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ARTICLE TYPE

Synthesis of (Z)-(arylamino)-pyrazolyl/isoxazolyl-2-propenones as Tubulin Targeting Anticancer Agents and Apoptotic Inducers

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A new class of pyrazole and isoxazole conjugates were synthesized and evaluated for their cytotoxic activity against various human cancer cell lines. These compounds have shown significant cytotoxicity with the lower IC_{50} values. FACS results revealed that A549 cells treated with these compounds arrested cells at G2/M phase of the cell cycle apart from activating cyclin-B1 protein levels. Particularly, compounds **9a** and **9b** demonstrated a remarkable inhibitory effect on tubulin polymerization and showed pronounced inhibitory effect on the tubulin polymerization with IC_{50} values of 1.28 μM and 0.28 μM respectively. Wereas, nocodazole a positive control have shown lower antitubulin activity with an IC_{50} value 2.64 μM. Furthermore, these compounds induced apoptosis by loss of mitochondrial membrane potential, propidium iodide (PI) staining and activation of caspase-3. A fluorescence based compititve colchicine binding assay results suggest that these conjugates bind successfully at the colchicene binding site of tubulin. These investigations reveal that such conjugates containing pyrazole with trimethoxy phenyl ring and indole moieties have the potential in the development of newer chemotherapeutic agents.

Introduction

Microtubules are long, stiff filamentous, tube-shaped polymers of protein; that are essential and ubiquitous in all eukaryotic cells and are considered as an important target for the development of anticancer agents.[1,2] They are crucial in the development and maintenance of cell shape, mitochondria, in the transport of vesicles and other components throughout the cells in cell signalling, cell division and mitosis.[3,4] Microtubules are polymers of α,β-tubulin heterodimer which are in dynamic equilibrium with tubulin dimers. Microtubule dynamics can be affected by treatment with some agents or under influence of some conditions, which destabilize the equilibrium between tubulin polymerization and depolymerisation thereby effect cellular replication. This leads to inhibition of the mitotic spindle formation thereby blocking mitosis at the metaphase transition and inducing cell death.[5] Thus, microtubules have become a significant target for invention of new anticancer agents. Drugs that inhibit polymerisation of microtubule are effective in the treatment of breast, lung, colon, cervix and other cancers. Nevertheless, occurrence of peripheral neuropathy is a most important complication in the development of microtubule depolymerising agents as drugs. For that reason, the discovery of new molecules to defeat neuropathies is required. There has been considerable interest in the invention and development of small molecules that inhibit tubulin polymerisation. Some of the important microtubule targeting agents are colchicines (**I**), combretastatin A-4(**II**) and nocodazole (**III**) (Figure 1A).[6]

Combretastatins A-4 is a well-known natural product, isolated from the bark of the South African tree *Combretum caffrum* exhibit potent cytotoxicity and tubulin polymerization inhibition.[7-11] Previously, a number of studies reported on structure–activity relationships of CA-4 indicating

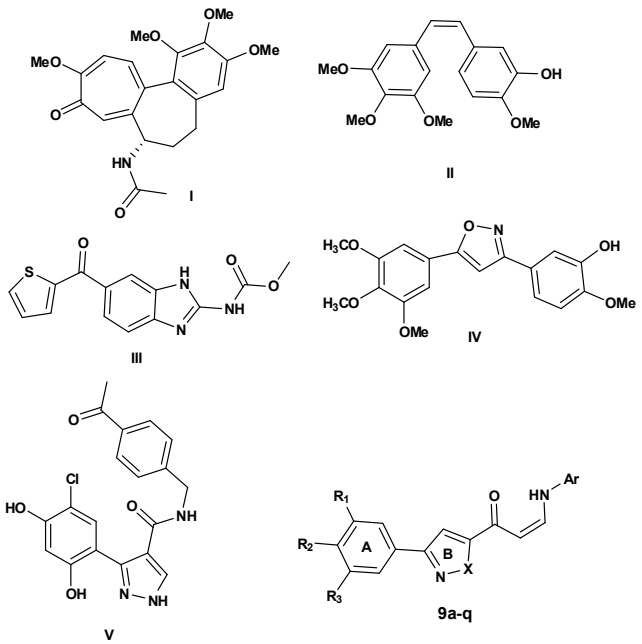


Figure 1A Chemical Structures of some anticancer molecules: Colchicines (**I**), combretastatin A-4 (**II**), nocodazole (**III**), isoxazole derivative (**IV**), pyrazole derivative (**V**) and (*Z*)-1-(3-aryl-1H-pyrazol-5-yl)-3-(arylamino) prop-2-en-1-one and (*Z*)-1-(3-arylisoxazol-5-yl)-3-(arylamino) prop-2-en-1-one conjugates (**9a-q**).

that *cis*-configuration at the olefinic bridge and 3,4,5-trimethoxy unit on the A-ring of are essential for activity.[12-14] To retain the *cis*-configuration, alternative bridge groups like; heteroaromatic rings (pyrazoles, thiazoles, isomeric triazoles, tetrazoles, oxazoles, imidazoles, furans, furanones and thiophenes) and non-heterocyclic (ethers, olefins, ketones, sulfonamides, sulfonates, amine, amide derivatives and cyclopentanes) were introduced as substitutes to olefinic group without loss of potency and tubulin depolymerisation activity.[15-19]

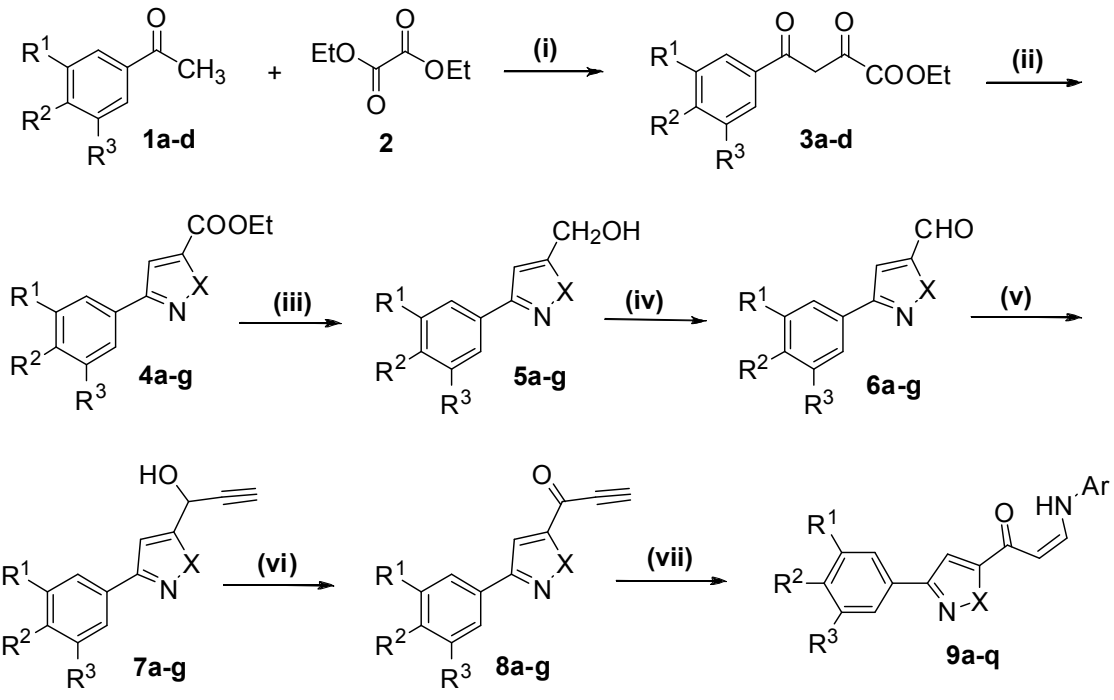
Individually, the isoxazole (**IV**) core moieties have been evaluated for antimicrobial activity, immunotropic activity, analgesic activity, antihypertensive activity, antifungal activity and antitumor activity.[20-25] Similarly, pyrazole (**V**)include derivatives are reported for different biological activities like antimicrobial, antifungal, anti-inflammatory including antiproliferative activity.[26-33] However, several clinically useful tubulin depolymerizing drugs have huge drawbacks. In the present work, the antitubulin compounds developed have not been proved in overcoming the problems of existing drugs. A new class of (*Z*)-(arylamino)-isoxazolyl prop-2-en-1-one and (*Z*)-(arylamino)-pyrazolyl prop-2-en-1-ones derivatives (**9a-q**) were synthesized by keeping structural aspects like *cis*-doble bond and bridge groups (isoxazole and pyrazole) of combretastatin A-4 and evaluated for their cytotoxicity against selected human cancer cell lines and they also showed significant inhibition of tubulin polymerization.

Results and Discussion

Chemistry

Synthesis of the (Z)-1-(3-aryl-1H-pyrazol-5-yl)-3-(arylamino) prop-2-en-1-one and (Z)-1-(3-arylisoxazol-5-yl)-3-(arylamino) prop-2-en-1-one conjugates is carried out as shown in Scheme 1. Diethyloxalate has been treated with substituted acetophenones in the presence of base to obtain β-ketoesters **3a-d**. These intermediates (**3a-d**) were reacted with hydroxylamine hydrochloride or hydrazine hydrochloride to provide aryl esters **4a-g**. Reduction of **4a-g** with LAH followed by oxidation with IBX give substituted isoxazole/pyrazole aldehydes (**6a-g**). These intermediate aldehydes **6a-g** were treated with ethynylmagnesium bromide in THF to obtain acetylene substituted aryl-pyrazole/isoxazole alcohols **7a-g**, further these alcohols (**7a-g**) were oxidized to the desired precursors **8a-g** with IBX in DMSO. Finally, we achieved the target conjugates **9a-t** by the reaction of appropriate precursors (**8a-g**) with corresponding aryl amines in ethanol. The compound structures were confirmed by means of ^{1}H NMR, ^{13}C NMR, HRMS and IR spectra. All the compounds were obtained in pure *Z*-isomeric forms and *Z*-form of compounds was confirmed based on their coupling constants[34].

Scheme:



Scheme: *Reagents and conditions*: (i) Na Metal, Ethanol, rt; 4-5 h; 85-90%; (ii) $NH_2NH_2.2HCl$ for **4a-c**, $NH_2OH.HCl$ for **4d-g**, EtOH, reflux, 2-3 h, 71-81%; (iii) LAH, THF, rt, 2-3 h, (yield 70-80%); (iv) IBX, DMSO, 0 °C, 2 h, 71-85%; (v) Ethynylmagnesium bromide, THF, 0°C, rt, 4-5 h, 59-69%; (vi) IBX, DMSO, 0°C, 2 h, 73-86%; (vii) aryl amines, EtOH, rt, 4-5 h, 69-82%.

Table 1. Structures of compounds **9a–q** and their isolated yield.

Comp	Ar	X	R^1	R^2	R^3	Yield % (mg)
9a	5-indolyl	NH	OCH_3	OCH_3	OCH_3	75 (120)
9b	6-indolyl	NH	OCH_3	OCH_3	OCH_3	78 (125)
9c	4-trifluoromethylphenyl	NH	OCH_3	OCH_3	OCH_3	76 (131)
9d	4-methoxyphenyl	NH	OCH_3	OCH_3	OCH_3	80 (126)
9e	3, 4, 5-trimethoxyphenyl	NH	OCH_3	OCH_3	OCH_3	77 (139)
9f	5-indolyl	NH	H	OCH_3	OCH_3	82 (137)
9g	3, 4, 5-trimethoxyphenyl	NH	H	F	H	80 (163)
9h	5-indolyl	O	OCH_3	OCH_3	OCH_3	77 (127)
9i	6-indolyl	O	OCH_3	OCH_3	OCH_3	72 (116)
9j	4-methoxyphenyl	O	OCH_3	OCH_3	OCH_3	75 (118)
9k	3, 4, 5-trimethoxyphenyl	O	OCH_3	OCH_3	OCH_3	72 (130)
9l	3, 4, 5-trimethoxyphenyl	O	H	OCH_3	OCH_3	76 (143)
9m	6-indolyl	O	H	OCH_3	H	78 (135)
9n	4-trifluoromethylphenyl	O	H	OCH_3	H	69 (130)
9o	4-methoxyphenyl	O	H	OCH_3	H	71 (120)
9p	3, 4, 5-trimethoxyphenyl	O	H	OCH_3	H	76 (151)
9q	5-indolyl	O	H	F	H	79 (140)

Biology

Antiproliferative activity

A new class of (Z)-1-(3-aryl-1H-pyrazol-5-yl)-3-(arylamino)prop-2-en-1-one and (Z)-1-(3-arylisoxazol-5-yl)-3-(arylamino)prop-2-en-1-one (**9a-q**) conjugates were synthesized and tested for their cytotoxic activity against various human cancer cell lines (MCF-7 (breast), A549 (lung), HCT116 (colon) and HeLa (cervix)) and the results are listed in Table 2.

