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ARTICLE

New (3-(1H-benzo[d]imidazol-2-yl)/(3-(3H-imidazo[4,5-b]pyridin-2-yl)-(1H-indol-5-yl)(3,4,5-trimethoxyphenyl)methanone conjugates as tubulin polymerization inhibitors

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A series of (3-(1H-benzo[d]imidazol-2-yl)/(3-(3H-imidazo[4,5-b]pyridin-2-yl)-(1H-indol-5-yl)(3,4,5-trimethoxyphenyl) methanone conjugates **4-6(a–i)** were synthesized and evaluated for their antiproliferative activity on selected human cancer cell lines such as prostate (DU-145), lung (A549), cervical (HeLa) and breast (MCF-7). Most of these conjugates showed considerable cytotoxicity with IC_{50} values ranging from 0.54 to 31.86 µM. Among them, compounds **5g** and **6f** showed significant activity against human prostate cancer cell line DU-145 with IC_{50} values of 0.68 µM and 0.54 µM respectively. Tubulin polymerization assay and immunofluorescence analysis results suggest that these compounds effectively inhibit microtubule assembly formation in DU-145. Further, the apoptosis inducing ability of these derivatives (**5g** and **6f**) was confirmed by Hoechst staining, measurement of mitochondrial membrane potential ROS generation and Annexin V-FITC assays.

Introduction

The Microtubules are dynamic in structures that together with actin microfilaments and intermediate filaments, constitute the cellular cytoskeleton. Besides their well-known role in cell division, their functions involve maintenance of cell shape and morphology, cellular motility, and trafficking of organelles and vesicles.[1] Microtubules are formed by the polymerization of heterodimers of α and β tubulin. In the mitotic phase, microtubules are in dynamic equilibrium with tubulin dimers as tubulin is assembled into microtubules and which are disassembled to tubulin. The essential role of microtubules in mitotic spindle formation and proper chromosomal separation makes them one of the most attractive targets for the design and development of many small natural and synthetic antitumor drugs.[1,2] Many of them exert their effects by inhibiting the noncovalent polymerization of tubulin into microtubules. Therefore, there has been great interest in identifying and developing novel antimicrotubule molecules.

Among the naturally occurring compounds, combretastatin A-4 (**1**, Fig. 1) is one of the best characterized antimitotic agents. Combretastatin A-4, isolated from the bark of the South African tree *Combretum caffrum*,[3] is a highly effective natural tubulin-binding molecule affecting microtubule

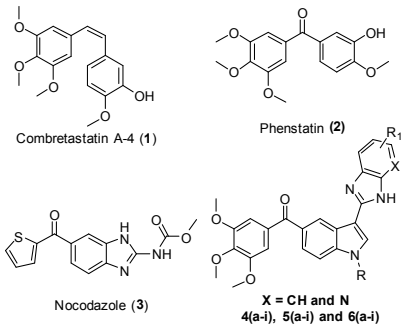

Fig. 1: Tubulin polymerization inhibitors.

dynamics by binding to the colchicine site.[4] It shows potent cytotoxicity against a wide variety of human cancer cell lines and MDR cell lines.[5] However, combretastatins are characterized by poor solubility and chemical instability. Importantly, the olefinic bond with *cis* configuration (*Z*-geometry) plays a fundamental role in binding at the colchicine site by positioning the rings at appropriate distance to maximize interactions.[6] Several attempts have been reported to modify this *cis*-olefinic bond to prevent its isomerisation under amenable conditions. This mostly included either modification of the olefinic bond by the introduction of saturation, substituents and its replacement with a three to six membered ring system, which resulted in *cis* restricted analogues of CA-4.[7-11] The replacement of the olefinic bridge of CA-4 with a carbonyl group furnished a benzophenone-type

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CA-4 analogue named phenstatin (**2**, Fig. 1), which has been found to be a potent cytotoxic agent and inhibitor of tubulin polymerization, with activities differing little from those of CA-4.[12] Benzophenone-type CA-4 analogues are attractive targets for anti-tubulin agents as the benzophenone backbone not only provides ease of synthesis without the need to control the geometric selectivity (*Z* and *E*-geometry) but also increases the pharmacological potential through increased drug stability and water solubility.[13]

