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Original article

4β-[(4-Alkyl)-1,2,3-triazol-1-yl] podophyllotoxins as anticancer compounds: Design, synthesis and biological evaluation

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ABSTRACT

A series of 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives were designed *in silico*, synthesised by employing click chemistry approach, and evaluated for cytotoxicity against a panel of human cancer cell lines (SF-295, A-549, PC-3, Hep-2, HCT-15 and MCF-7). Majority of the compounds proved to be more potent than etoposide and select compounds exhibited significant anticancer activity with IC_{50} values in the range of 0.001−1 μM. DNA fragmentation and flow-cytometric results reveals that 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives induce dose dependent apoptosis. Docking experiments showed a good correlation between their calculated interaction energies with the topoisomerase-II and the observed IC_{50} values of all these compounds.

1. Introduction

Podophyllotoxin (**1**), is a most abundant naturally occurring cyclolignan, mainly isolated from *Podophyllum peltatum* and *podophyllum hexandrum* [1,2]. Podophyllotoxin has cathartic, antirheumatic and antiviral properties but its antimitotic activity has proved to be the most attractive for researchers [3]. Podophyllotoxin is known as an antimicrotubule agent acting at the colchicine-binding site on tubulin [4]. Due to severe toxicity of **1**, it is not being used as an anticancer drug, but it's semi synthetic derivatives etoposide and teniposide (Fig. 1) are clinically useful drugs against various cancers, including small cell lung cancer, testicular carcinoma, lymphoma, and Kaposi's sarcoma [5–12]. The chemical modifications that led to etoposide, teniposide and other derivatives, also lead to the change in the mechanism of action of these ligands wherein podophyllotoxin act as antimicrotubule agent whereas its aforementioned derivatives act as topoisomerase-II inhibitors [13]. These derivatives block the catalytic activity of DNA topoisomerase-II by stabilizing a cleavage enzyme–DNA complex in which the DNA is cleaved and covalently linked to the enzyme. However, the therapeutic use of **2** and **3** is often hindered by problems such as acquired drug-resistance and poor water solubility. To get more potent analogues and to overcome drug-resistance recently several complex and more diverse analogues like Etopophos (**4**), GL-331, TOP-53, NK-611, NPF etc. have been synthesised (Fig. 1). Etopophos, is a water-soluble prodrug of **2**, is readily converted *in vivo* to the active drug, **2** and exhibits similar pharmacological and pharmacokinetic profiles that of **2**. NK-611, NPF and GL-331 are presently under clinical trial. According to structure–activity relationship (SAR) of podophyllotoxin, *trans*-lactone, 4β-substituted and 4′-demethyl moieties were essential to maintain the anticancer activity as topoisomerase-II inhibitors [14]. Particularly 4β-N-substituted derivatives of podophyllotoxin gained much importance owing to their improved cytotoxicity.

In recent years, we have been working on the chemical transformation of podophyllotoxin and focused libraries of potent aniline, phenol, thiophenol and carbohydrate based 1,2,3-triazole derivatives have been generated, some of which exhibited significant anticancer activity [15–17]. Even though aromatic substitution on the triazole moiety yielded podophyllotoxin analogues with good cytotoxicity, our recent docking studies revealed that 1,2,3-triazole derivatives with various aliphatic substituent's in triazole moiety showed better binding ability to topoisomerase-II enzyme than etoposide. This prompted us to synthesise a series of 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives using click reaction. Click chemistry enables a modular approach to generate these novel

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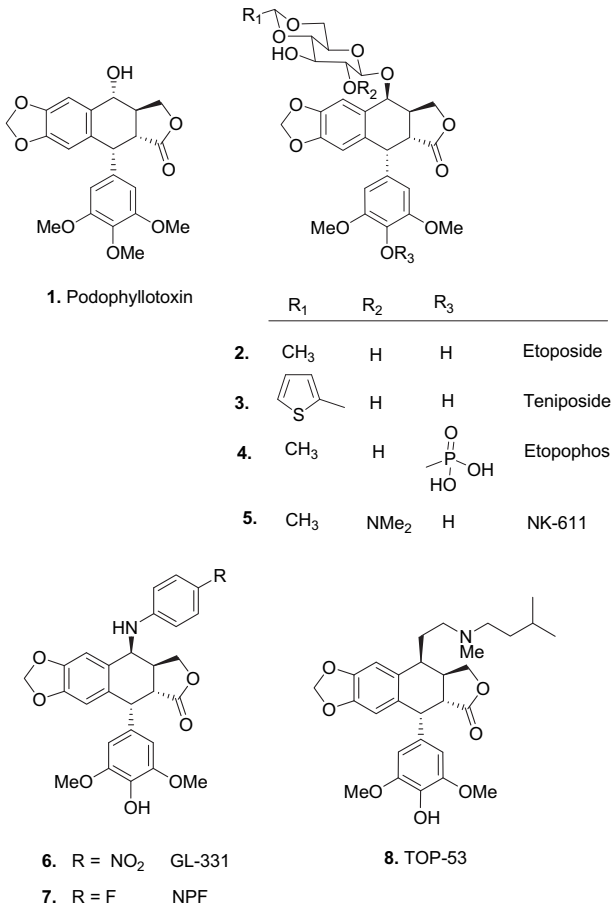


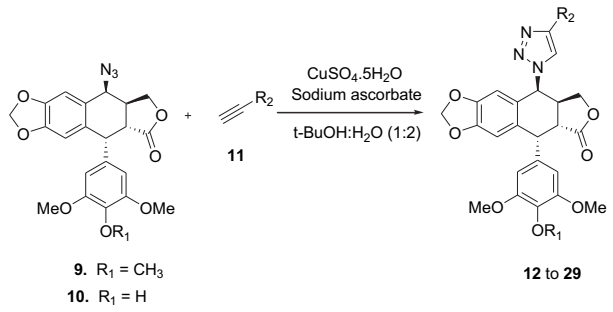
Fig. 1. Structures of some podophyllotoxin derivatives.

pharmacophores utilizing collection of reliable chemical reactions [18]. Of particular interest is the Huisgen [3 + 2] cycloaddition between a terminal alkyne and an azide to generate substituted 1,2,3-triazoles [19]. All the 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives were screened for anticancer activity against a panel of six human cancer cell lines. Most of the triazole derivatives exhibited better cytotoxicity than etoposide. Podophyllotoxin derivatives are known to exert its anti-tumour effect by promoting programmed cell death (apoptosis) [20]. DNA fragmentation and cell cycle analysis have also been evaluated on MCF-7 cell line. From the IC_{50} values and docking score it is clear that compounds which revealed good binding interaction with topoisomerase-II exhibited better cytotoxicity. Induction of apoptosis by these ligands has been confirmed by cell cycle and DNA fragmentation analysis.

