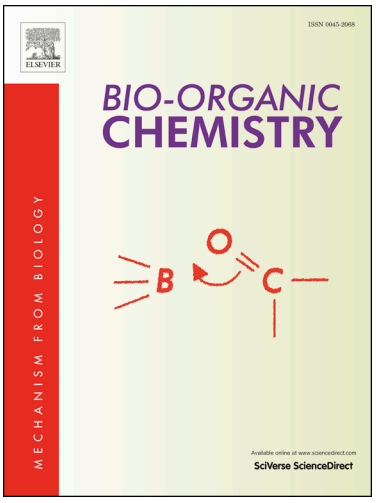

Imidazopyridine linked triazoles as tubulin inhibitors, effectively triggering apoptosis in lung cancer cell line

Ibrahim Bin Sayeed, M.V.P.S. Vishuwardhan, Apoorva Nagarajan, Srinivas Kantevari, Ahmed Kamal

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Imidazopyridine linked triazoles as tubulin inhibitors, effectively triggering apoptosis in lung cancer cell line

Ibrahim Bin Sayeed,[a,b] M.V.P.S. Vishuwardhan,[a] Apoorva Nagarajan,[b,d] Srinivas Kantevari,[b,d] Ahmed Kamal.[*a,b,c]

[a]*Medicinal Chemistry and Pharmacology Division, CSIR - Indian Institute of Chemical Technology, Hyderabad 500007, India*

[b]*Academy of Scientific and Innovative Research (AcSIR), CSIR - Indian Institute of Chemical Technology, Hyderabad 500007, India*

[c]*School of Pharmaceutical Education and Research, Jamia Hamdard University, New Delhi 110062, India*

[d]*Department of Crop Protection Chemicals, CSIR- Indian Institute of Chemical Technology, Hyderabad 500007, India*

Abstract: A library of new imidazopyridine linked triazole hybrid conjugates **(8a-r)** were designed, synthesized and evaluated for their cytotoxicity against four cancer cell lines namely, human lung (A549), human prostate (DU-145), human colon (HCT-116) and breast (MDA-MB 231) cancer. These conjugates exhibited good to moderate activity against the tested human cancer cell lines. Two of the conjugates (**8g** and **8j**) showed significant antitumor activity against human lung cancer cell line (A549) with IC_{50} values of **0.51**µM and **0.63**µM respectively. Flow cytometry analysis revealed that these conjugates arrested the cell cycle at G_2/M phase in human lung cancer cell line (A549). Immune-histochemistry and tubulin polymerization assay suggest inhibition of tubulin. Hoechst staining, annexin V and DNA fragmentation by tunnel assay suggested that these compounds induce cell death by apoptosis. Overall, the current study demonstrates that the synthesis of imidazopyridine linked triazole conjugates as promising anticancer agents causing G_2/M arrest and apoptotic-inducing ability.

Keywords: imidazopyridine, triazoles, tubulin, apoptosis, lung cancer

Introduction

Microtubules are important part of endoskeleton and play a key role in diverse cellular processes such as cell formation, cell division, regulation of motility; maintenance of cell shape, secretion and cytoplasmic transport, thus making microtubules an important target for anticancer drugs [1-3]. Furthermore, microtubules play a significant role in cell signalling pathways which are accountable for cellular apoptosis. The dynamics of this heterodimeric polymer is regulated by various important proteins such as dynein and kinesin [4]. Various

reports have established the indispensible role of microtubule in chromosomal translocation and spindle formation [5]. Colchicines bind to the soluble hetero dimers of tubulin at colchicine binding site which accommodate the diverse similar ligand structures. However, colchicine binding affinity varies depending on the source of tubulin; vinblastin in presence of increases the colchicine binding site affinity. Further, colchicine-tubulin complex decreases dimer dissociation constant by reducing the GTPase activity. Any aberration in dynamics of this protein results in the hindrance of cell division at metaphase; as a result, various efforts are made to block mitosis, like inhibition of tubulin polymerization by tubulin targeting agents has emerged as an effective approach for cancer treatment [6-7]. Many reports in literature recognise tubulin polymerization inhibition with subsequent arrest of cells during mitosis leading to apoptosis [8]. Microtubule binding agents are effective in the treatment of lung, breast, ovarian and other cancers. Colchicines (**I**) and nocodazole (**II**) are the prominent examples of compounds (**Figure 1**) that inhibit assembly of microtubule by binding to tubulin [9-10].

