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ARTICLE TYPE

Synthesis of 2-Anilinopyridyl-Triazole Conjugates as Antimitotic Agents

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A series of 2-anilinopyridyl-triazole conjugates (**6a-t**) were prepared and evaluated for their cytotoxic activity against a panel of three human cancer cell lines. Among them compounds **6q**, **6r** and **6s** showed significant cytotoxic activity with IC_{50} values ranging from 0.1 to 4.1 μM. Structure–activity relationships were elucidated with various substitutions on these conjugates. Flow cytometric analysis revealed that these compounds arrest the cell cycle at G2/M phase and induced cell death by apoptosis. The tubulin polymerization assay and immunofluorescence analysis showed that these compounds (**6q**, **6r** and **6s**)effectively inhibited the microtubule assembly in human prostate cancer cells (DU-145). The docking studies showed that **6s** interacts and binds efficiently with the tubulin protein at colchicine binding site. This was further confirmed by colchicine competitive binding assay. Moreover, compounds **6q**, **6r** and **6s** possessed anti-tubulin activity both *in vitro* and within cells as demonstrated by the ratio of soluble versus polymerized tubulin. Further the apoptotic effects of compounds were confirmed by Hoechst staining, caspase 3 activation, annexin-V FITC, mitochondrial membrane potential and DNA fragmentation analysis. Interestingly, these compounds did not effect the normal human embryonickidneycells,HEK-293.

Introduction

Tubulin-containing structures, such as microtubules are important for the formation of the mitotic spindle during the process of mitosis. They play a critical role in cell growth, division and cytoskeletal organization of normal as well as tumor cells, apart from being implicated in motility, shape and intracellular transport.[1] In the mitotic phase of the cell cycle, microtubules are in dynamic equilibrium with tubulin dimers by assembling the tubulin into microtubules or conversely disassembling microtubules to tubulin.[2] Disruption of the dynamic equilibrium can induce cell cycle arrest and ultimately lead to apoptosis.[3] Therefore, the compounds that could inhibit tubulin polymerization or interrupt microtubule depolymerization would be useful in the treatment of cancer. Tubulin is a major molecular target for antitumor drugs that disrupt cellular mitosis.[4] Three distinct classes of antimitotic agents are identified by their mechanism of action and their different binding sites on tubulin. The vinca alkaloids (vincristine, vinblastine, vindesine, and vinorelbine) and colchicine are known as microtubule-destabilizing agents or microtubule polymerization inhibitors but with a different binding site and depolymerization mechanism. Vinca alkaloids are used in the treatment of leukemias, lymphomas, small cell lung cancer and other cancers.[5,6] The taxanes are microtubule-stabilizing agents or tubulin polymerizing agents, which are used in the treatment of lung, breast, ovarian, head and neck, and bladder carcinomas among others. More recently, it has been established that some tubulin binding agents selectively target the vascular system of tumors. These compounds induce morphological changes in the endothelial cells of the blood vessels in the tumor, resulting in their occlusion and interruption of blood flow.[7,8]Therefore, there is a growing interest in identifying and developing newer molecules that could inhibit tubulin polymerization.

Combretastatin A-4 (CA-4, **1**; Fig. **1**), isolated from the bark of the South African tree *Combretum caffrum*,[9] is one of the well-known natural tubulin-binding molecules that affects microtubule dynamics. CA-4 strongly inhibits the polymerization of tubulin by binding to the colchicine site.[10]It inhibits cell growth at low nanomolar concentrations and exhibits inhibitory effects on multidrug resistant cancer cell lines. Nocodazole (**2**) is another well known inhibitor of tubulin polymerization which inhibits cell proliferation and largely used for pharmacological tool and positive control.[11] Small molecules with sulfonamide functionalities, such as *N*-pyridyl sulfonamide, E7010 (**3**) have been reported as potent antimitotic agents and potent anti-neoplastic activity against drug-resistant tumor cells (*in vitro* and *in vivo*). [12,13] E7010 binds reversibly to the colchicine-binding site of tubulin and arrests cells in the mitotic phase.[14–17] This compound exhibits good *in vivo* antitumor activity against several rodent and human tumors, and is presently in phase II clinical trials.[18]*N*-Phenyl nicotinamides (**4**) are a new class of apoptosis inducers that are known to arrest cells in G2/M phase and structure–activity relationship (SAR) studies indicate that the 3-pyridyl group is very important for their activity.[19] Similarly, in recent years, 1,2,3-triazoles have been reported to display a broad range of biological properties such as antifungal, anticancer, antibacterial, anti-HIV and antitubercular activities.[20-23]

As part of our ongoing efforts to discover newer anticancer agents, we previously reported 2-anilino-linked sulfonylhydrazides,[24] 2-aminobenzothiazole, triazolobenzothiadiazine[25] and 1,3,4-oxadiazole[26] conjugates as potent anticancer agents. Based on the E7010 scaffold, recently we have synthesized some new *N*-((1-benzyl-1*H*-1,2,3-triazol-4-

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yl)methyl)nicotinamides (**5**) as microtubule polymerization inhibitors that are active against certain cancer cell lines.[27]

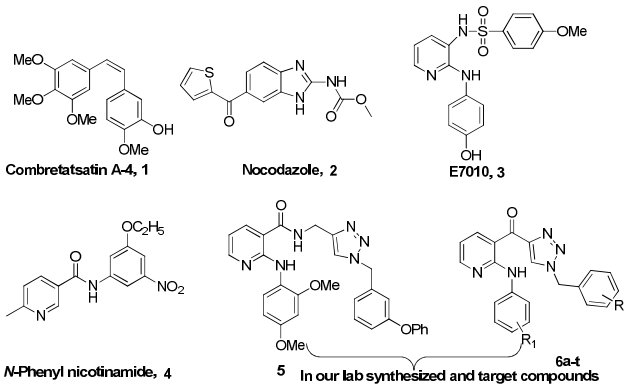


Figure 1: Tubulin polymerization inhibitors

In continuation to our efforts for identifying potent inhibitors of tubulin polymerization and selective anticancer agents, we have developed new molecules wherein 2-anilinopyridyl structural motif of E7010 have been directly attached to a triazole moiety resulting in (1-benzyl-1*H*-1,2,3-triazol-4-yl)(2-(arylamino)pyridin-3-yl)methanones (**6a–t**) (Figure **1**).These conjugates have been designed keeping in view the potent cytotoxicity of our previously reported E7010 based compounds (**5**) and are devoid of the methylene amino group present in **5** (Figure **2**). Therefore, to understand the effect of this structural modification conjugates 6a-t were evaluated for their cytotoxicity and for their effect on tubulin polymerization and cell cycle to understand their possible mechanism of action which is discussed herewith.

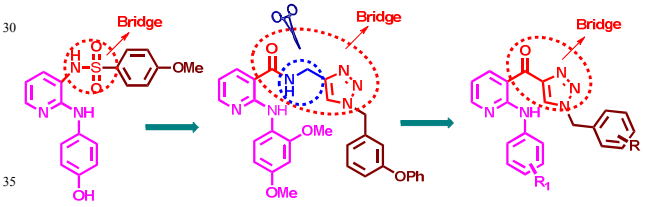
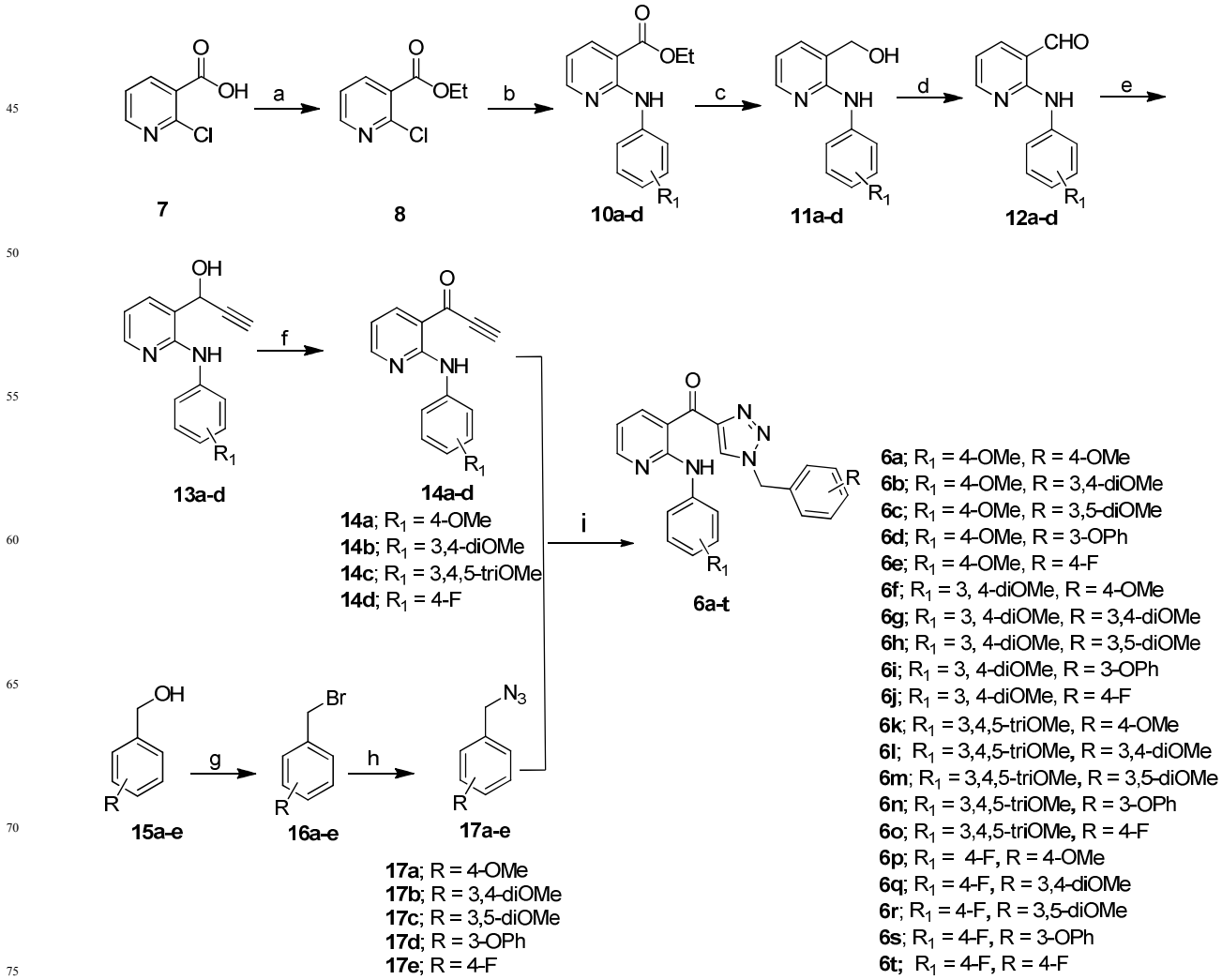


Figure 2: Strategy of the present work.

Results and discussions

Chemistry



Scheme 1: Synthesis of 2-anilinopyridyl-triazole conjugates; Reagents and Conditions: a) Cat. H_2SO_4, EtOH, reflux, 6 h, 79%; b) anilines (**9a-d**), ethylene glycol, 120 °C, 6 h, 73-81%; c) LAH, THF, 0 °C, 3 h, 71-78%; d) IBX, DMSO, rt, 30 min, 73-82%; e) ethynyl magnesium bromide, THF, 0 °C, 3 h, 69-76%; f) IBX, DMSO, rt, 30 min, 73-80%; g) PBr_3, DCM, 0 °C, 30 min; h) NaN_3, DMSO, rt, 12 h ; i) sodium ascorbate (10 mol %), $CuSO_4.5H_2O$ (5 mol %), H_2O/*t*-BuOH,

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(2:1), rt, 3 h, 72-84%.