2. Results and discussion

2.1. Chemistry

As illustrated in Scheme-1, 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives were synthesised by the cycloaddition reaction of C4β-azido podophyllotoxin, **9** and C4β-azido-4′-*O*-demethyl podophyllotoxin, **10** with various terminal aliphatic alkynes, **11**. Compounds **9** and **10** were synthesised according to literature procedures [21,22]. Compound **9** was obtained by the reaction of podophyllotoxin with CH_3SO_3H and NaI in acetonitrile followed by hydrolysis with $H_2O/Me_2CO/BaCO_3$ and reaction with NaN_3 in TFA. Compound **10** was obtained by the reaction of podophyllotoxin with CH_3SO_3H and NaI in dichloromethane followed by



Scheme-1. Click-chemistry strategy for the synthesis of 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives.

hydrolysis with $H_2O/Me_2CO/BaCO_3$ and reaction with NaN_3 in TFA. The azides **9** and **10** obtained were allowed to react with various terminal alkynes, in the presence of $CuSO_4 \cdot 5H_2O$ and sodium ascorbate, in t-butyl alcohol and water (1:2) at room temperature to yield selectively 4β-[(4-alkyl)-1,2,3-triazol-1-yl] derivatives in excellent yields (>96%). Using this click-chemistry protocol a focused library of analogues with different substitution has been generated (Table 1).

All the products were characterized by ^1H NMR, ^{13}C NMR, IR, ESI-MS. In the ^1H NMR spectra, the formation of triazoles was confirmed by the resonance of H–C(5) of the triazole ring in the aromatic region. The structure was further supported by the ^{13}C NMR spectra, which showed the C-atom signals corresponding to triazole derivatives. ESI-MS of all compounds showed [M + Na] or [M + 1].

2.2. Evaluation of biological activity

2.2.1. Anticancer activity

The *in vitro* cytotoxicity of all the compounds was evaluated against a panel of six human cancer cell lines *viz.*, SF-295 (Neuroblastoma), A-549 (Lung), PC-3 (Prostate), Hep-2 (Liver), HCT-15 (Colon) and MCF-7 (Breast). Etoposide was taken as reference compound. The IC_{50} values derived from *in vitro* screening studies revealed that all the compounds posses significant cytotoxicity against SF-295, PC-3, Hep-2, HCT-15 and MCF-7 cancer cell lines (Table 2). However, a lesser activity was observed against A-549 cancer cell line. Compounds **12**, **13**, **20**, **21**, **22** and **29** showed

Table 1
Various 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives and its docking score.

Entry	R_1	R_2	Yield%[a]	Glide score
12	CH_3	Ethyl	96	−3.53
13	CH_3	Propyl	97	−3.56
14	CH_3	Butyl	98	−3.25
15	CH_3	Pentyl	98	−3.21
16	CH_3	Hexyl	98	−3.38
17	CH_3	Heptyl	98	−3.21
18	CH_3	Octyl	99	−3.35
19	CH_3	Decyl	96	−3.41
20	CH_3	CH_2OH	99	−4.08
21	H	Ethyl	98	−4.32
22	H	Propyl	96	−3.45
23	H	Butyl	97	−3.16
24	H	Pentyl	99	−3.24
25	H	Hexyl	96	−3.37
26	H	Heptyl	98	−3.36
27	H	Octyl	96	−3.33
28	H	Decyl	99	−3.23
29	H	CH_2OH	97	−4.64
2				−3.02

[a] Isolated yields.

Table 2
IC_{50} values (μM) of various 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives.

Entry	SF-295	A-549	PC-3	Hep-2	HCT-15	MCF-7
12	1.8	35	0.03	0.06	0.4	0.4
13	3.6	39	5.1	1.6	0.03	1.4
14	8.1	82	6.6	9.6	11	9.8
15	24	39	8.4	7.7	11	9.5
16	10	35	6.4	10	27	8.6
17	25	36	23	9.8	45	6.4
18	14	>100	17	18	>100	9.3
19	6.2	>100	5.5	5.8	29	5.7
20	2.1	>100	0.06	0.06	15	0.01
21	4.8	17	0.06	0.05	1.9	0.04
22	2.3	27	0.2	2.9	0.8	5.7
23	21	35	12	17	34	20
24	4.3	26	4.7	21	2.1	14
25	18	26	8.7	>100	11	19
26	10	45	5.8	6	15	13
27	5.7	>100	5.1	3.8	13	11
28	13	30	19	19	15	11
29	15	18	8.2	6.7	18	0.6
2	13.5	5.62	17.5	2.15	7.15	19

significant cytotoxicity against PC-3, Hep-2, HCT-15 and MCF-7 cell lines. Compound **12** showed more potent cytotoxicity against SF-295 and PC-3 cell lines with IC_{50} values of 1.8 and 0.03 μM respectively. Compound **13** was found to show highest cytotoxicity against HCT-15 cell line with an IC_{50} value of 0.03. Compound **20** and **21** on the other hand showed highest cytotoxicity against HEP-2 cell line with IC_{50} values of 0.05 μM both. The IC_{50} values of the compounds revealed that methyl, ethyl and hydroxyl groups in triazole moiety increases the anticancer activity, further, it was found that increasing alkyl chain length of the compounds decreases the anticancer activity.

2.2.2. DNA fragmentation and cell cycle analysis

DNA fragmentation, which is a typical hallmark of the apoptotic cell death was analysed in human breast cancer cell line MCF-7. From the *in vitro* cytotoxicity studies it was found that compounds **12** and **13** significantly inhibits the growth of human breast cancer cell line MCF-7, and was therefore studied further to determine the mechanism of cell death in the same cell line. The

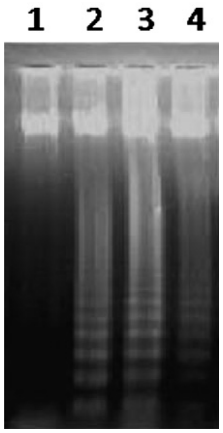


Fig. 2. Agarose gel electrophoresis of DNA extracted from MCF-7 cells. The figure represents MCF-7 cells treated with compounds **12** and **13** for 24 h. DNA from the cells was extracted and electrophoresed in 1% agarose gel and visualized by ethidium bromide staining under UV illumination. Lane 1, 2, and 3 are represent compound **12** at 0, 1, 5 μM. Lane 4: treated with compound **13** (5 μM).

DNA fragmentation analysis revealed that compound **12** and **13** induced a discrete ladder pattern in MCF-7 cell line at 1 μM after 24 h of incubation (Fig. 2). The cell cycle analysis of compound **12** and **13** showed a dose dependent increases in the sub-G1 (apoptotic) population (Fig. 3) in MCF-7 cell line after 24 h. Cell cycle analysis showed that the ratio of apoptosis in MCF-7 cells for compound **12** at 1 and 5 μM was 69.34% (24 h) and 80.10% (24 h) respectively, and for compound **13** at 1 and 5 μM it was found to be 55.34% and 78.16% respectively.