Most of the compounds have shown potential cytotoxicity against tested cancer cell lines. Among the series some compounds like **9a**, **9b** and **9f** showed significant cytotoxic potency with the IC_{50} values in the range of 0.36- 1.12 µM. Structure activity relationship (SAR) (Figure 1B) studies revealed that among these compounds, **9a**, **9b**, **9f**, **9h**, **9i**, **9m** and **9q** with indole group at aryl position are more active than other compounds. Moreover, **9a**, **9b** and **9f** that contain indolyl substitution at the aryl (Ar) and 3,4,5-trimethoxyphenyl substitution on the A-ring have shown a remarkable cytotoxic effect. Particularly, compound **9b** with 6-indolyl group is most potent than compound **9a** that possessing a 5-indolyl group (6-indolyl > 5-indolyl) (Figure 1B). Subsequently, compounds **9a** and **9b** exhibited notable inhibition effect on the tubulin polymerization, which certainly correlated with observed antiproliferative activity. In contrast, **9a** (IC_{50}: 0.61 µM) and **9b** (IC_{50}: 0.36 µM) with a trimethoxyphenyl substitution on ring A (similar to trimethoxy phenyl ring of colchicine) exhibited strong activity, whereas, the presence of dimethoxyphenyl substitution on ring A of **9f**, (IC_{50}: 0.76 µM) decreased the activity in A549 cells (trimethoxyphenyl > dimethoxyphenyl). Furthermore, the bridge rings (pyrazole and isoxazole) were played a key role in cytotoxic activity. The presence of pyrazole substitution on ring B of **9a** and **9b** are potent than the compounds **9h** and **9i** which include isoxazole substitution on ring B and this result shows that isoxazole substitution is decreasing activity and pyrazole substitution is essential for better cytotoxic activity. In addition, compounds (**9c** and **9n**) which are contain trifluorophenyl group at aryl showed moderate cytotoxic activities.

To study the cytotoxic effect on normal cell lines, compounds **9a**, **9b, 9c** and **9f** were screened on HEK-293 and the results suggested that these conjugates (**9a**, **9b, 9c** and **9f**) were 80-100 fold less toxic to normal cell (IC_{50} values 98.5, 86.3, 101.7 and 102.5 µM, respectively) when it compare with cancer cells (Table 2). Consequently, these results recommended that the presence of a pyrazole substitution on ring B with trimethoxyphenyl substitution on ring-A and indolyl group at aryl (Ar) resulted in promising molecules, which reveal effective anticancer activity and inhibition of tubulin polymerization activity.

Structure Activity Relation

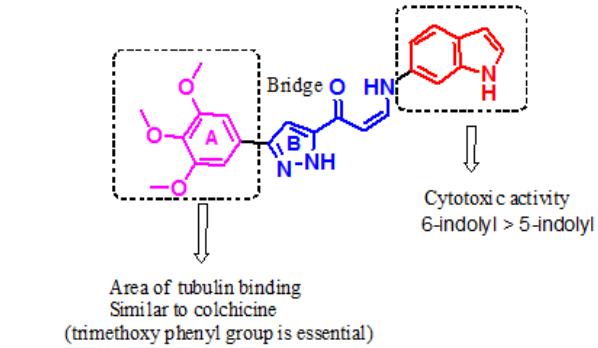


Figure 1B. Structure activity relationship of (Z)-(arylamino)-pyrazolyl/isoxazolyl-2-propenones (**9a-q**). The compounds that possessing 3,4,5-trimethoxyphenyl as A-ring and indolyl group showed significant anticancer activity. Moreover, 3,4,5-trimethoxyphenyl ring is a hall mark for tubulin binding.

Table 2. In vitro cytotoxic effect ([a]IC_{50} μM) of pyrazole/isoxazole - arylpropenone **(9a-q)** conjugates.

entry	Compd	[b]A549	[c]HeLa	[d]MCF-7	[e]HCT116
1	**9a**[f]	0.61±0.4	0.96±0.4	0.68±0.1	1.05±0.1
2	**9b**[f]	0.36±0.1	0.52±0.2	0.57±0.1	0.64±0.3
3	**9c**[f]	1.40±0.8	1.03±0.6	1.54±0.7	1.36±0.7
4	**9d**	2.52±0.2	2.99±0.9	1.38±1.5	2.50±0.6
5	**9e**	4.13±0.3	3.83±1.1	3.96±0.9	3.77±0.8
6	**9f**[f]	0.76±0.8	0.71±0.6	0.87±0.1	1.12±1.0
7	**9g**	2.43±0.6	2.81±0.9	3.77±0.6	1.86±0.6
8	**9h**	2.06±0.9	2.41±0.9	1.79±1.1	2.65±0.9
9	**9i**	1.68±1.1	1.41±1.0	1.55±0.8	1.29±0.6
10	**9j**	3.39±0.6	2.29±0.5	3.86±0.3	3.77±0.1
11	**9k**	2.93±0.6	3.69±0.5	2.80±0.4	3.74±0.3
12	**9l**	3.27±0.1	2.59±1.3	2.58±1.4	3.17±0.3
13	**9m**	2.46±1.1	2.18±0.8	3.03±1.1	2.67±0.7
14	**9n**	1.89±0.9	2.33±0.1	2.06±0.3	2.81±0.5
15	**9o**	2.89±0.6	2.43±1.1	2.62±0.6	3.44±1.2
16	**9p**	3.08±0.5	3.73±0.6	4.03±0.6	3.18±1.4
17	**9q**	2.19±0.9	1.08±0.3	2.51±0.2	1.36±0.4
18	**Noc**	0.9±0.2	1.2±0.7	1.25±0.9	1.3±0.7

[a] Concentration required to inhibition 50% cell growth and the values represent mean ± S.D. from three different experiments performed in triplicates. [b] A549: lung adenocarcinoma epithelial cell line. [c] HeLa: cervix cancer cell line [d] MCF-7: breast adenocarcinoma cell line. [e] HCT116: colon cell line. [f] **9a**, **9b**, **9c** and **9f** were inhibited normal cells (HEK-293 cell line) with IC_{50} values 98.5, 86.3, 101.7 and 102.5 μM, respectively

Effect on cell cycle arrest

To elucidate whether the cytotoxicity induced by the derivatives was due to cell cycle arrest, we performed flow cytometry analysis for derivatives that exhibited potent cytotoxicity. Thus, A-549 cells were treated with **9a**, **9b** and **9f** at 3 μM for 24 h and it was found that major population of cells accumulated in the G2/M phase of the cell cycle with 75.21, 75.61 and 74.36 % respectively (Figure 2).

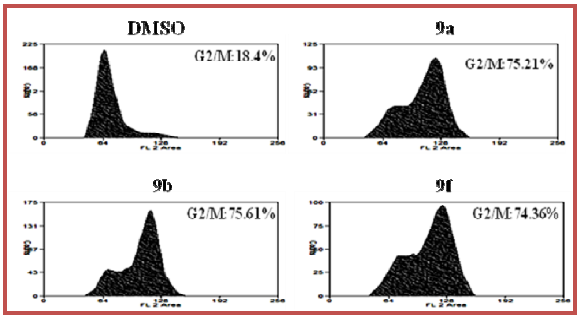


Figure 2. Anti-mitotic effect of 9a, 9b and 9f by FACS analysis: Induction of cell cycle arrest at G2/M by compounds **9a**, **9b** and **9f**. A549 cells were harvested after treatment at 3 μM for 24h. Untreated cells and DMSO treated cells served as control. The percentage of cells in each phase of cell cycle was quantified by flow cytometry.

Effect on Inhibition of tubulin polymerization

Cell viability assays revealed that all compounds showed potent cytotoxic activity against a majority of the cell lines tested. To investigate whether the antiproliferative activities of these conjugates are due to their antitubulin effects, we employed *in vitro* tubulin polymerization assay (Cytoskeleton, Inc.). For comparison, nocodazole was used as positive control in all experiments. Results demonstrated that compounds **9a**, **9b** and **9f** exhibited superior inhibition of tubulin assembly with IC_{50} values as 1.28 μM, 0.28 μM and 7.34 μM respectively; however, under similar experimental conditions, nocodazole showed lower antitubulin activity with an IC_{50} value 2.64 μM. Interestingly, compounds **9a** and **9b** which demonstrated strong inhibition of tubulin assembly, have the lowest IC_{50} values than the known tubulin assembly inhibitor, nocodazole. These results support the suggestion that compound **9b** is a potent inhibitor of tubulin assembly (Table 3).

Table 3. Effect of compounds **9a, 9b** and **9f** on Tubulin Polymerization

Compound	Inhibition of tubulinpolymerization,[a] IC_{50} (μM)
9a	1.28 ± 0.4
9b	0.28 ± 0.2
9f	7.34 ± 1.0
Noc (III)	2.64 ± 0.4

[a] Compounds (final concentration: 3 μM and final volume: 10μl) were pre-incubated with tubulin at a final conc. of 10μM

Effect on microtubule network

Compounds **9a**, **9b** and **9f** altered the cell-cycle factors with preferential G2/M phase and also these exhibit considerable effects on tubulin assembly. Furthermore, inhibition of tubulin polymerization is strongly correlated with G2/M cell-cycle arrest.[35] As **9a**, **9b** and **9f** showed G2/M cell-cycle arrest, it was considered of interest to study the microtubule inhibitory function of these compounds. Thus, A549 cells were treated with **9a**, **9b** and **9f** at 3 μM for 24 h and immunofluorescence analysis reveals that the cells exhibited a rounded or irregular morphology that is typical of mitotic arrest. Moreover, the chromatin was also condensed in the nuclei, suggesting that it is metaphase cell arrest. Whereas, cells treated with DMSO demonstrated a normal and intact tubulin organization. The A549 cells were also counterstained with DAPI to visualize the morphology of nuclei (Figure 3).

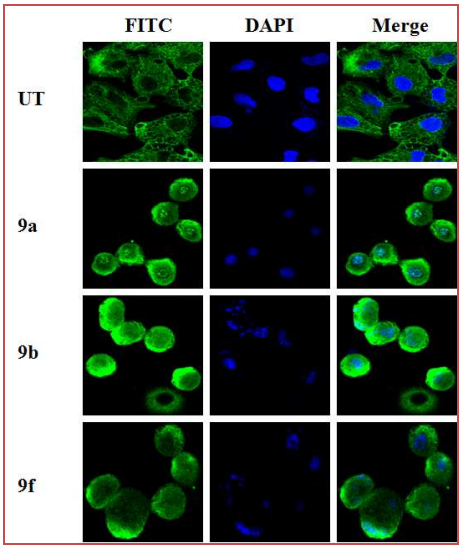


Figure 3. Effect of 9a, 9b and 9f on microtubules and nuclear condensation: A549 cells were treated with **9a**, **9b** and **9f** concentrations of 3 μM for 24 h, followed by fixation, permeabilization and indirect immunofluorescent analysis with an anti-α-tubulin-FITC. Nuclei were stained with DAPI. The merged images of cells stained for tubulin and DAPI are represented.

Analysis of free versus polymerized tubulin by western blotting

The microtubules continuously undergo polymerization and depolymerization which are mediated by α- and β-tubulin, and changing this dynamic balance is considered as a potential target for cancer drug development (e.g., paclitaxel or colchicines). In order to extend the *in vitro* effects of the compounds on tubulin polymerization to the cellular effects i.e., ratio of free vs polymerized tubulin, Western blot analysis of α-tubulin in A549 cells was performed by treating the cells with nocodazole (2 μM), paclitaxel (1 μM), **9a** and **9b** (2 μM) for 24 h. Immunoblot results show that the cells exposed to these compounds **9a** and **9b** contain more tubulin in the soluble fraction of cells, when compared to insoluble fraction. Hence, increased tubulin in soluble fraction of cells treated with these conjugates supported well with the inhibition of tubulin polymerization (Figure 4).

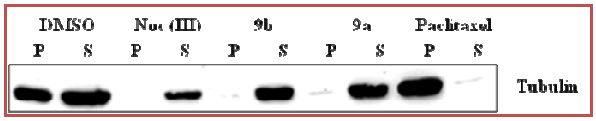


Figure 4. Distribution of tubulin in polymerized vs soluble fractions as analyzed by immunobloting: A549 cells were treated with 2 μM of **9a, 9b** and nocodazole (**III**) for 24 h. Paclitaxel was utilised at 1 μM concentrations for 24h treatments. The fractions containing soluble and polymerised tubulin were collected and separated by SDS-PAGE. Tubulin was detected by immunoblot analysis.

Effect on cellular cyclin-B1 levels by immunoblot analysis

During a normal cell cycle, the progression of cells in the G2 phase to M phase is triggered by the activation of the cyclin B1 dependent kinase.[36-37] So, we also investigated the expression level of cyclinB1. The A549 cells were exposed to **9a**, **9b** and **9f** and were then prepared for evaluation by western blotting. As shown in Figure 9, there was a marked increase in cyclin B1 protein levels for the compounds **9a**, **9b** and **9f** as compared with the control. These result showed that **9a**, **9b** and **9f** arrest cell cycle in G2/M phase involving cell-cycle regulators cyclin B1 (Figure 5).

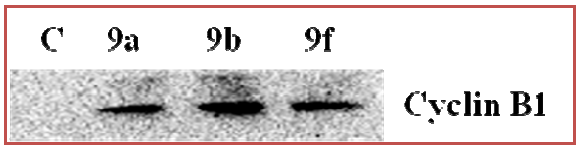


Figure 5. Western blot analysis of Cyclin-B1: Treatment of A549 cells with 2 μM concentrations of **9a, 9b** and **9f** for 24 h resulted an increase in Cyclin B1.

Competitive tubulin-binding assay

To confirm the above observations, we have investigated the effect of **9a**, **9b** and **9f** on tubulin polymerization using an *in vitro* tubulin polymerization assay. Compounds **9a**, **9b** and **9f** inhibited the polymerization of tubulin similar to that of nocodazole. Therefore we further assessed the ability of compounds **9a**, **9b** and **9f** to compete with the colchicine for binding to tubulin via competitive binding assays.[38] Nocodazole was used as a positive control and paclitaxel as a negative control. Because the intrinsic fluorescence of colchicine increases upon binding to tubulin, it was used as an index for **9a**, **9b** and **9f** competition with colchicine in tubulin binding. As shown in figure 6, paclitaxel did not affect the binding to tubulin. However, the fluorescence of colchicine-tubulin complex was reduced in the presence of compounds **9a**, **9b**, **9f** and nocodazole in a dose-dependent manner. These observations indicate that the compounds **9a**, **9b** and **9f** inhibit the binding of colchicine to tubulin, suggesting that the compounds **9a**, **9b**and **9f** binds at the colchicine binding site of tubulin (Figure 6).

