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Design, synthesis and biological evaluation of imidazopyridine–propenone conjugates as potent tubulin inhibitors†‡

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A library of imidazopyridine–propenone conjugates (**8a–8u**) were synthesized and evaluated for their anti-tumor activity against four human cancer cell lines, namely, prostate (DU-145), lung (A549), cervical (Hela) and breast (MCF-7) cancer cell lines. These conjugates showed good to moderate activity against the tested cell lines. Among them, two conjugates (**8m** and **8q**) showed significant antiproliferative activity against the human lung cancer cell line (A549) with IC₅₀ values of 0.86 μ M and 0.93 μ M, respectively. Flow cytometry analysis revealed that these compounds arrested the cell cycle at the G₂/M phase in the human lung cancer cell line (A549), inhibiting tubulin polymerization leading to apoptosis. Further, Hoechst staining, decrease in mitochondrial membrane potential and Annexin V-FITC assay suggested that the cell death was due to apoptosis induction. Overall, the present investigation demonstrated that the synthesized imidazopyridine–propenone conjugates are promising tubulin inhibitors and apoptotic inducers.

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Introduction

In eukaryotic cells, microtubules are key components and essential in a variety of elemental cellular processes such as cell formation, cell division, maintenance of cell shape, regulation of motility, secretion and cytoplasmic transport, making microtubules an important target for anticancer drugs.^{1–3} In addition, microtubules are also involved in cell signalling pathways which are accountable for cellular apoptosis. The microtubule dynamics are regulated by different important proteins such as dynein and kinesin.⁴ Various reports have recognized that microtubule-dependent force is essential for chromosomal translocation and spindle formation.⁵ The irregular microtubule dynamics result in the obstruction of cell division at the metaphase; as a result, various efforts aimed at blocking mitosis, like inhibition of tubulin polymerization by tubulin targeting agents, have emerged as an effective approach to treat cancer.^{6,7} Many literature reports are available on the inhibition of tubulin polymerization with subsequent arrest of cells during mitosis leading to apoptosis.⁸ Microtubule polymerization inhibitors are effective in the treatment of breast, lung, ovarian and other cancers. Colchicine (**I**) and

nocodazole (**II**) are prominent examples of compounds (Fig. 1) that inhibit assembly of microtubules by binding to tubulin.^{9,10} Among the said compounds, nocodazole shows preferential interference in the destabilization of microtubule polymerization.

Imidazopyridine is a fused bicyclic heterocycle that represents an important class of privileged scaffolds.¹¹ This scaffold displays a broad spectrum of biological activity such as inflammation, tumour suppression, viral, apoptosis, fungal and bacterial.¹² This heterocyclic core can also be found in drugs like olprinone, nicopidem, saripidem, zolpidem and zolimidine.¹³ Investigations demonstrated that this scaffold

Fig. 1 Chemical structures of microtubule targeting agents: colchicine (**I**), nocodazole (**II**), imidazopyridine guanylhydrazones (**III**), imidazopyridine–benzimidazoles (**IV**), aryl propenones (**V**) and imidazopyridine–propenone conjugates **8(a–u)**.

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can be extensively used owing to its various therapeutic benefits. Many attempts to discover new drugs through creative discovery of technologies have fallen short of producing the expected results. Henceforth, privileged structure-guided scaffold re-evolution is a primary strategy to identify structurally novel chemotypes by modifying either the central core of the scaffold or the side chain of existing active compounds.¹⁴ In this regard, our group previously reported imidazopyridine-benzimidazoles¹⁵ (IV) as apoptosis inducers that inhibit tubulin polymerization by binding at the colchicine binding site on tubulin. Thus imidazopyridine motifs provide immense opportunity to exploit undescribed bioactivities by making use of readily derivatized motifs with well established synthetic protocols of imidazopyridine.

Aryl-propenones¹⁶ are a new class of compounds reported recently as potent tubulin binders which block mitotic cell division leading to apoptotic cell death. This, combined with our previous work on aryl-propenones containing molecules as tubulin polymerization inhibitors,¹⁷ prompted us to combine an aryl-propenone chain with the imidazopyridine scaffold, which may exhibit an interesting cytotoxicity profile. Our continued efforts to discover effective anticancer agents through the combination of the said two scaffolds led us to design and synthesize a new series of twenty-one imidazopyridine-propenone conjugates that consist of a hybrid molecule with three rings. In addition, a comprehensive structure-activity relationship has been established by varying the substituents on the rings. These synthesized conjugates were tested for their antiproliferative effect on the human lung cancer cell line (A549). The two most active molecules in the series (conjugates **8m** and **8q**) were further investigated for their ability to inhibit tubulin assembly and induce apoptosis. The results of our investigations along this direction are presented in this work.

