammonium hydrogen carbonate via a significant in situ generation of isocyante.[7] However, to the best of our knowledge, the direct method for the synthesis of thiourea derivatives from organic azides has not been explored. In the literature, very few protocols are available for the synthesis of urea/thiourea derivatives by employing highly toxic phosgene/thiophosgene reagents or addition of nucleophilic compounds to isocyanates.[8] These methods are applicable to many synthetic conditions, but the majority of them have several drawbacks, including tedious work-up, long reaction times, toxic, harsh reaction conditions, and poor selectivity. In continuation of our earlier efforts in the development of novel approaches for the azido reductive cyclization towards bioactive pyrrolobenzodiazepines, podophyllotoxin, and fused quinazolinones,[9] herein we have reported a facile one-pot azido reduction with concomitant isothiocyanate coupling for the synthesis of novel podophyllotoxin–thiourea congeners by employing NH_2SO_3H/NaI reagent system.

Podophyllotoxin (**A**, Fig. 1) is an antimitotic aryltetralinlignane isolated from podophyllin, a resin produced by species of the genera *Podophyllum* such as *P. hexandrum* and *P. peltatum*.[10] It has displayed potent antimitotic activity against various cancer cell lines but the clinical use was complicated by severe gastrointestinal side effects.[11] Efforts made towards the discovery of less toxic podophyllotoxin congeners led to the development of several semi-synthetic derivatives such as etoposide (**B**, VP-16), teniposide (**C**, VM-26) and etopophos (**D**) as potent anticancer drugs, currently in clinical use against various malignancies including leukemia, neuroblastoma, non-Hodgkin's lymphoma, small-cell lung cancer,

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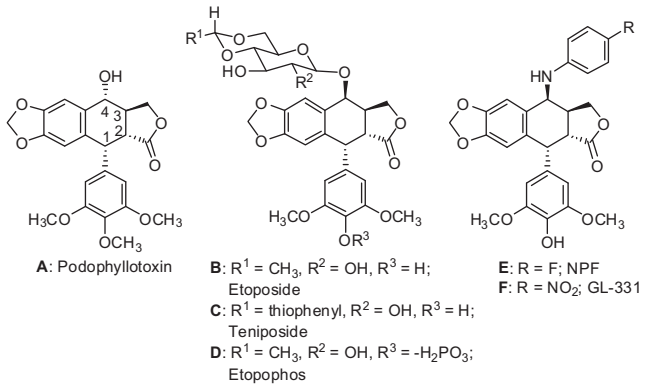


Figure 1. Representative structures of podophyllotoxin (A) and its semi-synthetic derivatives (B–F).

soft tissue sarcoma and Kaposi's sarcoma.[12–16] However, side effects associated with the clinical use of these drugs including poor bioavailability and the development of drug resistance by cancer cells has led to the need for the intensification of the research on novel anticancer drugs based on podophyllotoxin scaffold.[17–19] Studies have shown that podophyllotoxin blocks cell division by inhibition of tubulin assembly into microtubules through tubulin binding at the colchicine site,[20] whereas etoposide, teniposide and etopophos were potent irreversible inhibitors of DNA topoisomerase II.[21]

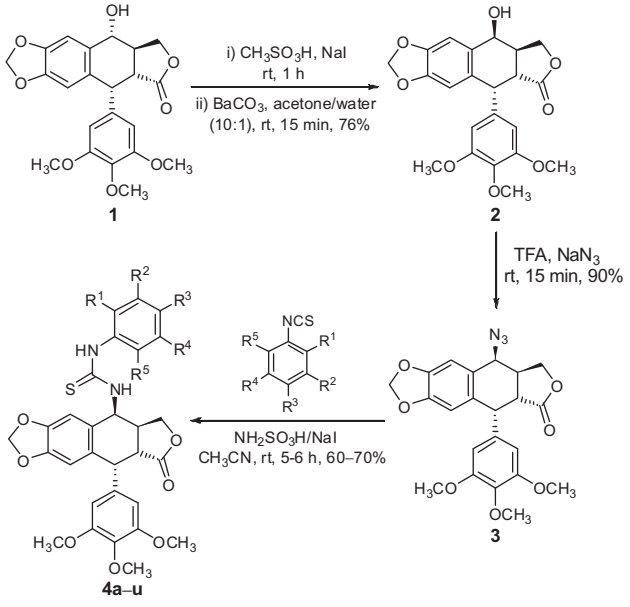
Based on the results of previous structure–activity relationship (SAR) studies on podophyllotoxin, 4β-stereochemistry and 4-N-linkage are essential for topoisomerase II inhibitory activity and the introduction of bulky groups at C4-position significantly potentiates the antitumor activity.[22] It was observed that, the sugar moiety of etoposide is not crucial for topoisomerase II inhibition.[23] Various research groups have carried out and reported extensive structural modifications at C4-position of podophyllotoxin,[12,24,25] which has led to the development of several N-linked congeners, such as NPF (**E**)[26] and GL-331 (**F**),[27] among which the compound GL-331 is currently in phase-II clinical trials against various resistant malignancies. Recently, we have reported a series of podophyllotoxin congeners as potential inhibitors of topoisomerase and tubulin polymerization.[28–30] On the whole, these compounds was found to overcome the limitations of Etoposide, with superior pharmacological profiles, suggesting the prospect of further optimization through rational C4 modifications.

