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Synthesis of chalcone-amidobenzothiazole conjugates as antimitotic and apoptotic inducing agents

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ABSTRACT

A series of chalcone-amidobenzothiazole conjugates (**9a–k** and **10a,b**) have been synthesized and evaluated for their anticancer activity. All these compounds exhibited potent activity and the IC_{50} of two potential compounds (**9a** and **9f**) against different cancer cell lines are in the range of 0.85–3.3 μM. Flow cytometric analysis revealed that these compounds induced cell cycle arrest at G2/M phase in A549 cell line leading to caspase-3 dependent apoptotic cell death. The tubulin polymerization assay (IC_{50} of **9a** is 3.5 μM and **9f** is 5.2 μM) and immuofluorescence analysis showed that these compounds effectively inhibit microtubule assembly at both molecular and cellular levels in A549 cells. Further, Annexin staining also suggested that these compounds induced cell death by apoptosis. Moreover, docking experiments have shown that they interact and bind efficiently with tubulin protein. Overall, the current study demonstrates that the synthesis of chalcone-amidobenzothiazole conjugates as promising anticancer agents with potent G2/M arrest and apoptotic-inducing activities via targeting tubulin.

1. Introduction

Tubulin is a α,β-heterodimeric protein and is the main constituent of microtubules. Self assembly of α and β tubulin heterodimers forms cytoskeletal structures of microtubules which involves in many cellular functions.[1] Their most important role is in the formation of the mitotic spindle, and are considered essential in the mitotic division of cells. Tubulin is the target of numerous small molecule antiproliferative ligands that act by interfering with microtubule dynamics.[2] These ligands can be broadly divided into two categories: those that inhibit the formation of the mitotic spindle such as colchicine (**1**, Fig. 1)[3,4] as well as vinblastine[5] and those that inhibit the disassembly of the mitotic spindle once it has formed, such as paclitaxel.[6] There are three known binding sites of tubulin namely, the taxane domain, the vinca domain, and the colchicine domain. Many compounds interact with tubulin at these known sites. Most of the tubulin binding compounds, such as paclitaxel and vinblastine, are in clinical use for various types of cancer.[2] Antimitotic agents are one of the major classes of cytotoxic drug for cancer treatment, and microtubules are a significant target for many natural product anticancer agents such as combretastatin A-4 (**2a**, Fig. 1)[7] and podophyllotoxin.[2,8] Combretastatin A-4 which is isolated from the bark of the South African Cape Bush willow (Combretum caffrum) exhibits potent anticancer activity against a number of human cancer cell lines including multidrug resistant cancer cell lines.[9] It acts as a microtubule destabilizing agent by binding to the tubulin at the site similar to that of colchicine (**1**).[10] The water-soluble prodrug of combretastatin A-4-phosphate (**2b**, Fig. 1), is now in clinical trials for thyroid cancer[11–13] and in patients with advanced cancer.[14] The amino derivative of CA-4 (**3a**, Fig. 1) is also in clinical trials as a water-soluble amino acid prodrug (**3b**, Fig. 1).[15] Being a potent inhibitor of colchicine binding, CA-4 is also shown to inhibit the growth and development of blood vessels, angiogenesis.[5,15–18] Studies on combretastatin have established that *cis*-orientation of ethenyl bridge is essential for strong cytotoxicity. However, during storage and administration, *cis*-olephine double bond is prone to isomerize into *trans*-form, resulting in dramatic reduction in both antitubulin activity and cytotoxicity.[19] Various structural modifications to CA-4 have been reported including variation of the A- and B-ring substituents.[20,21] Many conformationally restricted analogs of CA-4 are known and most of these compounds replace the *cis*-double bond with chalcone functionality or with a heterocyclic rigid ring scaffold structure.[20] These compounds inhibit cell growth of several human cancer cell lines, and many have been shown to be significant tubulin binding and

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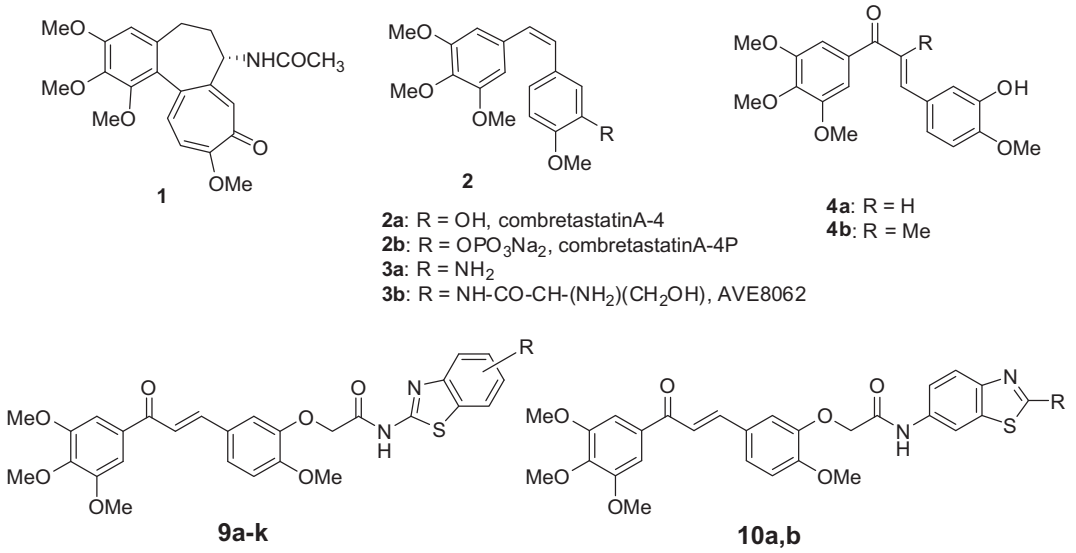
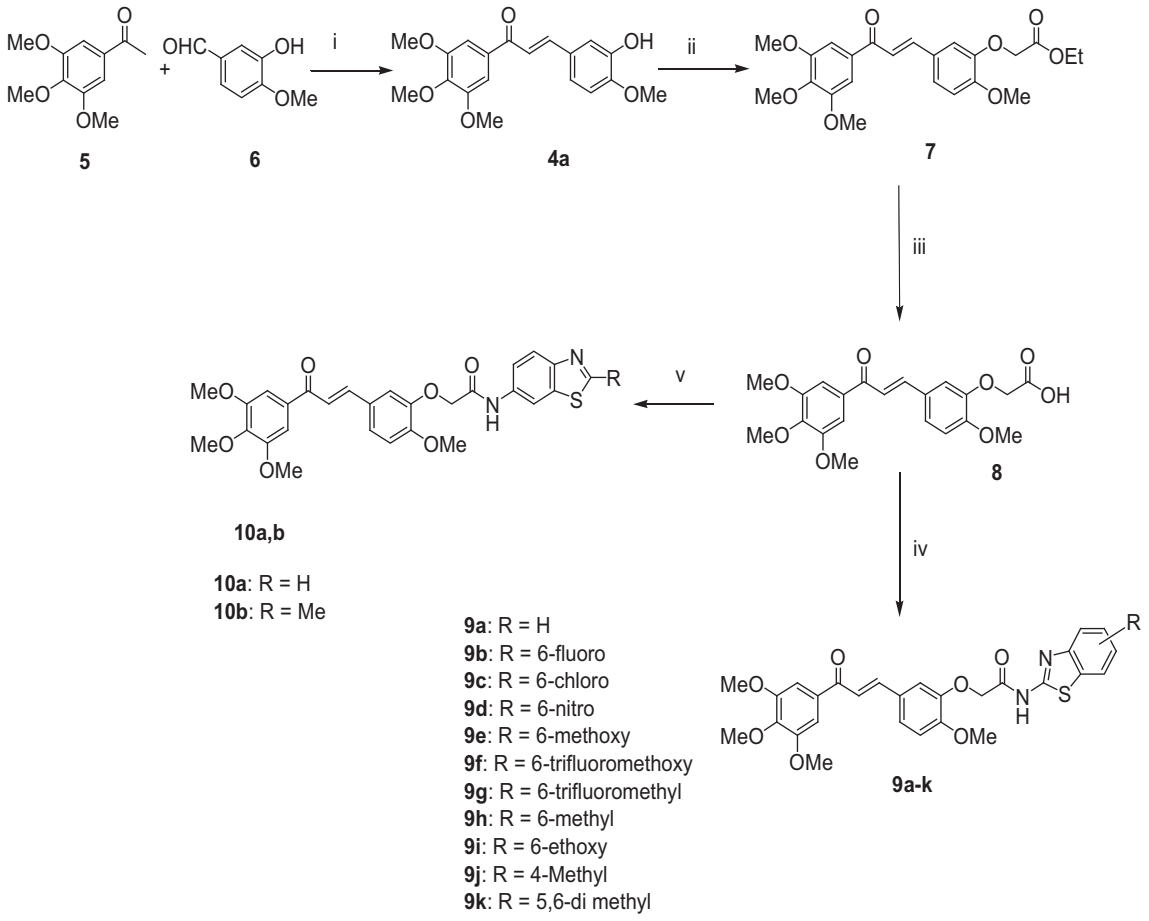


Figure 1. Structures of tubulin binding inhibitors and chalcone-benzothiazole conjugates (**9a–k** and **10a**,**b**).