1,4-Disubstituted triazoles can be prepared in high regioselectively by using Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction.[28,29] Following this strategy, the synthesis of compounds (6a–t) was carried out as shown in Scheme 1. Esterification of 2-chloronicotinic acid (7) by using conc. H_2SO_4 in ethanol at reflux for 6 h affords 2-chloronicotinic acid ethyl ester (8), this was treated with substituted anilines (9a–d) in ethylene glycol under refluxing conditions for 6 h to provide the coupled product of 2-anilinonicotinic acid esters (10a–d), these esters were reduced by LAH in dry THF to give the corresponding alcohols and which oxidized with 2-iodoxy benzoic acid in DMSO to provide 2-anilino nicotinaldehydes (12a–d). These aldehydes (12a-d) were treated with ethynyl magnesium bromide (0.5 M) in THF (0 °C to room temperature) for 3-4 h to produce 2-anilinonicotonyl-2-propyn-1-ol (13a-d). Oxidation of 13a-d with 2-iodoxybenzoic acid (IBX) dimethyl sulfoxide (DMSO) to give 2-anilinonicotonyl-2-propyn-1-one (14a-d). The azides 7a–e that are required as other precursors were obtained by the reaction of benzyl bromides with NaN_3 in DMSO.[30] The synthesis of 1,2,3-triazoles (6a-t) was carried out by exposing alkynes to benzyl azides in the presence of catalytic Cu(I) and sodium ascorbate in H_2O/*t*-BuOH mixture.

Biology

3.1 Antiproliferative activity

Preliminary screening of the synthesized conjugates (**6a-t**) was performed to evaluate cytotoxic activity against a panel of selected human cancer cell lines, namely HT-29 (colon cancer), DU-145 (prostate cancer) and A549 (lung cancer) and normal human embryonic kidney (HEK-293) cell lines, by employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[31] The results of this cytotoxicity data expressed as IC_{50} values in comparison with positive controls such as E7010 and nocodazole as well as compound **5** is summarized in Table **1**. Interestingly, all of the conjugates were selectively cytotoxic to cancer cells with a weak effect on normal cells. Moreover, all the compounds (**6a-t**) showed more pronounced activity against DU-145 cells compared to other cell lines tested with exception of **6k**, **6l**, **6m**, **6n** and **6p**. Some of the compounds such as **6n, 6o, 6q, 6r** and **6s** showed significant cytotoxicity, with IC_{50} values in micro to submicromolar range. Among them, **6s** exhibited enhanced cytotoxic activity than compound **5**, E7010 and nocodazole against all the tested cancer cell lines with IC_{50} values ranging from 0.1-1.1μM. Based on the cytotoxicity data, the structure activity relationship (SAR) for these 2-anilinopyridyl-triazole conjugates (**6a-t**) was elucidated. The introduction of substituents on the pyridyl ring as well as triazole ring were exemplified by preparing analogs possessing both electron donating as well as electron withdrawing substituents. These substituents include methoxy, dimethoxy, trimethoxy, phenyloxy phenyl and fluoro at various positions on the aromatic ring. It was observed that compounds with electron withdrawing substituents like the fluoro on the aryl ring of 2-arylpyridine moiety (**6q**, **6r** and **6s**) exhibited enhanced cytotoxic activity compared to their counterparts with electron releasing substituents. However, compounds possessing trimethoxy substituent on the aryl ring of 2-arylpyridine moiety (**6n** and **6o**) showed activity similar to the compounds **6q** and **6r**. In contrast, introduction of fluoro substituent on the benzyl ring of the triazole (**6e**, **6j** and **6t**) deleterious for the cytotoxic activity while electron donating substituents like methoxy and dimethoxy (**6q** and **6r)** showed moderate cytotoxic activities. Interestingly, **6o** possessing fluoro substituent on the benzyl ring of triazole is an exception to the above mentioned observation that exhibited significant cytotoxicity with an average IC_{50} value 2.96 μM. However, compounds possessing 3-phenoxy benzyl motif such as **6s** displayed excellent cytotoxic activity. (Mean IC_{50} = 0.46 μM), which is much better than compound **5**E7010 and nocodazole against all the tested cell lines particularly against DU-145 cells.Therefore, it can be concluded that electron withdrawing substituents on the aryl ring of 2-arylpyridine moiety and electron donating substituents on triazole are beneficial for the cytotoxic activity. Based on results obtained from the MTT assay, the most cytotoxic conjugates (**6q**, **6r** and **6s**) were used for further mechanistic studies, such as tubulin polymerization inhibition, immunohistochemistry and apoptosis induction. In addition, these conjugates were also investigated for competitive binding assay, as well as molecular docking studies.

Table**1**: Cytotoxicity of 2-anilinopyridyl-triazole conjugates (**6a-t**) against a panel of human cancer and normal cell lines.

	IC_{50} (μM)[a]			
Compound	**HT-29[b]**	**DU-145[c]**	**A549[d]**	**HEK-293[e]**
6a	8.8±0.2	6.3±0.26	7.5±2.6	41.2±1.5
6b	12.5±0.32	6.1±0.36	8.2±3.1	52.1±2.6
6c	12.8±0.25	9.1±0.54	9.5±0.2	60.1±0.3
6d	9.3±1.2	5.8±1.23	6.4±1.9	55.8±2.8
6e	13.8±1.6	9.4±1.51	11.6±1.4	74.6±3.2
6f	14.1±3.1	9.8±0.05	10.4±1.5	45.2±4.8
6g	12.5±1.8	4.9±1.6	6.5±0.7	65.8±5.1
6h	15.8±0.6	13.8±2.9	11.6±0.3	74.3±2.7
6i	13.4±1.2	10.1±1.8	12.1±0.7	52.6±6.3
6j	19.7±4.6	16±1.2	15.8±1.6	83.2±0.2
6k	10.7±0.9	10.9±0.02	9.5±4.3	74.6±2.9
6l	15.8±2.4	16.9±1.3	14.2±2.9	45.8±4.2
6m	19.5±2.9	17.7±3.6	16.5±0.4	65.3±5.1
6n	3.5±0.05	4.6±2.5	5.1±0.04	62.1±2.4
6o	3.3±0.09	2.5±0.08	3.1±0.08	74.6±1.6
6p	18.1±0.3	16.9±1.2	15.8±3.2	55.9±1.3
6q	3.7±0.05	3.1±0.02	4.1±1.3	64.3±2.7
6r	3.1±0.74	3.0±0.13	3.9±0.08	75.2±3.9
6s	0.2±0.01	0.1±0.06	1.1±0.04	59.6±0.8
6t	11.2±1.1	10.2±1.2	11.4±1.6	49.2±1.1
5	1.13±0.13	1.32±0.27	2.15±0.84	NT
nocodazole	2.8±0.05	3.1±0.61	2.2±1.6	NT
E7010	1.62±0.54	1.81±0.17	1.31±0.05	NT

[a]50% inhibitory concentration and mean ± SD of three individual experiments performed in duplicate. NT: not tested. [b]colon cancer, [c]prostate cancer and [d]lung cancer and [e]normal human embryonic kidney (HEK-293) cells. Nocodazole and E7010 were included as reference standards.

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Figure 3: SAR Analysis of 2-anilinopyridyl-triazole conjugates

Cell cycle analysis

To investigate the mechanism underlying the anticancer activity of these potential conjugates (**6q**, **6r**, **6s** and E7010), the cell cycle distribution in DU-145 cancer cell line was analyzed by flow cytometry.[32] In this study DU-145 cells were treated with **6q**, **6r** and **6s** at 3 µM concentration for 48 h. The data obtained clearly indicated that they show G2/M cell cycle arrest. Conjugates (**6q**, **6r** and **6s**) showed 65.69%, 60.30% and 62.15% of cell accumulation in G2/M phase respectively (Figure **4** and Table **2**). The positive control, E7010 showed 59.27% of cell accumulation in G2/M phase, whereas in control (untreated cells) 23.82% of G2/M phase was observed.

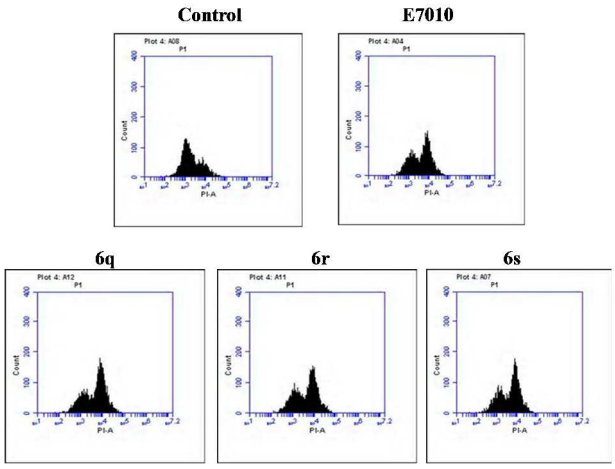

Figure 4:Flow cytometric analysis in DU-145 Prostrate cancer cell lines after treatment with compounds **6q**, **6r** and **6s** at 3 µM concentrations for 48 h. E7010 were employed as the positive controls.

Table **2**:Distribution of Cells (%) in G0/G1, G1, S and G2/M Phases of Cell Cycle.

Compound	G0/G1phase	G1phase	S phase	G2/M phase
Control	5.87	63.93	6.38	23.82
6q	4.23	24.72	5.36	65.69
6r	4.83	27.54	7.33	60.30
6s	3.91	26.47	7.47	62.15
E7010	3.26	29.81	7.65	59.27

Effect on tubulin polymerization

To investigate whether the anti-proliferative activities of these conjugates were related to the interaction with tubulin, we studied their effect on the tubulin polymerization in a cell-free system. Moreover these conjugates showed significantly induced on the G2/M cell cycle arrest, hence it was considered of interest to investigate the tubulin polymerization aspect.[33,34] As tubulin subunits heterodimerize and self-assemble to form microtubules in a time dependent manner, we have investigated the progression of tubulin polymerization 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 5 µM concentration. Conjugates **6q**, **6r** and **6s** inhibited tubulin polymerization by 58.2%, 59.5 % and 61.1%, respectively, compared to the control (Figure **5**). Tubulin polymerization inhibition was also observed in case of the standards like E7010 (58.5%) and nocodazole (59.5%). Furthermore, these three potential compounds were evaluated for their tubulin polymerization inhibition at different concentrations. They showed potent inhibition of tubulin polymerization with IC_{50} values 2.45, 2.76 and 1.84 µM respectively, as shown in Table **3**. Whereas, **5**, E7010 and nocodazole showed IC_{50} values of 1.93, 2.31 and 2.12 µM respectively.

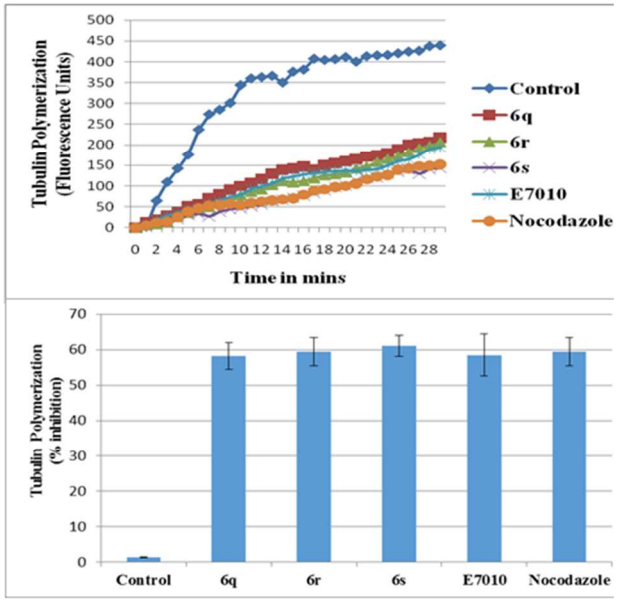

Figure 5: Effect of compounds on the tubulin polymerization: tubulin polymerization was monitored by the increase in fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37 °C. All the compounds were included at a final concentration of 5 µM. Values indicated are the mean ± SD of two different experiments performed in triplicates.

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Table **3**:Inhibition of tubulin polymerization (IC_{50}) of conjugate **6q, 6r and 6s** and **E7010** and **Nocodazole.**

Compound	IC_{50}[a] ± SD (in µM)
6q	2.45±0.12
6r	2.76±1.23
6s	1.84±0.58
5	1.93±0.23
E7010	2.31±0.36
Nocodazole	2.12±0.36

[a]Concentration of drug to inhibit 50% of tubulin assembly. Values indicated are the mean ± SD of two different experiments performed in triplicates. Statistical analysis was performed using Graph Pad Prism software version 5.01.

