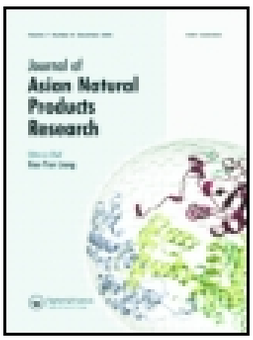

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Synthesis and antiproliferative activity of novel (+)- usnic acid analogues

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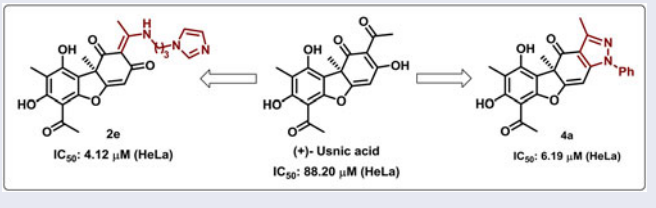
ABSTRACT

Twenty one novel (+)- usnic acid-based analogues belonging to three classes such as enamines, imines, and pyrazoles were synthesized. All the synthesized compounds were characterized by their spectral data (^{1}H NMR, ^{13}C NMR, IR, and HRMS). The synthesized compounds were evaluated for their antiproliferative activity against a panel of four human cancer cell lines including HeLa (cervix), MDA-MB-231 (breast), A549 (lung), and MiaPaca (pancreas) by employing SRB cell proliferation assay. Screening results indicated that all synthesized compounds showed enhanced activity than the parent compound. Most significantly, compounds **2e** and **4a** showed potent antiproliferative activity against all the cancer cell lines tested. Compounds **2e** and **4a** arrested the cell cycle in G2/M phase and induced apoptosis in HeLa cells. In view of significant antiproliferative activity, compounds **2e** and **4a** can be considered as lead molecules for further development.



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Antiproliferative activity; apoptosis; cell cycle arrest; enamine; pyrazole; (+)-usnic acid

1. Introduction

Usnic acid is a dibenzofuran derivative, found uniquely in lichens as secondary metabolite. It is especially abundant in lichen genera such as *Alectoria*, *Cladonia*, *Evernia, Lecanora*, *Ramalina,* and *Usnea*. In addition to its broad spectrum of biological activities such as antimicrobial, antiviral, antiprotozoal, antiproliferative, anti-inflammatory, and analgesic activities, the lichen extracts containing usnic acid have

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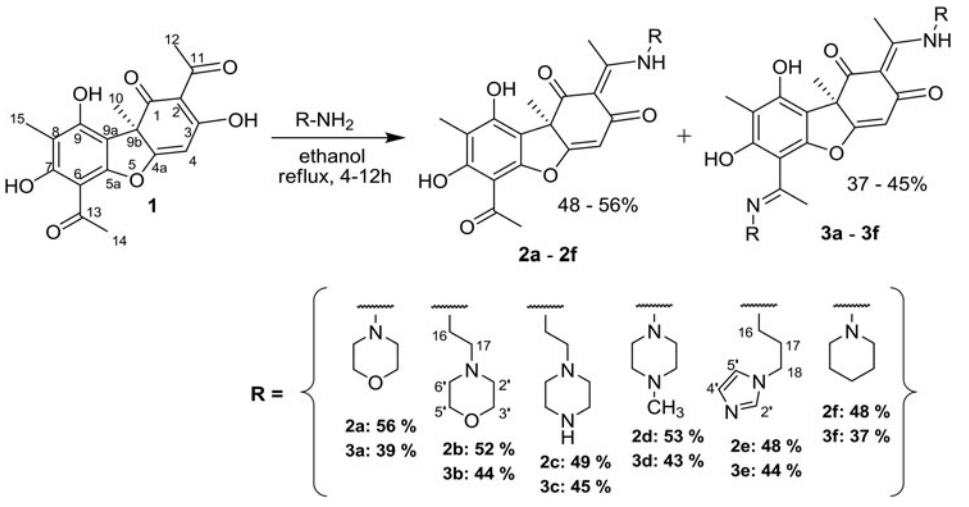
been used as crude drugs throughout the world. As a pure substance, it has been formulated in creams, toothpaste, mouthwash, deodorants, and sunscreen products [1–3]. High natural abundance, functional group diversity, and the broad spectrum of biological activities of usnic acid made it as a good target for chemists and biologists around the world. Till date various types of moieties such as enamines, chalcones, thiazoles, sulphides, aurones, flavones, and coumarins were introduced on ring A and ring C of usnic acid, of which usnic acid enamines showed promising biological activities [4–9]. Many research groups around the world synthesized a large variety of usnic acid enamines with potent biological activities such as antiviral and anticancer activities [5–7, 10–12]. Amines containing pharmacophoric moieties such as morpholines, piperazines, and pyridines were selected and coupled with usnic acid to obtain usnic acid enamines [13]. Pyrazole, a nitrogen-containing five membered heterocyclic ring, is an important pharmacophore in medicinal chemistry. Pyrazole moiety is present in many marketed drugs like celecoxib, antipyrine, phenylbutazone, novalgine, ramifenazone, apixaban, fipronil, rimonabant, and pyrazofurin. Pyrazole derivatives exhibit diverse biological activities such as anticancer, antimicrobial, analgesic, anti-inflammatory, antihypertensive, antiepileptic, and antidepressant [14]. Phenylhydrazines containing different electron releasing and withdrawing groups were selected and coupled with usnic acid to obtain usnic acid-based pyrazoles. Based on above facts, we have now synthesized some usnic acid-based enamines and pyrazoles and evaluated for their antiproliferative activity against four cancer cell lines.

