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New (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 as tubulin polymerization inhibitors†

 Kishore Mullagiri,^a V. Lakshma Nayak,^a Satish Sunkari,^a Geeta Sai Mani,^b
 Sravanthi Devi Guggilapu,^b Burri Nagaraju,^a
 Abdullah Alarifi^c and Ahmed Kamal *^{abc}

A series of new (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 **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 IC₅₀ 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 IC₅₀ 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 and ROS generation and annexin V-FITC assays.

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Introduction

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 the 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 tubulins. In the mitotic phase, microtubules are in dynamic equilibrium with tubulin dimers as tubulin is assembled into microtubules which are disassembled into 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 naturally occurring compounds, combretastatin A-4 (**1**, Fig. 1) is one of the best characterized antimetabolic 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 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 a *cis* configuration (*Z*-geometry) plays a fundamental role in binding at the colchicine site by

Fig. 1 Tubulin polymerization inhibitors.

^a Medicinal Chemistry and Biotechnology, CSIR-Indian Institute of Chemical Technology, Hyderabad 500 007, India. E-mail: ahmedkamal@iict.res.in

^b Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad 500 037, India

^c Catalytic Chemistry Research Chair, Chemistry Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia

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positioning the rings at an 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 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 affect 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 moieties constituted the core structure of antimetabolic compounds that have been reported as tubulin polymerization inhibitors.¹⁵ On the other hand, benzimidazoles are more privileged scaffolds due to their biological properties and have been reported to possess potential anticancer activity and anti-HIV activity,^{16–24} apart from their 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 possesses 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 hybrids³² and phenstatin/isocombrestatin-oxindole conjugates.³³ Considering the biological importance of these moieties (phenstatin, indole and benzimidazole), an 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 an indole moiety at the 3rd position. Thus, the rationale behind 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 their anti-proliferative activity and their structure–activity relationship (SAR) was examined 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, measure-

ment of mitochondrial membrane potential and annexin V assay were elucidated.

Results and discussion

Chemistry

The trimethoxy aroyl indole-benzimidazole conjugates 4–6(a–i) were synthesized in six straightforward 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 the corresponding 1-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°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 the TBDMS group with tetra-*n*-butylammonium fluoride (TBAF) provided 10a. Next, the 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 ($\text{Na}_2\text{S}_2\text{O}_5$) as shown in Scheme 1. All these new derivatives were characterized by spectral analysis such as $^1\text{H-NMR}$, ^{13}C NMR and HRMS.

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-trimethoxybenzaldehyde, *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) $\text{Na}_2\text{S}_2\text{O}_5$, EtOH : H_2O , 80°C , 2 h, 68–85%.

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 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 the MTT assay.³⁴ The results are summarized in Table 1 and the IC₅₀ values are expressed in μM and compared with nocodazole as the control. Some of the derivatives show significant activity against most of the cell lines tested with IC₅₀ 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 IC₅₀ values of <5 μM and the majority of derivatives showed good cytotoxicity against the prostate cancer cell line (DU-145). However, derivatives 5g and 6f were found to be very active against the DU-145 cell line with IC₅₀ 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.

Cell cycle analysis. Many anticancer compounds exert their cytotoxic effect either by arresting the cell cycle at a particular checkpoint of the cell cycle or by induction of apoptosis or by 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% cell accumulation in the G2/M phase at 0.5 μM concentration, whereas they exhibited 38.47 and 39.80% cell accumulation at 1 μM concentration, respectively (Fig. 2 and Table 2).

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 the 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 polymerization³⁶ was thus examined by monitoring the increase in fluorescence emission at 420 nm (excitation wavelength is 360 nm) in a 384-well plate for 1 h at 37 °C with and without the conjugates in comparison with the reference compound nocodazole. The test compounds (5g and 6f) significantly inhibited tubulin polymerization by 66.65 and 70.09%, respectively, whereas the reference compound (nocodazole) exhibited 65.47% inhibition (Fig. 3). Furthermore, these conjugates (5g and 6f) were evaluated for their *in vitro* tubulin polymerization activity at different concentrations. These molecules (5g and 6f) showed potent inhibition of tubulin polymerization with IC₅₀ values of 1.53 and 1.45 μM , respectively (Table 3), and nocodazole was employed as the reference compound.

Immunohistochemistry studies on tubulin. To examine *in vitro* tubulin polymerization, we investigated alterations in the microtubule network in DU-145 cells induced by conjugates 5g and 6f by using fluorescence microscopy in immunohistochemistry studies, as most antimitotic agents affect

Table 1 IC₅₀^a 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.

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).