Immunohistochemistry of tubulin (IHC)

In order to determine whether these conjugates interacts directly with tubulin through binding to the colchicine-binding site, we further examined the effect of **6q, 6r, 6s** and E7010 on cellular microtubule networks by using immunofluorescence techniques.[35] As shown in Figure **6,** the microtubule network exhibits normal arrangement and organization in DU-145 cells in the absence of drug treatment. However, after 48 hr drug treatment, when treated with 3µM of conjugates **6q**, **6r** and **6s** which indicates that these are significantly caused cellular microtubule depolymerization; we noted that most cells had disrupted microtubule fragments scattered throughout the cytoplasm. In contrast, 3 µM of E7010 dramatically promoted the disruption of microtubule depolymerization with a loss of microtubules with only a diffuse stain visible throughout the cytoplasm.

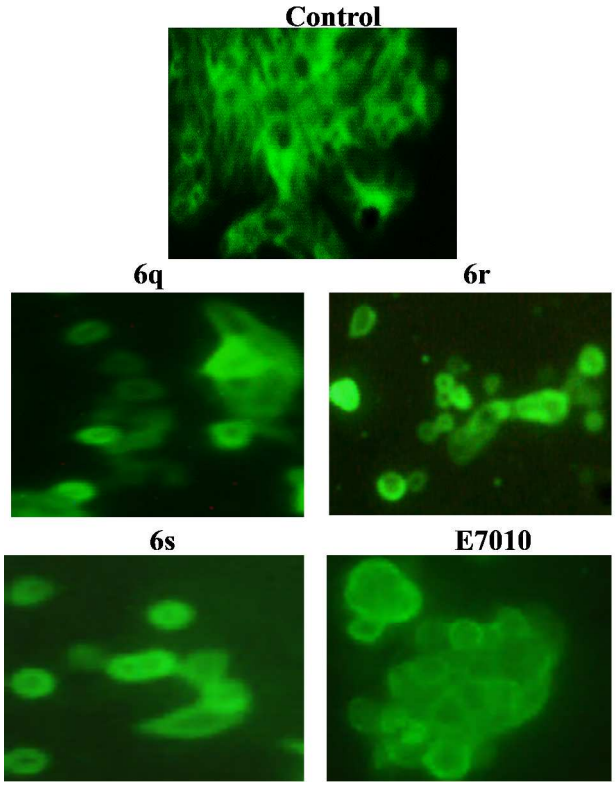

Figure 6: Immunohistochemistry of tubulin **(**IHC) analysis of compounds **6q**, **6r**, **6s** and **E7010** on the microtubule network in DU-145 Cells.

Competitive tubulin-binding assay

To confirm the above observations, we investigated the effect of **6q**, **6r** and **6s** on tubulin polymerization using an *in vitro* tubulin polymerization assay (Figure **5**). Therefore we further assessed the ability of compounds **6q**, **6r** and **6s** to compete with the colchicine for binding to tubulin via competitive binding assay, wherein E7010 was used as a positive control and paclitaxel as a negative control. Colchicine is known to show clear fluorescent properties when bound to tubulin (excitation at 350 nm and emission at 430 nm), although it is nonfluorescent by itself. As the intrinsic fluorescence of colchicine increases upon binding to the tubulin,[36] it was used as an index for **6q**, **6r** and **6s** competition with colchicine in tubulin binding. As shown in Figure **7**, paclitaxel did not affect the binding to the tubulin. However, the fluorescence of colchicine-tubulin complex was reduced in the presence of compounds **6q**, **6r**, **6s** and E7010 in a dose-dependent manner. These observations indicate that the compounds **6q**, **6r**, **6s** and E7010 inhibit the binding of colchicine to tubulin, thereby suggesting that the compounds **6q**, **6r** and **6s** have high affinity for the colchicine binding site.

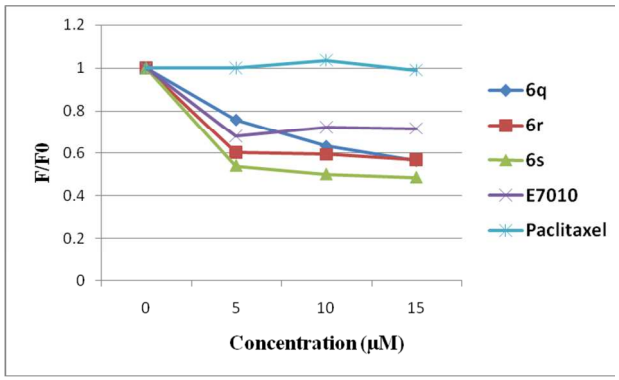

Figure 7: Fluorescence based colchicine competitive binding assay of conjugates **6q**, **6r** and **6s** were carried out at various concentrations containing 5µM of tubulin and colchicine for 60 min at 37 °C. E7010 was used as a positive control whereas paclitaxel was used as negative control which binds at taxane site. Fluorescence values are normalized to control.

Distribution of soluble versus polymerized tubulin in cells

The microtubules continuously undergo polymerization and depolymerization and inhibition of tubulin polymerization disturbs the assembly of microtubules, we analyzed the levels of soluble versus polymerized forms of tubulin in DU-145 cells. In order to extend the *in vitro* effects of the compounds **6q**, **6r**, **6s** and E7010 on tubulin polymerization to the cellular effects, Western blot analysis of α-tubulin in DU-145 cells was performed by treating with the compounds **6q**, **6r**, **6s** and E7010 at 3µM for 48 h. Following this incubation, the medium was removed, cells were washed with PBS, and soluble (containing free tubulin, supernant) and insoluble (containing tubulin from microtubules, pellet) fractions were collected as

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described in the Experimental Section.[37] Our results indicate that while the tubulin in the supernatant fraction showed an increased level of free tubulin at 3 μM of **6q**, **6r**, **6s** and E7010 whereas in the pellet shows a steady decrease in the polymerized tubulin. However, **6s** treated cells showed a more distinct shift in tubulin balance, with almost all tubulin present in the soluble fraction (Figure **8**).These results indicated that compounds, **6q**, **6r** and **6s** depolymerised microtubule mass into tubulin dimers under both *in vitro* and intracellular conditions.

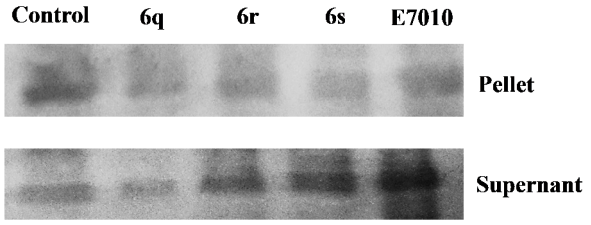

Figure 8: Western blot Analysis of compounds **6q**, **6r**, **6s** and E7010 in DU-145 cells on α-tubulin protein expression. Cells were treated with compounds **6q**, **6r**, **6s** and E7010 at 3 μM for 48 h. α-tubulin protein levels were examined in Western blot analysis.

Hoechst staining for morphological analysis of apoptosis

Apoptosis is one of the major pathways that lead to the process of cell death. Chromatin condensation and fragmented nuclei are known as the classic characteristics of apoptosis.[38] It was considered of interest to investigate the apoptosis inducing effect of these conjugates by Hoechst staining (H 33258) method in DU-145 cancer cell line. Therefore cells were treated with **6q**, **6r** and **6s** at 3μM concentrations for 24 h, wherein E7010 was used as the standard. Manual field quantification of apoptotic cells based on cytoplasmic condensation, presence of apoptotic bodies, nuclear fragmentation and relative fluorescence of the test compounds revealed that there was significant increase in the apoptotic cells as shown in Figure **9**.

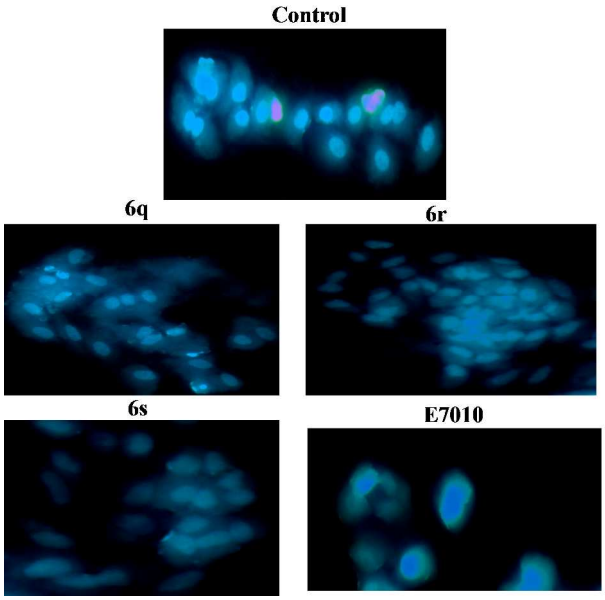

Figure 9: Hoechst staining of DU145 cells treated with compounds **6q**, **6r**, **6s**, and E7010.

Effect on caspase-3 Activity

It is well known that the cell cycle arrest at G2/M phase is shown to induce cellular apoptosis,[39,40] hence it was considered of interest to examine whether the cytotoxicity of **6q**, **6r** and **6s** is by virtue of apoptotic cell death.

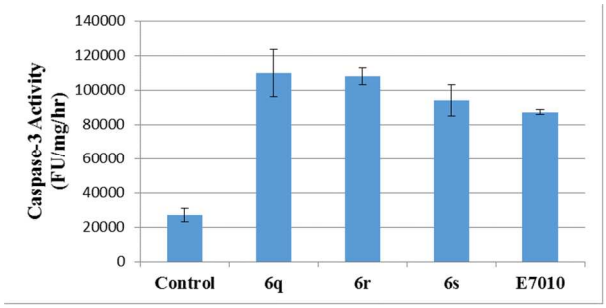

Figure 10: Effect of compounds **6q**, **6r**, **6s** and E7010 on caspase-3 activity: DU-145 cells were treated with compounds at 3 μM concentration for 48 h. Values indicated are the mean ± SD of two different experiments performed in triplicates.

The cysteine proteases (with specificity for aspartyl residues in substrates) 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 us to treat DU-145 cells with these compounds **6q**, **6r** and **6s** to examine the activation of caspase-3, wherein E7010 was used as positive control. The results indicate that there is nearly 4 to 9-fold induction in caspase-3 activity in cells treated with 3 μM concentration of these compounds (Figure **10**) thereby indicating that they have the ability to induce apoptosis in DU-145 cells.

Annexin V-FITC/Propidium iodide analysis of apoptosis

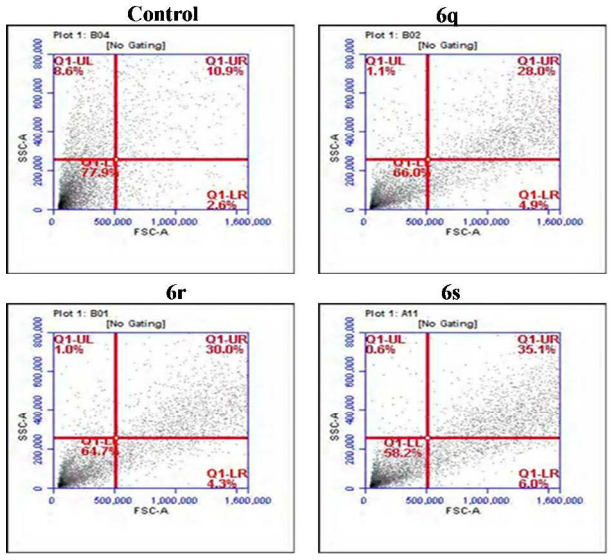

Figure 11: Representative histograms of DU-145 cells treated with compounds **6q**, **6r** and **6s** at 3 μM concentration for 48 h and analyzed by flow cytometry after double staining of the cells with Annexin-V-

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FITC and PI.

To characterize the mode of cell death induced by **6q**,**6r** and **6s**, a biparametriccytofluorimetric analysis was performed using propidium iodide (PI), which stains DNA and enters only dead cells, and fluorescent immunolabeling of the protein annexin-V, which binds to phosphatidyl serine (PS) in a highly selective manner.[41] Dual staining for annexin-V and with PI permits discrimination between live cells (annexin-V−/PI−), early apoptotic cells (annexin-V+/PI−), late apoptotic cells (annexin-V+/PI+), and necrotic cells (annexin-V−/PI+) . In this study DU-145 cells were treated with **6q**, **6r** and **6s** for 48 h at 3 μM concentration to examine the apoptotic effect. It was observed that they showed significant apoptosis against DU-145 cells. As shown in Figure**11**, results indicate that compounds **6q**, **6r** and **6s** showed 28.0%, 30.0% and 35.1% of apoptosis, respectively, whereas 10.9% of apoptosis was observed in control (untreated cells). This experiment suggests that these conjugates have the ability to significantly induce apoptosis in DU-145 cells.