2.3. Docking studies

To study the molecular basis of interaction and affinity of binding of the podophyllotoxin analogues, all the ligands were docked into the ATPase domain of Topoisomerase-II. ATPase domain is the probable binding site for etoposide as reported earlier [23,24]. Docking was done using Glide module of Schrodinger software. Docking results of these ligands are given in Table 1. Compounds **12**, **13**, **20**, **21**, **22** and **29** showed good interaction with TP-II and these results were matching with wet lab

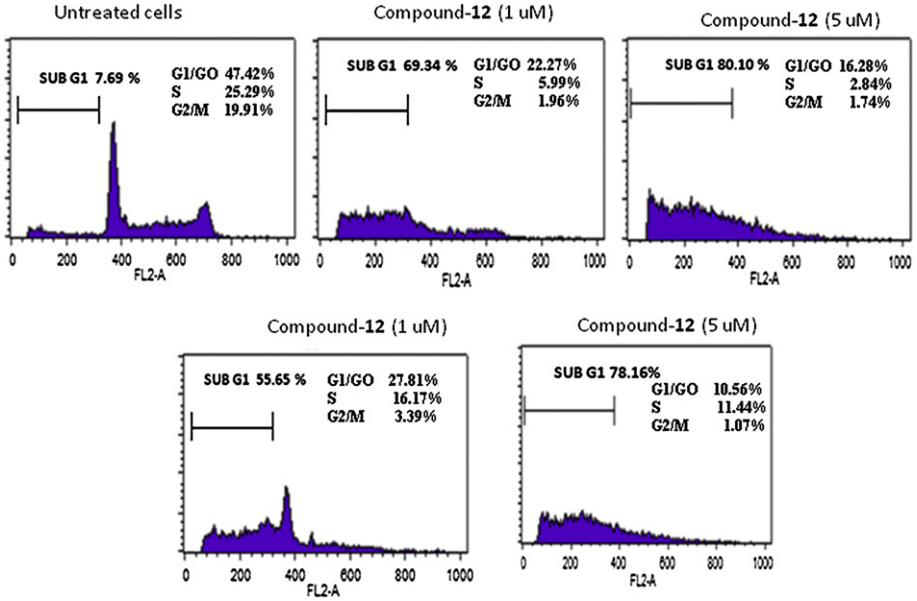


Fig. 3. DNA Cell Cycle analysis for compound **12** and **13** (1 and 5 μM).

findings. Compound **20** was found to have a score of −4.08 with 3 strong Hbonds with Arg98 and 2 Hbonds with Ser149 with strengths of 2.14 Å and 1.83 Å. Also they were found to have strong hydrophobic contacts with the residues of active site. Compound **21** was having single strong hydrogen bond with strength of 2.01 Ao with Lys131 along with strong hydrophobic contacts; this signifies a strong binding of the molecules to the receptor. The slight variation in dock score of compound **12** and **13** was observed with scores of −3.53 and −3.56 although both were having 2 hydrogen bonds with Arg98 with almost same strengths but the difference lies in their hydrophobic contacts. Compound **13** was having 231 contacts whereas **12** was having 220 contacts. The orientation of both the molecules was almost identical just the hydrophobic interactions were less in **12** as compared to **13**. The ligand receptor interactions of compound **29** and **21** are shown in Fig.4 and the interactions of compound **12** and **13** are shown in Fig. 5.

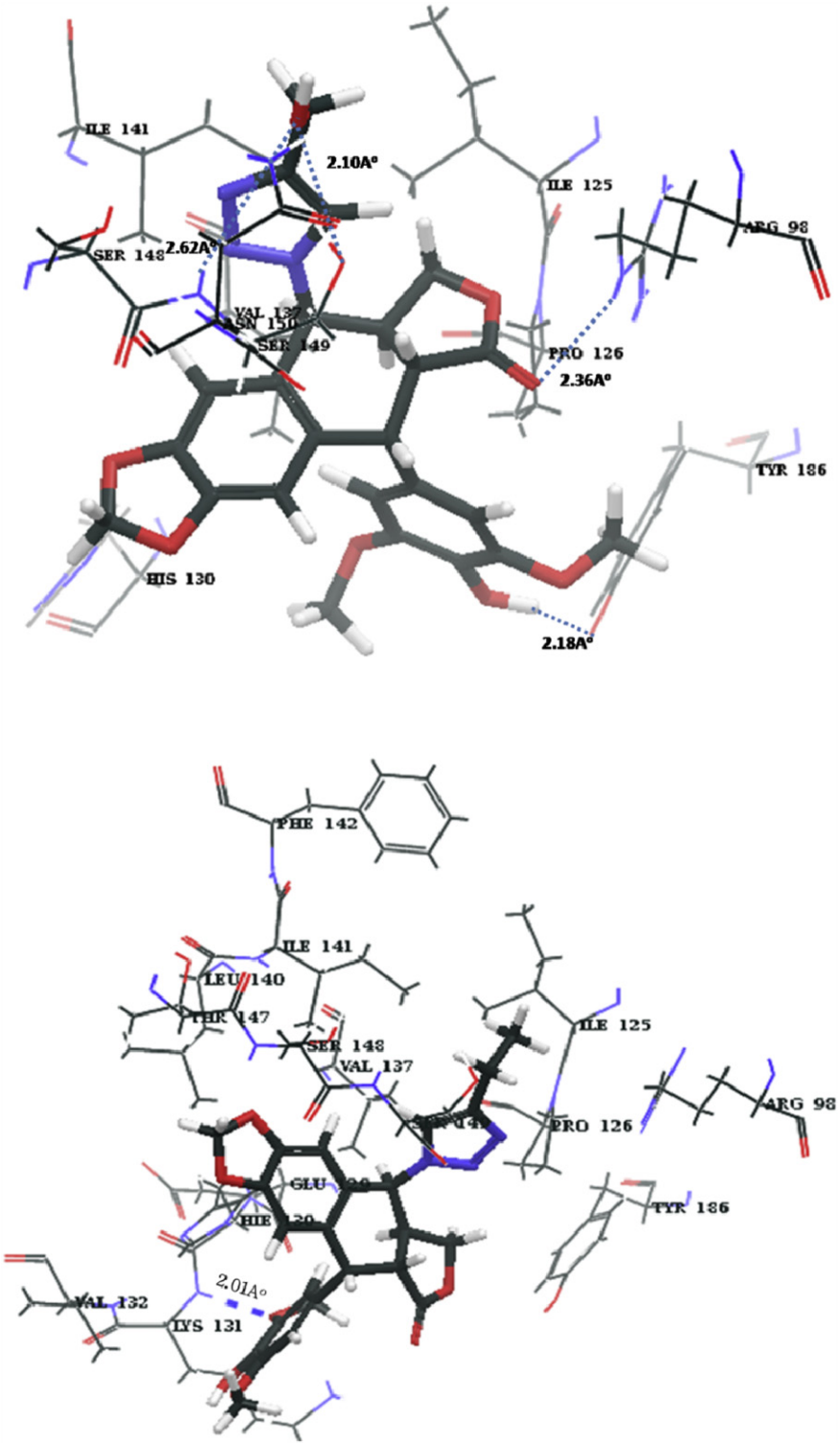


Fig. 4. Interaction of compound **29** and **21** with the residues within 5A of the receptor cavity.