Table 2 Distribution of DU-145 cells in various phases of the 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 μ M)	1.61	79.15	2.23	14.56
C: 5g (1 μ M)	0.65	57.53	2.07	38.47
D: 6f (0.5 μ M)	1.77	77.75	2.32	15.15
E: 6f (1 μ M)	0.48	57.05	1.83	39.80

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.

microtubules.³⁶ Therefore, DU-145 cells were treated with conjugates **5g** and **6f** at 0.5 μ M 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.

Hoechst staining for apoptosis. 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 apoptosis-inducing effect of the derivatives by the Hoechst staining (H33258) method on the DU-145 cell line. Therefore, DU-145 cells were treated with **5g** and **6f** each at 0.5 μ M concentration for 48 h. Manual field quantification of apoptotic cells based on cytoplasmic condensation, the presence of apoptotic bodies, nuclear fragmentation and relative fluorescence of these derivatives (**5g** and **6f**) revealed that there was a significant increase in the percentage of apoptotic cells (Fig. 5).

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$). The maintenance of mitochondrial membrane potential ($\Delta\Psi_m$) is significant for mitochondrial integrity and bio-

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

Compound	$IC_{50}^a \pm SD$ (in μ M)
5g	1.53 ± 0.02
6f	1.45 ± 0.05
Nocodazole	1.60 ± 0.03

^a 50% inhibitory concentration after 48 h of drug treatment.

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

energetic function.³⁷ Mitochondrial changes including loss of mitochondrial membrane potential ($\Delta\Psi_m$) 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 ($\Delta\Psi_m$). 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 μ M concentrations, a reduced mitochondrial membrane potential ($\Delta\Psi_m$) of DU-145 cells which was assessed by JC-1 staining (Fig. 6) was observed.

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 assay³⁸ to examine the occurrence of phosphatidylserine externalization and also to understand whether it is due to physiological apoptosis or nonspecific

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

Fig. 6 Derivatives **5g** and **6f** trigger mitochondrial injury. Drops in mitochondrial membrane potential ($\Delta\Psi_m$) were assessed by JC-1 staining of DU-145 cells treated with the derivatives and samples were then subjected to flow cytometry analysis on a FACScan (Becton Dickinson) in the FL1 and FL2 channels to detect the mitochondrial membrane potential. A: Untreated cells (control cells), B: **5g** (0.5 μM), C: **5g** (1 μM), D: **6f** (0.5 μM) and E: **6f** (1 μM).

necrosis (Table 4). In this study, DU-145 cells were treated with these derivatives for 48 h at 0.5 and 1 μM concentrations to examine their apoptotic effect. It was observed that these derivatives showed significant apoptosis against DU-145 cells and the results indicated that derivatives **5g** and **6f** showed 23.62 and 25.60% at 0.5 μM concentration, while they exhibited 36.43% and 42.17% apoptosis at 1 μM concentration, respectively, as shown in Fig. 7.

Effect on ROS generation

Many anticancer agents have been 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

Fig. 7 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).

Fig. 8 The effect of **5g** and **6f** on 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).

Table 4 Distribution of apoptotic cells in the 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

μM concentrations for 48 h. After 48 h of treatment, the ROS experiment was performed by using an oxidant-sensitive fluorescent probe, DCFDA (2',7'-dichlorofluorescein diacetate). The experimental results revealed that these compounds enhance the generation of ROS in DU-145 (Fig. 8).

Molecular docking studies

Molecular modeling studies were performed on Glide v6.0 (ref. 40) (Schrodinger, LLC, New York, NY) to investigate the

Fig. 9 (a) Docking of **5g** (coloured by atom) with protein (1SA0) into the colchicine-binding site of β -tubulin. (b) Docking of **6f** (coloured by atom) into the 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 the interaction with active site residues within a 4.5 Å sphere and H bonding with the amino acid backbone, respectively.

potential interactions between the 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) to study 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 the benzimidazole ring and the $C=O$ group displayed hydrogen bonding with Thr 179 and Cys 254, respectively. Interactions with other active site residues Gln 247, Leu 248, Leu 255, Asn 258, Met 259 and Lys 352 were also observed (Fig. 9a). Meanwhile, for compound **6f**, the *para* and *meta* positions of the trimethoxyphenyl ring formed hydrogen bonds with Cys 241. Moreover, interactions with active site 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. 9b).

Conclusion

In conclusion, we have synthesized trimethoxy aroyl indole-benzimidazole conjugates **4–6(a–i)** and evaluated their anti-proliferative activity against human cancer cell lines. Among them, conjugates **5g** and **6f** showed significant anti-proliferative activity against the human prostate cancer cell line (DU-145). The flow cytometric analysis revealed that these conjugates arrest the cell cycle at the G2/M phase. However, these derivatives effectively inhibited microtubule assembly and disrupt the microtubule network in the human prostate cancer cell line, DU-145. Further, apoptosis studies such as Hoechst staining, measurement of 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 into 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 suitable templates for the design of a 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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