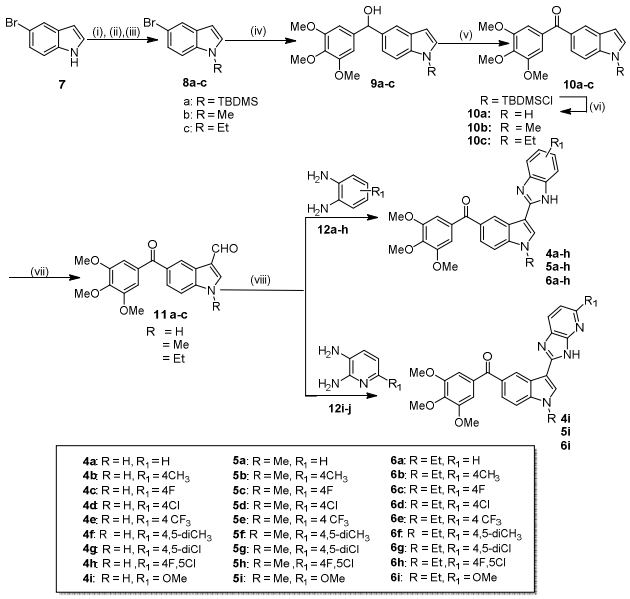
Agents that effect the tubulin polymerization having an indole as their core nucleus have been recently reviewed and in the past few years an ever increasing number of synthetic indoles as potent tubulin polymerization inhibitors have been reported.[14] Aroylindole moiety constituted the core structure of antimitotic compounds that have been reported as tubulin polymerization inhibitors.[15] On the other hand, benzimidazoles are more privileged scaffold due to their biological properties and have been reported to possess potential anticancer activity and anti-HIV activity,[16-24] apart from antibacterial,[25,26] antifungal, antiviral and antioxidant activities.[27-30] Nocodazole (NSC-238189, **3**, Fig. **1**) is another well-known inhibitor of tubulin polymerization that possess a benzimidazole moiety which inhibits cell proliferation and is largely used as a pharmacological tool and positive control.[31]As part of our ongoing efforts to discover newer tubulin inhibitors, we previously reported imidazopyridine-benzimidazole hybrids[32] and phenstatin/isocombretastatin-oxindole conjugates.[33] Considering the biological importance of these moieties (phenstatin, indole and benzimidazole) attempt has been made in the present study to synthesize indole-benzimidazole conjugates.

Based on these observations, we describe the modifications on the CA-4 scaffold which contains a trimethoxyphenyl moiety identical to the A-ring of CA-4. Further congeners were generated having indole moiety at 3rd position. Thus, the rationale of these moieties was to conserve the privileged structures of the trimethoxyphenyl and indole groups. Considering these modifications, an attempt has been made in the present study to synthesize indophenstatin-benzimidazole conjugates **4-6(a–i)** (Fig. 1). The resulting conjugates were evaluated for antiproliferative activity and examined their structure activity relationship (SAR) followed by studies to elucidate the mechanism of action which included cell cycle progression, tubulin polymerization assay and molecular docking studies. Further to confirm the induction of apoptotic cell death by the conjugates studies such as Hoechst staining, mitochondrial membrane potential and Annexin V elucidated.

Results and discussion

Chemistry

The trimethoxy aroyl indole-benzimidazole conjugates **4-6(a–i)** were synthesized in six straight forward reactions. Initially, the commercially available 5-bromoindole (**7**) was protected with tert-butylchlorodimethylsilane (TBDMSCl), alkylated with iodomethane (MeI) and bromoethane (EtBr) in the presence of sodium hydride and DMF to produce corresponding 1-



Scheme 1: ***Reagents and conditions*****:** (i) TBDMSCl, NaH, THF, 0 °C-rt, 3 h, 93%; (ii) MeI, NaOH,DMSO, 0°C-rt, 3 h, 95%; (iii) EtBr, NaH, THF,0°C-rt, 3 h, 95% (iv) 3,4,5-trimethoxy benzaldehyde, n-BuLi, THF, -78°C, 4 h, 73-75%, (v) IBX, DMSO, 0 °C–rt, 3 h, 95%; (vi) TBAF, THF, 0°C-rt, 4 h, 90% (vii) $POCl_3$, DMF, $CHCl_3$, Reflux, 12 h, 78-82%; (viii) $Na_2S_2O_5$, EtOH:H_2O, 80°C, 2 h, 68-85%.

