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Synthesis of a new 4-aza-2,3-didehydropodophyllotoxin analogues as potent cytotoxic and antimitotic agents

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ABSTRACT

A series of novel conjugates of 4-aza-2,3-didehydropodophyllotoxins (**11a–w**) were synthesized by a straightforward one-step multicomponent synthesis that demonstrated cytotoxicity against five human cancer cell lines (breast, oral, colon, lung and ovarian). All the twenty three compounds (**11a–w**) have been examined for the inhibition of tubulin polymerization. Among these compounds, **11a**, **11k** and **11p** exhibited inhibition of polymerization tubulin comparable to podophyllotoxin apart from disruption of microtubule organization within the cells. Flow cytometric analysis showed that these compounds (**11a**, **11k** and **11p**) arrested the cell cycle in the G2/M phase of cell cycle leading to caspase-3 dependent apoptotic cell death.

1. Introduction

Podophyllum peltatum L, also known as the American Mayapple, is the source of the natural product podophyllotoxin (**1**).[1] It has cytotoxic properties and is known to inhibit polymerization inhibition. The biological activity[2] of **1** has led to extensive structural modifications resulting in several clinically useful compounds like etoposide (**2**), teniposide (**3**) and the water-soluble prodrug, etopophos (**4**).[3–5] These compounds have shown to inhibit DNA topoisomerase-II (topo-II) by stabilizing the covalent topo-II DNA cleavable complex[6] and are used against a variety of cancers, including germ-cell malignancies, small-cell lung cancer, non-Hodgkin's lymphoma, leukemia, kaposi's sarcoma, neuroblastoma, and soft tissue sarcoma.[7–9] They have several limitations, such as poor water solubility, development of drug resistance, metabolic inactivation, and toxic effects.[10] To overcome such problems, extensive synthetic efforts have been carried out by a number of researchers. This has led to the development of NK-611 (**5**), and GL-331 (**6**). GL-331 contains a *p*-nitroanilino moiety at the 4β-position instead of a glycoside of etoposide, which proved to be more potent than etoposide. GL-331, is also an inhibitor of topo-II,[11] and induce apoptotic cell death through independent mechanism which contributes to its cytotoxicity. This compound has underwent phase II clinical trials for the treatment of various cancers,[12] however this did not proceed further. Recently, we have been involved in the development of new synthetic procedures[13] for the podophyllotoxin-based compounds and also in the design and synthesis of new analogs of podophyllotoxin as potential anticancer agents.[14]

Application of multicomponent reactions (MCRs) to the construction of natural product-based libraries would be most beneficial to this area of research. Such processes in which three or more reactants are combined together in a single reaction flask to produce a product incorporating most of the atoms contained in the starting materials have the advantages of the intrinsic atom economy, simpler procedures and equipment, time and energy savings, as well as environmental friendliness.[15–20] The structural complexity of podophyllotoxin **1**, arising from the presence of four stereogenic carbons in ring C (Fig 1). Most of the structure-activity relationship studies have been performed by derivatization of parent natural product rather than by de novo chemical synthesis.[21] In this connection, Itokawa and Takeya made an important contribution for the synthesis of 4-aza-podophyllotoxin (**7a**) and (**7b**) derivatives via three steps of condensation, cyclization, and reduction. Cytotoxicity of these compounds is comparable to that of podophyllotoxin.[22] The removal of the stereo centers at C-2 and C-3 solves the problem of epimerization at C-2 that has plagued the

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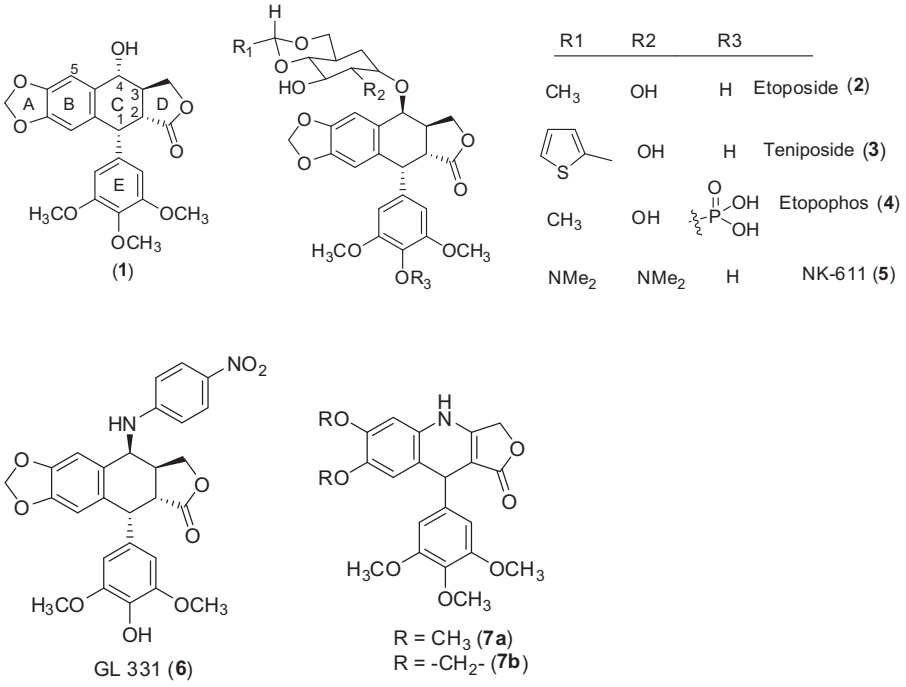


Figure 1.

clinical development of podophyllotoxin and its stereochemical complex derivatives, because the rapidly formed in vivo cis-lactone metabolite is significantly less potent.[23] Tu et al. reported a multicomponent reaction leading to the formation of N-substituted dihydropyridines, although no biological data for these compounds were provided.[24] Recently, Magedov et al. reported a multicomponent reaction leading to the formation of didehydropyridopyrazole analogues.[25]

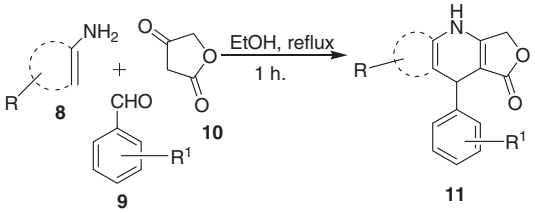
In continuation of these efforts and our interest in the structural modifications of the podophyllotoxin lignan, we would like to report herein efficient access to the construction of a new novel 4-aza-2,3-didehydropodophyllotoxin derivatives with improved cytotoxicity and inhibition of tubulin polymerization.

2. Chemistry

These new 4-aza-2,3-didehydropodophyllotoxin congeners (**11a–w**) were synthesized by applying a multicomponent route involving the condensation of substituted heteroaromatic amines, tetronic acid, and substituted aromatic aldehydes that proceeds smoothly in refluxing ethanol. In all cases, the resulting 4-aza-2,3-didehydropodophyllotoxins precipitate as the reaction mixtures are allowed to cool to room temperature and are isolated by simple filtration followed by recrystallized from ethanol to afford the pure compounds (Scheme 1). All the synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, mass spectral data and HRMS.