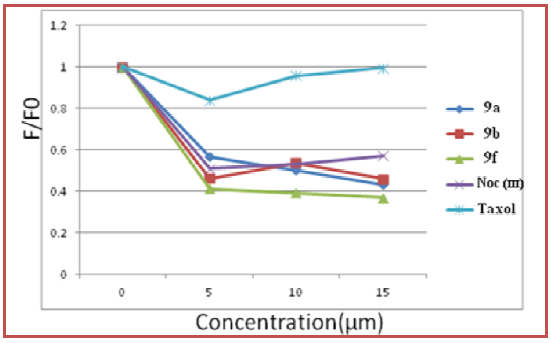


Figure 6. Fluorescence based colchicine competitive binding assay of conjugates 9a, 9b and 9f. It was carried out at various concentrations containing 3 μM of tubulin and colchicine for 60 min at 37 ^{0}C, whereas nocodazole was used as a positive control and taxol was used as negative control which binds at taxane site. Fluorescence values are normalized to control.

Effect on mitochondrial depolarization

Mitochondria play an essential role in the propagation of apoptosis. It is well established that at an early stage, apoptotic stimuli alter the mitochondrial trans membrane potential ($\Delta\psi_{mt}$).[39] This was monitored by the fluorescence of the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1). Wherein, JC-1 displays a red fluorescence (590 nm) with normal cells (high $\Delta\psi_{mt}$), which is caused by the spontaneous and local formation of aggregates that are associated with a large shift in the emission. In contrast, when the mitochondrial membrane is depolarized (low $\Delta\psi_{mt}$), JC-1 forms monomers that emit at 530 nm.

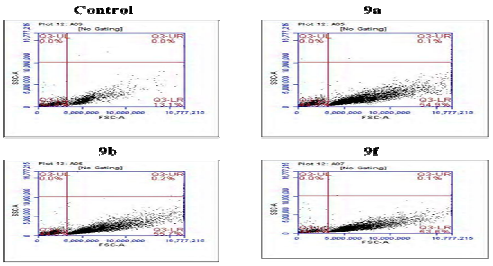


Figure 7. Assessment of $\Delta\psi_{mt}$ (mitochondrial depolarization) of compounds 9a, 9b and 9h in A549 cells: Representative histograms of control cells and cells incubated for 24 h in the presence of **9a, 9b** and **9f** as indicated and stained with the fluorescent probe JC-1 after treatment. The horizontal axis shows fluorescence intensity of the JC-1 monomer and the vertical axis shows fluorescence of JC-1 aggregates.

In this study, A549 cells were treated with **9a, 9b** and **9f** at 2 μM that exhibited a remarkable shift increase to 54.8%, 54.9% and 53.8% of fluorescence respectively, whereas control cells showed 13.1% and these result indicates depolarization of the mitochondrial membrane potential by these conjugates that results in the induction of mitochondrial apoptosis (Figure 7).

Caspase-3 activation

There are some reports that the cell cycle arrest at G2/M phase takes place by the induction of cellular apoptosis.[40] Hence, it was considered of interest to understand the correlation of cytotoxicity with that to apoptosis by **9a** and **9b**.

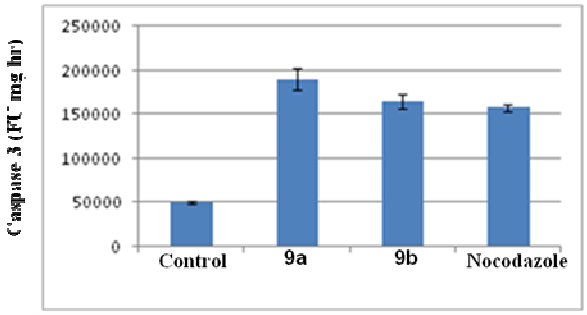


Figure 8. Effect of compounds 9a and 9b on caspase-3 activity: A-549 cells were treated for 48 h with 2 μM concentrations of compounds **9a** and **9b**.Values indicated are the mean _ SD of two different experiments performed in triplicates.

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 effecter caspase. Hence, we treated A-549 cells with **9a** and **9b** examined the activation of caspase-3. The results indicate that there is nearly 3-4 fold induction in caspase-3 activity in cells treated with these conjugates at 2 μM concentrations. Therefore activation of caspase-3 by **9a** and **9b** indicate that they have the capacity to induce apoptosis in A-549 (Figure 8).

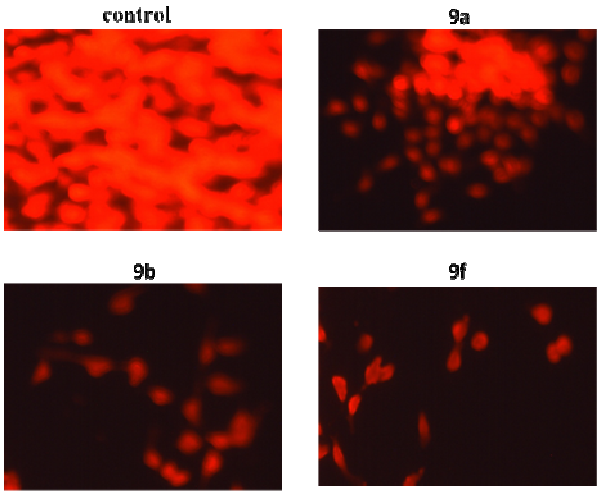


Figure 9. Assessment of apoptosis by PI: Cells were treated with compounds **9a**, **9b** and **9f** at 2 μM concentration for 24 h. Cells were examined under a fluorescence microscope.

Assessment of apoptosis by PI

To determine the apoptotic effect of **9a**, **9b** and **9f**, the PI-staining was studied by fluorescence microscopy and these induced morphological changes with characteristic of apoptosis in A549 cells.[41,42] Control cells displayed probable growth characteristics, but compounds **9a**, **9b** and **9f** showed typical apoptotic features of cells such as membrane blebbing and cell shrinkage using PI (Figure 9).

Conclusion

In conclusion, we have designed, synthesized a new type of conjugates (**9a-q**) and tested for their *in vitro* anticancer activities against a variety of different human cancer cell lines. All the compounds showed moderate to good cytotoxic potency against most of the tested cancer cell lines, however some of the compounds **9a**, **9b**, **9c** and **9f** exhibited significant cytotoxic potency with IC_{50} values in the range of 0.36 to 1.54 μM. FACS results reveal that these conjugates **9a**, **9b** and **9f** caused cell cycle arrest and accumulate cells in the G2/M phase. They inhibit tubulin assembly as shown by the tubilin polymerization assay. Interestingly, it was observed that IC_{50} value of **9b** (0.28 μM) was less than nocodazole (2.64 μM), a well known tubulin polymerization inhibitor and activated the cyclin-B1 protein levels and disrupted microtubule system. Moreover caspase-3 activation, loss of mitochondrial membrane potential and PI staining results suggest that they cause cell death by inducing apoptosis. The compounds **9a**, **9b** and **9f** inhibit the binding of colchicine to tubulin, thereby suggesting that the compounds **9a**, **9b**and **9f** binds at the colchicine binding site. Based on these results it is evident that these conjugates have the potential to be taken up for further detailed investigations either alone or in combination with the existing therapies as potential tubulin polymerization inhibitors.

Experimental protocols

General

All chemicals were purchased from Spectrochem Pvt. Ltd (Mumbai, India), Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) 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 attained by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker UXNMR/XWIN-NMR (300 M Hz) instruments. Chemical shifts are reported in parts per million (δ in ppm) relative to the peak for TMS (tetramethylsilane) as an intrernal standard, coupling constants are reported in hertz (Hz). ESI spectra were recorded on Micro mass, Quattro LC using ESI^{+} software with capillary voltage 3.98 kV and ESI mode positive ion trap detector. High-resolution mass spectra were recorded on a QSTAR XL Hybrid MS–MS mass spectrometer. Melting points were determined with an Electro thermal melting point apparatus, and are uncorrected. Purity was evaluated by analytical HPLC using waters 515 pump coupled to a waters 2487 dual λ absorbance UV detector. Mobile phase: H_2O-CH_3CN (10:90 v/v). (buffer ammonium acetate pH 4.4) UV detection at 254 nm. Column used was Luna 5u C18 with 5 μm

particle size. Flow rate: 0.7 mL/min: injection volume: 5 µL retention times are given in minutes.

Chemistry

Preparation of ethyl 2,4-dioxo-4-(substituted phenyl)butanoates (3a-d)

Diethyl oxalate (**2**) (1.0 mol) was added to freshly prepared sodium ethoxide at 0 °C and after 10 minutes substituted acetophenones (**1a-d**) (1.0 mol) were added slowly in small portions maintaining the temperature 0 °C. After completion of addition the stirring was continued at room temperature for 4-5 h. the reaction mixture was neutralized by diluted H_2SO_4 and extracted with ethyl acetate. It was recrystallized from methanol to offered solid products β-di ketoesters (**3a-d**) (yield 85-90%) which were taken as such for the next step.

Preparation of ethyl 3-substituted phenyl-1H-pyrazole-5-carboxylates (4a-c) and ethyl 3-(substituted phenyl)isoxazole-5-carboxylate (4d-g)

To each ethyl 2,4-dioxo-4-(substituted phenyl)butanoates (**3a-d**) obtained in the earlier step was added to hydrazine dihydrochloride (NH_2-NH_2.2HCl) for (**4a-c**) and hydroxylamine hydrochloride (NH_2OH.HCl) for (**4d-g**) in ethanol and heated to reflux for 2-3 h. The solvent was removed under vacuum then added water to the residue followed by extracted with ethyl acetate (50 ml x 4). The organic layer was dried on anhydrous Na_2SO_4 and evaporated the solvent to obtain crude product that was further purified by column chromatography using ethyl acetate and hexane. The pure aryl esters (**4a-g**) were eluted at 30-40% of ethyl acetate with good yields.

Ethyl 3-(3,4,5-trimethoxyphenyl)-1H-pyrazole-5-carboxylate (4a):

Ethyl 3-(3,4,5-trimethoxyphenyl)-1H-pyrazole-5-carboxylate (**4a**) was prepared using above method by the addition of ethyl 2,4-dioxo-4-(3,4,5-trimethoxyphenyl)butanoate (**3a**) (5.59 g, 18 mmol) and hydrazine dihydrochloride (2.81 g, 27 mmol) to ethanol (50 mL) as yellow colored solid; (4.14 g, yield 75%); mp 129-130 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.45 (t, 3H, J= 7.2 Hz, **-CH$_3$**), 3.91 (s, 6H, **-OCH$_3$**), 3.95 (s, 3H, **-OCH$_3$**), 4.43-4.53 (q, 2H, J=7.2 Hz, **CH$_2$**), 6.87 (s, 1H, **ArH**), 7.02 (s, 2H, **ArH**) ppm; MS (ESI): *m/z* 307 $[M+H]^+$.

Ethyl 3-(3,4-dimethoxyphenyl)-1*H*-pyrazole-5-carboxylate (4b):

Ethyl 3-(3,4-dimethoxyphenyl)-1*H*-pyrazole-5-carboxylate **4b** was prepared using above method by the addition of ethyl 2,4-dioxo-4-(3,4-trimethoxyphenyl)butanoate (**3b**) (4.95 g, 17.7 mmol) and hydrazine dihydrochloride (2.76 g, 26.5 mmol) to ethanol (45 mL) as yellow colored solid (3.90g, yield 80%); mp 116-117 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23-1.31 (t, 3H, J=7.1 Hz, -C**H**$_3$), 3.85 (s, 3H, -OC**H**$_3$), 3.90 (s, 3H, -OC**H**$_3$) 4.19-4.32 (q, 2H, J=7.1 Hz, -C**H**$_2$), 6.88 (d, 1H, J=7.1 Hz, -Ar**H**), 6.94 (s, 1H, Ar**H**), 7.21-7.28 (m, 1H, Ar**H**) 7.29-7.34 (m, 1H, Ar**H**) 9.67 (brs, 1H, -N**H**) ppm; MS (ESI): *m/z* 277 $[M+H]^+$.

Ethyl 3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxylate (4c)

Ethyl 3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxylate (**4c**) was prepared using above method by the addition of ethyl 2,4-dioxo-4-(4-fluorophenyl)butanoate (**3c**) (5.16g, 21.6 mmol) and hydrazine dihydrochloride (3.38g, 32.5 mmol) to ethanol (50 mL) as yellow colored solid (3.60g, yield 71%); mp 121-122 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.23-1.37 (t, 3H, J=7.5Hz, -C**H**$_3$), 4.17-4.36 (q, 2H, J=7.5Hz, C**H**$_2$), 6.8 (s, 1H, Ar**H**), 6.90 (d, 2H, J = 2.2Hz, Ar**H**), 7.62 (d, 2H, J = 9.0Hz, Ar**H**) ppm; MS (ESI): *m/z* 235 $[M+H]^+$.