Nitrogen-bridge head fused heterocycles containing an imidazole ring is common scaffold in various pharmacologically important lead molecules, exhibiting a wide range of biological activities by binding on different targets. The most widely implied heterocyclic scaffold from this group is imidazopyridine [11]. This bi-nitrogen fused heterocycle have a broad spectrum of biological activities such as, inhibitors of aromatase estrogen production suppressors [12a,b], potential positive inotropic agents [13], platelet aggregation, thromboxane synthetase inhibitors [14a,b], antiviral [15], and anticancer [16] being important amongst them. Imidazopyridine-guanylhydrazone (**III**) showed potent activity against various human cancer cell lines and it is considered as a promising lead. Imidazopyridine scaffold exhibit different types of molecular mechanisms in cancer chemotherapy such as down regulation of cell regulatory proteins like cyclin-D1, CDK2, up regulation of P27 and activation of Caspase-3 [17,18]. Similarly one of the imidazopyridine derivative namely 2,2,2-trifluoro-N-(6-(2-fluoro-5-methylbenzoyl)imidazo[1,2-*a*]pyridin-2-yl)acetamide (**IV**) induced cell cycle arrest at G_2/M phase suggesting tubulin arrest [19]. In this regard, we recently reported imidazopyridine-propenones [20] (**V**) as apoptosis inducers that inhibit assembly of tubulin by binding at the colchicines binding site of tubulin.

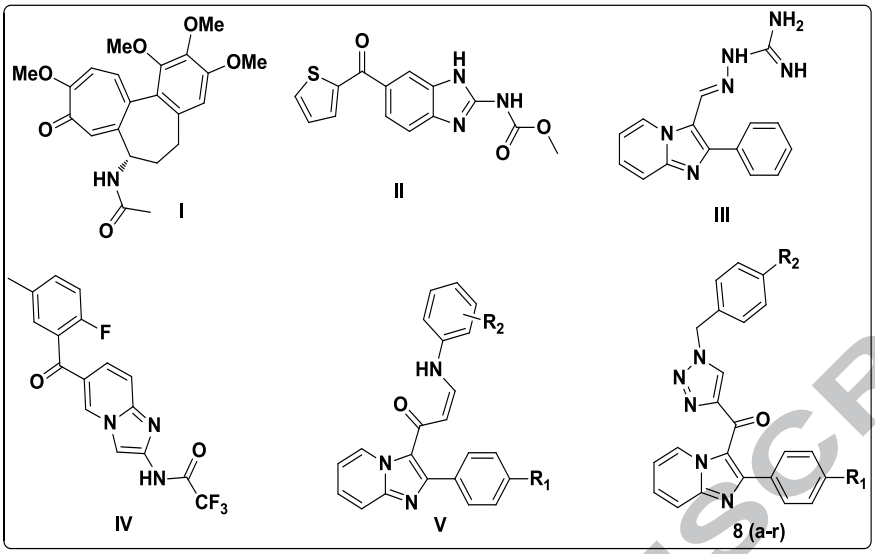

Figure 1: Chemical structures of microtubule targeting agents: colchicine (**I**), nocodazole (**II**), imidazopyridine guanylhydrazones (**III)**, 2,2,2-trifluoro-N-(6-(2-fluoro-5-methylbenzoyl)imidazo[1,2-*a*]pyridin-2-yl)acetamide (**IV**), imidazopyridine-propenones (**IV**) and imidazopyridine linked triazole hybrids **8 (a-r)**.

Similarly, 1,2,3-triazole derivatives have attracted the medicinal chemists worldwide, due to their wide range of biological activities, such as antifungal [21], antitubercular [22] and antitumor activity [23-25]. 1,2,3-Triazoles are also serve as building blocks for the synthesis of important bioorganic-conjugates. Moreover, triazoles possess a high dipole moment and are highly capable of forming hydrogen bonding. Considering the above biological significance about triazole scaffold, researchers have used this nucleus in the development of numerous biologically active compounds. Triazole hybrids of biological importance have been developed by various research groups across the globe.

Our continued efforts to discover new and effective anticancer agents targeting tubulin through the combination of biologically important pharmacophores prompted us to design synthesize and evaluate imidazopyridine linked triazole conjugates by combining two biologically significant pharmacophores such as imidazopyridine and triazole scaffold respectively. Our designed scaffold constitutes of three rings. In addition, a comprehensive structure activity relationship has been described by varying the substitutions on the rings. These synthesized conjugates were evaluated for their cytotoxic effect in human lung cancer cell line (A549). The two most active conjugates from the library namely (**8g** and **8j**) were further investigated for their ability to inhibit tubulin assembly and for their ability to induce apoptosis. The results of our investigation in this direction are presented in this work.