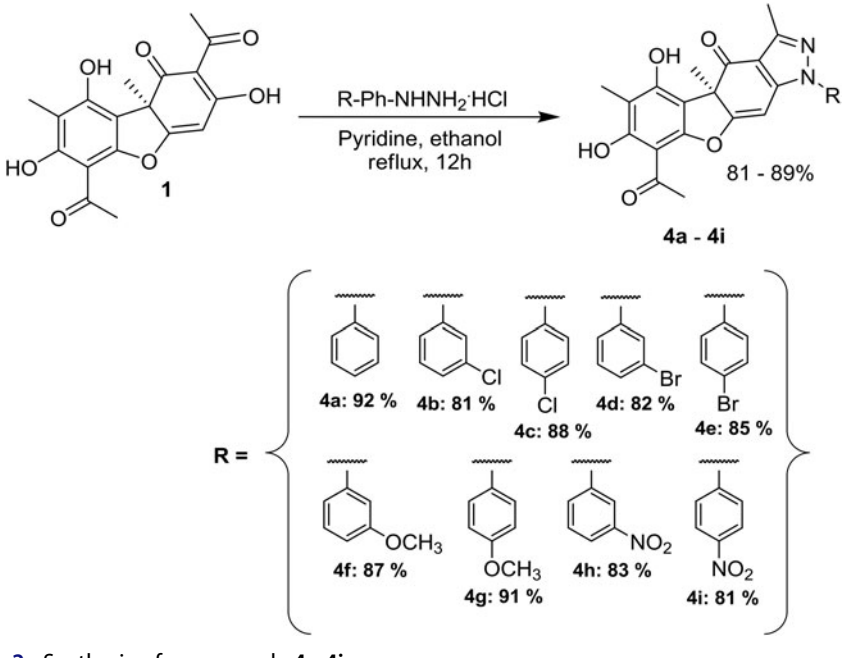
2. Results and discussion

2.1. Chemistry

(+)- Usnic acid (**1**) was isolated from the lichen *Usnea longissima* in 1% yield according to our reported procedure [15]. The protocols adopted for the synthesis of novel (+)- usnic acid-based enamines and imines are presented in Scheme 1. (+)- Usnic acid (1 eq.) was refluxed with appropriate alkylamines containing different heterocyclic rings (2.2 eq.) in ethanol to afford the enamines (**2a-2f**) in 48–56% yields and imines **3a-3f** in 37–45% yields [11, 16, 17]. Compounds **2a-2f** and **3a-3f** were thoroughly characterized by their spectral data (^{1}H & ^{13}C NMR, IR, and HRMS). In ^{1}H NMR spectra of compounds **2a-2f**, a three proton singlet appeared between δ 2.60 and 2.68 corresponding to C-12 methyl (enamine methyl) and in ^{13}C NMR spectra, the carbon signal appeared between δ 188.2 and 190.4 corresponding to C-3 carbonyl. In ^{1}H NMR spectra of compounds **3a-3f**, a three proton singlet appeared between δ 2.70 and 2.85 corresponding to C-14 methyl (imine methyl). The protocols adopted for the synthesis of (+)- usnic acid-based pyrazoles (**4a-4i**) are presented in Scheme 2. They were synthesized by following the previously reported procedure [18]. (+)- Usnic acid was refluxed with appropriate phenylhydrazines and pyridine in ethanol to get the (+)- usnic acid pyrazoles **4a-4i** in 81–92% yields. The synthesized pyrazoles were thoroughly characterized by their physical and spectral data and by comparing with the literature values [18]. In ^{13}C NMR spectra, the characteristic pyrazole ring carbon (carbon adjacent to sp^{3} nitrogen) appeared between δ 151.3 and 152.4.



Scheme 1. Synthesis of compounds **2a**-**2f** and **3a**-**3f.**



Scheme 2. Synthesis of compounds **4a**-**4i.**

2.2. Biological evaluation

2.2.1. In vitro cytotoxicity

The synthesized compounds **2a-2f**, **3a-3f** (enamines), and **4a-4i** (pyrazoles) were evaluated for *in vitro* cytotoxicity against a panel of four human cancer cell lines such as HeLa (cervix), MDA-MB-231 (breast), A549 (lung), and MIAPaCa (pancreas) by employing sulforhodamine B (SRB) cell proliferation assay [19]. Nocodazole was used as a positive control. The results are summarized in Table 1. The compounds were treated in

Table 1. IC_{50} values of synthesized compounds.

	IC_{50} (μM)			
Compound	HeLa	MDA MB-231	A549	MiaPaca
1	88.20 ± 0.31	79.62 ± 0.15	65.76 ± 0.82	61.49 ± 0.52
2a	22.71 ± 0.77	25.61 ± 0.29	26.64 ± 0.69	19.11 ± 0.35
2b	19.37 ± 0.91	27.37 ± 0.43	24.59 ± 0.98	40.73 ± 0.46
2c	30.51 ± 0.28	26.73 ± 0.46	27.52 ± 0.81	21.64 ± 0.78
2d	36.24 ± 0.46	30.56 ± 0.81	24.31 ± 0.27	30.62 ± 0.45
2e	**4.12 ± 0.31**	**3.90 ± 0.17**	**5.99 ± 0.27**	**4.93 ± 0.51**
2f	40.43 ± 0.48	29.73 ± 0.98	33.13 ± 0.78	26.53 ± 0.18
3a	28.56 ± 0.91	42.64 ± 0.67	49.98 ± 0.92	39.51 ± 0.61
3b	49.43 ± 0.34	70.67 ± 0.54	86.31 ± 0.28	69.49 ± 0.99
3c	46.10 ± 0.43	52.64 ± 0.32	41.10 ± 0.22	60.53 ± 0.96
3d	50.14 ± 0.32	45.31 ± 0.46	39.17 ± 0.23	40.4 ± 0.36
3e	33.31 ± 0.41	29.34 ± 0.41	35.0 ± 0.74	16.2 ± 0.38
3f	35.27 ± 0.87	39.11 ± 0.34	34.18 ± 0.29	29.94 ± 0.68
4a	**6.19 ± 0.76**	**5.90 ± 0.03**	**6.85 ± 0.94**	**7.39 ± 0.47**
4b	83.21 ± 0.57	61.71 ± 0.41	75.31 ± 0.5	58.21 ± 0.28
4c	41.28 ± 0.97	43.42 ± 0.29	58.12 ± 0.55	66.91 ± 0.25
4d	40.9 ± 0.21	51.41 ± 0.29	62.64 ± 0.62	55.91 ± 0.26
4e	43.64 ± 0.59	68.43 ± 0.56	41.34 ± 0.64	50.21 ± 0.33
4f	25.28 ± 0.95	34.63 ± 0.46	40.19 ± 0.27	24.52 ± 0.28
4g	28.34 ± 0.53	39.48 ± 0.96	30.27 ± 0.82	36.34 ± 0.51
4h	35.34 ± 0.61	38.81 ± 0.22	40.31 ± 0.24	37.46 ± 0.88
4i	43.14 ± 0.42	36.21 ± 0.31	40.2 ± 0.51	30.34 ± 0.59
Nocodazole	0.89 ± 0.99	0.91 ± 0.28	0.97 ± 0.84	0.95 ± 0.26

5-doses according to the NCI-60 protocol. Subsequently, after 48 h of treatment, the absorbance of the SRB was monitored and based on it IC_{50} values were determined by interpolation after plotting percent growth versus compounds concentrations. In addition, these results also indicate that the compounds bind a common target that this expressed in all the cell lines. Most of the synthesized compounds showed better antiproliferative activity than the parent compound. Among (+)- usnic acid enamines (**2a-2f** and **3a-3f)**, compound with imidazole substituent (**2e**) showed potent antiproliferative activity against all tested cell lines with IC_{50} values: 4.12 ± 0.31 μM (HeLa), 3.90 ± 0.17 μM (MDA-MB-231), 5.99 ± 0.27 μM (A549), and 4.93 ± 0.51 μM (MIAPaCa). Compounds **2a**, **2b**, **2c**, **2d**, and **2f** showed moderate IC_{50} values (19.37 ± 0.91 ~ 40.73 ± 0.46 μM) against all the tested cancer cell lines. From the close analysis of IC_{50} values (Table 1), it is evident that compounds with monosubstitution (**2a-2f**) showed better cytotoxicity than that of compounds with disubstitution (**3a-3b**) against all the tested cell cancer lines.

Among (+)- usnic acid pyrazoles (**4a-4i**), compound **4a** showed potent antiproliferative activity against all the tested cell lines with IC_{50} values: 6.19 ± 0.76 μM (HeLa), 5.90 ± 0.03 μM (MDA-MB-231), 6.85 ± 0.94 μM (A549), and 7.39 ± 0.47 μM (MIAPaCa). Compounds with methoxyphenyl and nitrophenyl groups (**4f**, **4g**, **4h**, and **4i)** showed moderate antiproliferative activity against all tested cell lines with IC_{50} values 24.52 ± 0.28–43.14 ± 0.42 μM. Compounds with chlorophenyl and bromophenyl substituents (**4b**, **4c**, **4d**, and **4e**) exhibited enhanced antiproliferative activity (IC_{50}: 40.9 ± 0.21–83.21 ± 0.57 μM) when compared to the parent compound against most of the tested cell lines. From the close analysis of IC_{50} values, it is evident that compounds with methoxyphenyl and nitrophenyl substituents (**4f**, **4g**, **4h**, and **4i)** exhibited better antiproliferative activity than the compounds with chlorophenyl and bromophenyl substituents (**4b**, **4c**, **4d**, and **4e**).