Scheme 1. Reagents and conditions: (i) 50% KOH, ethanol, rt, 8 h, 87%; (ii) 2-bromoethyl acetate, K_2CO_3, DMF, rt, 24 h, 88%; (iii) LiOH, THF, H_2O, rt, 12 h, 76%; (iv) 2-aminobenzothiazoles, EDCI, HOBt, CH_2Cl_2, rt, 24 h, 83–78%; (v) 6-amino benzothiazoles, EDCI, HOBt, CH_2Cl_2, rt, 24 h, 73–76%.

depolymerising agents. The chalcone analogs of combretastatin **4a,b** have been synthesized and developed by Ducki et al.[22] These chalcone analogs showed potent inhibition of tubulin assembly and possesses promising anticancer activity. The prodrugs of chalcones **4a** and **4b** are currently under preclinical evaluation.[23,24]

Benzothiazoles constitute an important scaffold of drugs, possessing several pharmacological functions,[25] and exhibit potent and intriguing antitumor properties.[26–28] Some of the 2-phenylamino derivatives have been reported to possess cytotoxicity on tumor cells which are comparable to that of cisplatin.[29] The

Table 1
Anticancer activity (IC_{50}) data of compounds **9a–k** and **10a,b**

Compound	IC_{50} values (μM)				
	A549[a]	A375[b]	MCF-7[c]	HT-29[d]	ACHN[e]
9a	0.85	1.84	2.4	1.53	2.3
9b	4.4	2.1	7.5	11.9	3.7
9c	6.9	13.1	8.5	6.1	9.7
9d	4.7	6.8	8.1	8.7	5.9
9e	3.6	2.8	2.2	3.4	1.5
9f	0.9	2.4	3.3	1.1	1.73
9g	1.7	2.3	2.6	3.7	2.4
9h	4.7	7.5	4.3	2.1	2.8
9i	8.1	5.8	4.2	2.4	2.6
9j	5.1	2.8	2.5	6.1	3.8
9k	6.3	5.3	6.2	5.9	3.1
10a	9.4	5.3	6.7	8.4	13.2
10b	8.7	6.8	4.9	9.5	8.5
CA-4	0.11	0.21	0.18	0.93	0.17
4a	0.76	0.19	0.12	1.9	1.62

[a] Lung cancer.
[b] Melanoma cancer.
[c] Breast cancer.
[d] Colon cancer.
[e] Renal cancer.

cantharidin-containing 2-aminobenzothiazoles possess antitumor properties against a number of different cancer cell lines and are effective in minimizing the original cytotoxicity, whilst maintaining potency towards cancer cell lines.[30] These considerations and encouraging anticancer profile with inhibition of tubulin polymerization greatly led us to synthesize these new conjugates. Herein we report the synthesis of chalcone-benzothiazole conjugates fused with amide bond and evaluation of their anticancer potential. These compounds were also evaluated for the cell cycle analysis, inhibition of tubulin polymerization and apoptotic nature. Moreover, the inhibition of tubulin polymerization exhibited by these compounds is supported by molecular docking investigations.

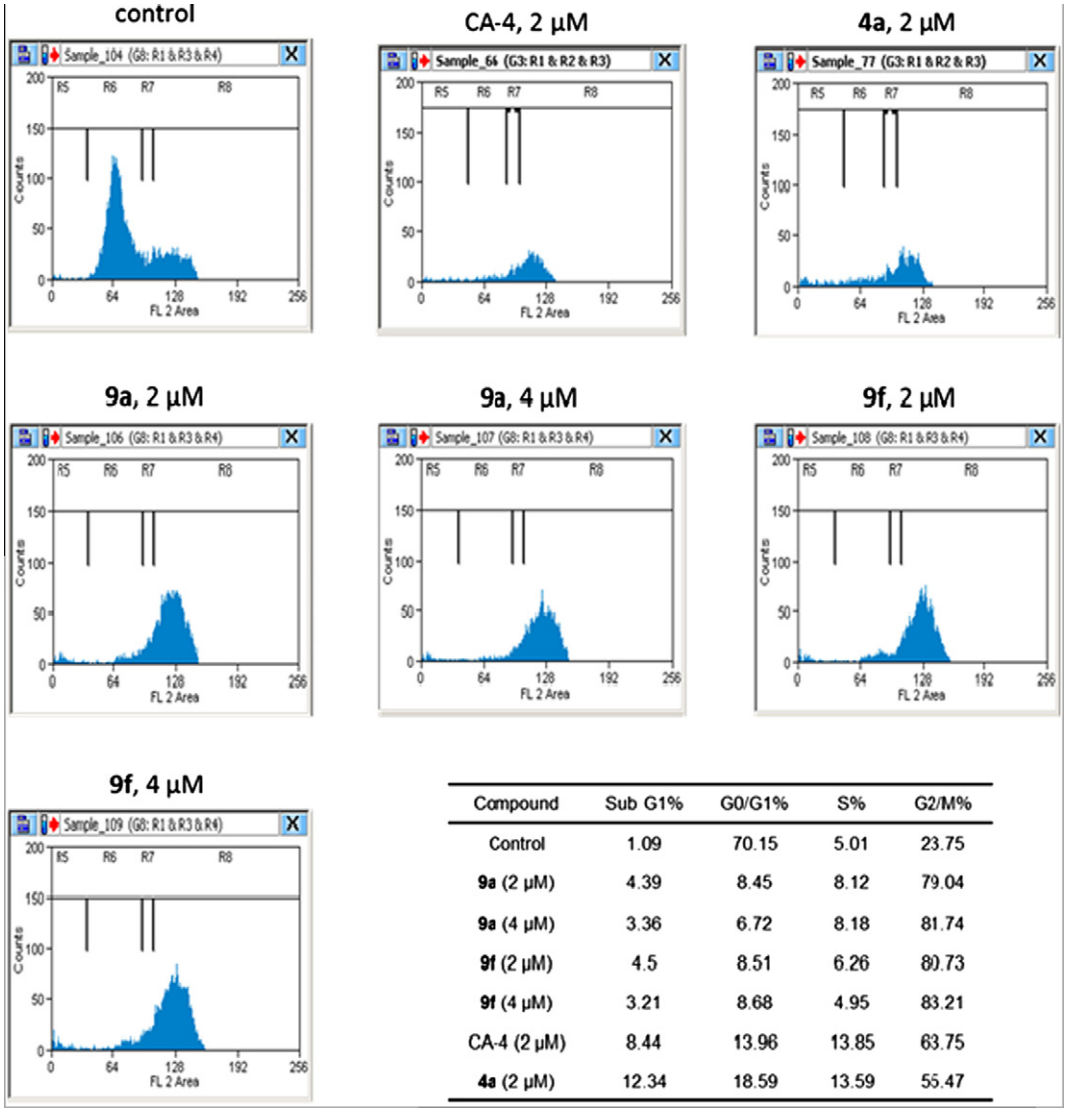
2. Chemistry

The synthesis of new chalcone-amidobenzothiazole conjugates (**9a–k** and **10a,b**) is shown in Scheme 1. The trimethoxychalcone **4a** was prepared in good yield by the Claisen–Schmidt condensation of trimethoxyacetophenone (**5**) and isovanilline (**6**) by using aqueous KOH in ethanol.[22] In this method, the prepared chalcone **4a** was predominantly with (*E*)-configuration, which was con-

Table 2
Anticancer activity (GI_{50} in μM) of chalcone-amidobenzothiazole conjugates against human cancer cell lines (data from NCI, USA)