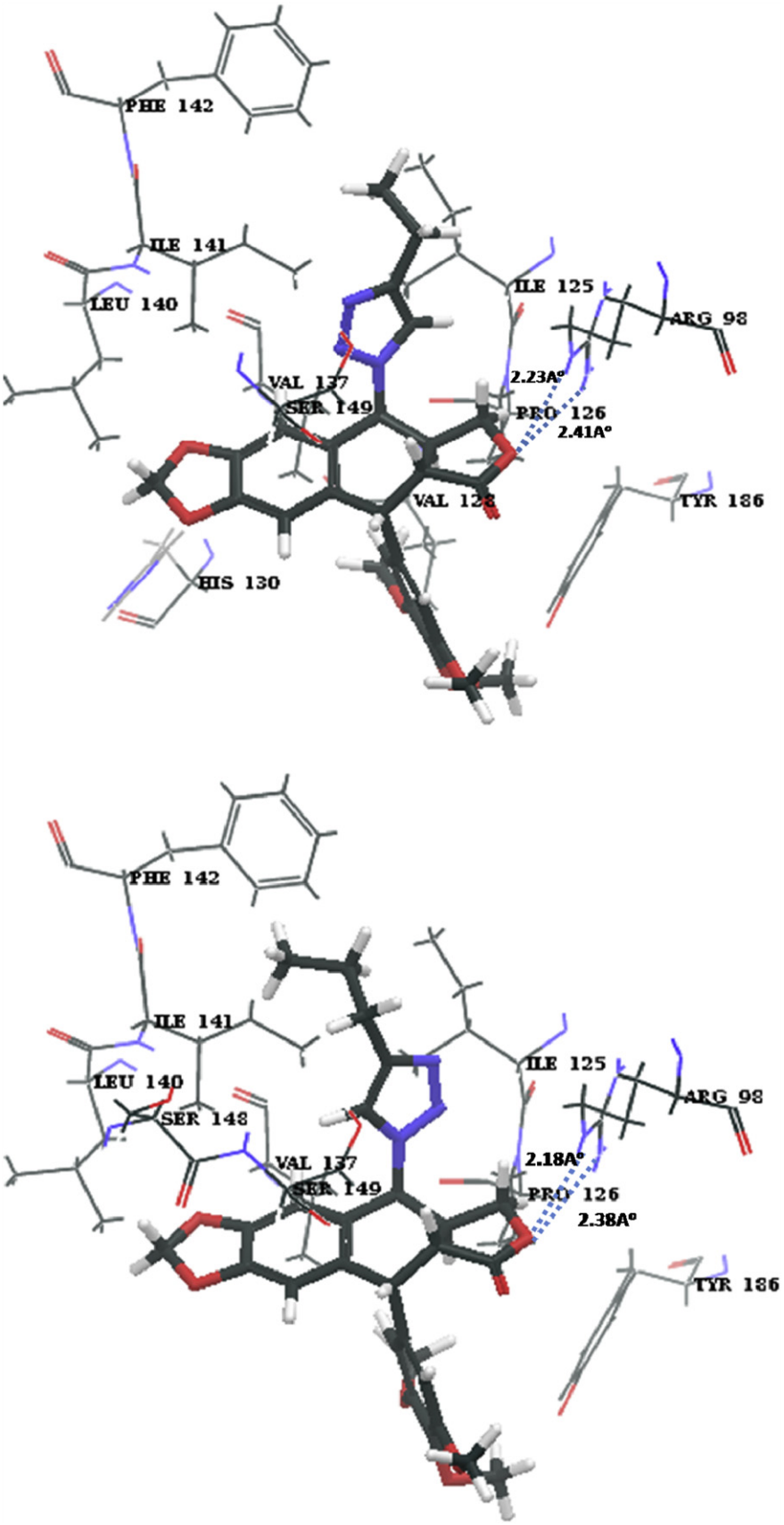


Fig. 5. Interactions of compound **12** and **13** with the residues within 5A of the receptor cavity.

From the results it was apparent that the *in silico* findings were well correlated with the data obtained through *in vitro* cytotoxicity assay.

3. Conclusions

In conclusion, a series of 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives were designed, synthesised and screened for anticancer activity against a panel of six human cancer cell lines. Most of the compounds exhibited improved anticancer activity as compared to etoposide and found to be pro-apoptotic molecules.

4. Experimental

Melting points were recorded on Buchi Melting point apparatus D-545 and IR spectra (KBr) on Bruker Vector 22 instrument. NMR spectra were recorded on Bruker DPX500 instrument in $CDCl_3$ with TMS as an internal standard. Chemical shift values are reported in δ (ppm) and coupling constants in hertz. Mass spectra were recorded on ESI-esquire 3000 Bruker Daltonics instrument. The progress of all reactions was monitored by TLC on 2 × 5 cm pre-coated silica gel 60 F_{254} plates of thickness 0.25 mm (Merck). The chromatograms were visualized under UV 254–366 nm and iodine. Purity was checked with Waters analytical HPLC.

4.1. Click chemistry-general procedure

To a solution of 1-pentyne (0.46 mmol) in t-butyl alcohol and water (1:2, 8 mL) was added $CuSO_4 \cdot 5H_2O$ (0.46 mmol), sodium ascorbate (1.1 mmol) followed by 4β-azido-podophyllotoxin (0.23 mmol). The reaction mixture was stirred at room temperature for 8 h (to synthesise compound **12** and **21** reaction mixture was stirred at 0 °C for 1 h, after 1 h reaction was allowed to reach room temperature). After completion, the reaction mixture was diluted with 80 mL of water and extracted with chloroform (3 × 30 mL). The combined extracts were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The crude product obtained was washed with n-hexane to remove excess alkyne and recrystallized with ether to yield the pure product (**12**–**29**). All the compounds were >98% pure, purity as confirmed by HPLC analysis (RP-18 column 4 × 250 mm, Merck, with a UV/vIS detector, mobile phase gradient mixture of methanol and water, flow rate of 0.8 mL/min).