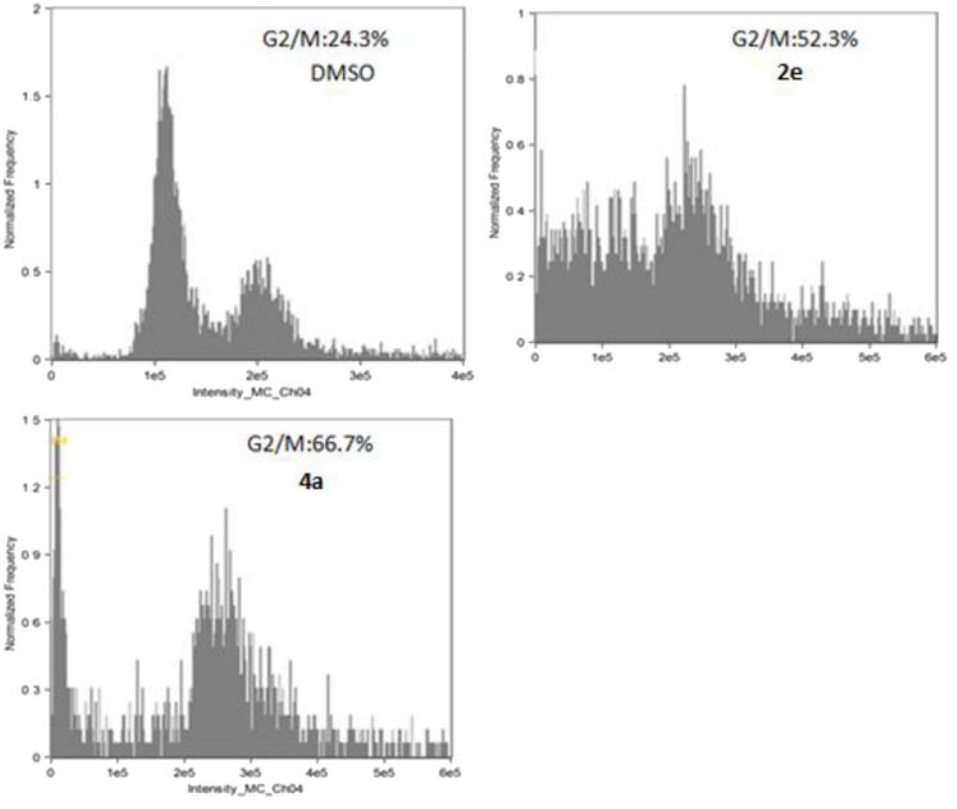


Figure 1. Anti-mitotic effect of 2e and 4a by FACS analysis: Induction of cell cycle G2/M arrest by compounds. HeLa cells were harvested after treatment at 10 μM for 24 h. DMSO treated cells were served as vehicle control, respectively. The percentage of cells in each phase of cell cycle was quantified by flow cytometry.

2.2.2. Cell cycle analysis

The screening results revealed that compounds **2e** and **4a** showed significant antiproliferative activity against human cervical cancer cell line HeLa with IC_{50} values $4.12 \pm 0.31\ \mu$M and $6.19 \pm 0.76\ \mu$M, respectively. To understand whether this cell growth inhibition of human cervical cancer cells was due to cell cycle arrest, we performed a cell cycle analysis study [20]. Compounds **2e** and **4a** were used at 10 μM concentrations for 24 h. Subsequently, the cells were harvested and fixed with ice cold 70% ethanol. The fixed cells were later stained with propidium-iodide and the cell cycle profile was monitored. Notably, **2e** (52.3%) and **4a** (66.7%) treated cells showed an accumulation in G2/M phase (Figure 1). Based on these results it can be concluded that **2e** and **4a** arrest cells in G2/M phase and possibly this arrest contributes to their anti-cancer effects.

2.2.3. Immunofluorescence of tubulin

HeLa cells were arrested in G2/M phase and showed altered morphology, wherein these cells rounded up with prominent disappearance of cytoplasmic staining, as compared to untreated cells. Since the compounds arrested in G2/M phase, we performed immunofluorescence of tubulin protein to understand the phenotype elicited by the compounds [19]. Interestingly, **2e** and **4a** treated cells showed typical G2/M

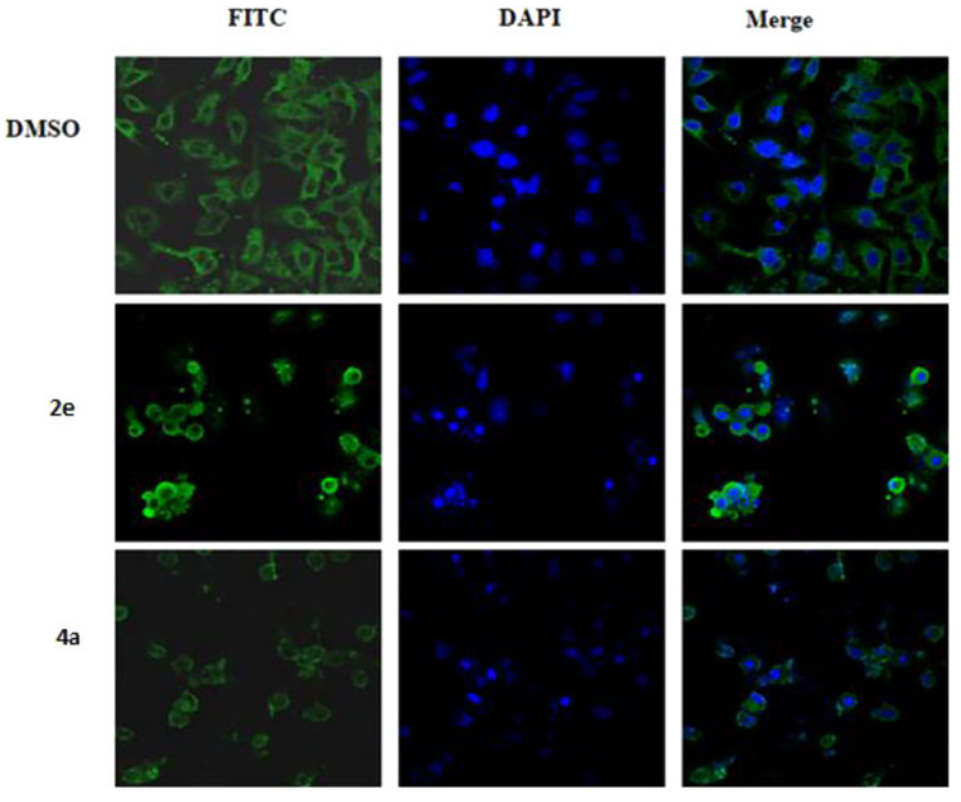


Figure 2. Effect of 2e and 4a on microtubules and nuclear condensation. HeLa cells were independently treated with **2e** and **4a** at 10 μM concentration for 24 h. Following the termination of experiment, cells were fixed, and stained for tubulin. DAPI was used as counter stain. The merged images of cells stained for tubulin and DAPI are represented. Paclitaxel was used as positive control.

arrest phenotype. Moreover, most of the compound treated cells displayed in the field have a centered nuclear staining as evidenced by 4',6-diamidino-2-phenylindole (DAPI). Thus, our results further corroborate with flow cytometry analysis that **2e** and **4a** indeed arrest cells in G2/M phase of cell cycle (Figure 2).