substituted indoles (**8a-c)** respectively. The products **8(a-c)** were further treated with n-butyllithium (*n*-BuLi) and 3,4,5-trimethoxybenzaldehyde at -78 ^{o}C to furnish the corresponding alcohols (**9a-c)** with moderate yields. The oxidized products (**10a-c)** were isolated with good yields from the reaction of **9(a-c)** with 2-iodoxybenzoic acid (IBX) in DMSO and after oxidation, the deprotection of TBDMS group with tetra-n-butylammonium fluoride (TBAF) provided **10a**. Next, Vilsmeier reaction of **10(a-c)** with phosphoryl chloride ($POCl_3$) and DMF afforded **11(a-c)** in good yields. Finally, the derivatives **4-6(a-i)** were generated by condensation of various aldehydes **11(a-c)** with substituted benzene-1,2-diamine (OPDs) in the presence of sodium metabisulfite ($Na_2S_2O_5$) as shown in Scheme **1**. All these new derivatives were characterized by spectral analysis such as ^{1}H-NMR, ^{13}C NMR and HRMS.

Biological studies

Cytotoxic activity

These conjugates **4-6(a-i)** were evaluated for their cytotoxic activity on a panel of deferent human cancer cell lines (All the cell lines were purchased from The National Centre for Cell Science (NCCS), Pune, India) such as, A549 (lung), HeLa (cervical), MCF-7 (breast) and DU-145 (prostate) by employing MTT assay.[34] The results are summarized in Table1 and the IC_{50} values expressed in µM and compared with nocodazole as control. Some of the derivatives show significant activity against most the cell lines tested with IC_{50} values ranging from 0.54 to 31.86 µM. Among them derivatives **4c-e**, **4i**, **5b**, **5c**, **5f-h**, **6a**, **6b**, **6d-f** and **6h** showed considerable activity with IC_{50}

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values of <5 μM and majority of derivatives show good cytotoxicity against prostate cancer cell line (DU-145). However, derivatives **5g** and **6f** were found to be very active against DU-145 cell line with IC_{50} values of 0.68 μM and 0.54 μM respectively. Therefore, the DU-145 cell line was chosen as a model cell line for subsequent experiments.

Table 1: [a]IC_{50} values (in μM) for derivatives **4-6(a-i)** on selected human cancer cell lines.

Compound	A549[b]	MCF-7[c]	DU-145[d]	HeLa[e]
4a	17.53	10.15	9.72	10.03
4b	26.79	11.88	11.09	12.07
4c	9.13	1.76	1.69	2.51
4d	16.28	3.89	2.47	3.84
4e	19.53	9.89	7.82	13.95
4f	27.00	8.97	4.83	11.12
4g	28.40	16.43	8.26	13.12
4h	31.86	15.00	10.42	17.52
4i	15.6	12.4	11.3	13.8
5a	18.69	14.44	11.88	14.75
5b	15.44	1.38	0.83	5.95
5c	3.16	1.73	1.20	9.80
5d	29.64	8.56	6.31	8.22
5e	27.08	2.11	1.95	6.44
5f	27.15	10.50	7.80	12.85
5g	**1.44**	**0.95**	**0.68**	**1.41**
5h	22.88	1.41	1.14	7.33
5i	28.54	14.73	7.26	15.16
6a	25.86	8.29	4.34	15.11
6b	2.19	1.65	1.53	2.00
6c	17.82	10.27	5.30	14.76
6d	1.69	1.41	1.26	6.48
6e	5.90	1.90	1.95	1.73
6f	**0.94**	**1.05**	**0.54**	**0.91**
6g	6.58	1.86	1.12	4.64
6h	14.36	11.38	5.95	11.25
6i	31.62	10.14	7.52	13.43
Nocodazole	1.05	1.48	1.39	1.05

[a]50% Inhibitory concentration after 48 h of drug treatment. [b]Human lung cancer, [c]Human breast cancer. [d]Human prostate cancer. [e]Human cervical cancer.