Cancer panel/cell line	[a]GI_{50} values (μM)					Cancer panel/cell line	[a]GI_{50} values (μM)				
	[b]**9a**	[c]**9b**	[d]**9e**	[e]**9f**	[f]**9g**		[b]**9a**	[c]**9b**	[d]**9e**	[e]**9f**	[f]**9g**
Leukemia						*CNS*					
CCRF-CEM	2.09	5.73	3.55	2.5	3.37	SF-268	0.95	3.78	4.16	1.2	3.28
K-562	0.44	4.32	3.52	0.43	3.67	SF-295	3.41	18.8	6.68	2.21	8.87
MOLT-4	3.03	29.9	3.5	2.89	4.31	SF-539	0.34	2.49	2.17	0.3	2.1
RPMI-8226	1.09	7.35	3.02	2.02	3.84	SNB-19	2.81	17.3	5.61	1.58	6.01
SR	0.38	3.17	2.53	0.39	0.85	SNB-75	0.53	1.67	2.04	0.34	2.2
						U251	0.96	6.08	3.08	0.7	3.27
Non-small cell lung						*Melanoma*					
A549/ATCC	2.63	23.2	11.7	1.99	14.3	LOX IMVI	0.52	3.37	2.59	0.47	2.88
EKVX	5.91	38.1	[g]NA	5.11	30.3	MALME-3M	6.42	27.7	9.62	2.69	8.75
HOP-62	6.11	11.7	4.11	2.12	6.57	M14	3.29	10.7	3.28	1.72	3.31
HOP-92	1.33	2.39	2.62	2.14	4.1	MDA-MB-435	0.3	1.93	1.51	0.3	1.16
NCI-H226	2.77	21.1	32.5	3.35	21.5	SK-MEL-2	5.39	NA	24.2	4.97	30
NCI-H23	3.4	11	5.06	2.15	3.68	SK-MEL-28	4.61	24.1	14.4	3.31	9.47
NCI-H322M	4.26	38.1	7.16	2.77	6.73	SK-MEL-5	1.76	4.49	4.56	2.03	2.88
NCI-H460	2.64	13.5	3.32	2.43	4.06	UACC-257	1.37	NA	20.4	8.08	NA
NCI-H522	1.32	3.2	2.16	0.67	2	UACC-62	7.87	33.6	14.5	9.52	22.7
Colon						*Ovarian*					
COLO-205	5.47	26.8	NA	6.37	37.7	IGROV1	3.6	16.2	7.73	2.54	15.6
HCC-2998	2.14	33	19.3	6.18	21.3	OVCAR-3	0.48	3.4	2.6	1.52	1.95
HCT-116	2.88	15.7	3.98	1.43	3.83	OVCAR-4	3.2	14.7	11.2	2.07	4.37
HCT-15	0.49	3.96	3.34	0.4	2.68	OVCAR-5	3.54	NA	NA	4.76	38.9
HT29	3.49	NA	32.4	3.66	21.5	OVCAR-8	2.77	10.2	3.49	1.54	3.85
KM12	0.36	2.97	2.4	1.51	1.77	NCI/ADR-RES	0.48	2.43	1.89	0.49	1.73
SW-620	0.62	3.84	4.07	1.08	3.82	SK-OV-3	2.54	15.2	7.32	2.16	5.08
Prostate						*Renal*					
PC-3	3.63	22.9	12.2	3.14	12	786-0	4.12	24.7	19.4	2.26	24.6
DU-145	1.94	4.05	2.49	1.56	2.62	A498	NA	NA	NA	7.4	NA
Breast						ACHN	3.46	12.4	4.94	1.62	7.48
MCF7	1.05	3.7	3.02	1.32	3.05	CAKI-1	3.78	30.7	25.1	2.2	10.9
MDA-MB-231	2.69	12.6	5.82	2.01	7.14	RXF 393	1.83	8.5	2.37	1.58	2.6
HS 578T	4.75	10.5	3.72	3.08	4.81	SN12C	3.02	17.4	5.19	1.74	5.41
BT-549	4.82	4.82	8.38	2.71	11.2	TK-10	3.3	20.9	16.4	2.08	15.6
T-47D	4.97	NA	14.7	3.08	15	UO-31	3.07	6.83	5.31	1.61	7.8
MDA-MB-468	2.3	13.1	10.5	3.52	13.5						

[a] Values are reported as GI_{50}, the μM concentration of the compound required to cause 50% inhibition of cell growth.
[b] (**9a**, NSC 753575).
[c] (**9b**, NSC 753577).
[d] (**9e**, NSC 753579).
[e] (**9g**, NSC 753585).
[f] (**9f**, NSC 753582).
[g] Not active (GI_{50} >50 μM).



Compound	Sub G1%	G0/G1%	S%	G2/M%
Control	1.09	70.15	5.01	23.75
9a (2 μM)	4.39	8.45	8.12	79.04
9a (4 μM)	3.36	6.72	8.18	81.74
9f (2 μM)	4.5	8.51	6.26	80.73
9f (4 μM)	3.21	8.68	4.95	83.21
CA-4 (2 μM)	8.44	13.96	13.85	63.75
4a (2 μM)	12.34	18.59	13.59	55.47

Figure 2. Flow cytometric analysis in A549 lung cancer cell lines after treatment with compounds **9a** and **9f** at 2 and 4 μM concentrations for 48 h. CA-4 and **4a** were employed as the positive controls.

firmed by ^1H–^1H coupling constant values of the ^1H NMR spectra. The chalcone **4a** upon etherification with α-bromoethyl acetate in the presence of K_2CO_3 in dry DMF gave ester **7**. This ester on hydrolysis with $LiOH{\cdot}H_2O$ afforded the acid **8**. The synthesis of conjugates **9a–k** was achieved by the formation of amide bond between the acid (**8**) and a variety of substituted 2-aminobenzothiazoles by using EDCI/HOBt. Similarly other analogs (**10a,b**) were synthesized using 6-aminobenzothiazoles as shown in Scheme 1.

3. Biological evaluation

3.1. Anticancer activity

The newly synthesized compounds (**9a–k** and **10a,b**) were evaluated for their anticancer activity against a panel of five human cancer cell lines, A549 (lung), A375 (melanoma), MCF-7 (breast), HT-29 (colon) and ACHN (renal) by employing MTT assay. CA-4 was used as the reference drug. The results are summarized in Table 1 and expressed as IC_{50} values. The in vitro screening results revealed that these compounds exhibited promising anticancer activity with IC_{50} values ranging from 0.85 to 13.2 μM against different cancer cell lines and comparable with the parent compound **4a**. The most active compounds **9a** and **9f** showed IC_{50} values ranging 0.85–2.4 and 0.9–3.3 μM, respectively. The conjugates **9a** and **9f** in HT-29 cell line and **9e** in ACHN cell line showed better activity compared to the parent compound **4a**. It is observed that the conjugates **10a,b** that have an amide bond at 6-position showed lesser activity compared to the analogs with an amide bond at 2-position of the benzothiazole moiety.

Further, five compounds (**9a**, **9b**, **9e**, **9f** and **9g**) from this series were selected for NCI-60 cell line anticancer screening programme by National Cancer Institute (NCI), USA. After preliminary screening on the tumor cell lines, these compounds were tested for five dose concentration on a panel of 59 human tumor cell lines derived from nine different cancer types: leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate and breast. The results expressed as GI_{50} values[31] for the test compounds are illustrated in Table 2. The test results revealed that all the synthesized compounds possess significant anticancer activity against different cancer cell lines (GI_{50} values range, 0.3–38.9 μM) and exhibited a wide spectrum of activity with mean GI_{50} values of 2.3, 15.4, 12.5, 5.3 and 19.3 μM, respectively. The most active compounds **9a** and **9f** in MTT assay were re-

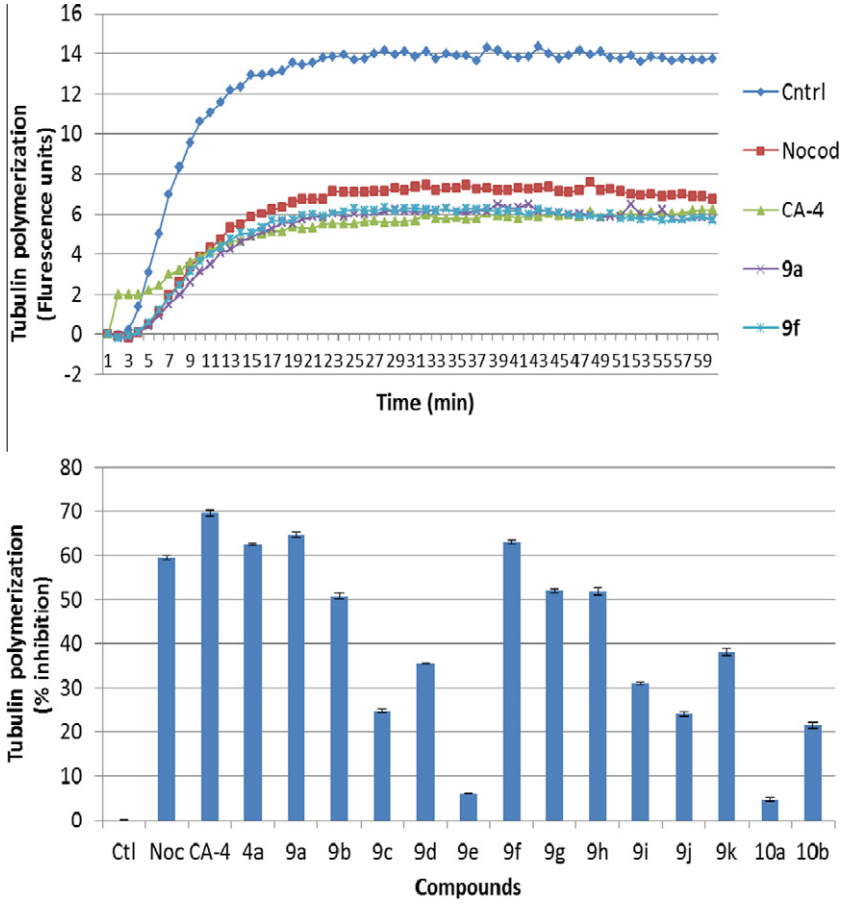


Figure 3. Effect of compounds on tubulin polymerization: tubulin polymerization was monitored by the increase in fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37 °C. All the compounds were included at a final concentration of 3 μM. Combretastatin A-4 (CA-4) and **4a** were used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

tained its activity in NCI screening data. These compounds exhibited promising activity with GI_{50} values ranging 0.3–7.87 and 0.3–9.52 μM, respectively. Whereas other compounds **9b**, **9e** and **9g** also showed good anticancer activity at micro molar level against all the cell lines tested. Particularly compound **9g** exhibited GI_{50} value of 0.85 μM against SR (leukemia) cell line.

