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PII: S0960-894X(15)00385-6
DOI: http://dx.doi.org/10.1016/j.bmcl.2015.04.053
Reference: BMCL 22636

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 3 December 2014
Revised Date: 5 April 2015
Accepted Date: 15 April 2015

Please cite this article as: Hyder, I., Yedlapudi, D., Kalivendi, S.V., Khazir, J., Nalla, N., Miryala, S., Sampath Kumar, H.M., Synthesis and Biological Evaluation of novel 4β-[(5-subtituted)-1, 2, 3, 4-tetrazolyl] podophyllotoxins as Anticancer compounds, *Bioorganic & Medicinal Chemistry Letters* (2015), doi: http://dx.doi.org/10.1016/j.bmcl.2015.04.053

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Synthesis and Biological Evaluation of novel 4β-[(5-subtituted)-1, 2, 3, 4-tetrazolyl] podophyllotoxins as Anticancer compounds

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Tetrazolyl Podophyllotoxin ,
Anticancer activity,
Tubulin polymerization

ABSTRACT

A series of novel 4β-[(5-susbtituted)-1,2,3,4-tetrazolyl] podophyllotoxin derivatives were synthesized by employing azide-nitrile click chemistry approach. All the derivatives were evaluated for their cytotoxicity against a panel of four human cancer cell lines and their IC_{50} values were found to be in the range of 2.4 μM to 29.06μM. The cytotoxicity exhibited by the majority of test compounds were found to comparable and often more effective than Doxorubicin and all compounds exhibited higher cytotoxicity on A-549 cell lines. Cell cycle analysis showed that the novel 4β-[(5-susbtituted)-1,2,3,4-tetrazolyl] podophyllotoxins resulted in cell cycle arrest at G2/M phase and were also found to be the potent inhibitors of tubulin polymerization *in vitro*.

Podophyllotoxin (**1**), is a most abundant naturally occurring cyclolignan, isolated from the different plants of the genus *Podophyllum.* [1] Over the last decade, there has been a plenty of research on podophyllotoxin primarily for antimitotic activity, even though wide range of other pharmacological attributes of podophyllotoxin derivatives *viz.,* cathartic, antirheumatic and antiviral properties have been reported. However, the anticancer activity of these cyclolignans proved to be promising and several research groups worldwide engaged in the structural modification around the podophyllotoxin scaffold resulting in the development of clinically approved cancer drugs like etoposide, teniposide and a soluble prodrug of etoposide-etopophos, against various types of cancers, including small cell lung cancer, testicular carcinoma, lymphoma, and Kaposi's sarcoma and some of them are in late stage clinical development like NK-611, NPF and GL-331(Figure.1).[2-4] The cytotoxic mechanism of these semisynthetic derivatives was explained by the inhibition of topoisomerase-II mechanism whereas its lead compound mechanism was explained by assembly of microtubule.[5] SAR of podophyllotoxin indicates that 4-β substituted, 4'-demethyl and trans lactone moieties are essential to maintain the antimitotic activity.[6] Previously our group designed and synthesized some potent anilino, phenol, thiophenol, carbohydrate, aliphatic substituted 1, 2, 3- triazole derivatives of podophyllotoxin and it was found that some of these derivatives are more potent than etoposide.[7] In continuation of our work, we synthesized a series of tetrazolyl derivatives of podophyllotoxin *via.* click chemistry approach by using azide and nitrile with a view to examine their effect on the cytotoxicity pattern on cancer cell lines and the interactions of these ligands with tubulins. Genesis of this chemistry is also derived from the fact that tetrazole containing moieties were found to be cytotoxic.[8] Microtubules are the cytoskeletal structures formed due to the self assembly of two homologues proteins, α and β tubulin which are present in all eukaryotic cells. Microtubules are significant target for many natural product anticancer agents. The taxane domain, the vinca domain and the colchicine domain are the characteristic binding sites of tubulin.[9] There are several reports proving that podophyllotoxin derivatives were known to inhibit tubulin. Recently Chunyan Zhao *et al* proved that these semi synthetic derivatives can act as tubulin polymerization inhibitors interacting with the colchicine-binding site.[10] Keeping all these reports in view, our attention was drawn towards tubulin inhibition of the novel podophylloxin analogues and interestingly these derivatives are also proved to be tubulin inhibitors.