Ethyl 3-(3,4,5-trimethoxyphenyl)isoxazole-5-carboxylate (4d)

Ethyl 3-(3,4,5-trimethoxyphenyl)isoxazole-5-carboxylate (**4d**) was prepared using above method by the addition of ethyl 2,4-dioxo-4-(3,4,5-trimethoxyphenyl)butanoate (**3a**) (5.62 g, 18.2 mmol) and hydroxylamine hydrochloride (1.87 g, 27.2 mmol) to ethanol (50 mL) as yellow colored solid; (4.51 g, yield 81%); mp 137-139 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.33-1.40 (t, 3H, J=6.7 Hz, J=7.5 Hz, -C**H**$_3$), 3.88 (s, 3H, -OC**H**$_3$), 3.91 (s, 6H, -OC**H**$_3$), 4.33-4.45 (q, 2H, J=6.7 Hz, J=7.5 Hz, C**H**$_2$), 7.01 (s, 2H, Ar**H**), 7.05 (s, 1H, Ar**H**) ppm; MS (ESI): *m/z* 308 $[M+H]^+$.

Ethyl 3-(3,4-dimethoxyphenyl)isoxazole-5-carboxylate (4e)

Ethyl 3-(3,4-dimethoxyphenyl)isoxazole-5-carboxylate (**4e**) was prepared using above method by the addition of ethyl 2,4-dioxo-4-(3,4-dimethoxyphenyl)butanoate (**3b**) (6.12g, 21.8 mmol) and hydroxylamine hydrochloride (2.26g, 32.7 mmol) to ethanol (60 mL) as yellow colored solid; (4.72g, yield 78%); mp 131-132 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.40 (t, 3H, J=7.4 Hz, **CH$_3$**), 3.81 (s, 6H, **OCH$_3$**), 4.39-4.46 (q, 2H, J=7.4 Hz, **CH$_2$**), 6.76 (s, 1H, **ArH**), 7.18 (d, 1H, J=7.8 Hz, **ArH**), 7.31 (s, 1H, **ArH**), 7.42 (dd, 1H, J=5.9 Hz, J=1.6 Hz, **ArH**) ppm; MS (ESI): *m/z* 278 $[M+H]^+$.

Ethyl 3-(4-methoxyphenyl)isoxazole-5-carboxylate (4f)

Ethyl 3-(4-methoxyphenyl)isoxazole-5-carboxylate (**4f**) was prepared using above described method by the addition of ethyl 2,4-dioxo-4-(4-methoxyphenyl)butanoate (**3d**) (5.89 g, 23.5 mmol) and hydroxylamine hydrochloride (2.44 g, 35.3 mmol) to ethanol (55 mL) as yellow colored solid; (4.36 g, yield 75%); mp 126-127 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (t, 1H, J=7.2 Hz, **CH$_3$**), 3.87 (s, 3H, **OCH$_3$**), 4.42-4.52 (q, 2H, J=7.2 Hz, **CH$_2$**), 6.81 (s, 1H, **ArH**), 7.00 (d, 2H, J=8.9 Hz, **ArH**), 7.75 (d, 2H, J=8.9 Hz, **ArH**) ppm; MS (ESI): *m/z* 248 $[M+H]^+$.

Ethyl 3-(4-fluorophenyl)isoxazole-5-carboxylate (4g)

Ethyl 3-(4-fluorophenyl)isoxazole-5-carboxylate (**4g**) was prepared using above explained method by the addition of ethyl 2,4-dioxo-4-(4-fluorophenyl)butanoate (**3c**) (5.29 g, 22.2mmol) and hydroxylamine hydrochloride (2.30 g 33.3 mmol) to ethanol (50 mL) as yellow colored solid; (3.81g, yield 73%); mp 115–116 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.39 (t, 3H, J=6.9 Hz, **CH$_3$**), 4.47-4.56 (m, 2H, **CH$_2$**), 6.69 (s, 1H, **ArH**), 7.21 (d, 2H, J=7.6 Hz, **ArH**), 7.47 (d, 2H, J=7.6 Hz, **ArH**) ppm; MS (ESI): *m/z* 236 $[M+H]^+$.

Preparation of (3-substitutedphenyl-1*H*-pyrazol-5-yl)methanols (5a-c) and (3-(substitutedphenyl)isoxazol-5-yl)methanol (5d-g)

To the ethyl 3-substituted phenyl-1*H*-pyrazole-5-carboxylates/ ethyl 3-substituted phenyl-isoxazole-5-carboxylates (**4a-g**), obtained in the above step was added to $LiAH_4$ (0.5 mol) in dry THF at 0 °C and stirred for 2-3 h at room temperature. Added saturated NH_4Cl solution drop wise to quench the unreacted $LiAlH_4$ and removed the THF under vacuum then extracted with ethyl acetate (100 ml X 4). The organic layer was dried on anhydrous Na_2SO_4 and evaporated ethyl acetate to obtain color less solid products of (3-substitutedphenyl-1*H*-pyrazol-5-yl)methanols/(3-substitutedphenyl-isoxazole-5-yl)methanols (**5a-g**) (yield 70-80%). The alcohols produced in this step were pure,

and no further purification was required. These compounds were taken as such for the next step.

Preparation of 3-subtitutedphenyl-1*H*-pyrazole-5-carbaldehydes (6a-c) and 3-(substitutedphenyl)isoxazole-5-carbaldehyde (6d-g)

To the (5-substitutedphenyl-1H-pyrazol-3-yl)methanols/(5-substitutedphenyl-isoxazole-3-yl)methanols (**5a-g**) (1 eq) produced in the above step was added IBX (1.5 eq) in DMSO and stirred for 2 h at room temperature. After completion reaction added ice cold water to the reaction mixture and extracted with ethyl acetate (50 ml X 4). The organic layer was dried on anhydrous Na_2SO_4 and evaporated the ethyl acetate. It was recrystallized from methanol to obtain pure corresponding 5-subtitutedphenyl-1H-pyrazole-3-carbaldehydes/5-subtitutedphenyl-isoxazole-3-carbaldehydes (**6a-g**) in good yields (71-85%).

3-(3,4,5-Trimethoxyphenyl)-1*H*-pyrazole-5-carbaldehyde (6a)

3-(3,4,5-Trimethoxyphenyl)-1*H*-pyrazole-5-carbaldehyde **6a** was prepared using above method by the addition of (3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)methanol **5a** (2.64 g, 10 mmol) and IBX (4.15 g, 15 mmol) to DMSO (20 mL) as brown colored solid (1.84 g, yield 71%); mp 128-129 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 3.90 (s, 3H, -OC**H**$_3$), 3.94 (s, 6H, -OC**H**$_3$), 6.86 (s, 1H, **ArH**), 7.06 (S, 2H, Ar**H**), 10.19 (s, 1H, C**H**O) ppm; MS (ESI): *m/z* 263 $[M+H]^+$.

3-(3,4-Dimethoxyphenyl)-1*H*-pyrazole-5-carbaldehyde (6b)

3-(3,4-Dimethoxyphenyl)-1*H*-pyrazole-5-carbaldehyde (**6b**) was prepared using above method by the addition of (3-(3,4-dimethoxyphenyl)-1*H*-pyrazol-5-yl)methanol (**5b**) (2.39g, 10.2 mmol) and IBX (4.28g 15.3 mmol) to DMSO (20 mL) as brown colored solid (1.94 g, yield 82%); mp 116-117 ^oC; ^1H NMR (300 MHz, $CDCl_3$): δ 3.87 (s, 6H, **OCH$_3$**), 6.86 (s, 1H, **ArH**), 7.02 (d, 1H, *J*=8.7 Hz, **ArH**), 7.34-7.39 (m, 2H, **ArH**), 10.02 (s, 1H, **CHO**) ppm; MS (ESI): *m/z* 233 $[M+H]^+$.

3-(4-Fluoroyphenyl)-1*H*-pyrazole-5-carbaldehyde (6c)

3-(4-Fluorophenyl)-1*H*-pyrazole-5-carbaldehyde (**6c**) was prepared using above method by the addition of (3-(4-fluorophenyl)-1*H*-pyrazol-5-yl)methanol (**5c**) (2.04 g. 10.6 mmol) and IBX (4.40 g, 15.8 mmol) to DMSO (20 mL) as brown colored solid (1.61 g, yield 80%); mp 121-122 ^oC; ^1H NMR (500 MHz, $CDCl_3$) *δ* 7.78-7.82 (m, 1H, Ar**H**), 7.86-7.93 (m, 2H, Ar**H**), 7.95-8.03 (m, 2H, Ar**H**) 9.95 (s, 1H, C**H**O) ppm; MS (ESI): *m/z* 191 $[M+H]^+$.

3-(3,4,5-Trimethoxyphenyl)isoxazole-5-carbaldehyde (6d)

3-(3,4,5-Trimethoxyphenyl)isoxazole-5-carbaldehyde (**6d**) was prepared using method by the addition of (3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)methanol (**5d**) (2.44 g, 9.2 mmol) and IBX (3.87 g, 13.8 mmol) to DMSO (20 mL) as white colored solid (2.06 g, yield 85%); mp 133-134 ^oC; ^1H NMR (400 MHz, $CDCl_3$): *δ* 3.86 (s, 3H, **OCH$_3$**), 3.89 (s, 6H, **OCH$_3$**), 6.86-7.24 (m, 3H, **ArH**), 9.98 (s, 1H, **CHO**); MS (ESI) *m/z* 264 $[M+H]^+$.

3-(3,4-Dimethoxyphenyl)isoxazole-5-carbaldehyde (6e)

3-(3, 4-Dimethoxyphenyl)isoxazole-5-carbaldehyde (**6e**) was prepared using above method by the addition of (3-(3,4-dimethoxyphenyl)isoxazol-5-yl)methanol (**5e**) (2.65 g, 11.3 mmol) and IBX (4.73 g, 16.9 mmol) to DMSO (20 mL) as white colored solid (1.99 g, yield 76%); mp 129-130 ^oC; ^1H NMR (300 MHz, $CDCl_3$): *δ* 3.96 (s, 6H, OCH_3), 6.79 (s, 1H, **ArH**), 6.97 (d, 1H, *J*=8.3 Hz, **ArH**), 7.32 (s, 1H, **ArH**), 7.41 (dd, 1H, *J*=6.4 Hz, *J*=1.9 Hz, **ArH**), 10.18 (s, 1H, **CHO**) MS (ESI) *m/z* 234 $[M+H]^+$.

3-(4-Methoxyphenyl)isoxazole-5-carbaldehyde (6f)

3-(4-Methoxyphenyl)isoxazole-5-carbaldehyde (**6f**) was prepared using method by the addition of (3-(4-methoxyphenyl)isoxazol-5-yl)methanol (**5f**) (2.32g, 11.3mmol) and IBX (4.75 g, 16.9 mmol) to DMSO (10 mL) as white colored solid (1.86 g, yield 81%); mp 114-115 ^oC; ^1H NMR (300 MHz, $CDCl_3$): *δ* 3.88 (s, 3H, **OCH$_3$**), 6.76 (s, 1H, **ArH**), 7.01 (d, 2H, *J*=8.9 Hz, **ArH**), 7.76 (d, 2H, *J*=8.9 Hz, **ArH**), 10.18 (s, 1H, **CHO**) ppm; MS (ESI) *m/z* 204 $[M+H]^+$.

3-(4-Fluorophenyl)isoxazole-5-carbaldehyde (6g)

3-(4-Fluorophenyl)isoxazole-5-carbaldehyde (**6g**) was prepared using method by the addition of (3-(4-fluorophenyl)isoxazol-5-yl)methanol (**5g**) (2.40 g, 12.4 mmol) and IBX (5.20 g, 18.6 mmol) to DMSO (12 mL) as white colored solid (1.88 g, yield 79%); mp 119-120 ^oC; ^1H NMR (300 MHz, $CDCl_3$): *δ* 6.71 (s, 1H, **ArH**), 7.10 (d, 2H, *J*=6.7 Hz, **ArH**), 7.53 (d, 2H, *J*=6.7 Hz, **ArH**), 10.12 (s, 1H, **CHO**) ppm; MS (ESI) *m/z* 192 $[M+H]^+$.

General procedure for the Synthesis of 1-(3-aryl-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (7a-c) and 1-(3-aryl isoxazol-5-yl)prop-2-yn-1-ol (7d-g)

A solution of 5-subtitutedphenyl-1H-pyrazole-3-carbaldehydes/5-subtitutedphenyl-isoxazole-3-carbaldehydes (**6a-g**) in dry tetrahydrofuran (THF) was added to stirred solution of ethynyl magnesium bromide in tetrahydrofuran (0.5 M) at 0 ^oC and then stirred at room temperature for 4-5 h. After completion of reaction saturated aqueous ammonium chloride solution (5-10 ml) was added, and the THF was removed in vacuum followed by ethyl acetate was added. The organic layer was extracted and washed with brine solution, dried over anhydrous Na_2SO_4 and evaporated in vacuum. It was recrystallized from methanol to obtain pure acetylene substituted aryl-pyrazole/isoxazole alcohols (**7a-g**) and these were used for next step.

1-(3-(3,4,5-Trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (7a)

1-(3-(3,4,5-Trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (**7a**) was obtained using above described method by adding 3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazole-5-carbaldehyde (**6a**) (1.65 g, 6.3 mmol) to the ethynyl magnesium bromide solution(18.8 ml, 9.5mmol) as brown solid (1.25 g, yield 69%); mp 131–133 ^oC; ^1H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): *δ* 2.85 (m, 1H, ≡**H**), 3.88 (s, 3H, **OCH$_3$**), 3.90 (s, 6H, **OCH$_3$**), 5.63 (d, 1H, *J*=2.1 Hz, **H-C-OH**), 6.62 (s, 1H, **ArH**), 6.86 (s, 2H, **ArH**) ppm; MS (ESI): *m/z* 289 $[M+H]^+$.