2.2.4. Immunoblot assays

Thus, based on our results that **2e** and **4a** show a G2/M arrest associated with an altered phenotype, we further investigated for the markers of G2/M by immunoblot analysis [19]. Cyclin-B1/Cdk1 is master regulator of mitosis, thus, we elucidated for the protein levels of Cyclin-B1 in **2e** and **4a** treated cells. In addition, nocodazole and paclitaxel were used as positive controls. Notably, **2e** and **4a** showed a robust induction of Cyclin-B1 protein similar to controls. Dimethyl sulfoxide (DMSO) treated cells did not exhibit any induction Cyclin-B1 protein (Figure 3). Further, we analyzed whether **2e** or **4a** target endogenous tubulin. As the results so far support the suggestion, to investigate this, we treated HeLa cells with **2e** or **4a** for 24 h and subsequently analyzed for tubulin protein in soluble and insoluble fractions. **2e** and **4a** markedly increased the soluble fraction to tubulin similar to nocodazole, a microtubule

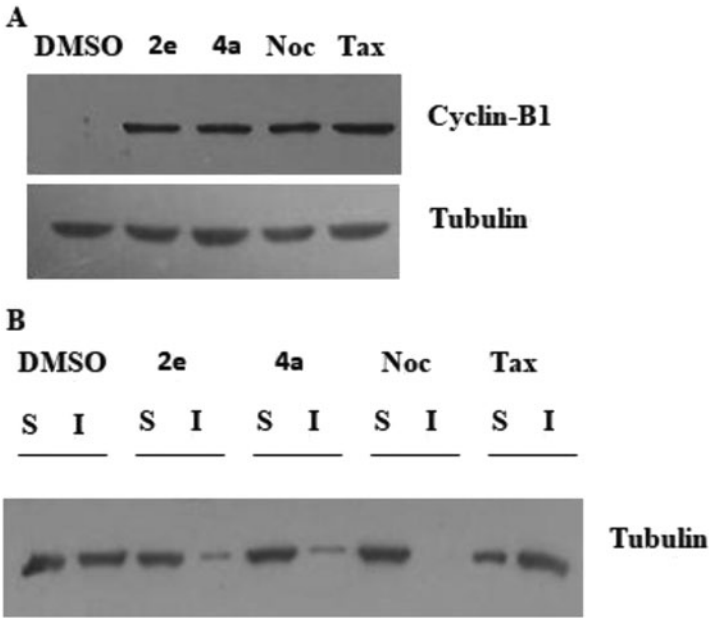


Figure 3. 2e and 4a induce cell cycle arrest. A. HeLa cells were treated with 10 μM concentration of compound (2e, 4a) for 24 h, nocodazole (Noc) and paclitaxel were used as controls. Cyclin-B1 and Tubulin were detected by specific antibodies. B. Distribution of tubulin analyzed by immunobloting in 2e and 4a treated HeLa cells: The cells were treated with 10 μM of 2e, 4a, Combretastatin and Paclitaxel for 24 h. Subsequently, the cells were permeabilized with Hepes-buffer containing triton-x-100 and analyzed for Tubulin protein by Western blotting analysis.

depolymerizing agent. In contrast, paclitaxel increased the polymerized or insoluble faction of tubulin in HeLa cells (Figure 3). Hence, it can be concluded that **2e** and **4a** are microtubule depolymerizing agents which exert their cytotoxicity by potently arresting cells in mitosis followed by apoptosis.

3. Conclusion

In total 21 (+)- usnic acid-based enamines, imines, and pyrazoles were synthesized in very good yields and evaluated for their antiproliferative activity against four cancer cell lines. All the synthesized compounds exhibited enhanced antiproliferative activity when compared to the parent compound. Especially, compounds **2e** and **4a** showed potent antiproliferative activity against all tested cell lines with IC_{50} values 3.90 ± 0.17–7.39 ± 0.47 μM. Compounds **2e** and **4a** arrested the cell cycle in G2/M phase in HeLa cells. It can be concluded that **2e** and **4a** are microtubule depolymerizing agents which exert their cytotoxicity by potently arresting cells in mitosis followed by apoptosis. These two molecules can be considered as lead compounds for further development.

4. Experimental

4.1. Chemistry

All the solvents (RANKEM, India) were distilled before use. All reagents were purchased from commercial sources (Sigma-Aldrich, Alfa Aesar, India) and used as

received. All reactions were monitored by silica gel 60 F_{254} glass TLC plates (Merck, India). Column chromatography was performed on ACME grade 60–120 mesh silica gel. Melting points were determined on a Büchi melting point apparatus and are uncorrected. IR spectra were recorded on Nicollet 740 FTIR spectrophotometer in KBr disks. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 300 MHz and Varian 500 MHz spectrometers using TMS as internal standard. Chemical shifts were reported as δ values in parts per million (ppm) and coupling constants (J) were reported in Hertz (Hz). The following abbreviations were used for NMR signals: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, brs = broad singlet, Ar-H = aryl protons. Mass spectra were obtained on Agilent ESI-QTOF instrument.

4.1.1. Isolation of (+)- usnic acid

(+)- Usnic acid was isolated from the lichen *Usnea longissima* in 1% yield according to our reported procedure [15].

4.1.2. General procedure for the synthesis of compounds 2a-2f and 3a-3f

To a solution of **1** (2.5 g, 0.726 mmol) in ethanol (18 ml) was added appropriate alkylamine (1.597 mmol) and the resulting mixture was refluxed for 4–12 h under stirring [11, 16]. The reaction mixture was then concentrated under reduced pressure and the resulting residue was chromatographed over a column of silica gel to afford pure compounds **2a-2f** and **3a-3f**.

4.1.2.1. (R,E)-6-Acetyl-2-[1-(morpholinoamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (2a). Pale yellow solid (0.174 g, 56%); mp: 93–96 °C; IR (KBr) ν_{max}: 2650–3428, 1698, 1626, 1557, 1458, 1369, 1280, 1187 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$+DMSO-d_6): δ 1.71 (3H, s, H-10), 2.07 (3H, s, H-15), 2.68 (6H, s, H-12, H-14), 2.92 (4H, t, J = 4.7 Hz, H-2', H-6'), 3.85 (4H, t, J = 4.7 Hz, H-3', H-5'), 5.80 (1H, s, H-4), 11.90 (1H, s, 9-OH), 13.36 (1H, s, 7-OH), 13.97 (1H,brs, NH); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6): δ 6.8 (C-15), 16.5 (C-12), 30.5 (C-14), 31.2 (C-10), 55.0 (C-2', C-6'), 56.4 (C-9b), 65.2 (C-3', C-5'),100.0 (C-6), 100.6 (C-4), 101.7 (C-2), 104.3 (C-9a), 107.0 (C-8), 155.1 (C-5a), 157.5 (C-9), 162.6 (C-7), 173.4 (C-11), 173.6 (C-4a), 189.2 (C-3), 197.5 (C-1), 199.9 (C-13); HRESIMS: *m/z* 429.1651 $[M+H]^+$ (cacld for $C_{22}H_{25}N_2O_7$, 429.1656).

4.1.2.2. (R,E)-6-Acetyl-2-[1-(2-morpholinoethylamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (2b). Pale yellow solid (0.172 g, 52%); mp: 83-85 °C; IR (KBr) ν_{max}: 2656–3449, 1699, 1624, 1555, 1460, 1370, 1286, 1188 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.71 (3H, s, H-10), 2.10 (3H, s, H-15), 2.55 (4H, t, J = 4.5 Hz, H-2', H-6'), 2.66–2.73 (8H, m, H-12, H-14, H-17), 3.55–3.58 (2H, m, H-16), 3.78 (4H, t, J = 4.5 Hz, H-3', H-5'), 5.79 (1H, s, H-4), 12.05 (1H, brs, 9-OH), 13.37 (1H, s, 7-OH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.5 (C-15), 18.6 (C-12), 31.0 (C-14), 31.8 (C-10), 40.4 (C-16), 52.7 (C-2', C-6'), 55.2 (C-17), 56.1 (C-9b), 66.2 (C-3', C-5'), 100.7 (C-6), 101.6 (C-4), 102.3 (C-2), 105.1 (C-9a), 106.2 (C-8), 155.7 (C-

5a), 157.6 (C-9), 162.5 (C-7), 172.6 (C-11), 174.3 (C-4a), 188.5 (C-3), 197.0 (C-1), 200.8 (C-13); HRESIMS: *m/z* 457.1961 $[M+H]^+$ (cacld for $C_{24}H_{29}N_2O_7$, 457.1969).