As illustrated in **Scheme-1**, 4β-[(5-susbtituted)-1,2,3,4-tetrazol-1-yl] podophyllotoxin derivatives were synthesized by the cycloaddition reaction of C4β-azidopodophyllotoxin, **9** and C4β-azido-4′-*O*-demethyl podophyllotoxin, **10** with various cyano compounds. Compounds **9, 10** were prepared by the previous literature procedure.[11] Compound **9** was synthesized by treating podophyllotoxin with $MeSO_3H$ and NaI in acetonitrile which forms the corresponding iododerivative followed by hydrolysis with $H_2O/Me_2CO/BaCO_3$ then treatment with NaN_3, TFA in chloroform. Similarly compound **10** was synthesized by treating podophyllotoxin with $MeSO_3H$ and NaI in presence dichloromethane which forms the corresponding iododerivative

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followed by hydrolysis with $H_2O/Me_2CO/BaCO_3$ which forms 4'-O-demethyl podophyllotoxin followed by treatment with NaN_3, TFA in presence of chloroform. [12, 13] Compounds **9, 10** were allowed to react with various cyanides in two different methods. Sulphonyl cyanides treated with compounds **9, 10** at 100 ^{0}C without solvent for 10 hours to form corresponding tetrazolyl derivatives. Whereas acyl cyanides treated with **9, 10** at RT in presence of $ZnBr_2$ to form corresponding tetrazolyl derivatives in moderate yields.[14-16]

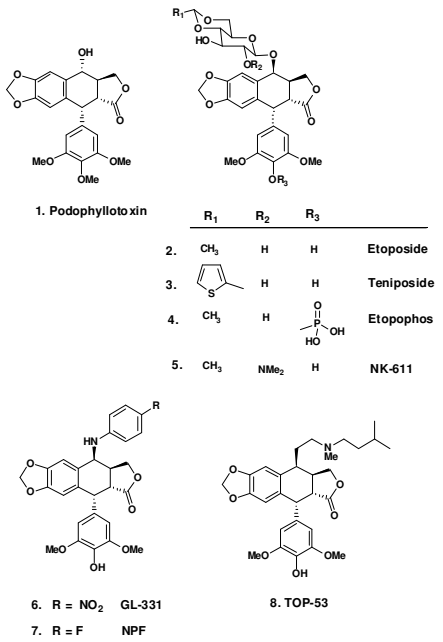
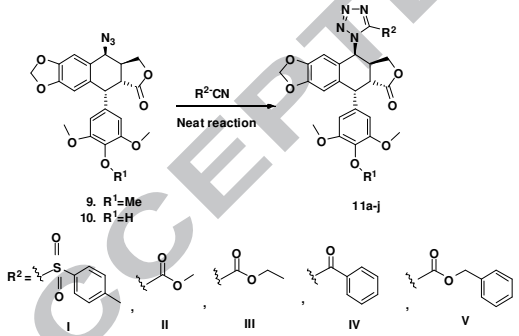


Figure.1; Structures of some podophyllotoxin derivatives



Reaction conditions: Method-I: Suphonyl cyanides, heat at 100 ^{0}c; Method-II: Acyl cyanides, $ZnBr_2$, RT, 48 hrs.

Scheme-1. Click-chemistry strategy for the synthesis of 4β-[(5-substituted)-1,2,3,4-tetrazolyl]podophyllotoxin derivatives.

All the synthesized compounds were characterized by IR, ^{1}H and ^{13}C NMR spectroscopy and ESI-MS.

All the 4β-[(5-susbtituted)-1,2,3,4-tetrazolyl] podophyllotoxin derivatives were screened for their cytotoxicity against a panel of four human cancer cell lines(SK-N-SH, A549,HeLa and MCF-7) and the results are summarized in Table1. Podophyllotoxin and Doxorubicin were taken as taken as positive controls. Majority of compounds exhibited IC_{50} values less than doxorubicin across the panel of all the four cell lines tested. While the IC_{50} values were in the range of 3.44 -6.5 µM in SK-NSH, the corresponding values were 2.4-4.29 µM, 3.05 – 5.25 µM and 4.85 – 9.44 µM in A549, HeLa and MCF-7 cells respectively. However, compound 11h exhibited either comparable (6.48 µM in A549 and 12.43 µM in MCF-7) or greater IC_{50} values (25.95 µM SK-N-SH and 29.06 µM in HeLa) than doxorubicin. Since a majority of compounds exhibited lower IC_{50} values in A549 cells amongst the cell lines employed hence further studies were carried out in this cell line.