Results and discussion

Chemistry

The synthesis of the imidazopyridine-propenones **8(a-u)** is shown in Scheme 1 (also given in the ESI†). To obtain 2-arylimidazopyridine **4(a-c)**, equimolar mixtures of substituted 2-bromoacetophenones and 2-aminopyridine were refluxed for 4–5 h, followed by addition of 2 N HCl under reflux conditions. The intermediates imidazopyridine alde-

hydes **5(a-c)** were prepared by means of Vilsmeier-Haack reaction on the corresponding 2-arylimidazopyridine **4(a-c)**. These aldehydes **5(a-c)** were further treated with ethynylmagnesium bromide in THF to obtain the intermediates **6(a-c)**, followed by oxidation with IBX in DMSO, providing the corresponding precursors **7(a-c)**. Subsequently, the desired compounds **8(a-u)** were prepared by reaction of the corresponding precursors **7(a-c)** with arylamines in ethanol. The list of substituents, along with their corresponding yields and respective melting points, are listed in Table 1.

Biological evaluation

Cytotoxic activity. The synthesized conjugates were evaluated for their cytotoxic potential against four human cancer cell lines, namely, prostate (DU-145), lung (A549), cervical (Hela) and breast (MCF-7) cancer cells, by employing MTT¹⁸ assay using nocodazole as a reference drug. The structure-activity relationship was analysed based on the cytotoxicity results given in Table 2. For ease of understanding, the rings in the molecule are labelled A, B and C, as shown in Fig. 2. Structural modifications were performed at the 4th position of rings B and C, respectively, whereas ring A was left as such. The IC₅₀ values of these conjugates showed considerable cytotoxic activity ranging from 0.86–22.78 μM. The synthesized conjugates fall into three categories depending on the substituents on ring B, as follows: compounds **8(a-h)** with 4-methoxy substitution, conjugates **8(i-m)** with no substitution and compounds **8(n-u)** with chlorine substituted at the 4th position. Among the conjugates **8(a-h)**, it can be observed from the data that conjugate **8g** (IC₅₀ – 1.05 μM) and conjugate **8h** (IC₅₀ – 1.21 μM) have displayed improved cytotoxicity on human lung cancer cells compared to nocodazole (IC₅₀ – 1.39 μM). Conjugate **8c** (IC₅₀ – 1.34 μM) is equally

Table 1 Structures of compounds **8(a-u)** and their yields

Compound	R ₂	R ₁	Yield %	Melting point (°C)
8a	3,5-Dimethoxyphenyl	OCH ₃	74%	152–154
8b	4-Methoxyphenyl	OCH ₃	75%	130–132
8c	4-Bromophenyl	OCH ₃	71%	174–176
8d	4-Fluorophenyl	OCH ₃	74%	118–120
8e	5-Indolyl	OCH ₃	71%	218–220
8f	6-Indolyl	OCH ₃	66%	198–200
8g	3-Quinolyl	OCH ₃	62%	210–212
8h	6-Quinolyl	OCH ₃	64%	232–234
8i	3,5-Dimethoxyphenyl	H	78%	151–153
8j	4-Methoxyphenyl	H	82%	172–174
8k	4-Bromophenyl	H	75%	160–162
8l	3-Quinolyl	H	65%	188–190
8m	6-Quinolyl	H	69%	204–206
8n	3,4,5-Trimethoxyphenyl	Cl	72%	194–196
8o	3,5-Dimethoxyphenyl	Cl	73%	120–122
8p	4-Methoxyphenyl	Cl	67%	96–98
8q	4-Bromophenyl	Cl	79%	208–210
8r	5-Indolyl	Cl	61%	251–253
8s	6-Indolyl	Cl	64%	219–221
8t	3-Quinolyl	Cl	69%	229–231
8u	6-Quinolyl	Cl	71%	214–216

Scheme 1 Synthesis of imidazopyridine-propenones.