4.1.1. 4β-[(4-Ethyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**12**)

White solid; mp: 149–150 °C; $[\alpha]_D^{25}$ −24.3 (*c* 0.45, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.93 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.07 (d, *J* = 4.79 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.76 (d, *J* = 4.98 Hz, 1H), 4.41–4.38 (t, *J* = 7.77 Hz, 1H), 3.82 (s, 3H), 3.75 (s, 6H), 3.28–3.16 (m, 2H), 3.08–3.04 (dd, *J* = 8.97 Hz, 4.98 Hz, 1H), 2.75–2.70 (t, *J* = 7.59 Hz, 2H), 1.28–1.25 (t, *J* = 7.54 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.33, 152.80, 149.32, 148.06, 137.52, 134.35, 133.11, 124.99, 110.44, 108.93, 108.20, 101.96, 67.54, 60.75, 58.46, 56.32, 43.69, 41.74, 37.22, 31.21, 29.68, 13.46; IR (KBr): 3424.95, 2926.66, 1779.70, 1589.47, 1505.77, 1485.17, 1236.15, 1125.91, 1036.07, 1001.91, 931.94 cm^{-1}; ESI-MS: 494.1 (M + 1).

4.1.2. 4β-[(4-Propyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**13**)

White solid; mp: 145 °C; $[\alpha]_D^{25}$ −28.8 (*c* 0.50, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.95 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.07 (d, *J* = 4.42 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.76 (d, *J* = 4.89 Hz, 1H), 4.41–4.39 (t, *J* = 7.28 Hz, 1H), 3.82 (s, 3H), 3.77 (s, 6H), 3.26–3.18 (m, 2H), 3.08–3.05 (dd, *J* = 9.06 Hz, 4.82 Hz, 1H), 2.68–2.65 (t, *J* = 7.6 Hz, 2H), 1.70–1.66 (m, 2H), 0.97–0.94 (t, *J* = 7.33 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.28, 153.09, 149.48, 148.45, 137.60, 134.33, 133.06, 125.11, 110.66, 109.05, 108.90, 101.90, 67.51, 60.68, 58.70, 56.77, 43.81, 42.00, 37.55, 31.23, 27.66, 22.45, 13.74; IR (KBr): 3424.55, 2926.56, 1779.20, 1589, 1505.97, 1485.27, 1236.15, 1125.92, 1036, 1001.95, 931.84 cm^{-1}; ESI-MS: 530 (M + Na).

4.1.3. 4β-[(4-Butyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**14**)

White solid; mp: 120–121 °C; $[\alpha]_D^{25}$ −33.3 (*c* 0.66, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.02 (s, 1H), 6.66 (s, 1H), 6.62 (s, 1H), 6.34 (s, 2H), 6.07 (d, *J* = 4.66 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.77 (d, *J* = 4.83 Hz, 1H), 4.4–4.37 (t, *J* = 7.69 Hz, 1H), 3.83 (s, 3H), 3.78 (s, 6H), 3.25–3.2 (m, 2H), 3.11–3.09 (dd, *J* = 8.99 Hz, 4.88 Hz, 1H), 2.7–2.67 (t, *J* = 7.71 Hz, 2H), 1.68–1.65 (p, *J* = 7.5 Hz, 2H), 1.60–1.56 (m, 2H), 0.95–0.93 (t, *J* = 7.41 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.30, 152.80, 149.31, 148.73, 137.53, 134.34, 133.10, 124.99, 110.42, 108.90, 108.01, 101.80, 67.51, 60.75, 58.40, 56.21, 43.68, 41.73, 37.22, 31.38, 25.38, 22.41, 14.05; IR (KBr): 3424.90, 2926.66, 1779.90, 1590, 1505.97, 1484.97, 1236.15, 1125.90, 1036.11, 1002.01, 931.84 cm^{-1}; ESI-MS: 522 (M + 1).

4.1.4. 4β-[(4-Pentyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**15**)

White solid; mp: 153–155 °C; $[\alpha]_D^{25}$ −36.3 (*c* 0.56, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.98 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.06 (d, *J* = 4.72 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.73 (d, *J* = 4.91 Hz, 1H), 4.40–4.38 (t, *J* = 7.8 Hz, 1H), 3.82 (s, 3H), 3.76 (s, 6H), 3.21–3.16 (m, 2H), 3.10–3.07 (dd, *J* = 9.03, 4.92 Hz, 1H), 2.65–2.62 (t, *J* = 7.71 Hz, 2H), 1.64–1.60 (m, 2H), 1.31–1.24 (m, 4H), 0.87–0.84 (t, *J* = 7.45 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.31, 152.79, 149.29, 148.05, 137.51, 134.35, 133.08, 125.03, 110.42, 108.90, 108.19, 101.96, 67.52, 60.75, 58.38, 56.31, 43.68, 41.74, 37.22, 31.91, 29.35, 25.68, 22.33, 14.12; IR (KBr): 3424.95, 2926.54, 1779.90, 1589.81, 1505.77, 1485.27, 1236.45, 1126.02, 1036, 1001.99, 931.95 cm^{-1}; ESI-MS: 536 (M + 1).

4.1.5. 4β-[(4-Hexyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**16**)

White solid; mp: 116 °C; $[\alpha]_D^{25}$ −25 (c 0.44, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.90 (s, 1H), 6.64 (s, 1H), 6.61 (s, 1H), 6.32 (s, 2H), 6.06 (d, *J* = 4.72 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.71 (d, *J* = 4.54 Hz, 1H), 4.42–4.39 (t, *J* = 7.42 Hz, 1H), 3.81 (s, 3H), 3.76 (s, 6H), 3.23–3.18 (m, 2H), 3.12–3.09 (dd, *J* = 8.091 Hz, 4.81 Hz, 1H), 2.66–2.63 (t, *J* = 7.90 Hz, 2H), 1.66–1.61 (m, 2H), 1.30–1.23 (m, 6H), 0.87–0.83 (t, *J* = 7.63 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.08, 153.18, 149.55, 148.30, 137.60, 134.32, 133.50, 125.31, 110.63, 109.22, 108.98, 102.00, 67.53, 60.78, 58.77, 56.71, 43.95, 42.02, 37.50, 31.88, 29.37, 29.21, 25.87, 22.65, 14.00; IR (KBr): 3425.27, 2926.64, 1779.63, 1590.09, 1505.77, 1485.27, 1236.01, 1125.81, 1036.27, 1001.90, 931.91 cm^{-1}; ESI-MS: 550 (M + 1).