Cell cycle analysis

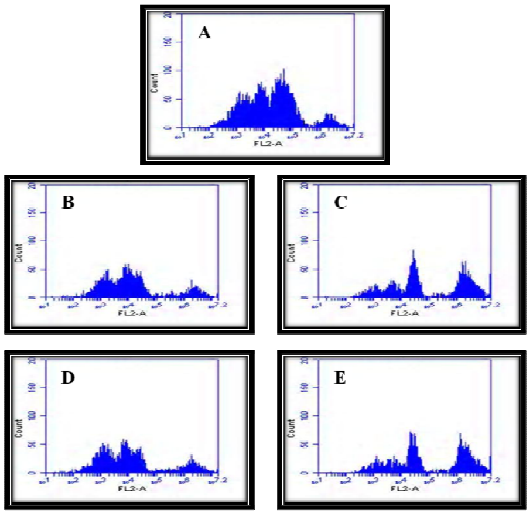

Fig. 2: Flow cytometric analysis in DU-145 prostate cancer cell lines after treatment with **5g** and **6f** at 0.5 and 1 μM concentrations for 48 h; A: Untreated cells (control cells), B: **5g** (0.5 μM), C: **5g** (1 μM), D: **6f** (0.5 μM) and E: **6f** (1 μM).

Many anticancer compounds exert their cytotoxic effect either by arresting the cell cycle at a particular checkpoint of cell cycle or by induction of apoptosis or a combined effect of both cycle block and apoptosis.[35] In this study DU-145 cells were treated with derivatives 5g and 6f at concentrations of 0.5 and 1 μM for 48 h. The data obtained clearly indicated that these derivatives show G2/M cell cycle arrest in comparison with the untreated cells. These derivatives 5g and 6f showed 14.56 and 15.15% of cell accumulation in G2/M phase at 0.5 μM concentration, whereas they exhibited 38.47 and 39.80 % of cell accumulation at 1 μM concentration respectively (Fig. 2 and Table 2).

Table 2. Distribution of DU-145 cells in various phases of cell cycle.

Sample	Sub G1%	G0/G1%	S%	G2/M%
A: Untreated cells (Control)	0.89	87.95	0.99	8.26
B: 5g (0.5 μM)	1.61	79.15	2.23	14.56
C: 5g (1 μM)	0.65	57.53	2.07	38.47
D: 6f (0.5 μM)	1.77	77.75	2.32	15.15
E: 6f (1 μM)	0.48	57.05	1.83	39.80

Effect of compounds on tubulin polymerization

In general G2/M cell cycle arrest is strongly associated with inhibition of tubulin polymerization and since compounds **5g** and **6f** cause cell cycle arrest at G2/M phase, it was considered of interest to investigate their microtubule inhibitory function. Tubulin subunits are known to heterodimerize and self-assemble to form microtubules in a time dependent manner. The progression of tubulin polymerization[36] was thus examined by monitoring the increase in fluorescence emission at 420 nm (excitation wavelength is 360 nm) in 384 well plate for 1 h at 37 °C with and without the conjugates in comparison with reference compound nocodazole. The test compounds (**5g** and **6f**) significantly inhibited tubulin polymerization by 66.65 and 70.09 % respectively, whereas the reference

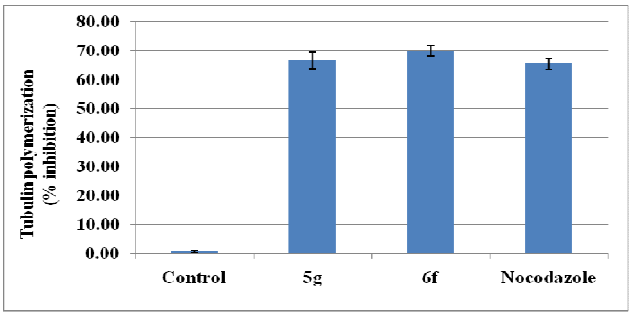

Fig. 3: Effect of compounds on tubulin polymerization: tubulin polymerization was monitored by the increase in fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37°C

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Table 3: Inhibition of tubulin polymerization (IC_{50}) of compound**5g** and**6f**

Compound	IC50[a] ± SD (in μM)
5g	1.53±0.02
6f	1.45±0.05
Nocodazole	1.60±0.03

compound (nocodazole) exhibited 65.47% inhibition (Fig. 3). Furthermore, these conjugates (**5g** and **6f**) were evaluated for their *in vitro* tubulin polymerization assay at different concentrations. These molecules (**5g** and **6f)** showed potent inhibition of tubulin polymerization with IC_{50} values of 1.53 and 1.45 μM respectively (Table 3) and nocodazole was employed as a reference compound.

