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Design, synthesis and biological evaluation of imidazopyridine/imidazopyrimidine-benzimidazole conjugates as potential anticancer agents

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A series of imidazopyridine/imidazopyrimidine-benzimidazole conjugates (**11a-t**) were synthesized and evaluated for their antiproliferative activity. All these conjugates showed moderate to better cytotoxic activity against the human cervical (Hela), lung (A549), prostate (DU-145) and melanoma (B-16) cancer cell lines. Among them, conjugates **11i** and **11p** showed significant antiproliferative activity against lung cancer cell line A549 with IC_{50} values 1.48 and 1.92 µM respectively. Flow cytometric analysis revealed that these conjugates induced cell cycle arrest at G_2/M phase in A549 cell line leading to caspase-3 dependent apoptotic cell death. The tubulin polymerization assay (IC_{50} of **11i** is 2.06 µM and **11p** is 2.26 µM) and immuofluorescence analysis displayed that these conjugates effectively inhibit microtubule assembly at both molecular and cellular levels in A549 cells. Further, Hoechst staining, caspase 3 activation assay, DNA fragmentation analysis and Annexin V-FITC assay also suggested that these compounds induced cell death by apoptosis. Furthermore, molecular docking studies indicated that these conjugates interact and bind efficiently with tubulin protein. Overall, the present study demonstrates that the synthesis of imidazopyridine/imidazopyrimidine-benzimidazole conjugates as promising tubulin inhibitors with G_2/M phase cell cycle arrest and apoptotic-inducing ability.

Introduction

According to WHO reports, cancer is an important cause of death worldwide and accounted for 8.2 million deaths (around 13% of all deaths) in 2012. Deaths due to cancer, are expected to rise to over 13.1 million in 2030.[1] The word cancer often invokes the spectral of an relentlessly lethal process. However, cancer is diverse and can follow different paths, not all of which develop to metastates and deaths and include lethargic disease that causes not much harm during the patient's lifetime. [2]

Microtubules are important cellular targets for anticancer therapy. The suppressing of microtubule dynamics, which is required for proper mitotic function, effectively blocks the cell cycle progression and results in apoptosis.[3] These microtubules have structural subunits of heterodimer as α and β-tubulin, which are cytoskeletal elements that are important for intracellular transfer as well as cell division in all eukaryotes.[4] Interfering with the energetic instability of microtubules, spindle poisons capture dividing cells in G_2/M phase of the cell cycle, causing mitotic catastrophy and this lead to apoptotic cell death. The well-known natural tubulin binding molecules affect by stabilizing (Paclitaxel, **1**) (Fig 1.) or destabilizing (nocodazole, **2**) microtubule assembly.[5-6] Paclitaxel and related compounds are important antitumor drugs, currently used for the treatment that induce tubulin polymerization and microtubule stabilization.[7-9] Nocodazole is another antimitotic drug that has high affinity to bind to tubulin and inhibit microtubule.[10] In addition, nocodazole exhibits significant effect on microtubule dynamic instability in interphase cells and purified brain tubulin.[11]