3. Biological evaluation

The biological activities of this series of 4-aza-2,3-didehydropodophyllotoxin derivatives were evaluated by an cytotoxicity assay, which was carried out in a panel of five human tumor cell lines comprising (breast, oral, colon, lung and ovarian) by using the sulforhodamine B (SRB) method. The results are summarized in Table 1, and compared to etopside as well as adriamycin. It is observed that all the compounds are significantly cytotoxic with the concentration of the compound that produced 50% inhibition of cell growth (GI_{50}) ranging from <0.1 to 2.9 μM. It appears from the data that 2-fluoro-4-methoxy substitutions in the 4-aryl pendent ring enhances the cytotoxicity as seen in compounds **11l** and **11t** for the breast cancer cell line (MCF-7). The presence of fluoro substituent adds the 4-position in the pendent aryl ring also



11a: R = 2,4 dimethoxy 5-pyrimidyl, R^1 = 3,4,5-trimethoxy,
11b: R = 2,4 dimethoxy 5-pyrimidyl, R^1 = 4-hydroxy-3-methoxy,
11c: R = 2,4 dimethoxy 5-pyrimidyl, R^1 = 3-hydroxy-4-methoxy,
11d: R = 2,4 dimethoxy 5-pyrimidyl, R^1 = 4-fluoro-3-methoxy,
11e: R = 5-indyl, R^1 = 3,4,5-trimethoxy,
11f: R = 5-indyl, R^1 = 4-hydroxy-3-methoxy,
11g: R = 5-indyl, R^1 = 3-hydroxy-4-methoxy,
11h: R = 5-indyl, R^1 = 4-fluoro-3-methoxy,
11i: R = 5-indyl, R^1 = 3-nitro-4-methoxy,
11j: R = 2-methyl-5-indyl, R^1 = 3,4,5-trimethoxy,
11k: R = 2-methyl-5-indyl, R^1 = 4-fluoro-3-methoxy,
11l: R = 2-methyl-5-indyl, R^1 = 2-fluoro-4-methoxy,
11m: R = 5-indazolyl, R^1 = 4-hydroxy-3-methoxy,
11n: R = 5-indazoly, R^1 = 3-hydroxy-4-methoxy,
11o: R = 5-indazolyl, R^1 = 4-fluoro-3-methoxy,
11p: R = 6-benzthiazolyl, R^1 = 3,4,5-trimethoxy,
11q: R = 2-methyl-6-benzthiazolyl, R^1 = 3-nitro-4-methoxy,
11r: R = 2-mercapto-5-imidazolyl, R^1 = 3-hydroxy-4-methoxy,
11s: R = 5-triazolyl, R^1 = 3,4,5-trimethoxy,
11t: R = 5-triazolyl, R^1 = 2-fluoro-4-methoxy,
11u: R = 3-(4-methoxyaryl)5-isoxazolyl, R^1 = 3,4,5-trimethoxy,
11v: R = 3-(4-chloroaryl)5-isoxazolyl, R^1 = 3-hydroxy-4-methoxy,
11w: R =2,3,4-trimethoxyaryl, R^1 = 4-hydroxy-3-methoxy;

Scheme 1.

Table 1
Cytotoxic activity (GI_{50} μM) of compounds **11a–2**

Compound	Breast MCF7	Oral KB	Colo Colo 205	Lung A-549	Ovarian A-2780
11a	2.4	0.17	0.16	2.1	–
11b	–	–	–	–	–
11c	–	–	–	–	–
11d	–	2.2	–	2.6	–
11e	–	2.0	2.3	2.4	2.7
11f	2.3	–	2.5	–	–
11g	2.9	2.2	0.19	2.3	2.7
11h	2.1	0.17	–	2.2	2.3
11i	–	–	2.4	–	–
11j	–	2.0	–	–	–
11k	2.6	–	–	2.1	2.4
11l	<0.1	2.2	2.7	–	2.3
11m	2.0	–	2.0	–	–
11n	–	–	2.6	–	–
11o	2.7	–	–	2.3	–
11p		2.1	2.5	2.4	2.3
11q	0.15	–	–	–	–
11r	2.7	–	–	–	–
11s	–	2.9	–	–	–
11t	<0.1	–	–	–	–
11u	–	–	–	–	–
11v	–	2.7	–	–	–
11w	2.9	–	–	–	–
Etoposide	2.1	0.3	0.13	3.08	1.3
ADR	0.13	0.13	<0.1	<0.1	<0.1

ADR = adriamycin is one of the control drug.

enhance the cytotoxicity in KB cell line as in case of **11h**. It is also observed that the change of A ring to hetero aryl or benzo hetero fused system have not shown much difference in the cytotoxicity.

3.1. Inhibition of tubulin polymerization

Since these 23 new compounds has structural resemblance to podophyllotoxin, it has been considered of interest to investigate their effect on tubulin polymerization. One of the possible explanations of compounds showing anticancer activity and cell cycle arrest is the inhibition of tubulin polymerization to functional microtubules as it is observed with antimitotic agents such as podophyllotoxin. As tubulin subunits heterodimerize and self-assemble to form microtubules in a time dependent manner, we have investigated the progression of tubulin polymerization by monitoring the increase in fluorescence emission at 460 nm (excitation wavelength is 360 nm) in 384 well plate for 1 h at 37 °C with and without the compounds at 3 μM concentration. Among the twenty three compounds examined, **11a**, **11k** and **11p** inhibited tubulin polymerization to nearly 63.7%, 45.3%, and 49.4%, respectively compared to control and a similar pattern of inhibition has been observed with the positive control, podophyllotoxin (80.7%) (Fig. 2). From this data it is evident that these structurally modified podophyllotoxin like molecules exhibit cytotoxicity probably involving a different mechanism compare to the typical podophyllotoxin ring system.

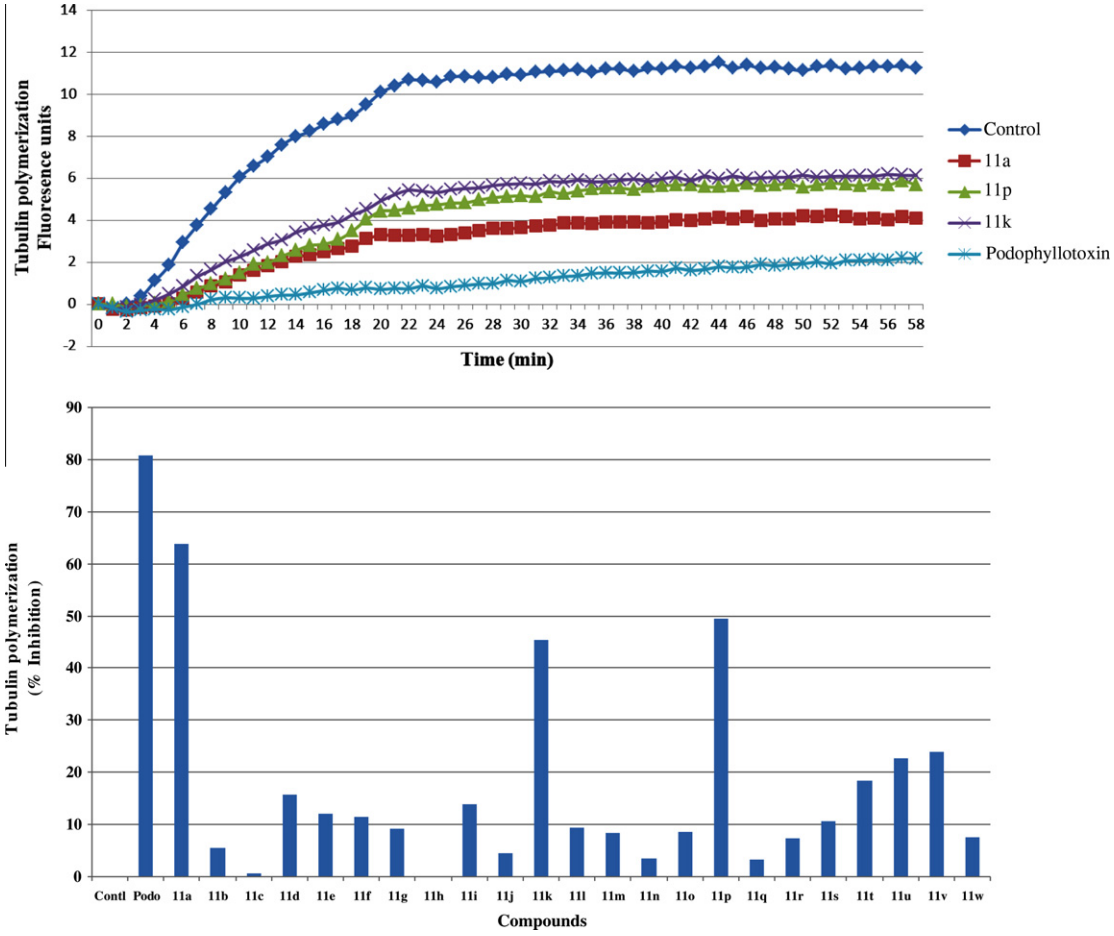


Figure 2. Effect of compounds on tubulin polymerization: Tubulin polymerization was monitored by the increase in fluorescence at 340 nm (excitation) and 460 nm (emission) for 1 h at 37 °C. All the compounds were included at a final concentration of 3 μM. Podophyllotoxin (Podo) was used as a positive control.