On the other hand, thiourea derivatives possess good protein tyrosine kinases (PTKs) inhibitory activity, which results in cytotoxic activity.[31,32] Novel aroylthiourea–podophyllotoxin derivatives have been reported and were found to induce G2/M cell cycle arrest in human colon cancer cells through ATR-Chk1-cdc25C and Weel signal pathways.[33] In order to investigate the propensity of thiourea moiety to enhance the cytotoxicity of podophyllotoxin scaffold by tethering, we have herein reported the design and a direct one-pot synthesis and cytotoxic evaluation of the new podophyllotoxin–thiourea derivatives **4a–u** by employing NH_2SO_3H–NaI reagent system.

Our synthetic strategy has been started from the preparation of 4β-deoxy-epipodophyllotoxin (**2**) stereoselectively from the commercially available podophyllotoxin (**1**) through iodination with CH_3SO_3H and NaI followed by nucleophilic substitution in the presence of $BaCO_3/H_2O$. 4β-Azido intermediate **3** has been obtained from podophyllotoxin (**2**) by employing NaN_3 in the presence of CF_3COOH. At this stage, we have screened the one-pot azido reduction of intermediate **3** with isothiocyanate by using different reagents such as $ZnBr_2$ and NH_2SO_3H in different solvents (THF, MeOH, CH_2Cl_2, DMF and acetonitrile) but no conversion was observed. Interestingly, when we attempted the same reaction with NH_2SO_3H in combination with NaI, the reactions proceeded smoothly in 5–6 h at room temperature and acetonitrile solvent gave the best conversion. Finally, the podophyllotoxin–thiourea congeners **4a–u** was successfully synthesized by reaction with various isothiocyanates in the presence of NH_2SO_3H and NaI in good yields (60–70%).[34] The synthetic plan for **4a–u** is depicted in Scheme 1 and the list of the synthesized analogues was shown in Table 1.

These compounds **4a–u** were evaluated for their in vitro cytotoxicity on A549 (human lung cancer), MDA MB-231 (human breast carcinoma), DU-145 (human prostate cancer), LNCaP (androgen-sensitive human prostate adenocarcinoma) and HGC-27 (Human gastric carcinoma) cell lines by employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay). The concentrations of the compounds which produce 50% inhibition of cell growth (IC_{50}) were compared with that of the standard drug, that is, etoposide. From close analysis of the IC_{50} values (Table 2), it is evident that the compounds **4a**, **4p**, **4s** and **4t** have selectively exhibited anticancer activity against the human prostate cancer (DU-145) cell line and the most potent compound was **4a** which has shown IC_{50} 0.50 ± 0.03 μM. In the androgen-sensitive human prostate adenocarcinoma cell line (LNCaP), the compounds **4a**, **4d**, **4e**, **4h**, **4j**, **4p**, **4s**, and **4t** have exhibited remarkable inhibitory activity with the compound **4p** being the most active (IC_{50} value of 3.56 ± 0.76 μM). Thus, it can be affirmed that the synthesized compounds are much effective towards human prostate cancer.