Table 2 IC₅₀ (μM) values^a for compounds **8(a–u)** on selected human cancer cell lines

Compound	DU-145 ^b	A549 ^c	Hela ^d	MCF-7 ^e
8a	6.261	4.467	4.571	14.86
8b	11.13	12.65	19.95	16.50
8c	1.452	1.346	4.545	9.833
8d	6.130	3.251	10.95	13.00
8e	11.33	10.97	13.60	18.94
8f	10.69	8.800	15.32	19.35
8g	5.280	1.052	9.440	14.95
8h	4.720	1.211	4.852	11.63
8i	2.924	2.239	3.090	10.22
8j	2.190	1.570	14.90	7.609
8k	1.820	1.349	2.042	8.667
8l	2.443	1.750	3.076	5.167
8m	1.076	0.861	1.219	1.807
8n	3.250	1.138	3.020	3.388
8o	11.97	9.897	14.56	13.58
8p	10.79	6.458	11.85	16.33
8q	1.500	0.933	1.479	2.692
8r	1.288	1.014	3.243	7.125
8s	2.630	2.291	10.93	9.600
8t	4.168	2.884	3.715	18.860
8u	13.630	13.040	16.350	22.780
Nocodazole	1.259	1.393	1.611	1.086

^a 50% inhibitory concentration after 48 h of drug treatment.^b Human prostate cancer. ^c Human lung cancer. ^d Human cervical cancer. ^e Human breast cancer. **Fig. 2** Structure–activity relationship of imidazopyridine–propenone conjugates.

active as nocodazole when tested on lung cancer cells. The presence of three methoxy groups in conjugate **8a** has caused moderate cytotoxicity on the four tested cancer cell lines. Among conjugates **8(i–m)**, particularly, conjugate **8m** with quinolyl substitution on ring C possessed significant activity against human lung cancer and prostate cancer cells with IC₅₀ values of 0.86 μM and 1.07 μM, respectively. Also, conjugate **8m** (IC₅₀ – 1.21 μM) was found to exert potent cytotoxicity on Hela cells in comparison with the rest of the conjugates. Conjugate **8k** (IC₅₀ – 1.34 μM) with bromine at the 4th position of ring C exerted cytotoxicity close to that of nocodazole. An overview on the cytotoxicity results of conjugates **8(n–u)** reveals that conjugate **8q**, having a chlorine atom at the 4th position of ring B and a bromine atom at the 4th position of ring C, displayed an IC₅₀ value of 0.93 μM on the human lung cancer cell line. Conjugate **8r** (IC₅₀ – 1.28 &

Fig. 3 Flow cytometric analysis of the A549 lung cancer cell line after treatment with compounds **8m** and **8q** at 0.5 and 1 μM concentrations for 48 h. A: Control cells (A549); B: nocodazole (1 μM); C: **8m** (0.5 μM); D: **8m** (1 μM); E: **8q** (0.5 μM) and F: **8q** (1 μM).

1.01 μM) with 5-indolyl substitution on ring C was found to display potent cytotoxicity against human prostate cancer cells and human lung cancer cells, respectively. A look at the substituents on ring B shows that the potency is maximum when the ring is unsubstituted, followed by a good profile with an electron withdrawing halogen (chlorine), and less potency with an electron donating substituent (methoxy). Similarly on ring C, heterocyclic substituents (quinolyl, indolyl) were found to be highly active, followed by withdrawing substituents (bromine, fluorine), compared to a donating substituent (methoxy) on ring C.

Effect on cell cycle arrest

Many anticancer compounds exert their growth inhibitory effect either by arresting the cell cycle at a particular checkpoint or by induction of apoptosis or a combination of both.^{19,20} The screening results revealed that both the compounds **8m** and **8q** showed improved antiproliferative activity against the lung cancer cell line (A549). To understand that the cell growth inhibition was due to cell cycle arrest, we performed cell cycle analysis using human lung cancer (A549) cells. In this study, A549 cells were treated with compounds **8m** and **8q** at concentrations of 0.5 and 1 μM for 48 h. The data obtained clearly indicated that these compounds exhibited G₂/M cell cycle arrest in comparison with the untreated cells. The compounds **8m** and **8q** showed 15.69% and 14.40% cell accumulation in the G₂/M phase at 0.5 μM concentration, whereas they exhibited 30.50 and 29.84% cell accumulation at 1 μM concentration, respectively (Fig. 3 and Table 3).