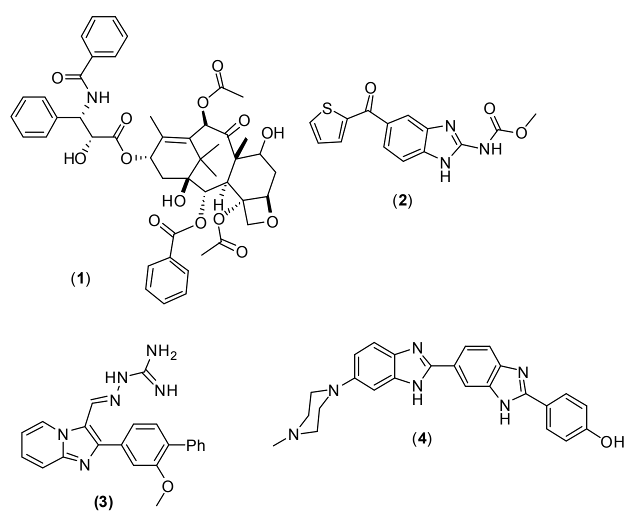
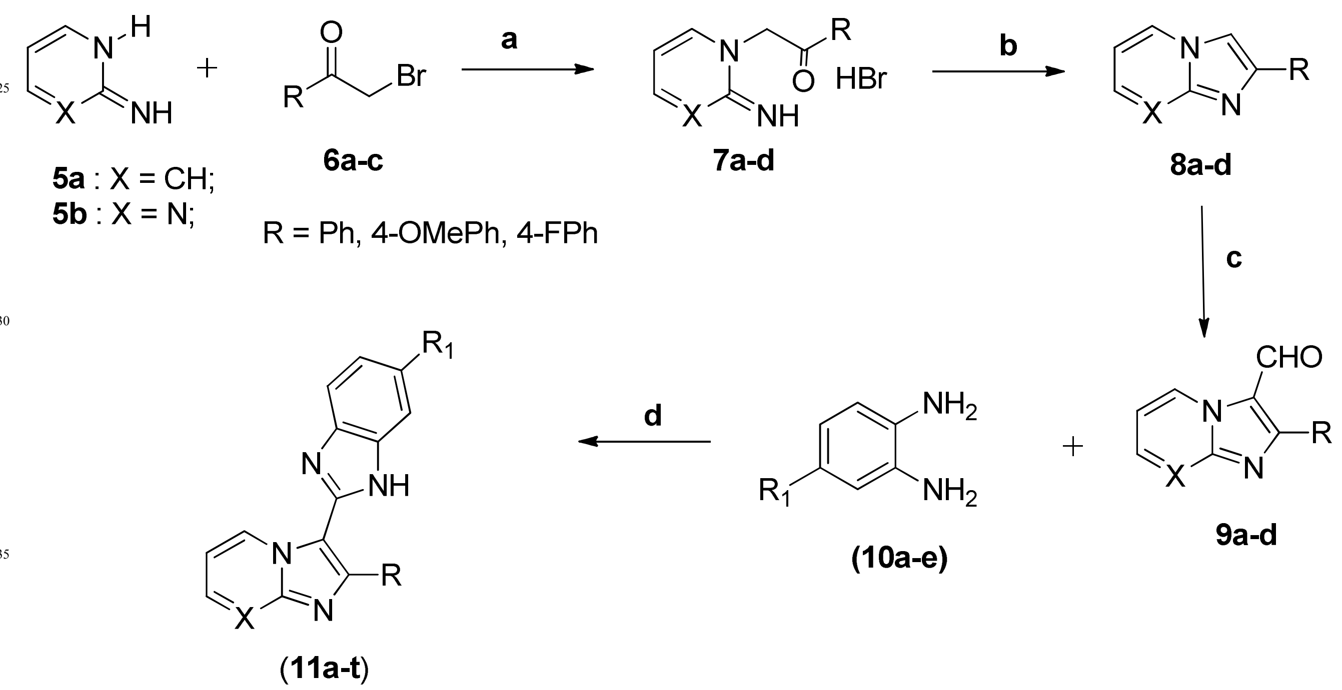


Fig 1. Chemical structures of microtubule targeting agents. Paclitaxel (**1**) Nocodazole (**2**), imidazopyridines (**3**), Hoechst 32258 (**4**).

Over the past few years there is considerable interest in the synthesis and pharmacological studies of heteroaromatic organic compounds like imidazopyridines (**3**), possess promising anticancer activity[12] (Fig 1.). Recently we reported that imidazopyridine-oxindole derivatives and conjugates of pyrazole-oxindole exhibit excellent antiproliferative activity by inhibiting the polymerization of tubulin and inducing apoptosis.[13,14] Benzimidazoles are important due to their wide range of biological activities such as antitumor,[15,16] anti-angiogenesis[17] and anti vascular activity.[18]

Some of the benzimidazole pharmacophores like bisbenzimidazole Hoechst 33258 (**4**) displayed broad spectrum of antiproliferative activity with DNA minor groove binding and inhibition of topoisomerase ability (Fig 1.).[19] Some of the novel benzimidazole-2-carbamate derivatives (BzCs) inhibit the microtubule polymerization mechanism through selectively binding to the β-tubulin subunit in which mutations have been identified that lead to drug resistance.[20]

The excellent biological activity exhibited by these conjugates prompted us to explore some newer conjugates by linking two pharmacophores such as imidazopyridine and benzimidazoles scaffolds to enhance the antimitotic activity. The results of our investigations along this direction are presented below.