Fascinatingly, compound **4a** has exhibited significant cytotoxicity against all the five cell lines compared to the standard etoposide. However, the compounds **4b**, **4f**, **4g**, **4k**, **4m**, **4n**, **4o**, **4r**, and **4u** were found to be inactive in all the tested cancer cell lines. It is interesting to observe that compound **4p** exhibited potent growth inhibition activity on human lung cancer cell line (A549), with IC_{50} value of 14.84 ± 2.46 μM. Similarly, **4a** and **4e** have also shown good growth inhibition effect against this cell line with IC_{50} values of 37.6 ± 17.1 μM and 35.14 ± 5.31 μM, respectively, in comparison with etoposide (38.5 ± 3.6 μM) and podophyllotoxin (128.61 ± 15.1 μM). Compound **4a** was only found to display



Scheme 1. Synthesis of novel podophyllotoxin–thiourea (**4a–u**) congeners.

Table 1
A series of novel podophyllotoxin–thiourea derivatives **4a–u**

Entry	R^1	R^2	R^3	R^4	R^5	Yield[a] (%)
4a	H	H	OCH_3	H	H	60
4b	H	H	CN	H	H	67
4c	H	H	NO_2	H	H	70
4d	CH_3	H	CH_3	H	CH_3	62
4e	NO_2	H	OCH_3	H	H	67
4f	H	OCH_3	H	OCH_3	H	64
4g	OCH_3	H	NO_2	H	H	60
4h	H	Cl	Cl	H	H	65
4i	H	OCH_3	OCH_3	H	H	62
4j	H	CF_3	H	H	H	66
4k	OCH_3	H	OCH_3	H	H	68
4l	F	H	H	H	H	60
4m	H	F	H	H	H	60
4n	OCH_3	H	H	OCH_3	H	67
4o	H	H	F	H	H	62
4p	H	H	CF_3	H	H	69
4q	H	H	$CH(CH_3)_2$	H	H	65
4r	H	H	$OCH_2C_6H_5$	H	H	62
4s	CF_3	H	H	H	H	61
4t	H	H	$COOC_2H_5$	H	H	64
4u	H	OCH_3	NO_2	H	H	60

[a] Isolated yields.

significant cytotoxicity in the human gastric carcinoma (HGC-27) cell line with IC_{50} value of 3.85 ± 0.27 μM. Likewise, compounds **4a**, **4c**, **4d**, **4e**, **4h**, **4j** and **4p** were found to be effective in the human breast carcinoma (MDA MB-231) cell line when compared to etoposide, with **4p** being the most active showing IC_{50} value of 7.58 ± 0.26.

It is our conjecture to explain the SAR, where the substitution at R^3 position is significant for anticancer activity. The presence of methoxy substitution at either R^1 (**4g**, **4k** and **4n**, Table 1) or R^2 (**4i** and **4u**) or R^4 (**4n**) or both R^2 and R^4 (**4f**) resulted in the loss of cytotoxicity. Bulky substituents at the R^3 position of phenyl ring as seen in **4q** and **4r** resulted in no cytotoxic activity. In the human prostate cancer (DU-145) cell line, presence of small groups capable of hydrogen-bonding such as methoxy (**4a**) and trifluoromethyl (**4p**) at R^3 position, has led to the most potent compounds with IC_{50} of 0.50 ± 0.03 μM and 1.96 ± 0.19 μM, respectively. Compound **4p** possessing trifluoromethyl substituent at R^3 position was found to be the most active against A549 (human lung cancer), LNCaP (androgen-sensitive human prostate adenocarcinoma) and MDA MB-231 (human breast carcinoma) cell lines with IC_{50} of 14.84 ± 2.46, 3.56 ± 0.76 and 7.58 ± 0.26, respectively.

The synthesized compounds **4a**, **4c**, **4p**, **4s** and **4t** which showed IC_{50} values less than 10 μM in the human prostate cancer (DU-145) cell line were further assayed for cytotoxicity on normal human prostate cell line (RWPE-1) with etoposide as reference standard, to determine the specificity towards cancer cells. From the results, compound **4a** was found to be highly specific towards DU-145 cell line (IC_{50} values are 81.7 fold higher in RWPE-1 cells) when compared to normal prostate epithelial cells; whereas the IC_{50} value for the standard drug etoposide was only 5.3 fold higher in RWPE-1 cells. Interestingly, the IC_{50} values for the compounds **4c**, **4d**, **4e**, **4p**, **4s** and **4t** are also 7.2, 6.8, 6.4, 8.6, 19 and 27 fold, respectively higher in RWPE-1 cells when compared to DU-145 cells. Hence, all these new compounds were found to be more specific towards prostate cancer cells than the normal prostate cells in comparison to the standard drug etoposide, with optimal safety window.