1-(3-(3,4-Dimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (7b)

1-(3-(3,4-Dimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (**7b**) was obtained using above described method by adding 3-(3,4-dimethoxyphenyl)-1*H*-pyrazole-5-carbaldehyde (**6b**) (1.72 g, 7.4 mmol) to the ethynyl magnesium bromide solution (22.2 ml, 11 mmol) as brown solid (1.13 g, yield 59%); mp 127-128 ^oC ^1H NMR (300 MHz, $CDCl_3$): *δ* 2.73 (d, 1H, *J*=2.8 Hz, ≡ **H**), 3.96 (s, 3H, –OCH_3), 3.93 (s, 3H, –OC**H**$_3$), 5.66 (d, 1H, *J*=2.8 Hz, **H-C-OH**), 6.89 (s, 1H, **ArH**), 6.96 (d, 1H, *J*=6.9 Hz, **ArH**), 7.19-7.25 (m, 2H, **ArH**) ppm; MS (ESI): *m/z* 259 $[M+H]^+$.

1-(3-(4-Fluorophenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (7c)

1-(3-(4-fluorophenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (**7c**) was obtained using above described method by adding 3-(4-fluorophenyl)-1*H*-pyrazole-5-carbaldehyde (**6c**) (1.44 g, 7.6 mmol) to the ethynyl magnesium bromide solution (22.7 ml, 11.4 mmol) as brown solid (1.06g, yield 65%); mp 119-120 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.70 (d, 1H, *J*=2.3 Hz, ≡**H**), 5.71 (m, 1H, **H-C-OH**), 6.61 (s, 1H, **ArH**), 7.23 (d, 2H, *J*=7.3 Hz, **ArH**), 7.38 (d, 2H, *J*=7.3 Hz, **ArH**) ppm; MS (ESI): *m/z* 217 $[M+H]^+$.

1-(3-(3,4,5-Trimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-ol (7d)

1-(3-(3,4,5-Trimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-ol (**7d**) was obtained using above described method by adding 3-(3,4,5-trimethoxyphenyl)isoxazole-5-carbaldehyde (**6d**) (1.87 g, 7.1 mmol) to the ethynyl magnesium bromide solution (21.3 ml, 10.5 mmol) as white solid (1.25 g, yield 61%); mp 128–129 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.70 (d, 1H, J=2.3 Hz, ≡**H**), 3.90 (s, 3H, –OC**H**$_3$), 3.93 (s, 6H, –OC**H**$_3$), 5.68 (d, 1H, *J*=2.3 Hz, **H**-C-OH), 6.64 (s, 1H, **ArH**). 6.99 (s, 2H, **ArH**) ppm; MS (ESI): *m/z* 290 $[M+H]^+$.

1-(3-(3,4-Dimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-ol (7e)

1-(3-(3,4-Dimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-ol (**7e**) was obtained using above described method by adding 3-(3,4,-dimethoxyphenyl)isoxazole-5-carbaldehyde (**6e**) (1.80 g, 7.7 mmol) to the ethynyl magnesium bromide solution (23.2 ml, 11.2 mmol) as white solid (1.30g, yield 65%); mp 122-123 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.66 (d, 1H, *J*=2.6, ≡**H**), 3.91 (s, 3H, –OC**H**$_3$), 3.94 (s, 3H, –OC**H**$_3$), 5.63 (d, 1H, *J*=2.5 Hz, **H-C-OH**), 6.69 (s, 1H, **ArH**), 7.01 (d, 1H, *J*=8.0 Hz, **ArH**), 7.19 (s, 1H, **ArH**), 7.33 (dd, 1H, *J*=7.9 Hz, *J*=1.2 Hz, **ArH**) ppm; MS (ESI): *m/z* 260 $[M+H]^+$.

1-(3-(4-Methoxyphenyl)isoxazol-5-yl)prop-2-yn-1-ol (7f)

1-(3-(4-Methoxyphenyl)isoxazol-5-yl)prop-2-yn-1-ol (**7f**) was obtained using above described method by adding 3-(4-methoxyphenyl)isoxazole-5-carbaldehyde (**6f**) (1.61 g, 7.9 mmol) to the ethynyl magnesium bromide solution (23.8 ml, 12 mmol) as white solid (1.13 g, yield 62%); mp 120-121 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.59 (d, 1H, *J*=2.1 Hz, ≡**H**), 3.83 (s, 3H, –OC**H**$_3$), 5.80 (d, 1H, *J*=2.1 Hz, **H-C-OH**), 6.56 (s, 1H, **ArH**), 7.08 (d, 2H, *J*=8.1 Hz, **ArH**), 7.41 (d, 2H, *J*=8.1 Hz, **ArH**) ppm; MS (ESI): *m/z* 230 $[M+H]^+$.

1-(3-(4-Fluorophenyl)isoxazol-5-yl)prop-2-yn-1-ol (7g)

1-(3-(4-Fluorophenyl)isoxazol-5-yl)prop-2-yn-1-ol (**7g**) was obtained using above described method by adding 3-(4-fluorophenyl)isoxazole-5-carbaldehyde (**6g**) (1.60 g, 8.4 mmol) to the ethynyl magnesium bromide solution (25.1 ml, 12.6 mmol) as white solid (1.09 g, yield 60%); mp 127-128 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.59 (d, 1H, *J*=1.9 Hz, ≡**H**), 5.41 (d, 1H, *J*=1.7 Hz, **H-C-OH**), 6.81 (s, 1H, **ArH**), 7.29 (d, 2H, *J*=8.6 Hz, **ArH**), 7.36 (d, 2H, *J*=8.6 Hz, **ArH**) ppm; MS (ESI): *m/z* 218 $[M+H]^+$.

General method for synthesis of 1-(3-Aryl-1*H*-pyrazol-5-yl)prop-2-yn-1-one (8a-c) and 1-(3-Aryl isoxazol-5-yl)prop-2-yn-1-one (8d-g)

A solution of 2-iodoxy benzoic acid (IBX) (1.5 eq) and dimethyl sulfoxide (DMSO) was stirred for 10 min at room temperature until homogeneous solution. A solution of 1-(3-aryl-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (**7a-c**) and 1-(3-aryl isoxazol-5-yl)prop-2-yn-1-ol (**7d-g**) (1 eq) in dimethyl sulfoxide was added slowly, and it was stirred for 2 h. After completion of reaction, ice water was added to reaction mixture and the mixture was stirred for another 10 min. To this mixture ethyl acetate was added and filtered through celite. The organic layer was separated and washed with water subsequently saturated Na_2CO_3 solution and brine, after that dried over anhydrous Na_2SO_4 and evaporated in vacuum. It was recrystallized from methanol to attain the pure compound and was used directly for next step.

1-(3-(3,4,5-Trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (8a)

1-(3-(3,4,5-Trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8a**) was obtained using above described method by adding 1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (**7a**) (1.15 g, 4 mmol) to the 2- iodoxy benzoic acid (1.68 g, 6 mmol) in DMSO solution(10 mL) as pale yellow solid (856 mg, yield 75%); mp 125-126 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.58 (s, 1H, ≡**H**), 3.91 (s, 3H, –OC**H**$_3$), 3.94 (s, 6H, –OC**H**$_3$), 6.89 (s, 1H, **ArH**), 7.02 (s, 2H, **ArH**) ppm; MS (ESI): *m/z* 287 $[M+H]^+$.

1-(3-(3, 4-Dimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (8b)

1-(3-(3,4-Dimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8b**) was obtained using above described method by adding 1-(3-(3,4-dimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (**7b**) (1.06 g, 4.1 mmol) to the 2- iodoxy benzoic acid (1.72 g, 6.2 mmol) in DMSO solution (10 mL) as pale yellow solid (904 mg, yield 86%); mp 119-120 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.36 (brs, 1H, ≡**H**), 3.93 (s, 3H, –OC**H**$_3$), 3.96 (s, 6H, –OC**H**$_3$), 6.93-7.06 (m, 2H, **ArH**), 7.23-7.32 (m, 1H, **ArH**), 7.44 (s, 1H, **ArH**), 9.96 (brs, 1H, **NH**) ppm; MS (ESI): *m/z* 257 $[M+H]^+$.

1-(3-(4-Fluorophenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (8c)

1-(3-(4-Fluorophenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8c**) was obtained using above described method by adding 1-(3-(4-fluorophenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-ol (**7c**) (920 mg, 4.3 mmol) to the 2- iodoxy benzoic acid (1.79g, 6.4 mmol) in DMSO solution(10 mL) as pale yellow solid (720 mg, yield 79%); mp 114-115 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.59 (s, 1H, ≡**H**), 6.91 (s, 1H, **ArH**), 7.29 (d, 2H, *J*=7.8 Hz, **ArH**), 7.53 (d, 2H, *J*=7.8 Hz, **ArH**) ppm; MS (ESI): *m/z* 215 $[M+H]^+$.

1-(3-(3,4,5-Trimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (8d)

1-(3-(3,4,5-Trimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8d**) was obtained using above described method by adding 1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-ol (**7d**) (1.12 g, 3.9 mmol) to the 2- iodoxy benzoic acid (1.64g, 5.8 mmol) in DMSO (10 mL) solution as pale yellow solid (960 mg, yield 81%); mp 135-136 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.60 (s, 1H, ≡**H**), 3.89 (s, 3H, –OC**H**$_3$), 3.94 (s, 6H, –OC**H**$_3$), 6.99 (s, 2H, **ArH**), 7.36 (s, 1H, **ArH**) ppm; MS (ESI): *m/z* 288 $[M+H]^+$.

1-(3-(3, 4-Dimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (8e)

1-(3-(3,4-Dimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8e**) was obtained using above described method by adding 1-(3-(3,4-dimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-ol (**7e**) (1.17 g, 4.5 mmol) to the 2- iodoxy benzoic acid (1.90 g, 6.8 mmol) in DMSO (10 mL) solution as pale yellow solid (847 mg, yield 73%); mp 127-128 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.57 (s, 1H, ≡**H**), 3.95 (s, 3H, –OC**H**$_3$), 3.97 (s, 3H, –OC**H**$_3$), 6.84 (s, 1H, **ArH**), 6.97 (d, 1H, *J*=8.5 Hz, **ArH**), 7.30 (dd, 1H, *J*=6.4 Hz,

J=2.0 Hz, **ArH**), 7.42 (s, 1H, **ArH**) ppm; MS (ESI): *m/z* 258 $[M+H]^+$.

1-(3-(4-Methoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (8f)

1-(3-(4-Methoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8f**) was obtained using above described method by adding 1-(3-(4-methoxyphenyl)isoxazol-5-yl)prop-2-yn-1-ol (**7f**) (1.01 g, 4.4 mmol) to the 2- iodoxy benzoic acid (1.85 g, 6.6 mmol) in DMXO (10 mL) solution as pale yellow solid (751 g, yield 75%); mp 119-120 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.69 (s, 1H, ≡**H**), 3.87 (s, 3H, –O**CH**$_3$), 6.76 (s, 1H, **ArH**), 7.00 (d, 2H, *J*=8.3 Hz, **ArH**), 7.74 (d, 2H, *J*=8.3 Hz, **ArH**) ppm; MS (ESI): *m/z* 228 $[M+H]^+$.

1-(3-(4-Fluorophenyl)isoxazol-5-yl)prop-2-yn-1-one (8g)

1-(3-(4-Fluorophenyl)isoxazol-5-yl)prop-2-yn-1-one (8g) was obtained using above described method by adding 1-(3-(4-fluorophenyl)isoxazol-5-yl)prop-2-yn-1-ol (**7g**) (960 mg, 4.4 mmol) to the 2- iodoxy benzoic acid (1.86 g, 6.6 mmol) in DMSO (10 mL) solution as pale yellow solid (732mg, yield 77%); mp 116-117 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.57 (s, 1H, ≡**H**), 6.78 (s, 1H, **ArH**), 7.21 (d, 2H, *J*=7.6 Hz, **ArH**), 7.41 (d, 2H, *J*=7.6 Hz, **ArH**) ppm; MS (ESI): *m/z* 216 $[M+H]^+$.

General method for synthesis of (*Z*)-3-Arylamino-1-(5-aryl-1*H*-pyrazol-3-yl)prop-2-en-1-one (9a-g) and (*Z*)-3-Arylamino-1-(5-aryl isoxazol-3-yl)prop-2-en-1-one (9h-q)

The 1-(3-Aryl-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8a-c**) and 1-(3-Aryl isoxazol-5-yl)prop-2-yn-1-one (**8d-g**) and aryl amine were dissolved in absolute ethanol and the mixture was stirred for 4-5 h at room temperature. After the completion of the reaction (confirm by TLC), water was added to the reaction mixture and the crude product filtered. It was recrystallized from methanol to afford pure (*Z*)-3-Arylamino-1-(5-aryl-1*H*-pyrazol-3-yl)prop-2-en-1-ones (**9a-g**) and (*Z*)-3-Arylamino-1-(5-aryl isoxazol-3-yl)prop-2-en-1-ones (**9h-q**).