Immunohistochemistry studies on tubulin

To find out *in vitro* tubulin polymerization, we investigated alterations in the microtubule network in DU-145 cells induced by conjugates **5g** and **6f** by using fluorescence microscope of immunohistochemistry studies, as most antimitotic agents affect microtubules.[36] Therefore, DU-145 cells were treated with conjugates **5g** and **6f** at 0.5 μM concentration for 48 h. The results demonstrated a well-organized microtubular network in control cells. However, cells treated with conjugates **5g** and **6f** showed disrupted microtubule organization as shown in Fig. 4, thus confirming the inhibition of tubulin polymerization.

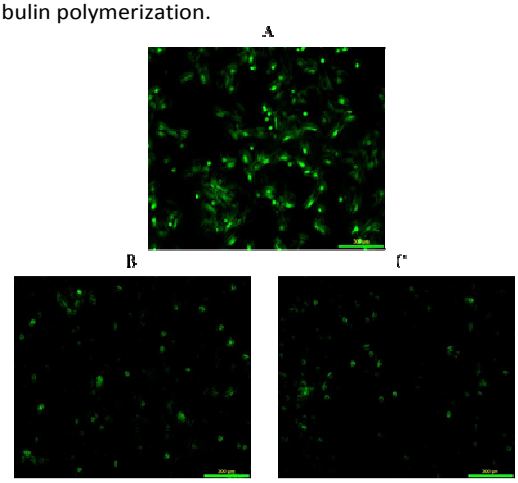

Fig. 4. Immunohistochemistry analysis of derivativeson microtubule network. DU-145 cells were treated with conjugates **5g** and**6f** at 0.5 μM concentrations for 48 h followed by staining with an antitubulin antibody and FITC conjugated secondary antibody.A: Untreated cells (control cells), B: **5g** (0.5 μM) and C:**6f** (0.5 μM).

Hoechst staining for apoptosis

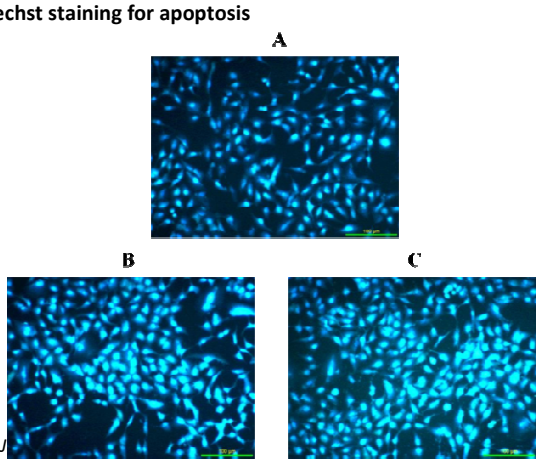

Fig. 4. Hoechst staining in DU-145 prostate cancer cell line.A: Untreated cells (control cells), B: **5g** (0.5 μM) and C:**6f** (0.5 μM).

Apoptosis is one of the major pathways that lead to the process of cell death. Chromatin condensation and fragmented nuclei are known as the classic characteristics of apoptosis. It was considered of interest to investigate the apoptotic inducing effect of the derivatives by Hoechst staining (H33258) method in DU-145 cell line. Therefore, DU-145 cells were treated with **5g** and **6f** at each 0.5 μM concentrations for 48 h. Manual field quantification of apoptotic cells based on cytoplasmic condensation, presence of apoptotic bodies, nuclear fragmentation and relative fluorescence of these derivatives (**5g** and **6f**) revealed that there was significant increase in the percentage of apoptotic cells (Fig. 5).