On the contrary, compound **4a** was 2.3 fold more specific towards androgen-sensitive human prostate adenocarcinoma (LNCaP) cell line when compared to normal prostate epithelial cells; whereas the IC_{50} value for the standard drug etoposide was only 1.8 fold higher in RWPE-1 cells. Interestingly, compound **4c** has the similar safety window as that of etoposide while the IC_{50} values for the compounds **4d**, **4e**, **4p**, **4s** and **4t** are 1.8, 3.1, 4.7, 3.4 and 11.6 fold, respectively, higher in RWPE-1 cells when compared to LNCaP cells.

The morphological changes in DU-145 cells after treatment with the compound **4a** for 48 h were studied under a phase-contrast microscope. Cells treated with 125, 250 and 500 nM of compound **4a** for 48 h showed obvious morphological changes, with chromatin condensation, fragmentation and formation of apoptotic bodies. However, the control group (without test compound)

Table 2
IC_{50} (μM) values for the new podophyllotoxin–thiourea derivatives **4a–u** by MTT assay

Compounds	A549	DU-145	RWPE-1	LNCaP	HGC	MDA MB-231
4a	37.6 ± 17.1	0.50 ± 0.03*	40.85 ± 0.78	17.56 ± 1.45	3.85 ± 0.27	18.0 ± 0.97
4b	NA	NA	–	NA	NA	NA
4c	44.01 ± 3.15	7.89 ± 1.18	56.69 ± 4.31	53.15 ± 1.11	69.61 ± 1.34	31.77 ± 3.11
4d	NA	10.59 ± 2.39	71.67 ± 4.7	39.76 ± 0.77	60.33 ± 0.03	19.38 ± 0.03
4e	35.14 ± 5.31	12.24 ± 0.68	78.44 ± 3.6	25.6 ± 1.61	33.37 ± 3.18	39.76 ± 0.77
4f	NA	NA	NA	NA	NA	NA
4g	NA	NA	NA	NA	NA	NA
4h	NA	14.13 ± 0.56	>100	20.02 ± 0.97	86.14 ± 8.02	11.86 ± 0.57
4i	65.53 ± 9.35	58.76 ± 1.02	>100	>100	23.67 ± 1.43	>100
4j	NA	20.96 ± 1.61	>100	22.23 ± 2.11	12.91 ± 1.15	17.92 ± 3.42
4k	NA	NA	NA	NA	NA	NA
4l	NA	63.33 ± 1.87	>100	>100 μM	22.43 ± 1.04	>100
4m	NA	NA	NA	NA	NA	NA
4n	NA	NA	NA	NA	NA	NA
4o	NA	NA	NA	NA	NA	NA
4p	14.84 ± 2.46	1.96 ± 0.19	16.94 ± 1.93	3.56 ± 0.76	56.83 ± 3.88	7.58 ± 0.26
4q	61.97 ± 9.75	59.94 ± 1.23	>100	84.78 ± 2.33	>100	>100
4r	NA	NA	NA	NA	NA	NA
4s	>100	2.34 ± 0.04	44.50 ± 0.30	12.93 ± 1.07	86.02 ± 0.23	85.66 ± 2.18
4t	>100	2.37 ± 0.11	64.01 ± 3.79	5.49 ± 0.17	45.38 ± 0.18	>100
4u	NA	NA	NA	NA	NA	NA
Etoposide	38.5 ± 3.6	5.21 ± 0.02	27.64 ± 2.76	50.33 ± 1.04	10.90 ± 1.37	49.35 ± 1.6
Podophyllotoxin	128.61 ± 5.1	ND	ND	ND	ND	ND

NA: Compounds that have not shown 50% inhibition at 100 μM concentration.
ND: Cytotoxicity studies were not done.
* $p < 0.05$, when compared to IC_{50} values with RWPE cells.

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showed normal healthy shape with intact nuclei and without any abnormalities (Fig. 2a). Most of the treated cells in all the test concentrations exhibited similar morphological changes of apoptosis but the damage was severe in the cells exposed to the highest concentration of 500 nM (Fig. 2d).

