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ARTICLE TYPE

Synthesis and biological evaluation of imidazo[1,5-*a*]pyridine-benzimidazole hybrids as inhibitors of both tubulin polymerization and PI3K/Akt pathway

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A series of imidazo[1,5-*a*]pyridine-benzimidazole hybrids **(5a-aa)** were prepared and evaluated for their cytotoxic activity against a panel of sixty human tumor cell lines. Among them compounds **5d** and **5l** showed significant cytotoxic activity with GI_{50} values ranging from 1.06 to 14.9 μM and 0.43 to 7.73 μM respectively. Flow cytometric analysis revealed that these compounds arrest the cell cycle at G2/M phase and induced cell death by apoptosis. The tubulin polymerization assay (IC_{50} of **5d** is 3.25 μM and **5l** is 1.71 μM) and immunofluorescence analysis showed that these compounds effectively inhibited the microtubule assembly in human breast cancer cells (MCF-7). Further the apoptotic effects of compounds were confirmed by Hoechst staining, mitochondrial membrane potential, cytochrome c release, ROS generation, caspase 9 activation and DNA fragmentation analysis. After treatment with these compounds for 48 h, p-PTEN and p-AKT levels were markedly decreased. Moreover these compounds did not significantly inhibit the normal human embryonic kidney cells, HEK-293.The molecular docking simulations predicted the binding interactions of **5d** and **5l** with colchicine binding site of the tubulin which is in compliance with the antiproliferative activity data.

Introduction

Cancer is one of the major causes of death across the world for decades. The effectiveness of many existing anticancer drugs is limited due to their toxicity to normal rapidly growing cells and may develop resistance to that drug. Another drawback is that majority of the drugs currently in the market are not specific. Microtubule system of eukaryotic cells is essential for cell division since microtubules are the key components of the mitotic spindle.[1] Microtubules are in dynamic equilibrium with tubulin dimers as tubulin is assembled into microtubules, which in turn are disassembled to tubulin.[2-4] This dynamicity is characterized by the continuous turnover of α,β-tubulin heterodimers in the polymeric microtubules. Thus microtubules play an important role in fundamental cellular processes, such as regulation of motility, cell signaling, formation and maintenance of cell shape, secretion and intracellulartransport.[5] Therefore, microtubules have become an important target and there has been considerable interest in the discovery and development of small molecules that affect tubulin polymerization.

In recent years different heterocyclic compounds have been identified through molecular biology; empirical screening and rational drug design for the development of newer anticancer agents.[6] Nitrogen-bridgehead fused heterocycles containing an imidazole ring are common structural components in many pharmacologically important molecules that display a wide range of activities for diverse number of targets. One of the most widely used heterocyclic system from this group is imidazopyridines[7] that shows broad spectrum of biological activities like inhibitors of aromatase, estrogen production suppressors,[8] positive inotropic agents,[9] platelet aggregation inhibitors, thromboxane synthetase inhibitors,[10] antiviral,[11,12] and antibacterial.[13] Imidazopyridines exhibit anticancer activity through different molecular mechanisms such as vascular endothelial growth factor (VEGF)-receptor KDR inhibition and induction of apoptosis.[14,15] Recently, we have reported the synthesis of imidazopyridine-oxindole derivates (**4**) as apoptosis inducing agents.[16]

Similarly, benzimidazoles are of great importance due to their biological properties and have been reported to possess potential anticancer activity and anti-HIV activity,[17-25]apart from antibacterial,[26-27] antifungal, antiviral and antioxidant activities.[27-31] Compounds such as nocodazole (NSC-238189) (**1**),[32] FB642 (**2**),[33] Hoechst-33258 (**3**)[34] are some biologically active antitumor agents that possess a benzimidazole moiety.

Some of the potent hybrid molecules that have been recently developed as new anticancer agents were obtained by the combination of different pharmacophores.[35,36] Benzimidazole attached to other heterocyclic moieties including fused rings resulted in compounds (hybrid molecules) with improved pharmacological profile. The promising biological activity exhibited by these hybrids prompted us to develop some newer hybrid molecules by linking the imidazopyridine pharmacophore with benzimidiazole scaffold with a view to enhance their

anticancer activity. In this context we have designed and synthesized imidazopyridine-benzimdiazole hybrids (**5a–aa**) that were evaluated for their anticancer potential in the Development Therapeutic Program (DTP) of the National Cancer Institute (NCI), USA, against a panel of sixty cell lines, apart from their effect on inhibition of tubulin polymerization and apoptotic cell death. (Table **1**).

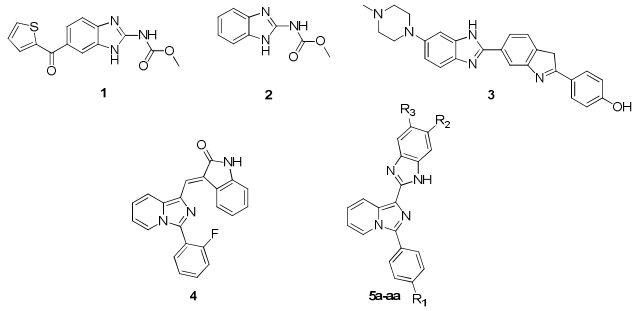
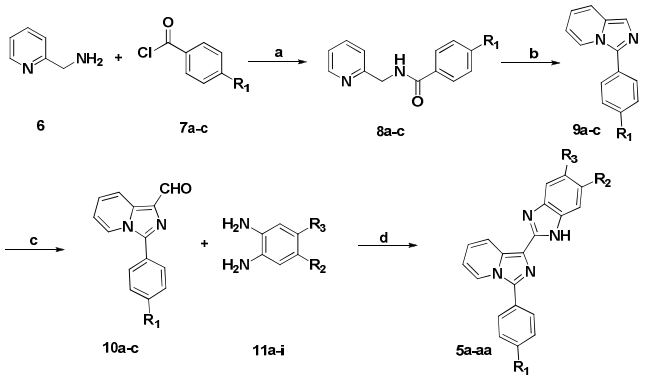


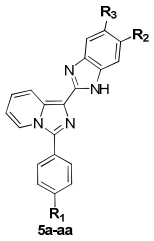
Figure 1: Lead structures of biologically active antitumor agents having benzimidazole scaffold: Nocodazole (**1**), FB642 (**2**), Hoechst-33258 (**3**) and synthesized in our laboratory Imidiazopyridine derivative (**4**) as well as proposed library of Imidazopyridine-benzimidazole hybrids **(5a-aa)**.

Chemistry

Synthesis of the imidazo[1,5-*a*]pyridine-benzimidazole hybrids **(5a-aa)** was accomplished as described in Scheme 1. Differently substituted benzoylchlorides (**7a-c**) were reacted with 2-pyridylmethanamine (**6**) in presence of the base triethylamine to give *N*1-(2-pyridylmethyl)-substituted benzamides **(8a-c)** which were cyclized to corresponding imidazo[1,5-*a*]pyridines (**9a-c**) by refluxing in $POCl_3$. Formylation of these imidazo[1,5-*a*]pyridine derivatives (**9a-c**) under Vilsmieir-Haack conditions with $POCl_3$ in dry DMF yielded the intermediates 3-(substituted phenyl)imidazo[1,5-*a*]pyridine-1-carbaldehydes (**10a-c**). The reaction of **(10a-c)** with substituted *o*-phenylediamines (**11a-i**) in ethanol using sodium metabisulphate afforded the final benzimidiazole-derived imidazo[1,5-*a*]pyridines (**5a-aa**) in excellent yields.



Scheme Reagents and conditions: (a) TEA, dry THF,0 °C to rt, 3h; (b) $POCl_3$, reflux, 3 h; (c) DMF, $POCl_3$,0 °C to 100 °C, 3 h; (d) $Na_2S_2O_4$, EtOH, reflux, 8 h.



Compound	R_1	R_2	R_3	Compound	R_1	R_2	R_3	Compound	R_1	R_2	R_3
5a	H	H	H	5j	OMe	H	H	5s	CF_3	H	H
5b	H	OMe	H	5k	OMe	OMe	H	5t	CF_3	OMe	H
5c	H	Me	H	5l	OMe	Me	H	5u	CF_3	Me	H
5d	H	F	H	5m	OMe	F	H	5v	CF_3	F	H
5e	H	Cl	H	5n	OMe	Cl	H	5w	CF_3	Cl	H
5f	H	CF_3	H	5o	OMe	CF_3	H	5x	CF_3	CF_3	H
5g	H	F	Cl	5p	OMe	F	Cl	5y	CF_3	F	Cl
5h	H	Cl	Cl	5q	OMe	Cl	Cl	5z	CF_3	Cl	Cl
5i	H	Me	Me	5r	OMe	Me	Me	5aa	CF_3	Me	Me

Biological evaluation

Cytotoxic activity

All the newly synthesized imidazo[1,5-*a*]pyridine-benzimidazole hybrids **(5a-aa)** were evaluated by the National Cancer Institute (NCI), for their anticancer activity at single concentration of 10^{-5}M against a panel of sixty cancer cell lines.[37-39] The human tumor cell lines were derived from nine different cancer types: leukemia, melanoma, lung, colon, CNS, ovarian, renal, prostate and breast cancers. The compounds were added at a single concentration and the cell cultures were incubated for 48 h and the end point determinations were made with a protein binding dye, sulforhodamine B (SRB). Ten among the 17 compounds tested, exhibited significant growth inhibition at a single dose of 10 μM concentration which were further evaluated against the sixty cell line panel at five dose concentration, and the results are summarized in Table **1**. From the five dose assay results, it is observed that, most of the tested compounds exhibited significant antiproliferative activity with distinct selectivity on different cell lines. Two compounds **5d** and **5l** emerged as hit compounds that show remarkable growth inhibition on all the tested cell line

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Table 1: In vitro anticancer activity (GI_{50} μM)[a] of compounds **5d**, **5f, 5k-p**, and **5z** on 60 human cancer cell lines