3.2. Effect of compounds on cell cycle

To investigate the mechanism underlying the anticancer activity of these potential compounds (**9a** and **9f**), the cell cycle distribution in A549 cancer cell line was analyzed by flow cytometry. In this study A549 cells were treated with compounds **9a** and **9f** at 2 and 4 μM concentrations for 48 h. The data obtained clearly indicated that these compounds show G2/M cell cycle arrest in comparison to the untreated control. These compounds (**9a** and **9f**) showed 79% and 80% of cell accumulation in G2/M phase at 2 μM concentration, whereas they exhibited 81% and 83% of cell accumulation at 4 μM concentration, respectively (Fig. 2). The positive controls, CA-4 as well as **4a** showed 63% and 55% of cell accumulation in G2/M phase, respectively, at 2 μM concentration whereas in control (untreated cells) 23% of G2/M phase was observed.

3.3. Effect of compounds on tubulin polymerization

One of the possibilities that these compounds exhibit anticancer activity as well as G2/M cell cycle arrest is by the inhibition of tubulin polymerization as this has been observed in many antimitotic agents such as combretastatins. These compounds showed significant effect on the G2/M cell cycle arrest, 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[32,33] by monitoring the increase in 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 13 compounds examined, **9a** and **9f** inhibited tubulin polymerization by 64.7% and 63.1%, respectively, compared to control (Fig. 3). Tubulin polymerization inhibition was also observed in case of standards like nocodazole, CA-4 and **4a** (59.6%, 69.7% and 62.6%).

Furthermore, these two potential compounds (**9a** and **9f**) were evaluated for their in vitro tubulin polymerization assay at different concentrations. These molecules showed potent inhibition of tubulin polymerization with IC_{50} values 3.5 and 5.2 μM, respectively, compared to the control (Table 3). CA-4 and **4a** were showed IC_{50} values 2.1 and 3.7 μM, respectively.

Table 3
Inhibition of tubulin polymerization (IC_{50}) of compounds **9a** and **9f**

Compound	IC_{50}[a] ± SD (in μM)
9a	3.5 ± 0.17
9f	5.2 ± 0.2
CA-4	2.1 ± 0.12
4a	3.7 ± 0.14

[a] Concentration of drug to inhibit 50% of tubulin assembly.

3.4. Immunohistochemistry of tubulin

In order to substantiate the observed in vitro effects of these compounds on the inhibition of tubulin polymerization to functional microtubules immunohistochemistry studies have been carried out to examine the in situ effects of compounds **9a** and **9f** on cellular microtubules in A549 cancer cells.[33] Therefore, A549 cells were treated with **9a**, **9f**, **4a** and CA-4 at 2 μM concentration for 48 h. In this study, untreated human lung cancer cells displayed the normal distribution of microtubules (Fig. 4). However, cells treated with compounds **9a** and **9f** showed disrupted microtubule organization as seen in Figure 4, thus demonstrating the inhibition of tubulin polymerization. However, the controls CA-4 and **4a** also showed disrupted microtubule organization. This immunofluorescence study showed that the level of tubulin polymerization inhibition was comparable to that of CA-4 and **4a** for the compounds **9a** and **9f**.

3.5. Caspase-3 activity

It is well known[34–36] that the cell cycle arrest at G2/M phase is shown to induce cellular apoptosis, hence it was considered of interest to examine whether the cytotoxicity of **9a** and **9f** is by virtue of apoptotic cell death. Cysteine aspartase group, namely, caspases play a crucial role in the induction of apoptosis and amongst them caspase-3 happens to be one of the effector caspase. This prompted to treat A549 cells with compounds **9a**, **9f**, **4a**, CA-4, BT (2-aminobenzothiazole) and BT-OCF_3 (6-trifluoromethoxy-2-aminobenzothiazole) to examine the activation of caspase-3, wherein, CA-4 and **4a** are used as controls. The results indicate that

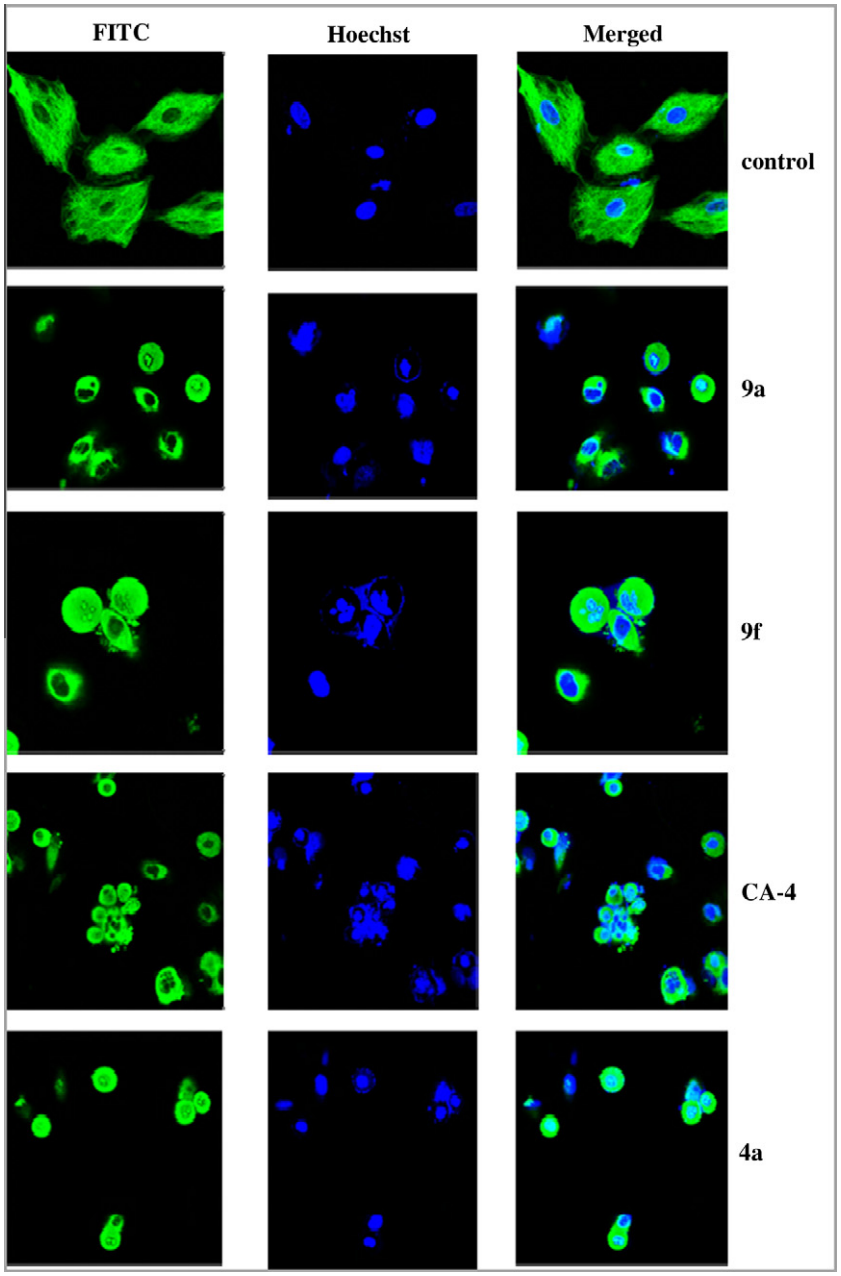


Figure 4. IHC analysis of compounds on the microtubule network: A549 cells were treated with compounds **9a**, **9f**, **4a** and CA-4 at 2 μM concentration for 48 h followed by staining with α-tubulin antibody. Microtubule organization was clearly observed by green color tubulin network like structures in control cells and was found to be disrupted in cells treated with compounds **9a** and **9f**. Combretastatin A-4 CA-4) and **4a** were used as controls.

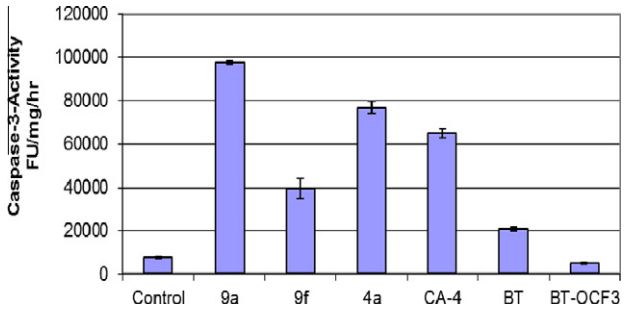


Figure 5. Effect of compounds **9a** and **9f** on caspase-3 activity: A549 lung cancer cells were treated for 48 h with 2 μM concentration of compounds **9a**, **9f**, BT and BT-OCF_3. CA-4 and **4a** were used as positive controls. Values indicated are the mean ± SD of two different experiments performed in triplicates.

there is nearly 4- to 9-fold induction in caspase-3 activity in cells treated with 2 μM concentration by these compounds (Fig. 5). Under similar conditions, both CA-4 and **4a** induced the caspase activity by nearly 6- to 8-fold as compared to control. However induction was not observed with aminobenzothiazoles, thus suggesting the activation of caspase-3 by **9a** and **9f** indicate that they have the ability to induce apoptosis in A 549 cells.