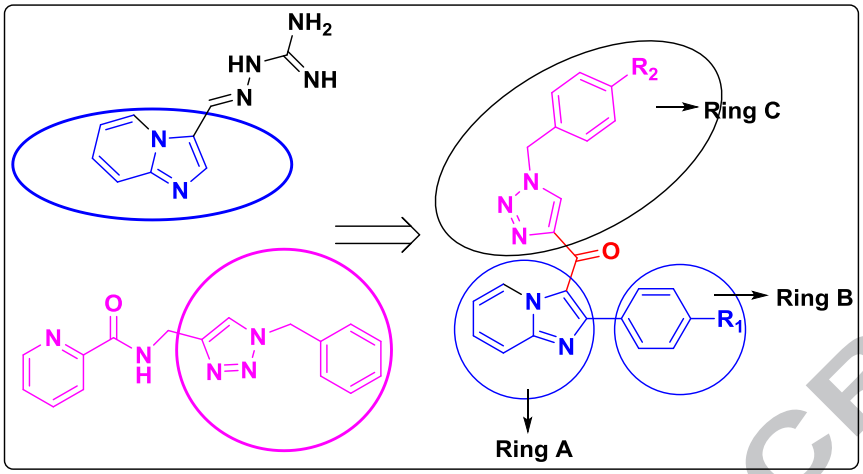

Figure 2: Design concept for Imidazopyridine linked triazole derivatives

Results and Discussions

Chemistry

Synthesis of the imidazopyridine linked triazoles (**8a-r**) is shown in **Scheme 1**. To obtain 2-arylimidazopyridine (**4a-c**), equimolar mixtures of substituted 2-bromoacetophenones and 2-aminopyridine were refluxed for 4-5h followed by 2N HCl under reflux conditions. The intermediates, imidazopyridine aldehydes (**5a–c**) were prepared by means of Vilsmeier-Haack reaction on the corresponding 2-arylimidazopyridine (**4a–c**). These aldehydes (**5a-c**) were further treated with ethynylmagnesium bromide in THF to obtain the intermediates (**6a-c**) followed by oxidation with 2-iodoxybenzoic acid (IBX) in DMSO providing the corresponding precursors (**7a-c**). Subsequently, the desired compounds (**8a-r**) were prepared by reaction of corresponding precursors (**7a-c**) with benzyl azide in tertiary butanol and water using click chemistry. The list of substitutions along with their corresponding yields and respective melting points are listed in **Table1**.