	Growth inhibition (GI_{50} μM)[a]									
Cancer panel/cell line	5d[d]	5f[e]	5k[f]	5l[g]	5m[h]	5n[i]	5o[j]	5p[k]	5q[l]	5z[m]
Leukaemia										
CCRF-CEM	5.28	5.64	2.53	1.97	4.39	27.3	7.40	3.91	NA[c]	NA[c]
HL-60(TB)	14.9	27.8	2.54	2.58	39.8	49.8	NA[c]	4.97	NA[c]	NA[c]
K-562	3.47	11.0	3.60	2.92	14.0	5.40	35.3	4.84	NT[b]	NA[c]
MOLT-4	3.60	28.3	3.37	2.17	3.83	NA[c]	2.81	4.21	NA[c]	NA[c]
RPMI-8226	2.60	3.84	1.72	1.47	2.86	3.55	NT[b]	3.02	3.74	NA[c]
SR	4.45	3.65	3.21	2.77	6.63	12.6	32.3	3.87	6.26	NA[c]
Non-small lung										
A549/ATCC	2.86	3.12	3.17	2.67	3.96	1.82	28.5	4.04	2.35	NT[b]
HOP-62	1.94	2.46	5.07	3.72	7.41	1.96	38.0	19.8	1.81	3.04
HOP-92	1.73	3.66	2.31	0.416	3.67	2.19	NA[c]	3.24	1.82	2.97
NCI-H226	1.80	10.9	6.57	3.88	2.72	1.94	NT[b]	14.5	2.19	2.16
NCI-H23	3.54	3.21	10.26	3.85	1.87	7.52	NA[c]	29.1	NT[b]	6.57
NCI-H322M	3.18	3.35	3.22	2.38	9.53	5.63	NA[c]	6.87	NT[b]	9.45
NCI-H460	2.07	2.93	3.23	2.28	6.83	2.23	9.73	5.43	3.01	NT[b]
NCI-H522	2.43	2.99	2.89	1.95	4.49	4.24	NA[c]	4.39	3.37	10.6
Colon										
COLO 205	3.42	4.88	7.19	3.23	7.81	3.61	NA[c]	7.10	NT[b]	NA[c]
HCC-2998	4.11	5.98	3.96	2.93	6.60	18.6	51.8	4.03	NA[c]	NA[c]
HCT-116	2.00	1.85	3.63	3.26	3.61	2.41	26.1	6.09	2.65	2.92
HCT-15	4.29	3.50	3.76	2.83	3.36	3.89	NA[c]	5.72	30.1	30.5
HT29	3.25	3.47	3.75	3.36	7.13	3.66	58.2	4.26	4.42	NT[b]
KM12	3.31	5.11	3.88	2.38	7.03	7.68	36.4	4.56	NA[c]	NT[b]
SW-620	3.97	3.17	4.26	3.12	2.24	4.07	NA[c]	5.38	8.36	NA[c]
CNS										
SF-268	3.28	5.63	4.53	2.80	7.20	2.93	NA[c]	9.11	2.59	4.19
SF-295	NT[b]	3.85	NT[b]	NT[b]	NT[b]	NT[b]	NA[c]	NT[b]	NT[b]	3.90
SF-539	2.32	1.69	9.71	3.32	1.04	1.76	NA[c]	39.8	1.86	2.08
SNB-19	2.37	6.44	5.26	2.97	1.12	3.60	NA[c]	8.35	2.64	6.88
SNB-75	2.09	1.68	24.0	5.49	3.40	1.87	48.1	5.31	1.73	1.45
U251	2.22	1.98	4.42	3.27	1.36	3.16	NA[c]	6.67	3.05	3.13
Melanoma										
LOX IMVI	4.86	1.89	4.80	3.03	10.8	2.85	31.3	5.36	3.60	NT[b]
MALME-3M	2.07	2.71	13.3	2.83	5.32	2.35	NA[c]	20.0	2.51	3.56
M14	3.83	17.7	3.74	3.15	12.9	4.24	83.1	51.5	4.92	NT[b]
MDA-MB-435	2.59	5.47	3.14	2.30	8.05	2.30	47.0	3.80	5.77	NT[b]
SK-MEL-2	2.85	1.49	4.07	2.51	10.1	2.71	NA[c]	6.86	NT[b]	9.49
SK-MEL-28	2.13	9.22	1.85	4.61	22.8	2.14	NA[c]	NA[c]	NT[b]	NT[b]
SK-MEL-5	1.55	1.90	1.60	1.39	2.20	4.27	47.0	2.22	2.91	4.20
UACC-257	2.16	13.1	4.39	2.41	9.15	2.63	NA[c]	3.15	5.77	NA[c]
UACC-62	1.96	3.19	4.51	2.14	5.41	4.89	51.1	6.05	5.59	15.6
Ovarian										
IGROV1	1.95	4.03	2.70	2.18	6.63	4.95	NA[c]	4.91	3.73	16.1
OVCAR-3	4.29	4.08	4.97	2.99	6.14	2.61	NA[c]	8.75	3.18	3.74
OVCAR-4	1.93	1.81	4.53	3.81	6.92	1.89	NT[b]	7.93	2.00	1.87
OVCAR-5	3.07	6.77	11.4	4.69	4.52	7.33	NA[c]	NA[c]	NA[c]	NA[c]
OVCAR-8	1.87	2.70	2.53	2.05	3.76	2.40	22.7	3.45	2.53	6.61
NCI/ADR-RES	2.23	3.71	3.86	2.71	6.50	2.78	85.3	5.50	3.90	6.10
SK-OV-3	2.40	1.86	36.1	6.95	10.3	2.24	NA[c]	NA[c]	2.65	2.74
Renal										
786-0	2.23	1.92	8.47	3.67	8.82	1.60	NA[c]	8.94	1.79	2.19
A498	2.08	1.69	4.28	2.47	3.83	5.61	NA[c]	2.31	4.20	4.58
ACHN	2.25	2.23	3.57	2.76	4.28	1.86	63.5	7.34	2.61	3.90
CAKI-1	3.38	5.07	3.81	2.83	12.1	2.09	NA[c]	5.35	3.32	NT[b]
SN12C	4.50	2.59	3.91	3.08	13.3	3.75	NA[c]	11.2	NA[c]	NA[c]
TK-10	3.25	2.86	2.65	3.39	16.1	2.01	NA[c]	5.25	2.36	8.15
UO-31	1.75	4.36	2.47	1.67	6.99	1.74	NA[c]	2.62	1.87	NT[b]
RXF 393	2.99	2.06	24.2	7.73	6.05	1.94	30.5	4.65	1.76	2.26
Prostate										

PC-3	1.82	4.11	3.01	1.88	6.14	2.13	4.73	4.58	2.58	NT[b]
DU-145	5.56	7.50	4.11	2.85	10.7	3.27	NA[c]	6.58	NT[b]	NT[b]
Breast										
MCF7	1.37	2.40	3.40	2.70	6.15	3.15	26.3	4.57	7.20	NT[b]
MDA-MB231/ATCC	2.45	2.04	5.65	3.09	4.34	2.77	50.6	5.82	2.79	2.49
HS 578T	3.56	3.65	2.57	2.17	8.54	2.76	NA[c]	12.2	2.68	3.99
BT-549	2.76	4.23	3.20	2.55	4.84	2.74	NA[c]	4.91	3.08	8.69
T-47D	1.06	1.71	2.70	2.27	3.55	3.66	5.33	3.53	4.06	4.05
MDA-MB-468	1.49	2.02	4.08	2.32	4.12	1.99	13.4	2.93	5.04	3.05
Non-small lung										
A549/ATCC	2.86	3.12	3.17	2.67	3.96	1.82	28.5	4.04	2.35	NT[b]
HOP-62	1.94	2.46	5.07	3.72	7.41	1.96	38.0	19.8	1.81	3.04
HOP-92	1.73	3.66	2.31	0.416	3.67	2.19	NA[c]	3.24	1.82	2.97
NCI-H226	1.80	10.9	6.57	3.88	2.72	1.94	NT[b]	14.5	2.19	2.16
NCI-H23	3.54	3.21	10.26	3.85	1.87	7.52	NA[c]	29.1	NT[b]	6.57
NCI-H322M	3.18	3.35	3.22	2.38	9.53	5.63	NA[c]	6.87	NT[b]	9.45
NCI-H460	2.07	2.93	3.23	2.28	6.83	2.23	9.73	5.43	3.01	NT[b]
NCI-H522	2.43	2.99	2.89	1.95	4.49	4.24	NA[c]	4.39	3.37	10.6
Colon										
COLO 205	3.42	4.88	7.19	3.23	7.81	3.61	NA[c]	7.10	NT[b]	NA[c]
HCC-2998	4.11	5.98	3.96	2.93	6.60	18.6	51.8	4.03	NA[c]	NA[c]
HCT-116	2.00	1.85	3.63	3.26	3.61	2.41	26.1	6.09	2.65	2.92
HCT-15	4.29	3.50	3.76	2.83	3.36	3.89	NA[c]	5.72	30.1	30.5
HT29	3.25	3.47	3.75	3.36	7.13	3.66	58.2	4.26	4.42	NT[b]
KM12	3.31	5.11	3.88	2.38	7.03	7.68	36.4	4.56	NA[c]	NT[b]
SW-620	3.97	3.17	4.26	3.12	2.24	4.07	NA[c]	5.38	8.36	NA[c]
CNS										
SF-268	3.28	5.63	4.53	2.80	7.20	2.93	NA[c]	9.11	2.59	4.19
SF-295	NT[b]	3.85	NT[b]	NT[b]	NT[b]	NT[b]	NA[c]	NT[b]	NT[b]	3.90
SF-539	2.32	1.69	9.71	3.32	1.04	1.76	NA[c]	39.8	1.86	2.08
SNB-19	2.37	6.44	5.26	2.97	1.12	3.60	NA[c]	8.35	2.64	6.88
SNB-75	2.09	1.68	24.0	5.49	3.40	1.87	48.1	5.31	1.73	1.45
U251	2.22	1.98	4.42	3.27	1.36	3.16	NA[c]	6.67	3.05	3.13
Melanoma										
LOX IMVI	4.86	1.89	4.80	3.03	10.8	2.85	31.3	5.36	3.60	NT[b]
MALME-3M	2.07	2.71	13.3	2.83	5.32	2.35	NA[c]	20.0	2.51	3.56
M14	3.83	17.7	3.74	3.15	12.9	4.24	83.1	51.5	4.92	NT[b]
MDA-MB-435	2.59	5.47	3.14	2.30	8.05	2.30	47.0	3.80	5.77	NT[b]
SK-MEL-2	2.85	1.49	4.07	2.51	10.1	2.71	NA[c]	6.86	NT[b]	9.49
SK-MEL-28	2.13	9.22	1.85	4.61	22.8	2.14	NA[c]	NA[c]	NT[b]	NT[b]
SK-MEL-5	1.55	1.90	1.60	1.39	2.20	4.27	47.0	2.22	2.91	4.20
UACC-257	2.16	13.1	4.39	2.41	9.15	2.63	NA[c]	3.15	5.77	NA[c]
UACC-62	1.96	3.19	4.51	2.14	5.41	4.89	51.1	6.05	5.59	15.6

*(a) Values are reported as GI_{50}, the μM concentration of the compound required to cause 50% inhibition of cell growth; (b) NT: Not Tested; (c) NA: Not Active; (d) **5d** (NSC 766340); (e) **5f** (NSC 774952); (f) **5k** (NSC 766330); (g) **5l** (NSC 766338); (h) **5m** (NSC 766325); (i) **5n** (NSC 766332); (j) **5o** (NSC 766331); (k) **5p** (NSC 766333); (l) **5q** (NSC 766337); (m) **5z** (NSC 774957)*

with GI_{50} values in the range of 1.06–14.9 μM and 0.46–7.73 μM respectively.

The in vitro cytotoxicity values of these ten selected compounds allow a rudimentary picture of the structure-activity relationship (SAR) to be drawn. Four compounds (**5d**, **5f**, **5l** and **5n**) exhibited better cytotoxicity profiles when compared to the other congeners. It is interesting to note that the benzimidazole half of these four compounds carry a single substituent ($R_2 \neq H$, $R_3 = H$) the benzenoid subunit. A comparison of the most inactive member of the series **5o** (R_1 = OMe, R_2 = CF_3, R_3 = H) with the significantly active compounds **5f** (R_1 = H, R_2 = CF_3, R_3 = H) and **5l** (R_1= OMe, R_2 = Me, R_3 =H) reveals certain interesting observations (Figure **2**). **5o** and **5f** differ only in the nature of the R_1 substituent (OMe and H respectively). **5l** has the same R_1 substituent (OMe) as **5o** but a different R_2 substituent (Me instead of CF_3). Thus the near- complete loss of activity for **5o** cannot be attributed to the individual substituents R_1 or R_2, but if may rather be the result of a different binding pattern for individual compounds to their cellular targets. Some evidence in support of this inference has been obtained in the docking experiments, where two active compounds **5d** and **5l** showed very different binding poses with the colchicine binding site of tubulin (vide infra). In view of the possibility of such an opportunistic binding pattern for individual compounds with the same binding site, a correlation of the cytotoxicity to substituents (R_1, R_2 and R_3) on the compounds is not attempted at this stage.

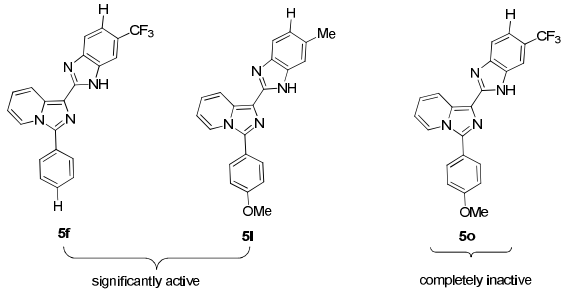

Figure 2: SAR of selected compounds **5f**, **5l** and **5o**

Specific killing of cancer cells without affecting normal cell growth is a key safety feature of cancer chemotherapy. Therefore these compounds (**5d** and **5l**) were evaluated for possible cytotoxicity towards normal human embryonic kidney cells, HEK-293 by MTT assay. The assay results suggested that these compounds did not significantly (Compound **5d** and **5l** showed 75.85 and 97.72 μM IC_{50} values respectively) affect the growth of normal human embryonic kidney cells, HEK-293.