4.1.2.3. (R,E)-6-Acetyl-2-[1-(2-(piperazin-1-yl)ethylamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (2c). Pale yellow solid (0.161 g, 49%); mp: 103-105 °C; IR (KBr) ν_{max}: 2660–3427, 1700, 1623, 1555, 1460, 1370, 1287, 1188 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.70 (3H, s, H-10), 2.10 (3H, s, H-15), 2.57 (4H, t, $J = 4.5$ Hz, H-2', H-6'), 2.65–2.71 (8H, m, H-12, H-14, H-17), 3.00 (4H, t, $J = 4.4$ Hz, H-3', H-5'), 3.56–3.60 (2H, m, H-16), 5.79 (1H, s, H-4), 13.35 (1H, brs, 7-OH); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6): δ 6.5 (C-15), 17.1 (C-12), 30.1 (C-14), 30.9 (C-10), 40.7 (C-16), 45.5 (C-3', C-5'), 54.2 (C-2', C-6'), 55.0 (C-17), 55.7 (C-9b), 100.1 (C-6), 101.0 (C-4), 101.4 (C-2), 104.1 (C-9a), 106.2 (C-8), 154.8 (C-5a), 157.2 (C-9), 162.1 (C-7), 172.4 (C-11), 173.7 (C-4a), 188.6 (C-3), 197.4 (C-1), 199.5 (C-13); HRESIMS: *m/z* 456.2112 $[M+H]^+$ (cacld for $C_{24}H_{30}N_3O_6$, 456.2129).

4.1.2.4. (R,E)-6-Acetyl-2-[1-(4-methylpiperazin-1-ylamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (2d). Pale yellow solid (0.169 g, 53%); mp: 78-80 °C; IR (KBr) ν_{max}: 2642–3422, 1699, 1629, 1553, 1457, 1365, 1286, 1188 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.70 (3H, s, H-10), 2.09 (3H, s, H-15), 2.33 (3H, S, H-7'), 2.56–2.61 (4H, m, H-3', H-5'), 2.67 (3H, s, H-12), 2.78 (3H, s, H-14), 2.93 (4H, t, $J = 4.4$ Hz, H-2', H-6'), 5.70 (1H, s, H-4), 11.96 (1H, brs, 9-OH), 13.35 (1H, s, 7-OH), 14.09 (1H, brs, NH); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6): δ 6.2 (C-15), 15.8 (C-12), 29.8 (C-14), 30.5 (C-10), 44.2 (C-7'), 52.3 (C-3', C-5'), 53.7 (C-2', C-6'), 55.5 (C-9b), 99.2 (C-6), 99.7 (C-4), 101.0 (C-2), 103.6 (C-9a), 105.9 (C-8), 154.4 (C-5a), 156.7 (C-9), 161.8 (C-7), 172.4 (C-11), 172.8 (C-4a), 188.2 (C-3), 196.6 (C-1), 199.2 (C-13); HRESIMS: *m/z* 442.1958 $[M+H]^+$ (cacld for $C_{23}H_{28}N_3O_6$, 442.1972).

4.1.2.5. (R,E)-6-Acetyl-2-[1-(3-(1H-imidazol-1-yl)propylamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (2e). Pale yellow solid (0.157 g, 48%); mp: 80-82 °C; IR (KBr) ν_{max}: 2650–3444, 1701, 1626, 1555, 1467, 1368, 1283, 1186 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$+DMSO-d_6): δ 1.71 (3H, s, H-10), 2.06 (3H, s, H-15), 2.22–2.28 (2H, m, H-17), 2.59 (3H, s, H-12), 2.68 (3H, s, H-14), 3.44–3.50 (2H, m, H-16), 4.17 (2H, t, $J = 6.6$ Hz, H-18), 5.79 (1H, s, H-4), 7.01 (1H, S, H-4'), 7.07 (1H, S, H-5'), 7.55 (1H, S, H-2'), 11.90 (1H, s, 9-OH), 13.36 (1H, s, 7-OH), 13.51 (1H, brs, NH); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6): δ 6.9 (C-15), 17.6 (C-12), 29.6 (C-17), 30.6 (C-14), 31.4 (C-10), 39.9 (C-16), 43.2 (C-18), 56.5 (C-9b), 100.6 (C-6), 101.7 (C-4), 101.8 (C-2), 104.4 (C-9a), 107.1 (C-8), 118.1 (C-5'), 129.3 (C-4'), 136.5 (C-2'), 155.2 (C-5a), 157.6 (C-9), 162.7 (C-7), 173.4 (C-11), 174.7 (C-4a), 189.5 (C-3), 197.6 (C-1), 200.1 (C-13); HRESIMS: *m/z* 452.1798 $[M+H]^+$ (cacld for $C_{24}H_{26}N_3O_6$, 452.1816).

4.1.2.6. (R,E)-6-Acetyl-2-[1-(piperidin-1-ylamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (2f). Pale yellow solid (0.170 g, 55%); mp: 75-77 °C; IR (KBr) ν_{max}: 2650–3420, 1701, 1628, 1554, 1460, 1363, 1286, 1188 cm^{-1};

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.50 (2H, brs, H-4'), 1.71–1.77 (7H, m, H-10, H-3', H-5'), 2.09 (3H, s, H-15), 2.67 (3H, s, H-12), 2.76–2.87 (7H, m, H-14, H-2', H-6'), 5.78 (1H, s, H-4), 12.04 (1H, brs, 9-OH), 13.35 (1H, s, 7-OH), 13.98 (1H, brs, NH); ^{13}C NMR (125 MHz, $CDCl_3$+DMSO-d_6): δ 7.4 (C-15), 17.2 (C-12), 22.9 (C-4'), 24.9 (C-3', C-5'), 31.2 (C-14), 31.9 (C-10), 56.7 (C-9b, C-2', C-6'), 100.4 (C-6), 101.2 (C-4), 102.2 (C-2), 105.0 (C-9a), 107.9 (C-8), 155.8 (C-5a), 158.2 (C-9), 163.4 (C-7), 173.7 (C-11), 174.3 (C-4a), 190.4 (C-3), 198.1 (C-1), 200.5 (C-13); HRESIMS: *m/z* 427.1869 $[M+H]^+$ (cacld for $C_{23}H_{27}N_2O_6$, 427.1863).

4.1.2.7. (R,E)-2-[1-(Morpholinoamino)ethylidene]-6-[(E)-1-(morpholinoimino)ethyl]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (3a). Pale yellow solid (0.145 g, 39%); mp: 100-102 °C; IR (KBr) ν_{max}: 2650–3430, 1694, 1623, 1554, 1457, 1366, 1268, 1188 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.69 (3H, s, H-10), 2.13 (3H, s, H-15), 2.59 (3H, s, H-12), 2.79 (3H, s, H-14), 2.86 - 2.91 (8H, m, H-2', H-6', H-2'', H-6''), 3.84 - 3.88 (8H, m, H-3', H-5', H-3'', H-5''), 5.73 (1H, s, H-4), 11.40 (1H, brs, 9-OH), 14.14 (1H, brs, NH); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6): δ 7.3 (C-15), 16.5 (C-12), 17.3 (C-14), 31.3 (C-10), 54.8 (C-2', C-6'), 55.1 (C-2'', C-6''), 56.5 (C-9b), 65.2 (C-3', C-5'), 65.3 (C-3'', C-5''), 98.5 (C-6), 100.1 (C-4), 100.9 (C-2), 103.6 (C-9a), 107.0 (C-8), 153.3 (C-5a), 153.5 (C-9), 160.3 (C-7), 167.2 (C-13) 173.4 (C-11), 174.2 (C-4a), 189.5 (C-3), 198.0 (C-1); HRESIMS: *m/z* 513.2330 $[M+H]^+$ (cacld for $C_{26}H_{33}N_4O_7$, 513.2343).