11k : R = 4-fluoro phenyl, R_1=H, X=N;
11l : R = 4-fluoro phenyl, R_1=F, X=N;
11m : R = 4-fluoro phenyl, R_1=Cl, X=N;
11n: R = 4-fluoro phenyl, R_1=Me, X=N;
11o : R = 4-fluoro phenyl, R_1=OMe, X=N;
11p : R = phenyl, R_1=H, X=N;
11q : R = phenyl, R_1=F, X=N;
11r : R = phenyl, R_1=Cl, X=N;
11s : R = phenyl, R_1=Me, X=N;
11t: R = phenyl, R_1=OMe, X=N;

11a : R = 4-methoxy phenyl, R_1=H, X=CH;
11b: R = 4-methoxy phenyl , R_1=F, X=CH;
11c : R = 4-methoxy phenyl, R_1=Cl, X=CH;
11d : R = 4-methoxy phenyl, R_1=Me, X=CH;
11e : R = 4-methoxy phenyl, R_1=OMe, X=CH;
11f : R = 4-methoxy phenyl, R_1=H, X=N;
11g : R = 4-methoxy phenyl, R_1=F, X=N;
11h : R = 4-methoxy phenyl, R_1=Cl, X=N;
11i : R = 4-methoxy phenyl, R_1=Me, X=N;
11j : R = 4-methoxy phenyl, R_1=OMe, X=N;

Scheme 1 *Reagents & conditions*: (a) acetone, reflux, 6-8 h; (b) 2N HCl, reflux,1-2 h, 85-92%; (c) $POCl_3$, DMF, reflux,12 h, 80-85%; (d) $Na_2S_2O_5$, EtOH, 8 h, reflux, 70-77%.

Results and discussion

Chemistry

The imidazopyridine / imidazopyrimidinebenzimidazole conjugates (**11a–t**) were prepared by the oxidative cyclization of substituted o-phenylenediamines (**10a-e**) and imidazo[1,2-a]pyridine/ imidazo[1,2-a]pyrimidine-3-aldehyde (**9a-d**) with sodium metabisulphite in ethanol. as shown in **Scheme 1**. The imidazo[1,2-a]pyridine/ imidazo[1,2-a] pyrimidine -3-aldehyde (**9a-d**) were obtained by the Vilsmeier reaction with the corresponding imidazo[1,2-a] pyridine/ imidazo[1,2-a] pyrimidine (**8a-d**), that were obtained from compounds (**7a-d**). The crucial intermediates such as compounds (**7a-d)** were obtained by the reaction of appropriate 2-bromo-1-arylethanone

(**6a-c**) and 2-aminopyridine/pyrimidine. (**5a, b**)

Evaluation of biological activity

cytotoxic activity

To explore the structure activity relationship of imidazopyridine/imidazopyrimidine-benzimidazole conjugates (**11a-t**) consisting A, B, C, D-rings (Fig 2.) derived from **scheme 1**. These 20 compounds prepared with respect to different modifications made on the C-ring and D-ring were evaluated for cytotoxic activity against a panel of four human cancer cell lines such as A549 (lung), Hela (cervical), DU-145 (prostate cancer) and B-16 (mouse melanoma) by employing MTT assay.[21] Nocodazole was used as reference drug. The results are summarized in Table 1 and expressed as IC_{50} values. The *in vitro* screening results revealed that these conjugates possess considerable cytotoxic activity with IC_{50} values ranging from 1.48 µM to >30 µM. particularly, the conjugate **11i** having electron donating substituents like *para*-methoxy group on C-ring and *para*-methyl group on D-ring possess significant cytotoxicity against lung and cervical cancer cells with IC_{50} value 1.48 µM and 1.92 µM respectively.

Table 1: *in vitro* antiproliferative activity IC_{50} (in µM)[a] imidazopyridine/imidazopyrimidine-benzimidazole conjugates (**11a-11t**)

	IC_{50} values (in µM)			
Compound	Hela[b]	A549[c]	DU-145[d]	B-16[e]
11a	20.38	23.15	24.0	> 30
11b	17.58	14.57	15.88	14.79
11c	14.45	19.37	19.49	16.02
11d	26.36	> 30	> 30	> 30
11e	> 30	> 30	> 30	> 30
11f	9.06	10.51	13.25	16.67
11g	16.96	15.07	13.80	19.35
11h	13.97	10.22	19.89	26.80
11i	2.57	1.48	7.08	3.63
11j	15.84	6.91	5.62	19.95
11k	8.19	10.98	7.78	16.60
11l	15.26	19.19	14.42	15.56
11m	20.31	19.68	12.58	> 30
11n	12.89	> 30	10.18	15.11
11o	19.95	18.62	16.0	> 30
11p	2.25	1.92	2.34	10.47
11q	6.91	8.51	4.89	16.98
11r	> 30	> 30	> 30	> 30
11s	7.58	2.19	2.34	8.91
11t	9.33	2.12	3.71	10.23
Nocodazole	2.23	1.62	0.25	0.69

In vitro antiproliferative activity of imidazopyridine/imidazopyrimidine- benzimidazole conjugates determined by MTT assay. [a] 50% Inhibitory concentration and the values are average of three individual experiments after 48 h of drug treatment. [b]Cervical cancer, [c]Lung cancer, [d]Prostate cancer, [e]Mouse melanoma cancer

In contrast, substitution with fluoro at *para* position of C-ring and chloro at *para* position of D-ring as in **11r** proved deleterious for its antiproliferative activity against same cell lines like A549 cells (IC_{50} value is >30 µM) and Hela cells - (IC_{50} value is >30 µM). However, imidazopyrmidine conjugates (**11f-t**) were exhibited better cytotoxicity compared to imidazopyridine conjugates (**11a-e**) against tested cell lines.