Cell cycle analysis

The data from MTT assay showed that the test compounds **5d** and **5l** induced significant inhibition of breast cancer cells with IC_{50} values 1.79 and 2.85 μM respectively. It was of interest to examine cell-cycle alterations caused by these conjugates in MCF-7 cancer cells to understand the phase distribution.[40] Thus, cells were treated with these conjugates at concentrations of 1 and 3 μM for 48 h and the results indicated that these compounds showed cell cycle arrest in G2/M phase at 1 μM concentration **(**Figure **3**). Interestingly, when the concentration was increased from 1 μM to 3 μM, it was observed that the percentage of accumulation of cells in subG1 phase increased, which indicates the onset of apoptosis. [41]

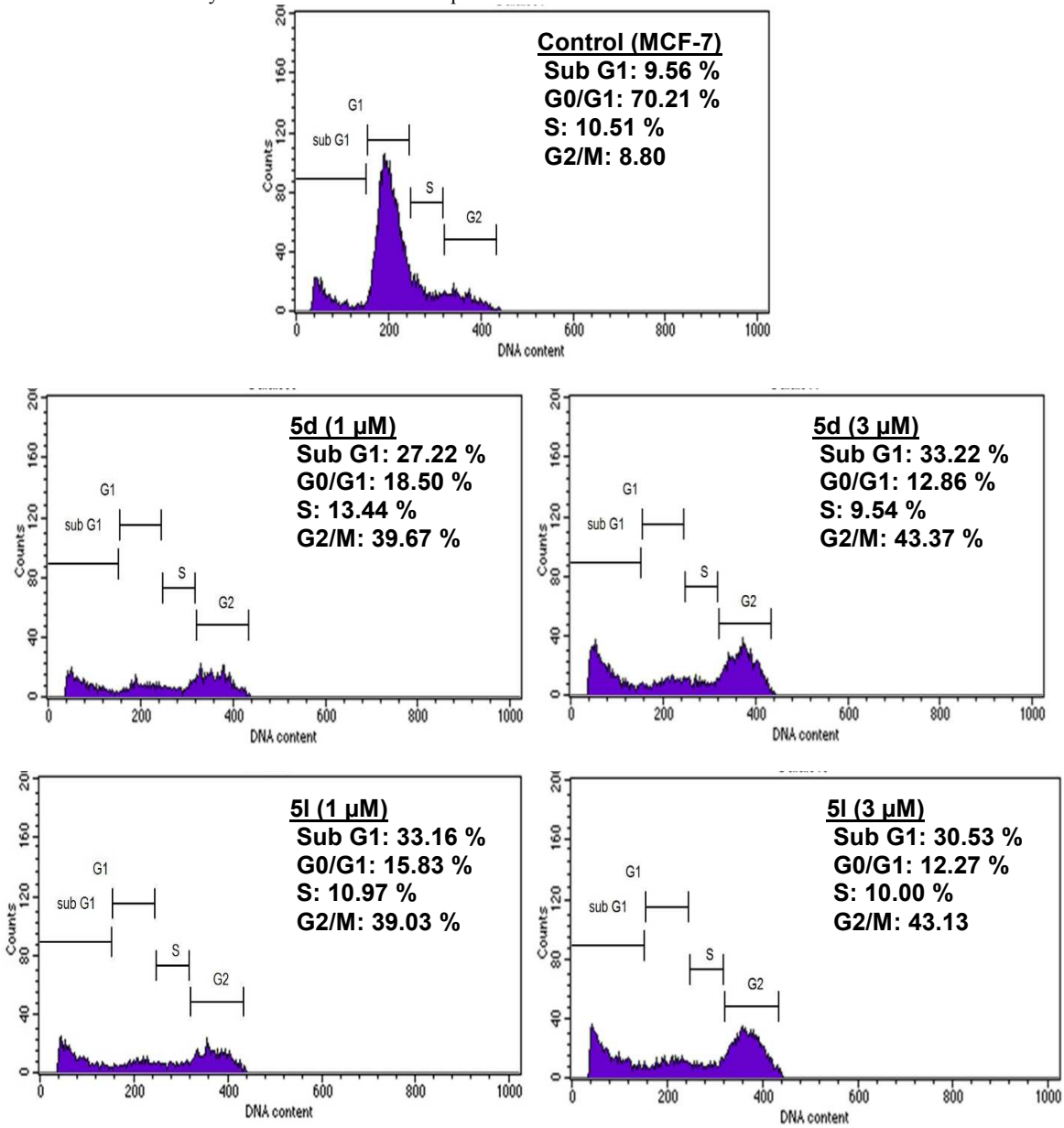

Figure 3: Flow cytometric analysis in MCF-7 breast cancer cell line; MCF-7 cells were treated with the **5d** and **5l** at concentrations of 1 and 3 μM for 48 h.

Inhibition of tubulin polymerization

Compounds that alter cell-cycle parameters with preference to G2/M blockade are known to exhibit effects on tubulin assembly. Moreover, inhibition of tubulin polymerization is strongly associated with G2/M cell-cycle arrest.[42] These compounds arrest the cell cycle at G2/M phase at 1 μM concentration, hence it was considered of interest to investigate the tubulin polymerization aspect. As tubulin subunits heterodimerize and self-assemble to form microtubules in a

time dependent manner, we have investigated the progression of tubulin polymerization[43] by monitoring the increase in fluorescence emission at 420 nm (excitation wavelength is360 nm) in 384 well plate for 1 h at 37 °C with and without the compounds at 3 µM concentration.

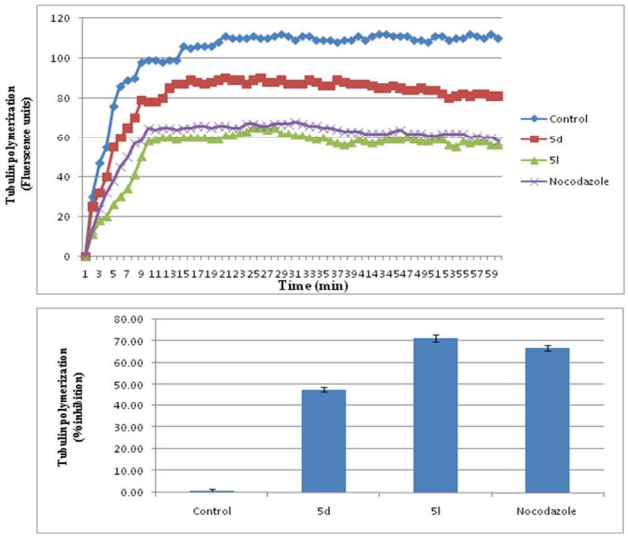

Figure 4: Effect of compounds on tubulin polymerization: tubulin polymerization was monitored by the increase in fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37°C. All the compounds were included at a final concentration of 3 µM. Nocodazole was used as a positive controls. Values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

Table 2 Inhibition of tubulin polymerization (IC_{50}) of compounds **5d** and **5l**.

Compound	IC_{50}[a] ± SD (µM)
5d	3.25±0.42
5l	1.71±0.08
Nocodazole	1.75±0.08

(a) Concentration of drug to inhibit 50% of tubulin assembly.

Compounds, **5d** and **5l** inhibited tubulin polymerization by 47.92 and 71.27%, respectively, compared to control (Figure **4**). Tubulin polymerization inhibition was also observed in case of standard nocodazole (66.98%).Furthermore, these conjugates (**5d**, **5l** and nocodazole) were evaluated for their inhibition of tubulin polymerization assay at different concentrations. It was observed that compound **5l** showed potent inhibition of tubulin polymerization with an IC_{50} value 1.71 µM which is similar to that of nocodazole (1.75 µM), whereas compound **5d** inhibited with an IC_{50} value of 3.25 µM as shown in Table **2**.

Immunohistochemistry of tubulin

In addition to *in vitro* tubulin polymerization studies, we investigated alterations in the microtubule network induced by compounds **5d** and **5l** in MCF-7 cells by immunofluoresence microscopy of immunohistochemistry studies, as most antimitotic agents affect microtubules.[44] Therefore, MCF-7 cells were treated with compounds 5d and 5l at 1 µM concentration for 48 h. Nocodazole was used as reference compound in this study. Results revealed that, untreated human breast cancer cells displayed the normal distribution of microtubules (Figure **5**). However, cells treated with these compounds (**5d** and **5l**) as well as nocodazole showed disrupted microtubule organization, as shown in Figure **5**, thus demonstrating the inhibition of tubulin polymerization.

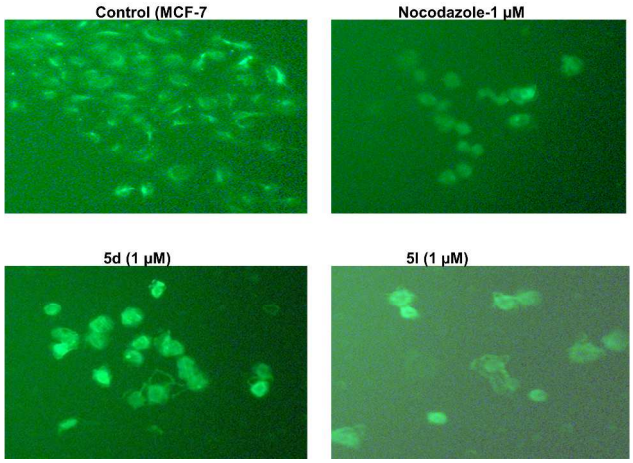

Figure 5: IHC analysis of compounds on the microtubule network: MCF-7 cells were treated with compounds **5d**, **5l** and Nocodazole at 1 µM concentration for 48 h.

Hoechst staining for apoptosis

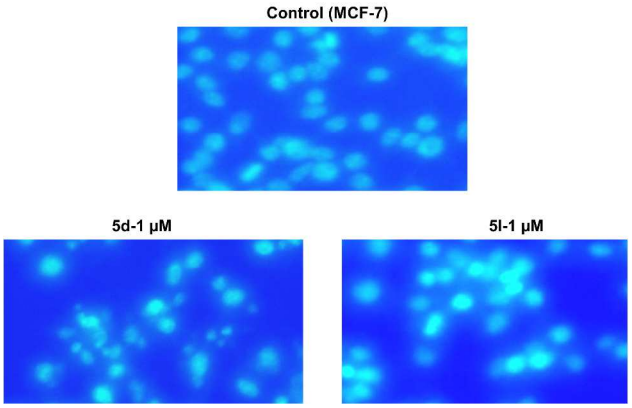

Figure 6: Hoechst staining in MCF-7 breast cancer cell line.

Apoptosis is one of the major pathways that lead to cell death, wherein chromatin condensation and DNA fragmentation are known as the classic characteristics of apoptosis. It was considered of interest to investigate the apoptotic inducing effects of these compounds (**5d** and **5l**) by Hoechst staining (H 33258) in MCF-7 cancer cells. Therefore cells were treated with these compounds at 1 µM concentration for 48 h. Based on chromatin condensation, fragmented nuclei, presence of apoptotic bodies and relative fluorescence of the test compounds (**5d** and **5l**) revealed a significant increase in the percentage of apoptotic cells relative to that of untreated control cells (Figure **6**).

Measurement of mitochondrial membrane potential (ΔΨm)

The maintenance of mitochondrial membrane potential (ΔΨm) is significant for mitochondrial integrity and bio energetic function.[45] Mitochondrial changes, including loss of mitochondrial membrane potential (ΔΨm), are key events that

take place during drug-induced apoptosis. To determine the changes in mitochondrial membrane potential (ΔΨm), we examined the lipophilic dye JC-1, specific for mitochondria. Mitochondria that maintain normal mitochondrial membrane potential (ΔΨm) concentrate JC-1 into aggregates indicates red fluorescence.

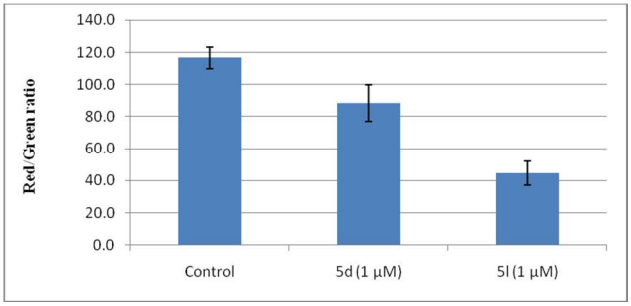

Figure 7: Figure represents the loss of mitochondrial membrane potential in human breast cancer cells, MCF-7 after 48 h of drug treatment with compounds **5d** and **5l** at 1 µM concentration.

However, JC-1 forms monomers (green fluorescence) in depolarized mitochondria. MCF-7 cells were treated with compounds **5d and 5l** at 1 µM concentration and incubated for 48 h. After 48 h of treatment with these compounds, it was observed that decrease in the mitochondrial membrane potential (ΔΨm) when compared to untreated control (Figure **7**).

Cytochrome c release

An important consequence of apoptotic pathway is the mitochondrial dysfunction and cytochrome c release.[46,47] In this context, human breast cancer cells, MCF-7 were treated with test compounds (**5d** and **5l**) at 1µM concentration for 48 h and Western blot analysis was performed to examine the effect on cytochrome c level. After treatment with these compounds (**5d** and **5l**), it was observed that there was an increase in the cytochrome c protein levels (Figure **11**), suggesting that the cytosolic cytochrome c release from mitochondria might be responsible for the induction of apoptosis by these compounds in the human breast cancer cells (MCF-7).