3.2. Immunohistochemistry of tubulin

In order to substantiate the observed in vitro effects of the compounds on the inhibition of tubulin polymerization to functional microtubules, immunohistochemistry studies have been carried out to examine the in situ effects of compounds **11a**, **11k** and **11p** on cellular microtubules and their special arrangement. In this study, untreated human lung cancer cells (A-549) displayed the normal distribution of microtubules (Fig. 3). However, cells treated with 5 μM concentration of compounds **11a**, **11k** and **11p** and podophyllotoxin demonstrated disrupted microtubule organization as seen in Fig. 3A–E, thus exemplifying the inhibition of tubulin polymerization.

3.3. Cell cycle analysis (flow cytometry)

Since the compounds **11a**, **11k** and **11p** mediate their cytotoxic effects by inhibiting the polymerization of tubulin, we have examined whether the same could be reflected in the pattern of cell cycle arrest. Towards this, the cell cycle dependent DNA content was determined by flow cytometry using propidium iodide staining according to the standard protocol. The effect of compounds **11a**, **11k** and **11p** (10 μM) along with DMSO control were examined in A-549 lung cancer cells treated individually with these compounds for 48 h. Podophyllotoxin was used as standard comparison as shown in (Fig. 4).

3.4. Caspase-3 activation assay

Since in several instances cell cycle arrest at G2/M phase has been shown to induce cellular apoptosis, we have examined whether the cytotoxicity by **11a**, **11k** and **11p** is by virtue of apoptotic cell death. Cysteine aspartase group, namely, caspases play a crucial role in the induction of apoptosis and amongst them caspase-3 happens to be one of the effector caspase. Hence, we have treated A-549 cells with **11a**, **11k** and **11p** along with the positive control podophyllotoxin and examined for the activation of caspase-3. Results indicate that there was nearly 2–3.5-fold induction in caspase-3 activity in cells treated with 5 μM concentration of compounds. Under similar conditions, podophyllotoxin (1 μM) induced caspase activity to nearly 4-fold as compared to controls as shown in (Fig. 5).

4. Conclusion

These new heterocyclic compounds (**11a–w**) are accessible by a straightforward one-step multicomponent synthesis, which involves simple isolation of the products by filtration and requires no further purification. These compounds (**11a–w**) were evaluated for their cytotoxic activity a five human cancer cell lines. All the twenty three compounds (**11a–w**) have been examined for the tubulin polymerization assay. Among these compounds **11a**, **11k** and **11p** exhibited inhibition of polymerization tubulin comparable to podophyllotoxin apart from disruption of microtubule organization within the cells. Further, flow cytometric analysis showed that these **11a**, **11k** and **11p** compounds arrested the cell cycle in the G2/M phase of cell cycle leading to caspase-3 dependent apoptotic cell death. These results provide an insight for future direction in the development of such molecules.

5. Experimental

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) and were used without further purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 F-254, and visualization on TLC was achieved by UV light or iodine indicator. ^{1}H and ^{13}C NMR spectra were recorded on INOVA (400 MHz) or Gemini Varian-VXR-unity (200 MHz) or Bruker UXNMR/XWIN-NMR (300 MHz) instruments.

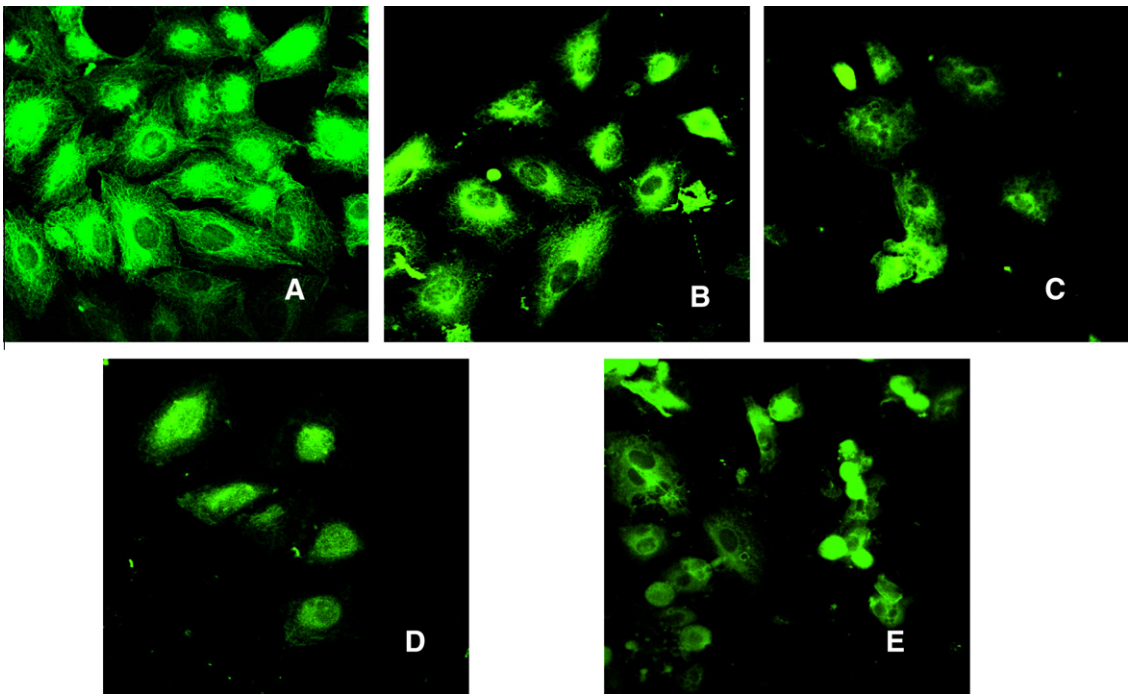
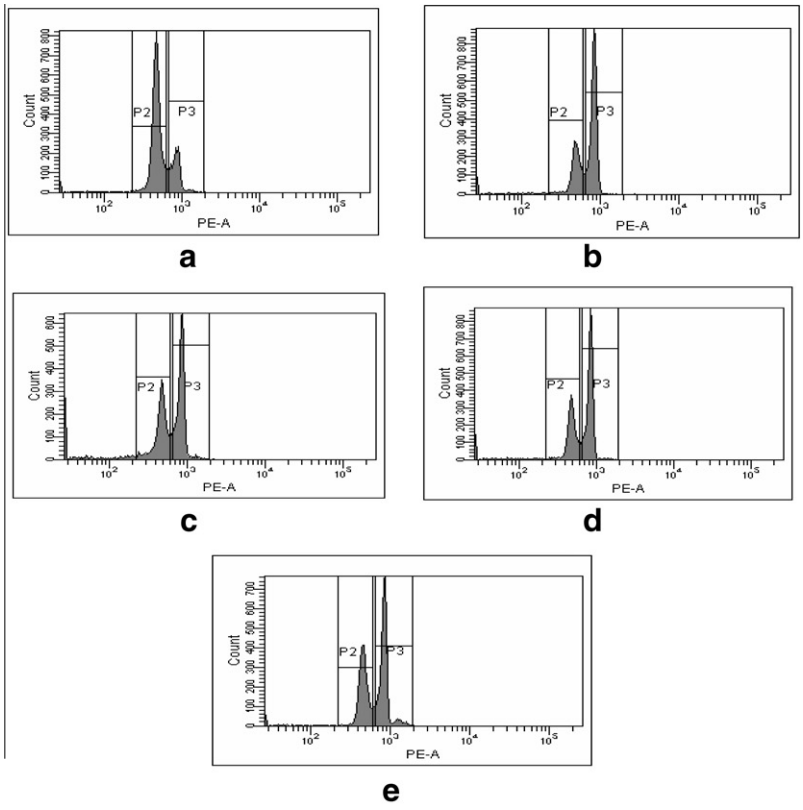


