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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, cytotoxic activity and docking studies of new 4-aza-podophyllotoxin derivatives

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Abstract The synthesized nine aza-podophyllotoxin derivatives (**8a–f**, **10**, **12 and 14**) have been evaluated for their cytotoxicity in a panel of tumor cancer cell lines (Zr-75-1, MCF7, KB, Gurav, DWD, Colo-205, A-549 and Hop62). Among them, **8a** and **8b** compounds show stronger growth inhibition activity than the standard drug etoposide. Further, molecular docking simulations were carried out against human topoisomerase II, a putative target for these classes of molecules.

Keywords Podophyllotoxin · Knoevenagel condensation · Etoposide · Cytotoxicity · Docking studies

Introduction

Cancer is the second leading cause of mortality in developed countries, and cancer chemotherapy commonly involves the use of cytotoxic agents that destroy rapidly dividing cells. Within the past decade, advances in our understanding of the cell cycle have presented new targets that may allow for the development of more selective chemotherapeutic agents—agents that target only cancer cells. Despite this progress, cytotoxic agents will remain a mainstay in cancer chemotherapy for the near future. Podophyllotoxin **1** is a naturally occurring aryl tetralin lignan obtained from a number of plant species of the podophyllum family (Lee *et al.*, 1993; MacRae *et al.*, 1993) and is known as an antimicrotubule agent (*Loike et al.*, 1978).The biological activity of podophyllotoxin has led to extensive structural modifications, resulting in several clinically useful compounds. Several semisynthetic derivatives of podophyllotoxin **1**, namely etoposide **2** (Stähblin, 1973), teniposide **3** (Stähblin, 1970) and etopophos **4** (Schacter, 1996; Witterland *et al.*, 1996), are in clinical use for the treatment of a variety of malignancies including lung and testicular carcinoma, lymphoma, non-lymphocytic leukemia (Belani *et al.*, 1994; Hande, 1998) shown in Fig. 1. The cytotoxic mechanism of these drugs is the inhibition of topoisomerase II (Macdonald *et al.*, 1991) unlike the lead compound that inhibits mitosis. Podophyllotoxin derivatives are widely used as anticancer agents, but they still have several secondary effects such as poor water solubility, development of drug resistance, metabolic inactivation, and toxic effects (Kobayashi and Ratain, 1994). Because of the structural complexity of **1**, arising from the presence of four stereogenic carbons in ring C, most of the SAR studies have been performed by derivatization of the parent natural product rather than by de novo chemical synthesis (Youngjae, 2005; Gordaliza *et al.*, 2000; Hitotsuyanagi *et al.*, 1997, 2000).

Heterocyclic compounds are widely distributed in nature and are essential to life in various ways due to their different physiological activities. Pyrazole derivatives have been found to possess a broad spectrum of biological activities, which stimulated the research activity in this field. Several pyrazole derivatives proved to have anticancer activity (George *et al.*, 2013). Recently, the research and development of new pyrazoles for cancer therapy have

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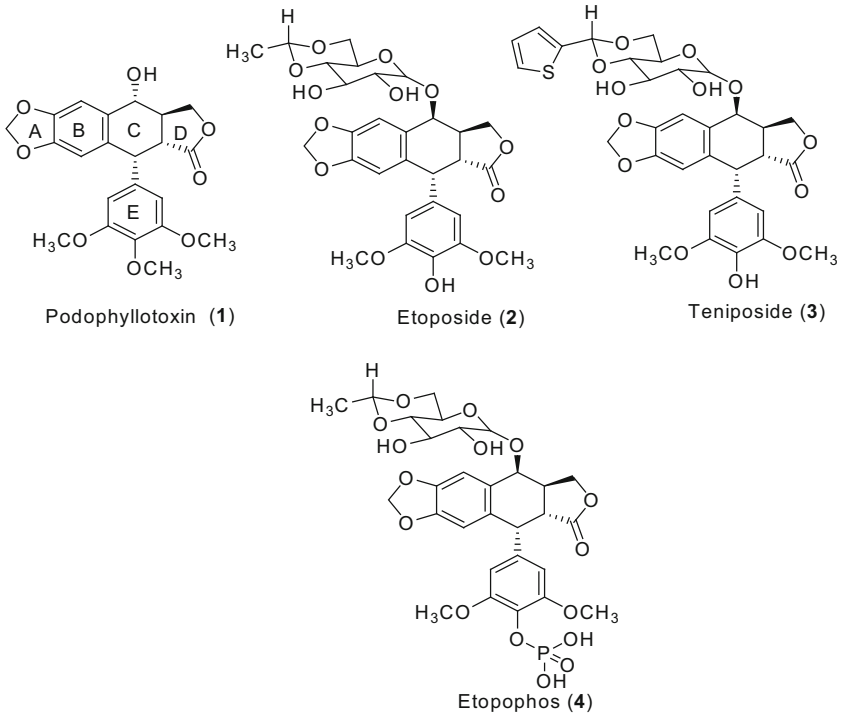


Fig. 1 Structures of podophyllotoxin and semi synthetic derivatives of podophyllotoxin

been one of the major focuses in anticancer drug design. The indole scaffold is also found in a manifold of naturally occurring plant-based alkaloids, and its derivatives have displayed versatile pharmacological properties including antitumor (Nguyen *et al.*, 1990). Furthermore, indazole (Showalter *et al.*, 1998) and isoxazole (Dengler *et al.*, 1995) heterocyclic compounds also shown potent anticancer activity on human cancer cell lines.