The results of light microscopy were consistent with that of fluorescence microscopy using Acridine Orange and Ethidium Bromide (AO/EB) staining (Fig. 3a–d). The bright condensed chromatin identified by the staining was a clear indication of early apoptosis leading to margination of chromatin into a horseshoe shaped structure. Cells treated with high concentration, that is, 500 nM of compound **4a** exhibited intense nuclear fragmentation followed by the formation of apoptotic bodies.

In tumors, there are many more cells that are in active cell division compared with the normal tissues. As a result, cell cycle arrest becomes a critical target in oncotherapy. The effect of compound **4a** on cell cycle progression was examined by propidium iodide staining method at concentrations of 125, 250 and 500 nM for 48 h. Compound **4a** has shown significant increase (1.85 fold) in the accumulation of cells at G2/M phase at concentration of 500 nM when compared to the untreated cells. These results indicated that the compound **4a** controlled the growth of the prostate cancer cells by inhibiting the cell cycle at G2/M-phase as shown in Figure 4.

Apoptosis, the process of programmed cell death (PCD), is an important target for cancer chemotherapy. The biochemical events lead to characteristic cell changes, including nuclear fragmentation, chromatin condensation and fragmentation of chromosomal DNA. Externalization of phosphatidyl serine is the characteristic feature of cell undergoing apoptosis which was confirmed by Annexin V FITC staining technique. After treatment with 0, 125, 250 and 500 nM different concentrations of compound **4a** for 48 h to the DU 145 cells, there is an increase in the percentage of apoptotic cells (Annexin V positive only) in a dose dependent manner with % percentage population of 1.42%, 5.31%, 15.35%, 44.38%, respectively (Fig. 5). These results confirm that compound **4a** inhibits cell proliferation by inducing apoptosis in prostate cancer cells.

Topoisomerase II inhibition assay was performed by using Topoisomerase II Drug Screening Kit (TG 1009, Topogen, USA) with etoposide as the standard. From the results, it was found that catenated DNA treated with topoisomerase II (5 units) has shown

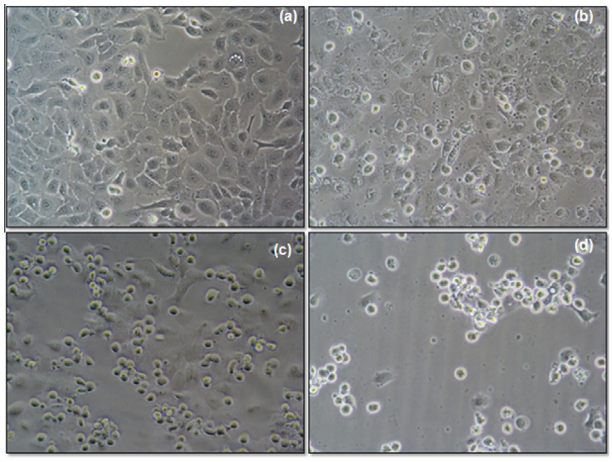

Figure 2. Morphological changes observed in light microscopy in DU-145 cells treated with and without compound **4a** for 48 h. (a) Control, cells with intact nuclei; (b) early signs of apoptosis characterized at 125 nM by extremely condensed chromatin; (c) cell membrane blebbing, nuclear fragmentation and chromatin condensation at 250 nM. (d) Decreased cell population and late apoptotic cells at 500 nM.

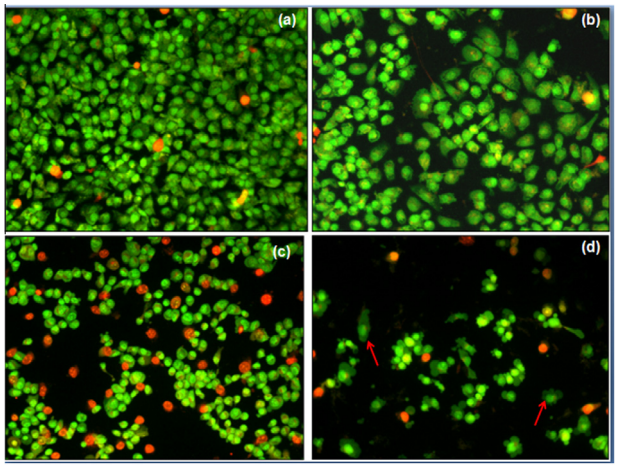

Figure 3. Morphological changes in DU-145 cells treated with and without compound **4a** for 48 h. Fluorescence microscopy: (a) green live cells show normal morphology of control; (b) early signs of apoptosis characterized at 125 nM by extremely condensed chromatin, which marginated into a horseshoe-shaped structure and cell membrane blebbing, (c) destructive fragmentation of the nuclei at 250 nM and irregular distribution of chromatin, (d) late apoptotic cells at 500 nM exhibited condensed chromatin and their nuclei, stained red with EB.