Figure 3. IHC analysis of tubulin polymerization (A) control untreated (B), (C), and (D) cells treated with compounds **11a**, **11k**, and **11p** at 5 μM for 48 h, respectively, (E) podophyllotoxin (1 μM).



Samples	G1%	G2/M%	S%
Control	71.6	23.1	5.2
Podo	31.4	61.5	7.1
11a	38.9	48.2	12.7
11k	35.4	55.6	8.8
11p	42.4	53.1	4.4

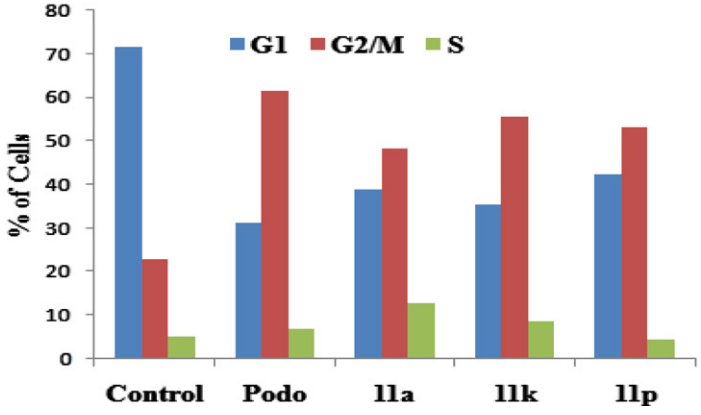


Figure 4. FACS analysis of cell cycle distribution of A-549 cells (a) control untreated (b) Podophyllotoxin (Podo) was used at 1 μM concentration, (c–e) compounds **11a**, **11p**, and **11k** treated cells at 10 μM concentration.

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. High-resolution mass spectra (HRMS) were recorded on QSTAR XL Hybrid MS/MS mass spectrometer. Melting points were determined with an electrothermal melting point apparatus, and are uncorrected.

5.1. 2,4-Dimethoxy-5-(3,4,5-trimethoxyphenyl)-5,6,8,9-tetrahydrofuro[3′,4′:5,6]pyrido[2,3-*d*]pyrimidin-6-one (11a)

The compound tetronic acid (102 mg, 1.02 mmol) was dissolved in 4 mL of ethanol, followed by addition of 3,4,5-trimethoxybenzaldehyde (200 mg, 1.02 mmol) and 2,4-dimethoxypyrimidine-5-amine (158 mg, 1.02 mmol). The reaction mixture was reflux at

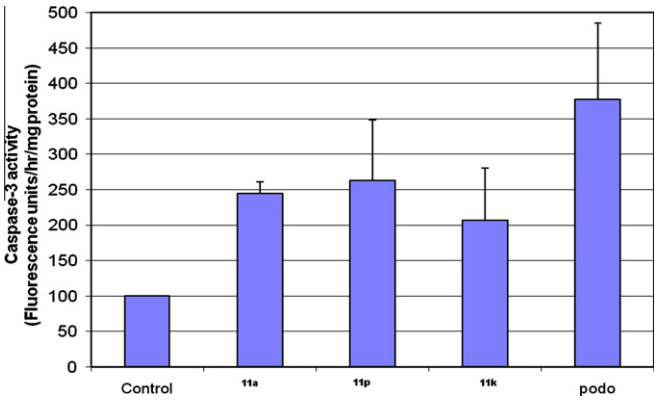


Figure 5. Effect of compounds **11a**, **11p**, and **11k** on caspase-3 activity: A-549 cells were treated for 48 h with 5 mm concentration of compounds **11a**, **11p** and **11k**. Following termination of experiment, cellular caspase-3 activity was measured by fluorimetric assay as described in the 'Section 5'. Podophyllotoxin (Podo) was used as a positive control. Values indicated are the mean ± SD of two different experiments performed in triplicates.

ethanol temperature for 1 h. Then reaction mixture was allowed to cool to room temperature, and the precipitated product was collected by vacuum filtration and washed with ethanol (3 mL) then recrystallized from ethanol to afford pure compound **11a** as a white solid in 390 mg, 92% yield. Mp: 299–300 °C, ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.61 (s, 3H), 3.70 (s, 6H), 3.81 (s, 3H), 3.85 (s, 3H), 4.79–5.02 (m, 3H), 6.43 (s, 2H), 10.61 (s,1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 34.3, 53.8, 54.3, 55.7, 59.7, 65.2, 93.8, 99.6, 104.7, 136.1, 139.9, 152.4, 156.8, 163.2, 168.8, 171.1; MS (ESI): 416 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{22}N_3O_7$ $[M+Na]^+$ 416.1457; found: 416.1447.

5.2. 5-(4-Hydroxy-3-methoxyphenyl)-2,4-dimethoxy-5,6,8,9-tetrahydrofuro[3′,4′:5,6]pyrido[2,3-*d*]pyrimidin-6-one (11b)

This compound **11b** was prepared follwing the method described for the preparation of the compound **11a**, employing tetronic acid (127 mg, 1.27 mmol) with 4-hydroxy-3-methoxybenzaldehyde (200 mg, 1.27 mmol) and 2,4-dimethoxypyrimidine-5-amine (197 mg, 1.27 mmol) to afford pure compound **11b** as a white solid in 450 mg, 92% yield. Mp: 294–296 °C, ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.77 (s, 3H), 3.84 (s, 3H), 3.90 (s, 3H), 4.82–5.03 (dd, 2H, J = 16.8, 16.8 Hz), 6.47–6.52 (dd, 1H, J = 2.1, 2.1 Hz), 6.66 (d, 1H, J = 8.0 Hz), 6.84 (d, 1H, J = 2.1 Hz), 10.64 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.5, 53.8, 54.2, 55.4, 65.1, 94.3, 100.2, 111.8, 115.1, 119.4, 135.6, 145.0, 146.9, 156.6, 157.5, 163.0, 168.7, 171.1; MS (ESI): 372 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{16}N_2O_4Na$ $[M+Na]^+$ 371.1007; found: 371.0995.

5.3. 5-(3-Hydroxy-4-methoxyphenyl)-2,4-dimethoxy-5,6,8,9-tetrahydrofuro[3′,4′:5,6]pyrido[2,3-*d*]pyrimidin-6-one (11c)

This compound **11c** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (127 mg, 1.27 mmol) with 3-hydroxy-4-methoxybenzaldehyde (200 mg, 1.27 mmol) and 2,4-dimethoxypyrimidine-5-amine (197 mg, 1.27 mmol) to afford pure compound **11c** as white solid in 460 mg, 94% yield. Mp: 284–285 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.71 (s, 3H), 3.77 (s, 3H), 3.85 (s, 3H), 4.65 (s, 1H), 4.76– 4.95 (dd, 2H, J = 15.9, 16.8 Hz), 9.10 (s, 1H), 10.60 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.4, 53.9, 54.3, 55.5, 65.1, 74.1, 82.8, 94.3, 100.4, 111.8, 114.7, 115.1, 117.9, 137.4, 146.2, 156.0, 157.2, 168.7; MS (ESI): 372 $[M+H]^+$; HRMS (ESI) calcd for $C_{18}H_{17}N_3O_6Na$ $[M+Na]^+$ 394.1015; found: 394.1000.