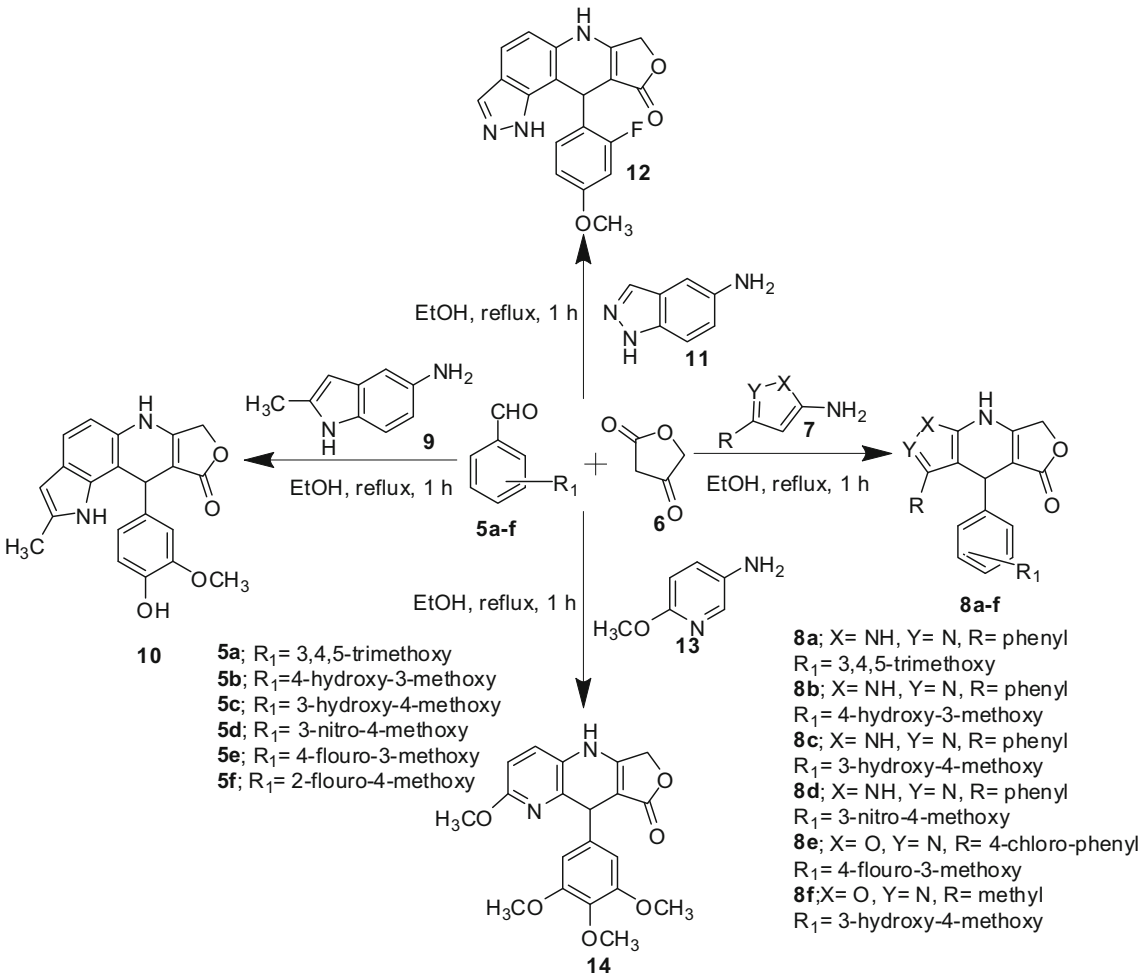
The analogues of podophyllotoxins such as etoposide and teniposide have fewer side effects. Further, various scientists from worldwide have been paying great attention to the synthesis of 4-aza-podophyllotoxin analogues and observed very little side effects (Shi *et al.*, 2011a, b; Kamal *et al.*, 2011, 2014). However, the podophyllotoxins are known as the DNA topoisomerase II inhibitors with respect to their mechanism of actions, having the greatest significance in the use of several FDA-approved anticancer drugs.

Some heterocyclic-fused podophyllotoxin analogues potently induce apoptosis in cancerous Jurkat cells even after a short 24-h exposure (Magedov *et al.*, 2007). In the view of pharmaceutical importance of heterocyclic-fused 4-aza-podophyllotoxin analogues and as continuation of our research work toward the synthesis of potential anticancer agents, here we report the synthesis of a series of aza-podophyllotoxin derivatives with pyrazole, isoxazole, indole, indazole and pyridine moieties as an inner ring part of podophyllotoxins. The few podophyllotoxins are shown in Fig. 1. All these congeners have been evaluated for their anticancer activity against a panel of human cancer cell lines. The probable binding modes of present podophyllotoxin derivatives are explained through the molecular docking simulations due to the history of the podophyllotoxins which behaves as DNA topoisomerase II inhibitors.

Results and discussion

The synthesis of these aza-podophyllotoxin derivatives (**8a–f**, **10**, **12** and **14**) was done by using the Knoevenagel condensation as shown in Scheme 1. In this condensation reaction, the substituted heterocyclic amines, tetronic acid and substituted aromatic aldehydes were refluxed in ethanol. In all cases, the resulting aza-podophyllotoxins were precipitated by cooling the reaction mixtures after the completion of the reaction. Now, the precipitated compounds were filtered and recrystallized from acetone to afford the pure compounds. The structures of all synthetic compounds were confirmed by ^{1}H NMR, ^{13}C NMR and mass spectral data.