(*Z*)-3-(1*H*-Indol-5-ylamino)-1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-en-1-one (9a)

(*Z*)-3-(1*H*-indol-5-ylamino)-1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-en-1-one **9a** was obtained according to above described method by the addition of 1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8a**) (110 mg, 0.38 mmol) and 5-amino indole (51 mg, 0.38 mmol) as yellow solid, mp 162-164 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.91 (s, 3H, **-OCH$_3$**), 3.95 (s, 6H, **-OCH$_3$**), 6.23 (d, 1H, *J*=6.8 Hz, **COCH=**), 6.55 (brs, 1H, **ArH**), 6.91 (s, 1H **ArH**), 7.00-7.08 (m, 3H, **ArH**), 7.24-7.33 (m, 1H, **ArH**), 7.37-7.48 (m, 2H, **ArH**), 7.64 (dd, 1H, *J*=6.8 Hz, 12.1 Hz, **=CH-**NH), 8.25 (brs, 1H, **NH**), 12.20 (d, 1H, *J*=12.8 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ 181.6, 169.9, 163.7, 154.3, 146.4, 135.9, 127.9, 117.8, 116.3, 98.0, 94.6, 94.3, 61.1, 56.2 ppm; HPLC purity: *t*R 5.86 min (97.5 %); IR (KBr) (ν_{max}/cm$^-$): 3291, 2919, 1639, 1523, 1502, 1425, 1294, 1240, 1130, 1003, 816; MS (ESI): *m/z* 419 $[M+H]^+$; HRMS (ESI) calcd for $C_{23}H_{23}N_4O_4$ $[M+H]^+$ 419; $C_{23}H_{22}N_4O_4Na$ $[M+Na]^+$ 441.15333; found: 441.15341.

(*Z*)-3-(1*H*-Indol-6-ylamino)-1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-en-1-one (9b)

(*Z*)-3-(1*H*-Indol-6-ylamino)-1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-en-1-one (**9b**) was obtained according to above described method by the addition of 1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8a**) (110 mg, 0.38 mmol) and 6-amino indole (51 mg, 0.38 mmol) as yellow solid, mp 227-229 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.89 (s, 3H, **-OCH$_3$**), 3.95 (s, 6H, **-OCH$_3$**), 5.87 (d, 1H, *J*=7.5 Hz, **COCH=**), 6.53 (brs, 1H, **ArH**), 6.94 (s, 1H, **ArH**), 7.03 (dd, 1H, *J*=2.5 Hz, J=6.6 Hz, **ArH**), 7.08 (s, 2H, **ArH**), 7.24-7.28 (m, 1H, **ArH**), 7.38 (d, 1H, *J*=8.5 Hz, **ArH**), 7.42 (s, 1H, **ArH**), 7.61 (dd, 1H, *J*=7.5 HZ, *J*=12.6 Hz, **=CH-**NH), 8.24 (brs, 1H, **NH**), 12.18 (d, 1H, *J*=12.5 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ 181.6, 153.5, 151.4, 144.6, 142.5, 137.5, 127.6, 127.2, 116.0, 102.8, 95.7, 60.8, 56.1 ppm; HPLC purity: *t*R 6.99 min (98.3 %); IR (KBr) (ν_{max}/cm$^-$): 3364, 2922, 2849, 1635, 1514, 1434, 1287, 1143, 1081, 1020, 981, 885; MS (ESI): *m/z* 419 $[M+H]^+$; HRMS (ESI) calcd for $C_{23}H_{23}N_4O_4$ $[M+H]^+$ 419.17138; found: 419.17139 and $C_{23}H_{22}N_4O_4Na$ $[M+Na]^+$ 441.15333; found: 441.15349.

(*Z*)-3-(4-(Trifluoromethyl)phenylamino)-1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-en-1-one (9c)

(*Z*)-3-(4-(Trifluoromethyl)phenylamino)-1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-en-1-one (**9c**) was obtained according to above described method by the addition of 1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8a**) (110 mg, 0.38 mmol) and 4-(trifluoromethyl)aniline (63 mg, 0.38 mmol) as yellow solid, mp 238-240 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.92 (s, 3H, **-OCH$_3$**), 3.96 (s, 6H, **-OCH$_3$**), 6.02 (d, 1H, *J*=7.9 Hz, **COCH=**), 6.99 (s, 1H, **ArH**), 7.03 (s, 2H, **ArH**), 7.20 (d, 2H, *J*=8.5 Hz, **ArH**), 7.56 (dd, 1H, *J*=7.9 Hz, *J*=12.3, **=CH-**NH), 7.63 (d, 2H, *J*=8.5 Hz, **ArH**), 11.97 (d, 1H, *J*=12.3 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ 191.7, 155.3, 154.1, 151.6, 144.7, 137.7, 136.1, 122.6, 115.0, 112.8, 94.1, 93.3, 60.9, 56.0 ppm; ppm; HPLC purity: *t*R 5.86 min (97.7 %)IR (KBr) (ν_{max}/cm$^-$): 3363, 2933, 2836, 1634, 1573, 1500, 1425, 1289, 1247, 1126, 1091, 980, 886; MS (ESI): *m/z* 448 $[M+H]^+$; HRMS (ESI) calcd for $C_{22}H_{20}F_3N_3O_4Na$ $[M+Na]^+$ 470.12981; found: 470.13022.

(*Z*)-3-(4-Methoxyphenylamino)-1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-en-1-one (9d)

(Z)-3-(4-Methoxyphenylamino)-1-(3-(3,4,5-trimethoxyphenyl)-1H-pyrazol-5-yl)prop-2-en-1-one (**9d**) was obtained according to above described method by the addition of 1-(3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8a**) (110 mg, 0.38 mmol) and 4-methoxyaniline (47 mg, 0.38 mmol) as yellow solid, mp 260-262 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.81 (s, 3H, **-OCH$_3$**), 3.89 (s, 3H, **-OCH$_3$**), 3.95 (s, 6H, **-OCH$_3$**), 5.86 (d, 1H, *J*=7.6 Hz, **COCH=**), 6.68-6.97 (m, 3H, **ArH**), 7.04-7.13 (m, 4H, **ArH**), 7.48 (dd, 1H, *J*=7.6 Hz, *J*=12.8 Hz, **=CH-**NH), 12.02 (d, 1H, *J*=12.8 Hz, **NH-**CH=) ppm; ^{13}C NMR (400 MHz,$CDCl_3$): δ 181.4, 170.7, 163.7, 156.9, 153.63, 147.2, 139.9, 133.1, 122.5, 11.3, 115.1, 103.2, 97.9, 93.7, 61.0, 56.3, 55.6 ppm; HPLC purity: *t*R 13.16 min (98.0 %); HPLC purity: *t*R 7.13 min (95.2 %)IR (KBr) (ν_{max}/cm$^-$): 3390, 2921, 2850, 1628, 1504, 1436, 1285, 1256, 1179, 1078, 794; MS (ESI): *m/z* 410 $[M+H]^+$; HRMS (ESI) calcd for $C_{22}H_{23}N_3O_5Na$ $[M+Na]^+$ 432.15324; found: 432.15335

(*Z*)-1-(3-(3,4,5-Trimethoxyphenyl)-1*H*-pyrazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (9e)

(*Z*)-1-(3-(3,4,5-Trimethoxyphenyl)-1*H*-pyrazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (**9e**) was obtained according to above described method by the addition of 1-(3-

(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8a**) (110 mg, 0.38 mmol) and 3,4,5-trimethoxyaniline (70 mg, 0.38 mmol) as yellow solid, mp 234-236 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.83 (s, 3H, **-OCH$_3$**), 3.89 (s, 9H, **-OCH$_3$**), 3.95 (s, 6H, **-OCH$_3$**), 5.90 (d, 1H, *J*=7.4 Hz, **COCH=**), 6.34 (s, 2H, **ArH**), 6.94 (s, 1H, **ArH**), 7.04 (s, 2H, **ArH**), 7.48 (dd, 1H, *J*=7.4 Hz, *J*=12.6 Hz, **=CH-**NH), 11.96 (d, 1H, *J*=12.3 Hz, **NH-**CH=) ppm; ^{13}C NMR (500 MHz, $CDCl_3$): δ 188.1, 152.8, 147.4, 140.5, 136.5, 132.9, 129.5, 125.7, 123.2, 121.9, 118.6, 112.7, 110.5, 105.9, 96.4, 60.9, 55.9 ppm; HPLC purity: *t*R 5.24 min (97.1 %); IR (KBr) (ν_{max}/cm^{-}): 3270, 3118, 1632, 1585, 1432, 1284, 1253, 1218, 1081, 985, 800; MS (ESI): *m/z* 470 $[M+H]^+$; HRMS (ESI) calcd for $C_{24}H_{28}N_3O_7$ $[M+H]^+$ 470.19243, found: 470.19251.

(*Z*)-3-(1*H*-Indol-5-ylamino)-1-(3-(3,4-dimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-en-1-one (9f)

(*Z*)-3-(1*H*-Indol-5-ylamino)-1-(3-(3,4-dimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-en-1-one (**9f**) was obtained according to above described method by the addition of 1-(3-(3,4-dimethoxyphenyl)-1*H*-pyrazol-5-yl)prop-2-yn-1-one (**8b**) (110 mg, 4.3 mmol) and 5- amino indole (57 mg, 4.3 mmol) as yellow solid, mp 139-141 °C; ^{1}H NMR (500MHz, $CDCl_3$): δ 3.86 (s, 3H, **-OCH$_3$**), 3.90 (s, 3H, **-OCH$_3$**), 6.22 (d, 1H, *J*=7.4 Hz, **COCH=**), 6.40 (d, 1H, *J*=8.8 Hz, **ArH**), 6.85-7.02 (m, 1H, **ArH**), 7.07 (d, 1H, *J*=7.4 Hz, **ArH**), 7.17 (d, 1H, *J*=9.9 Hz, **ArH**), 7.20-7.31 (m, 1H, **ArH**), 7.36 (brs, 1H, **ArH**), 7.42-7.58 (m, 3H, **ArH**), 7.97 (dd, 1H, *J*=7.3 Hz, *J*=12.8 Hz, **=CH-**NH), 8.18 (s, 1H, **NH**), 11.03 (d, 1H, *J*=13.7 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 182.3, 171.0, 161.9, 161.2, 145.9, 141.4, 127.5, 115.8, 114.3, 96.7, 96.3, 95.2, 94.7, 55.4 ppm; HPLC purity: *t*R 5.39 min (97.4 %); IR (KBr) (ν_{max}/cm^{-}): 3389, 2922, 2850, 1631, 1513, 1465, 1259, 1136, 1025, 799; MS (ESI): *m/z* 389 $[M+H]^+$, HRMS (ESI) calcd for $C_{22}H_{21}N_4O_3$ $[M+H]^+$ 389.16121; found: 389.16135.

(*Z*)-1-(3-(4-Fluorophenyl)-1*H*-pyrazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (9g)

(*Z*)-1-(3-(4-Fluorophenyl)-1*H*-pyrazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (**9g**) was obtained according to above described method by using starting materials 1-(3-(4-fluorophenyl)-1H-pyrazol-5-yl)prop-2-yn-1-one (**8c**) (110 mg, 5.3 mmol) and 3,4,5-trimethoxyaniline (94 mg, 5.3 mmol) as yellow solid, mp 197-199 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.81 (s, 3H, **-OCH$_3$**), 3.83 (s, 6H, **-OCH$_3$**), 5.89 (d, 1H, *J*=7.5 Hz, **COCH=**), 5.94 (s, 2H, **ArH**), 6.34 (s, 1H, **ArH**), 7.08-7.19 (m, 2H, **ArH**), 7.49 (dd, 1H, *J*=7.5 Hz, *J*=12.3 Hz, **=CH-**NH), 7.75-7.84 (dd, 2H, *J*=3.2 Hz, *J*=6.6 Hz, **ArH**), 11.93 (d, 1H, *J*=12.4 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ 181.8, 170.0, 163.0, 154.3, 146.5, 135.8, 128.1, 116.4, 116.1, 97.9, 94.6, 94.3, 61.0, 56.3 ppm; HPLC purity: *t*R 6.77 min (96.2 %); IR (KBr) (ν_{max}/cm^{-}):3323, 2928, 2837, 1634, 1537, 1464, 1371, 1287, 1125, 992, 802; MS (ESI): *m/z* 398 $[M+H^+$; HRMS (ESI) calcd for $C_{21}H_{21}N_3O_4F$ $[M+H]^+$ 398.15139; found: 398.15146.

(*Z*)-3-(1*H*-Indol-5-ylamino)-1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-en-1-one (9h)

(*Z*)-3-(1*H*-Indol-5-ylamino)-1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-en-1-one (**9h**) was obtained according to above described method by the addition of 1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8d**) (110 mg, 0.38 mmol) and 5- amino indole (51 mg, 0.38 mmol) as yellow solid, mp 242-244 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.90 (s, 3H, **-OCH$_3$**), 3.95 (s, 6H, **-OCH$_3$**), 5.89 (d, 1H, *J*=7.6 Hz, **COCH=**), 6.55 (brs, 1H, **ArH**), 6.91-7.00 (m, 2H, **ArH**), 7.07 (s, 2H, **ArH**), 7.14-7.23 (m, 2H, **ArH**), 7.57-7.69 (m, 2H, **ArH**), 8.17-8.26 (brs, 1H, **NH**), 12.19 (d, 1H, *J*=12.1 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz, $CDCl_3$+DMSO-d_6): δ 171.3, 161.3, 153.2, 147.3, 143.9, 132.2, 128.0, 125.7, 111.9, 107.4, 102.7, 102.2, 101.0, 92.3, 60.4, 55.4 ppm; HPLC purity: *t*R 5.58 min (96.8 %) IR (KBr) (ν_{max}/cm^{-}):3240, 2920, 2849, 1638, 1589, 1554, 1489, 1281, 1235, 1128, 991, 875; MS (ESI): *m/z* 420-$[M+H]^+$; HRMS (ESI) calcd for $C_{23}H_{22}N_3O_5$ $[M+H]^+$ 420.15540; found: 420.15520 and $C_{23}H_{21}N_3O_5Na$ $[M+Na]^+$ 442.13734, found: 442.13739.