On over view, electron donating substitution on D-ring like methyl and hydrogen shows significant cytotoxity compared to halo substitution like fluoro and chloro on D-ring.

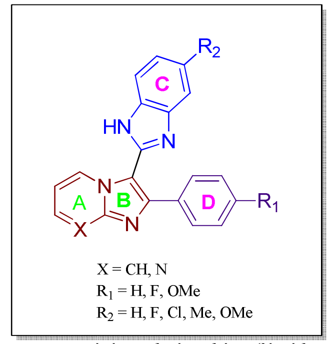


Fig 2. Structure activity relationship of imidazopyridine /imidazopyrimidine-benzimidazole conjugates

Effect on cell cycle arrest

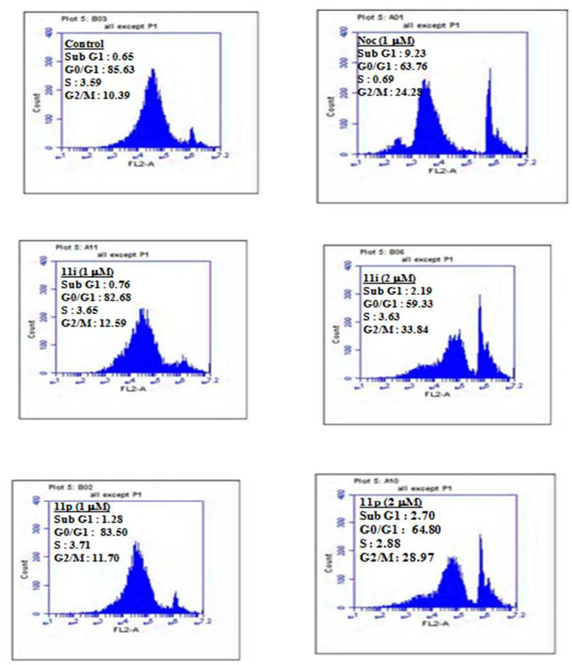


Fig 3. Flow cytometric analysis in A549 lung cancer cell line: Control (A549), Nocodazole (**2**) (1 µM), **11i** (1µM), **11i** (2µM), **11p** (1µM), and **11p** (2µM).

The blockade of cell cycle progress by anti-cancer agents prevents the proliferation of cancer cells, which is also exploited for cancer therapy. In our *in vitro* screening results revealed that compound **11i** and **11p** showed significant activity against human lung cancer cells A549. It was of interest to understand whether this inhibition of cell growth was on account of cell cycle arrest. Hence, we studied the cell cycle distribution by flow cytometry in A549 lung cancer cells. In this study A549 cells were treated with compounds **11i** and **11p** for 48 h at concentrations 1 and 2 µM. The data obtained clearly indicated that these compounds show G_2/M cell cycle arrest in comparison to the untreated control. These compounds (**11i** and **11p**) showed 11.70 and 12.59 % of cell accumulation in G_2/M phase at 1 µM concentration, whereas they exhibited 28.97 and 33.84 % of cell accumulation at 2 µM concentration, respectively (Fig 3.).[26]

Effect on Tubulin polymerization

Compounds that alter cell-cycle parameters with preferential G_2/M blockade are known to exhibit effects on tubulin assembly Moreover, inhibition of tubulin polymerization is strongly associated with G2/M cell-cycle arrest.[27] The cell cycle analysis results revealed that these compounds (**11i** and **11p**) arrested the cell cycle at G_2/M phase as compared to control. Thus it was considered of interest to investigate the effect on tubulin polymerization. As tubulin subunits heterodimerize and self-assemble to form microtubules in a time dependent manner, we have investigated the progression of tubulin polymerization[22, 23] by monitoring the increase of fluorescence emission at 420 nm (excitation wavelength is 360 nm) in 384 well plate for 1 h at 37 °C with and without the compounds at 3 µM concentration. Among the two compounds examined, **11i** and **11p** compounds inhibited tubulin polymerization by 61.12% and 60.49% respectively, whereas the standard compound nocodazole inhibited 66.93% of tubulin polymerization (Fig 4.).