The biological activities of this series of aza-podophyllotoxin (**8a–f**, **10**, **12** and **14**) derivatives were evaluated by an in vitro cytotoxicity test, which was carried out with a panel of four tumor cell lines that comprise human breast cancer (Zr-75-1, MCF7), human oral cancer (KB, Gurav and DWD), human colon cancer (Colo-205) and human lung cancer (A-549 and Hop62), using etoposide as reference compound. The screening procedure was based on the



Scheme 1 Synthesis of various aza podophyllotoxin derivatives

standard SRB method (Skehan *et al.*, 1990), and the GI_{50} values are given in Table 1. We have observed that all the compounds are significantly cytotoxic with the concentration ranging from 0.11 to 2.98 μM and produced 50 % inhibition of cell growth (GI_{50}). Where the drug etoposide exhibited cytotoxic with GI_{50} concentration ranging from 0.13–3.08 μM. Among them, **8a** and **8b** compounds showed a basically stronger inhibition than etoposide.

In the present study, a series of novel aza-podophyllotoxin derivatives **8a–f**, **10**, **12** and **14** have been designed, synthesized and evaluated for their cytotoxic activity against a panel of four types of human tumor cell lines. The compounds **8a–8d** comprises of the five-membered pyrazole ring along with phenyl group. The substituted phenyl ring present on the adjacent six-membered ring to the pyrazole makes them potent than the standard drug etoposide. Almost all four cell lines were shown the susceptibility to compounds **8a** and **8b** (except Hop62 i.e., for **8b**). The substitution of trimethoxy phenyl ring with vanillin on six-membered ring makes it slightly lesser potent (in case of Zr-75-1 and Hop62) than compound **8a**. The substitution of vanillin with isovanillin ring reduces the potency of compound **8c** when compared with **8b**. The presence of nitro and methoxy at the third and fourth position on the phenyl ring of six-membered nucleus slightly increases the anticancer activity of compound **8d** in colon and lung cancer cell lines. However, the replacement of pyrazole nucleus with five-membered ring, i.e., oxazole (**8e** and **8f**), pyrrole (**10**, **12**) and six-membered ring, i.e., pyridine (**14**) is responsible for depleting them to moderate actives. The compound **14** has the trimethoxy substituted phenyl ring as compound **8a**, the replacement of six-membered ring with pyrazole made it least active. Among the series, the compounds **8a** and **8b** exhibited potent cytotoxicities in vitro than etoposide, and we believed that these compounds act as promising novel anticancer agents in cancer chemotherapy. Further, the molecular docking studies were carried out against the human topoisomerase

Table 1 Cytotoxic activity (GI_{50} μM) of compounds **8a–f**, **10**, **12** and **14**

Compound	Zr-75-1[a]	MCF7[a]	KB[b]	Gurav[b]	DWD[b]	Colo-205[c]	A-549[d]	Hop62[d]
8a	0.17	0.13	0.19	0.15	0.14	0.11	0.12	2.01
8b	2.98	0.18	0.18	0.14	0.11	0.12	0.19	NA
8c	2.91	2.76	NA	2.09	2.30	NA	2.43	2.98
8d	NA	2.20	NA	NA	2.36	0.19	2.56	NA
8e	2.47	2.27	NA	2.28	2.45	2.37	2.64	NA
8f	2.31	2.43	2.22	NA	NA	NA	2.36	2.38
10	2.26	2.71	NA	2.07	NA	2.90	2.67	NA
12	2.39	NA	2.93	NA	2.12	NA	2.78	2.95
14	2.15	2.78	NA	NA	2.54	2.87	NA	NA
Etoposide[e]	0.20	2.11	0.31	0.51	0.62	0.13	3.08	0.80

Growth inhibition of 50 % and the values are the mean of three determinations

NA not active

[a] Breast cancer

[b] Oral cancer

[c] Colon cancer

[d] Lung cancer

[e] Etoposide

II-complexed DNA, in order to understand the approximate binding modes of the present compounds.

Binding mode analysis

The complex of DNA with human topoisomerase II along with etoposide (PDB: 3QX3) was employed; the chain A along with DNA complex was selected for the present molecular docking studies through the extra-precision docking mode of GLIDE 5.0. The docking protocol was validated by redocking that resulted in 0.48 Å RMSD. The etoposide shows the H-bonding interactions with Asp479 and DGF13 and hydrophobic interactions with DF13F and DAF12 of DNA. Simulation study was carried out for only two most potent molecules (**8a** and **8b**) in this series. Ligand preparation through LigPrep module in Maestro 8.5 (Schrodinger LLC) provided four structures for each molecule: two isomers (R and S) each with two different protonation states (in pyrazole moiety). All the four structures were docked on the target (PDB: 3QX3). In case of **8a**, only one structure with configuration of R and protonation at 2 N of pyrazoline docked into the target protein, while all the other three structures failed to dock. Two structures of **8b** were found to dock well against the target protein: one with R configuration having protonation at 1 N of pyrazole and one with S configuration having protonation at 2 N of the pyrazole. Both are having the docking score almost equal. This clearly indicates that substitution on the phenyl ring on dihydropyridyl ring plays a major role in determining the activity of the isomers. Increasing the bulkiness of the substitution on phenyl ring makes the S-isomer to loose its activity.