3.6. Annexin V-FITC for apoptosis

The apoptotic effect of these compounds (**9a** and **9f**) was further evaluated by Annexin V FITC/PI (AV/PI) dual staining assay[37] 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

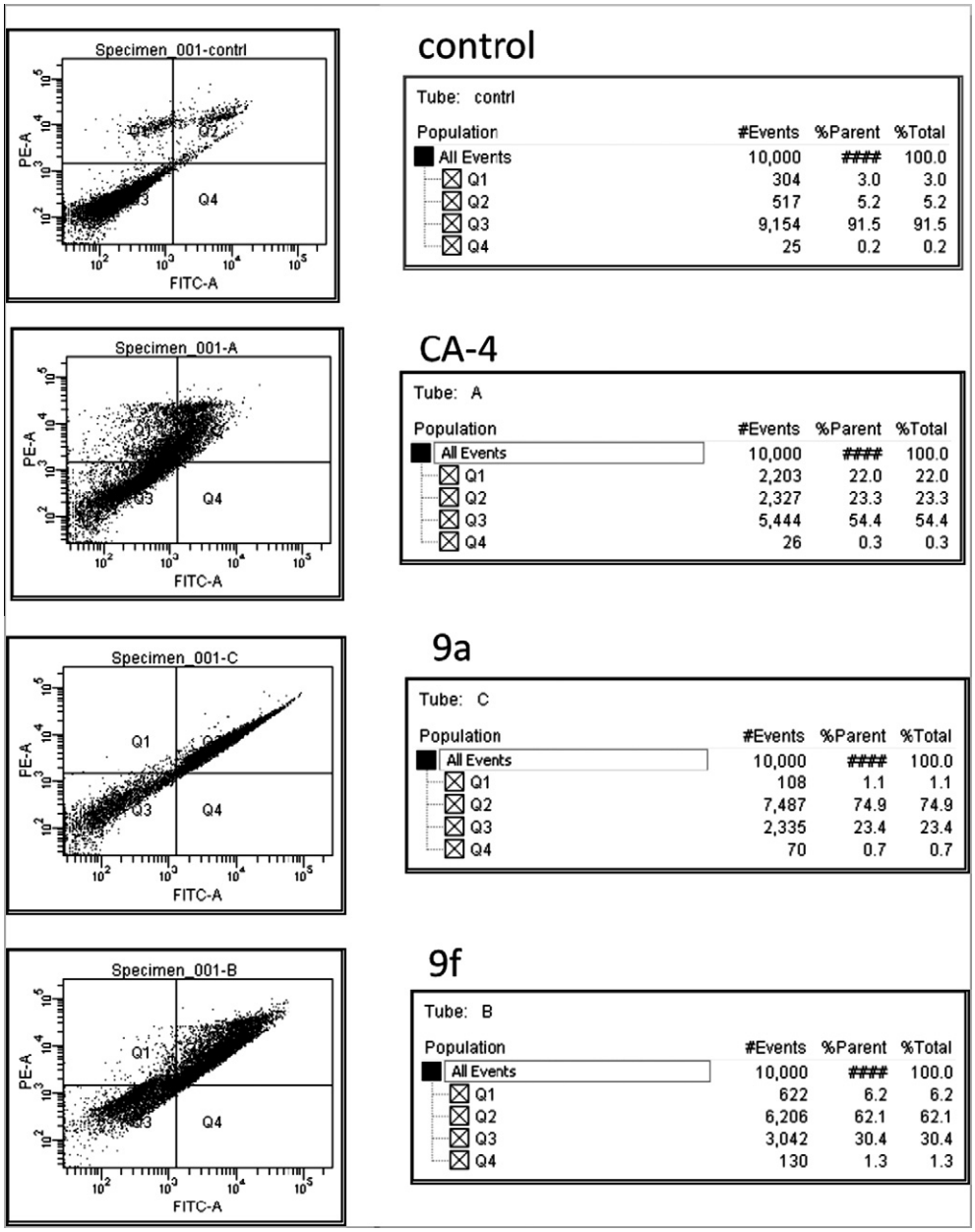


Figure 6. Annexin V-FITC staining. A549 cells were treated with compounds **9a**, **9f**, and CA-4 at 2 μM concentration for 48 h.

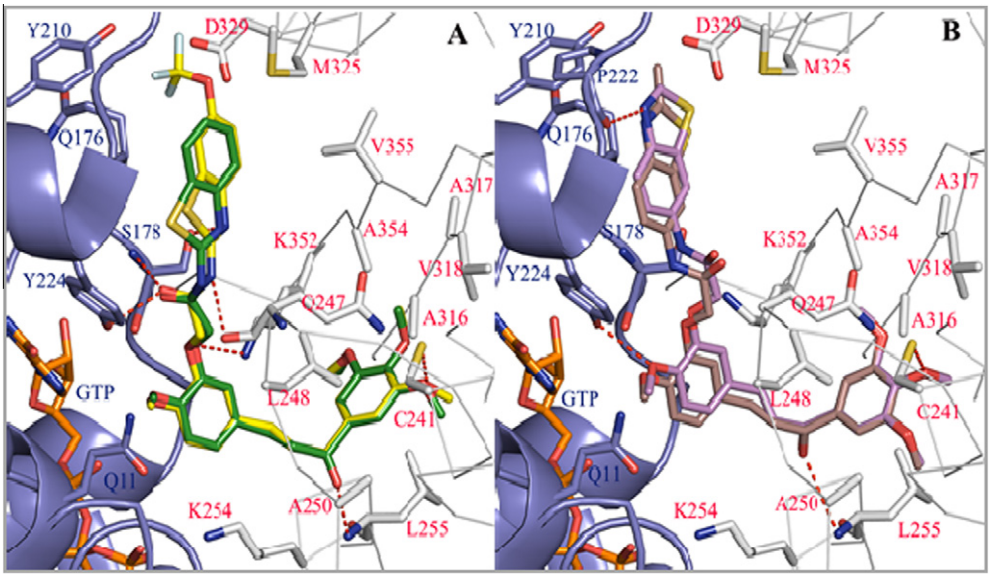


Figure 7. (A) Docking position of **9a** (green) **9f** (yellow) in the colchicine-binding site of tubulin; (B) Docking position of **10a** (purple), **10b** (gray) in the colchicine-binding site of tubulin. Red dotted lines represent possible hydrogen bond between protein and ligands.

compounds **9a** and **9f** for 48 h at 2 μM concentration to examine the apoptotic effect. It was observed that these compounds showed significant apoptosis against A549 cells as shown in Figure 6. Results indicated that compounds **9a** and **9f** showed 62.1% and 74.9% of apoptosis, respectively, whereas 5.2% of apoptosis was observed in control (untreated cells). The standard CA-4 showed 23.3% of apoptosis at 2 μM concentration. From this experiment it was suggested that these compounds significantly induce apoptosis in A549 cells.

3.7. Molecular Modeling

To elucidate the mode of binding with tubulin, it was considered of interest to examine these compounds (**9a–k** and **10a,b**) by directly docking to the colchicine binding site of tubulin. The proposed binding site for **9a** and **9f** is located at the interface with the α-β subunit (Fig. 7). The trimethoxy phenyl ring of these compounds is surrounded by Cys241, Lys352, Val318, Leu255, Leu242, Leu248, Ala250, Ala316, Ile378 and Met259 of β subunit. Most of the interactions are of Van der Waals type interactions, similar to colchicine and podophyllotoxin with tubulin.[38] Cys241 form a S–H···O (3.5 Å) hydrogen bond with one of the methoxy oxygen atoms of **9a** (3.5 Å) or **9f** (3.5 Å). The –C═O, near to trimethoxy phenyl ring interacts with the main chain amide of Leu255 (2.9 Å). The benzothiazole amide and the linker region of molecules **9a** and **9f** were positioned between α and β sub-units and making a series of contacts with both the domains. The amide nitrogen atom of benzothiazole group forms a hydrogen bond with –OH of Ser178, while the corresponding carbonyl of the inhibitors interact with the main chain amide of Ser178 and side chain –OH of Tyr224 of the α subunit. The variable region of the molecules **9a–k** and, **10a,b** extends into the interface of α-β-chains surrounded by Gln176, Ser178, Tyr210 and Pro255 of α-tubulin and Asp229, Met325 and Val355 of β-tubulin (Fig. 7). Based on the modeling, it is clear that these molecules fit well in the tubulin α-β interface thereby allocating favorable interactions. However it is not that clear to conclude about the actual molecular basis for the compounds **9a** and **9f** to be slightly better than others with respect to their activity, probably it could be due to their enhanced bioavailability. The major difference between the compounds of series **9** and **10** is in the position of linkage of benzothiazole moiety with the chalcone moiety. Although the positioning of this benzothiazole ring is inverted in the series of compounds **10** with respect to the chalcone moiety, this group however remains at the same place but with reversed polarities, thereby explaining about their lower activity compared to the compounds of series **9** (Fig. 7 and Fig. S1 in Supplementary data).