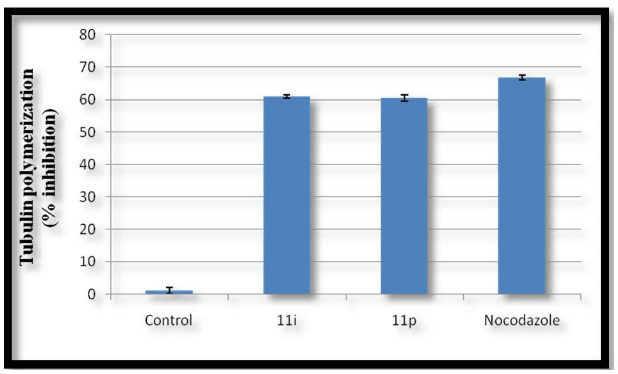


Fig. 4 Effect of compounds on tubulin polymerization: tubulin polymerization was monitored by the increase in fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37 ºC. All the compounds were included at a final concentration of 3 µM. Nocodozole was used as a positive controls. Values indicated are the mean ± SD of two different experiments performed in triplicates.

Table 2. Inhibition of tubulin polymerization (IC_{50}) of compound **11i** and **11p**.

Compound	IC_{50} [a] ± SD (in µM)
11i	2.06±0.09
11p	2.26±0.38
Nocodazole	1.82±0.06

Effect of congeners on tubulin polymerization. IC_{50} (µM) values for **11i** and **11p** were determined from the tubulin polymerization assays. [a] Concentration of drug to inhibit 50% of tubulin assembly

Furthermore, these three potential conjugates (**11i** and **11p**) were evaluated for their *in vitro* tubulin polymerization assay at different concentrations. These molecules showed potent inhibition of tubulin polymerization with IC_{50} values 2.06 and 2.26 µM, respectively (Table 2). Nocodazole was used as positive control.

Immunohistochemistry of tubulin

In addition to *in vitro* tubulin polymerization studies, we investigated alterations in the microtubule network induced by conjugates **11i** and **11p** in A549 cell culture by immunofluoresence microscopy of immunohistochemistry studies, as most antimitotic agents affect microtubules.[28] Therefore, A549 cells were treated with **11i** and **11p** at 1 µM concentration for 48 h. Nocodazole was used as reference compound. The test results, demonstrated a well organized microtubular network in control cells. However, cells treated with test conjugates showed disrupted microtubule organization as seen in Fig 5, thus demonstrating the aspect of inhibition of tubulin polymerization.

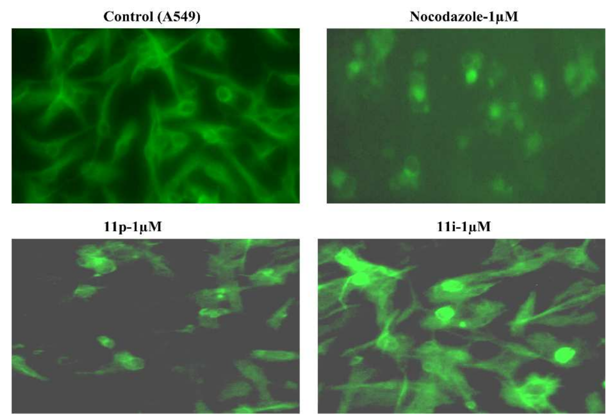


Fig 5. IHC analysis of compounds on the microtubule network: A549 cells were treated with compounds **11i** and **11p** at 1 µM concentrations for 48 h followed by staining with α–tubulin antibody. Nocodazole was used as the reference compound.

Hoechst staining

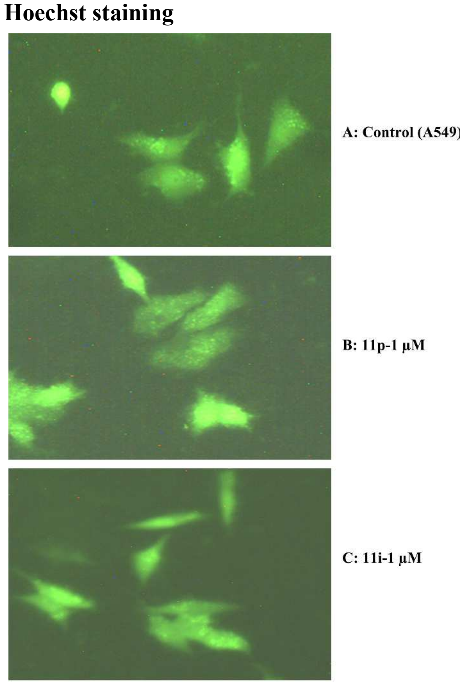


Fig 6. Hoechst staining in A549 lung cancer cell line. **A:** Control cells (A549), **B:** 10x at 0.5 µM concentration and **C:** 10x at 1 µM concentration