(*Z*)-3-(1*H*-Indol-6-ylamino)-1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-en-1-one (9i)

(*Z*)-3-(1*H*-Indol-6-ylamino)-1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-en-1-one (**9i**) was obtained according to above described method by the addition of 1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8d**) (110 mg, 0.38 mmol) and 6- amino indole (51 mg, 0.38 mmol) as yellow solid, mp 134-136 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.90 (s, 3H, **-OCH$_3$**), 3.93 (s, 6H, **-OCH$_3$**), 6.25 (d, 1H, *J*=7.4 Hz, **COCH=**), 6.55 (brs, 1H, **ArH**), 6.90 (s, 1H, **ArH**), 6.95-7.00 (dd, 1H, *J*=1.7 Hz, *J*=8.3 Hz, **ArH**), 7.05 (s, 2H, **ArH**), 7.16-7.24 (m, 2H, **ArH**), 7.59-7.69 (m, 2H, **ArH, =CH-**NH), 8.15-8.23 (brs, 1H, **NH**), 12.19 (d, 1H, *J*=13.7 Hz, **NH-**CH=) ppm; ^{13}C NMR (500 MHz, $CDCl_3$): δ 187.6, 153.1, 140.7, 137.6, 134.7, 133.2, 132.2, 129.6, 126.4, 122.6, 121.8, 109.6, 106.3, 96.7, 60.9, 55.9 ppm; HPLC purity: *t*R 6.61 min (97.3 %); IR (KBr) (ν_{max}/cm^{-}): 3433, 2925, 2850, 1635, 1565, 1500, 1444, 1278, 1236, 1131, 1009, 873; MS (ESI): *m/z* 420 $[M+H]^+$; HRMS (ESI) calcd for $C_{23}H_{22}N_3O_5$ $[M+H]^+$ 420.15540; found: 420.15552 and $C_{23}H_{21}N_3O_5Na$ $[M+Na]^+$ 442.13734, found: 442.13788.

(*Z*)-3-(4-Methoxyphenylamino)-1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-en-1-one (9j)

(*Z*)-3-(4-Methoxyphenylamino)-1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-en-1-one (**9j**) was obtained according to above described method by the addition of 1-(3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8d**) (110 mg, 0.38 mmol) and 4-methoxyaniline (47 mg, 0.38 mmol) as yellow solid, mp 290-292 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.82 (s, 3H, **-OCH$_3$**), 3.91 (s, 3H, **-OCH$_3$**), 3.94 (s, 6H, **-OCH$_3$**), 6.22 (d, 1H, *J*=7.5 Hz, **COCH=**), 6.88 (s, 1H, **ArH**), 6.92 (d, 2H, *J*=8.8 Hz, **ArH**), 7.04 (s, 2H, **ArH**), 7.09 (d, 2H, *J*=8.8 Hz, **ArH**), 7.48 (dd, 1H, *J*=7.6 Hz, *J*=12.8 Hz, **=CH-**NH), 12.01 (d, 1H, *J*=12.7 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ 182.2, 171.0, 163.5, 161.3, 154.2, 146.3, 135.9, 127.5, 119.9, 114.4, 96.8, 94.4, 61.0, 56.2, 55.4 ppm; HPLC purity: *t*R 7.03 min (95.7 %); IR (KBr) (ν_{max}/cm^{-}): 3428, 2938, 1642, 1567, 1500, 1462, 1270, 1123, 1078, 1006, 872; MS (ESI): *m/z* 411 $[M+H]^+$; HRMS (ESI) calcd for $C_{22}H_{23}N_2O_6$ $[M+H]^+$ 411.15506; found: 411.15525 and $C_{22}H_{22}N_2O_6Na$ $[M+Na]^+$ 433.13701, found: 433.13742.

(*Z*)-1-(3-(3,4,5-Trimethoxyphenyl)isoxazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (9k)

(*Z*)-1-(3-(3,4,5-Trimethoxyphenyl)isoxazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (**9k**) was obtained according to above described method by the addition of 1-(3-

(3,4,5-trimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8d**) (110 mg, 0.38 mmol) and 3,4,5-trimethoxyaniline (70 mg, 0.38 mmol) as yellow solid, mp 291-293 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.90 (s, 12H, **-OCH$_3$**), 3.91 (s, 6H, **-OCH$_3$**), 6.27 (d, 1H, *J*=7.6 Hz, **COCH=**), 6.35 (s, 2H, **ArH**), 6.88 (s, 1H, **ArH**), 7.04 (s, 2H, **ArH**), 7.51 (dd, 1H, *J*=7.5 Hz, *J*=12.5 Hz, **=CH-**NH), 11.95 (d, 1H, *J*=12.1 Hz, **NH-**CH=) ppm; ^{13}C NMR (500 MHz, $CDCl_3$): δ 188.9, 152.9, 143.8, 137.6, 133.5, 132.4, 130.4, 129.1, 120.3, 107.9, 106.6, 105.8, 94.0, 60.8, 55.8 ppm; HPLC purity: *t*R 6.60 min (96.2 %); IR (KBr) (ν_{max}/cm^{-}): 3318, 2924, 2839, 1645, 1569, 1285, 1173, 1156, 1077, 864; MS (ESI): *m/z* 471 $[M+H]^+$; HRMS (ESI) calcd for $C_{24}H_{27}N_2O_8$ $[M+H]^+$ 471.17619; found: 471.17667 and $C_{24}H_{26}N_2O_8Na[M+Na]^+$ 493.15814, found: 493.15856.

(*Z*)-1-(3-(3,4-Dimethoxyphenyl)isoxazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (9l)

(*Z*)-1-(3-(3,4-Dimethoxyphenyl)isoxazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (**9l**) was obtained according to above described method by the addition of 1-(3-(3,4-dimethoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8e**) (110 mg, 0.43 mmol) and 3,4,5-trimethoxyaniline (78 mg, 0.43 mmol) as yellow solid, mp 152-154 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.83 (s, 3H, **-OCH$_3$**), 3.89 (s, 6H, **-OCH$_3$**), 3.95 (s, 3H, **-OCH$_3$**), 3.98 (s, 3H, **-OCH$_3$**), 6.27 (d, 1H, *J*=7.7 Hz, **COCH=**), 6.35 (s, 2H, **ArH**), 6.84 (s, 1H, **ArH**), 6.97 (d, 1H, *J*=8.5 Hz, **ArH**), 7.33 (s, 1H, **ArH**), 7.42 (dd, 1H, *J*=1.9 Hz, *J*=6.4 Hz, **ArH**), 7.46-7.55 (dd, 1H, *J*=7.6 Hz, *J*=12.6 Hz, **=CH-**NH), 11.96 (d, 1H, *J*= 12.6 Hz, **NH-**CH=) ppm; ^{13}C NMR (500 MHz, $CDCl_3$): δ 186.9, 154.9, 152.9, 147.6, 143.8, 139.2, 133.1, 130.0, 129.4, 129.1, 124.9, 113.4, 110.7, 105.8, 93.3, 60.9, 56.7, 55.9 ppm; HPLC purity: *t*R 5.8 min (98.0 %); IR (KBr) (ν_{max}/cm^{-}): 3376, 2930, 1631, 1589, 1468, 1289, 1236, 1125, 991, 889; MS (ESI): *m/z* 441 $[M+H]^+$; HRMS (ESI) calcd for $C_{23}H_{25}N_2O_7$ $[M+H]^+$ 441.16563; found: 441.16368 and $C_{23}H_{24}N_2O_7Na$ $[M+Na]^+$ 463.14757, found: 463.14629.

(*Z*)-3-(1*H*-Indol-6-ylamino)-1-(3-(4-methoxyphenyl)isoxazol-5-yl)prop-2-en-1-one (9m)

(*Z*)-3-(1*H*-Indol-6-ylamino)-1-(3-(4-methoxyphenyl)isoxazol-5-yl)prop-2-en-1-one (**9m**) was obtained according to above described method by the addition of 1-(3-(4-methoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8f**) (110 mg, 0.48 mmol) and 6-aminoindole (64 mg, 0.48 mmol) as yellow solid, mp 136-138 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.86 (s, 3H, -**OCH$_3$**), 6.13 (d, 1H, *J*=7.4 Hz, **COCH=**), 6.40 (d, 1H, *J*=7.9 Hz, **ArH**), 6.85-7.11 (m, 4H, **ArH**), 7.18-7.26 (m, 1H, **ArH**), 7.33 (s, 1H, **ArH**), 7.52 (dd, 1H, *J*=6.2 Hz, *J*=8.4 Hz, **ArH**), 7.78-7.96 (m, 3H, **ArH, =CH-**NH), 8.08 (s, 1H, **-NH**), 10.97 (d, 1H, *J*=12.6 Hz, **NH-**CH=) ppm; ^{13}C NMR (500 MHz, $CDCl_3$ + DMSO-d_6): δ 180.2, 170.1, 163.2, 160.6, 147.5, 133.4, 131.8, 127.9, 126.9, 125.9, 119.3, 113.9, 111.9, 107.5, 101.0, 96.3, 92.6, 54.8 ppm; HPLC purity: *t*R 9.34 min (96.3 %); MS (ESI): *m/z* 360 $[M+H]^+$; HRMS (ESI) calcd for $C_{21}H_{18}N_3O_3$ $[M+H]^+$ 360.13427; found: 360.13449 and $C_{21}H_{17}N_3O_3Na$ $[M+Na]^+$ 382.11621, found: 382.11648.

(*Z*)-1-(3-(4-Methoxyphenyl)isoxazol-5-yl)-3-(4-(trifluoromethyl)phenylamino)prop-2-en-1-one (9n)

(*Z*)-1-(3-(4-Methoxyphenyl)isoxazol-5-yl)-3-(4-(trifluoromethyl)phenylamino)prop-2-en-1-one (**9n**) was obtained according to above described method by the addition of 1-(3-(4-methoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8f**) (110 mg, 0.48 mmol) and 4-(trifluoromethyl)aniline (78 mg, 0.48 mmol) as yellow solid, mp 129-130 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.85 (s, 3H, **-OCH$_3$**), 6.21 (d, 1H, *J*=7.7 Hz, **COCH=**), 6.51 (d, 1H, *J*=8.4 Hz, **ArH**), 7.03-7.16 (m, 2H, **ArH**), 7.34 (d, 1H, *J*=8.4 Hz, **ArH**), 7.54 (d, 1H, *J*=8.9 Hz, **ArH**), 7.58-7.72 (m, 2H, **ArH**), 7.86 (d, 2H, *J*=8.6 Hz, **ArH**), 8.37 (t, 1H, *J*=12.8 Hz, **=CH-**NH), 10.63 (d, 1H, *J*=12.8 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 178.2, 161.6, 144.4, 142.7, 142.1, 132.3, 125.6, 124.9, 117.3, 114.8, 113.9, 112.7, 95.7, 93.3, 53.4 ppm; HPLC purity: *t*R 6.40 min (96.7 %); MS (ESI): *m/z* 389 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{16}F_3N_2O_3$ $[M+H]^+$ 389.11075; found: 389.11105 and $C_{20}H_{15}F_3N_2O_3Na$ $[M+Na]^+$ 411.09270, found: 411.09257.

(*Z*)-1-(3-(4-Methoxyphenyl)isoxazol-5-yl)-3-(4-methoxyphenylamino)prop-2-en-1-one (9o)

(*Z*)-1-(3-(4-Methoxyphenyl)isoxazol-5-yl)-3-(4-methoxyphenylamino)prop-2-en-1-one (**9o**) was obtained according to above described method by the addition of 1-(3-(4-methoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8f**) (110 mg, 0.48 mmol) and 4-methoxyaniline (60 mg, 0.48 mmol) as yellow solid, mp 117-119 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.82 (s, 3H, -**OCH$_3$**), 3.87 (s, 3H, **-OCH$_3$**), 6.22 (d, 1H, *J*=7.6 Hz, **COCH=**), 6.82 (s, 1H, **ArH**), 6.92 (d, 2H, *J*=9.1 Hz, **ArH**), 7.00 (d, 2H, *J*=8.3 Hz, **ArH**), 7.08 (d, 2H, *J*=8.3 Hz, **ArH**), 7.49 (dd, 1H, *J*=7.6 Hz, *J*=12.8 Hz, **=CH-**NH), 7.76 (d, 2H, *J*=9.1 Hz, **ArH**), 12.01 (d, 1H, *J*=12.8 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz, DMSO-d_6): δ 179.9, 170.1, 163.8, 160.8, 148.2, 136.2, 133.6, 127.6, 125.0, 121.1, 119.1, 114.1, 109.8, 109.0, 99.3, 97.6, 93.2, 55.2 ppm; HPLC purity: *t*R 7.30 min (97.2 %)IR (KBr) (ν_{max}/cm^{-}): 3412, 2933, 1638, 1498, 1434, 1232, 1125, 1075, 1007, 871, 805; MS (ESI): *m/z* 351 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{19}N_2O_4$ $[M+H]^+$ 351.13393; found: 351.13399 and $C_{20}H_{18}N_2O_4Na$ $[M+Na]^+$ 373.11588, found: 373.11604.