Effect on intracellular ROS generation

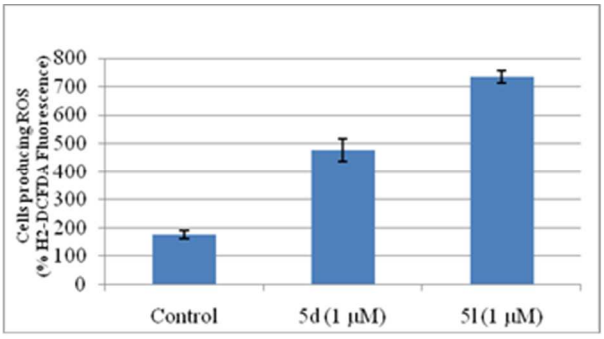

Figure 8: The effect of **5d** and **5l** on the ROS production in human breast cancer cells (MCF-7).

Many anticancer agents, have demonstrated to exert their cytotoxic effects by the generation of reactive oxygen species (ROS),[48,49] which is considered as one of the key mediators of apoptotic signaling. Therefore, we decided to investigate the role of these compounds (**5d** and **5l**) in inducing the production of ROS that could potentially lead to the cytotoxic effect in the MCF-7 cells. In order to demonstrate this production of ROS was examined by using an oxidant-sensitive fluorescent probe, DCFDA (2', 7'dichlorofluorescin diacetate).After treatment with these compounds at 1µM concentration, it was observed that **5d** and **5l** significantly increased the production of ROS in MCF-7 cells (Figure **8**).

Activation of caspase 9

The activation of caspases play an important role in the process of programmed cell death or apoptosis. Caspases, or cysteine-aspartic protease, are a family of cysteine proteases, which are crucial mediators of apoptosis. The MCF-7cells lack endogenous caspase-3, whereas caspase-9 plays an important role in mediating drug-induced apoptosis.[50]

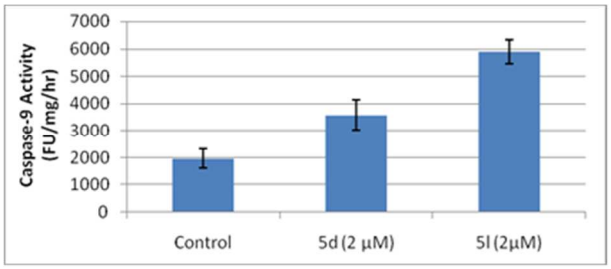

Figure 9: Effect of compounds **5d** and **5l** on caspase-9 activity; MCF-7 breast cancer cells were treated with these compounds at 2 µM concentration for 48 h. Values indicated are the mean ± SD of two different experiments performed in triplicates.

MCF-7 cells were treated with these compounds (**5d** and **5l)** at2 µM concentration for 48 h. The results demonstrate that there was 2-3 fold induction in caspase-9 activity when compared to untreated control (Figure **9**) which suggests that they have the ability to induce cell death by apoptosis in MCF-7 cells.

DNA fragmentation assay

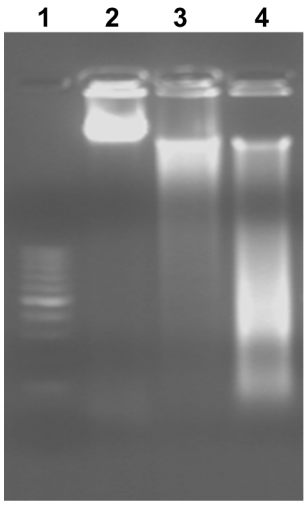

Figure 10: DNA fragmentation assay: Lane-1: Marker (100 bp), Lane-2: Control DNA, Lane-3: **5d**-1µM and Lane-4: **5l**-1 µM.

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ARTICLE TYPE

DNA laddering was carried out in order to elucidate the mode of action of these compounds especially for their ability to induce oligonucleosomal DNA fragmentation (DNA ladder), which is a characteristic feature of the programmed cell death or apoptosis.[51,52] During apoptosis, DNA is cleaved into smaller fragments by endonucleases that can be observed by gel electrophoresis as ladders. MCF-7 cells were treated with these compounds (**5d** and **5l**) at 1 µM concentration 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 they produced significant DNA fragmentation (Figure **10**), which is indicative of apoptosis

Western blot analysis

The PI3K pathway plays a key role in the cell signal transduction. AKT is the downstream target of PI3K, which controls angiogenesis and tumor growth.[53]The PTEN geneis frequently mutated or inactivated in a high proportion of human cancers, including up to 30% of breast cancers, resulting in hyper-activation of the PI3K/Akt signaling pathway.[54] Increasing PTEN expression and down-regulating PTEN phosphorylation are important steps in retarding tumor growth.[55] Therefore, controlling the PTEN or AKT signal pathway may be an important anticancer mechanism.[56]In order to understand the effect of these compounds on phosphorylated levels of p-PTEN and p-Akt, we have performed western blot analysis. In this study, MCF-7 cells were treated with these compounds (**5d** and **5l)** at 1 µM concentration for 48 h. After treatment with these compounds, phospho-PTEN and phospho-Akt levels were significantly reduced (Figure **11**). This result suggests that these compounds inhibition of cancer-cell growth may be via the inhibition of PI3K/AKT signaling pathway.

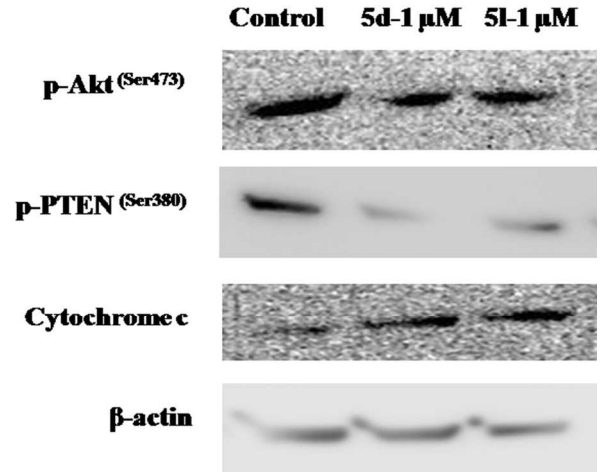
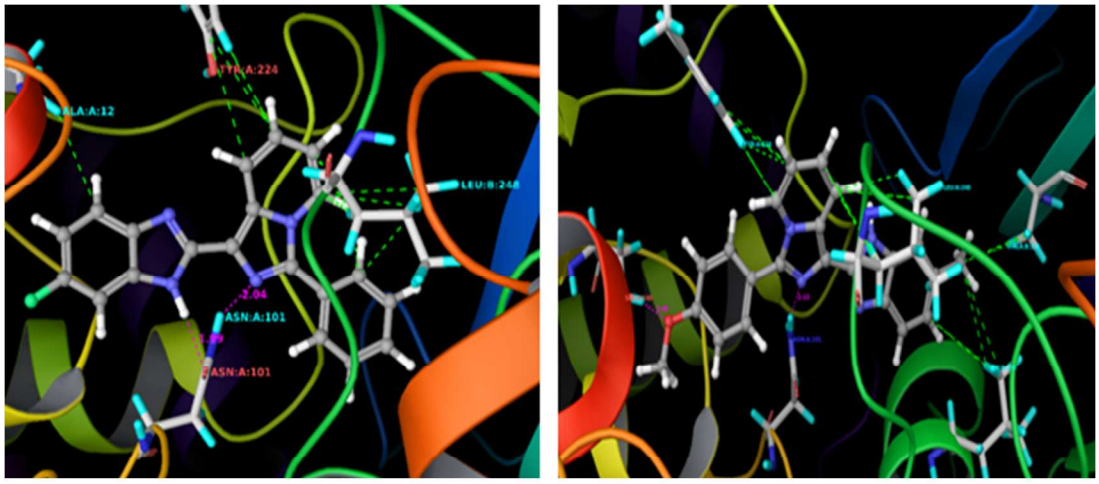


Figure 11: Effect of compounds on p-Akt, p-PTEN level and Cytochrome c release. MCF-7 cells were treated with compounds **5d** and **5l** at 1 µM concentration for 48 h. The cell lysates were collected and the expression levels of p-Akt, p-PTEN and Cytochrome c release were determined by Western blot analysis.*β*-Actin was used as a loading control.

Molecular modelling studies



5d **5l**

Figure 12: Docking pose and interacting aminoacids of compound **5d**, **5l**. Green dotted lines: hydrophobic interaction, pink dotted lines: Hydrogen bond interaction.

The crystal co-ordinates of αβ-tubulin subunits were taken from protein data bank (PDB ID: 3E22).[57] As the colchicine binds at the interface of αβ-tubulin subunits, both the chains were considered for molecular docking studies. The multi-step Schrödinger's (Schrödinger L.L.C., USA) protein preparation tool (PPrep) has been used for final preparation of receptor model. PPrep neutralizes side chains and residues which are not

involved in salt bridges. This step is then followed by restrained minimization using the OPLS 2005 force field to RMSD of 0.3 A°. The synthesized compounds **5d** and **5l** were sketched by using 2D sketcher and prepared for docking using Ligprep,[58] module of Schrödinger and a total of 10 conformations were generated for **5d** and **5l.** Active site pocket of colchicine was considered for grid generation with coordinates of X:-187.5857; Y:-54.3614 Z:-39.887 and GLIDE (3)-XP (Extra Precision) flexible program was used for docking.[59]

Molecular docking studies revealed that these compounds (**5d** and **5l**) bind to the colchicine binding site of the tubulin with a binding affinity of -6.58 and -7.00 respectively compared to colchicine (-5.76). NH group of benzimidazole ring and nitrogen of imidazopyridine group in **5d** showed a hydrogen bond interaction with Asn: A: 101 at distance of 1.99 A° and 2.14 A° respectively (Figure **12**). In case of **5l,** NH group of benzimidazole ring showed hydrogen bond interaction with aminonitrogen of Asn:A: 101 at distance of 2.04 A° and substitution with methoxy phenyl group contributed for the hydrogen bond interactions with hydroxyl group of Ser: A: 140 at a distance of 2.09A° which is responsible for the flipping of structure wherein the orientation of benzimidazole group is on opposite side of the imidazopyridine core. This orientation is fruitful for extensive interactions such as hydrophobic interactions with Tyr: A: 224, Leu: B: 248, Ala: B: 316 and Leu: B: 255 within 3A°distance of the active site pocket with the amino acid residues present in the protein cavity. Therefore, substitution with methoxyphenyl in **5l** resulted in improved docking score which contributed for the enhanced antiproliferative activity. Hence, from the molecular modelling studies it is clear that compound **5l** exhibits significant binding with colchicine binding site of the tubulin and this correlates well with the observed antiproliferative activity.

Conclusion

In conclusion, a series of imidazo[1,5-*a*]pyridine-benzimidazole hybrids **(5a-5aa)** were synthesized and evaluated for their anticancer activity against a panel of sixty human tumor cell lines derived from nine different cancer types: leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate and breast. Among them ten compounds **(5d, 5f, 5k-p, and 5z)** with promising activity were taken up for five dose concentration assay by the NCI. Interestingly some of these compounds exhibit promising anticancer activity and two of them (**5d** and **5l**) showed significantly GI_{50} values ranging from 1.06 to 14.9 µM and 0.43 to 7.73 µM, respectively against the cell lines tested. FACS analysis revealed that these compounds arrest the cell cycle at G2/M phase and induced cell death by apoptosis. These potential compounds **(5d** and **5l)** showed significant inhibition of tubulin polymerization with IC_{50} values 3.25 and 1.75 µM, respectively. Immunohistochemistry study results also suggest that these compounds disrupt the microtubule assembly in MCF-7 cells. The induction of apoptosis was further confirmed by Hoechst staining, loss of mitochondrial membrane potential, cytochrome c release, ROS generation, activation of caspase 9 and DNA fragmentation analysis. Moreover, these compounds significantly suppressed the expression levels of p-Akt and p-PTEN. Overall, the current study demonstrates that the synthesis of imidazo[1,5-*a*]pyridine-benzimidazole hybrids as promising cytotoxic agents with apoptotic-inducing ability by inhibiting both tubulin polymerization as well as PI3K/Akt pathway. Moreover, these compounds did not affect the normal human embryonic kidney cells (HEK-293), suggesting that these molecules selectively inhibits the growth of cancer cells.