Analysis of interaction of **8a** (R-isomer) with target protein revealed the existence of two H-bonding interactions and a π–π stacking interaction involving DNA (DA12F and DG7C). The phenyl ring on pyrazole ring established a hydrophobic interaction with Gln778A (Fig. 2a, b). Similarly, analysis of **8b** (R-isomer) with target protein revealed the existence of π–π stacking interaction involving DA12F similar to **8a**, but here the phenyl ring pyrazoline is involved (in **8a,** it is phenyl ring at dihydropyridyl). There is no H-bonding interaction too. It is observed that R-isomers interact with the DNA rather than the protein part. Analysis of **8b** (S-isomer) revealed that the isomer orients in a different way and it interacts mainly with the protein part of the target and there is no π–π stacking interaction DNA bases (Fig. 3a, b). In summary, the R-isomers exhibited interaction with DNA (DA12F) similar to etoposide and we supposed that these isomers be the active isomers. The superpose of all ligands in active site is shown in Fig. 4.

Experimental

All the chemicals and reagents were obtained from Aldrich (Sigma-Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA),

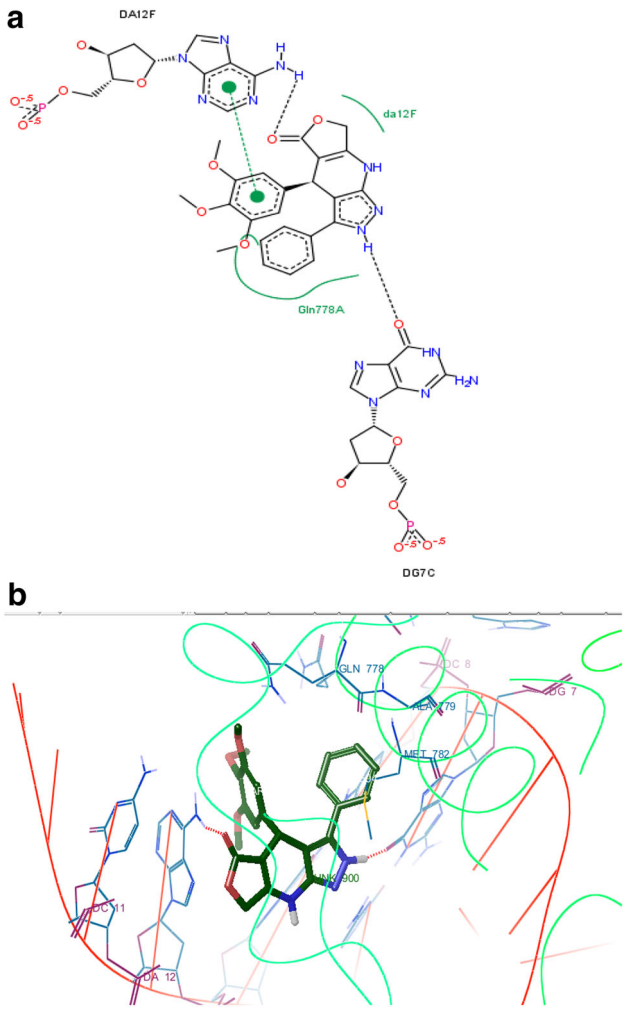


Fig. 2 **a** 2D Poseviwer plot of **8a**. **b** *Red line* indicates DNA, and *curved line* indicates protein; *green thick* residue indicates **8a** and *others* active-site residues (Color figure online)

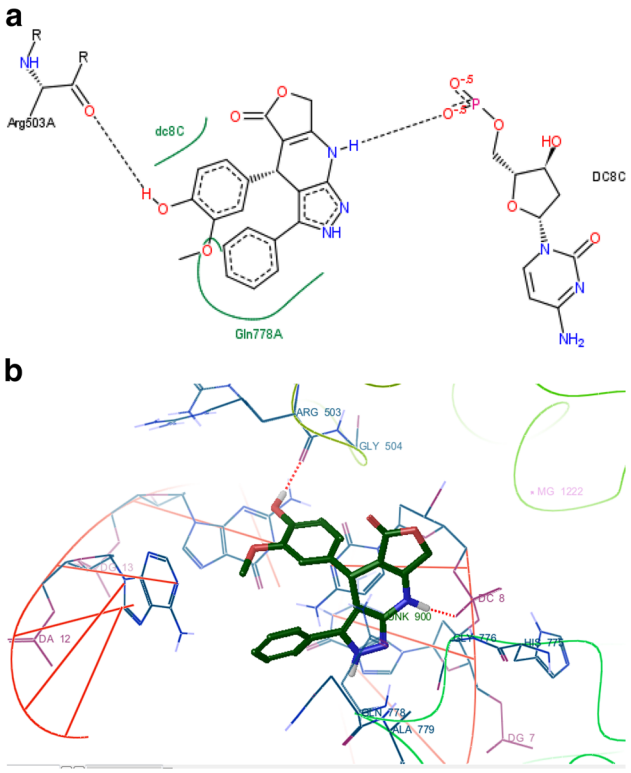


Fig. 3 **a** 2D Poseviwer plot of **8b**. **b** *Red line* indicates DNA, and *curved line* indicates protein; *green thick* residue indicates **8b** and *others* active-site residues (Color figure online)

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 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.