(*Z*)-1-(3-(4-Methoxyphenyl)isoxazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (9p)

(*Z*)-1-(3-(4-Methoxyphenyl)isoxazol-5-yl)-3-(3,4,5-trimethoxyphenylamino)prop-2-en-1-one (**9p**) was obtained according to above described method by the addition of 1-(3-(4-methoxyphenyl)isoxazol-5-yl)prop-2-yn-1-one (**8f**) (110 mg, 0.48 mmol) and 3,4,5-trimethoxyaniline (90 mg, 0.48 mmol) as yellow solid, mp 149-151 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.83 (s, 3H, **-OCH$_3$**), 3.88 (s, 3H, **-OCH$_3$**), 3.89 (s, 6H, **-OCH$_3$**), 6.27 (d, 1H, *J*=7.7 Hz, **COCH=**), 6.34 (s, 2H, **ArH**), 6.82 (s, 1H, **ArH**), 7.00 (d, 2H, *J*=8.9 Hz, **ArH**), 7.50 (dd, 1H, *J*=7.7 Hz, *J*=12.6 Hz, **=CH-**NH), 7.77 (d, 2H, *J*=8.9 Hz, **ArH**), 11.97 (d, 1H, *J*=12.1 Hz, **NH-**CH=) ppm; ^{13}C NMR (300 MHz,$CDCl_3$): δ 179.0, 159.7, 152.65, 150.9, 144.1, 135.9, 133.6, 128.1, 121.4, 120.2, 114.7, 110.9, 60.9, 56.2, 55.5 ppm; HPLC purity: *t*R 9.34 min (95.7 %); IR (KBr) (ν_{max}/cm^{-}): 3244, 2921, 2847, 1649, 1615, 1563, 1437, 1285, 1111, 1066, 838, 771; MS (ESI): *m/z* 411 $[M+H]^+$; HRMS (ESI) calcd for $C_{22}H_{23}N_2O_6$ $[M+H]^+$ 411.15506; found: 411.15496 and $C_{22}H_{22}N_2O_6Na$ $[M+Na]^+$ 433.13701, found: 433.13668.

(*Z*)-3-(1*H*-Indol-5-ylamino)-1-(3-(4-fluorophenyl)isoxazol-5-yl)prop-2-en-1-one (9q)

(*Z*)-3-(1*H*-Indol-5-ylamino)-1-(3-(4-fluorophenyl)isoxazol-5-yl)prop-2-en-1-one (**9q**) was obtained according to above

described method by the addition of 1-(3-(4-fluorophenyl)isoxazol-5-yl)prop-2-yn-1-one (**8g**) (110 mg, 0.5 mmol) and 5-aminoindole (68 mg, 0.5 mmol) as yellow solid, mp 130-131 ^{o}C; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 6.18 (dd, 1H, *J*=7.3 Hz, *J*=1.7 Hz **COCH=**), 6.49 (s, 1H, **ArH**), 6.93 (s, 1H, **ArH**), 6.99 (td, 1H, *J*=8.5 Hz, **ArH**), 7.16-7.23 (m, 2H, **ArH**), 7.39-7.45 (m, 3H, **ArH**), 7.67 (dd, 1H, *J*=7.3 Hz, *J*=12.8 Hz, **=CH-**NH), 7.80-7.86 (dd, 2H, *J*=2.3 Hz, *J*=7.6 Hz, **ArH**), 10.14 (brs, 1H, **NH**), 12.22 (d, 1H, *J*=12.7 Hz, **NH**-CH=); ^{13}C NMR (500 MHz, DMSO-d_6): δ 170.0, 148.3, 147.5, 147.2, 141.9, 138.8,128.5, 126.3, 123.2, 121.7, 119.4, 109.4, 108.8, 105.7, 101.4 ppm; HPLC purity: *t*R 9.04 min (98.8 %); IR (KBr) (ν_{max}/cm^{-}): 3247, 2934, 2833, 1632, 1588, 1487, 1368, 1290, 1247, 1126, 991, 829, 790; MS (ESI): *m/z* 348 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{15}FN_3O_2$ $[M+H]^+$ 348.11428; found: 348.11454.

Biology

Cell Cultures, Maintenance and Antiproliferative Evaluation

All cell lines used in this study were purchased from the American Type Culture Collection (ATCC, United States). A-549, HeLa, MCF-7 and HCT-116 were grown in Dulbecco's modified Eagle's medium (containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C). A-549 cells were cultured in Eagle's minimal essential medium (MEM) containing non-essential amino acids, 1 mM sodium pyruvate, 10 mg/mL bovine insulin, and 10% FBS. Cells were trypsinized when sub-confluent from T25 flasks/60 mm dishes and seeded in 96-wel plates. The synthesized test compounds were evaluated for their *in vitro* antiproliferative in four different human cancer cell lines. A protocol of 48 h continuous drug exposure was used, and a MTT cell proliferation assay was used to estimate cell viability or growth. The cell lines were grown in their respective media containing 10% fetal bovine serum and were seeded into 96-well microtiter plates in 200 µL aliquots at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. Aliquots of 2 µL of the test compounds were added to the wells already containing 198 µL of cells, resulting in the required final drug concentrations. For each compound, four concentrations (0.1, 1, 2, 10 and 50 µM) were evaluated, and each was done in triplicate wells. Plates were incubated further for 48 h, and the assay was terminated by the addition of 10 µL of 5% MTT and incubated for 60 min at 37 °C. Later, the plates were air-dried. Bound stain was subsequently eluted with 100 µL of DMSO, and the absorbance was read on multimode plate reader (Tecan M200) at a wavelength of 560 nm. Percent growth was calculated on a plate by plate basis for test wells relative to control wells. The above determinations were repeated thrice. The growth inhibitory effects of the compounds were analyzed by generating dose response curves as a plot of the percentage surviving cells versus compound concentration. The sensitivity of the cancer cells to the test compound was expressed in terms of IC_{50}, a value defined as the concentration of compound that produced 50% reduction as compared to the control absorbance. IC_{50} values are indicated as means ± SD of three independent experiments.

Cell Cycle Analysis

A-549 cells in 60 mm dishes were incubated for 24 h in the presence or absence of test compounds **9a**, **9b** and **9f** at 3 µM concentrations. Cells were harvested with trypsin-EDTA, fixed with ice-cold 70% ethanol at 4 ^{o}C for 30 min, ethanol was removed by centrifugation and cells were stained with 1 mL of DNA staining solution [0.2 mg of propidium Iodide (PI), and 2 mg RNase A] for 30 min as described earlier . The DNA contents of 20,000 events were measured by flow cytometer (BD FACS Canto II). Histograms were analyzed using FCS express 4 plus.

Tubulin Polymerization Assay

An *in vitro* assay for monitoring the time-dependent polymerization of tubulin to microtubules was performed employing a fluorescence-based tubulin polymerization assay kit (BK011, Cytoskeleton, Inc.) according to the manufacturer's protocol. The reaction mixture in a final volume of 10 µl in PEM buffer (80 mM PIPES, 0.5 mM EGTA, 2 mM $MgCl_2$, pH 6.9) in 384 well plates contained 2 mg/mL bovine brain tubulin, 10 µM fluorescent reporter, 1 mM GTP in the presence or absence of test compounds at 37 ^{o}C. Tubulin polymerization was followed by monitoring the fluorescence enhancement 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 for 1 h at 1-min intervals in a multimode plate reader (Tecan M200). To determine the IC_{50} values of the compounds against tubulin polymerization, the compounds were pre-incubated with tubulin at varying concentrations (0.1, 1, 2, 5 and 10 µM). Assays were performed under similar conditions as employed for polymerization assays as described above.

Immunohistochemistry of Tubulin and Analysis of Nuclear Morphology

A549 cells were seeded on glass cover slip, incubated for 24 h in the presence or absence of test compounds **9a**, **9b** and **9f** at concentrations of 3 µM. Cells grown on cover slips were fixed in 3.5% formaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 10 minutes at room temperature. Cells were permeablized for 6 minutes in PBS containing 0.5% Triton X-100 (Sigma) and 0.05% Tween-20 (Sigma). The permeablized cells were blocked with 2% BSA (Sigma) in PBS for 1h. Later, the cells were incubated with primary antibody for tubulin from (sigma) at (1:200) diluted in blocking solution for 4h at room temperature. Subsequently the antibodies were removed and the cells were washed thrice with PBS. Cells were then incubated with FITC labeled anti-mouse secondary antibody (1:500) for 1h at room temperature. Cells were washed thrice with PBS and mounted in medium containing DAPI. Images were captured using the Olympus confocal microscope and analyzed with Provision software.

Western blot analysis of soluble versus polymerized tubulin

A549 cells were seeded in 12-well plates at 1×105 cells per well in complete growth medium. Following treatment of cells with respective compounds **9a, 9b** and nocodazole for duration of 24 h, cells were washed with PBS and subsequently soluble and insoluble tubulin fractions were collected. To collect the soluble tubulin fractions, cells were permeablized with 200 µL of pre-warmed lysis buffer [80 mM Pipes-KOH (pH 6.8), 1 mM MgCl2, 1 mM EGTA, 0.2% Triton X-100, 10% glycerol, 0.1% protease inhibitor cocktail (Sigma-Aldrich)] and incubated for 3 min at 30 oC. Lysis buffer was gently removed, and mixed with 100 µL of 3×Laemmli's sample buffer (180 mM Tris-Cl pH 6.8, 6% SDS, 15% glycerol, 7.5% β-mercaptoethanol and 0.01% bromophenol

blue). Samples were immediately heated to 95 oC for 3 min. To collect the insoluble tubulin fraction, 300 µL of 1×Laemmli's sample buffer was added to the remaining cells in each well, and the samples were heated to 95oC for 3 min. Equal volumes of samples were run on an SDS-10 % polyacrylamide gel and were transferred to a nitrocellulose membrane employing semidry transfer at 50 mA for 1h. Blots were probed with mouse anti-human α-tubulin diluted 1:2,000 ml (Sigma) and stained with rabbit anti-mouse secondary antibody coupled with horseradish peroxidase, diluted 1:5000 ml (Sigma). Bands were visualized using an enhanced Chemiluminescence protocol (Pierce) and radiographic film (Kodak).

Dot-blot assay

Cells were trypsinized when sub-confluent from T25 flasks/60 mm dishes and seeded in 6-well plates. The pyrazole-oxadiazole conjugates were evaluated for their activity against Cyclin B1. A549 cells were treated with 2 µM concentrations of (**9a, 9b** and **9f**) for 24 h. Susbequently, cells were harvested and proteins were quantified using Amido Black followed by densitomerty analysis. Equal amount of protein were blotted on nitrocellulose membrane using Bio-Dot SF microfiltration apparatus (Bio-Rad). Briefly, nitrocellulose membrane and 3 filters papers (Whatmann 3) were soaked in IX TBS solution for 10 min. Later, the filter papers, membrane were arranged in the apparatus and connected to vacuum pump (Millipore). The membranes were rehydrated using 100 µl of 1XTBS by vacuum filtration. Subsequently, 50 µl volumes of equal protien samples were blotted on the membrane and washed with 200 µl of 1X TBS through application of vacuum. The blot was blocked with 5% blotto for 1 h at room temperature. Immunoblot analysis was performed as described previously using UVP, biospectrum 810 imaging system.

Competitive tubulin binding assay

The various concentrations (0 µM, 5 µM, 10µM and 15 µM) of conjugates **9a, 9b** and **9f** were coincubated with 3 µM colchicines in 30 mM Tris buffer containing 3 µM tubulin at 37oC for 60 min. The standard nocodazole (**III**) was employed as a positive control whereas peclitaxel was used as the negative control. After incubation, the fluorescence of tubulin-colchicine complex was measured by using Tecan multimode reader at excitation wavelength of 380 nm and emission wavelength of 435 nm; whereas 30 mM Tris buffer was used as a blank. The raw fluorescence values were normalized first by subtracting the fluorescence of the buffer and then setting the fluorescence of 3 µM tubulin with 3 µM colchicine to 100%. Values represented were ± SD of at least three independent experiments.

Assessment of $\Delta\psi_{mt}$ (mitochondrial depolarization)

The mitochondrial membrane potential was measured with the lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1, Molecular Probes). A549 cells were treated with compounds **9a**, **9b** and **9f** at 2 µM concentrations. After treatment, cells were collected by centrifugation and resuspended in HBSS (Hank's balanced salt solution) containing 1 µM JC-1. The shift of the membrane potential was measured by flow cytometry using JC-1 (Molecular Probes).

Caspase-3 activity assay

AFC conjugated Ac-DEVD substrate was used to determine the caspase-3 activity of the potent compounds **9a, 9b** and nocodazole at 2 µM. A549 cells were seeded in six well plates with the confluence of per (2.5×10^{-6}) well and are treated with the compounds at 2 µM concentration. The cells were washed with PBS after incubation for 48 h and then scraped in to the PBS and centrifuged at 2000 rpm for 10 min at 4 °C. Pellet was resuspended in 80 ml of lysis buffer. Then the pellet was passed through insulin syringe followed by incubation of suspension on ice for 20–30 min centrifuged the lysate at 13,200 rpm for 20 min at 4 °C and transferred the supernatant to fresh tubes. In a 96 well black polystyrene plate, 50 ml cell lysate, 50 ml of 2X assay buffer and 2 ml of caspase-3substrate 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 consequently.

Assessment of apoptosis by PI

The A549 cells exposed to compound **9a**, **9b** and **9f** at 2 µM concentration after 24 h and cells were washed twice with PBS and fixed with 4% formaldehyde for10 min. The fixed cells were then washed again with PBS and stained with and 10 µg/mL of PI for 10 min. After washed once with PBS the cells were examined under a fluorescence microscope.

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Notes and references

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