4. Conclusion

In conclusion, new series of chalcone-amidobenzothiazole conjugates (**9a–k** and **10a,b**) have been synthesized and evaluated for their anticancer potential against five human cancer cell lines (A549, A375, MCF-7, HT-29 and ACHN). Furthermore, five compounds (**9a**, **9b**, **9e**, **9f** and **9g**) from this series were selected for the NCI-60 cell line anticancer screening programme by NCI, USA. Interestingly some of these compounds exhibit promising anticancer activity and two of them (**9a** and **9f**) showed IC_{50} values 0.85 and 0.9 μM, respectively, against A549 cancer cell line. Flow cytometric analysis of these compounds showed the arrest of the cell cycle in the G2/M phase leading to caspase-3 dependent apoptotic cell death. These active compounds (**9a** and **9f**) showed potent inhibition of tubulin polymerization with IC_{50} values 3.5 and 5.2 μM, respectively. The immunohistochemistry study of tubulin showed that the level of tubulin polymerization inhibition was comparable to that of CA-4 and **4a** for the compounds **9a** and **9f**. Activation of caspase-3 and Annexin staining experiments revealed that these compounds induce cell death by apoptosis. Autodock results suggest that the docking position of the trimethoxyphenyl group of chalcone-amidobenzothiazole conjugates showed a similar binding mode to that of the A-ring of colchicine, forming extensive hydrophobic contacts with the binding pocket of the β-chain. Cys241 forms a hydrogen bond with one of the methoxy group of trimethoxy phenyl functionality. The variable region of benzothiazole ring extended to the interface of α-β-chains. From molecular modeling studies it is demonstrated that these molecules bind well with the tubulin protein and are involved in a series of interactions with the protein providing the molecular basis for inhibition of tubulin polymerization. Overall, the current study demonstrates that the synthesis of chalcone-amidobenzothiazole conjugates as promising anticancer agents with potent G2/M arrest and apoptotic-inducing activities via targeting tubulin deserving further research and development, and helps provide data for exploiting new tubulin binding agents.

5. Experimental section

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) or Spectrochem Pvt. Ltd (Mumbai, India) and were used without further purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 GF-254, and visualization on TLC was achieved by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H and ^{13}C spectra were recorded on Gemini Varian-VXR-unity (200 MHz) or Bruker UXNMR/XWIN-NMR (300 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from internal TMS standard. ESI spectra were recorded on Micromass, Quattro LC using positive ion-mode with capillary voltage 3.98 kV. High-resolution mass spectra (HRMS) were recorded on QSTAR XL Hybrid MS/MS mass spectrometer. Melting points were determined with an Electro thermal melting point apparatus, and are uncorrected.

5.1. (*E*)-3-(3-Hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (4a)

To a stirred mixture of 3,4,5-trimethoxyacetophenone (**5**) (210 mg, 1 mmol) and 3-hydroxy-4-methoxybenzaldehyde (**6**) (152 mg, 1 mmol) in ethanol (10 ml) was added 50% aqueous solution of potassium hydroxide (1 ml) and stirred for 8 h at room temperature. After completion of the reaction as checked by TLC, the solvent was evaporated, neutralized with dilute HCl and extracted with ethyl acetate (2 × 50 ml). The combined organic fractions were washed with water followed by brine solution, dried over Na_2SO_4 and purified by column chromatography using (30% EtOAC/hexane) to obtain the pure product **4a**. (300 mg, 87% yield); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (d, 1H, J = 16.1 Hz), 7.36 (d, 1H, J = 16.1 Hz), 7.25–7.32 (m, 3H), 7.14 (dd, 1H, J = 8.7, 2.1 Hz), 6.89 (d, 1H, J = 8.7 Hz), 5.75 (bs, 1H), 3.96 (s, 9H), 3.94 (s, 3H); MS (ESI): m/z 345 $[M+H]^+$.

5.2. (*E*)-Ethyl-2-(2-methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy) acetate (7)

To a solution of compound **6** (344 mg, 1 mmol) in dry DMF (15 ml) was added, anhydrous K_2CO_3 (276 mg, 2 mmol), α-bromo-ethyl acetate (250 mg, 1.5 mmol) and the mixture was stirred at room temperature for 24 h. The reaction was monitored by TLC using ethyl acetate–hexane (6:4). After completion of the reaction as indicated by the TLC, K_2CO_3 was removed by filtration, diluted with water and extracted with dichloromethane (2 × 20 ml). The combined organic phases were washed with water followed by brine solution, dried over Na_2SO_4 and evaporated under vacuum. The residue, thus obtained was purified by column chromatography using ethyl acetate and hexane (1:1) to afford pure compound **7** as yellow solid. (380 mg, 88% yield); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.73 (d, 1H, J = 15.6 Hz), 7.36 (d, 1H, J = 15.6 Hz), 7.27–7.33 (m, 3H), 7.14–7.18 (dd, 1H, J = 7.8, 1.5 Hz), 6.94 (d, 1H, J = 7.8 Hz), 4.74 (s, 2H), 4.24–4.32 (q, 2H), 3.96 (s, 9H), 3.94 (s, 3H), 1.31 (t, 3H, J = 7.0 Hz); MS-ESI(+): m/z 431 $[M+H]^+$.

5.3. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)acetic acid (8)

To a solution of compound **7** (430 mg, 1 mmol) in THF (15 ml) and water (2 ml), $LiOH.H_2O$ (84 mg, 2 mmol) was added and the mixture was stirred at room temperature for 12 h. The reaction was monitored by TLC using ethyl acetate. After completion of the reaction as indicated by the TLC, the solvent was removed under vacuum and neutralized with dilute HCl up to pH 7. After neutralization the reaction mixture was extracted with dichloromethane (2 × 20 ml). The combined organic phases were washed with water followed by brine solution, dried over Na_2SO_4 and evaporated under vacuum to obtain compound **8**. This crude compound was purified by recrystallization by using ethyl acetate as solvent to obtain the pure product **8** as yellow solid. (307 mg, 76% yield); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.96 (bs, 1H), 7.75 (d, 1H, J = 15.6 Hz), 7.37 (d, 1H, J = 15.6 Hz), 7.28–7.33 (m, 3H), 7.14–7.19 (dd, 1H, J = 7.8, 1.5 Hz), 6.96 (d, 1H, J = 7.8 Hz), 4.75 (s, 2H), 3.97 (s, 9H), 3.95 (s, 3H); MS-ESI(+): m/z 403 $[M+H]^+$.

5.4. (*E*)-*N*-(Benzo[*d*]thiazol-2-yl)-2-(2-methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)acetamide (9a)

To a solution of 2-aminobenzothiazole (150 mg, 1 mmol) in dichloromethane (20 ml) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) (210 mg, 1.1 mmol) and 1-hydroxy-1,2,3-benzotriazole (HOBt) (13.5 mg, 0.1 mmol). Then compound **8** (402 mg, 1 mmol) was added and the reaction mixture was stirred at room temperature for 14 h and the reaction was monitored by TLC. After completion of reaction, water was added to reaction mixture and extracted with dichloromethane (2 × 30 ml). The organic layer was dried with Na_2SO_4 and evaporated under vacuum to afford the crude product. This was further purified by column chromatography using ethyl acetate and hexane (1:1) as solvent system to obtain the pure product **9a** as yellow solid. (449 mg, 84% yield); mp 163–165 °C; 1H NMR (200 MHz, $CDCl_3$): δ 11.93 (bs, 1H), 7.81–7.84 (dd, 1H, J = 7.8, 1.9 Hz), 7.72–7.75 (dd, 1H, J = 7.8, 1.9 Hz), 7.67 (d, 1H, J = 15.6 Hz), 7.52–7.6 (m, 2H), 7.38–7.43 (m, 1H), 7.34–7.37 (dd, 1H, J = 7.8, 1.9 Hz), 7.31 (s, 2H), 7.25–7.29 (m, 1H), 7.01 (d, 1H, J = 7.8 Hz), 4.74 (s, 2H), 3.98 (s, 3H), 3.91 (s, 6H), 3.85 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 187.7, 167.5, 156.3, 152.7, 151.3, 147.4, 147.3, 143.8, 141.6, 133.1, 131.0, 129.8, 127.4, 126.5, 124.1, 123.5, 119.9, 119.0, 113.9, 112.2, 105.9, 67.1, 60.0, 56.0, 55.7; MS-ESI(+): m/z 557 $[M+Na]^+$; HRMS-ESI(+) for $C_{28}H_{26}N_2O_7SNa$ Calcd 557.1358, Found 557.1347 $[M+Na]^+$.

5.5. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(6-fluoro benzo[d]thiazol-2-yl)acetamide (9b)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-6-fluorobenzothiazole (168 mg, 1 mmol) to obtain the pure product **9b** as yellow solid. (465 mg, 84% yield); mp 165–166 °C; 1H NMR (200 MHz, $CDCl_3$): δ 10.57 (bs, 1H), 7.65–7.77 (m, 2H), 7.44–7.53 (m, 2H), 7.31–7.39 (m, 2H), 7.25 (s, 2H), 7.24 (d, 1H, J = 15.4 Hz), 7.12 (d, 1H, J = 8.3 Hz), 4.82 (s, 2H), 4.08 (s, 3H), 3.96 (s, 6H), 3.94 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.8, 167.3, 158.2, 152.4, 151.3, 147.7, 147.2, 143.8, 141.6, 133.1, 131.2, 129.9, 127.3, 124.7, 124.1, 123.5, 119.8, 119.0, 113.5, 112.2, 105.8, 67.0, 60.2, 56.1, 55.5; MS-ESI(+): m/z 553 $[M+H]^+$; HRMS-ESI(+) for $C_{28}H_{26}FN_2O_7S$ Calcd 553.5724, Found 553.5712 $[M+H]^+$.