Compound	R^1	R^2	IC_{50} values (µM)			
			SK-NSH	A-549	HeLa	MCF-7
11a	**Me**	**I**	**5.31±0.12**	**2.89±0.43**	**3.15±0.11**	**6.99±0.08**
11b	**Me**	**II**	**6.5±0.23**	**3.11±0.31**	**5.25±0.06**	**6.97±0.36**
11c	**Me**	**III**	**3.61±0.30**	**2.4±0.05**	**4.76±0.73**	**7.47±0.05**
11d	**Me**	**IV**	**4.68±0.19**	**4.29±0.89**	**4.76±0.45**	**6.89±0.04**
11e	**Me**	**V**	**4.58±0.02**	**2.48±0.25**	**4.01±0.08**	**4.85±0.46**
11f	**H**	**I**	**3.44±0.09**	**3.29±0.28**	**3.05±0.02**	**5.29±0.10**
11g	**H**	**II**	**5.64±0.57**	**2.44±0.01**	**3.95±0.33**	**5.27±0.51**
11h	**H**	**III**	**25.95±5.11**	**6.49±0.77**	**29.06±4.18**	**12.43±1.08**
11i	**H**	**Iv**	**3.49±0.06**	**2.71±0.53**	**3.61±0.18**	**7.02±0.92**
11j	**H**	**V**	**6.16±0.78**	**2.98±0.48**	**4.77±0.11**	**9.44±0.42**
1			**7.92±1.23**	**2.55±0.27**	**1.92±0.06**	**3.34±0.15**
Doxorubicin			**7.36±0.01**	**6.13±0.54**	**10.78±0.69**	**12.34±2.57**

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

Cell viability assays revealed that a majority of derivatives exhibited effective antiproliferative activity against all the four cell lines tested. To investigate further, the anti-tubulin activity of the compounds (5 µM) was evaluated using podophyllotoxin as a positive control. Results as shown in Figure.2/Table-2, demonstrate that podophyllotoxin inhibited the polymerization of tubulin to nearly 90% at 5 µM concentration. Under similar experimental conditions, compound **11c** inhibited *in vitro* tubulin polymerization to 99%, whereas, **11d, 11e, 11f, 11g** and **11j** exhibited 72, 57, 22, 28 and 29% inhibition respectively. However, no significant anti-tubulin activity was observed with compounds, **11a, 11b, 11h** and **11i.**

Molecules that inhibit tubulin polymerization are known to induce cell cycle arrest at G2/M or mitotic phase.[13] To elucidate the down-stream consequence of anti-tubulin effects of derivatives, we analysed the effect of individual compounds on cell cycle by flow cytometry, which is used to quantitatively determine the population of cells in each phase of the cell cycle by measuring the DNA content of individual cells. A549 cells were treated individually with compounds (5 µM concentration) for duration of 24 h and found that compounds that exhibited anti-tubulin activity such as, **11c, 11d, 11e, 11f, 11g** and **11j** induced cell cycle arrest at G2/M phase(Figure.3). Even, the compounds that did not affect tubulin polymerization (**11a, 11b, 11h** and **11i**) also induced mitotic arrest. Further, **11a** and **11b** which possess either negligible / no anti-tubulin activity induced cell cycle arrest at both G2/M to a lesser extent of 54 and 45% respectively as compared to other derivatives, but significantly induced arrest even at sub-G0 phase. The observed findings could be due to the possible topoisomerase inhibition of the derivatives as it has been shown previously that modification at C-4 position on podophyllotoxin leads to the generation of topo II inhibitors capable of inducing cell cycle arrest at G2/M as well as in sub G0 phase of the cell cycle.

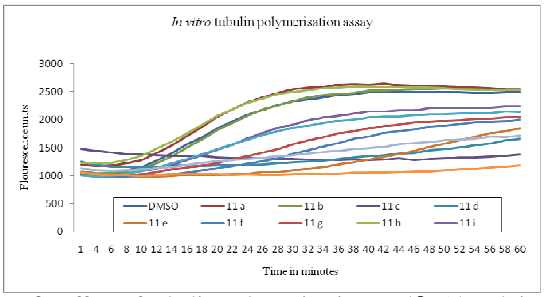


Figure.2; Effect of tubulin Polymerization on 4β-[(5-susbtituted) -1,2,3,4-tetrazol-1-yl] podo-phyllotoxin derivatives.