Effect on mitochondrial depolarization

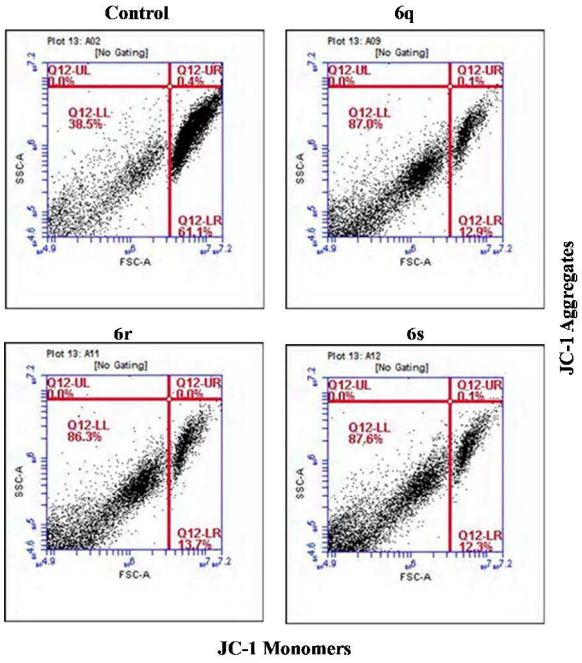

Figure 12: Assessment of $\Delta\psi_{mt}$(mitochondrial depolarization) of compounds **6q**, **6r** and **6s** in DU-145 cells. Representative histograms of control cells and cells incubated for 48 h in the presence of **6q**, **6r** and **6s** as indicated, and stained with the fluorescent probe JC-1 after treatment. The horizontal axis shows fluorescence intensity of the JC-1 monomer, and the vertical axis shows fluorescence of JC-1 aggregates.

Mitochondria play an essential role in the propagation of apoptosis. It is well established that at an early stage, apoptotic stimuli alter the mitochondrial transmembrane potential ($\Delta\psi_{mt}$).[42]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. In this study DU-145 cells were treated with **6q**, **6r** and **6s** at 3 μM that exhibited a remarkable shift increase to 37.8%, 38.8% and 42.8% of fluorescence respectively. In comparison control cells show 14.7%, which indicates depolarization of the mitochondrial membrane potential by these conjugates that results in the induction of mitochondrial apoptosis (Figure **12**).

DNA fragmentation assay

Apoptosis was also assessed by electrophoresis of extracted genomic DNA from cells.[43]Endonuclease mediated cleavage of nuclear DNA results in the formation of oligonucleosomal DNA fragment (180–200 base pairs long) a biochemical hallmark of apoptosis in many cell types. DNA laddering assay was performed with DU-145 cells by treatment of **6q**, **6r** and **6s** at 3 μM concentration for 48 h, then the genomic DNA was isolated and electrophoreses was carried out in 1.8% agarose gel. All the tested compounds induce DNA fragmentation that is, a characteristic ladder pattern was observed in DU-145 at 3 μM, while no laddering was observed in the control cells as shown in the Figure **13**.

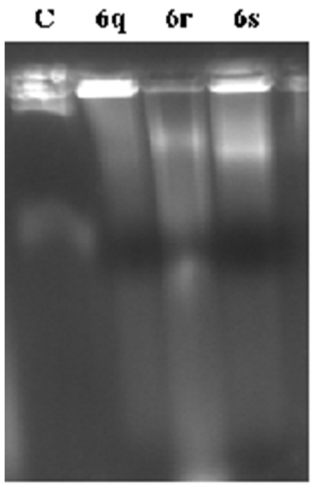

Figure 13: DNA fragmentation of the compounds (**6q, 6r** and **6s**) at 48 h in DU-145 Cells.

Molecular Docking

A series of 2-anilinopyridyl-triazole conjugates (**6a-t**) were synthesized that exerts its cytotoxic effect by inhibiting tubulin polymerization. With a view to understand and the mode of binding of these molecules molecular docking studies were performed on the tubulin protein at colchicine binding domain. Crystal structure of the protein was obtained from Protein Data Bank (PDB ID 3E22).[44] and necessary corrections to the protein were carried out using Protein Preparation Wizard from Schrodinger package. Geometry of the molecule was optimized in Gaussian 09 using PM3 semi-empirical method.[45]Further docking studies were performed using Auto Dock 4.2 docking

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software[46] and the docking results were visualized using PyMOL.[47]

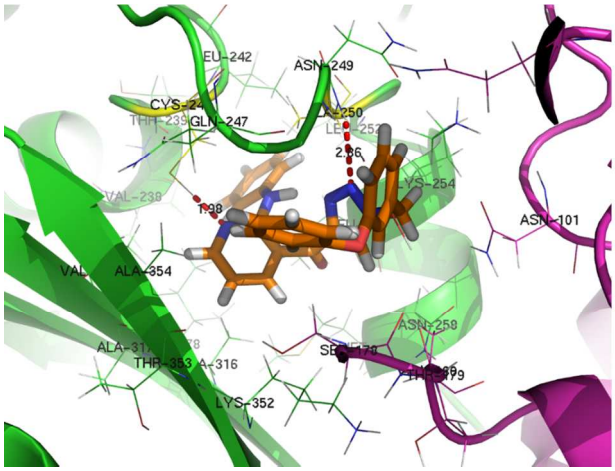

Figure 14: Docking pose for **6s** where α chain shown in pink color and β chain in green color, hydrogen bonding interactions in red dotted line and amino acid forming hydrogen bonding interactions in yellow color.

Docking studies were performed on **6s** being the most active molecule. Docking studies show that these molecule bind well in the colchicine binding domainas shown in the Figure **14**. Docking pose of **6s** displayed that it binds at the interface of α and β chains where 2-anilinopyridinyl group lies deep in the hydrophobic site of the β chain and benzyltriazole part at the interface of the α and β chains. Compound **6s** binds at the colchicine binding site with two hydrogen bonding interactions and hydrophobic interactions by the nearby amino acids. Hydrogen bonding interactions include nitrogen of the pyridine ring with the thiol of Cys241 and nitrogen of triazole with the backbone amide of Ala250. Besides this, benzyltriazole group has hydrophobic interactions with Gln11, Asn101, Ser178, Thr179, Ala180 and Tyr224 amino acids of α chain and Gln247, Asn249, Ala250, Lys254 amino acids of β chain. Whereas, 2-aminopyridyl group has hydrophobic interactions with Tyr202, Val238, Thr239, Leu242, Leu252, Ala255, Asn258, Met259, Ala316, Ala317, Val318k, Lys352, Thr353, Ala354, Ile378 amino acids of the β chain.

Conclusion

A series of 2-anilinopyridyl-triazole conjugates (**6a-t**) were synthesized using Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction and were evaluated for their cytotoxic and tubulin polymerization inhibitory potential. These compounds exhibit significant cytotoxic activity against three human cancer cell lines HT-29 (colon cancer), DU-145 (prostate cancer) and A549 (lung cancer) with IC_{50} values ranging from 0.1 to 19.1 µM. Amongst them some of these conjugates like 6q, 6r and 6s showed potent cytotoxic efficacy against DU-145 human cancer cell line. Moreover, preliminary studies showed that they arrest cells in the G2/M phase of the cell cycle and tubulin polymerization assay showed that the level of tubulin inhibition for **6s** (1.84 µM) was similar to that of E7010 (2.31 µM). The docking experiments were able to rationalize a binding mode for the interaction of **6s** with the colchicine binding site. This was further confirmed by colchicine competitive binding assay. Moreover, **6q**, **6r** and **6s** possessed anti-tubulin activity both *in vitro* and within cells as demonstrated by the ratio of soluble versus polymerized tubulin. Detailed biological studies like Hoechst staining, caspase-3 activation, annexin-V FITC, mitochondrial depolarization and DNA fragmentation analysis demonstrate that these compounds induce apoptotic cell death. These results suggest that such conjugates have the potential to be developed as tubulin polymerization inhibitors for the treatment of cancer.

Experimental

Chemistry

Materials and Methods.

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 plate containing 60 GF-254, and visualization was achieved by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H and ^{13}C NMR spectra were determined in $CDCl_3$ by using Varian and Avance instruments. Chemical shifts are expressed in parts per million (δ in ppm) downfield from internal TMS and coupling constants are expressed in Hz. ^{1}H NMR spectroscopic data are reported in the following order: multiplicity (s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet), coupling constants in Hz, number of protons. ESI mass spectra were recorded on a Micro mass Quattro LC using ESI+ software with capillary voltage 3.98 kV and an ESI mode positive ion trap detector. Melting points were determined with an Electro thermal melting point apparatus, and are uncorrected.

Preparation of ethyl 2-chloronicotinate (8)

A solution of 2-chloro nicotinic acid (10 g, 64 mmol) and ethanol (100 ml) was stirred at 0 °C for 15 min. To this solution concentrated sulfuric acid (3 mL) was added drop wise and refluxed for 4 h. After completion of reaction (checked by TLC), ethanol was removed by vacuum followed by neutralization with sodium bicarbonate solution and ethyl acetate was added. The organic layer was extracted, washed with water, dried over anhydrous sodium sulphate and evaporated in vacuum to obtain pure ethyl 2-chloronicotinate (**8**) and it was used directly to next step without further purification.

Preparation of ethyl 2-(aryl amino)nicotinate (10a-d):

A solution of ethyl 2-chloronicotinate (**8**) and substituted anilines (**9a-d**) were dissolved in ethylene glycol and heated at 120 °C for 5-6 h. After completion of reaction, the reaction mixture was cooled to room temperature followed by water and ethyl acetate was added. The organic layer was separated, dried over anhydrous Na_2SO_4, evaporated in vacuum and it was

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purified by column chromatography (20% ethyl acetate/hexane) to get pure compounds (**10a-d**).

Ethyl 2-(4-methoxyphenylamino)nicotinate(10a)

This compound was obtained by using above described method by dissolving ethyl 2-chloronicotinate (**2**) (3 g, 16.2 mmol) and 4-methoxyaniline (**9a**) (1.99 g, 16.2 mmol) in ethylene glycol (20 mL). Yellow solid; 3.57g; 81% yield; Mp: 88-90 °C; ^{1}H NMR (300 MHz, $CDCl_3$):δ9.96 (brs, 1H), 8.32 (dd, *J* = 4.7, 2.0 Hz, 1H), 8.22 (dd, *J* = 7.8, 2.1 Hz, 1H), 7.53 (d, *J* = 9.0 Hz, 2H), 6.90 (d, *J* = 9.0 Hz, 2H), 6.65 (dd, *J* = 7.8, 4.7 Hz, 1H), 4.44-4.37 (q, *J* = 7.1 Hz, 2H), 3.81 (s, 3H), 1.40 (t, *J* = 7.1 Hz, 3H) ppm; MS (ESI) *m/z* 273 $(M+H)^+$.

Ethyl 2-(3,4-dimethoxyphenylamino)nicotinate (10b)

This compound was obtained by using above described method by dissolving ethyl 2-chloronicotinate (**2**) (3 g, 16.2 mmol) and 3, 4-dimethoxyaniline (**9b**) (1.99 g, 16.2 mmol) in ethylene glycol (20 mL) as yellow colored solid, 3.57; 81% yield; Mp: 88-90 °C;^{1}H NMR (500 MHz, $CDCl_3$): δ 9.98 (s, 1H), 8.36 – 8.33 (m, 1H), 8.23 – 8.19 (m, 1H), 7.27 – 7.25 (m, 2H), 7.17 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.86 (d, *J* = 8.6 Hz, 1H), 6.67 (ddd, *J* = 7.7, 4.5, 1.8 Hz, 1H), 3.90 (s, 4H), 3.88 (s, 4H), 4.41-4.35 (q, *J* = 7.1 Hz, 2H), 1.41 (t, *J* = 7.1 Hz, 3H) ppm.MS (ESI) *m/z* 303 $(M+H)^+$.