4.1.6. 4β-[(4-Heptyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**17**)

White solid; mp: 112 °C; $[\alpha]_D^{25}$ −66.3 (c 0.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.81 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.07 (d, *J* = 4.63 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.82 (d, *J* = 4.42 Hz, 1H), 4.45–4.42 (t, *J* = 7.28 Hz, 1H), 3.82 (s, 3H), 3.77 (s, 6H), 3.28–3.24 (m, 2H), 3.12–3.09 (dd, *J* = 9.01 Hz, 4.72 Hz, 1H), 2.76–2.73 (t, *J* = 7.66 Hz, 2H), 1.76–1.72 (m, 2H), 1.30–1.25 (m, 10H), 0.86–0.83 (t, *J* = 7.51 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.79, 152.04, 149.41, 148.14, 137.80, 134.25, 133.23, 124.94, 110.50, 108.91, 108.40, 101.91, 67.51, 60.77, 58.62, 56.43, 43.73, 41.81, 37.25, 31.46, 29.70, 29.25, 28.94, 25.77, 22.49, 13.99; IR (KBr): 3428.5, 2926.36, 1780.27, 1589.77, 1505.27, 1485.55, 1236.01, 1126.06, 1036.27, 1001.90, 932.03 cm^{-1}; ESI-MS: 564 (M + 1).

4.1.7. 4β-[(4-Octyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**18**)

White solid; mp: 125 °C; $[\alpha]_D^{25}$ −49.2 (*c* 0.60, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.96 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s,

2H), 6.08 (d, J = 4.81 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.81 (d, J = 4.72 Hz, 1H), 4.42–4.39 (t, J = 7.21 Hz, 1H), 3.82 (s, 3H), 3.77 (s, 6H), 3.26–3.19 (m, 2H), 3.08–3.04 (dd, J = 8.72 Hz, 4.72 Hz, 1H), 2.71–2.67 (t, J = 7.36 Hz, 2H), 1.75–1.63 (m, 4H), 1.42–1.26 (m, 8H), 0.89–0.84 (t, J = 7.54 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.52, 152.11, 149.39, 148.22, 137.68, 134.33, 133.63, 124.99, 110.45, 109.00, 108.60, 101.95, 67.55, 60.71, 58.66, 56.40, 43.76, 41.79, 37.30, 31.51, 29.79, 29.29, 29.11, 28.90, 25.69, 22.52, 13.98; IR (KBr): 3425.90, 2926.03, 1779.79, 1589.87, 1505.99, 1485.01, 1236.54, 1120.24, 1036.57, 1001.51, 932.06 cm^{-1}; ESI-MS: 590 (M + Na).

4.1.8. 4β-[(4-Decyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**19**)

White solid; mp: 108 °C; $[\alpha]_D^{25}$ −55.2 (c 0.4, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.90 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.07 (d, J = 4.81 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.75 (d, J = 4.63 Hz, 1H), 4.42–4.39 (t, J = 7.21 Hz, 1H), 3.82 (s, 3H), 3.77 (s, 6H), 3.26–3.19 (m, 2H), 3.08–3.04 (dd, J = 8.72 Hz, 4.72 Hz, 1H), 2.71–2.67 (t, J = 7.36 Hz, 2H), 1.75–1.63 (m, 4H), 1.42–1.26 (m, 10H), 0.89–0.84 (t, J = 7.54 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.30, 152.82, 149.33, 148.07, 137.60, 134.37, 133.14, 125.09, 110.43, 108.92, 108.29, 101.96, 67.54, 60.74, 58.49, 56.34, 43.70, 41.77, 37.22, 31.89, 29.69, 29.57, 29.36, 29.11, 28.77, 25.75, 22.68, 18.45, 14.11; IR (KBr): 3425.54, 2926.77, 1779.70, 1589.54, 1505.90, 1485.03, 1236.45, 1125.81, 1036.07, 1001.91, 932.09 cm^{-1}; ESI-MS: 606 (M + 1).

4.1.9. 4β-[(4-Hydroxy methyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**20**)

White solid; mp: 148–150 °C; $[\alpha]_D^{25}$ −45 (c 0.6, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.24 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.07 (d, J = 4.69 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.75 (d, J = 4.97 Hz, 1H), 4.41–4.38 (t, J = 6.74 Hz, 1H), 3.82 (s, 3H), 3.75 (s, 6H), 3.74 (s, 2H), 3.28–3.16 (m, 2H), 3.08–3.04 (dd, J = 8.97 Hz, 4.98 Hz, 1H), ^{13}C NMR ($CDCl_3$): δ 173.04, 153.11, 149.62, 148.14, 137.75, 134.29, 133.28, 124.74, 110.64, 109.19, 108.83, 102.03, 67.47, 59.85, 58.94, 56.69, 43.88, 41.83, 37.39, 29.71; IR (KBr): 3330.21, 2926.75, 1763.60, 1613.10, 1517.54, 1483.50, 1233.36, 1110.90, 1033.63, 997.10, 930.11 cm^{-1}; ESI-MS: 496 (M + 1).

4.1.10. 4′-O-Demethyl-4β-[(4-ethyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**21**)

White solid; mp: 160 °C; $[\alpha]_D^{25}$ −19.2 (c 0.54, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.92 (s, 1H), 6.64 (s, 1H), 6.61 (s, 1H), 6.31 (s, 2H), 6.06 (d, J = 4.90 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.72 (d, J = 4.81 Hz, 1H), 4.40–4.37 (t, J = 7.72 Hz, 1H), 3.76 (s, 6H), 3.26–3.15 (m, 2H), 3.07–3.03 (dd, J = 9.03 Hz, 4.72 Hz, 1H), 2.73–2.68 (t, J = 7.66 Hz, 2H), 1.26–1.23 (t, J = 7.82 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.39, 149.35, 148.00, 146.65, 134.46, 133.36, 129.82, 125.09, 110.40, 108.93, 108.80, 101.94, 67.72, 58.51, 56.45, 43.51, 41.89, 37.04, 31.22, 29.62, 13.34; IR (KBr): 3388.06, 2927.36, 1766.09, 1610.99, 1515.77, 1483.69, 1228.15, 1109.47, 1034.63, 995.41, 926.94 cm^{-1}; ESI-MS: 480 (M + 1).

4.1.11. 4′-O-Demethyl-4β-[(4-propyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**22**)

White solid; mp: 154 °C; $[\alpha]_D^{25}$ −24.0 (c 0.46, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.90 (s, 1H), 6.64 (s, 1H), 6.61 (s, 1H), 6.32 (s, 2H), 6.07 (d, J = 4.63 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.71 (d, J = 4.66 Hz, 1H), 4.40–4.38 (t, J = 7.36 Hz, 1H), 3.78 (s, 6H), 3.25–3.17 (m, 2H), 3.08–3.05 (dd, J = 8.90 Hz, 4.91 Hz, 1H), 2.67–2.64 (t, J = 7.81 Hz, 2H), 1.69–1.65 (m, 2H), 0.98–0.95 (t, J = 7.21 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.12, 149.29, 148.02, 146.86, 134.95, 133.41, 129.90, 125.29, 110.40, 108.80, 108.49, 101.79, 67.42, 58.50, 56.66, 43.61, 41.92, 37.25, 31.16, 27.63, 22.38, 13.60; IR (KBr): 3388.54, 2926.96, 1765.9, 1611.09, 1515.57, 1484.21, 1228.05, 1108.97, 1034.63, 995.01, 926.64 cm^{-1}; ESI-MS: 494 (M + 1).