Measurement of mitochondrial membrane potential (ΔΨm)

The maintenance of mitochondrial membrane potential (ΔΨm) is significant for mitochondrial integrity and bioenergetic function.[37] Mitochondrial changes including loss of mitochondrial membrane potential (ΔΨm) are key events that take place during drug-induced apoptosis. Mitochondrial injury by **5g** and **6f** was evaluated by detecting drops in mitochondrial membrane potential (ΔΨm). In this study we have investigated the involvement of mitochondria in the induction of apoptosis by these derivatives. After 48 h of treatment with these derivatives at 0.5 and 1 μM concentrations, it was observed that reduced mitochondrial membrane potential (ΔΨm) of DU-145 cells which assessed by JC-1 staining (Fig. 5).

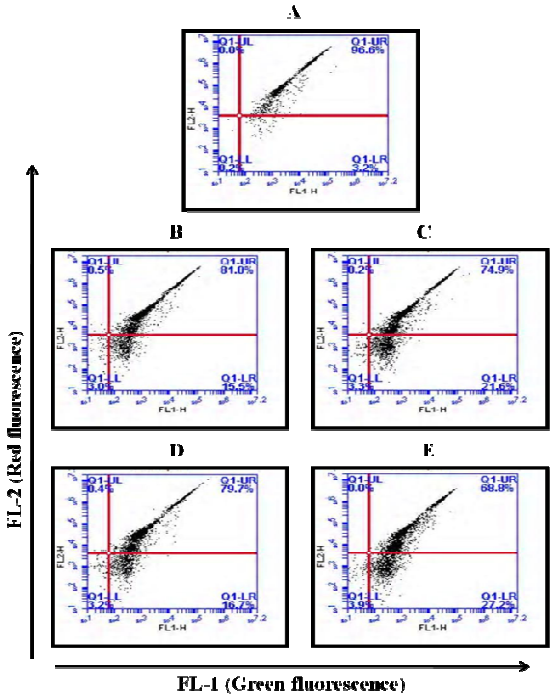

Fig. 5: Derivatives **5g** and**6f** triggers mitochondrial injury. Drops in mitochondrialmembrane potential (ΔΨm) was assessed by JC-1 staining of DU-145 cells treated with derivativesand samples were then subjected to flow cytometry analysis on a FACScan (Becton Dickinson) in the FL1, FL2 channel to detect mitochondrialmembranepotential.A: Untreated cells (control cells), B: **5g** (0.5 μM), C: **5g** (1 μM), D: **6f** (0.5 μM) and E: **6f** (1 μM).

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Annexin V-FITC assay for apoptosis

The apoptotic effect of **5g** and **6f** was further evaluated by Annexin V FITC/PI (AV/PI) dual staining assay[38] to examine the occurrence of phosphatidyl serine externalization and also to understand whether it is due to physiological apoptosis or nonspecific necrosis. In this study DU-145 cells were treated with these derivatives for 48 h at 0.5 and 1 µM concentrations to examine the apoptotic effect. It was observed that these derivatives showed significant apoptosis against DU-145 cells and results indicated that derivatives **5g** and **6f** showed 23.62 and 25.60% at 0.5µM concentration, while they exhibited 36.43% and 42.17% apoptosis at 1 µM concentration respectively as shown in Fig. 6.

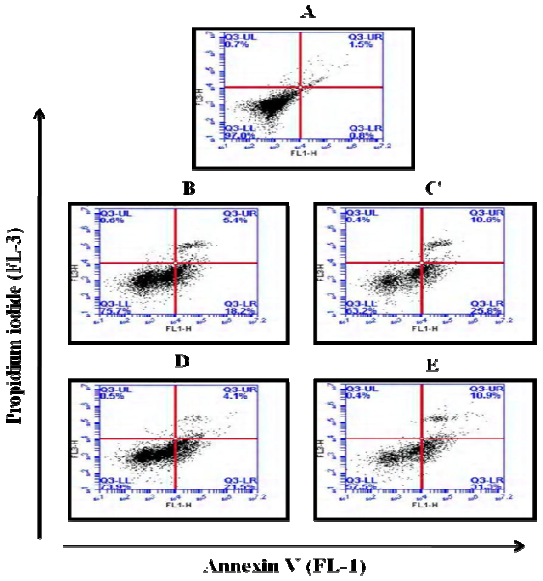

Fig. 6: Annexin V-FITC staining assay. Quadrants; Upper left (necrotic cells), Lower left (live cells), Lower right (early apoptotic cells) and Upper right (late apoptotic cells). A: Untreated cells (control cells), B: **5g** (0.5 µM), C: **5g** (1 µM), D: **6f** (0.5 µM) and E: **6f** (1 µM).