5.4. 5-(4-Fluoro-3-methoxyphenyl)-2,4-dimethoxy-5,6,8,9-tetrahydrofuro[3′,4′:5,6]pyrido[2,3-*d*]pyrimidin-6-one (11d)

This compound **11d** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (88 mg, 0.88 mmol) with 4-fluoro-3-methoxybenzaldehyde (200 mg, 0.88 mmol) and 2,4-dimethoxypyrimidine-5-amine (137 mg, 0.88 mmol) to afford pure compound **11d** as a white solid in 470 mg, 97% yield. Mp: 267–270 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.00 (s, 3H), 3.02 (s, 3H), 3.07 (s, 3H), 3.94– 4.12 (m, 3H), 5.77–5.84 (m, 1H), 6.15–6.29 (m, 2H), 9.78 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 33.9, 53.8, 54.3, 55.8, 65.2, 93.7, 99.6, 113.2, 115.2, 115.5, 119.4, 141.1, 156.7, 157.9, 163.3, 168.7, 171.0; MS (ESI): 397 $[M+Na]^+$; HRMS (ESI) calcd for $C_{18}H_{16}N_3O_5FNa$ $[M+Na]^+$ 396.0971; found: 396.0961.

5.5. 10-(3,4,5-Trimethoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrrolo[2,3-*f*]quinolin-9-one (11e)

This compound **11e** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (102 mg, 1.02 mmol) with 3,4,5-trimethoxybenzaldehyde (200 mg, 1.02 mmol) and 1*H*-indol-5-amine (134 mg, 1.02 mmol) to afford pure compound **11e** as a brown solid in 386 mg, 96% yield. Mp: 299–301 °C, ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.61 (s, 3H), 3.69 (s, 6H), 4.83–5.06 (dd, 2H, J = 15.1, 15.1 Hz), 5.29 (s, 1H), 6.39 (br s,1H), 6.58 (s, 2H), 6.87 (d, 1H, J = 9.0 Hz), 7.25 (t, 1H, J = 3.0, 2.2 Hz), 7.29 (d, 1H, J = 9.0 Hz), 9.92 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 55.7, 55.9, 59.7, 64.9, 94.7, 105.2, 109.9, 113.2, 117.3, 122.1, 129.2, 135.9, 141.9, 152.5, 157.7, 172.1. MS (ESI): 393 $[M+H]^+$. HRMS (ESI) calcd for $C_{22}H_{20}N_2O_5Na$ $[M+Na]^+$ 415.1269; found: 415.1257.

5.6. 10-(4-Hydroxy-3-methoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrrolo[2,3-*f*]quinolin-9-one (11f)

This compound **11f** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (127 mg, 1.27 mmol) with 4-hydroxy-3-methoxybenzaldehyde (200 mg, 1.27 mmol) and 1*H*-indol-5-amine (168 mg, 1.27 mmol) to afford pure compound **11f** as a broun colour solid in 440 mg, 96% yield. Mp: 301–303 °C, ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.77 (s, 3H), 4.52– 5.03 (dd, 2H, J = 16.0, 15.3 Hz), 4,52 (s, 1H), 5.31 (s, 1H), 6.06 (d, 1H, J = 8.7 Hz), 6.40–6.49 (m, 2H), 6.56 (d, 1H, J = 8.7 Hz), 6.80–6.99 (dd, 1H, J = 2.1, 2.1 Hz), 6.79 (t, 1H, J = 2.1 Hz), 9.12 (s, 1H), 10.21(s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 56.0, 64.7, 68.8, 94.9, 100.1, 104.9, 110.8, 112.3, 114.9, 120.1, 122.2, 124.4, 125.2, 127.0, 132.7, 137.8, 144.5, 146.8, 157.4, 172.4; MS (ESI): 349 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{16}N_2O_4Na$ $[M+Na]^+$ 371.1007; found: 371.0995.

5.7. 10-(3-Hydroxy-4-methoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrrolo[2,3-*f*]quinolin-9-one (11g)

This compound **11g** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (127 mg, 1.27 mmol) with 3-hydroxy-4-methoxybenzaldehyde (200 mg, 1.27 mmol) and 1*H*-indol-5-amine (168 mg, 1.27 mmol) to afford pure compound **11g** as a broun colour solid in 447 mg, 97% yield. Mp: 297–299 °C, ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.77 (s, 3H), 4.52– 5.03 (dd, 2H, J = 16.0, 15.3 Hz), 4,52 (s, 1H), 5.31 (s, 1H), 6.06 (d, 1H, J = 8.7 Hz), 6.40– 6.49 (m, 2H), 6.56 (d, 1H, J = 8.7 Hz), 6.80–6.99 (dd, 1H, J = 2.1, 2.1 Hz), 6.79 (t, 1H, J = 2.1 Hz), 9.12 (s, 1H), 10.21 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 37.8, 55.5, 64.7, 94.8, 99.9, 110.9, 111.2, 111.5, 113.8, 115.3, 118.3, 125.2, 127.0, 128.6, 132.7, 139.4,

145.7, 145.9, 157.3, 172.3; MS (ESI): 349 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{16}N_2O_4Na$ $[M+Na]^+$ 371.1007; found: 371.0995.

5.8. 10-(4-Fluoro-3-methoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrrolo[2,3-*f*]quinolin-9-one (11h)

This compound **11h** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (88 mg, 0,88 mmol) with 4-fluoro-3-methoxybenzaldehyde (200 mg, 0.88 mmol) and 1*H*-indol-5-amine (116 mg, 0.88 mmol) to afford pure compound **11h** as a white solid in 441 mg, 97% yield. Mp: 170–172 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.76 (s, 3H), 4.71–4.87 (dd, 2H, J = 15.2, 15.2 Hz), 5.27 (s, 1H), 6.11 (br s, 1H), 6.51–6.56 (m, 1H), 6.72–6.77 (dd, 1H, J = 2.6, 2.6 Hz), 6.84–6.89 (m, 1H), 7.07–7.09 (m, 2H), 7.20 (d, 1H, J = 8.9 Hz), 9.78 (s, 1H), 10.92 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 55.7, 55.9, 64.8, 94.3, 100.0, 111.2, 113.1, 115.1, 115.3, 119.9, 125.5, 126.9, 128.6, 132.7, 143.2, 146.1, 146.3, 148.1, 151.3, 157.7, 172.3; MS (ESI): 351 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{15}N_2O_3FNa$ $[M+Na]^+$ 373.0964; found: 373.0950.