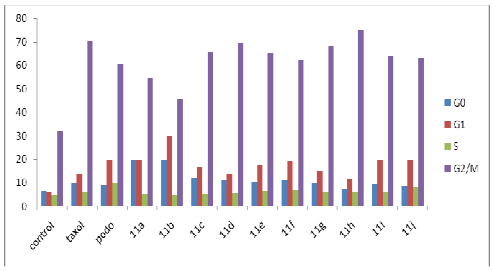


Figure.3; Antimitotic activity of compounds by FACS analysis: A549 cells were harvested after treatment with compounds at 5μM for 12h. Untreated cells and DMSO treated cells served as controls. The percentage of cells in each phase of cell cycle was quantified by Flow cytometry and the values indicate the number of cells stalled at G2/M phase.

Most of the sulphonyl and acyl tetrazolyl podophyllotoxin derivatives exhibited uniform cytotoxicity against four different cell lines without profound effect of the substituion on tetrazolyl ring on the derived cell viability with an exception of compound **11h** bearing ethyl substitution on tetrazole moiety with free hydroxyl on the phenyl ring of podophyllotoxin scaffold(R^1=H), exhibiting least cytotoxicity amongst all the compounds studied. Except compound **11h**, all compounds shown comparable IC_{50} values with parent compound podophyllotoxin against human neural bone marrow metastasis cancer cel line(SK-NSH) and human lung adenocarcinoma epithelial cell line (A-549). However, the observed IC_{50} values for the compounds were slightly higher than that of podophyllotoxin in cervical cancer (HeLa) and breast cancer (MCF-7) cell lines. Again, except **11h**, most of the compounds exhibited lower IC_{50} values in all four cell lines when compared to doxorubicin. Based on this premise, all compounds were tested for their *in vitro* inhibition of tubulin polymerization and it was observed that compound **11c** shown 99 % inhibition as compared to podophyllotoxin (93% inhibition) followed by other derivatives. However, cell cycle arrest at both G2/M phase and very significant induction of cell cycle arrest at sub-G0 phase by the compounds viz.,**11a, 11b** and **11i** which did not otherwise inhibit tubulin polymerization, may be attributed to the topoisomerase inhibition which contribute to apoptosis. Thus, the tetrazolyl derivatives of podophyllotoxin might be inducing apoptosis in cancer cell lines through dual target interaction i.e., tubulin polymerization inhibition as well as topoisomerase-II inhibition as observed previously by other C-4 substituted podophyllotixin analogues leading to induction of apoptosis.

Entry	Compound	% Inhibition of tubulin polymerization
1	11a	0.73
2	11b	12.40
3	11c	99.15
4	11d	72.50
5	11e	57.47
6	11f	22.46
7	11g	28.29
8	11h	2.68
9	11i	11.52
10	11j	29.79
11	1	93.90
12	Nocodozole	65.27

Table 2. *In vitro* tubulin polymerization assay. Values are indicates as % inhibition with respect to control.

In summary, a series of novel 4β-[(5-susbtituted)-1,2,3,4-tetrazolyl] podophyllotoxin derivatives were synthesized and evaluated for their cytotoxicity against a panel of four cell lines. The IC50 values varied from 2.4μM to 29.06 μM. These derivatives showed inhibition of tubulin polymerization in vitro and arrested the cells at G2/M phase

Acknowledgments
The authors thank CSIR, New Delhi for financial support as part of XII Five Year plan programme under title DENOVA (CSC-0205), ORIGIN (CSC-0108). I.H thanks CSIR for the award of fellowship.

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17. **General procedure for preparation of suphonyl tetrazoles (I);** A mixture of Azido- podophyllotoxin and paratoulene sulphonyl cyanide were taken in a sealed tube and stir at 100 °c for 12 hours in the absence of solvent. After completion of reaction, the reaction mixture was chromatographed on silica gel eluting with ethyl acetate/hexane (24-30%) to get the desired products **11a** and **11f** as white solids.