Scheme 1: Synthesis of imidazopyridine-triazole hybrids

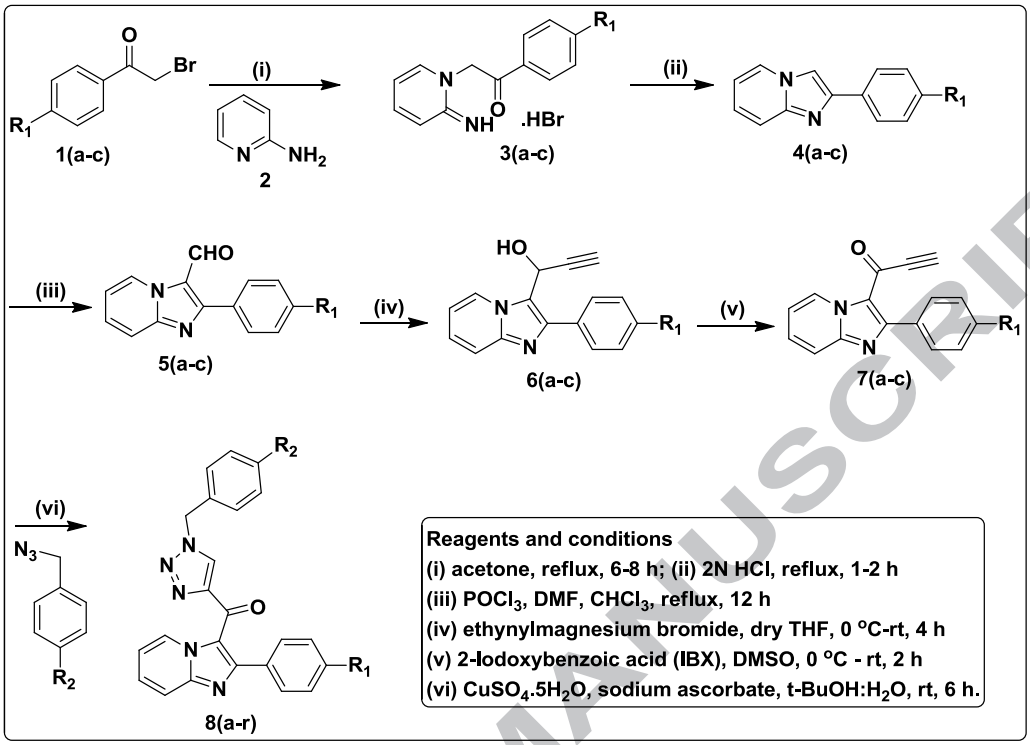

Table 1: Structures of compounds **8(a–r)** and their yields

Compound	R_2	R_1	Yield %
8a	3,4,5-trimethoxyphenyl	OCH_3	77%
8b	3,4-dimethoxyphenyl	OCH_3	75%
8c	4-methoxyphenyl	OCH_3	85%
8d	4-flourophenyl	OCH_3	83%
8e	4-chlorophenyl	OCH_3	81%
8f	4-bromophenyl	OCH_3	78%
8g	3,4,5-trimethoxyphenyl	H	86%
8h	3,4-dimethoxyphenyl	H	78%
8i	4-methoxyphenyl	H	77%
8j	4-chlorophenyl	H	82%
8k	4-flourophenyl	H	84%
8l	4-bromophenyl	H	77%
8m	3,4,5-trimethoxyphenyl	Cl	77%
8n	3,4-dimethoxyphenyl	Cl	79%
8o	4-methoxyphenyl	Cl	81%
8p	4-flourophenyl	Cl	82%
8q	4-chlorophenyl	Cl	80%
8r	4-bromophenyl	Cl	82%

Biological evaluation

Table 2: IC_{50} (μM) values[a] for compounds **8(a-r)** on selected human cancer cell lines

Compound	A549[b]	DU-145[c]	HCT 116[d]	MDA MB-231[e]	HEK 293[f]	SI[g]
8a	22.41±0.27	47.64±0.08	12.50±0.63	19.10±0.37	86.53±0.58	3.86
8b	12.75±0.35	19.17±1.67	20.55±0.09	6.30±0.95	65.32±0.57	5.12
8c	12.15±0.88	14.09±0.07	19.40±0.59	15.75±0.88	77.35±0.37	6.37
8d	10.22±0.95	5.02±0.92	14.46±0.08	15.28±0.59	99.12±1.04	9.70
8e	16.16±2.74	15.11±0.17	6.16±0.38	3.09±0.67	87.35±0.81	5.41
8f	1.17±0.52	1.47±0.63	22.55±0.44	10.51±0.07	75.45±0.43	64.50
8g	**0.51**±0.32	1.04±0.51	1.05±0.19	1.75±0.33	63.53±1.33	124.57
8h	12.86±0.49	8.91±0.88	19.53±0.71	3.16±0.92	72.54±0.94	5.64
8i	1.91±0.76	1.30±0.13	9.48±0.81	1.62±0.88	88.68±0.72	46.43
8j	0.63±0.49	1.11±0.84	2.83±0.35	3.98±1.05	91.25±2.18	144.84
8k	1.93±0.08	1.71±0.73	10.62±0.06	1.94±0.16	93.26±0.94	48.32
8l	14.63±0.55	11.4±0.39	18.96±0.22	8.88±0.37	98.65±0.82	6.74
8m	14.13±1.18	26.99±1.58	10.67±0.86	25.84±0.46	77.65±0.94	5.49
8n	23.79±0.58	18.15±0.16	16.53±0.17	10.90±0.27	64.28±0.17	2.70
8o	47.92±0.19	14.73±0.99	35.05±0.17	11.07±0.82	73.56±0.67	1.54
8p	26.40±0.86	11.14±0.42	32.78±0.93	15.40±0.73	79.62±0.39	3.02
8q	11.13±0.94	12.99±0.67	10.37±1.06	12.84±0.61	59.65±1.18	5.36
8r	3.79±0.73	8.14±0.29	11.53±0.84	10.13±0.71	90.36±1.83	23.84
Nocodazole	1.47±0.14	1.38±0.16	1.65±0.94	1.56±0.54	-	-
Taxol	0.04±0.07	0.009±0.04	0.015±0.09	0.005±0.08	-	-
Colchicine	0.1±0.03	0.7±0.02	0.5±0.83	0.3±0.95	-	-

[a]50% Inhibitory concentration after 48 h of drug treatment; [b]human lung cancer; [c]human prostate cancer; [d]human colon cancer; [e]human breast cancer; [f]Human embryonic kidney cells (normal cell line); [g]SI-selective index corresponding to A549 cell line

Cytotoxic activity

To better understand the structure activity relationships of imidazopyridine linked triazole hybrid congeners **8**(**a-r**), the IC_{50} values were obtained by employing MTT cytotoxicity assay [26]. Our designed scaffold consists of 3 rings as depicted in the **Figure 3**. For convenience the rings are labeled **A**, **B** and **C**. For SAR study, changes were made at rings **B** and **C** respectively whereas ring **A** was kept unchanged. The cytotoxicity of the synthesized congeners was evaluated against four human cancer cell lines namely lung (A549), prostate (DU-145), colon (HCT116) and breast (MDA-MB-231) cancer cells. The assay was performed using taxol and colchicine as reference drugs. The results are summarized in **Table 2**. The IC_{50} values of these conjugates shows considerable cytotoxic activity ranging from 0.51µM – 47.94 µM. Particularly congener **8g**, having an H atom at ring **B** and 3,4,5-trimethoxy group at ring **C** posses significant activity against human lung cancer and prostate cancer cells with IC_{50} values of **0.51**µM and **1.04**µM respectively. Another congener **8j** having H atom at 4th position of ring **B** and chlorine atom at 4th position on ring **C**, displayed IC_{50} value 0.63µM and 1.11µM respectively. An overview on the substituents on ring **B** shows that the potency is high when the ring is unsubstituted followed by good profile with an electron withdrawing halogen (chlorine) and less with an electron donating substituent (methoxy) is present on ring **B**. Similarly on ring **C** trimethoxy group was showed to have maximum potency among the donating group and in case of withdrawing groups, chlorine atom showed maximum potency on ring **C**.