4.1.2.8. (R,E)-2-[1-(2-Morpholinoethylamino)ethylidene]-6-[(E)-1-(2-morpholino-ethylamino)ethyl]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (3b). Pale yellow solid (0.181 g, 44%); mp: 80-83 °C; IR (KBr) ν_{max}: 2664–3450, 1695, 1623, 1553, 1461, 1367, 1286, 1188 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.70 (3H, s, H-10), 2.11 (3H, s, H-15), 2.60 (8H, t, J = 4.6 Hz, H-2', H-6', H-2'', H-6''), 2.68–2.74 (10H, m, H-12, H-14, H-17, H-19), 3.52–3.67 (4H, m, H-16, H-18), 3.85 (8H, t, J = 4.6 Hz, H-3', H-5', H-3'', H-5''), 5.80 (1H, s, H-4), 11.75 (1H, brs, 9-OH), 13.47 (1H, s, 7-OH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 7.3 (C-15), 18.6 (C-12), 19.1 (C-14), 32.0 (C-10), 40.9 (C-16), 43.3 (C-18), 52.8 (C-2', C-6'), 52.9 (C-2'', C-6''), 55.3 (C-17), 56.3 (C-19), 57.2 (C-9b), 66.2 (C-3', C-5'), 66.3 (C-3'', C-5''), 99.0 (C-6), 101.5 (C-4), 103.1 (C-2), 105.1 (C-9a), 107.0 (C-8), 155.8 (C-5a), 157.7 (C-9), 170.3 (C-7), 170.6 (C-13) 173.2 (C-11), 174.5 (C-4a), 188.6 (C-3), 197.5 (C-1); HRESIMS: *m/z* 569.2953 $[M+H]^+$ (cacld for $C_{30}H_{41}N_4O_7$, 569.2969).

4.1.2.9. (R,E)-2-[1-(2-(Piperazin-1-yl)ethylamino)ethylidene]-6-[(E)-1-(2-(piperazin-1-yl)ethylamino)ethyl]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (3c). Pale yellow solid (0.185 g, 45%); mp: 92–95 °C; IR (KBr) ν_{max}: 2650–3430, 1697, 1623, 1554, 1458, 1369, 1287, 1188 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.73 (3H, s, H-10), 2.07 (3H, s, H-15), 2.50 - 2.62 (8H, m, H-2', H-6', H-2'', H-6''), 2.65–2.85 (10H, m, H-12, H-14, H-17, H-19), 2.93 (8H, t, J = 4.4 Hz, H-3', H-5', H-3'', H-5''), 3.55–3.70 (4H, m, H-16, H-18), 5.80 (1H, s, H-4), 12.20 (1H, brs, 9-OH), 13.22 (1H, brs, 7-OH), 13.37 (1H, brs, NH); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6): δ 6.7 (C-15), 17.2 (C-12), 17.6 (C-14), 31.0 (C-10), 40.9 (C-16), 43.2 (C-18), 45.7 (C-3', C-5'),

45.9 (C-3'', C-5''), 53.8 (C-2', C-6'), 54.0 (C-2'', C-6''), 55.0 (C-17), 55.9 (C-19), 56.3 (C-9b), 98.6 (C-6), 100.5 (C-4), 102.3 (C-2), 104.2 (C-9a), 107.0 (C-8), 154.6 (C-5a), 157.4 (C-9), 169.5 (C-7), 170.5 (C-13) 172.7 (C-11), 173.7 (C-4a), 188.4 (C-3), 197.9 (C-1); HRESIMS: *m/z* 567.3288 $[M+H]^+$ (cacld for $C_{30}H_{43}N_6O_5$, 567.3289).

4.1.2.10. (R,E)-2-[1-(4-Methylpiperazin-1-ylamino)ethylidene]-6-[(E)-1-(4-methylpiperazin-1-ylimino)ethyl]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (3d). Pale yellow solid (0.167 g, 43%); mp: 87–90 °C; IR (KBr) ν_{max}: 2720–3426, 1695, 1624, 1553, 1457, 1365, 1280, 1187 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$+DMSO-d_6): δ 1.68 (3H, s, H-10), 2.10 (3H, s, H-15), 2.33 (6H, S, H-7', H-7''), 2.58 - 2.63 (11H, m, H-12, H-3', H-5', H-3'', H-5''), 2.78 (3H, s, H-14), 2.89 - 2.95 (8H, m, H-2', H-6', H-2'', H-6''), 5.71 (1H, s, H-4), 11.43 (1H, s, 9-OH), 13.94 (1H, brs, 7-OH), 14.87 (1H, brs, NH); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6): δ 6.7 (C-15), 15.7 (C-12), 16.6 (C-14), 30.5 (C-10), 44.2 (C-7'), 44.3 (C-7''), 52.3 (C-3', C-5'), 52.5 (C-3'', C-5''), 53.5 (C-2', C-6'), 53.7 (C-2'', C-6''), 55.6 (C-9b), 97.7 (C-6), 99.2 (C-4), 100.1 (C-2), 102.7 (C-9a), 105.8 (C-8), 152.4 (C-5a), 152.7 (C-9), 159.6 (C-7), 165.9 (C-13) 172.5 (C-11), 173.1 (C-4a), 188.4 (C-3), 196.9 (C-1); HRESIMS: *m/z* 539.2968 $[M+H]^+$ (cacld for $C_{28}H_{39}N_6O_5$, 539.2976).

4.1.2.11. (R,E)-2-[1-(3-(1H-Imidazol-1-yl)propylamino)ethylidene]-6-[(E)-1-(3-(1H-imidazol-1-yl)propylamino)ethyl]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (3e). Pale yellow solid (0.178 g, 44%); mp: 88–91 °C; IR (KBr) ν_{max}: 2644–3452, 1698, 1624, 1553, 1467, 1365, 1284, 1186 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$+DMSO-d_6): δ 1.72 (3H, s, H-10), 2.06 (3H, s, H-15), 2.24–2.29 (4H, m, H-17, H-20), 2.60 (3H, s, H-12), 2.69 (3H, s, H-14), 3.42–3.50 (4H, m, H-16, H-19), 4.22–4.36 (4H, m, H-18, H-21), 5.80 (1H, s, H-4), 7.02 (2H, S, H-4', H-4''), 7.08 (2H, S, H-5', H-5''), 7.55 (2H, S, H-2', H-2''), 12.00 (1H, s, 9-OH), 13.38 (1H, s, 7-OH), 13.52 (1H, brs, NH); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6): δ 7.4 (C-15), 17.6 (C-12), 18.6 (C-14), 29.8 (C-17), 30.8 (C-20), 31.6 (C-10), 40.5 (C-16), 43.0 (C-19), 43.6 (C-18), 44.2 (C-21), 57.0 (C-9b), 99.1 (C-6), 101.8 (C-4), 101.9 (C-2), 103.5 (C-9a), 108.1 (C-8), 118.1 (C-5'), 118.3 (C-5''), 129.4 (C-4'), 129.5 (C-4''), 136.4 (C-2'), 136.6 (C-2''), 154.5 (C-5a), 155.2 (C-9), 170.0 (C-7), 170.6 (C-13) 173.9 (C-11), 174.9 (C-4a), 189.8 (C-3), 198.5 (C-1); HRESIMS: *m/z* 559.2653 $[M+H]^+$ (cacld for $C_{30}H_{35}N_6O_5$, 559.2663).