5.9. 10-(4-Methoxy-3-nitrophenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrrolo[2,3-*f*]quinolin-9-one (11i)

This compound **11i** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (110 mg, 1.10 mmol) with 4-methoxy-3-nitrobenzaldehyde (200 mg, 1.10 mmol) and 1*H*-indol-5-amine (145 mg, 1.10 mmol) to afford pure compound **11i** as a yellow solid in 400 mg, 96% yield. Mp: 280–281 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.08 (s, 3H), 4.03–4.14 (dd, 2H, J = 16.1, 16.1 Hz), 4.62 (s, 1H), 5.34 (s, 1H), 6.05 (d, 1H, J = 8.7 Hz), 6.36–6.39 (m, 2H), 6.49 (d, 1H, J = 8.0 Hz), 6.72–6.74 (dd, 1H, J = 2.1, 1.4 Hz), 6.83 (d, 1H, J = 2.1 Hz), 9.12 (s, 1H), 10.23 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 37.2, 56.4, 65.0, 93.7, 99.7, 111.3, 111.5, 112.3, 113.8, 123.5, 125.7, 126.7, 128.7, 132.7, 133.5, 138.6, 138.8, 150.2, 157.7, 172.2; MS (ESI): 429 $[M+Na]^+$; HRMS (ESI) calcd for $C_{20}H_{15}N_3O_5Na$ $[M+Na]^+$ 400.0909; found: 400.0911.

5.10. 2-Methyl-10-(3,4,5-trimethoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrrolo[2,3-*f*]quinolin-9-one (11j)

This compound **11j** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (102 mg, 1.02 mmol) with 3,4,5-trimethoxybenzaldehyde (200 mg, 1.02 mmol) and 2-methyl-1*H*-indol-5-amine (193 mg, 1.02 mmol) to afford pure compound **11j** as a broun solid in 409 mg, 98% yield. Mp: 280–281 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.27 (s, 3H), 3.55 (s, 3H), 3.63 (s, 6H), 4.77–4.94 (dd, 2H, J = 15.2, 15.2 Hz), 5.13 (s, 1H), 6.00 (br s, 1H), 6.48 (s, 2H), 6.69 (d, 1H, J = 8.9 Hz), 7.13 (d, 1H, J = 8.0 Hz), 9.83 (s, 1H), 10.85 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.3, 38.5, 55.7, 59.7, 64.8, 94.4, 98.2, 105.2, 109.9, 110.1, 112.4, 128.0, 128.6, 132.8, 135.6, 135.7, 142.2, 152.3, 157.7, 172.3; MS (ESI): 401 $[M+Na]^+$; HRMS (ESI) calcd for $C_{20}H_{15}N_3O_5Na$ $[M+Na]^+$ 400.0909; found: 400.0911.

5.11. 10-(4-Fluoro-3-methoxyphenyl)-2-methyl-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrrolo[2,3-*f*]quinolin-9-one (11k)

This compound **11k** was prepared following the method described for the preparation of the compound **11a,** employing tetronic acid (129 mg, 1.29 mmol) with 4-fluoro-3-methoxybenzaldehyde (200 mg, 1.29 mmol) and 2-methyl-1*H*-indol-5-amine (246 mg, 1.29 mmol) to afford pure compound **11k** as a white solid in 461 mg, 97% yield. Mp: 198–199 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.80 (s, 3H, $-CH_3$), 4.30 (s, 3H), 5.22–5.32 (dd, 2H, J = 15.5, 15.5 Hz), 5.73 (s, 1H), 6.27 (s, 1H), 7.04–7.07 (m, 1H), 7.16 (d, 1H, J = 8.6 Hz), 7.32–7.37 (dd, 1H, J = 7.7, 7.7 Hz), 7.55 (d, 1H, J = 6.9 Hz), 7.61 (d, 1H, J = 8.6 Hz), 10.13 (s, 1H), 11.13 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.2, 18.4, 38.0, 55.8, 64.7, 94.2, 98.0, 110.0, 112.1, 113.4, 115.2, 119.8, 127.9, 128.6, 132.8, 135.8, 143.1, 146.2, 157.5, 172.3; MS (ESI): 365 $[M+H]^+$; HRMS (ESI) calcd for $C_{21}H_{17}N_2O_3FNa$ $[M+Na]^+$ 387.1120; found: 387.1115.

5.12. 10-(2-Fluoro-4-methoxyphenyl)-2-methyl-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrrolo[2,3-*f*]quinolin-9-one (11l)

This compound **11l** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (129 mg, 1.29 mmol) with 2-fluoro-4-methoxybenzaldehyde (200 mg, 1.29 mmol) and 2-methyl-1*H*-indol-5-amine (246 mg, 1.29 mmol) to afford pure compound **11l** as a white solid in 469 mg, 99% yield. Mp: 205–207 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.80 (s, 3H), 4.30 (s, 3H), 5.22–5.32 (dd, 2H, J = 15.5, 15.5 Hz), 5.73 (s, 1H), 6.27 (s, 1H), 7.04–7.07 (m, 3H), 7.16 (d, 1H, J = 8.6 Hz), 7.61 (d, 1H, J = 8.6 Hz), 10.13 (s, 1H), 11.13 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.2, 18.4, 38.0, 55.8, 64.7, 94.2, 98.0, 110.0, 112.1, 113.4, 115.2, 119.8, 127.9, 128.6, 132.8, 135.8, 143.1, 146.2, 157.5, 172.3; MS (ESI): 365 $[M+H]^+$; HRMS (ESI) calcd for $C_{21}H_{17}N_2O_3FNa$ $[M+Na]^+$ 387.1120; found: 387.1134.

5.13. 10-(4-Hydroxy-3-methoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrazolo[3,4-*f*]quinolin-9-one (11m)

This compound **11m** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (127 mg, 1.27 mmol) with 4-hydroxy-3-methoxybenzaldehyde (200 mg, 1.27 mmol) and 1*H*-indazol-5-amine (168 mg, 1.27 mmol) to afford pure compound **11m** as a broun solid in 439 mg, 95% yield. Mp: 238–240 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.73 (s, 3H), 4.77– 4.89 (dd, 2H, J = 14.7, 15.6 Hz), 5.27 (s, 1H), 6.49–6.58 (m, 2H), 6.91 (s, 1H), 7.02 (d, 1H, J = 8.7 Hz), 7.34 (d, 1H, J = 8.7 Hz), 7.72 (s, 1H), 9.89 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 37.8, 55.6, 64.9, 95.1, 109.8, 112.3, 113.9, 115.2, 117.3, 120.2, 122.2, 129.2, 132.2, 137.3, 137.5, 144.8, 147.0, 157.5, 172.3; MS (ESI): 350 $[M+H]^+$; HRMS (ESI) calcd for $C_{19}H_{15}N_3O_4Na$ $[M+Na]^+$ 372.0960; found: 372.0954.

5.14. 10-(3-Hydroxy-4-methoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrazolo[3,4-*f*]quinolin-9-one (11n)

This compound **11n** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (127 mg, 1.27 mmol) with 3-hydroxy-4-methoxybenzaldehyde (200 mg, 1.27 mmol) and 1*H*-indazol-5-amine (168 mg, 1.27 mmol) to afford pure compound **11n** as a white solid in 442 mg, 96% yield. Mp: 243–245 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.73 (s, 3H), 4.77– 4.89 (dd, 2H, J = 14.7, 15.6 Hz), 5.27 (s, 1H), 6.49– 6.58 (m, 2H), 6.91 (s, 1H), 7.02 (s, 1H, J = 8.7 Hz), 7.34 (d, 1H, J = 8.7 Hz), 7.72 (s, 1H), 9.89 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 37.7, 55.5, 64.9, 95.0, 109.8, 111.6, 113.8, 115.3, 117.3, 118.4, 122.2, 129.2, 132.1, 137.3, 139.0, 146.1, 146.1, 157.4, 172.2; MS (ESI): 350 $[M+H]^+$; HRMS (ESI) calcd for $C_{19}H_{15}N_3O_4Na$ $[M+Na]^+$ 372.0960; found: 372.0954.