Table 4. Distribution of apoptotic cells in Annexin-V FITC experiment.

Sample	Upper left%	Upper right%	Lower left%	Lower right%
A:Untreated cells (Control)	0.69	1.55	96.99	0.77
B: 5g (0.5 µM)	0.65	5.38	75.73	18.24
C: 5g (1 µM)	0.38	10.58	63.19	25.85
D: 6f (0.5 µM)	0.52	4.09	73.88	21.51
E: 6f (1 µM)	0.36	10.87	57.47	31.30

Effect on ROS generation

Many anticancer agents have demonstrated to exert their cytotoxic effects by the generation of reactive oxygen species (ROS)[39] which is considered as one of the key mediators of apoptotic signalling. In this connection, DU-145 cells were treated with these compounds (**5g** and **6f**) at 0.5 and 1 µM concentrations for 48 h. After 48 h of treatment, ROS experiment was performed by using an oxidant-sensitive fluorescent probe, DCFDA (2',7'-dichlorofluorescindiacetate). Experimental results revealed that these compounds enhance the generation of ROS in DU-145 (Fig. 7).

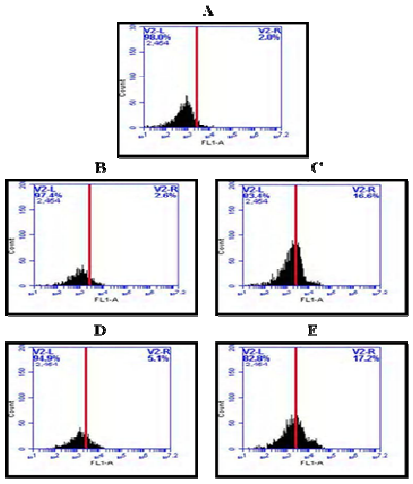

Fig. 7 The effect of **5g** and **6f** on the ROS production in DU-145 cells; A: Untreated cells (Control cells), B: **5g** (0.5 µM), C: **5g** (1 µM), D: **6f** (0.5 µM) and E: **6f** (1 µM).

Molecular docking studies

Molecular modeling studies were performed on Glide v6.0[40] (Schrodinger, LLC, New York, NY) to investigate the potential interactions between new series of synthesized compounds and protein (PDB code: 1SA0). Compounds **5g** and **6f** were docked into the colchicine binding site of β tubulin (PDB 1SA0) for studying the binding mode of these compounds for antitumor activity. The trimethoxyphenyl ring of compound **5g** formed hydrogen bonding interactions with Ser 140 and Gln 11. Additionally, the –NH group of benzimidazole ring and C=O group displayed hydrogen bonding with Thr 179 and Cys 254

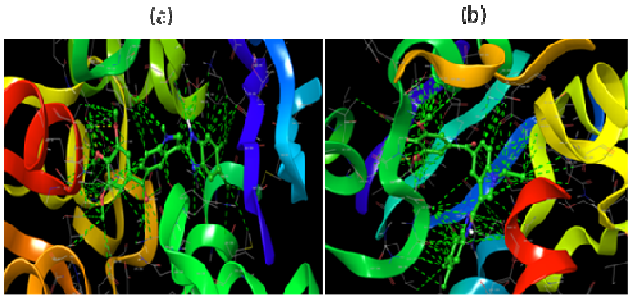

Fig. 7: (a) Docking of 5g (coloured by atom) with protein (1SA0) on colchicine-binding site of β tubulin.(b) Docking of 6f (coloured by atom) with colchicine-binding site of β tubulin. The backbone of tubulin is shown using a ribbon representation, and the interacting amino acids are shown as stick models. Green and Yellow dashed lines represent interaction with active site residues within a 4.5 Å sphere, H bonding with amino acid backbone respectively.

respectively. The interactions with other active site residues Gln 247, Leu 248, Leu 255, Asn 258, Met 259 and Lys 352 were also observed (Fig. 7a). While in compound 6f; the para, meta positions of trimethoxyphenyl ring formed hydrogen bonding with Cys 241. Moreover, the interaction with active site

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residues Gln 247, Leu 248, Ala 250, Asp 251, Leu 252, Lys 254, Leu 255, Asn 258, Ala 316, Ala 317, Lys 352, Thr 353, Ala 354, Val 355 and Ile 378 were also observed (Fig 7b).