4.1.2.12. (R,E)-2-[1-(Piperidin-1-ylamino)ethylidene]-6-[(E)-1-(piperidin-1-ylimino)ethyl]-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione (3f). Pale yellow solid (0.136 g, 37%); mp: 72–74 °C; IR (KBr) ν_{max}: 2655–3439, 1697, 1624, 1555, 1459, 1365, 1280, 1187 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.48–1.53 (4H, m, H-4', H-4''), 1.72–1.79 (11H, m, H-10, H-3', H-5', H-3'', H-5''), 2.11 (3H, s, H-15), 2.67 (3H, s, H-12), 2.85–2.95 (11H, m, H-14, H-2', H-6', H-2'', H-6''), 5.71 (1H, s, H-4), 11.95 (1H, brs, 9-OH), 13.36 (1H, S, 7-OH), 13.92 (1H, brs, NH); ^{13}C NMR (125 MHz, $CDCl_3$+DMSO-d_6): δ 7.3 (C-15), 17.1 (C-12), 18.2 (C-14), 22.8 (C-4'), 22.9 (C-4''), 24.8 (C-3', C-5'), 24.9 (C-3'', C-5''), 31.8 (C-10), 56.6 (C-9b, C-2', C-6'), 56.7 (C-2'', C-6''), 99.0 (C-6), 100.4 (C-4), 101.2 (C-2), 104.1 (C-9a), 108.0 (C-8), 153.6 (C-5a),

153.8 (C-9), 161.3 (C-7), 168.4 (C-13) 173.9 (C-11), 174.4 (C-4a), 190.0 (C-3), 198.6 (C-1); HRESIMS: *m/z* 509.2749 $[M+H]^+$ (cacld for $C_{28}H_{37}N_4O_5$, 509.2758).

4.1.3. General procedure for the synthesis of compounds 4a-4i

The (+)- usnic acid-based pyrazole hybrids (**4a**-**4i**) were synthesized by following the previously reported procedure [18,21]. (+)- Usnic acid (1 eq) was refluxed with appropriate phenyl hydrazine (1 eq) and pyridine (1 eq) in ethanol for 12 h. After completion, the reaction mixture was concentrated under reduced pressure and chromatographed over silica gel column to get the pure compounds **4a**-**4i** in 81–92% yields.

4.1.3.1. (R)-8-Acetyl-5,7-dihydroxy-3,4a,6-trimethyl-1-phenyl-1H-benzofuro[3,2-f]indazol-4(4aH)-one (4a). Pale yellow solid, yield: 92%, mp: 196–198 °C; IR (KBr) ν_{max}: 3083, 2984, 2926, 1675, 1626, 1511, 1477, 1364, 1288, 1170, 1046, 760 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.81 (3H, s), 2.11 (3H, s), 2.59 (3H, s), 2.64 (3H, s), 6.24 (1H, s), 7.44-7.58 (5H, m), 11.12 (1H, s), 13.31 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 7.4, 13.2, 30.4, 31.1, 60.3, 89.2, 101.4, 103.9, 108.2, 110.6, 123.9, 128.7, 129.6, 137.8, 148.1, 151.4, 156.2, 157.6, 163.5, 172.9, 196.1, 200.3; HRESIMS: *m/z* 417.1442 $[M+H]^+$ (cacld for $C_{24}H_{21}N_2O_5$, 417.1445).

4.1.3.2. (R)-8-Acetyl-1-(3-chlorophenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1H-benzofuro[3,2-f]indazol-4(4aH)-one (4b). Pale yellow solid, yield: 81%, mp: 101–103 °C; IR (KBr) ν_{max}: 3082, 2984, 2927, 1675, 1625, 1475, 1370, 1289, 1178, 1046, 777 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.81 (3H, s), 2.10 (3H, s), 2.58 (3H, s), 2.65 (3H, s), 6.22 (1H, s), 7.44-7.51 (3H, m), 7.62-7.63 (1H, m), 11.02 (1H, s), 13.32 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 7.4, 13.1, 30.4, 31.1, 60.4, 89.2, 101.4, 103.9, 108.2, 110.7, 121.9, 124.2, 128.7, 130.4, 135.5, 138.6, 148.1, 151.6, 156.2, 157.6, 163.5, 173.3, 196.1, 200.3; HRESIMS: *m/z* 451.1057 $[M+H]^+$ (cacld for $C_{24}H_{20}N_2O_5Cl$, 451.1055).

4.1.3.3. (R)-8-Acetyl-1-(4-chlorophenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1H-benzofuro[3,2-f]indazol-4(4aH)-one (4c). Pale yellow solid, yield: 88%, mp: 109–110 °C; IR (KBr) ν_{max}: 3083, 2985, 2926, 1675, 1625, 1498, 1370, 1289, 1178, 1046, 830 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.81 (3H, s), 2.11 (3H, s), 2.57 (3H, s), 2.64 (3H, s), 6.21 (1H, s), 7.53 (4H, brs), 11.04 (1H, s), 13.31 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 7.4, 13.2, 30.4, 31.1, 60.3, 88.9, 101.4, 103.8, 108.3, 110.7, 125.0, 129.8, 134.5, 136.3, 148.2, 151.6, 156.1, 157.5, 163.5, 173.2, 196.1, 200.3; HRESIMS: *m/z* 451.1042 $[M+H]^+$ (cacld for $C_{24}H_{20}N_2O_5Cl$, 451.1055).

4.1.3.4. (R)-8-Acetyl-1-(3-bromophenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1H-benzofuro[3,2-f]indazol-4(4aH)-one (4d). Pale yellow solid, yield: 82%, mp: 122–124 °C; IR (KBr) ν_{max}: 3082, 2985, 2926, 1675, 1624, 1473, 1362, 1289, 1178, 1046, 756 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.81 (3H, s), 2.11 (3H, s), 2.57 (3H, s), 2.66 (3H, s), 6.24 (1H, s), 7.41-7.63 (3H, m), 7.75–7.80 (1H, m), 11.02 (1H, s), 13.33 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 7.4, 13.2, 30.4, 31.2, 60.4, 88.9, 101.5, 103.8, 108.3, 110.8, 122.3,

123.1, 126.9, 130.8, 131.7, 138.9, 148.3, 151.7, 156.1, 157.5, 163.5, 173.4, 196.1, 200.3; HRESIMS: *m/z* 495.0536 $[M+H]^+$ (cacld for $C_{24}H_{20}N_2O_5Br$, 495.0550).

4.1.3.5. (R)-8-Acetyl-1-(4-bromophenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1H-benzofuro[3,2-f]indazol-4(4aH)-one (4e). Yellow solid, yield: 85%, mp: 130–132 °C; IR (KBr) ν_{max}: 3082, 2985, 2927, 1675, 1624, 1507, 1367, 1289, 1046, 826 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.80 (3H, s), 2.10 (3H, s), 2.57 (3H, s), 2.64 (3H, s), 6.21 (1H, s), 7.47 (2H, d, $J = 6.8$ Hz), 7.69 (2H, d, $J = 6.8$ Hz), 11.03 (1H, s), 13.31 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 7.4, 13.2, 30.4, 31.1, 60.4, 88.9, 101.5, 103.8, 108.3, 110.8, 122.4, 125.2, 132.8, 136.8, 148.2, 151.7, 156.1, 157.5, 163.5, 173.3, 196.1, 200.3; HRESIMS: *m/z* 495.0541 $[M+H]^+$ (cacld for $C_{24}H_{20}N_2O_5Br$, 495.0550).

4.1.3.6. (R)-8-Acetyl-1-(3-methoxyphenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1H-benzofuro[3,2-f]indazol-4(4aH)-one (4f). Pale yellow solid, yield: 87%, mp: 157–159 °C; IR (KBr) ν_{max}: 3083, 2985, 2926, 1675, 1624, 1473, 1365, 1289, 1046, 778 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.81 (3H, s), 2.11 (3H, s), 2.58 (3H, s), 2.64 (3H, s), 3.89 (3H, s), 6.27 (1H, s), 6.99-7.02 (1H, m), 7.12-7.14 (2H, m), 7.43-7.46 (1H, m), 11.10 (1H, s), 13.32 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 7.4, 13.2, 30.4, 31.1, 55.6, 60.3, 89.3, 101.5, 103.9, 108.2, 109.7, 110.6, 114.4, 115.9, 130.3, 138.8, 148.2, 151.3, 156.2, 157.6, 160.5, 163.5, 172.9, 196.1, 200.3; HRESIMS: *m/z* 447.1540 $[M+H]^+$ (cacld for $C_{25}H_{23}N_2O_6$, 447.1550).