Table 3 Distribution of A549 cells in various phases of the cell cycle

Sample	Sub G ₁ %	G ₀ /G ₁ %	S%	G ₂ /M%
A: Control (A549)	1.11	90.52	1.92	7.40
B: Nocodazole (1 μM)	0.79	66.92	4.55	29.68
C: 8m (0.5 μM)	0.50	82.37	2.77	15.69
D: 8m (1 μM)	0.91	66.32	4.28	30.50
E: 8q (0.5 μM)	0.66	83.28	2.68	14.40
F: 8q (1 μM)	0.86	65.94	5.62	29.84

Effect of compounds on tubulin polymerization

In general, G₂/M cell cycle arrest is strongly associated with inhibition of tubulin polymerization²¹ and since compounds **8m** and **8q** caused 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 polymerization^{22,23} 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 **8m** and **8q** inhibited tubulin polymerization by 68.21% and 66.53%, respectively, whereas the reference compound (nocodazole) inhibited 66.83% of tubulin polymerization (Fig. 4). This was followed by the evaluation of the IC₅₀ values for these conjugates, and the results are shown in Table 4. It was observed that these conjugates (**8m** and **8q**) showed tubulin assembly inhibition with IC₅₀ values of 1.82 and 1.93 μM, respectively.

Immunohistochemistry studies on tubulin

As most antimitotic agents affect microtubules, the alterations in the microtubule network in A549 cells induced by **8m** and **8q** by using a fluorescence microscope were investigated. The cells were treated with **8m** and **8q** at 0.5 μM concentration for 48 h. The results demonstrated a well-organized microtubular network in the control cells; however, cells treated with these compounds and nocodazole resulted

Fig. 4 Effect of conjugates on tubulin polymerization: tubulin polymerization was monitored by means of the increasing fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37 °C.

Table 4 Inhibition of tubulin polymerization (IC₅₀) by compounds **8m** and **8q**

Compound	IC ₅₀ ^a ± SD (in μM)
8m	1.82 ± 0.06
8q	1.93 ± 0.03
Nocodazole	1.98 ± 0.04

^a Concentration of drug to inhibit 50% of tubulin assembly.

in disrupted microtubule organization (Fig. 5), thus confirming the inhibition of tubulin polymerization.

Hoechst staining for apoptosis

Apoptosis, with its classic characteristics of chromatin condensation and fragmented nuclei, is one of the major pathways that lead to the process of cell death. The study was performed using the A549 cell line by the Hoechst staining (H33258) method. In the study, A549 cells were treated with the test compounds at 0.5 μM concentration for 48 h. Manual field quantification of apoptotic cells based on cytoplasmic condensation, presence of apoptotic bodies, nuclear fragmentation and relative fluorescence of the test compounds (**8m** and **8q**) revealed that the compounds exerted apoptotic activity, as evident from the increase in the percentage of apoptotic cells (Fig. 6).

Measurement of mitochondrial membrane potential (ΔΨm)

Maintenance of mitochondrial membrane potential (ΔΨm) is considered vital for mitochondrial integrity and bioenergetic function.²⁴ Loss of mitochondrial membrane potential (ΔΨm) is a key event that takes place during drug-induced apoptosis. Mitochondrial injury by compounds **8m** and **8q** was evaluated by measuring the drop in mitochondrial membrane potential

Fig. 5 Immunohistochemistry analysis of the microtubule network in A549 cells treated with conjugates **8m** and **8q** at 0.5 μM concentration for 48 h followed by staining with an antitubulin antibody and an FITC conjugated secondary antibody. A: Control (A549); B: nocodazole (0.5 μM); C: **8m** (0.5 μM) and D: **8q** (0.5 μM).

Fig. 6 Hoechst staining of the A549 lung cancer cell line. A: Control cells (A549); B: nocodazole (0.5 μ M); C: **8m** (0.5 μ M) and D: **8q** (0.5 μ M). The arrows indicate apoptotic cells.

Fig. 7 Compounds **8m** and **8q** trigger mitochondrial injury. The drop in membrane potential ($\Delta\Psi$ m) was assessed by JC-1 staining of A549 cells treated with the test compounds, and the samples were then subjected to flow cytometry analysis using a FAC scan (Becton Dickinson) in the FL1 and FL2 channels to determine the mitochondrial potential. A: Control cells (A549); B: nocodazole (1 μ M); C: **8m** (0.5 μ M); D: **8m** (1 μ M); E: **8q** (0.5 μ M) and F: **8q** (1 μ M).

($\Delta\Psi$ m). In this study, we investigated the involvement of mitochondria in the induction of apoptosis by these compounds. Af-

Fig. 8 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: Control cells (A549); B: nocodazole (1 μ M); C: **8m** (0.5 μ M); D: **8m** (1 μ M); E: **8q** (0.5 μ M) and F: **8q** (1 μ M).

ter 48 h of drug treatment with these compounds at 0.5 and 1 μ M concentrations, it was observed that there was a considerable reduction in the mitochondrial membrane potential ($\Delta\Psi$ m) of the A549 cells, as assessed by JC-1 staining (Fig. 7).