Experimental

Chemistry

Materials and Methods

^{1}H NMR spectra were recorded on Avance 300, Inova 400, Avance 500, and Bruker 600 MHz spectrometers using tetramethyl silane (TMS) as the internal standard. Chemical shifts are reported in parts per million (ppm) downfield from tetramethyl silane. Spin multiplicities are described as s (singlet), brs (broad singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), and or m (multiplet). Coupling constants are reported in Hertz (Hz). Melting points were determined in a capillary tube using an Electrothermal apparatus (Model IA9200) and are uncorrected. The IR spectra were recorded by employing a Nicolet FTIR model MX-1spectrophotometer. Analytical thin layer chromatography (TLC) was performed on MERCK precoated silica gel 60- F254 (0.5-mm) glass plates. Visualization of the spots on TLC plates was achieved either by exposure to iodine vapour or UV light or by dipping the plates into methanolic sulphuric acid-*β*-naphthol or to ethanolic anisaldehyde-sulphuric acid-acetic acid or to ethanolic ninhydrin solution and heating the plates to 120 °C. Column chromatography was performed using silica gel 60-120 and 100-200 mesh. Moisture sensitive reactions were carried out using standard syringe septum Techniques and under inert atmosphere of nitrogen. All solvents and reagents were purified by standard techniques. *O*-Phenylene di-amines **(11a-i)** and substituted benzoyl chlorides **(7a-c)** are commercially available and used as received. All evaporation of solvents was carried out under reduced pressure on Laborota-4000 rotary evaporator below 45 °C. The names of all the compounds given in the experimental section were taken from Chem Ultra, Version 11.0.

General procedure for the synthesis of *N*1-(2-Pyridylmethyl) substituted benzamides (**8a-c**):

Method A.

To a stirred solution of 2-pyridylmethanamine **6** (1 mmol) in dry THF was added triethylamine (3.0 mmol) followed by substituted benzoylchlorides **7a-c (**1.1 mmol) at 0 °C. The reaction mixture was stirred for 3 h and the reaction was monitored by TLC. After completion of reaction, THF was removed under vacuum to get the crude products. This was further purified by column chromatography (EtOAc-Hexane) to obtain the pure compounds **(8a-c).**

***N*1-(2-Pyridylmethyl) benzamide** (**8a**). This compound was prepared according to the method **A**, employing benzoylchloride **7a** (1 mmol) to obtain the pure product **8a**. Yield: 76%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.53 (d, J = 4.7 Hz,1H), 7.88 (d, J = 6.4 Hz, 2H), 7.67 (t, J = 7.7 Hz, 2H), 7.47-

7.39 (m, 3H), 7.32 (d, *J* = 7.9 Hz, 1H), 7.20 (t, *J* = 5.0 Hz,1H), 4.73 (d, *J* = 4.7 Hz, 2H); MS (ESI): *m/z* 213 $[M+H]^+$.

N*1-(2-Pyridylmethyl)-4-methoxybenzamide (8b*). This compound was prepared according to the method A, employing 4-methoxy benzoylchloride **7b** (1 mmol) to obtain the pure product **8b**. Yield: 80%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.56 (d, *J* = 4.8 Hz,1H), 7.83 (d, *J* = 8.7 Hz, 2H), 7.68 (t, *J* = 7.8 Hz,1H), 7.47 (brs, 1H), 7.32 (d, *J* = 7.8 Hz,1H), 7.21 (t, *J* = 5.8 Hz,1H), 6.93 (d, *J* = 8.7 Hz, 2H), 4.75 (d, *J* = 4.8 Hz, 2H), 3.85 (s, 3H); MS (ESI): *m/z* 243 $[M+H]^+$.

***N*-(Pyridin-2-ylmethyl)-4-(trifluoromethyl)benzamide (8c)**. This compound was prepared according to the method A, employing 4-trifluoromethyl benzoylchloride **7c** (1 mmol) to obtain the pure product **8c**. Yield: 77%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.51 (d, *J* = 5.2 Hz, 1H), 8.15 (brs, 1H), 7.96 (d, *J* = 7.5 Hz, 2H), 7.63 (d, *J* = 8.3Hz, 2H), 7.30 (d, *J* = 7.5 Hz,1H), 7.19 (q, *J*= 5.2, 1.5Hz,1H), 4.71 (d, *J* = 4.5 Hz, 2H); MS (ESI): *m/z* 281 $[M+H]^+$.

General procedure for the synthesis of substituted phenylimidazo[1,5-*a*]pyridine (9a-c):

Method B.

To *N1*-(2-Pyridylmethyl)-substituted benzamides (**8a-c)** (1 mmol), add 4 mL of $POCl_3$ and refluxed for 3 hrs. This was poured into cold water and neutralized with $NaHCO_3$ solution. This water layer was extracted three times with ethylacetate. The combined organic phases were dried over anhydrous Na_2SO_4 and evaporated under vacuum. The residue, thus obtained was purified by column chromatography using ethylacetate and hexane as solvent system to afford the pure compounds **(9a-c).**

3-Phenylimidazo[1,5-*a*]pyridine (**9a**). This compound was prepared according to the method B. Yield: 78%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.24 (d, *J* = 7.5 Hz,1H), 7.76 (d, *J* = 6.7 Hz, 2H), 7.50-7.36 (m, 5H), 6.67 (dd, *J* = 9.0, 6.7 Hz, 1H), 6.51 (t, *J* = 6.7 Hz, 1H); MS (ESI): *m/z* 195 $[M+H]^+$.

3-(4-Methoxyphenyl)imidazo[1,5-*a*]pyridine (**9b**). This compound was prepared according to the method B. Yield: 80%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.34 (d, *J* = 8.7 Hz,1H), 8.32 (d, *J* = 6.8 Hz, 1H), 7.71 (d, *J* = 8.7 Hz, 2H), 7.32-7.17 (m, 1H), 7.07 (d, *J* = 8.7 Hz, 2H), 6.98 (s, 1H), 6.88 (t, *J* = 6.8 Hz, 1H), 3.90 (s, 3H); MS (ESI): *m/z* 225 $[M+H]^+$.

3-(4-(Trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridine (9c). This compound was prepared according to the method B. Yield: 78%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.28 (d, *J* = 7.5 Hz,1H), 7.91 (d, *J* = 8.3 Hz, 2H), 7.73 (d, *J* = 8.3 Hz, 2H), 7.54 (s, 1H), 7.47 (d, *J* = 9.0 Hz,1H), 6.73 (q, *J* = 6.7, 2.2 Hz,1H), 6.59 (t, *J* = 6.0 Hz, 1H); MS (ESI): *m/z* 263 $[M+H]^+$.

General procedure for the synthesis of3-(substituted Phenyl)imidazo[1,5-*a*]pyridine-1-carbaldehydes (10a-c) :

Method C. To an ice-water cooled solution of substituted phenyl imidazo[1,5-*a*]pyridines (**9a-c**) (1mmol) in DMF (1.4 mmol) was added drop wise $POCl_3$ (1.4 mmol)with stirring and then reaction mixture was heated to 100 °C for 2 h. After completion of the reaction, the reaction mixture was poured into ice water and quenched with ammonium hydroxide solution. The aqueous solution was extracted with ethyl acetate and the resultant organic layer was dried over anhydrous sodium sulphate and evaporated under vacuum. The residue, thus obtained was purified by column chromatography using ethylacetate and hexane as solvent system to afford pure compounds **(10a-c)**.

3-Phenylimidazo[1,5-*a*]pyridine-1-carbaldehyde (**10a**). This compound was prepared according to the method C. Yield: 80%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.10 (s, 1H), 8.39 (d, *J* = 8.3 Hz, 2H), 7.77 (d, *J* = 6.6 Hz, 2H), 7.58-7.46 (m, 3H), 7.22 (dd, *J* = 9.4, 6.7 Hz,1H), 6.86 (t, *J* = 6.7 Hz, 1H); MS (ESI): *m/z* 223 $[M+H]^+$.

3-(4-Methoxyphenyl)imidazo[1,5-*a*]pyridine-1-carbaldehyde(10b). This compound was prepared according to the method C. Yield: 76%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.16 (s, 1H), 8.34 (d, *J* = 8.7 Hz, 2H), 8.31 (d, *J* = 6.8 Hz, 1H), 7.26-7.23 (dd, *J* = 8.7 Hz, 2H), 7.07 (d, *J* = 8.7 Hz, 2H), 6.88 (t, *J* = 7.8 Hz, 1H), 3.90 (s, 3H); MS (ESI): *m/z* 253 $[M+H]^+$.

3-(4-(Trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridine-1-carbaldehyde (10c). This compound was prepared according to the method C. Yield: 80%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.16 (s, 1H), 8.42-8.38 (m, 2H), 7.95 (d, *J* = 8.3 Hz, 2H), 7.83 (d, *J* = 8.3 Hz, 2H), 7.34-7.29 (q, *J* = 6.0, 3.0 Hz, 1H), 6.97 (t, *J* = 7.5 Hz, 1H); MS (ESI): *m/z* 291 $[M+H]^+$.

General procedure for synthesis of compounds (5a-aa):

Method D

To a solution appropriate aldehyde (**10a-c)** in ethanol (10 mL), appropriate *o*-phenylene diamines (**11a-c)** (1 mmol) and sodium meta bisulphate (2 mmol) dissolved in H_2O (5 mL) were added sequentially and then reaction mixture was refluxed for 4 h. After completion of reaction, solvent was removed by vacuo to give crude product which was extracted with ethyl acetate. The combined organic extracts were dried over anhydrous $Na_2SO_4$and concentrated to give crude products which were further purified by column chromatography by using ethylacetate and hexane as solvent system to afford pure compounds **(5a-aa)**.

2-(3-Phenylimidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5a).

This compound was prepared according to the method D. Yield (166 mg, 78%); Yellow solid, mp: 205-210 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.29 (br s, 1H), 8.62 (d, *J* = 9.2 Hz, 1H), 8.34 (d, *J* = 7.1 Hz, 1H), 7.79 (d, *J* = 6.9 Hz, 3H), 7.6-7.47 (m, 4H), 7.23 (dd, *J* = 5.6, 2.6 Hz, 2H), 7.05 (dd, *J* = 9.6, 6.6 Hz, 1H), 6.75 (td, *J* = 7.3, 1.3 Hz, 1H); ^{13}C NMR (75 MHz, DMSO): δ 148.44, 143.50, 138.34, 129.27, 129.11, 129.13, 129.01, 128.92, 128.14, 127.99, 122.27, 122.07, 121.78, 120.80, 114.56; IR (KBr, cm^{-1}): 3310 (NH), 2952, 2352, 2327, 1634, 1519, 1251, 1031; MS (ESI): *m/z* 311 $[M+H]^+$; HRMS calcd for $C_{20}H_{15}N_4$ $[M+H]^+$ 311.12912, found 311.12877.

6-Methoxy-2-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5b).

This compound was prepared according to the method D. Yield (172 mg, 75%); Yellow solid, mp: 195-200 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.19 (br s, 1H), 8.63 (d, *J* = 8.2 Hz, 1H), 8.28 (d, *J* = 7.3 Hz, 1H), 7.81 (d, *J* = 7.3 Hz, 2H), 7.50 (td, *J* = 15.6, 7.3 Hz, 5H), 7.03 (t, *J* = 6.4 Hz, 1H), 6.87 (d, *J* = 8.2 Hz, 1H), 6.73 (t, *J* = 7.3 Hz, 1H), 3.87 (s, 3H); ^{13}C NMR (75 MHz, DMSO): δ 155.38, 148.11, 137.65, 129.90, 129.18, 129.04, 128.42, 127.75, 127.41, 122.67, 122.38, 122.32, 119.66, 114.66, 110.64, 55.36; IR (KBr, $\bar{v}$): 3416 (NH), 2352, 2318, 1642, 1581, 1453, 1344, 1154 cm^{-1}; MS (ESI): *m/z* 341

$[M+H]^+$; HRMS calcd for $C_{21}H_{17}N_4O$ $[M+H]^+$ 341.13969, found 341.13905.

6-Methyl-2-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5c).