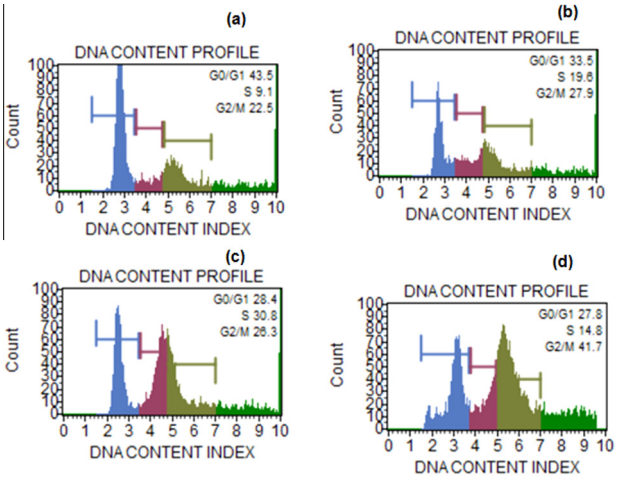

Figure 4. Effect of compound **4a** on cell cycle progression of DU-145 cells, (a) control cells; (b–d) cells treated with 125, 250 and 500 nM for 48 h followed by analysis of cell cycle distribution using propidium iodide cell staining method and analyzed by Muse cell analyser. All assays were done in duplicate.

nicked circular and relaxed circular DNA in the Lane E (Fig. 6). Catenated DNA in the presence of topoisomerase II incubated with etoposide at a concentration of 100 μM (Lane D) has shown clear linear DNA formation, indicating that it acts as Interfacial poison (IFP) which blocks the resealing of the dsDNA. Catenated DNA in the presence of topoisomerase II incubated with compound **4p** at 100 μM, moderately inhibited the topoisomerase II activity whereas that incubated with **4a** at 50 and 100 μM strongly inhibited the catalytic activity, as evident from the highly super coiled catenated DNA in the wells.

To visualize the binding mode of the compounds **4a**–**u**, molecular docking studies were performed with ATP-binding domain of human topoisomerase-IIα. The docking analysis has shown that the scaffold of all the synthesized compounds occupied the ATP-binding active site with similar orientations, formed hydrogen-bonding with several surrounding residues and the results are in correlation with the DNA topoisomerase-IIα inhibition assay.

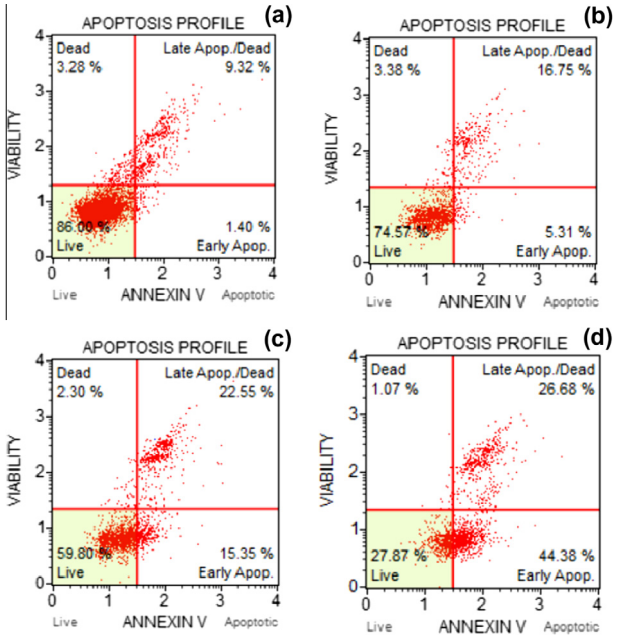

Figure 5. Annexin V binding assay by Muse™ Cell Analyzer (a) control cells; (b–d) cells treated with 125, 250 and 500 nM of **4a** after 48 h of treatment in DU-145. Data represents one of three independent experiments.