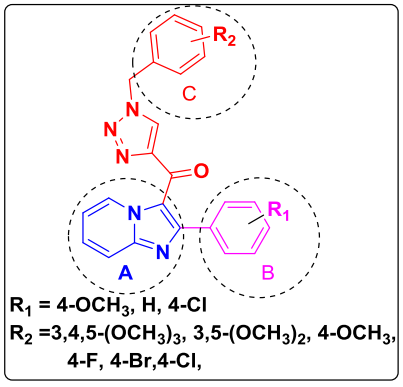

Figure 3: Structure activity relationship of imidazopyridine linked triazole hybrids

Cell cycle analysis

Cell cycle analysis plays an important role in studies for anticancer screening, mechanism and cytokinetic research [27]. To gain further insight into the mode of action of **8g** and **8j** as the most potent compounds, we examined their effect on the cell cycle by flow cytometry in A549 cells. These results clearly demonstrate that the treatment of cells for 48h with 1 µM of compound **8g** and **8j** causes cell cycle arrest at G_2/M phase as seen in **Figure 4** (see supplementary information file) and **Table 3**.

Table 3: Cell cycle distribution of A549 cells (%) for **8g** and **8j**

S.No	**Sub G_1**	**G_1**	**S**	**G_2/M**
Control	7.5	78.2	8.6	5.7
8g	3.5	18.8	7.5	**70.2**
8j	5.5	19.5	6.5	**68.5**

Effect on microtubule network

To substantiate the observed cytotoxic effects of these conjugates **8g** and **8j** on the inhibition of tubulin polymerization to functional microtubules, immunocytochemistry studies have been carried further to examine the *insitu* effects of conjugates **8g** and **8j** on cellular microtubules. Therefore, A549 cancer cells were treated with **8g** and **8j** at 1µM concentration for 48h. From the data, the results revealed that untreated human lung cancer cells displayed normal distribution of microtubules as seen in **Figure 5**. However, cells treated with **8g** and **8j** showed disrupted microtubule organization as observed thereby demonstrating the inhibition of tubulin polymerization. However, the positive control nocodazole showed disrupted microtubule organization. This immunocytochemistry study indicated that the level of tubulin polymerization inhibition was similar to that of standard nocodazole for these conjugates **8g** and **8j** on lung cancer cells.