5.6. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(6-chlorobenzo[*d*]thiazol-2-yl)acetamide (9c)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-6-chlorobenzothiazole (184 mg, 1 mmol) to obtain the pure product **9c** as yellow solid. (445 mg, 78% yield); mp 163–

165 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.59 (bs, 1H), 7.76–7.81 (m, 1H), 7.71 (d, 1H, J = 15.1 Hz), 7.65–7.69 (m, 2H), 7.46 (d, 1H, J = 2.0 Hz), 7.37–7.41 (dd, 1H, J = 8.5, 2.0 Hz), 7.25 (s, 2H), 7.21 (d, 1H, J = 15.1 Hz), 6.86 (d, 1H, J = 8.3 Hz), 4.83 (s, 2H), 4.07 (s, 3H), 3.95 (s, 6H), 3.93 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.8, 167.2, 157.8, 152.3, 151.2, 147.5, 147.3, 143.7, 141.7, 133.2, 131.1, 129.8, 127.4, 124.6, 124.1, 123.5, 119.7, 119.1, 113.6, 112.2, 105.9, 67.0, 60.1, 56.1, 55.6; MS-ESI(+): m/z 570 $[M+H]^+$; HRMS-ESI(+) for $C_{28}H_{26}ClN_2O_7S$ Calcd 570.0253, Found 570.0239 $[M+H]^+$.

5.7. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(6-nitro benzo[d]thiazol-2-yl)acetamide (9d)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-6-nitrobenzothiazole (195 mg, 1 mmol) to obtain the pure product **9d** as yellow solid. (482 mg, 83% yield); mp 169–170 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 10.98 (bs, 1H), 8.77 (d, 1H, J = 2.1 Hz), 8.31–8.38 (dd, 1H, J = 8.3, 2.1 Hz), 7.79 (d, 1H, J = 15.6 Hz), 7.72 (d, 1H, J = 8.3 Hz), 7.28–7.34 (m, 2H), 7.25 (d, 1H, J = 15.6 Hz), 7.23 (s, 2H), 6.95 (d, 1H, J = 8.3 Hz), 4.83 (s, 2H), 4.01 (s, 3H), 3.92 (s, 6H), 3.9 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$):δ 187.8, 168.6, 160.8, 152.8, 151.3, 147.7, 147.2, 143.9, 141.7, 133.2, 131.1, 129.9, 127.7, 126.2, 124.2, 121.5, 119.9, 119.2, 113.5, 112.2, 105.9, 67.1, 60.2, 56.1, 55.3; MS-ESI(+): m/z 580 $[M+H]^+$; HRMS-ESI(+) for $C_{28}H_{26}N_3O_9S$ Calcd 580.5667, Found 580.5661 $[M+H]^+$.

5.8. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(6-meth oxybenzo[*d*]thiazol-2-yl)acetamide (9e)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-6-methoxybenzothiazole (180 mg, 1 mmol) to obtain the pure product **9e** as yellow solid. (475 mg, 84% yield); mp 166–167 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.62 (bs, 1H), 7.75 (d, 1H, J = 15.6 Hz), 7.71 (d, 1H, J = 8.0 Hz), 7.34–7.4 (m, 2H), 7.28–7.34 (m, 2H), 7.23 (d, 1H, J = 15.6 Hz), 7.22 (s, 2H), 6.89 (d, 1H, J = 8.3 Hz), 4.82 (s, 2H), 4.02 (s, 3H), 3.96 (s, 6H), 3.95 (s, 3H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.7, 167.3, 156.1, 155.1, 152.8, 151.3, 147.3, 143.9, 142.4, 141.6, 133.1, 132.7, 127.4, 124.2, 121.2, 119.8, 114.9, 113.5, 112.1, 105.9, 104.6, 67.0, 60.1, 56.0, 55.7, 55.5; MS-ESI(+): m/z 565 $[M+H]^+$; HRMS-ESI(+) for $C_{29}H_{29}N_2O_8S$ Calcd 565.6061, Found 565.6054 $[M+H]^+$.

5.9. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(6-trifluoromethoxybenzo[d]thiazol-2-yl) acetamide (9f)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-6-trifluoromethoxybenzothiazole (234 mg, 1 mmol) to obtain the pure product **9f** as yellow solid. (502 mg, 81% yield); mp 168–170 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.62 (bs, 1H), 7.77 (d, 1H, J = 9.0 Hz), 7.66–7.73 (m, 2H), 7.27–7.39 (m, 3H), 7.24 (d, 1H, J = 15.1 Hz), 7.22 (s, 2H), 6.98 (d, 1H, J = 8.3 Hz), 4.83 (s, 2H), 4.07 (s, 3H), 3.95 (s, 6H), 3.91 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 187.8, 168.2, 160.5, 152.7, 151.3, 151.1, 147.2, 143.9, 141.69, 133.1, 131.9, 127.4, 126.2, 124.2, 122.9, 122.6, 121.0, 119.8, 113.7, 112.2, 105.9, 67.0, 60.1, 56.0, 55.7; MS-ESI(+): m/z 619 $[M+H]^+$; HRMS-ESI(+) for $C_{29}H_{26}F_3N_2O_8S$ calcd 619.5724, found 619.5710 $[M+H]^+$.

5.10. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(6-trifl uoromethylbenzo[*d*]thiazol-2-yl)acetamide (9g)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-6-trifluoromethylbenzothiazole (218 mg, 1 mmol) to obtain the pure product **9g** as yellow solid. (490 mg, 81% yield); mp 167–169 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.74 (bs, 1H), 8.11 (s, 1H), 7.87 (d, 1H, J = 8.3 Hz), 7.64–7.74 (m, 2H), 7.28–7.40 (m, 3H), 7.22 (s, 2H), 6.99 (d, 1H, J = 8.3 Hz), 4.84 (s, 2H), 4.09 (s, 3H), 3.95 (s, 6H), 3.91 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 187.7, 167.9, 158.7, 152.7, 151.3, 147.2, 144, 143.8, 141.7, 133.1, 132.6, 128.5, 127.4, 124.2, 121.5, 119.8, 118.3, 114.9, 113.7, 112.2, 105.9, 67.0, 60.0, 56.0, 55.7; MS-ESI(+): m/z 603 $[M+H]^+$; HRMS-ESI(+) for $C_{29}H_{26}F_3N_2O_7S$ Calcd 603.5776, Found 603.5761 $[M+H]^+$.

5.11. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(6-methylbenzo[d]thiazol-2-yl)acetamide (9h)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-6-methylbenzothiazole (164 mg, 1 mmol) to obtain the pure product 9 h as yellow solid. (450 mg, 82% yield); mp 162–164 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (d, 1H, J = 15.6 Hz), 7.70 (d, 1H, J = 8.3 Hz), 7.63 (s, 1H), 7.31–7.43 (m, 2H), 7.23–7.30 (m, 4H), 7.01 (d, 1H, J = 8.4 Hz), 4.84 (s, 2H), 4.04 (s, 3H), 3.96 (s, 3H), 3.94 (s, 3H), 2.48 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.7, 167.5, 153.1, 151.8, 147.3, 143.7, 141.6, 133.1, 131.4, 127.5, 126.1, 124.3, 123.6, 121.7, 120.5, 119.9, 113.2, 112.2, 105.8, 67.2, 60.2, 56.7, 55.8, 28.3; MS-ESI(+): m/z 549 $[M+H]^+$; HRMS-ESI(+) for $C_{29}H_{29}N_2O_7S$ Calcd 549.1695, Found 549.1696 $[M+H]^+$.

5.12. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(6-ethoxybenzo[d]thiazol-2-yl)acetamide (9i)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-6-ethoxybenzothiazole (194 mg, 1 mmol) to obtain the pure product **9i** as yellow solid. (460 mg, 79% yield); mp 165–167 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.75 (d, 1H, J = 15.8 Hz), 7.70 (d, 1H, J = 9.0 Hz), 7.36–7.41 (m, 2H), 7.29–7.34 (m, 2H), 7.25–7.28 (m, 3H), 7.01 (d, 1H, J = 8.3 Hz), 4.84 (s, 2H), 4.06–4.15 (q, 2H), 4.04 (s, 3H), 3.96 (s, 6H), 3.95 (s, 3H), 1.46 (t, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.4, 166.4, 156.4, 155.3, 152.9, 151.8, 147.2, 143.3, 142.5, 141.7, 133.3, 132.7, 127.7, 124.1, 121.5, 119.9, 115.0, 113.4, 112.3, 105.4, 104.7, 67.1, 60.7, 56.3, 55.9, 55.1, 31.5; MS-ESI(+): m/z 579 $[M+H]^+$; HRMS-ESI(+) for $C_{30}H_{31}N_2O_8S$ Calcd 579.1801, Found 579.1781 $[M+H]^+$.

5.13. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(4-methylbenzo[*d*]thiazol-2-yl)acetamide (9j)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-4-methylbenzothiazole (164 mg, 1 mmol) to obtain the pure product **9j** as yellow solid. (430 mg, 78% yield); mp 163–165 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 10.71 (br s, 1H), 7.76

(d, 1H, J = 15.4 Hz), 7.65–7.70 (m, 1H), 7.32–7.43 (m, 4H), 7.28 (s, 2H), 7.22–7.26 (m, 1H), 7.02 (d, 1H, J = 8.4 Hz), 4.86 (s, 2H), 4.09 (s, 3H), 3.96 (s, 6H), 3.94 (s, 3H), 2.67 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.7, 167.6, 152.7, 151.3, 147.2, 143.8, 141.6, 133.1, 131.3, 127.4, 126.1, 124.2, 123.6, 121.6, 120.5, 119.8, 113.7, 112.2, 105.9, 67.1, 60.0, 56.0, 55.7, 28.6; MS-ESI(+): m/z 549 $[M+H]^+$; HRMS-ESI(+) for $C_{29}H_{29}N_2O_7S$ Calcd 549.1695, Found 549.1696 $[M+H]^+$.