Disruption of microtubule formation leads to cell-cycle arrest in the G_2/M phase, followed by apoptotic cell death.[24] Chromatin condensation and fragmented nuclei are known as the classic characteristics of apoptosis and apoptosis is one of the major pathways that lead to the process of cell death. It was considered of interest to investigate the apoptotic inducing effect of the two potent conjugates (**11p** and **11i**) by Hoechst staining (H 33342) method in A549 cancer cell line. Therefore cells were treated with **11i** and **11p** at 1 µM concentration for 48 h wherein nocodazole was used as the reference compound. Apoptotic cells were observed based on cytoplasmic condensation, presence of apoptotic bodies, nuclear fragmentation and relative fluorescence of the test compounds (**11p** and **11i**) revealed that there is significant increase in the percentage of apoptotic cells (Fig 6.).

Caspases-3 activation

Caspases or cysteine-aspartic proteases are a family of cysteine proteases, which are crucial mediators of apoptosis. Among them, caspase-3 is the best understood in the mammalian caspases in terms of its specificity and role in apoptosis. Caspase-3 is also required for some typical hallmarks of apoptosis.[22] A549 cells were treated with **11p** and **11i** at 1 and 2 µM concentrations for 48 h and were examined for the activation of caspase-3 activity. Results indicated that there was nearly 2 to 5-fold induction in caspase- 3 levels compared to control (Fig 7.).

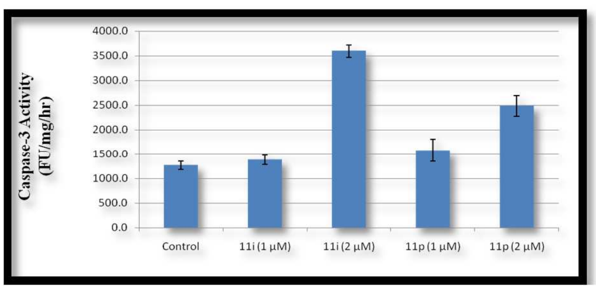


Fig. 7 Effect of compounds **11i** and **11p** on caspase-3 activity: A549 cells were treated with compounds **11i** and **11p** at 1 and 2 µM concentrations for 48 h. Values indicated are the mean ± SD of two different experiments performed in triplicates.

DNA fragmentation analysis

DNA laddering was carried out in order to elucidate the mode of action of the compound especially for their ability to induce oligonucleosomal DNA fragmentation (DNA ladder), which is a characteristic feature of the programmed cell death or apoptosis.[25, 26] During apoptosis, DNA is cleaved into smaller fragments and fragmented DNA produces a series of bands which are described as DNA ladders. These fragments can be observed by gel electrophoresis as ladders. A549 cells were treated with **11i** and **11p** at 1µM concentrations for 48 h and DNA was isolated from these cells. The DNA was run on 2% agarose gel electrophoresis after staining with ethidium bromide under UV illumination. It was observed that compounds produced significant DNA fragmentation (Fig 8.), which is indicative of apoptosis.

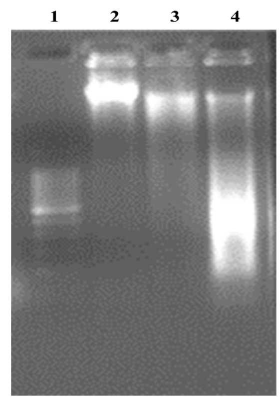


Fig. 8 DNA fragmentation of compounds 11i and 11p in A-549 lung cancer cells: Lane-1: Marker (100 bp), Lane-2: Untreated control DNA, Lane-3: 11p 1 µM, Lane-4: 11i at 1µM.

Annexin V-FITC for apoptosis

The apoptotic effect of these compounds (**11p** and **11i**) was further evaluated by Annexin V FITC/PI (AV/PI) dual staining assay[29] 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 **11p** and **11i** for 48 h at 1 and 2 µM concentration to examine the apoptotic effect. It was observed that these compounds showed significant apoptosis against A549 cells as shown in Fig 6.