3-phenyl-4-(3,4,5-trimethoxyphenyl)-4,5,7,8-tetrahydro-1H-furo[3,4-b]pyrazolo[4,3-e] pyridin-5-one (**8a**) The compound tetronic acid (100 mg, 1 mmol) was dissolved in 4 mL of ethanol, followed by the addition of 3,4,5-trimethoxybenzaldehyde (**5a**) (196 mg, 1 mmol) and 3-phenyl-1H-pyrazol-5-amine (159 mg, 1 mmol). The reaction mixture was reflux at ethanol temperature for 1 h. The reaction mixture was allowed to cool to room temperature. Now, the precipitated product was collected by vacuum filtration, washed with ethanol (3 mL) and then recrystallized from acetone to afford pure compound **8a** as in 390 mg, 93 % yield. Mp: 189–191 °C, ^{1}H NMR (200 MHz, DMSO-d6): δ 3.61 (s, 3H, –OC**H**$_3$), 3.62 (s, 6H, (–OC**H**$_3$)$_2$), 4.83–5.02 (dd, 2H, $J = 16.1$, 15.4 Hz, –C**H**$_2$–O), 5.27 (s, 1H, –C**H**–), 6.43 (s, 2H, Ar**H**), 7.35–7.58 (m, 5H, Ar**H**), 8.72 (s, 1H, –O**H**), 10.32 (s, 1H, –N**H**); ^{13}C NMR (75 MHz, DMSO-d6): δ 34.8(–CH–, C_4), 55.5(–CH_3, C_4''), 59.7(–CH_3, C_3'', C_5''), 64.7(–CH_2–, C_7), 96.1(–C–, C_{10}), 101.7(–C–, C_{11}), 104.7(–C–, C_2', C_6'), 126.7(–CH–, C_2'', C_6''), 127.9(–CH–, C_4'), 128.4(–CH–,C_3', C_5'), 129.3(–CH–, C_1''), 135.6(–C–, C_1'), 138.8(–C–, C_3), 140.5(–C–, C_5''), 148.1(–C–, C_3''), 152.1(–C–, C_9), 159.3(–C–, C_{12}), 171.9(–C–, C_5); MS (ESI): 420 $[M + H]^{+}$.

4-(4-hydroxy-3-methoxyphenyl)-3-phenyl-4,5,7,8-tetrahydro-1H-furo[3,4-b] pyrazolo[4,3-e]pyridin-5-one (**8b**) This compound **8b** was prepared following the method described for the preparation of the compound **8a**, employing tetronic acid (100 mg, 1 mmol), 4-hydroxy-3-

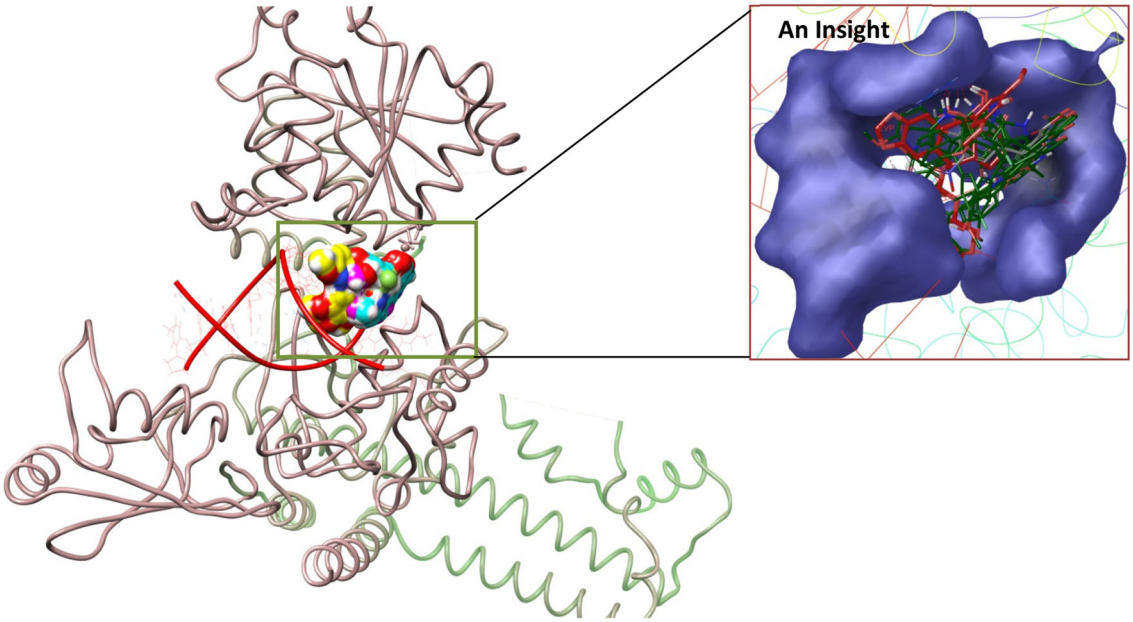


Fig. 4 Superpose: *Red lines* indicate DNA, and other *curved lines* indicate protein; *red color stick* indicates etoposide and *green stick* residues indicate compounds (Color figure online)