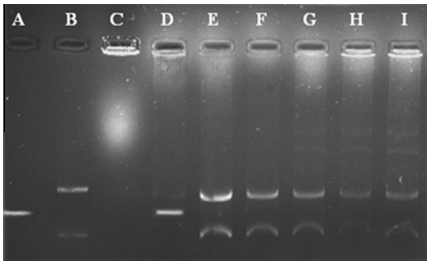

Figure 6. Effect of the synthesized compounds **4a** and **4p** on topoisomerase II. Lane A: Linear DNA; Lane B: Decatenated DNA; Lane C: Catenated DNA; Lane D: Catenated DNA + Topoisomerase II (5 units) + (Etoposide: 100 μM); Lane E: Catenated DNA + Topoisomerase II (5 units); Lane F: Catenated DNA + Topoisomerase II (5 units) + (**4p**: 50 μM); Lane G: Catenated DNA + Topoisomerase II (5 units) + (**4p**: 100 μM); Lane H: Catenated DNA + Topoisomerase II (5 units) + (**4a**: 50 μM); Lane I: Catenated DNA + Topoisomerase II (5 units) + (**4a**: 100 μM).

Figure 7 represents the binding pose of **4a** superimposed on etoposide in the binding pocket, where it is surrounded and forms strong hydrophobic interactions with Asn91, Asn95, Arg98, Gly124, Ile125, Pro126, Ile141, Gly166, and Gly164 residues similar to etoposide. The methoxy oxygen at R^3 in **4a** forms hydrogen bonds simultaneously with the back bones of three amino acid residues: Gly166, Gly164 and Tyr165 whereas the hydroxy group of etoposide hydrogen bonded with only Ile141. This explains the higher cytotoxicity of **4a** in comparison with etoposide. All these strong interactions of the ligands with the magnesium ion and residues below the ATP binding site (Asn46, Asp73, Ile78, Arg136 and Tyr165) suggest that the binding of our ligands with topoisomerase II impedes the entry of ATP into it. The molecular docking here can provide an insight for the design of the novel podophyllotoxin derivatives in order to improve the binding affinity of the compounds and to compete effectively with ATP.

In conclusion, the present study has led to the development of a direct one-pot azido reduction tandem isothiocyanates coupling for the synthesis of novel podophyllotoxin–thiourea derivatives

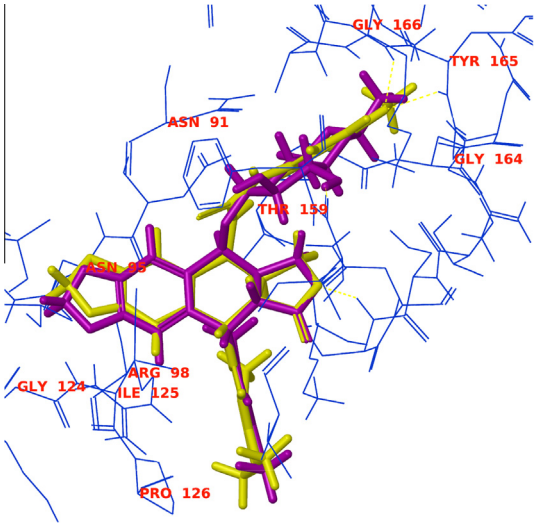

Figure 7. View of compound **4a** (yellow) and etoposide (purple) docked in the ATP binding domain of human topoisomerase-IIα. The amino acids involved in the interaction are depicted in stick representation.

by employing NH_2SO_3H/NaI reagent system. Further, these compounds have been evaluated for their in vitro antitumor activity on selected human cancer cell lines. Some of the representative compounds have shown promising activity in comparison to etoposide with optimal safety window. One of the compounds **4a** exhibited broad spectrum of anticancer activity against all the tested cancer cell lines and induced apoptosis in human prostate cancer (DU-145) cell line with DNA–topoisomerase II inhibitory activity and strong G2/M cell cycle arrest. The docking results have also shown similar orientation and interactions at the ATPse domain of topoisomerase-IIα compared to the standard etoposide. Overall, these preliminary results of podophyllotoxin–thiourea derivatives offer an impetus for the future endeavors in the drug discovery as potent antitumour agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.07.100.

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34. Typical reaction procedure, experimental data for all the compounds and general methods are provided in the supporting information.