Ethyl 2-(3, 4, 5-trimethoxyphenylamino)nicotinate(10c)

This compound was obtained by using above described method by dissolving ethyl 2-chloronicotinate (**2**) (3 g, 16.2 mmol) and 3,4,5-trimethoxyaniline (**9c**) (2.97 g, 16.2 mmol) in ethylene glycol (20 mL). Yellow solid; 4.2g; 78% yield; Mp: 104-106 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.01 (brs, 1H), 8.31 (dd, *J* = 2.8, 1.9 Hz, 1H), 8.22 (dd, *J* = 5.7, 2.2 Hz, 1H), 7.01 (s, 2H), 6.63 (dd, *J* = 5.7, 2.8 Hz, 1H), 4.29-4.21 (q, *J* =7.1 Hz, 2H), 3.91 (s, 6H), 1.39 (t, *J* =7.1 Hz, 3H) ppm; MS (ESI) *m/z* 333 $(M+H)^+$.

Ethyl 2-(4-fluorophenylamino)nicotinate (10d)

This compound was obtained by using above described method by dissolving ethyl 2-chloronicotinate (**2**) (3 g, 16.2 mmol) and 4-fluoroaniline (**9d**) (1.80 g, 16.2 mmol) in ethylene glycol (20 mL). Yellow solid; 3.08g; 73% yield; Mp: 75-77 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.12 (brs, 1H),8.34 (dd, *J* = 2.7, 1.9 Hz, 1H), 8.24 (dd, *J* = 5.6, 2.1 Hz, 1H), 7.62 (dd, *J* = 4.7, 4.3 Hz, 2H), 7.00-7.06 (m, 2H), 6.69 (dd, *J* = 5.6, 1.9 Hz, 1H), 4.41-4.36 (q, *J* = 7.2 Hz, 2H), 1.42 (t, *J* = 7.2 Hz, 3H) ppm; MS (ESI) *m/z* 261$(M+H)^+$.

General method for synthesis of (2-(aryl amino) pyridin-3-yl) methanol (11a-d):

To the ethyl 2-(arylamino)nicotinate (**10a-d**), obtained in the above step was added $LiAlH_4$ (0.5 mol) in dry THF at 0 °C and stirred for 3-4 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. The organic layer was dried on anhydrous Na_2SO_4 and evaporated ethyl acetate to obtain brown solid products of (2-(aryl amino) pyridin-3-yl) methanols (**11a-d**) (yield 70-79%). The alcohols produced in this step were pure, and no further purification was required. These compounds were taken as such for the next step.

(2-(4-Methoxyphenylamino)pyridin-3-yl)methanol (11a)

This compound was obtained according to above described method by adding ethyl 2-(4-methoxyphenylamino)nicotinate (**10a**) (3.5 g, 12.8 mmol) to $LiAlH_4$ (675 mg, 19.2 mmol) in THF (30 mL). Brown coloured solid; 2.15g; 72% yield; Mp: 90-92 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.09 (dd, *J* =3.2 ,1.9 Hz, 1H,), 7.40 (d, *J* =9.0 Hz, 2H), 7.46 (brs, 1H), 7.27 (dd, *J* =5.1, 1.7 Hz, 1H), 6.87 (d, *J* =9.0 Hz, 2H), 6.62 (dd, *J* = 5.1, 2.1 Hz, 1H), 4.60 (s, 2H), 3.78 (s, 3H) ppm; MS (ESI): *m/z* 231 $(M+H)^+$.

(2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)methanol (11b)

This compound was obtained according to above described method by adding ethyl 2-((3,4-dimethoxyphenyl)amino)nicotinate (**10b**) (3.5 g, 11.5 mmol) to $LiAlH_4$ (486 mg, 13.9 mmol) in THF (30 mL). Brown coloured solid; 2.28g; 76% yield; Mp: 90-92 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10 (dd, *J* = 5.0, 1.6 Hz, 1H), 7.54 (s, 1H), 7.26 (dd, *J* = 5.7, 4.2 Hz, 1H), 7.15 (d, *J* = 2.3 Hz, 1H), 6.99 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.82 (d, *J* = 8.6 Hz, 1H), 6.63 (dd, *J* = 7.1, 5.1 Hz, 1H), 4.58 (s, 2H), 3.85 (d, *J* = 6.3 Hz, 6H) ppm; MS (ESI): *m/z*261 $(M+H)^+$.

(2-(3,4,5-Trimethoxyphenylamino)pyridin-3-yl)methanol (11c)

This compound was obtained according to above described method by adding ethyl 2-(3,4,5-trimethoxyphenylamino)nicotinate (**10c**) (4.0 g, 12 mmol) to $LiAlH_4$ (506mg, 14.4 mmol) in THF (30 mL). Brown coloured solid; 2.48g; 71% yield; Mp: 99-101 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ8.16 (dd, *J* = 3.1, 1.9 Hz, 1H), 7.80 (brs, 1H), 7.21-7.26 (m, 1H), 6.92 (s, 2H), 6.63 (dd, *J* = 6.6, 3.1 Hz, 1H), 4.62 (s, 2H), 3.89 (s, 6H), 3.83 (s, 3H) ppm; MS (ESI): *m/z* 291 $(M+H)^+$.

2-(4-Fluorophenylamino)pyridin-3-yl)methanol (11d)

This compound was obtained according to above described method by adding ethyl 2-(4-fluorophenylamino)nicotinate (**10d**) (3.0 g, 11.5 mmol) to $LiAlH_4$ (219 mg, 6 mmol) in THF (30 mL). Brown coloured solid; 2.28g; 78% yield; Mp: 94-96 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ8.10 (dd, *J* = 3.0, 2.3 Hz, 1H),7.61 (brs, 1H), 7.45 (dd, *J* = 9.0, 4.5 Hz, 2H),c7.27 (dd, *J* = 5.3, 1.5 Hz, 1H), 7.04-6.94 (m, 2H), 6.67 (dd, *J* = 4.5, 2.3 Hz, 1H), 4.59 (s, 2H) ppm; MS (ESI): *m/z* 219 $(M+H)^+$.

General method for synthesis of 2-(Aryl amino)nicotinaldehyde(12a-d)

To the (2-(aryl amino) pyridin-3-yl)methanol(**11a-d**) produced in the above step was added IBX (1.2 mol) in DMSO and stirred for 2 h at room temperature. Added ice cold water to the reaction mixture and extracted with ethyl acetate. The organic layer was dried on anhydrous $NaSO_4$ and evaporated the ethyl acetate to obtain pure corresponding 2-(Aryl

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amino)nicotinaldehyde (**12a-d**) in good yields.

2-(4-Methoxyphenylamino)nicotinaldehyde(12a)

This compound was prepared according to above explained method by the addition of (2-(4-methoxyphenylamino)pyridin-3-yl)methanol (**11a**) (2.5 g, 10.86 mmol) to IBX (3.6 g 12.85 mmol) in DMSO (25 mL) solution. Yellow coloured solid; 1.8g; 73% yield; Mp: 73-75 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ10.35 (brs,1H), 9.81 (s, 1H), 8.39-8.36 (dd, *J* = 3.1, 1.8 Hz, 1H), 7.87-7.82 (dd, *J* = 6.0, 1.8 Hz, 1H), 7.58 (d, *J* = 9.0 Hz, 2H), 6.92 (d, *J* = 9.0 Hz, 2H), 6.81-6.75 (dd, *J* = 5.3, 2.3 Hz, 1H), 3.82 (s, 3H), MS (ESI): *m/z* 229 $(M+H)^+$.

2-((3,4-dimethoxyphenyl)amino)nicotinaldehyde (12b)

This compound was prepared according to above explained method by the addition of (2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)methanol (**11b**) (2.5 g. 9.6 mmol) to IBX (3.2 g 11.5 mmol) in DMSO (25 mL) solution. Yellow coloured solid; 1.97g; 79% yield; Mp: 70-73 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.30 (s, 1H), 9.88 (s, 1H), 8.40 (dd, *J* = 4.8, 2.0 Hz, 1H), 7.86 (dd, *J* = 7.6, 2.0 Hz, 1H), 7.30 (d, *J* = 2.4 Hz, 1H), 7.22 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.87 (d, *J* = 8.6 Hz, 1H), 6.81 (dd, *J* = 7.6, 4.8 Hz, 1H), 3.91 (s, 3H), 3.88 (s, 3H); MS (ESI): *m/z* 259 $(M+H)^+$.

2-(3,4,5-Trimethoxyphenylamino)nicotinaldehyde(12c)

This compound was prepared according to above explained method by the addition of (2-(3,4,5-trimethoxyphenylamino)pyridin-3-yl)methanol (**11c**) (2.5 g. 8.6 mmol) to IBX (2.89 g 10.3 mmol) in DMSO (25 mL) solution. Yellow coloured solid; 2.03g; 82% yield; Mp: 95-97 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.38 (s, 1H), 9.89 – 9.87 (m, 1H), 8.44 – 8.41 (m, 1H), 7.90 – 7.86 (m, 1H), 7.04 (d, *J* = 0.7 Hz, 2H), 6.87 – 6.83 (m, 1H), 3.89 (s, 6H), 3.84 (s, 3H) ppm; MS (ESI): *m/z* 289 $(M+H)^+$.

2-(4-Fluorophenylamino)nicotinaldehyde(12d)

This compound was prepared according to above explained method by the addition of (2-(4-fluorophenylamino)pyridin-3-yl)methanol (**11d**) (2.5 g. 11.47 mmol) to IBX (3.85 g 13.7 mmol) in DMSO (25 mL) solution. Yellow coloured solid; 1.90g; 77% yield; Mp: 59-61 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ10.27 (brs, 1H), 9.87 (s, 1H), 8.36-8.39 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.85 (dd, *J* = 6.0, 1.5 Hz, 1H), 7.58 (d, *J* = 9.0 Hz, 2H), 6.92 (d, *J* = 9.0 Hz, 2H), 6.78 (dd, *J* = 5.3, 2.3 Hz, 1H) ppm; MS (ESI): *m/z* 217 $(M+H)^+$.

General procedure for the Synthesis of 1-(2-(arylphenylamino)pyridin-3-yl)prop-2-yn-1-ol (13a-d)

A solution of aldehydes (**12a-d**) in dry tetrahydrofuran (THF) was added to stirred solution of ethynyl magnesium bromide (0.5 M) in tetrahydrofuran at 0 °C and then stirred at room temperature for 3-4 h. After completion of reaction saturated aqueous ammonium chloride solution 5-10 ml was added, and the THF was removed in vaccum 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 vaccum to obtain pure compounds and these were used for next step without purification.

1-(2-(4-Methoxyphenylamino)pyridin-3-yl)prop-2-yn-1-ol (13a)

This compound was obtained using above described method by adding 2-(4-methoxyphenylamino)nicotinaldehyde (**12a**) (2.00 g, 9.2 mmol) to the ethynyl magnesium bromide (34.4 ml, 18 mmol) in dry tetrahydrofuran solution. Brown coloured solid; 1.65g; 72% yield; Mp: 117-119 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.14 (dd, *J* = 5.0, 1.8 Hz, 1H), 7.71 (dd, *J* = 7.4, 1.6 Hz, 1H), 7.41 (s, 1H), 7.39 – 7.36 (m, 2H), 6.90 – 6.86 (m, 2H), 6.70 (dd, *J* = 7.4, 5.0 Hz, 1H), 5.44 (d, *J* = 2.2 Hz, 1H), 3.79 (s, 3H), 2.77 (d, *J* = 2.3 Hz, 1H) ppm; MS (ESI): *m/z* 255 $(M+H)^+$.

1-(2-(3,4-dimethoxyphenylamino)pyridin-3-yl)prop-2-yn-1-ol (13b)

This compound was obtained using above described method by adding 2-((3,4-dimethoxyphenyl)amino)nicotinaldehyde (**12b**) (2.0g, 7.75 mmol) to the ethynyl magnesium bromide (28.8 ml, 15.5 mmol) in dry tetrahydrofuran solution. Brown coloured solid; 1.67g; 76% yield; Mp: 125-127 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.14 (dd, *J* = 5.0, 1.8 Hz, 1H), 7.68 (dd, *J* = 7.4, 1.4 Hz, 1H), 7.48 (s, 1H), 7.12 (d, *J* = 2.4 Hz, 1H), 6.95 (dd, *J* = 8.6, 2.5 Hz, 1H), 6.81 (d, *J* = 8.6 Hz, 1H), 6.70 (dd, *J* = 7.4, 5.0 Hz, 1H), 5.40 (d, *J* = 2.2 Hz, 1H), 3.86 (s, 3H), 3.84 (s, 3H), 2.76 (d, *J* = 2.3 Hz, 1H) ppm; MS (ESI): *m/z* 285 $(M+H)^+$.