4.1.12. 4′-O-Demethyl-4β-[(4-butyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**23**)

White solid; mp: 124–125 °C; $[\alpha]_D^{25}$ −25.2 (c 0.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.00 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.34 (s, 2H), 6.06 (d, J = 4.90 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.74 (d, J = 4.66 Hz, 1H), 4.40–4.37 (t, J = 7.82 Hz, 1H), 3.78 (s, 6H), 3.23–3.18 (m, 2H), 3.10–3.07 (dd, J = 8.72 Hz, 4.65 Hz, 1H), 2.69–2.66 (t, J = 7.51 Hz, 2H), 1.66–1.63 (p, J = 7.33 Hz, 2H), 1.58–1.54 (m, 2H), 0.94–0.92 (t, J = 7.41 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.38, 149.31, 147.99, 146.66, 134.46, 133.34, 129.81, 125.05, 110.89, 109.55, 108.89, 101.93,67.46, 58.59, 56.27, 43.50, 41.85, 37.05, 31.28, 25.41, 22.68, 13.99; IR (KBr): 3387.96, 2927.66, 1766.29, 1611.23, 1515.97, 1483.39, 1227.95, 1109.67, 1034.93, 995.45, 926.99 cm^{-1}; ESI-MS: 508 (M + 1).

4.1.13. 4′-O-Demethyl-4β-[(4-pentyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**24**)

White solid; mp: 168 °C; $[\alpha]_D^{25}$ −29.1 (c 0.45, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.0 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.06 (d, J = 4.51 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.75 (d, J = 4.72 Hz, 1H), 4.41–4.38 (t, J = 7.91 Hz, 1H), 3.76 (s, 6H), 3.20–3.17 (m, 2H), 3.09–3.06 (dd, J = 8.72 Hz, 4.88 Hz, 1H), 2.63–2.60 (t, J = 7.79 Hz, 2H), 1.64–1.60 (m, 2H), 1.31–1.24 (m, 4H), 0.88–0.85 (t, J = 7.45 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.35, 149.35, 148.02, 146.67, 134.50, 133.36, 129.82, 125.10, 110.92, 109.54, 108.90, 101.94, 67.55, 58.61, 56.57, 43.80, 41.90, 37.09, 31.554, 29.69, 25.72, 22.35, 13.99; IR (KBr): 3388.16, 2927.67, 1765.30, 1610.99, 1515.52, 1483.60, 1228.71, 1109.47, 1034.53, 994.41, 927.07 cm^{-1}; ESI-MS: 522 (M + 1).

4.1.14. 4′-O-Demethyl-4β-[(4-hexyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**25**)

White solid; mp: 124–126 °C; $[\alpha]_D^{25}$ −21.6 (c 0.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.93 (s, 1H), 6.65 (s, 1H), 6.61 (s, 1H), 6.32 (s, 2H), 6.06 (d, J = 4.46 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.71 (d, J = 4.63 Hz, 1H), 4.43–4.40 (t, J = 7.75 Hz, 1H), 3.76 (s, 6H), 3.21–3.16 (m, 2H), 3.11–3.08 (dd, J = 8.64 Hz, 4.89 Hz, 1H), 2.65–2.62 (t, J = 8.10 Hz, 2H), 1.65–1.60 (m, 2H), 1.31–1.24 (m, 6H), 0.88–0.84 (t, J = 7.77 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.28. 149.14, 148.17, 146.99, 135.05, 133.54, 130.02, 125.41, 110.55, 108.96, 108.57, 101.95, 67.57, 58.65, 56.80, 43.75, 42.06, 37.38, 31.51, 29.24, 28.95, 25.78, 22.51, 13.93; IR (KBr): 3388.21, 2927.12, 1765.42, 1610.90, 1515.54, 1483.33, 1228.91, 1109.45, 1035.03, 994.45, 926.97 cm^{-1}; ESI-MS: 558 (M + 23).

4.1.15. 4′-O-Demethyl-4β-[(4-heptyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**26**)

White solid; mp: 118 °C; $[\alpha]_D^{25}$ −51 (c 0.75, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.85 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.07 (d, J = 4.88 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.78 (d, J = 4.66 Hz, 1H), 4.44–4.41 (t, J = 7.54 Hz, 1H), 3.78 (s, 6H), 3.27–3.23 (m, 2H), 3.10–3.07 (dd, J = 8.72 Hz, 4.81 Hz, 1H), 2.75–2.72 (t, J = 7.51 Hz, 2H), 1.75–1.71 (m, 2H), 1.30–1.25 (m, 10H), 0.87–0.84 (t, J = 7.90 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.28, 149.14, 148.17, 146.99, 135.05, 133.54, 130.02, 125.41, 110.55, 108.96, 108.57, 101.95, 67.57, 58.69, 56.66, 43.75, 41.96, 37.29, 31.51, 29.77, 29.30, 28.99, 25.70, 22.55, 13.96; IR (KBr): 3388.01, 2927.77, 1766.3, 1610.99, 1515.54, 1482.9, 1228.99, 1109.15, 1034.93, 994.55, 926.90 cm^{-1}; ESI-MS: 550 (M + 1).

4.1.16. 4′-O-Demethyl-4β-[(4-octyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**27**)

White solid; mp: 128–130 °C; $[\alpha]_D^{25}$ −45 (c 0.60, $CHCl_3$); δ 6.94 (s, 1H), 6.64 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.07 (d, J = 4.91 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.80 (d, J = 4.46 Hz, 1H), 4.43–4.40 (t, J = 7.51 Hz, 1H), 3.78 (s, 6H), 3.25–3.18 (m, 2H), 3.08–3.04 (dd,

J = 8.96, 4.79 Hz, 1H), 2.70–2.66 (t, J = 7.66 Hz, 2H), 1.74–1.63 (m, 4H), 1.41–1.25 (m, 8H), 0.88–0.83 (t, J = 7.77 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.40, 149.35, 147.99, 146.70, 134.52, 133.39, 129.86, 125.22, 110.40, 108.92, 107.98, 101.94, 67.56, 58.60, 56.57, 43.52, 41.88, 37.00, 31.81, 29.68, 29.26, 29.17, 28.76, 25.73, 22.63, 13.93; IR (KBr): 3388.15, 2928.27, 1765.60, 1612.03, 1515.27, 1483.9, 1230.01, 1109.55, 1034.63, 995.12, 927.30 cm^{-1}; ESI-MS: 564 (M + 1).