methoxybenzaldehyde (**5b**) (152 mg, 1 mmol) and 3-phenyl-1H-pyrazol-5-amine (159 mg, 1 mmol) to afford a pure compound **8b** as in 310 mg, 28 % yield. Mp: 304–306 °C, ^{1}H NMR (200 MHz, DMSO-d6): δ 3.58 (s, 3H, –OC**H**$_3$), 4.73–4.85 (dd, 2H, J = 15.2, 15.2 Hz, –C**H**$_2$–O), 5.08 (s, 1H, –C**H**–), 6.40 (d, 1H, J = 9.3 Hz, Ar**H**), 6.52 (d, 1H, J = 8.4 Hz, Ar**H**), 6.73 (s, 1H, Ar**H**), 7.22–7.36 (m, 3H, Ar**H**), 7.46 (d, 2H, J = 7.6 Hz, Ar**H**), 12.55 (s, 1H, –N**H**); ^{13}C NMR (75 MHz, DMSO-d6): δ 34.4 (–CH–, C_4), 55.3 (–CH_3, OCH_3, C_3''), 64.7 (–CH_2, C_7), 96.8 (–C–, C_{10}, C_{11}), 102.3 (–C–, C_1', C_1''), 112.0 (–CH–, C_1'), 115.1(–CH–, C_6'), 119.5(–CH–, C_4'), 126.5(–CH–, C_5'), 127.9 (–CH–, C_3'), 128.5 (–CH–, C_2''), 129.3 (–CH–, C_6''), 136.3(–CH–, C_5''), 138.5(–C–, C_4''), 144.5 (–C–, C_3), 146.6 (–C–, C_3''), 148.3 (–C–, C_9), 158.8 (–C–, C_{12}), 172.0 (–C–, C_5); MS (ESI): 376 $[M + H]^+$.

4-(3-hydroxy-4-methoxyphenyl)-3-phenyl-4,5,7,8-tetrahydro-1H-furo[3,4-b] pyrazolo[4,3-e]pyridin-5-one (**8c**) This compound **8c** was prepared following the method described for the preparation of the compound **8a**, employing tetronic acid (100 mg, 1 mmol), 3-hydroxy-4-methoxybenzaldehyde (**5c**) (152 mg, 1 mmol) and 3-phenyl-1H-pyrazol-5-amine (159 mg, 1 mmol) to afford a pure compound **8c** as in 290 mg, 77 % yield. Mp: 304–306 °C, ^{1}H NMR (400 MHz, DMSO-d6): δ 3.70 (s, 3H, –OC**H**$_3$), 4.88–5.02 (dd, 2H, J = 15.6, 16.3 Hz, –C**H**$_2$–O), 5.49 (s, 1H, –C**H**–), 6.41–6.43 (dd, 1H, J = 1.7, 1.7 Hz, Ar**H**), 6.54 (d, 1H, J = 7.8 Hz, Ar**H**), 7.05–7.07 (m, 4H, Ar**H**), 7.83 (d, 1H, J = 7.8 Hz, Ar**H**), 8.72 (s, 1H, –O**H**), 10.18 (s, 1H, –N**H**); ^{13}C NMR (75 MHz, DMSO-d6): δ 34.4 (–CH–, C_4), 55.3 (–CH_3, O–CH_3, C_4''), 64.6 (–CH_2, C_7), 97.2 (–C–, C_{10}, C_{11}), 102.0 (–C–, C_1', C_1''), 111.4 (–CH–, C_2''), 114.9 (–CH–, C_2'), 118.2 (–CH–, C_6'), 126.1 (–CH–, C_4'), 127.8 (–CH–, C_3', C_5'), 128.5 (–CH–, C_6''), 129.1 (–CH–, C_5''), 137.9 (–C–, C_3), 138.3 (–C–, C_3''), 145.9 (–C–, C_4''), 148.6 (–C–, C_9), 158.4 (–C–, C_{12}), 171.8(–C–, C_5); MS (ESI): 376 $[M + H]^+$.

4-(4-methoxy-3-nitrophenyl)-3-phenyl-4,5,7,8-tetrahydro-1H-furo[3,4-b]pyrazolo[4,3-e]pyridin-5-one (**8d**) This compound **8d** was prepared following the method described for the preparation of the compound **8a**, employing tetronic acid (100 mg, 1 mmol), 4-methoxy-3-nitrobenzaldehyde (**5d**) (181 mg, 1 mmol) and 3-phenyl-1H-pyrazol-5-amine (159 mg, 1 mmol) to afford a pure compound **8d** as in 380 mg, 94 % yield. Mp: 268–270 °C, ^{1}H NMR (400 MHz, DMSO-d6): δ 3.79 (s, 3H, –OC**H**$_3$), 4.73–4.83 (dd, 2H, J = 15.5, 15.5 Hz, –C**H**$_2$–O), 5.29 (s, 1H, –C**H**–), 7.04 (d, 1H, J = 8.6 Hz, Ar**H**), 7.22 (t, 1H, Ar**H**), 7.29 (t, 2H, Ar**H**), 7.36–7.43 (m, 3H, Ar**H**), 7.51 (d, 1H, J = 2.5 Hz, Ar**H**), 10.30 (s, 1H, –N**H**); ^{13}C NMR (75 MHz, DMSO-d6): δ 33.8 (–CH–, C_4), 56.3 (–CH_3, O–CH_3, C_4''), 64.8 (–CH_2, C_7), 95.7 (–C–, C_9), 101.0 (–C–, C_{10}), 113.7 (–CH–, C_2', C_6'), 123.7 (–CH–, C_4'), 126.4 (–CH–, C_3', C_5'), 128.1 (–CH–, C_5''), 128.4 (–CH–,C_2''), 133.5 (–CH–, C_6''), 137.2 (–C–, C_1', C_1''), 138.2 (–C–, C_3''), 138.8 (–C–, C_3), 148.1 (–C–, C_4''), 150.3 (–C–,C_9), 159.2 (–C–, C_{12}), 171.7 (–C–, C_5); MS (ESI): 405 $[M + H]^+$.