1-(2-(3,4,5-Trimethoxyphenylamino)pyridin-3-yl)prop-2-yn-1-ol (13c)

This compound was obtained using above described method by adding 2-(3,4,5-trimethoxyphenylamino)nicotinaldehyde (**12c**) (2.0g, 7 mmol) to the ethynyl magnesium bromide (25.89 ml, 14 mmol) in dry tetrahydrofuran solution as brown solid (1.32g, 69%); mp 125-127 °C; Brown coloured solid; 1.51g; 69% yield; Mp: 117-119 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.21 (dd, *J* = 4.9, 1.8 Hz, 1H), 7.77 (dd, *J* = 7.4, 1.7 Hz, 1H), 7.57 (brs, 1H), 6.81 (s, 2H), 6.77 (dd, *J* = 7.4, 5.0 Hz, 1H), 5.52 (d, *J* = 2.2 Hz, 1H), 3.86 (s, 6H), 3.82 (s, 3H), 2.81 (d, *J* = 2.3 Hz, 1H) ppm; MS (ESI): *m/z* 315 $(M+H)^+$.

1-(2-((4-fluorophenyl)amino)pyridin-3-yl)prop-2-yn-1-ol (13d)

This compound was obtained using above described method by adding 2-((4-fluorophenyl)amino)nicotinaldehyde (**12d**) (2.0g, 9.2 mmol) to the ethynyl magnesium bromide (34.2 ml, 18.5 mmol) in dry tetrahydrofuran solution. Brown coloured solid; 1.49g; 72% yield; Mp: 107-109 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.14 (dd, *J* = 5.0, 1.7 Hz, 1H), 7.68 (dd, *J* = 7.4, 1.6 Hz, 1H), 7.55 (s, 1H), 7.43 – 7.38 (m, 2H), 7.02 – 6.96 (m, 2H), 6.73 (dd, *J* = 7.4, 5.0 Hz, 1H), 5.40 (d, *J* = 2.1 Hz, 1H), 2.76 (d, *J* = 2.2 Hz, 1H) ppm; MS (ESI): *m/z* 225 $(M+H)^+$.

General method for synthesis of 1-(2-(Arylamino)pyridin-3-yl)prop-2-yn-1-one (14a-d)

A solution of 2-iodoxy benzoic acid and dimethyl sulfoxide (DMSO) was stirred for 10 min at room temperature until homogeneous solution. A solution of alcohols (**13a-d**) in

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dimethyl sulfoxide was added slowly, and it was stirred for 2-3 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 to attain the crude compounds and these were purified by column chromatography to get pure compounds (**14a-d**).

1-(2-(4-Methoxyphenylamino)pyridin-3-yl)prop-2-yn-1-one (14a)

This compound was obtained using above described method by adding 1-(2-(4-methoxyphenylamino)pyridin-3-yl)prop-2-yn-1-ol (**13a**) (1.2g, 4.7.2 mmol) to the 2-iodoxy benzoic acid (1.58g, 5.6 mmol) in dimethyl sulfoxide (25 mL) solution. Yellow coloured solid; 950 mg; 80% yield; Mp: 98-100 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.63 (s, 1H), 8.48 – 8.42 (m, 1H), 8.41-8.36 (m, 1H), 7.55 (d, *J* = 8.9 Hz, 2H), 6.92 (d, *J* = 8.9 Hz, 2H), 6.71-6.78 (m, 1H), 3.81 (s, 3H), 3.53 (s, 1H) ppm; MS (ESI): *m/z* 253 $(M+H)^+$.

1-(2-((3,4-Dimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (14b)

This compound was obtained using above described method by adding 1-(2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-ol (**13b**) (1.5g, 5.2 mmol) to the 2-iodoxy benzoic acid (1.77g, 6.33 mmol) in dimethyl sulfoxide (25 mL) solution. Yellow coloured solid; 1.13g; 76% yield; Mp: 104-106 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.67 (s, 1H), 8.48 – 8.45 (m, 1H), 8.41 (dd, *J* = 4.7, 2.0 Hz, 1H), 7.27 – 7.26 (m, 1H), 7.18 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.87 (d, *J* = 8.6 Hz, 1H), 6.76 (dd, *J* = 7.9, 4.7 Hz, 1H), 3.90 (s, 3H), 3.89 (s, 3H), 3.53 (s, 1H); MS (ESI): *m/z* 283 $(M+H)^+$.

1-(2-(3,4,5-Trimethoxyphenylamino)pyridin-3-yl)prop-2-yn-1-one (14c)

This compound was obtained using above described method by adding 1-(2-(3,4,5-trimethoxyphenylamino)pyridin-3-yl)prop-2-yn-1-ol (**13c**) (1.20 g, 3.8 mmol) to the 2-iodoxy benzoic acid (1.28 g, 4.5 mmol) in dimethyl sulfoxide (25 mL) solution. Yellow coloured solid; 868 mg; 73% yield; Mp: 149-150 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.74 (brs, 1H,), 8.49 (dd, *J* = 6.0, 2.3 Hz, 1H), 8.44 (dd, *J* = 2.3, 2.2 Hz, 1H), 6.99 (s, 2H), 6.81 (dd, , *J* = 5.3, 3.0 Hz, 1H), 3.89 (s, 6H,), 3.84 (s, 3H), 3.55 (s, 1H) ppm; MS (ESI): *m/z* 313 $(M+H)^+$.

1-(2-(4-Fluorophenylamino)pyridin-3-yl)prop-2-yn-1-one (14d)

This compound was obtained using above described method by adding 1-(2-(4-fluorophenylamino)pyridin-3-yl)prop-2-yn-1-ol (**13d**) (1.3g, 5.37 mmol) to the 2-iodoxy benzoic acid (1.89g, 6.4 mmol) in dimethyl sulfoxide (25 mL) solution. Yellow coloured solid; 992 mg; 77% yield; Mp: 148-150 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.72 (brs, 1H), 8.48 (dd, *J* = 6.0, 1.5 Hz, 1H), 8.41 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.64 (dd, *J* = 9.0, 3.7 Hz, 2H), 7.00-7.11 (m, 2H,), 6.79 (dd, *J* = 4.5, 3.8 Hz, 1H,), 3.54 (s, 1H), ppm; MS (ESI): *m/z* 241 $(M+H)^+$.

General method for synthesis of (1-benzyl-1H-1,2,3-triazol-4-yl)(2-(phenylamino)pyridin-3-yl)methanone (6a-t)

To a solution of substituted ethynyl ketones (**14a-d**) (0.59 mmol) and substituted benzyl azides (**17a-e**) (0.65 mmol) in 2:1 mixture of water and tert-butyl alcohol, sodium ascorbate (0.06 mmol) and copper (II) sulfate (0.03 mmol) were added sequentially. The reaction was stirred at room temperature for overnight, TLC analysis indicated completion of reaction. The solvent was concentrated under vacuum and extracted with EtOAc to give crude product. The crude products were purified by column chromatography to afford pure products **(6a-t)** as yellow solids.

(1-(4-methoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((4-methoxyphenyl)amino)pyridin-3-yl)methanone (6a)

This compound **6a** was prepared by method described in above general method employing 1-(2-((4-methoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14a**, 150 mg, 0.59 mmol) and 1-(azidomethyl)-4-methoxybenzene (**17a**, 97 mg, 0.59 mmol) to affords **6a** as yellow solid; 192 mg; 78% yield; Mp: 102-104 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.92 (s, 1H), 9.37 (dd, *J* = 8.0, 1.7 Hz, 1H), 8.38 (dd, *J* = 4.5, 1.7 Hz, 1H), 8.09 (s, 1H), 7.55 (d, *J* = 8.9 Hz, 2H), 7.33 – 7.23 (m, 2H), 6.92 (t, *J* = 8.3 Hz, 4H), 6.77 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.54 (s, 2H), 3.82 (d, *J*= 4.1 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 185.59, 160.26, 156.87, 156.06, 154.29, 149.12, 143.95, 132.37, 130.04, 128.12, 125.60, 123.83, 114.74, 114.16, 112.85, 112.82, 55.54, 55.41, 54.08; MS (ESI): *m/z* 416 $[M+H]^+$; HRMS calcd for $C_{23}H_{22}O_3N_5$ $[M+H]^+$416.17172, found 416.17065.

(1-(3,4-dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((4-methoxyphenyl)amino)pyridin-3-yl)methanone (6b)

This compound **6b** was prepared by method described in above general method employing 1-(2-((4-methoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14a**, 150 mg, 0.59 mmol) and 4-(azidomethyl)-1,2-dimethoxybenzene (**17b**, 114 mg, 0.59 mmol) to affords **6b** as a yellow solid; 217 mg; 82% yield; Mp: 142-143 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.92 (s, 1H), 9.38 (dd, *J* = 8.0, 1.8 Hz, 1H), 8.38 (dd, *J* = 4.6, 1.8 Hz, 1H), 8.11 (s, 1H), 7.56 (t, *J*= 6.4 Hz, 2H), 6.96 – 6.84 (m, 5H), 6.77 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.53 (s, 2H), 3.90 (s, 3H), 3.87 (s, 3H), 3.81 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.55, 161.51, 156.90, 156.08, 154.35, 143.92, 132.35, 128.35, 128.13, 125.92, 123.85, 121.32, 114.16, 112.85, 111.48, 111.41, 106.46, 100.71, 56.03, 55.54, 55.49, 54.58, 54.48; MS (ESI): *m/z* 446 $[M+H]^+$; HRMS calcd for $C_{24}H_{24}O_4N_5$ $[M+H]^+$446.18228, found 446.18060.

(1-(3,5-dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((4-methoxyphenyl)amino)pyridin-3-yl)methanone (6c)

This compound **6c** was prepared by method described in above general method employing 1-(2-((4-methoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14a**, 150 mg, 0.59 mmol) and 1-(azidomethyl)-3,5-dimethoxybenzene (**17c**, 114 mg, 0.59 mmol) to affords **6c** as a yellow solid yield; 208 mg; 79% yield; Mp: 120-122 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.93 (s, 1H), 9.38 (dd, *J* = 8.0, 1.9 Hz, 1H), 8.38 (dd, *J* = 4.6, 1.9 Hz, 1H), 8.15 (s, 1H), 7.58 – 7.54 (m, 2H), 6.93 – 6.89 (m, 2H), 6.77 (dd, *J* = 8.0, 4.7 Hz, 1H), 6.46 (s, 3H), 5.52 (s, 2H), 3.81 (s, 3H), 3.78 (s, 6H); ^{13}C

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NMR (75 MHz, $CDCl_3$): δ 185.61, 161.43, 156.37, 154.04, 153.12, 148.98, 147.16, 143.92, 135.50, 135.33, 134.00, 128.38, 113.21, 113.06, 106.39, 100.55, 99.26, 60.89, 56.00, 55.39, 54.50; MS (ESI): *m/z* 446 $[M+H]^+$; HRMS calcd for $C_{24}H_{24}O_4N_5$ $[M+H]^+$446.18228, found 446.18116.

(2-((4-methoxyphenyl)amino)pyridin-3-yl)(1-(3-phenoxybenzyl)-1H-1,2,3-triazol-4-yl)methanone (6d)

This compound **6d** was prepared by method described in above general method employing 1-(2-((4-methoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14a**, 150 mg, 0.59 mmol) and 1-(azidomethyl)-3-phenoxybenzene (**17d**, 133 mg, 0.59 mmol) to affords **6d** as a yellow solid; 237 mg; 84% yield; Mp: 110-113 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.93 (s, 1H), 9.37 (dd, *J* = 8.0, 1.9 Hz, 1H), 8.38 (dd, *J* = 4.6, 1.9 Hz, 1H), 8.15 (s, 1H), 7.58 – 7.54 (m, 2H), 7.36 (tdd, *J* = 8.0, 4.8, 2.8 Hz, 3H), 7.17 – 7.13 (m, 1H), 7.05 – 6.96 (m, 5H), 6.94 – 6.89 (m, 2H), 6.77 (dd, *J* = 8.0, 4.6 Hz, 1H), 5.57 (s, 2H), 3.81 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 158.38, 156.92, 156.30, 156.11, 154.39, 149.29, 143.95, 135.53, 132.34, 130.76, 130.00, 128.34, 124.05, 123.90, 122.66, 119.44, 118.90, 118.24, 114.18, 112.87, 112.79, 55.55, 54.18; MS (ESI): *m/z* 478 $[M+H]^+$; HRMS calcd for $C_{21}H_{15}N_4ClF$ $[M+H]^+$478.18479, found 478.18484.