4.1.3.7. (R)-8-Acetyl-1-(4-methoxyphenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1H-benzofuro[3,2-f]indazol-4(4aH)-one (4g). Pale yellow solid, yield: 91%, mp: 168–171 °C; IR (KBr) ν_{max}: 3083, 2980, 2928, 1676, 1624, 1520, 1478, 1365, 1289, 1047, 829 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.80 (3H, s), 2.10 (3H, s), 2.57 (3H, s), 2.63 (3H, s), 3.88 (3H, s), 6.17 (1H, s), 7.05 (2H, d, $J = 8.8$ Hz), 7.48 (2H, d, $J = 8.8$ Hz), 11.16 (1H, s), 13.32 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 7.4, 13.2, 30.4, 31.1, 55.6, 60.2, 89.1, 101.4, 103.9, 108.1, 110.3, 114.7, 125.4, 130.8, 148.1, 151.1, 156.2, 157.6, 159.7, 163.4, 172.6, 196.0, 200.3; HRESIMS: *m/z* 447.1528 $[M+H]^+$ (cacld for $C_{25}H_{23}N_2O_6$, 447.1550).

4.1.3.8. (R)-8-Acetyl-1-(3-nitrophenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1H-benzofuro[3,2-f]indazol-4(4aH)-one (4h). Yellow solid, yield: 83%, mp: 116–118 °C; IR (KBr) ν_{max}: 3083, 2980, 2928, 1675, 1624, 1473, 1367, 1289, 1044, 769 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 1.84 (3H, s), 2.11 (3H, s), 2.59 (3H, s), 2.65 (3H, s), 6.28 (1H, s), 7.78 (1H, t, $J = 8.19$ Hz), 7.98–8.02 (1H, m), 8.81–8.85 (1H, m), 8.49 (1H, t, $J = 2.07$ Hz), 10.92 (1H, s), 13.31 (1H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 7.4, 13.1, 30.4, 31.2, 60.6, 88.5, 101.5, 103.7, 108.4, 111.1, 118.4, 122.9, 129.2, 130.7, 138.9, 148.5, 148.7, 152.2, 156.1, 157.3, 163.5, 174.1, 196.1, 200.3; HRESIMS: *m/z* 462.1289 $[M+H]^+$ (cacld for $C_{24}H_{20}N_3O_7$, 462.1295).

4.1.3.9. (R)-8-Acetyl-1-(4-nitrophenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1H-benzofuro[3,2-f]indazol-4(4aH)-one (4i). Pale yellow solid, yield: 81%, mp: 125–127 °C; IR (KBr) ν_{max}: 3083, 2980, 2928, 1675, 1626, 1513, 1473, 1344, 1284, 1044, 847 cm^{-1}; ^1H NMR

(300 MHz, $CDCl_3$): δ 1.83 (3H, s), 2.10 (3H, s), 2.60 (3H, s), 2.65 (3H, s), 6.32 (1H, s), 7.83 (2H, d, $J = 9.06$ Hz), 8.45 (2H, d, $J = 9.06$ Hz), 10.89 (1H, s), 13.31 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 7.4, 13.2, 30.3, 31.1, 60.6, 88.8, 101.5, 103.6, 108.5, 111.3, 123.7, 125.2, 142.8, 146.7, 148.7, 152.4, 156.1, 157.3, 163.6, 174.1, 196.1, 200.2; HRESIMS: m/z 462.1288 $[M + H]^+$ (cacld for $C_{24}H_{20}N_3O_7$, 462.1295).

4.2. Biology

4.2.1. Cytotoxicity assay

All cell lines (HeLa, MDA-MB-231, A549, and MiaPaca) used in this study were purchased from the American Type Culture Collection (ATCC), United States. The synthesized test compounds were evaluated for their in vitro anti proliferative activity in these four different human cancer cell lines. A protocol of 48 h continuous drug exposure was used, and a SRB cell proliferation assay was used to estimate cell viability or growth. All the cell lines were grown in Dulbecco's modified Eagle's medium (containing 10% Fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37 °C). Cells were trypsinized when sub-confluent from T25 flasks/60 mm dishes and seeded in 96-well plates in 100 μL aliquots at plating densities depending on the doubling time of individual cell lines. The microliter plates were incubated at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs and were incubated for 48 h with different doses (0.01, 0.1, 1, 10, 100μM) of synthesized compounds. After 48 h of incubation at 37 °C, cell monolayers were fixed by the addition of 10% (wt/vol) cold trichloroacetic acid and incubated at 4 °C for 1 h and were then stained with 0.057% SRB dissolved in 1% acetic acid for 30 min at room temperature. Unbound SRB was washed with 1% acetic acid. The protein–-bound dye was dissolved in 10 mM Tris base solution for OD determination at 510 nm using a microplate reader (Enspire, Perkin Elmer) [19].

4.2.2. Cell cycle analysis

HeLa cells in 60 mm dishes were incubated for 24 h in the presence or absence of test compounds **2e** and **4a** at 10 μM concentrations. 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 1 ml of DNA staining solution [0.2 mg of propidium iodide (PI), and 2 mg RNase A] for 30 min as described earlier. The DNA contents of 20,000 events were measured by flow cytometer (BD FACSCanto II). Histograms were analyzed using FCS express 4 plus [22].

4.2.3. Immunohistochemistry of tubulin and analysis of nuclear morphology

HeLa cells were seeded on glass cover slip, incubated for 24 h in the presence or absence of test compounds **2e** and **4a** at a concentration of 10 μM. Cells grown on cover slips were fixed in 3.5% formaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 10 minutes at room temperature. Cells were pre-mobilized for 6 minutes in PBS containing 0.5% Triton X-100 (Sigma) and 0.05% Tween-20 (Sigma). The pre-mobilized cells were blocked with 2% Bovine serum albumin (BSA) (Sigma) in PBS for 1 h. Later, the cells were incubated with primary antibody for tubulin from

(Sigma) at (1:200) diluted in blocking solution for 4 h at room temperature. Subsequently the antibodies were removed and the cells were washed thrice with PBS. Cells were then incubated with FITC labeled anti-mouse secondary antibody (1:500) for 1 h at room temperature. Cells were washed thrice with PBS and mounted in medium containing DAPI. Images were captured using the Olympus confocal microscope FLOW VIEW FV 1000 series and analyzed with FV10ASW 1.7 series software [19].

4.2.4. Western blot analysis of soluble versus polymerized tubulin

Cells were seeded in 12-well plates at 1×10^5 cells per well in complete growth medium. Following treatment of cells with compounds **2e** and **4a** for duration of 24 h, cells were washed with PBS and subsequently soluble and insoluble tubulin fractions were collected. To collect the soluble tubulin fractions, cells were permeablized with 200 µl of pre-warmed lysis buffer [80 mM Pipes-KOH (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA, 0.2% Triton X-100, 10% glycerol, 0.1% protease inhibitor cocktail (Sigma-Aldrich)] and incubated for 3 min at 30 °C. Lysis buffer was gently removed, and mixed with 100 µl of 3 × Laemi's sample buffer (180 mM Tris-Cl pH 6.8, 6% Sodium dodecyl sulphate (SDS), 15% glycerol, 7.5% β-mercaptoethanol and 0.01% bromophenol blue). Samples were immediately heated to 95 °C for 3 min. To collect the insoluble tubulin fraction, 300 µl of 1 × Laemi's sample buffer was added to the remaining cells in each well, and the samples were heated to 95 °C for 3 min. Equal volumes of samples were run on an SDS-10% polyacrylamide gel and were transferred to a nitrocellulose membrane employing semidry transfer at 50 mA for 1 h. Blots were probed with mouse anti-human α-tubulin diluted 1:2000 ml (Sigma) and stained with rabbit anti-mouse secondary antibody coupled with horseradish peroxidase, diluted 1:5000 ml (Sigma). Bands were visualized using an enhanced Chemiluminescence protocol (Pierce) and radiographic film (Kodak) [19].

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Disclosure statement

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