3-(4-chlorophenyl)-4-(4-fluoro-3-methoxyphenyl)-4,5,7,8-tetrahydrofuro[3′,4′:5,6] pyrido[3,2-d]isoxazol-5-one (**8e**) This compound **8e** was prepared following the method described for the preparation of the compound **8a**, employing tetronic acid (100 mg, 1 mmol), 4-fluoro-3-methoxybenzaldehyde (**5e**) (154 mg, 1 mmol) and 3-(4-chlorophenyl)isoxazol-5-amine (194 mg, 1 mmol) to afford a pure compound **8e** as in 385 mg, 93 % yield. Mp: 244–246 °C, ^{1}H NMR (300 MHz, DMSO-d6): δ 3.72 (s, 3H, –OC**H**$_3$), 4.67–4.79 (dd, 2H, $J = 16.6$, 16.2 Hz, –C**H**$_2$–O), 4.92 (s, 1H, –C**H**–), 6.55–6.64 (m, 3H, Ar**H**), 7.24 (d, 2H, $J = 8.4$ Hz, Ar**H**), 7.39 (d, 2H, $J = 8.4$ Hz, Ar**H**), 8.32 (s br, 1H, Ar**H**), 11.16 (s, 1H, –N**H**); ^{13}C NMR (75 MHz, DMSO-d6): δ 34.7 (–CH–, C_4), 55.6 (–CH_3, O–CH_3, C_3''), 64.8 (–CH_2,C_7), 94.0 (–C–, C_{10}), 100.8 (–C–, C_{11}), 113.5 (–CH–, C_2''), 115.4 (–CH–, C_5''), 115.7 (–CH–, C_6''), 120.0 (–CH–, C_2'), 126.9 (–CH–, C_6'), 128.6 (–C–, C_5'), 129.2 (–CH–, C_3'), 134.6 (–CH–, C_1'), 140.0 (–C–, C_1''), 146.2 (–C–, C_4'), 148.4 (–C–, C_4''), 151.6 (–C–, C_3''), 157.2 (–C–, C_{12}), 159.5 (–C–, C_3), 162.0 (–C–, C_9), 170.7 (–C–, C_5); MS (ESI): 435 $[M + Na]^+$.

4-(3-hydroxy-4-methoxyphenyl)-3-methyl-4,5,7,8-tetrahydrofuro[3′,4′:5,6]pyrido[3,2-d]isoxazol-5-one (**8f**) This compound **8f** was prepared following the method described for the preparation of the compound **8a**, employing tetronic acid (100 mg, 1 mmol), 3-hydroxy-4-methoxybenzaldehyde (**5c**) (152 mg, 1 mmol) and 3-methylisoxazol-5-2amine (98 mg, 1 mmol) to afford a pure compound **8f** as in 420 mg, 88 % yield. Mp: 205–207 °C, ^{1}H NMR (300 MHz, DMSO-d6): δ 3.80 (s, 3H, –C**H**$_3$), 3.86 (s, 3H, –OC**H**$_3$), 5.38–5.62 (m, 3H, –C**H**$_2$–O, –C**H**–), 6.97–7.11 (m, 3H, Ar**H**), 9.35 (s, 1H, –O**H**); ^{13}C NMR (75 MHz, DMSO-d6): δ 12.7 (–CH_3, C_3), 37.7 (–CH–, C_4), 55.5 (–CH_3, O–CH_3, C_4'), 68.6 (–CH_2, C_7), 111.1 (–CH–, C_2', C_5'), 116.9 (–CH–, C_6'), 121.3 (–C–, C_{10}, C_{11}, C_1'), 121.9 (–C–, C_3'), 145.6 (–C–, C_4'), 149.2 (–C–, C_{12}), 149.5 (–C–, C_9), 157.0 (–C–, C_3), 170.1 (–C–, C_5); MS (ESI): 314 $[M - H]^+$.

10-(4-hydroxy-3-methoxyphenyl)-2-methyl-6,7,9,10-tetrahydro-1H-furo[3,4-b]pyrrolo [2,3-f]quinolin-9-one (**10**) This compound **10** was prepared following the method described for the preparation of the compound **8a**, employing tetronic acid (100 mg, 1 mmol), 4-hydroxy-3-methoxybenzaldehyde (**5b**) (152 mg, 1 mmol) and 2-methyl-1H-indol-5-amine(146 mg, 1 mmol) to afford a pure compound **10** as in 310 mg, 85 % yield. Mp: 313–315 °C, ^{1}H NMR (200 MHz, DMSO-d6): δ 1.21 (s, 3H, –C**H**$_3$), 3.80 (s, 3H, –OC**H**$_3$), 4.95–5.12 (dd, 2H, $J = 15.69$, 15.69 Hz, –C**H**$_2$–O), 5.28 (s, 1H, –C**H**–), 5.59 (d, 1H, Ar**H**), 5.71 (d, 1H, Ar**H**), 6.21 (s, 1H, Ar**H**), 7.00 (d, 1H, Ar**H**), 7.92 (s, 1H, Ar**H**), 9.48 (s, 1H, –O**H**); ^{13}C NMR (75 MHz, DMSO-d6): δ 13.7 (–CH_3, C_2), 36.0 (–CH–, C_{10}), 55.5 (–CH_3, O–CH_3, C_3'), 65.0 (–CH_2, C_7), 96.3 (–CH–,C_3), 112.2 (–C–, C_{14},C_{15}), 115.2 (–CH–, C_4, C_5), 119.7 (–CH–, C_2', C_5', C_6'), 136.0 (–C–, C_2, C_{11}, C_{12}), 145.0 (–C–, C_1'), 146.9 (–C–, C_3', C_4'), 157.7 (–C–, C_{13}, C_{16}), 171.9(–C–, C_9); MS (ESI): 363 $[M + H]^+$.