5.14. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(5,6-dimethylbenzo[*d*]thiazol-2-yl)acetamide (9k)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 2-amino-5,6-dimethylbenzothiazole (178 mg, 3 mmol) to obtain the pure product **9k** as yellow solid. (470 mg, 83% yield); mp 164–166 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.21 (s, 2H), 7.62–7.72 (m, 2H), 7.44–7.57 (m, 2H), 7.34 (s, 2H), 7.07 (d, 1H, J = 8.6 Hz), 4.99 (s, 2H), 3.91 (s, 3H), 3.86 (s, 6H), 3.78 (s, 3H), 2.35 (s, 6H), 2.34 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.8, 167.6, 153.7, 152.4, 147.7, 144.7, 141.3, 133.3, 130.6, 127.4, 126.2, 124.2, 123.4, 121.3, 120.2, 119.5, 113.4, 111.9, 104.8, 67.7, 61.2, 56.6, 55.5, 28.1, 27.6; MS-ESI(+): m/z 585 $[M+Na]^+$; HRMS-ESI(+) for $C_{30}H_{30}N_2O_7SNa$ Calcd 585.6335, Found 585.6320 $[M+Na]^+$.

5.15. (*E*)-*N*-(Benzo[*d*]thiazol-6-yl)-2-(2-methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)acetamide (10a)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 6-aminobenzothiazole (150 mg, 1 mmol) to obtain the pure product **10a** as yellow solid. (410 mg, 76% yield); mp 161–163 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.72 (bs, 1H), 8.93 (s, 1H), 8.55 (d, 1H, J = 1.9 Hz), 7.94 (d, 1H, J = 8.7 Hz), 7.62 (d, 1H, J = 15.6 Hz), 7.54–7.58 (dd, 1H, J = 8.7, 1.9 Hz), 7.49–7.54 (m, 2H), 7.27–7.31 (dd, 1H, J = 8.7, 1.9 Hz), 7.26 (s, 2H), 6.94 (d, 1H, J = 8.7 Hz), 4.73 (s, 2H), 3.93 (s, 3H), 3.88 (s, 6H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.5, 162.7, 158.7, 153.2, 150.7, 147.6, 147.2, 142.4, 141.7, 132.2, 131.5, 129.4, 127.8, 126.4, 124.4, 123.6, 120.3, 119.2, 113.5, 112.7, 106.2, 66.3, 61.2, 56.2, 55.1; MS-ESI(+): m/z 535 $[M+H]^+$; HRMS-ESI(+) for $C_{28}H_{27}N_2O_7S$ Calcd 535.1538, Found 535.1515 $[M+H]^+$.

5.16. (*E*)-2-(2-Methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-enyl)phenoxy)-*N*-(2-methylbenzo[*d*]thiazol-6-yl)acetamide (10b)

This compound was prepared according to the method described for compound **9a** by employing compound **8** (402 mg, 1 mmol), EDCI (210 mg, 1.1 mmol), HOBt (13.5 mg, 0.1 mmol) and 6-amino-2-methylbenzothiazole (164 mg, 1 mmol) to obtain the pure product **10b** as yellow solid. (419 mg, 73% yield); mp 164–165 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.01 (bs, 1H), 8.03 (d, 1H, J = 2.2 Hz), 7.78–7.83 (dd, 1H, J = 9.0, 2.2 Hz), 7.75 (s, 1H), 7.69 (d, 1H, J = 15.8 Hz), 7.33 (d, 1H, J = 15.8 Hz), 7.27–7.31 (m, 2H), 7.22 (s, 2H), 6.94 (d, 1H, J = 8.3 Hz), 4.69 (s, 2H), 4.01 (s, 3H), 3.94 (s, 6H), 3.91 (s, 3H), 2.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.5, 162.6, 159.6, 153.1, 150.6, 147.6, 147.1, 142.3, 141.7, 132.4, 131.6, 129.3, 127.8, 126.3, 124.3, 123.6, 120.5, 119.3, 113.6, 112.8, 106.4, 66.2, 61.5, 56.3, 55.3, 29.7; MS-ESI(+): m/z 549 $[M+H]^+$; HRMS-ESI(+) for $C_{29}H_{29}N_2O_7S$ Calcd 549.1695, Found 549.1696 $[M+H]^+$.

6. Materials and methods

6.1. MTT assay

The cytotoxic activity of the compounds was determined using MTT assay.[39] 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 540 nm wavelength was recorded.

6.2. In vitro growth inhibition

The screening of anticancer activity is evaluated by the NCI, USA, according to standard procedures (http://dtp.nci.nih.gov/branches/btb/ivclsp.html).[31]

6.3. Cell cycle analysis

To determine the effect of compounds on the stages of cell cycle, A549 cells (1×10^6) were seeded in six-well plates and treated with compounds **9a** and **9f** at concentrations of 2 and 4 μM/ml. After 48 h treatment, both floating and trypsinized adherent cells were collected and fixed with 70% ethanol. After fixation cells were washed with PBS and stained with 50 mg/ml propidium iodide in hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X-100) containing DNase-free RNase-A for 20 min. Stained cells were analyzed using fluorescence-activated cell sorter caliber (Becton Dickinson).[39]

6.4. Tubulin polymerization assay

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.). Combretastatin A-4 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 mM), in the presence or absence of test compounds at 2.5, 5, 10, and 15 mM concentrations. Polymerization was monitored by increase in the Fluorescence as mentioned above at 37 °C.[32,33]

6.5. Immunohistochemistry

A549 cells were seeded on glass cover slips, incubated for 48 h in the presence or absence of test compounds **9a**, 9f (2.5 mM). 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 (−20 °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 confocal microscope, equipped with FITC settings and the pictures were analyzed for the integrity of microtubule network. In parallel experiments, CA-4 (2.5 mM) was used as positive control for analyzing microtubule integrity.[33]

6.6. Caspase assay

To determine the caspase-3 activity of the active compounds AFC conjugated Ac-DEVD substrate was used. Lung cancer cells (A549) were seeded in six well plates with the confluence of 2.5×10^5 per well and are treated with the compounds at 2 μM concentration along with standard etoposide. After incubation for 24 h cell were washed with PBS and then cells were scraped in to the PBS and centrifuged at 2000 rpm for 10 min at 4 °C. Pellet was resuspended in 80 μl of lysis buffer, pellet was passed through insulin syringe followed by incubation of suspension on ice for 20–30 min. centrifuged the lysate at 13200 rpm (G-force value: 16100 G) for 20 min at 4 °C and transferred the supernatant to fresh tubes. In a 96 well black polystyrene plate, 50 μl of 2× assay buffer, 50 μl cell lysate and 2 μl of caspase-3 substrate were taken. The reaction was allowed to take place for 1 h. The fluorescence generated by the release of the fluorogenic group AFC on cleavage by caspase-3 was measured by excitation at 400 nm and emission at 505 nm for every 5 min over 1 h. Protein was estimated by Bradford's method and normalized accordingly.[39]

6.7. Annexin staining

A549 cells (1×10^6) were seeded in six-well plates and allowed to grow overnight. The medium was then replaced with complete medium containing 6.5 μM concentration of compound, for 24 and 48 h with vehicle alone (0.001% DMSO) as control. After 24 and 48 h treatment, cells from the supernatant and adherent monolayer cells were harvested by trypsinization, washed with PBS at 3000 rpm. Then the cells (1×10^6) were stained with Annexin V-FITC and propidium iodide using the Annexin-V-PI apoptosis detection kit (Invitrogen). Flow cytometry was performed using a FACScan (Becton Dickinson) equipped with a single 488-nm argon laser as described earlier.[34] Annexin V-FITC was analysed using excitation and emission settings of 488 and 535 nm (FL-1 channel); PI, 488 and 610 nm (FL-2 channel). Debris and clumps were gated out using forward and orthogonal light scatter. The experiment was repeated three times.

6.8. Molecular modeling

Tubilin bound with colchicine (PDB code: 3E22) was selected as the receptor for docking simulation. After removing the ligand and solvent molecules, hydrogen atoms and Kollman charge were added to each protein atom. Coordinates of each compound were obtained from Chemdraw11 followed by MM2 energy minimization. Docking of All compounds was carried out by AutoDock4 in a predefined colchicine binding domain.[38,40,41] Grids map was created in Autodock4 that defines interaction of protein and the ligand in the binding pocket. We used this grid map with 60 points in each *x*, *y*, and *z* direction, equally spaced at 0.375 Å. Docking was performed using the Lamarckian genetic algorithm in AutoDock4.[42] Each docking experiment was performed 100 times, yielding 100 docked conformations. Parameters used for the docking were as follows: population size of 150; random starting position and conformation; maximal mutation of 2 Å in translation and 50° in rotations; elitism of 1; mutation rate of 0.02 and crossover rate of 0.8; and local search rate of 0.06. Simulations were performed with a maximum of 1.5 million energy evaluations and a maximum of 27,000 generations. Final docked conformations were clustered using a tolerance of 1 Å RMSD.

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Supplementary data

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