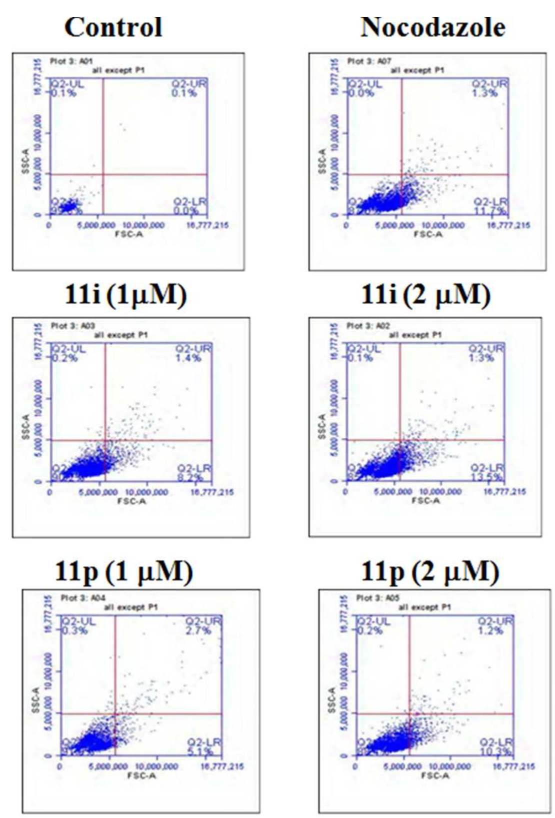


Fig. 9 Annexin V-FITC staining. A549 cells were treated with compounds **11p**, **11i** and Nocodazole (**2**).

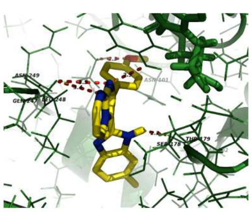
Results indicated that compounds **11p** and **11i** showed 7.77 and 9.61 % of apoptosis at 1 µM concentration, whereas they showed 11.45 and 14.79 % of apoptosis at 2 µM concentration respectively (Fig 9.). In this study the untreated control cells showed 0.14 % of apoptosis from this experiment it was suggested that these compounds induced cell death by apoptosis (Table 3.).

Table 3. Annexin V-FITC staining of A549 cells in the presence of compounds **11p, 11i** and **nocodazole**.

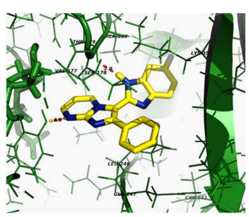
Sample	UL %	UR %	LL %	LR %
Control	0.07	0.14	99.79	0.00
Noc (1µM)	0.04	1.29	86.96	11.71
11p (1 µM)	0.35	2.69	91.88	5.08
11p (2 µM)	0.18	1.20	88.37	10.25
11i (1 µM)	0.22	1.43	90.17	8.18
11i (2 µM)	0.07	1.30	85.14	13.49

Molecular modelling

The conjugates that are synthesised in the present study are designed on the basis of nocodazole which is an aminobenzimidazole with antimiotic and antitumoral activity by tubulin inhibition. Nocodazole and colchicine show resemblance in binding pattern at *αβ* -interface of the tubulin.[30] To investigate the possible binding interactions for the conjugates (**11p** and **11k**), we performed molecular docking studies at the colchicine binding pocket. Our docking studies suggests that these conjugates occupy the binding site of the colchicine and interacted with both *α*- and *β*-tubuline interface.The imidazopyrimidine ring of **11i** binds to the hydrophobic region with *β*Gln247, *β*Leu248. Apart from this the 4-methoxyphenyl group at the imidazopyrimidine ring involved in hydrogen bond interaction with *β*Lys254 and hydrophobic interactions with *α*Asn101, *α*Gly143. The benzimidazole ring of conjugates (**11i** and **11p)** involved in the hydrogen binding interactions with *α*Thr179 and these interactions are in the range of 1.7-2.2Å.



11i



11p

Fig. 10 A predicted mode of compound **11i** and **11p** (yellow stick model) binding within colchicine binding site in the tubulin (PDB ID code: **3E22**).Surrounding amino acid side chains are shown in green stick format and labelled in black. The hydrogen bonds are shown by dashed lines in red color and the distance between the ligands and protein is less than 3 Å

The benzimidazole ring of conjugates **11p** involved in hydrophobic interaction with αAla180. The imidazopyrimidine ring of **11p** and **11i** establishes hydrogen bond with *α*Tyr224. Some hydrophobic interactions are observed with phenyl group of the conjugates **11p** and **11s** with *β*Gln247, *β*Leu248, *α*Val355, *β*Ala354, *β*Thr353. All the conjugates interact with both *αβ* interface tubulin in the colchicine binding pocket (*α*Ser178, *α*Thr179, *α*Val181, *β*Lys352, *β*Thr353, *α*Asn101, *β*Gln247, *β*Leu248). In case of **11i** methoxy substituent on phenyl group induced change in the pose orientation that caused the formation of hydrogen bond interaction with *β*Lys254, this in turn suggest a structural justification for increased activity of **11i** over **11p**.These results support the tubulin polymerization inhibition potential of these conjugates at the molecular level.