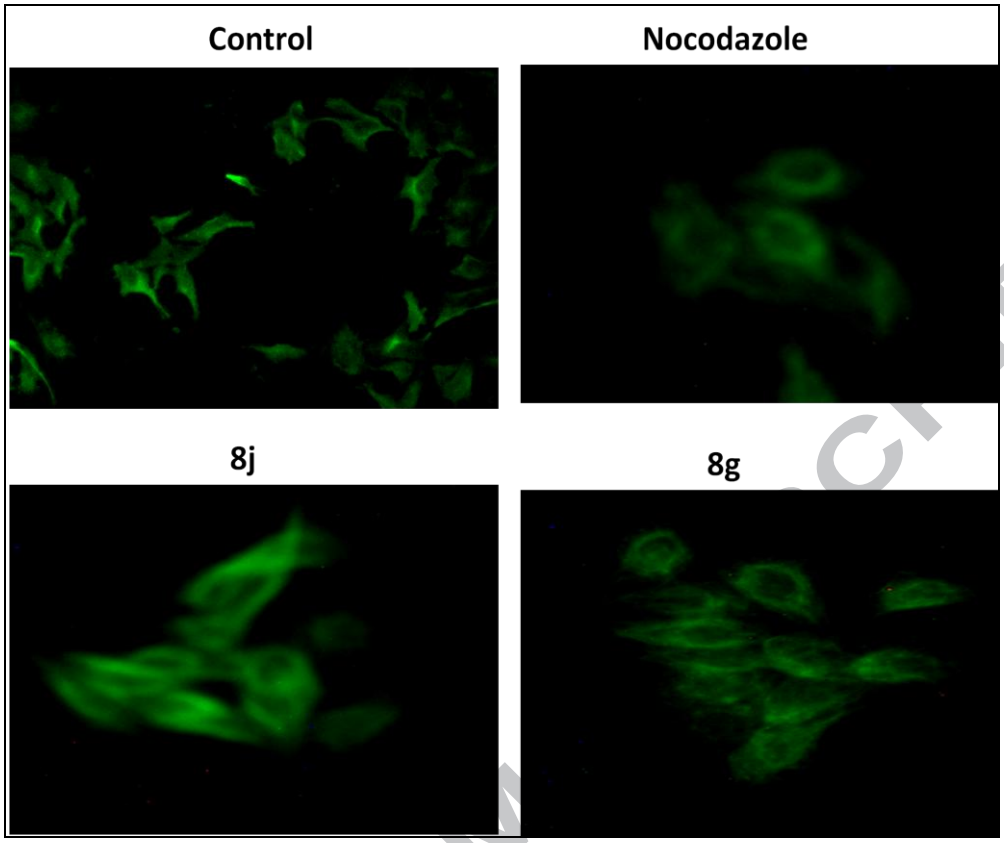

Figure 5: Immunocytochemistry (ICC) analyses of compounds on the microtubule network; A549 cells were treated with compounds **8g** and **8j** and nocodazole at 1µM concentration for 48 h followed by staining with α-tubulin antibody with nocodazole as positive control.

Effect of compounds on tubulin polymerization

To elucidate whether the antiproliferative activities of these conjugates 8g and 8j were related to the interaction with tubulin, we studied their effects on the tubulin polymerization in a cell-free system [28,29]. As tubulin subunits heterodimerize and self-assemble to form microtubules in a time dependent manner, we investigated the progression of tubulin polymerization by monitoring the increase in fluorescence emission at 420 nm (excitation wavelength is 360 nm) in a 384 well plate for 30 min at 37 ^{0}C with and without the compounds at 3µM concentration. Conjugates **8g** and **8j** inhibited tubulin polymerization by 59.46%, 56.08% respectively, compared to the control **Figure 6**, (supplementary information file). Tubulin polymerization inhibition was also observed in the case of the standard like nocodazole (54.73%).

Effect on DNA (tunel assay and hoechst staining)

A terminal transferase dUTP nick end labelling assay was carried out with a view to understand the effect of **8g** and **8j** on DNA integrity by treating A549 cells at 1µM for 24 h.

Figure 7 (given in supplementary information file) illustrates that DNA fragmentation (green FITC staining) was observed in the treated cells whereas there is no fragmentation in the untreated cells, thereby suggesting apoptotic cell death. Moreover, Hoechst DNA staining also indicated the formation of apoptotic bodies (blebbing) in cells treated with conjugate **8g** and **8j**, which is more prominent than untreated cells, as apparent in **Figure 7** and the results demonstrated that **8g** and **8j** have apoptotic-inducing ability.

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 on A549 cell line by Hoechst staining [30] (H33258) method. In the study, A549 cells were treated with the test compounds at 1µM concentrations for 48 h. Manual field quantification of apoptotic cells based on cytoplasm condensation, presence of apoptotic bodies, nuclear fragmentation and relative fluorescence of the test compounds (**8g** and **8j**) revealed that the compounds exerted apoptotic activity as evident from the increase in the percentage of apoptotic cells (**Figure 8,** see supplementary file).

Measurement on reactive oxygen species (ROS)

Accumulation of ROS in human lung cancer (A549) cells treated with compound **8g** and **8j** was estimated. Therefore we investigated whether ROS production has increased after treatment with the test compounds. Apoptosis can be initiated by oxidative stress which may be mediated by the generation of reactive oxygen species (ROS). As these conjugates **8g** and **8j** is cytotoxic and induces apoptosis in A549 cells, it was considered of interest to understand its effect of conjugates on the generation of ROS [31]. Therefore, cells were incubated with **8g** and **8j** for 24 h at 1µM concentration and the intracellular generation of ROS was examined using DCFH-DA dye by fluorescent microscopy. As shown in **Figure 9**, there was a significant increase in DCF positive cell population by **8g** and **8j** as compared to the control which reveals that it induces apoptosis through intracellular ROS in A549 cells. Altogether, these results indicate that this compound induced apoptosis through the mitochondrial pathway.