10-(2-fluoro-4-methoxyphenyl)-6,7,9,10-tetrahydro-1H-furo[3,4-b]pyrazolo[3,4-f] quinolin-9-one (**12**) This compound **12** was prepared following the method described for the preparation of the compound **8a**, employing tetronic acid (100 mg, 1 mmol), 2-fluoro-4-methoxybenzaldehyde (**5f**) (154 mg, 1 mmol) and 1H-indazol-5-amine (133 mg, 1 mmol) to afford a pure compound **12** as in 305 mg, 86 % yield. Mp: 298–300 °C, ^{1}H NMR (400 MHz, DMSO-d6): δ 3.69 (s, 3H, –OC**H**$_3$), 4.80–4.91 (dd, 2H, $J = 15.2$, 15.2 Hz, –C**H**$_2$–O), 5.55 (s, 1H, –C**H**–), 6.56–6.64 (m, 2H, Ar**H**), 6.98 (d, 1H, $J = 9.3$ Hz, Ar**H**), 7.11 (t, 1H, Ar**H**), 7.32 (d, 1H, $J = 9.3$ Hz, Ar**H**), 7.60 (s, 1H, Ar**H**), 9.96 (s, 1H, –N**H**); ^{13}C NMR (75 MHz, DMSO-d6): δ 31.2 (–CH–, C_{10}), 55.3 (–CH_3, O–CH_3, C_4'), 64.9 (–CH_2, C_7), 93.5 (–C–, C_{14}, C_{15}), 100.6 (–CH–, C_3'), 100.9 (–CH–, C_5'), 110.0 (–C–, C_{12}), 110.5 (–CH–, C_5), 112.6 (–C–, C_{11}), 117.3 (–C–, C_1'), 129.4 (–CH–, C_4), 131.0 (–CH–,C_3), 137.3 (–CH–,C_6'), 157.8 (–C–, C_{13}, C_{16}), 158.9 (–C–, C_4'), 161.0 (–C–, C_2'), 171.9 (–C–, C_9); MS (ESI): 352 $[M + H]^+$.

2-methoxy-9-(3,4,5-trimethoxyphenyl)-5,6,8,9-tetrahydrofuro[3,4-b][1,5]naphthyridin-8-one (**14**) This compound **14** was prepared following the method described for the preparation of the compound **8a**, employing tetronic acid (100 mg, 1 mmol), 3,4,5-trimethoxybenzaldehyde (**5a**) (196 mg, 1 mmol) and 6-methoxypyridin-3-amine (124 mg, 1 mmol) to afford a pure compound **14** as in 340 mg, 88 % yield. Mp: 205–207 °C, ^{1}H NMR (400 MHz, DMSO-d6): δ 3.85 (s, 6H, (–OC**H**$_3$)$_2$), 3.88 (s, 6H, (–OC**H**$_3$)$_2$), 4.65 (s, 2H, –C**H**$_2$–O), 4.77 (s, 1H, –C**H**–), 7.88 (d, 1H, $J = 6.0$ Hz, Ar**H**), 8.07–8.09 (m, 3H, Ar**H**); ^{13}C NMR (75 MHz, DMSO-d6): δ 36.1 (–CH–, C_9), 60.9 (–CH_3, O–CH_3, C_2), 61.2 (–CH_3, C_3'), 65.1 (–CH_3, C_4'), 65.4 (–CH_3, C_5'), 72.0 (–CH_2, C_6), 104.2 (–CH–, C_2', C_6'), 110.2 (–CH–, C_3), 111.8 (–C–, C_{13}), 111.9 (–CH–, C_4), 136.8 (–C–, C_1'), 141.0 (–C–, C_{11}), 141.8 (–C–, C_4'), 147.9 (–C–, C_{10}), 157.5 (–C–, C_3', C_5'), 157.6 (–C–, C_2), 170.7 (–C–, C_{12}), 174.9 (–C–, C_8); MS (ESI): 383 $[M - H]^+$.

Procedure of the SRB assay

The synthesized compounds (**8a–f**, **10**, **12** and **14**) 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 μL 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 for further 48 h, and assay was terminated by the addition of 50 μL of cold trichloroacetic 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. Percent 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.

Molecular docking simulations

The molecular docking studies were carried out using the Maestro 8.5 (Schrodingers LLC) installed on RHEL 5.0. The X-ray crystallographic structure of the human topoisomerase II beta along with etoposide and DNA complex (PDB Code: 3QX3) was downloaded from the PDB (http://www.rcsb.org/pdb/explore.do?structureId=3qx3). The protein was prepared by using the protein preparation wizard. It contains two chains; the A chain along with the DNA, Mg^{2+} ion and etoposide was selected for further docking studies. The receptor grid around the reference ligand, i.e., etoposide, was generated with default parameters. The ligands were drawn using the ChemDraw and imported into project table, prepared by LigPrep. The molecular docking studies were carried out by employing extra-precision docking of Glide 5.0.

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