(1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)(2-((4-methoxyphenyl)amino)pyridin-3-yl)methanone (6e)

This compound **6e**was prepared by method described in above general method employing 1-(2-((4-methoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14a**, 150 mg, 0.59 mmol) and 1-(azidomethyl)-4-fluorobenzene (**17e**, 98 mg, 0.82 mmol) to affords **6e** as a yellow solid yield. 187 mg; 78% yield; Mp: 116-118 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.92 (s, 1H), 9.38 (dd, *J* = 8.0, 1.7 Hz, 1H), 8.38 (dd, *J* = 4.5, 1.7 Hz, 1H), 8.13 (s, 1H), 7.55 (d, *J* = 8.9 Hz, 2H), 7.35 (dd, *J* = 8.4, 5.2 Hz, 2H), 7.11 (t, *J* = 8.5 Hz, 2H), 6.91 (d, *J* = 8.9 Hz, 2H), 6.77 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.58 (s, 2H), 3.81 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.40, 164.71, 161.41, 156.86, 156.06, 154.39, 149.30, 143.89, 132.26, 130.37, 130.26, 129.59, 129.55, 128.18, 123.85, 116.55, 116.26, 114.12, 112.84, 112.70, 55.50, 53.72; MS (ESI): *m/z* 404 $[M+H]^+$; HRMS calcd for $C_{21}H_{15}N_4ClF$ $[M+H]^+$404.15015, found 404.15018.

2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)(1-(4-methoxybenzyl)-1H-1,2,3-triazol-4-yl)methanone (6f)

This compound **6f** was prepared by method described in above general method employing 1-(2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14b**, 150 mg, 0.53 mmol) and 1-(azidomethyl)-4-methoxybenzene (**17a**, 82 mg, 0.53 mmol) to affords **6f** as a yellow solid; 177 mg; 75% yield; Mp: 112-114 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.96 (s, 1H), 9.39 (dd, *J* = 8.0, 1.9 Hz, 1H), 8.39 (dd, *J* = 4.6, 1.9 Hz, 1H), 8.09 (s, 1H), 7.30 (dt, *J* = 4.9, 2.8 Hz, 3H), 7.19 (dt, *J* = 8.6, 3.1 Hz, 1H), 6.95 – 6.92 (m, 2H), 6.88 – 6.85 (m, 1H), 6.79 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.54 (s, 2H), 3.90 (s, 3H), 3.88 (s, 3H), 3.82 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 185.64, 160.27, 156.78, 154.27, 149.09, 148.99, 145.51, 143.98, 132.81, 130.05, 128.13, 125.56, 114.74, 114.24, 112.95, 112.90, 111.52, 106.95, 56.18, 55.93, 55.41, 54.09; MS (ESI): *m/z* 446 $[M+H]^+$; HRMS calcd for $C_{24}H_{24}O_4N_5$ $[M+H]^+$446.18228, found 446.18067.

(1-(3,4-dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((3,4dimethoxyphenyl)amino)pyridin-3-yl)methanone (6g)

This compound **6g** was prepared by method described in above general method employing 1-(2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14b**, 150 mg, 0.53 mmol) and 4-(azidomethyl)-1,2-dimethoxybenzene (**17b**, 102 mg, 0.53 mmol) to affords **6g** as a yellow solid. 194 mg; 77% yield; Mp: 148-150 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.97 (s, 1H), 9.40 (dd, *J* = 8.0, 1.6 Hz, 1H), 8.40 (dd, *J* = 4.5, 1.6 Hz, 1H), 8.11 (s, 1H), 7.31 – 7.26 (m, 1H), 7.19 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.97 – 6.83 (m, 4H), 6.79 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.53 (s, 2H), 3.90 (s, 6H), 3.87 (d, *J* = 2.3 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 185.59, 156.77, 154.29, 149.82, 149.64, 149.11, 148.98, 145.52, 143.94, 132.79, 128.18, 125.91, 121.32, 114.24, 112.94, 112.88, 111.51, 111.47, 111.42, 106.95, 56.18, 56.02, 55.92, 54.46; MS (ESI): *m/z* 476 $[M+H]^+$; HRMS calcd for $C_{25}H_{26}O_5N_5$ $[M+H]^+$476.19285, found 476.19090.

(1-(3,5-dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((3,4dimethoxyphenyl)amino)pyridin-3-yl)methanone (6h)

This compound **6h** was prepared by method described in above general method employing 1-(2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14b**, 150 mg, 0.53 mmol) and 1-(azidomethyl)-3,5-dimethoxybenzene (**17c**, 102 mg, 0.53 mmol) to affords **6h** as a yellow solid. 199 mg; 79% yield; Mp: 104-107 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.97 (s, 1H), 9.39 (dd, *J* = 8.0, 1.8 Hz, 1H), 8.40 (dd, *J* = 4.6, 1.8 Hz, 1H), 8.15 (s, 1H), 7.29 (d, *J* = 2.3 Hz, 1H), 7.20 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.87 (d, *J* = 8.6 Hz, 1H), 6.79 (dd, *J* = 8.0, 4.7 Hz, 1H), 6.46 (s, 3H), 5.52 (s, 2H), 3.90 (s, 3H), 3.88 (s, 3H), 3.78 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.54, 161.46, 156.76, 154.27, 149.13, 148.95, 145.49, 143.93, 135.66, 132.75, 128.40, 114.25, 112.92, 111.46, 106.92, 106.43, 100.62, 56.14, 55.89, 55.45, 54.52; MS (ESI): *m/z* 476 $[M+H]^+$; HRMS calcd for $C_{25}H_{26}O_5N_5$ $[M+H]^+$476.19285, found 476.19086.

2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)(1-(3-phenoxybenzyl)-1H-1,2,3-triazol-4-yl)methanone (6i)

This compound **6i** was prepared by method described in above general method employing 1-(2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14b**, 150 mg, 0.53 mmol) and 1-(azidomethyl)-3-phenoxybenzene (**17d**, 119 mg, 0.53 mmol) to affords **6i** as a yellow solid. 223 mg; 79% yield; Mp: 78-80 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.97 (s, 1H), 9.39 (dd, *J* = 8.0, 1.8 Hz, 1H), 8.40 (dd, *J* = 4.6, 1.8 Hz, 1H), 8.16 (s, 1H), 7.40 – 7.29 (m, 4H), 7.23 – 7.12 (m, 2H), 7.01 (dd, *J* = 13.0, 5.5 Hz, 5H), 6.87 (d, *J* = 8.6 Hz, 1H), 6.79 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.57 (s, 2H), 3.90 (s, 3H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.52, 158.33, 156.78, 156.24, 154.32, 149.20, 148.95, 145.52, 143.96, 135.49, 132.72, 130.73, 129.97, 128.36, 124.02, 122.64, 119.40,

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118.85, 118.21, 114.29, 112.94, 112.84, 111.46, 106.96, 56.15, 55.90, 54.13; MS (ESI): *m/z* 508 $[M+H]^+$; HRMS calcd for $C_{29}H_{26}O_4N_5$ $[M+H]^+$508.19793, found 508.19653.

(2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)(1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)methanone (6j)

This compound **6j** was prepared by method described in above general method employing 1-(2-((3,4-dimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14b**, 150 mg, 0.53 mmol) and 1-(azidomethyl)-4-fluorobenzene (**17e**, 73 mg, 0.53 mmol) to affords **6j** as a yellow solid. 174 mg; 79% yield; Mp: 108-110 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.96 (s, 1H), 9.40 (dd, *J* = 8.0, 1.7 Hz, 1H), 8.40 (dd, *J* = 4.5, 1.7 Hz, 1H), 8.13 (s, 1H), 7.34 (dd, *J* = 8.3, 5.2 Hz, 2H), 7.28 (d, *J* = 2.2 Hz, 1H), 7.17 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.11 (t, *J* = 8.4 Hz, 2H), 6.88 (d, *J* = 8.6 Hz, 1H),6.78, (q, *J* = 7.9,4.7 Hz, 1H), 5.59 (s, 2H), 3.89 (d, *J* = 5.4 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.49, 156.82, 154.38, 149.32, 149.01, 145.59, 143.95, 132.75, 130.40, 130.28, 129.54, 128.19, 116.59, 116.30, 114.32, 112.95, 112.83, 111.53, 107.03, 56.19, 55.93, 53.77; MS (ESI): *m/z* 434 $[M+H]^+$; HRMS calcd for $C_{23}H_{21}O_3N_5F[M+H]^+$434.16229, found 434.16050.

(1-(4-methoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)methanone (6k)

This compound **6k** was prepared by method described in above general method employing 1-(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14c**, 150 mg, 0.48 mmol) and 1-(azidomethyl)-4-methoxybenzene (**17a**, 78 mg, 0.48 mmol) to affords **6k** as a yellow solid yield. 166 mg; 73% yield; Mp: 99-100 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.04 (s, 1H), 9.42 (dd, *J* = 8.0, 1.9 Hz, 1H), 8.42 (dd, *J* = 4.6, 1.9 Hz, 1H), 8.10 (s, 1H), 7.32 – 7.29 (m, 2H), 7.01 (s, 2H), 6.95 – 6.92 (m, 2H), 6.83 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.55 (s, 2H), 3.88 (s, 6H), 3.83 (s, 3H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.77, 160.32, 156.48, 154.07, 153.24, 149.01, 144.03, 135.44, 134.23, 130.05, 128.18, 125.50, 114.76, 113.28, 113.19, 99.45, 60.97, 56.12, 55.40, 54.12; MS (ESI): *m/z* 476 $[M+H]^+$; HRMS calcd for $C_{25}H_{26}O_5N_5$ $[M+H]^+$476.19285, found 476.19111.

(1-(3,4-dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)methanone (6l)

This compound **6l** was prepared by method described in above general method employing 1-(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14c**, 150 mg, 0.48 mmol) and 4-(azidomethyl)-1,2-dimethoxybenzene (**17b**, 92 mg, 0.48 mmol) to affords **6l** as a yellow solid. 184 mg; 76% yield; Mp: 108-110 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 11.04 (s, 1H), 9.42 (d, *J* = 7.8 Hz, 1H), 8.43 (d, *J* = 3.0 Hz, 1H), 8.12 (s, 1H), 7.01 (s, 2H), 6.98 – 6.79 (m, 4H), 5.54 (s, 2H), 3.89 (d, *J* = 7.3 Hz, 12H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.73, 156.47, 154.11, 153.23, 149.87, 149.67, 149.03, 144.00, 135.42, 134.20, 128.25, 125.85, 121.35, 113.29, 113.17, 111.48, 99.43, 60.97, 56.12, 56.03, 54.49; MS (ESI): *m/z* 506 $[M+H]^+$; HRMS calcd for $C_{26}H_{28}O_6N_5$ $[M+H]^+$506.20341, found 506.20305.

(1-(3,5-dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)methanone (6m)

This compound **6m** was prepared by method described in above general method employing 1-(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14c**, 150 mg, 0.48 mmol) and 1-(azidomethyl)-3,5-dimethoxybenzene (**17c**, 92 mg, 0.48 mmol) to affords **6m** as a yellow solid. 174 mg; 72% yield; Mp: 101-103°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 11.04 (s, 1H), 9.42 (dd, *J* = 8.0, 1.9 Hz, 1H), 8.43 (dd, *J* = 4.6, 1.9 Hz, 1H), 8.16 (s, 1H), 7.01 (s, 2H), 6.84 (dd, *J* = 8.0, 4.7 Hz, 1H), 6.46 (s, 3H), 5.53 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.72, 161.51, 156.48, 154.15, 153.22, 149.09, 144.02, 135.56, 135.39, 128.43, 113.29, 113.15, 106.49, 100.66, 99.39, 60.97, 56.09, 55.47, 54.60; MS (ESI): *m/z* 506 $[M+H]^+$; HRMS calcd for $C_{26}H_{28}O_6N_5$ $[M+H]^+$506.20341, found 506.20205.