This compound was prepared according to the method D. Yield (169 mg, 77%); Yellow solid, mp: 182-184 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.65-8.62 (m, 1H), 8.28 (t, *J* = 6.5 Hz, 1H), 7.82-7.80 (m, 3H), 7.55 (td, *J* = 7.3, 1.8 Hz, 2H), 7.50-7.46 (m, 1H), 7.22 (dd, *J* = 5.9, 3.0 Hz, 1H), 7.06-7.01 (m, 2H), 6.75-6.71 (m, 1H), 2.47 (s, 3H); ^{13}C NMR (75 MHz, DMSO): δ 148.08, 137.68, 130.41, 130.08, 129.16, 128.98, 128.20, 122.76, 122.70, 122.48, 122.23, 119.67, 114.66, 21.30; IR (KBr, cm^{-1}): 3444 (NH), 2352, 2327, 1633, 1593, 1445, 1317, 1275, 1031; MS (ESI): *m/z* 325 $[M+H]^+$; HRMS calcd for $C_{21}H_{17}N_4$ $[M+H]^+$ 325.14360, found 325.14371.

6-Fluoro-2-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5d).

This compound was prepared according to the method D. Yield (163 mg, 73%); Yellow solid, mp: 234-235 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.60 (br s, 1H), 8.63 (d, *J* = 9.0 Hz, 1H), 8.29 (d, *J* = 7.3 Hz, 1H), 7.82 (d, *J* = 8.3 Hz, 2H), 7.56-7.43 (m, 4H), 7.06 (dd, *J* = 8.6, 6.6 Hz, 2H), 6.94 (td, *J* = 9.8, 1.8 Hz, 1H), 6.73 (t, *J* = 6.4 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO): δ 158.43, 155.32, 148.18, 136.29, 128.81, 127.65, 127.34, 127.21, 126.52, 120.57, 120.53, 118.30, 112.85, 107.55, 107.22; IR (KBr, cm^{-1}): 3416 (NH), 3010, 2353, 2327, 1632, 1510, 1391,1284, 1126; MS (ESI): *m/z* 329 $[M+H]^+$; HRMS calcd for $C_{20}H_{14}N_4F$ $[M+H]^+$ 329.11970, found 329.11907.

6-Chloro-2-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5e).

This compound was prepared according to the method D. Yield (159 mg, 68%); Yellow solid, mp: 236-238 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.70 (br s, 1H), 8.62 (d, *J* = 9.0 Hz, 1H), 8.34 (d, *J* = 7.3 Hz, 1H), 7.79 (d, *J* = 7.1 Hz, 3H), 7.55-7.43 (m, 4H), 7.15 (d, *J* = 7.7 Hz, 1H), 7.08 (dd, *J* = 8.4, 6.4 Hz, 1H), 6.80 (t, *J* = 6.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$+ DMSO): δ 148.38, 137.00, 129.60, 127.96, 127.88, 127.62, 126.96, 125.20, 121.17, 120.84, 120.44, 118.74, 114.02, 113.98, 113.28, 112.92; IR (KBr, cm^{-1}): 3421 (NH), 3062, 2354, 2327, 1639, 1504, 1381, 1273, 1039; MS (ESI): *m/z* 345 $[M+H]^+$; HRMS calcd for $C_{20}H_{14}N_4Cl$ $[M+H]^+$ 345.09015, found 345.08968.

2-(3-Phenylimidazo[1,5-*a*]pyridin-1-yl)-6-(trifluoromethyl)-1H-benzo[*d*]imidazole (5f).

This compound was prepared according to the method D. Yield (166 mg, 65%), Yellow solid, mp: 222-225 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.90 (br s, 1H), 8.64 (d, *J* = 9.0 Hz, 1H), 8.32 (d, *J* = 7.1 Hz, 1H), 7.81 (dd, *J* = 8.3, 1.3 Hz, 2H), 7.54-7.41 (m, 5H), 7.26 (s, 1H), 7.11 (dd, *J* = 9.0, 6.6 Hz, 1H), 6.80 (t, *J* = 7.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$+ DMSO): δ 150.10, 137.35, 130.12, 128.27, 128.19, 127.89, 127.28, 125.89, 122.29, 122.17, 121.76, 121.42, 120.99, 120.91, 119.17, 117.13, 117.09, 113.52; IR (KBr): cm^{-1}: 3428 (NH), 3072, 2356, 2328, 1595, 1330, 1248, 1112; MS (ESI): *m/z* 379 $[M+H]^+$; HRMS calcd for $C_{21}H_{14}N_4F_3$ $[M+H]^+$ 379.11651, found 379.11632.

5-Chloro-6-fluoro-2-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5g).

This compound was prepared according to the method D. Yield (164 mg, 67%); Yellow solid, mp: 256-257 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.83 (br s, 1H), 8.58 (d, *J* = 9.2 Hz, 1H), 8.32 (d, *J* = 7.1 Hz, 1H), 7.78 (d, *J* = 6.9 Hz, 3H), 7.55-7.43 (m, 4H), 7.09 (dd, *J* = 8.8, 6.6 Hz, 1H), 6.80 (t, *J* = 6.7 Hz, 1H); ^{13}C NMR (75 MHz, DMSO): δ 155.22, 151.96, 150.98, 143.98, 141.72, 138.68, 133.17, 130.79, 129.01, 128.25, 123.28, 122.94, 121.25, 119.33, 118.41, 114.81, 113.09, 111.35, 105.06, 98.87; IR (KBr, cm^{-1}): 3416 (NH), 3060, 2354, 2327, 1633, 1462, 1351, 1146; MS (ESI): *m/z* 363 $[M+H]^+$; HRMS calcd for $C_{21}H_{14}N_4F_3$ $[M+H]^+$ 363.08073, found 363.08047.

5, 6-Dichloro-2-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5h).

This compound was prepared according to the method D. Yield (161 mg, 63%); Yellow solid, mp: 266-268 °C; ^{1}H NMR (300 MHz, DMSO): δ 8.65 (d, *J* = 6.9 Hz, 1H), 8.56 (d, *J* = 9.0 Hz, 1H), 8.43 (s, 1H), 8.50 (d, *J* = 6.9 Hz, 1H), 7.98 (s, 1H), 7.76-7.65 (m, 4H), 7.35 (dd, *J* = 9.0, 6.6 Hz, 1H), 7.06 (t, *J* = 6.7 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO): δ 149.57, 137.02, 129.76, 127.86, 127.58, 126.94, 122.91, 121.29, 120.86, 120.26, 118.65, 113.27; IR (KBr, cm^{-1}): 3410 (NH), 2954, 2355, 2321, 1633, 1565, 1381, 1135; MS (ESI): *m/z* 379 $[M+H]^+$; HRMS calcd for $C_{20}H_{13}N_4Cl_2$ $[M+H]^+$ 379.05118, found 379.05130.

5, 6-Dimethyl-2-(3-phenylimidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5i).

This compound was prepared according to the method D. Yield (166 mg, 72%); Yellow solid, mp: 218-220 °C; ^{1}H NMR (300 MHz, DMSO): δ 10.11 (br s, 1H), 8.67 (d, *J* = 9.0 Hz, 1H), 8.29 (d, *J* = 7.1 Hz, 1H), 7.82 (d, *J* = 7.1 Hz, 2H), 7.59-7.47 (m, 4H), 7.07-7.00 (m, 2H), 6.73 (t, *J* = 6.4 Hz, 1H), 2.4 (s, 6H); ^{13}C NMR (125 MHz, DMSO): δ 137.58, 130.09, 129.21, 128.97, 128.21, 123.58, 122.61, 122.48, 122.32, 119.84, 114.66, 19.03; IR (KBr, cm^{-1}): 3443 (NH), 2920, 2352, 2327, 1578, 1504, 1373, 1251, 1031; MS (ESI): *m/z* 339 $[M+H]^+$; HRMS calcd for $C_{22}H_{19}N_4$ $[M+H]^+$ 339.16042, found 339.16011.

2-(3-(4-Methoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5j).

This compound was prepared according to the method D. Yield (175 mg, 86%); Yellow solid, mp: 165-167 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.63 (d, *J* = 9.1 Hz, 1H), 8.20 (d, *J* = 7.3 Hz, 1H), 7.77 (d, *J* = 8.6 Hz, 3H), 7.20 (dd, *J* = 5.7, 2.8 Hz, 2H), 7.03 (d, *J* = 8.6 Hz, 3H), 7.01-6.99 (m, 1H), 6.71 (dt, *J* = 7.4, 1.2 Hz, 1H), 3.85 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO): δ 157.97, 146.69, 136.07, 128.22, 127.86, 120.54, 120.11, 119.99, 119.70, 119.39, 117.96, 112.48, 53.36; IR (KBr, cm^{-1}): 3418 (NH), 2952, 2358, 1594, 1457, 1345, 1251, 1030; MS (ESI): *m/z* 341 $[M+H]^+$; HRMS calcd for $C_{21}H_{17}N_4O$ $[M+H]^+$ 341.13969, found 341.13924.

6-Methoxy-2-(3-(4-methoxyphenyl) imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5k).

This compound was prepared according to the method D. Yield (176 mg, 80%); Yellow solid, mp: 153-154 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.62 (d, *J* = 9.0 Hz, 1H), 8.22 (d, *J* = 7.3 Hz, 1H), 7.74 (d, *J* = 8.6 Hz, 2H), 7.06 – 6.98 (m, 5H), 6.84 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.70 (t, *J* = 6.4 Hz, 1H), 3.85 (d, *J* = 6.4 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.38, 156.14, 148.33, 138.41, 130.23, 129.73, 122.15, 121.74, 121.57, 121.52, 120.86, 114.64, 114.44, 111.43, 55.81, 55.43; IR (KBr, cm^{-1}): 3462 (NH), 2935, 2360, 1617, 1590, 1327, 1129, 1068; MS

(ESI): *m/z* 371 $[M+H]^+$; HRMS calcd for $C_{22}H_{19}N_4O$ $[M+H]^+$ 371.15025, found 371.15007.

2-(3-(4-Methoxyphenyl) imidazo[1,5-*a*]pyridin-1-yl)-6-methyl-1H-benzo[*d*]imidazole (5l).

This compound was prepared according to the method D. Yield (178 mg, 84%); Yellow solid, mp: 199-201 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.23 (br s, 1H), 8.64 (d, *J* = 9.2 Hz, 1H), 8.19 (d, *J* = 7.3 Hz, 1H), 7.70 (d, *J* = 8.8 Hz, 3H), 7.06-7.98 (m, 5H), 7.02 (dt, *J* = 7.3, 1.1 Hz, 1H), 6.70 (t, *J* = 7.1 Hz, 1H), 3.87 (s, 3H), 2.46 (s,3H); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO): δ 158.85, 146.98, 136.98, 136.38, 129.93, 129.01, 128.53, 121.92, 120.72, 120.54, 120.42, 118.94, 113.32, 113.09, 113.01, 112.87, 112.63, 54.04, 20.34; IR (KBr, cm^{-1}): 3340 (NH), 2952, 2361, 1578, 1520, 1332, 1237, 1132, 928; MS (ESI): *m/z* 355 $[M+H]^+$; HRMS calcd for $C_{22}H_{19}N_4O_2$ $[M+H]^+$ 355.15534, found 355.15507.

6-Fluoro-2-(3-(4-methoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5m).

This compound was prepared according to the method D. Yield (173 mg, 81%); Yellow solid, mp: 210-215 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.61 (d, *J* = 9.2 Hz, 1H), 8.26 (d, *J* = 7.3 Hz, 1H), 7.69 (d, *J* = 8.8 Hz, 3H), 7.06 – 7.00 (m, 4H), 6.91 (dt, *J* = 9.4, 1.7 Hz, 1H), 6.73 (dt, *J* = 7.1, 1.7 Hz, 1H), 3.85 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.80, 160.39, 157.66, 138.63, 130.58, 129.66, 122.02, 121.82, 121.34, 120.73, 114.64, 114.49, 110.02, 109.69, 55.38; IR (KBr, cm^{-1}): 3432 (NH), 2930, 2352, 1609, 1583, 1504, 1411, 1248, 1131, 1043; MS (ESI): *m/z* 359 $[M+H]^+$; HRMS calcd for $C_{21}H_{16}N_4OF$ $[M+H]^+$ 359.13027, found 359.12957.

6-Chloro-2-(3-(4-methoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5n).