Conclusion

In summary, twenty congeners of imidazopyridine /imidazopyrimidine-benzimidazole conjugates (**11a-t**) have been synthesized and evaluated for their cytotoxic activity against four human cancer cell lines such as A-549 (lung), Hela (cervical), DU-145 (prostate) and B-16 (melonoma). Some of these conjugates exhibited significant cytotoxic activity at micromolar (µM) concentration. Two of the most potent compounds (**11i** and **11p**) exhibited promising cytotoxic activity (IC_{50}, 1.48 and 1.92 µM respectively) against A-549 cancer cell line. Flow cytometric analysis revealed that these conjugates arrested the cell cycle at the G_2/M phase. These potent conjugates (**11i** and **11p**) exert their cytotoxic activity by inhibition of tubulin polymerization, with an IC_{50} value of 2.06 µM and 2.26 µM respectively. Further, Hoechst staining, DNA fragmentation assay and activation of caspase-3 and Annexin V-FITC assay suggested that these conjugates induce cell death by apoptosis. Furthermore, molecular modeling analysis suggests that these conjugates preferably bind to the colchicine binding site of tubulin. Overall, this investigation describes the synthesis of imidazopyridine/imidazopyrimidine-benzimidazoles conjugates as potential anticancer agents with apoptosis inducing ability by targeting tubulin.

Acknowledgements

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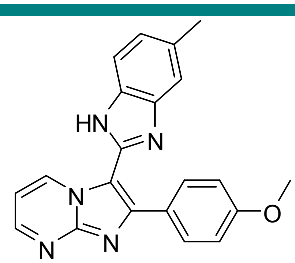

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Design, synthesis and biological evaluation of imidazopyridine/imidazopyrimidine-benzimidazole conjugates as potential anticancer agents

Graphical Abstract

Design, Synthesis and Biological Evaluation of Imidazopyridine/imidazopyrimidine-Benzimidazole Conjugates as Potential Anticancer Agents

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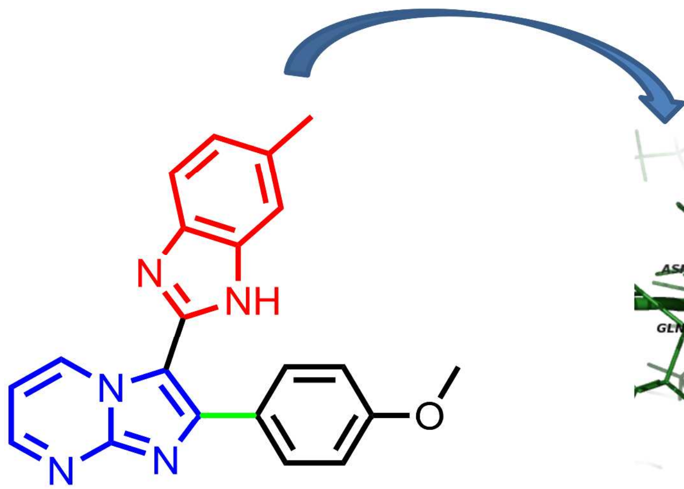

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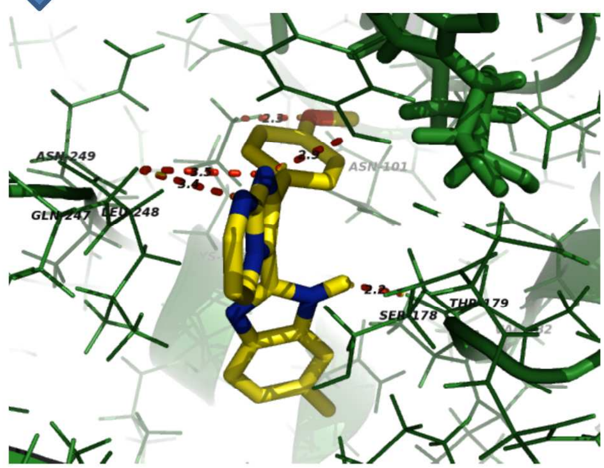

Lead microtubule targeting inhibitor