5.15. 10-(4-Fluoro-3-methoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]pyrazolo[3,4-*f*]quinolin-9-one (11o)

This compound **11o** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (88 mg, 0.88 mmol) with 4-fluoro-3-methoxybenzaldehyde (200 mg, 0.88 mmol) and 1*H*-indazol-5-amine (116 mg,

0.88 mmol) to afford pure compound **11o** as a white solid in 433 mg, 95% yield. Mp: 307–309 °C, ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.79 (s, 3H), 4.85–5.01 (dd, 2H, J = 15.6, 15.6 Hz), 5.42 (s, 1H), 6.64–6.69 (m, 1H), 6.97–7.08 (m, 2H), 7.22–7.26 (dd, 1H, J = 1.8, 1.8 Hz), 7.39 (d, 1H, J = 8.8 Hz), 7.87 (s, 1H), 10.01 (s,1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 42.8, 60.8, 69.9, 99.5, 118.0, 118.4, 120.3, 120.5, 122.3, 124.9, 127.0, 134.2, 147.8, 147.9, 151.2, 153.6, 156.0, 162.7, 177.1; MS (ESI): 352 $[M+H]^+$; HRMS (ESI) calcd for $C_{19}H_{14}N_3O_3FNa$ $[M+Na]^+$ 374.0916; found: 374.0900.

5.16. 10-(3,4,5-Trimethoxyphenyl)-6,7,9,10-tetrahydrofuro[3,4-*b*][1,3]thiazolo[5,4-*f*]quinolin-9-one (11p)

This compound **11p** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (102 mg, 1.02 mmol) with 3,4,5-trimethoxybenzaldehyde (200 mg, 1.02 mmol) and benzo[*d*]thiazol-5-amine (153 mg, 1.02 mmol) to afford pure compound **11p** as a broun solid in 393 mg, 94% yield. Mp: 309–310 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.59 (s, 3H), 3.67 (s, 6H), 4.86–5.00 (dd, 2H, J = 16.1, 16.1 Hz), 5.09 (s, 1H), 6.48 (s, 2H), 7.22 (d, 1H, J = 8.7 Hz), 7.92 (d, 1H, J = 8.7 Hz), 9.06 (s, 1H), 10.33 (s, 1H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 40.9, 55.7, 59.8, 64.9, 95.5, 105.9, 116.1, 116.2, 122.4, 134.0, 135.1, 136.2, 138.9, 149.4, 152.5, 153.7, 157.2, 171.6; MS (ESI): 411 $[M+H]^+$; HRMS (ESI) calcd for $C_{21}H_{19}N_2O_5$ $[M]^+$ 411.1014; found: 411.1021.

5.17. 10-(3-Methoxy-4-nitrophenyl)-2-methyl-6,7,9,10-tetrahydrofuro[3,4-*b*][1,3]thiazolo[5,4-*f*]quinolin-9-one (11q)

This compound **11q** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (110 mg, 1.10 mmol) with 3-methoxy-4-nitrobenzaldehyde (200 mg, 1.10 mmol) and 2-methyl-benzo[*d*]thiazol-5-amine (182 mg, 1.10 mmol) to afford pure compound **11q** as a yellow solid in 430 mg, 95% yield. Mp: 190–191 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.71 (s, 3H), 3.83 (s, 3H), 4.80–4.96 (dd, 2H, J = 15.5, 16.4 Hz), 5.64 (s, 1H), 7.00–7.08 (m, 2H), 7.49–7.51 (dd, 1H, J = 2.5, 2.5 Hz), 7.66–7.71 (m, 2H), 8.08 (s, 1H), 10.11 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 19.9, 35.9, 55.9, 56.4, 65.1, 95.2, 113.8, 114.4, 115.1, 121.3, 123.7, 129.6, 133.4, 134.9, 138.4, 150.4, 158.3, 167.7, 171.7; MS (ESI): 410 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{15}N_3O_5NaS$ $[M+Na]^+$ 432.0630; found: 432.0610.

5.18. 10-(3-Hydroxy-4-methoxyphenyl)-2-sulfanyl-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*]imidazo[4,5-*f*]quinolin-9-one (11r)

This compound **11r** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (127 mg, 1.27 mmol) with 3-hydroxy-4-methoxybenzaldehyde (200 mg, 1.27 mmol) and 5-amine-1*H*-benzo[*d*]imidazole-2-thiol (210 mg, 1.27 mmol) to afford pure compound **11r** as a broun solid in 452 mg, 90% yield. Mp: 334–337 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.00 (s, 3H), 7.07–7.17 (m, 4H), 7.49 (d, 1H, J = 8.9 Hz), 8.19–8.22 (dd, 1H, J = 2.2, 2.2 Hz), 8.40 (d, 1H, J = 2.2 Hz), 8.69 (s, 1H), 12.58 (s, 1H), 12.63 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 55.6, 69.0, 103.9, 105.2, 111.3, 111.5, 117.1, 121.1, 122.8, 124.5, 133.5, 137.5, 145.9, 147.5, 148.3, 149.2, 162.5, 167.8, 173.5; MS (ESI): 381 $[M+H]^+$; HRMS (ESI) calcd for $C_{19}H_{13}N_3O_4NaS$ $[M+Na]^+$ 402.0524; found: 402.0537.

5.19. 10-(3,4,5-Trimethoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*][1,2,3]triazolo[4,5-*f*]quinolin-9-one (11s)

This compound **11s** was prepared following the method described for the preparation of the compound **11a,** employing tetronic acid (102 mg, 1.02 mmol) with 3,4,5-trimethoxybenzaldehyde (200 mg, 1.02 mmol) and 1*H*-benzo[*d*][1,2,3]triazol-5-amine (136 mg, 1.02 mmol) to afford pure compound **11s** as a white solid in 378 mg, 94% yield. Mp: 324–325 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.56 (s, 3H), 3.66 (s, 6H), 4.88–5.14 (dd, 2H, J = 15.6, 15.6 Hz), 5.27 (s, 1H), 6.59 (s, 2H), 7.12 (d, 1H, J = 8.5 Hz), 7.87 (d, 1H, J = 8.5 Hz), 10.41 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 36.7, 55.7, 59.7, 65.1, 96.0, 104.8, 115.2, 118.3, 132.6, 135.3, 136.1, 140.2, 141.6, 152.6, 158.0, 171.8; MS (ESI): 395 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{18}N_4O_5Na$ $[M+Na]^+$ 417.1174; found: 417.1176.

5.20. 10-(2-Fluoro-4-methoxyphenyl)-6,7,9,10-tetrahydro-1*H*-furo[3,4-*b*][1,2,3]triazolo[4,5-*f*]quinolin-9-one (11t)

This compound **11t** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (88 mg, 0.88 mmol) with 2-fluoro-4-methoxybenzaldehyde (200 mg, 0.88 mmol) and 1*H*-benzo[*d*][1,2,3]triazol-5-amine (118 mg, 0.88 mmol) to afford pure compound **11t** as a white solid in 439 mg, 96% yield. Mp: 308–310 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.68 (s, 3H), 4.85–4.95 (dd, 2H, J = 15.6, 15.6 Hz), 5.06 (s, 1H), 6.55–6.61 (m, 2H), 7.08 (d, 1H, J = 8.7 Hz), 7.20 (br s, 1H), 7.69–7.71 (m, 1H), 10.29 (s, 1H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 29.8, 55.3, 65.0, 94.6, 101.5, 101.8, 109.8, 111.6, 115.0, 118.2, 130.6, 130.9, 158.9, 159.3, 171.6; MS (ESI): 353 $[M+H]^+$; HRMS (ESI) calcd for $C_{18}H_{13}N_4O_3FNa$ $[M+Na]^+$ 375.0869; found: 375.0870.