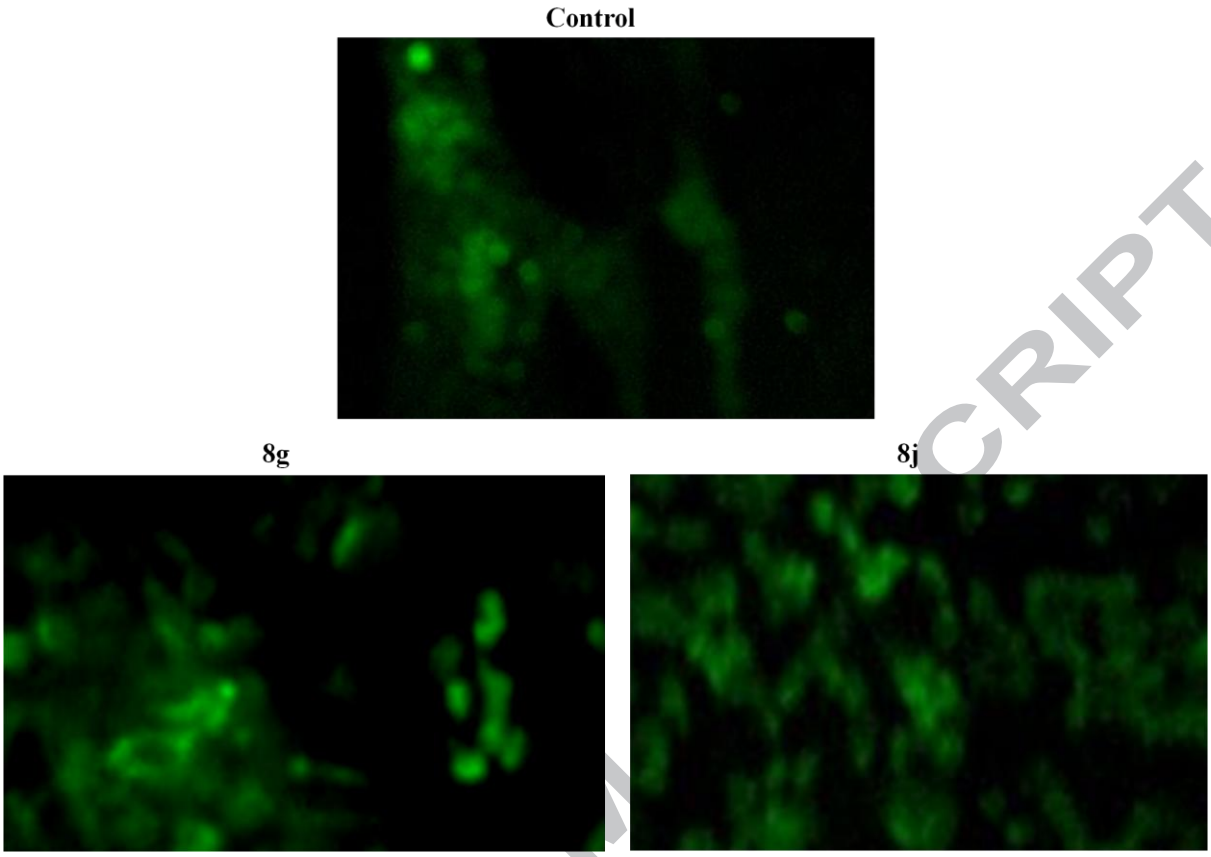

Figure 9: Effect of conjugates **8g** and **8j** on reactive oxygen species in Lung cancer cells.

Annexin v-fitc for apoptosis

The apoptotic inducing ability is also investigated by Annexin V FITC/PI (AV/PI) dual staining [32] assay to examine the occurrence of phosphatidylserine externalization and also to understand whether it is due to physiological apoptosis or nonspecific necrosis. In this study A549 cells were treated with compounds **8g** and **8j** for 48 h at 1µM concentration to examine (**Figure 10**) the apoptotic effect. It was observed that these compounds showed significant apoptosis against A549 cells as shown in. Results indicated that compounds **8g** and **8j** showed **57.6%** and **62.7%** of apoptosis, respectively whereas **14.4%** of apoptosis was observed in control (untreated cells). From this experiment it was suggested that these conjugates significantly induce apoptosis in A549 cells.

Molecular docking studies

In*silico* docking studies were performed to gather information about the binding profile of the two most potent molecules of the series, viz., compounds **8g** and **8j** in the colchicine binding site of tubulin dimer. The three-dimensional crystal structure of tubulin was taken from RCSB Protein Data Bank (PDB ID: 3E22) [33] for docking studies. Auto dock 4.2 [34]

was used for the docking study. Docking results were viewed in Discovery Studio 2016 [35]. The study indicated that both these compounds bind at the interface of α/β chains of the tubulin dimer. Compound **8g** exhibited various interactions with the residues in the binding pocket. Conventional hydrogen bonds were seen between the carbonyl oxygen and Thr 349, nitrogen atom of imidazopyridine ring and Ile332, nitrogen atom of the triazole ring and Gly350. The oxygen atoms in the methoxy substituents were also involved in hydrogen bonding with amino acid residues Asp179, Asn329 and Ile341. Other bonds stabilizing the compound in the binding site include π-alkyl stacking between the phenyl substituent and Ala333, imidazopyridine ring and Lys336. The docking image is shown in **Figure 11**.