4.1.17. 4′-O-Demethyl-4β-[(4-decyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**28**)

White solid; mp: 115–116 °C; $[\alpha]_D^{25}$ −46.2 (*c* 0.4, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.93 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.06 (d, J = 4.91 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.74 (d, J = 4.72 Hz, 1H), 4.43–4.40 (t, J = 7.33 Hz, 1H), 3.78 (s, 6H), 3.25–3.18 (m, 2H), 3.08–3.04 (dd, J = 9.01, 4.79 Hz, 1H), 2.70–2.66 (t, J = 7.46 Hz, 2H), 1.76–1.64 (m, 4H), 1.41–1.25 (m, 10H), 0.90–0.85 (t, J = 7.84 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.23, 149.48, 148.19, 147.02, 135.09, 133.61, 130.03, 125.36, 110.56, 108.96, 108.64, 101.96, 67.55, 58.71, 56.81, 43.76, 42.06, 37.36, 31.94, 29.60, 29.35, 29.15, 28.83, 25.87, 22.68, 18.52, 14.02; IR (KBr): 3388.18, 2927.42, 1765.60, 1610.59, 1515.24, 1483.99, 1228.99, 1109.45, 1035.03, 994.45, 926.91 cm^{-1}; ESI-MS: 592 (M + 1).

4.1.18. 4′-O-Demethyl-4β-[(4-hydroxymethyl)-1,2,3-triazol-1-yl]-4-desoxypodophyllotoxin (**29**)

White solid; mp: 160–162 °C; $[\alpha]_D^{25}$ −45 (c 0.60, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25(s, 1H), 6.66 (s, 1H), 6.62 (s, 1H), 6.32 (s, 2H), 6.06 (d, J = 4.89 Hz, 1H), 6.03 (s, 1H), 6.01 (s, 1H), 4.76 (d, J = 4.77 Hz, 1H), 4.42–4.39 (t, J = 6.79 Hz, 1H), 3.77 (s, 6H), 3.74 (s, 2H), 3.28–3.17 (m, 2H), 3.09–3.05 (dd, J = 8.57 Hz, 4.88 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ 173.01, 149.54, 148.10, 137.63, 134.27, 133.21, 124.60, 110.18, 109.07, 108.72, 101.90, 67.36, 59.81, 58.91, 56.65, 43.86, 41.81, 37.36, 29.68; IR (KBr): 3384.21, 2926.74, 1763.60, 1611.10, 1516.99, 1483.59, 1232.06, 1109.97, 1033.67, 997.06, 928.11 cm^{-1}; ESI-MS: 482 (M + 1).

4.2. Evaluation of anticancer activity

The effect of 4β-[(4-alkyl)-1,2,3-triazol-1-yl] podophyllotoxin derivatives on the growth of cancer cell lines was evaluated according to the procedure adopted by the National Cancer Institute for *in vitro* anticancer drug screening that uses the protein-binding dye sulforhodamine B to estimate cell growth. Briefly, cells in their log phase of growth were harvested, counted and seeded (10^4 cells/well in 100 mL medium) in 96-well microtitre plates. After 24 h of incubation at 37 °C and 5% CO_2 to allow cell attachment, cultures were treated with varying concentrations (0.1–100 mM) of test samples made with 1:10 serial dilutions. Four replicate wells were set up for each experimental condition. Test samples were left in contact with the cells for 48 h under same conditions. Thereafter cells were fixed with 50% chilled TCA and kept at 4 °C for 1 h, washed and air-dried. Cells were stained with sulforhodamine B dye. The adsorbed dye was dissolved in tris-buffer and the plates were gently shaken for 10 min on a mechanical shaker. The optical density (OD) was recorded on ELISA reader at 540 nm. The cell growth was calculated by subtracting mean OD value of the respective blank from the mean OD value of experimental set. Percentage of growth in the presence of test material was calculated considering the growth in the absence of any test material as 100% and the results are reported in terms of IC_{50} values. Etoposide was taken as positive control.

4.3. DNA fragmentation assay

DNA fragmentation was determined by electrophoresis of extracted genomic DNA from breast cancer cell line MCF-7. Cells (2×10^6/6 mL medium/60 mm tissue culture plate) were treated with compound **12** and **13** at 1 μM for 24 h. Cells were harvested, washed with PBS, pellets were dissolved in lysis buffer (10 mM EDTA, 50 mM tris pH 8.0, 0.5% w/v) SDS and proteinase K (0.5 mg/mL) and incubated at 50 °C for 1 h. Finally the DNA obtained was heated rapidly to 70 °C, supplemented with loading dye and immediately resolved on to 1.5% agarose gel at 50 V for 2–3 h.

4.4. Cell cycle analysis

Effect of Compound **12** and **13** on DNA content by cell cycle phase distribution was assessed using MCF-7 cells by incubating the cells 1×10^6 mL/well with compound **12** and **13** (1 & 5 μM each) for 24 h. The cells were then washed twice with ice-cold PBS, harvested, fixed with ice-cold PBS in 70% ethanol and stored at −20 °C for 30 min. After fixation, these cells were incubated with RNase A (0.1 mg/mL) at 37 °C for 30 min, stained with propidium iodide (50 μg/mL) for 30 min on ice in dark, and then measured for DNA content using BD-LSR flow cytometer (Becton Dickinson, USA) equipped with electronic doublet discrimination capability using blue (488 nm) excitation from argon laser. Data were collected in list mode on 10,000 events for FL2-A vs. FL2-W.

4.5. Molecular docking

A compound library of 18 podophyllotoxin analogues were built and minimized in Schrödinger using the parental structure of podophyllotoxin as a template. Each structure was assigned an appropriate bond order using ligprep script shipped by Schrödinger and optimized by means of the OPLS-2005 [25] force field using default settings. Different conformations were generated for input ligands to cover every possibility of best conformations using confgen module (ConfGen, version 2.1, Schrodinger, LLC, New York, NY, 2009) of Schrodinger. The starting coordinates of the human topoisomerase-II ATPase-AMP-PNP complex [PDB: 1ZXM] was taken from the Protein Data Bank (www.rcsb.org) and further modified for docking calculations. For Glide (Schrödinger) calculations, TP-II complex was imported to Maestro (Schrödinger), the co-crystallized ligands were identified and removed from the structure and the protein was minimized using the protein preparation wizard (Schrödinger) by applying OPLS-2001 force field [26]. Water molecules were removed and H atoms were added to the structure. Minimizations were performed until the average root mean square deviation of the non hydrogen atoms reached 0.3. Docking was performed using Glide. After ensuring that the protein and ligands are in correct form for docking, the receptor-grid files were generated using a grid-receptor generation program using default settings. The ligands were docked with the binding site using the 'extra precision' Glide algorithm in Schrodinger.

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