(*5R,5aR,8aS,9S*)-9-(5-Tosyl-1H-tetrazol-1-yl)-5-(3,4,5-trimethoxyphenyl)-5,5a,8a,9-tetrahydrofuro[3',4':6,7]naphtho[2,3-d][1,3]dioxol-6(8*H*)-one (11a):

This compound **11a** was prepared by the method **(I),** employing 4β- Azido podophyllotoxin (90 mg, 0.20 mmol) and para toulene sulphonyl cyanide (0.20 mmol) The crude product was was chromatographed on a silica gel column with ethyl acetate/hexane (24%) as eluent afford the pure compound **11a,** 86 mg in 69 % yield as White solid. m.p: 216 ºC; $[\alpha]_D^{25}$ - 29 (*c* 0.5, $CHCl_3$), R_f =0.5 (50% Ethyl acetate/hexane); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.01 (d, *J* = 8.3 Hz, 2H), 7.41 (d, *J* = 8.3 Hz, 2H), 6.64 (s, 1H), 6.55 (s, 1H), 6.37 (d, *J* = 5.1 Hz , 1H), 6.32 (s, 2H), 6.02 (d, *J* = 1.2 Hz, 1H), 5.97 (d, *J* = 1.2 Hz, 1H), 4.81 (d, *J* = 5.1 Hz, 1H), 4.30 (m, 1H), 3.82 (s, 3H), 3.77 (s, 6H), 3.69 (dd, *J* = 14.4, 5.1 Hz, 1H), 3.36-3.28 (m, 1H), 2.47 (s, 3H); ^{13}C NMR(75 MHz, $CDCl_3$) δ 21.7, 36.2, 40.9, 43.5, 56.3, 60.7, 63.2, 66.2, 102.0, 108.1, 108.2, 110.7, 123.0, 128.8, 130.3, 133.3, 134.0, 134.8, 137.5, 146.7, 147.9, 149.6, 152.7, 166.8, 172.6; IR (KBr) 3436, 2929, 1788, 1726, 1594, 1498, 1124, 1040 cm^{-1}; ESI–MS: *m/z* 643[M +Na] $^+$;

18. **General procedure for preparation of aromatic/aliphatic tetrazoles (II)** A mixture of Azido- podophyllotoxin, acyl cyanide and zinc bromide were taken in a round bootom flask under nitrogen atmosphere and the reagents were stirred vigorously for 48 h at room temperature. To the reaction mixture add ethyl acetate and water and stir for 2 h. The organic layer was then isolated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were dried over NaSO4. After filtration and concentration, the crude product was purified by column chromatography with ethyl acetate/ hexane afford the pure compounds (**11b, 11c, 11d, 11e, 11g, 11h, 11i, 11j**).

Methyl1-((*5S,5aS,8aR,9R*)-8-oxo-9-(3,4,5-trimethoxyphenyl)-5,5a,6,8,8a,9-hexahydrofuro[3',4':6,7]naphtho[2,3-d][1,3]dioxol-5-yl)-1*H*-tetrazole-5-carboxylate (11b):

This compound **11b** was prepared by the method **(II)**, employing 4β- Azido podophyllotoxin (100 mg, 0.22 mmol) and methyl cyano formate (2.86 mmol) and Zinc bromide(0.22 mmol). The crude product was chromatographed on a silica gel column with ethyl acetate/hexane (28 %) as eluent) afford the pure compound **11b,** 93 mg in 77 % yield as White solid. mp 126 ºC; $[\alpha]_D^{25}$ - 37.7 (*c* 0.5, $CHCl_3$); R_f =0.4 (50% Ethyl acetate/hexane); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.64 (s, 1H), 6.57 (s, 2H), 6.30 (d, *J* = 10.8 Hz, 2H), 6.20 (s, 1H), 5.98 (d, *J* = 1.2 Hz , 1H), 5.96 (d, *J* = 1.2 Hz , 1H), 4.73 (d, *J* = 4.4 Hz , 1H), 4.25 (m, 1H), 4.06 (s, 3H), 3.85 (s, 6H), 3.83 (s, 3H), 3.74-3.68 (m,1H), 3.09 (dd, *J* = 14.3, 4.4 Hz 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.7, 44.0, 45.4, 55.9, 60.3, 66.3, 69.4, 101.7, 106.2, 107.7, 110.3, 125.5, 128.6, 130.8, 133.1, 133.8, 134.1, 148.8, 152.9, 163.3, 172.1; IR (KBr): 3414, 2924, 2853, 1783, 1748, 1591, 1496, 1238, 1123 cm^{-1}; ESI–MS**:** *m/z* 547 [M +Na] $^+$;

Supplementary Material:

Copies of ^{1}H, ^{13}C NMR and mass spectra of all the new compounds can be obtained free of charge from the internet.

Graphical Abstract

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Synthesis and Biological Evaluation of novel 4β-[(5-subtituted)-1,2,3,4-tetrazolyl] podophyllotoxins as Anticancer compounds

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