5.21. 3-(4-Methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-4,5,7,8-tetrahydrofuro[3',4':5,6]pyrido[3,2-*d*]isoxazol-5-one (11u)

This compound **11u** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (102 mg, 1.02 mmol) with 3,4,5-trimethoxybenzaldehyde (200 mg, 1.02 mmol) and 3-(4-methoxyphenyl)isoxazol-5-amine (193 mg, 1.02 mmol) to afford pure compound **11u** as a white solid in 436 mg, 94% yield. Mp: 218–219 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.54 (s, 3H), 3.59 (s, 6H), 3.75 (s, 3H), 4.89–5.04 (dd, 2H, J = 16.4, 16.4 Hz), 5.23 (s, 1H), 6.40 (s, 2H), 6.94 (d, 2H, J = 8.6 Hz), 7.52 (d, 2H, J = 8.6 Hz), 11.53 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 35.1, 55.6, 59.7, 64.9, 94.1, 100.8, 105.1, 127.2, 128.6, 129.3, 134.6, 136.0, 138.9, 152.3, 157.4, 159.7, 161.8, 170.7; MS (ESI): 451 $[M+H]^+$; HRMS (ESI) calcd for $C_{24}H_{22}N_2O_7Na$ $[M+Na]^+$ 473.1324; found: 473.1330.

5.22. 3-(4-Chlorophenyl)-4-(3-hydroxy-4-methoxyphenyl)-4,5,7,8-tetrahydrofuro[3',4':5,6]pyrido[3,2-*d*]isoxazol-5-one (11v)

This compound **11v** was prepared following the method described for the preparation of the compound **11a,** employing tetronic acid (127 mg, 1.27 mmol) with 3-hydroxy-4-methoxybenzaldehyde (200 mg, 1.27 mmol) and 3-(4-chlorophenyl) isoxazol-5-amine (246 mg, 1.27 mmol) to afford pure compound **11v** as a pale yellow solid in 459 mg, 85% yield. Mp: 245–246 °C, ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.72 (s, 3H), 4.67–4.79 (dd, 2H, J = 16.6, 16.2 Hz), 4.92 (s, 1H), 6.55– 6.64 (m, 3H), 7.24 (d, 2H, J = 8.4 Hz), 7.39 (d, 2H, J = 8.4 Hz), 8.32 (s, 1H), 11.16 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 34.4, 55.4, 64.6, 94.1, 101.7, 111.4, 115.1, 118.4, 127.0, 128.3, 128.6, 129.0, 134.6, 136.1, 146.0, 146.3, 156.3, 159.4, 162.2, 167.1, 170.7; MS (ESI): 433 $[M+Na]^+$; HRMS (ESI) calcd for $C_{21}H_{15}N_2O_5NaCl$ $[M+Na]^+$ 433.0567; found: 433.0554.

5.23. 9-(4-Hydroxy-3-methoxyphenyl)-5,6,7-trimethoxy-1,3,4,9-tetrahydrofuro[3,4-*b*]quinolin-1-one (11w)

This compound **11w** was prepared following the method described for the preparation of the compound **11a**, employing tetronic acid (127 mg, 1.27 mmol) with 4-hydroxy-3-methoxybenzaldehyde (200 mg, 1.27 mmol) and 3,4,5-trimethoxybenzenamine (232 mg, 1.27 mmol) to afford pure compound **11w** as a yellow solid in 447 mg, 85% yield. Mp: 290–291 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.38 (s, 3H), 3.63 (s, 3H), 3.70 (s, 3H), 3.78 (s, 3H), 4.75– 4.91 (m, 3H), 6.37–6.41(m, 2H), 6.60 (d, 1H, J = 7.5 Hz), 6.76 (d, 1H, J = 1.5 Hz), 8.72 (s, 1H), 9.86 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 34.7, 55.6, 59.9, 60.2, 64.8, 95.4, 96.2, 110.4, 112.0, 115.0, 119.7, 133.0, 137.4, 138.2, 144.6, 146.8, 151.7, 152.6, 157.4, 172.1; MS (ESI): 400 $[M+H]^+$.

6. Procedure of the SRB-assay

The synthesized compounds (**11a–w**) 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 mL 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 mL of the drug dilutions were added to the appropriate microtiter wells already containing 90 mL of cells, sulting in the required final drug concentrations. For each compound four concentrations (0.1, 1, 10 and 100 μM) were evaluated and each was done in triplicate wells. Plates were incubated further for 48 h and assay was terminated by the addition of 50 mL of cold trichloro acetic acid (TCA) (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The plates were washed five times with tap water and airdried. 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 airdried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm with 690 nm reference wavelengths. Percent growth was calculated on a plate by plate basis for test wells relative to control wells. The above determinations were repeated three times. Percentage growth was expressed as the (ratio of average absorbance of the test well to the average absorbance of the control wells) × 100. Growth inhibition of 50% (GI50) was calculated from $[(\mathrm{Ti}-\mathrm{Tz})/(\mathrm{C}-\mathrm{Tz})]\times 100\frac{1}{4}50$, 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 1/4 Optical density at time zero, OD of control 1/4 C, and OD of test growth in the presence of drug 1/4 Ti.[26]

7. Tubulin polymerization assay

A fluorescence based in vitro tubulin polymerization assay was performed according to the manufacturer's protocol (BK011, Cytoskeleton, Inc.). Briefly, the reaction mixture in a total volume of 10 μL contained PEM buffer, GTP (1 mM) in the presence or absence of test compounds **11a** to **11w** (final concentration of 3 μM).Tubulin polymerization was followed by a time dependent increase in fluorescence due to the incorporation of a fluorescence reporter into microtubules as polymerization proceeds. Fluorescence emission at 460 nm (excitation wavelength is 360 nm) was measured by using a Varioscan multimode plate reader (Thermo scientific inc.). Podophyllotoxin was used as positive control in each assay.[27,28]

8. Immunohistochemistry and microscopy

A-549 cells were seeded on glass cover slips, incubated for 48 h in the presence or absence of test compounds **11a**, **11k** and **11p** (5 μM). Following the termination of incubation, cells were fixed with 3% paraformaldehyde, 0.02% glutaraldehyde in PBS and permeabilized by dipping the cells in 100% methanol (–20 °C). Later, cover slips were blocked with 1% BSA in phosphate buffered saline for 1 hr. followed by incubation with a primary anti tubulin (mouse monoclonal) antibody and FITC conjugated secondary mouse anti Ig G antibody. Photographs were taken using the confocal microscope, equipped with FITC settings and the pictures were analyzed for the integrity of microtubule network. In parallel experiments, podophyllotoxin (1 μM) is used as positive control.[29]

9. Analysis of cell cycle

Human lung cancer cell line (A-549) in 6 well plates were incubated for 48 hrs in the presence or absence of test compounds **11a**, **11k** and **11p** (10 μM) and podophyllotoxin (1 μM). Cells were harvested with Trypsin-EDTA, fixed with ice-cold 70% ethanol at 4 °C for 30 min, ethanol was removed by centrifugation and cells were stained with 250 μL of DNA staining solution [10 mg of propidium iodide (PI), 0.1 mg of trisodium citrate, and 0.03 mL of Triton X-100 were dissolved in 100 mL of sterile water] at room temperature for 30 min in the dark. The DNA contents of 20,000 events were measured by flow cytometer (BD CANTO II, San Jose, CA). Histograms were analyzed using Summit Software.

10. Caspase assay

A-549 cells were plated in 6-well plates, grown to 60–80% confluence, and treated with either no drug or test compound **11a**, **11k** and **11p** (5 μM). Podophyllotoxin (1 μM) was used as positive control. Following 24 h of incubation, cells were collected by scraping, washed with PBS and briefly centrifuged to collect the pellet. Cells were lysed in 200 μL of 1X lyses buffer by purging at least ten times through an insulin syringe followed by incubation on ice for 10–20 min. The lysate was centrifuged at 13,200 rpm for 20 min at 4 °C and the obtained clear supernatant was used for caspase activity measurements employing AMC-conjugated substrate for caspase-3 as described earlier.

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