This compound was prepared according to the method D. Yield (169 mg, 76%); Yellow solid, mp: 224-226 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 11.54 (br s, 1H), 8.58 (d, *J* = 9.2 Hz, 1H), 8.27 (d, *J* = 7.1 Hz, 1H), 7.67 (d, *J* = 8.6 Hz, 3H), 7.07 (dd, *J* = 9.2, 6.4 Hz, 2H), 6.93 (d, *J* = 8.6 Hz, 3H), 6.79 (td, *J* = 7.3, 1.1 Hz, 1H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.52, 157.88, 155.76, 152.58, 138.87, 130.87, 129.69, 122.51, 121.94, 121.42, 120.96, 120.72, 115.07, 114.70, 114.59, 55.41; IR (KBr, cm^{-1}): 3444 (NH), 3121, 2359, 2341, 1611, 1575, 1409, 1349, 1251, 1141, 1039; MS (ESI): *m/z* 375 $[M+H]^+$; HRMS calcd for $C_{21}H_{16}N_4OCl$ $[M+H]^+$ 375.10072, found 375.10135.

2-(3-(4-Methoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-6-(trifluoromethyl)-1H-benzo[*d*]imidazole (5o).

This compound was prepared according to the method D. Yield (164 mg, 67%); Yellow solid, mp: 224-226 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.65 (d, *J* = 9.0 Hz, 1H), 8.27 (d, *J* = 7.1 Hz, 1H), 7.68 (d, *J* = 8.7 Hz, 3H), 7.39 (d, *J* = 7.6 Hz, 1H), 7.09 (dd, *J* = 8.8, 6.4 Hz, 1H), 6.95 (d, *J* = 8.5 Hz, 3H), 6.78 (t, *J* = 7.1 Hz, 1H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 159.77, 150.68, 138.07, 130.49, 129.33, 126.44, 123.01, 122.82, 122.59, 121.71, 121.40, 121.12, 121.06, 119.86, 117.82, 113.88, 54.83; IR (KBr, cm^{-1}): 3435 (NH), 3083, 2358, 2340, 1612, 1523, 1331, 1287,1113, 1040; MS (ESI): *m/z* 409 $[M+H]^+$; HRMS calcd for $C_{22}H_{16}N_4F_3O$ $[M+H]^+$ 409.12707, found 409.12652.

5-Chloro-6-fluoro-2-(3-(4-methoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5p).

This compound was prepared according to the method D. Yield (168 mg, 72%); Yellow solid, mp: 210-213 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.97 (br s, 1H), 8.58 (d, *J* = 9.2 Hz, 1H), 8.26 (d, *J*= 7.1 Hz, 1H), 7.77 (d, *J* = 8.8 Hz, 3H), 7.10 (d, *J* = 7.5 Hz, 1H), 7.07 – 7.04 (m, 1H), 7.02 (d, *J* = 8.6 Hz, 2H) 6.74 (dt, *J*= 7.5, 1.3 Hz, 1H), 3.84 (s, 3H); ^{13}C NMR (75 MHz, DMSO): δ 159.79, 150.04, 149.61, 145.40, 143.25, 138.11, 134.93, 132.97, 130.33, 130.23, 129.78, 125.58, 125.49, 122.76, 121.31, 121.07, 119.38, 118.98, 117.20, 114.49, 114.37, 112.05, 110.57, 55.24; IR (KBr, cm^{-1}): 3444 (NH), 3121, 2356, 2339, 1611, 1591, 1454, 1317, 1275, 1038; MS (ESI): *m/z* 393 $[M+H]^+$; HRMS calcd for $C_{21}H_{15}N_4ClF$ $[M+H]^+$ 393.09129, found 393.09085.

5,6-Dichloro-2-(3-(4-methoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5q).

This compound was prepared according to the method D. Yield (154 mg, 63%); Yellow solid, mp: 220-222 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.54 (d, *J* = 9.0 Hz, 1H), 8.29 (d, *J* = 7.1 Hz, 1H), 7.77-7.68 (m, 4H), 7.11-7.05 (m, 3H), 6.81 (t, *J* = 6.3 Hz, 1H), 3.91 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$+ DMSO): δ 158.70, 149.63, 137.04, 129.43, 128.38, 122.74, 120.95, 120.79, 120.07, 119.83, 118.53, 112.94, 53.85; IR (KBr, cm^{-1}): 3427 (NH), 3129, 2357, 2339, 1592, 1518, 1444, 1307, 1290; MS (ESI): *m/z* 409 $[M+H]^+$; HRMS calcd for $C_{21}H_{15}N_4OCl_2$ $[M+H]^+$ 409.06174, found 409.06079.

2-(3-(4-Methoxyphenyl)imidazo[1,5-*a*]pyridin-1-yl)-5,6-dimethyl-1H-benzo[*d*]imidazole (5r).

This compound was prepared according to the method D. Yield (157 mg, 71%); Yellow solid, mp: 250-252 °C; ^{1}H NMR (300 MHz, DMSO): δ 8.58 (d, *J* = 9.0 Hz, 1H), 8.30 (d, *J* = 7.1 Hz, 1H), 7.77 (d, *J* = 8.6 Hz, 2H), 7.33 (d, *J* = 8.1 Hz, 1H), 7.09 (d, *J* = 8.6 Hz, 2H), 7.07 – 6.97 (m, 2H), 6.79 (t, *J* = 6.9 Hz, 1H), 3.91 (s, 3H), 2.58 (s, 3H), 2.39 (s, 3H); IR (KBr, cm^{-1}): 3361 (NH), 2919, 2353, 2319, 1588, 1313, 1236, 1121; MS (ESI): *m/z* 369 $[M+H]^+$; HRMS calcd for $C_{23}H_{21}N_4O$ $[M+H]^+$ 369.17099, found 369.17051.

2-(3-(4-(Trifluoromethyl)phenyl)imidazo[1,5-a]pyridin-1-yl)-1H-benzo[*d*]imidazole (5s).

This compound was prepared according to the method D. Yield (156 mg, 79%); Yellow solid, mp: 176-180 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.21 (br s, 1H), 8.67 (d, *J* = 9.0 Hz, 1H), 8.31 (d, *J* = 7.1 Hz, 1H), 7.98 (d, *J* = 8.1 Hz, 2H), 7.82 (d, *J* = 8.1 Hz, 3H), 7.26 (s, 3H), 7.10 (dd,*J* = 8.8, 6.4 Hz, 1H), 6.82 (t, *J* = 7.1 Hz, 1H); ^{13}C NMR (75 MHz, DMSO): δ 159.90, 156.79, 149.82, 136.27, 132.99, 131.01, 130.77, 128.93, 128.59, 125.80, 125.76, 123.29, 122.98, 122.36, 119.52, 115.12; IR (KBr, cm^{-1}): 3381 (NH), 2952, 2353, 2327, 1642, 1580, 1411, 1326, 1165, 1110, 1068; MS (ESI): *m/z* 379 $[M+H]^+$; HRMS calcd for $C_{23}H_{21}N_4O$ $[M+H]^+$ 379.11651, found 379.11652.

6-Methoxy-2-(3-(4-(trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5t).

This compound was prepared according to the method D. Yield (154 mg, 73%); Yellow solid, mp: 180-187 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.64 (d, *J* = 9.0 Hz, 1H), 8.30 (d, *J* = 7.1 Hz, 1H), 7.98 (d, *J* = 8.1 Hz, 3H), 7.82 (d, *J* = 8.1 Hz, 2H), 7.52 (br s, 1H), 7.08 (dd, *J* = 6.4, 2.6 Hz, 2H), 6.89 (dd, *J* = 8.8, 2.2 Hz, 1H), 6.80 (t, *J* = 6.9 Hz, 1H), 3.87 (s, 3H); ^{13}C NMR (125 MHz, DMSO): δ 155.39, 133.11, 130.46, 128.75, 128.59, 128.57, 128.50, 125.82, 125.79, 125.16, 123.00, 122.96, 122.88, 122.81, 119.78, 119.63, 115.08, 110.70, 55.33; IR

(KBr, cm^{-1}): 3417 (NH), 3018, 2355, 2328, 1633, 1583, 1410, 1325, 1128, 1066;MS (ESI): *m/z* 409 $[M+H]^+$; HRMS calcd for $C_{22}H_{16}N_4F_3O$ $[M+H]^+$ 409.12707, found 409.12646.

6-Methyl-2-(3-(4-(trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5u).

This compound was prepared according to the method D. Yield (151 mg, 74%); Yellow solid, mp: 124-128 °C; ^1H NMR (300 MHz, $CDCl_3$): 8.66 (d, *J* = 9.2 Hz, 1H), 8.29 (d, *J* = 7.1 Hz, 1H), 7.98 (d, *J* = 8.1 Hz, 3H), 7.83 (d, *J* = 8.3 Hz, 3H), 7.05 (d, *J* = 8.8 Hz, 2H), 6.81 (t, *J* = 6.2 Hz, 1H), 2.49 (s, 3H); ^{13}C NMR (75 MHz, DMSO): δ 143.39, 136.09, 134.57, 133.93, 133.51, 133.12, 132.56, 130.86, 130.67, 129.05, 128.86, 128.63, 128.50, 127.81, 125.83, 122.95, 120.40, 119.77, 117.71, 115.13, 113.92, 111.00, 110.70, 21.29; IR (KBr, cm^{-1}): 3436 (NH), 3123, 2354, 2328, 1616, 1519, 1325, 1284,1125, 1067; MS (ESI): *m/z* 393 $[M+H]^+$; HRMS calcd for $C_{22}H_{16}N_4F_3$ $[M+H]^+$ 393.13216, found 393.13193.

6-Fluoro-2-(3-(4-(trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5v).

This compound was prepared according to the method D. Yield (156 mg, 76%); yellow solid, mp: 230-232 °C; ^1H NMR (300 MHz, $CDCl_3$+DMSO): δ 8.63 (d, *J* = 9.2 Hz, 1H), 8.52 (d, *J* = 6.9 Hz, 1H), 8.12 (d, *J*= 8.1 Hz, 2H), 7.89 (t, *J* = 6.4 Hz, 2H), 7.54 (dd, *J* = 8.6, 4.9 Hz, 1H), 7.30 (dd, *J* = 9.2, 1.8 Hz, 1H), 7.16 (dd, *J* = 8.6, 6.6 Hz, 1H), 6.93 (dt, *J* = 8.3, 2.2 Hz, 2H); ^{13}C NMR (75 MHz, DMSO): δ 159.90, 156.79, 149.82, 136.27, 132.99, 131.01, 130.77, 128.93, 128.59, 125.80, 125.76, 123.29, 122.98, 122.36, 119.52, 115.12, 111.38, 109.17; IR (KBr, cm^{-1}): 3388 (NH), 3021, 2356, 2338, 1619, 1582, 1410, 1325, 1130, 1068; MS (ESI): *m/z* 397 $[M+H]^+$; HRMS calcd for $C_{22}H_{16}N_4F_3$ $[M+H]^+$ 397.10709, found 397.10688.

6-Chloro-2-(3-(4-(trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5w).

This compound was prepared according to the method D. Yield (145 mg, 68%); Yellow solid, mp: 229-230 °C; ^1H NMR (300 MHz, $CDCl_3$): δ 10.34 (br s, 1H), 8.67 (d, *J* = 9.2 Hz, 1H), 8.35 (d, *J* = 7.3 Hz, 1H), 7.98 (d, *J* = 8.3 Hz, 3H), 7.80 (d, *J* = 8.1 Hz, 3H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.12 (dd, *J* = 9.0, 6.6 Hz, 1H), 6.84 (t, *J* = 6.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO): δ 148.37, 135.28, 131.89, 130.12, 128.99, 128.56, 127.19, 125.30, 124.62, 124.57, 121.65, 121.47, 120.76, 120.52, 119.14, 113.90; IR (KBr, cm^{-1}): 3400 (NH), 3029, 2357, 1617, 1574, 1409, 1326, 1131, 1068; MS (ESI): *m/z* 413 $[M+H]^+$; HRMS calcd for $C_{21}H_{13}N_4F_3Cl$ $[M+H]^+$413.07754, found 413.07716.

6-(Trifluoromethyl)-2-(3-(4-(trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5x).

This compound was prepared according to the method D. Yield (146 mg, 63%); Yellow solid, mp: 200-202 °C; ^1H NMR (300 MHz, $CDCl_3$): δ 10.49 (br s, 1H), 8.67 (d, *J* = 8.6 Hz, 1H), 8.36 (d, *J* = 7.1 Hz, 1H), 7.95 (d, *J* = 8.1 Hz, 2H), 7.81 (d, *J* = 5.4 Hz, 3H), 7.63-7.46 (m, 2H), 7.16 (dd, *J* = 8.8, 6.4 Hz, 1H), 6.87 (t, *J* = 6.9 Hz, 1H); ^{13}C NMR (75 MHz, DMSO): δ 150.69, 136.69, 132.92, 131.52, 129.13, 128.77, 128.29, 125.89, 125.84, 123.93, 123.21, 122.30, 121.91, 121.75, 119.44, 118.27, 118.23, 115.33; IR (KBr, cm^{-1}): 3444 (NH), 2903, 2352, 1620, 1596, 1410, 1327, ,1249,1112, 1038; MS (ESI): *m/z* 447 $[M+H]^+$; HRMS calcd for $C_{22}H_{13}N_4F_6$ $[M+H]^+$ 447.10389, found 447.10339.