Annexin V-FITC for apoptosis

The apoptotic effect of **8m** and **8q** was further evaluated by Annexin V FITC/PI dual staining assay²⁵ to examine the occurrence of phosphatidyl-serine externalization and also to understand whether it was due to physiological apoptosis or nonspecific necrosis. In this study, A549 cells were treated with these compounds for 48 h at 0.5 and 1 μ M concentrations to examine the apoptotic effect. It was observed that compounds **8m** and **8q** showed good apoptotic effect against A549 cells, as shown in Fig. 8. Results indicated that compounds **8m** and **8q** showed 34.27% and 31.85% apoptosis at 0.5 μ M concentration, whereas they exhibited 53.51% and

Table 5 Distribution of apoptotic cells in the Annexin-V FITC experiment

Sample	Upper left %	Upper right %	Lower left %	Lower right %
A: Control(A549)	1.17	3.83	93.97	1.03
B: Nocodazole (1 μ M)	1.11	16.23	51.48	31.19
C: 8m (0.5 μ M)	0.75	11.35	64.98	22.92
D: 8m (1 μ M)	1.07	18.52	45.42	34.99
E: 8q (0.5 μ M)	1.64	9.94	66.51	21.91
F: 8q (1 μ M)	1.38	18.06	46.59	33.97

Fig. 9 Binding model of ligands at the interface of the α,β -tubulin heterodimer. Ligands are shown in ball and stick models (yellow colour). Hydrogen bonding interactions are shown as green coloured dashed lines and the residues involved are represented by thin tube models. 9A) Represents the binding mode of **8m**; 9B) represents the binding mode of **8q**.

52.03%, respectively, at 1 μ M concentration (Fig. 8 and Table 5).

Molecular modelling study

Molecular docking studies were performed on the two selected compounds **8m** and **8q** to authenticate the obtained experimental results. These compounds were successfully docked in the colchicine binding site of tubulin (PDB code: 3UT5)²⁶ using the Schrödinger docking program.²⁷ The binding mode was predicted and analysed using Glide docking which showed that these compounds bind in the colchicine

binding pocket at the interface of the α - and β -chains. For **8m**, the N atom in the imidazopyridine ring has shown H-bonding with the Asn A101 (1.96 Å) residue of the α -chain. Further, in the case of **8m** the protein ligand complex was stabilized by hydrophobic interactions with other residues, like Ala A180, Val A181, Tyr A224, Met B259, Leu B255, Cys B241, Ala B250, Ile B378, Ile B318, Ala B354, Ala B316, Tyr B202, Val B238. The N atom in the imidazopyridine ring and the O atom of the carbonyl group of **8q** have shown H-bondings with Asn A101 (1.97 Å) and Ser A178 (2.62 Å) residues, respectively. Other residues like Val A181, Ala A180, Tyr A224, Ala B250, Cys B241, Leu B255, Ala B354, Ala B316, Ile B318, Ile B378 and Met B259 were found to be involved in hydrophobic interactions with **8q**. The binding models for compounds **8m** and **8q** are shown in Fig. 9.

Conclusion

In the present study, we synthesized 21 conjugates of imidazopyridine-propenone (**8a–8u**) and evaluated their anti-proliferative activity against four human cancer cell lines, namely, prostate (DU-145), lung (A549), cervical (Hela) and breast (MCF-7) cancer. Among them, compounds **8m** and **8q** showed significant antiproliferative activity (IC_{50} , 0.86 μ M and 0.93 μ M, respectively) against the human lung cancer cell line (A549). Flow cytometry analysis revealed that these conjugates caused cell cycle arrest at the G₂/M phase. These conjugates (**8m** and **8q**) exerted their cytotoxicity by inhibiting tubulin polymerization, with an IC_{50} value of 1.82 μ M and 1.93 μ M, respectively. Further, Hoechst staining, mitochondrial membrane potential and Annexin V FITC assay studies suggested that these compounds induced cell death by apoptosis in human lung cancer cells (A549). The docking studies gave an insight that these compounds bind at the colchicine site of the tubulin. Overall, the encouraging biological profiles exhibited by imidazopyridine-propenone conjugates make them promising tubulin polymerization inhibitors and apoptosis inducers.

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