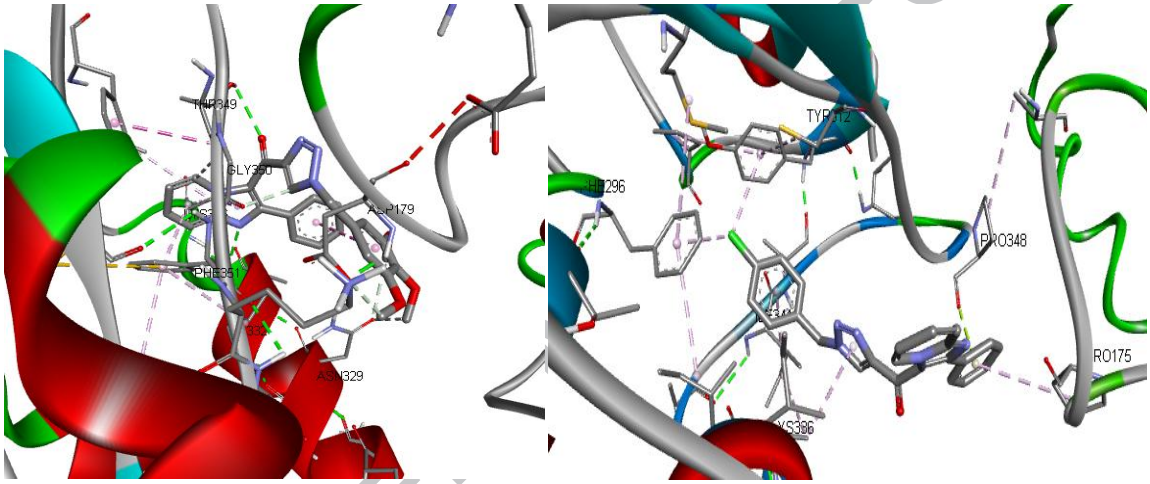

Figure 11: Binding interaction of compound **8g** (left) and binding interactions of compound **8j** (right)

Compound **8j** showed substantial interactions such as hydrogen bonding between nitrogen on triazole ring and Lys336 and Ile341. Pro348 and Pro175 were seen to form π-π interactions with the phenyl substituent on the imidazole ring. More such bonds were seen between Ile332, Ile335 and imidazopyridine ring and the benzyl ring bearing the chloro- substituent respectively. Phe226 and Tyr312 formed similar kinds of bonds with the chloro- substituent. The docking image is shown in **Figure 11**.

Conclusion

In the present study, we synthesized 18 hybrid conjugates of imidazopyridine linked triazole **8(a-r)** and evaluated for their cytotoxic activity against four human cancer cell lines namely prostate (DU-145), lung (A549), HCT-116 (Colon) and breast (MCF-7) cancer. Among them, compounds **8g** and **8j** showed significant cytotoxic activity ($IC_{50,}$ 0.51 µM and 0.63

μM respectively) against human lung cancer cell line (A549). Flow cytometry analysis revealed that these conjugates caused cell cycle arrest at G_2/M phase suggesting inhibition of tubulin polymerization, Hoechst 33258 staining and DNA fragmentation by tunnel, ROS and Annexin V also suggests that **8g** and **8j** induces cell death by apoptosis. Overall, the current study demonstrates that these new imidazopyridine linked triazolehybrids, particularly **8g** and **8j** have the potential to be developed as new class of anticancer agents by further structural modifications.

Notes

Refer to the supplementary information file for experimental procedures, spectral data and biological assay procedures and the relevant figures.

Conflicts of interest

There are no conflicts of interest to declare.

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Research Highlights

- Imidazopyridine-triazole conjugates **8(a-r)** were synthesized and evaluated for their anti-proliferative activity.
- Conjugates **8g** and **8j** showed IC_{50} values 0.51 & 0.63µm respectively
- Conjugates arrest cell growth in G_2/M phase indicating tubulin arrest.
- Triggering of apoptosis was confirmed through Hoescht staining and annexin V assay.

Imidazopyridine linked triazoles as tubulin inhibitors, effectively triggering apoptosis in lung cancer cell line

Ibrahim Bin Sayeed,[a,b] M.V.P.S. Vishuwardhan,[a] Apoorva Nagarajan,[b,d] Srinivas Kantevari,[b,d] Ahmed Kamal.[*a,b,c]

[a]*Medicinal Chemistry and Pharmacology Division, CSIR - Indian Institute of Chemical Technology, Hyderabad 500007, India*

[b]*Academy of Scientific and Innovative Research (AcSIR), CSIR - Indian Institute of Chemical Technology, Hyderabad 500007, India*

[c]*School of Pharmaceutical Education and Research, Jamia Hamdard University, New Delhi 110062, India*

[d]*Department of Crop Protection Chemicals, CSIR- Indian Institute of Chemical Technology, Hyderabad 500007, India*