5-Chloro-6-fluoro-2-(3-(4-(trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5y).

This compound was prepared according to the method D. Yield (152 mg, 68%); Yellow solid, mp: 260-262 °C; ^1H NMR (300 MHz, $CDCl_3$+DMSO): δ 10.82 (br s, 1H), 8.60 (d, *J* = 9.0 Hz, 1H), 8.46 (d, *J* = 7.3 Hz, 1H), 8.07 (d, *J* = 8.1 Hz, 2H), 7.88 (d, *J* = 8.3 Hz, 2H), 7.60 (d, *J* = 6.6 Hz, 1H), 7.40 (d, *J* = 9.4 Hz, 1H),7.16 (dd, *J* =6.4, 2.6 Hz, 1H), 6.91 (t, *J* = 7.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO): δ 154.69, 151.54, 150.33, 136.45, 132.89, 131.16, 128.99, 128.63, 125.81, 125.76, 123.64, 123.09, 122.25, 121.82, 119.37, 115.20, 113.21, 112.94; IR (KBr, cm^{-1}): 3438 (NH), 3049, 2358, 1618, 1581, 1407, 1324, 1173, 1067; MS (ESI): *m/z* 431 $[M+H]^+$; HRMS calcd for $C_{22}H_{13}N_4F_6$ $[M+H]^+$ 431.06811, found 431.06707.

5,6-Dichloro-2-(3-(4-(trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5z).

This compound was prepared according to the method D. Yield (142 mg, 61%); Yellow solid, mp: 300-302 °C; ^1H NMR (300 MHz, $CDCl_3$): δ 8.54 (dd, *J* = 9.0, 7.1 Hz, 2H), 8.11 (d, *J* = 8.1 Hz, 2H), 7.87 (d, *J* = 8.1 Hz, 2H), 7.71 (s, 2H), 7.20 (dd, *J* = 8.6, 6.6 Hz, 1H), 6.95 (t, *J* = 6.9 Hz, 1H); IR (KBr, cm^{-1}): 3457 (NH), 2923, 2358, 1617, 1566, 1444, 1329, 1287, 1109, 1068; MS (ESI): *m/z* 447 $[M+H]^+$; HRMS calcd for $C_{21}H_{12}N_4F_3Cl_2$ $[M+H]^+$ 447.03856, found 447.03875.

5,6-Dimethyl-2-(3-(4-(trifluoromethyl)phenyl)imidazo[1,5-*a*]pyridin-1-yl)-1H-benzo[*d*]imidazole (5aa).

This compound was prepared according to the method D. Yield (140 mg, 66%); Yellow solid, mp: 245-147 °C; ^1H NMR (300 MHz, $CDCl_3$): δ 10.15 (br s, 1H), 8.70 (d, *J* = 8.8 Hz, 1H), 8.30 (d, *J* = 7.1 Hz, 1H), 7.98 (d, *J* = 8.1 Hz, 2H), 7.82 (d, *J* = 8.3 Hz, 2H), 7.07(dd, *J* = 8.4, 6.0 Hz, 2H), 6.79 (dd, *J* = 7.1, 0.9 Hz, 1H), 2.41 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 147.46, 136.49, 132.94, 131.03, 128.24, 126.21, 126.16, 124.56, 122.28, 121.38, 121.27, 115.24, 19.38; IR (KBr, cm^{-1}): 3443 (NH), 2919, 2357, 2317, 1675, 1562, 1478, 1334, 1239, 1101; MS (ESI): *m/z* 407 $[M+H]^+$; HRMS calcd for $C_{23}H_{18}N_4F_3$ $[M+H]^+$ 407.14781, found 407.14713.

6.2 Biology

Anticancer activity

The cytotoxic activity of the compounds was determined using MTT assay .[60-61] 1×10^4 cells/well were seeded in 200 µl DMEM, supplemented with 10% FBS in each well of 96-well microculture plates and incubated for 24 h at 37 °C in a CO_2 incubator. Compounds, diluted to the desired concentrations in culture medium, were added to the wells with respective vehicle control. After 48 h of incubation, 10 µl MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added to each well and the plates were further incubated for 4 h. Then the supernatant from each well was carefully removed, formazon crystals were dissolved in 100 µL of DMSO and absorbance at 570 nm wavelength was recorded.

Cell cycle analysis

Flow cytometric analysis (FACS) was performed to evaluate the distribution of the cells through the cell cycle phases. MCF-7, breast cancer cells were incubated with compounds (**5d** and **5l**) at 1 and 3 µM concentrations for 48 h. Untreated and treated cells were harvested, washed with PBS, fixed in ice-cold 70% ethanol and stained with propidium iodide (Sigma

Aldrich). Cell cycle was performed by flow cytometry (Becton Dickinson FACS Caliber) as earlier described.[62]

Inhibition of tubulin polymerization

A fluorescence based in vitro tubulin polymerization assay was performed according to the manufacturer's protocol (BK011, Cytoskeleton, Inc.). Briefly, the reaction mixture in a total volume of 10 µL contained PEM buffer, GTP (1 µM) in the presence or absence of test compounds (final concentration of 3 µM). Tubulin polymerization was followed by a time dependent increase in fluorescence due to the incorporation of a fluorescence reporter into microtubules as polymerization proceeds. Fluorescence emission at 420 nm (excitation wavelength is 360 nm) was measured by using a Varioscan multimode plate reader (Thermo scientific Inc.). Nocodazole was used as positive control in each assay. The IC_{50} value was defined as the drug concentration required inhibiting 50% of tubulin assembly compared to control. The reaction mixture for these experiments include: tubulin (3 mg/ml) in PEM buffer, GTP (1 µM), in the presence or absence of test compounds at 2.5, 5, 10, and 15 µM concentrations. Polymerization was monitored by increase in the Fluorescence as mentioned above at 37 °C.

Immunohistochemistry

MCF-7 cells were seeded on glass cover slips, incubated for 48 h in the presence or absence of test compounds 5**d** and **5l** at 1 µM concentration. Following the termination of incubation, cells were fixed with 3% paraformaldehyde, 0.02% glutaraldehyde in PBS and permeabilized by dipping the cells in 100% methanol followed by overnight incubation at 4 °C. Later, cover slips were blocked with 1% BSA in phosphate buffered saline for 1 h followed by incubation with a primary anti tubulin (mouse monoclonal) antibody and FITC conjugated secondary mouse anti IgG antibody. Photographs were taken using the fluorescence microscope, equipped with FITC settings and the pictures were analyzed for the integrity of microtubule network. In parallel experiments, nocodazole (1 µM) was used as positive control for analyzing microtubule integrity.

Hoechst staining

Cells were seeded at a density of 10,000 cells over 18-mm cover slips and incubated for 24 h. After incubation, cells were treated with the compounds **5d** and **5l** at 1 µM concentration for 48 h. Hoechst 33258 (Sigma Aldrich) was added to the cells at a concentration of 0.5 mg/mL and incubated for 30 min at 37 °C. Later, cells were washed with phosphate buffered saline (PBS). Cells from each cover slip were captured from randomly selected fields under fluorescent microscope (Leica, Germany) to qualitatively determine the proportion of viable and apoptotic cells based on their relative fluorescence and nuclear fragmentation.[63]

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential (ΔΨm) was determined by using JC-1 dye. Cell-permeable cationic carbocyanine dye JC-1 (Sigma aldrich India, Catlog- CS0390), also known as 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodine, emits green fluorescence (525 nm) in its monomeric form. However, upon transfer to the membrane environment of a functionally active mitochondrion, it exhibits an aggregation dependent orange-red fluorescence (emission at 590 nm). Briefly, cultures were treated with the test drugs for 48 h. After drug treatment the cells were incubated with JC-1 dye for 20 min at 37 °C. After incubation cultures were used for the measuring mitochondrial membrane potiential (ΔΨm), according to the manufacturer's instructions.

ROS Generation

To evaluate intracellular reactive oxygen species (ROS) levels, 2', 7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) fluorescent dye was used to clarify this issue. The nonpolar DCFH-DA is converted to the polar derivative DCFH by esterases when it is taken up by the cell. DCFH is nonfluorescent but is rapidly oxidized to the highly fluorescent DCF by intracellular H_2O_2 or nitric oxide. MCF-7 cells were treated with compound 5**d** and **5l** at 1 µM concentration for 48 h. To determine the production of ROS, cells were treated with 1 µM of DCFH2-DA at 37 °C for 30 min, and the fluorescence of DCF was measured at 530 nm after excitation at 485 nm (DCFH2-DA, after deacetylation to DCFH2, is oxidized intracellular to its fluorescent derivative, DCF).[64]

Activation of Caspase 9

Caspase-9 assay was conducted for detection of apoptosis in breast cancer cell line (MCF-7). The commercially available apoptosis detection kit (Sigma-Caspase 9 Assay kit, Colorimetric) was used. MCF-7 cells were treated with compounds 5**d** and **5l** at 1 µM concentrations for 48 h. After 48 h of treatment, cells were collected by centrifugation, washed once with PBS, and cell pellets were collected. Suspended the cell pellet in lysis buffer and incubated for 15 min. After incubation, cells were centrifuge at 20,000 rpm for 15 min and collected the supernatant. Supernatants were used for measuring caspase 9 activity using an ELISA-based assay, according to the manufacturer's instructions.

DNA fragmentation analysis

Cells were seeded (1×10^6) in six well plates and incubated for 24 h. After incubation, cells were treated with compounds **5d** and**5l** at 1 µM concentration for 48 h. After 48 h of drug treatment cells were collected and centrifuged at 2500 rpm for 5 min at 4°C. Pellet was collected and washed with Phosphate buffered saline (PBS), added 100 µl of Lysis buffer, centrifuged at 3000 rpm for 5 min at 4 °C and collected supernant and add 10 µl of 10% SDS and 10 µl of (50 mg/mL) RNase-A and incubated for 2 h at 56 °C. After that 10 µl of Proteinase K (25 mg/mL) was added and incubated at 37°C for 2 h. After incubation, 65 µl of 10 M Ammonium acetate and 500 µl of ice cold ethanol was added and mixed well. And this sample was incubated at 80 °C for 1 h. After that samples were centrifuged at 12000 rpm for 20 min at 4°C, washed with 80% ethanol followed by air dried for 10 min at room temperature. The pellet was dissolved in 50 µl TE buffer, and DNA laddering was determined by using 2% agarose gel electrophoresis inTE buffer.[65]

Protein extraction and western blot analysis

MCF-7 cells were treated with compounds **5d** and **5l** at 1 µM concentration for 48 h. The cell lysates were obtained by lysing the cells in ice-cold radio immune precipitation assay (RIPA) buffer (1×PBS, 1% NP-40 detergent,0.5% sodium deoxycholate, and 0.1% SDS) containing100 mg/mLphenylmethanesulfonyl fluoride (PMSF), 5 mg/mLaprotinin, 5 mg/mL leupeptin, 5 mg/mL pepstatin,

and100 mg/mL NaF. After centrifugation at 12 000 rpm for 10 min, the protein in the supernatant was quantified by the Bradfordmethod (BIO-RAD) by using a Multimode Varioskan instrument (Thermo Fischer Scientifics Ltd.). Protein (50 mg per lane) was appliedin 12% SDS polyacrylamide gel. After electrophoresis, the protein was transferred to a polyvinylidinedifluoride (PVDF) membrane (Thermo Scientific Inc.). The membrane was blocked at room temperature for 2 h in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% blocking powder (Santa Cruz). The membrane was washed with TBST for 5 min, then primary antibody was added and the membrane was incubated at 48 °C overnight. Cytochrome c, p-Akt,$^{(ser473)}$ p-PTEN$^{(ser380)}$ and β-actin antibodies were purchased from Cell Signaling Technology (CST). The membrane was incubated withthe corresponding horseradish peroxidase labeled secondary antibody (1:2000; CST) at room temperature for 1 h. Membranes were washed with TBST three times for 15 min, and the blots were visualized with chemiluminescence reagent (Thermo Fischer Scientifics Ltd). Images were captured by using the UVP ChemiDoc imager(BIO-RAD).[66]

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