Conclusion

In conclusion, we have synthesized trimethoxy aroyl indole-benzimidazole conjugates **4-6(a–i)** and evaluated for their antiproliferative activity against human cancer cell lines. Among them, conjugates **5g** and **6f** showed significant antiproliferative activity against human prostate cancer cell line (DU-145). The flow cytometric analysis revealed that these conjugates arrest cell cycle at G2/M phase. However, these derivatives effectively inhibited microtubule assembly and disrupt microtubule network in human prostate cancer cell line, DU-145. Further, apoptosis studies such as Hoechst staining, mitochondrial membrane potential and Annexin V FITC assay suggested that these derivatives induced cell death by apoptosis in DU-145 cells. Moreover, docking studies provided some molecular insights about the binding mode of these conjugates **5g** and **6f** that bind at the colchicine-binding site in α,β interfaces of the tubulin. Based on the above results it is evident that conjugates of this structural class, particularly conjugates **5g** and **6f** are acquiescent to further modifications and function as a suitable template for the design of new class of tubulin polymerization inhibitors and apoptosis inducers for the treatment of cancer.

Conflicts of interest

The authors declare no competing interest.

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New (3-(1*H*-benzo[d]imidazol-2-yl)/(3-(3*H*-imidazo[4,5-*b*]pyridin-2-yl)-(1H-indol-5-yl)(3,4,5-trimethoxyphenyl)methanone conjugates as tubulin polymerization inhibitors

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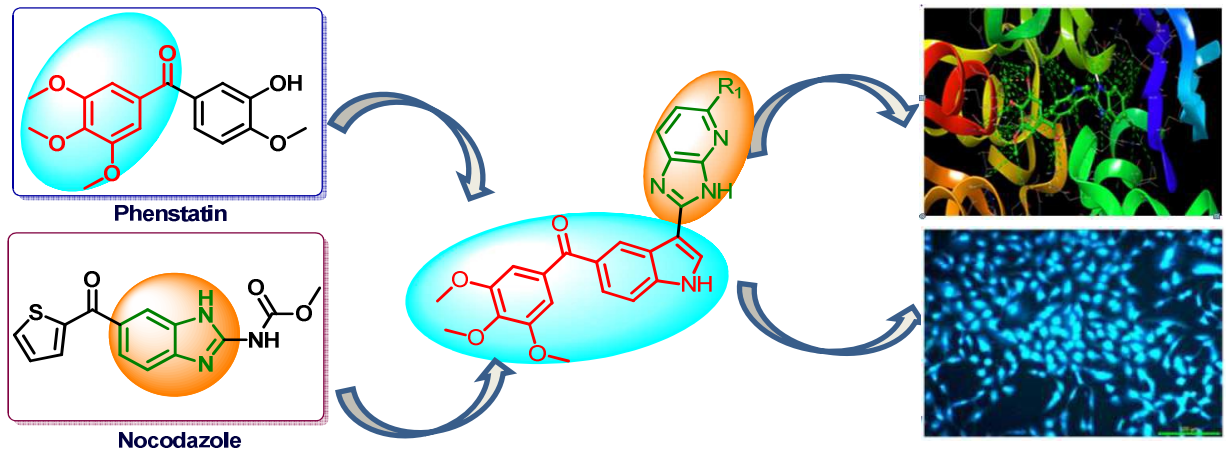

A series of (3-(1*H*-benzo[*d*]imidazol-2-yl)/(3-(3*H*-imidazo[4,5-*b*]pyridin-2-yl)-(1*H*-indol-5-yl)(3,4,5-trimethoxyphenyl) methanone conjugates were synthesized and evaluated for their antiproliferative activity on selected human cancer cell lines. Tubulin polymerization assay and immunofluorescence analysis results suggest that these compounds effectively inhibit microtubule assembly formation in DU-145.