(1-(3-phenoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)methanone (6n)

This compound **6n**was prepared by method described in above general method employing 1-(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14c**, 150 mg, 0.48 mmol) and 1-(azidomethyl)-3-phenoxybenzene (**17d**, 108 mg, 0.48 mmol) to affords **6n** as a yellow solid. 209 mg; 81% yield; Mp: 105-107°C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.05 (s, 1H), 9.41 (dd, *J* = 8.0, 1.3 Hz, 1H), 8.43 (dd, *J* = 4.5, 1.6 Hz, 1H), 8.16 (s, 1H), 7.36 (td, *J* = 7.9, 2.3 Hz, 3H), 7.15 (t, *J* = 7.4 Hz, 1H), 7.06 – 6.97 (m, 7H), 6.84 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.58 (s, 2H), 3.88 (s, 6H), 3.84 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.66, 158.35, 156.47, 156.22, 154.16, 153.21, 149.12, 144.03, 135.42, 135.38, 134.11, 130.75, 129.98, 128.43, 124.04, 122.66, 119.41, 118.87, 118.23, 113.30, 113.12, 99.38, 60.97, 56.09, 54.17; MS (ESI): *m/z* 538 $[M+H]^+$; HRMS calcd for $C_{30}H_{28}O_5N_5$ $[M+H]^+$538.20850, found 538.20727.

(1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)methanone (6o)

This compound **6o**was prepared by method described in above general method employing 1-(2-((3,4,5-trimethoxyphenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14c**, 150 mg, 0.48 mmol) and 1-(azidomethyl)-3-phenoxybenzene (**17e**, 66 mg, 0.48 mmol) to affords **6o** as a yellow solid; 175 mg; 79% yield; Mp: 68-70°C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.04 (s, 1H), 9.44 – 9.38 (m, 1H), 8.43 (dd, *J* = 4.6, 1.9 Hz, 1H), 8.14 (s, 1H), 7.35 (dd, *J* = 8.5, 5.2 Hz, 2H), 7.12 (t, *J* = 8.6 Hz, 2H), 7.01 (s, 2H), 6.83 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.60 (s, 2H), 3.88 (s, 7H), 3.84 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.64, 156.49, 154.22, 153.23, 149.21, 144.02, 135.36, 130.43, 130.32, 129.51, 128.27, 116.61, 116.32, 113.31, 113.09, 99.44, 60.99, 56.11, 53.80; MS (ESI): *m/z* 464 $[M+H]^+$; HRMS calcd for $C_{24}H_{23}O_4N_5F[M+H]^+$464.17286, found 464.17094.

(2-((4-fluorophenyl)amino)pyridin-3-yl)(1-(4-methoxybenzyl)-1H-1,2,3-triazol-4-yl)methanone (6p)

This compound **6p** was prepared by method described in above general method employing 1-(2-((4-

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fluorophenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14d**, 150 mg, 0.62 mmol) and 1-(azidomethyl)-4-methoxybenzene (**17a**, 101 mg, 0.62 mmol) to affords **6p** as a yellow solid; 193 mg; 77% yield; Mp: 148-150 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.01 (s, 1H), 9.41 (dd, *J* = 8.0, 1.9 Hz, 1H), 8.39 (dd, *J* = 4.6, 1.7 Hz, 1H), 8.09 (s, 1H), 7.66 – 7.61 (m, 2H), 7.32 – 7.28 (m, 2H), 7.07 – 7.01 (m, 2H), 6.96 – 6.91 (m, 2H), 6.82 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.54 (s, 2H), 3.82 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.72, 160.56, 160.25, 157.35, 156.44, 154.00, 148.98, 143.98, 135.36, 135.33, 130.04, 128.19, 125.48, 123.44, 123.34, 115.59, 115.29, 114.72, 113.39, 113.07, 55.39, 54.09; MS (ESI): *m/z* 404 $[M+H]^+$; HRMS calcd for $C_{22}H_{19}O_2N_5F[M+H]^+$404.15173, found 404.15112.

(1-(3,4-dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((4-fluorophenyl)amino)pyridin-3-yl)methanone (6q)

This compound **6q** was prepared by method described in above general method employing 1-(2-((4-fluorophenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14d**, 150 mg, 0.62 mmol) and 4-(azidomethyl)-1,2-dimethoxybenzene (**17b**, 120 mg, 0.62 mmol) to affords **6q** as a yellow solid; 213 mg; 79% yield; Mp: 175-176 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.01 (s, 1H), 9.41 (dd, *J* = 8.0, 1.9 Hz, 1H), 8.39 (dd, *J* = 4.6, 1.9 Hz, 1H), 8.12 (s, 1H), 7.66 – 7.61 (m, 2H), 7.07 – 7.02 (m, 2H), 6.94 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.89 (d, *J* = 8.2 Hz, 1H), 6.86 (d, *J* = 1.9 Hz, 1H), 6.83 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.54 (s, 2H), 3.90 (s, 3H), 3.87 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.67, 160.54, 157.33, 156.43, 154.02, 149.79, 149.59, 143.94, 128.23, 125.82, 123.42, 123.32, 121.30, 115.58, 115.28, 113.38, 111.40, 111.34, 55.99, 54.46; MS (ESI): *m/z* 434 $[M+H]^+$; HRMS calcd for $C_{23}H_{21}O_3N_5F$ $[M+H]^+$ 434.16052, found 434.16048.

(1-(3,5-dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)(2-((4-fluorophenyl)amino)pyridin-3-yl)methanone (6r)

This compound **6r** was prepared by method described in above general method employing 1-(2-((4-fluorophenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14d**, 150 mg, 0.62 mmol) and 1-(azidomethyl)-3,5-dimethoxybenzene (**17c**, 120 mg, 0.62 mmol) to affords **6r** as a yellow solid; 202 mg; 75% yield; Mp: 150-151 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.01 (s, 1H), 9.41 (dd, *J* = 8.0, 1.8 Hz, 1H), 8.40 (dd, *J* = 4.5, 1.7 Hz, 1H), 8.16 (s, 1H), 7.64 (dt, *J* = 6.9, 4.4 Hz, 2H), 7.07 – 7.01 (m, 2H), 6.83 (dd, *J* = 8.0, 4.7 Hz, 1H), 6.46 (s, 3H), 5.52 (s, 2H),3.79 (d, *J* = 4.9 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.65, 161.48, 160.57, 156.46, 154.03, 143.96, 135.60, 135.35, 135.32, 128.45, 123.47, 123.36, 115.59, 115.29, 113.39, 106.44, 100.64, 55.46, 54.57; MS (ESI): *m/z* 434 $[M+H]^+$; HRMS calcd for $C_{23}H_{21}O_3N_5F$ $[M+H]^+$434.16082, found 434.16084.

(2-((4-fluorophenyl)amino)pyridin-3-yl)(1-(3-phenoxybenzyl)-1H-1,2,3-triazol-4-yl)methanone (6s)

This compound **6s** was prepared by method described in above general method employing 1-(2-((4-fluorophenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14d**, 150 mg, 0.62 mmol) and 1-(azidomethyl)-3-phenoxybenzene (**17d**, 140 mg, 0.62 mmol) to affords **6s** as a yellow solid; 241 mg; 83% yield; Mp: 100-102 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.02 (s, 1H), 9.41 (dt, *J* = 8.0, 1.7 Hz, 1H), 8.40 (dd, *J* = 4.7, 1.9 Hz, 1H), 8.16 (s, 1H), 7.68 – 7.62 (m, 2H), 7.39 – 7.33 (m, 3H), 7.17 – 7.13 (m, 1H), 7.07 – 6.96 (m, 7H), 6.83 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.58 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.66, 160.62, 158.39, 157.41, 156.51, 156.29, 154.09, 149.17, 143.99, 135.49, 135.37, 130.77, 129.99, 128.44, 124.05, 123.50, 123.40, 122.65, 119.43, 118.91, 118.24, 115.61, 115.31, 113.42, 113.08, 54.19; MS (ESI): *m/z* 466 $[M+H]^+$; HRMS calcd for $C_{27}H_{21}O_2N_5F[M+H]^+$466.16738, found 466.16571.

(1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)(2-((4-fluorophenyl)amino)pyridin-3-yl)methanone (6t)

This compound **6t** was prepared by method described in above general method employing 1-(2-((4-fluorophenyl)amino)pyridin-3-yl)prop-2-yn-1-one (**14d**, 150 mg, 0.62 mmol) and 1-(azidomethyl)-4-fluorobenzene(**17e**, 86 mg, 0.62 mmol) to affords **6t** as a yellow solid; 178 mg; 73% yield; Mp: 171-172 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.01 (s, 1H), 9.41 (dd, *J* = 8.0, 1.9 Hz, 1H), 8.40 (dd, *J* = 4.6, 1.9 Hz, 1H), 8.14 (s, 1H), 7.67 – 7.61 (m, 2H), 7.35 (dd, *J* = 8.6, 5.2 Hz, 2H), 7.14 – 7.08 (m, 2H), 7.07 – 7.01 (m, 2H), 6.82 (dd, *J* = 8.0, 4.7 Hz, 1H), 5.59 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.58, 164.77, 161.47, 160.61, 157.40, 156.49, 154.09, 149.22, 143.94, 135.37, 130.38, 130.27, 129.58, 129.54, 128.29, 123.47, 123.37, 116.58, 116.29, 115.59, 115.30, 113.39, 113.04, 53.77; MS (ESI): *m/z* 392 $[M+H]^+$; HRMS calcd for $C_{21}H_{16}ON_5F_2[M+H]^+$392.13174, found 392.13156.

Biology

Anticancer activity

The cytotoxicity activity of the compounds was determined using MTT assay. 1×10^4 cells/well were seeded in 200 µl DMEM, supplemented with 10% FBS in each well 96 well microculture plates and incubated for 24h 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 of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium 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 wavelengths was recorded.

Cell cycle analysis

Flow cytometric analysis (FACS) was performed to evaluate the distribution of the cells through the cell cycle phases. Human prostate cancer cells (DU-145) were incubated with compounds (**6q**, **6r** and **6s**)at 3 µM concentrations for 48 h. Untreated and treated cells were harvested, washed with PBS, fixed in ice-cold 70% ethanol and stained with propidium iodide (Sigma Aldrich). Cell cycle was performed by flow cytometry (Becton Dickinson FACS Caliber) as earlier described.

***In Vitro*Tubilin polymerization assay**

A fluorescence based in vitro tubulin polymerization assay was

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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 5 µM). Tubulin polymerizationwas 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.). E7010 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 various concentrations. Polymerization was monitored by increase in the Fluorescence as mentioned above at 37 °C.

Immunohistochemistry

DU-145 cells were seeded on glass cover slips, incubated for 48 h in the presence or absence of test compounds **6q**, **6r** and **6s** at 3 µMconcentration. Following the termination of incubation, cells were fixed with 3% paraformaldehyde, 0.02% glutaraldehyde in PBS and permeabilized by dipping the cells in 100% methanol followed by overnight incubation at 4°C. Later, cover slips were blocked with 1% BSA in phosphate buffered saline for 1 h followed by incubation with a primary anti tubulin (mouse monoclonal) antibody and FITC conjugated secondary mouse anti IgG antibody. Photographs were taken using the fluorescence microscope, equipped with FITC settings and the pictures were analyzed for the integrity of microtubule network. In parallel experiments, E7010 (3 µM) was used as positive control for analyzing microtubule integrity.

Competitive tubulin-binding assay

For colchicine competitive binding assay, tubulin was coincubated with indicated concentrations of MPSP-001 and taxol at 37 °C for 1 h. Then colchicine was added to a final concentration of 5 µmol/L. Fluorescence was determined using a Hitachi F-2500 spectrofluorometer (Tokyo, Japan) at excitation wavelengths of 365 nm and emission wavelengths of 435 nm. Blank values (buffer alone) as background were subtracted from all samples. Then the inhibition rate (IR) was calculated as follows: IR=F/F0 where F0 is the fluorescence of the 5 µmol/L colchicine-tubulin complex, and F is the fluorescence of a given concentration of E7010 or taxol (12.5µmol/L, 25 µmol/L, 50 µmol/L and 100 µmol/L) competition with the 5 µmol/L colchicine-tubulin complex. taxol, not binding in the colchicine-site of tubulin, was added as a negative control.[48]

Analysis of soluble versus polymerized tubulin in cells:

The cellular tubulin polymerization was quantified by a modified method which was originally described by Minottiet al.[49] Cultured DU-145 cells were treated with 3µM of compounds for 48 h. Then the cells were washed twice with PBS and harvested by trypsinization. Cells were lysed at 37 °C for 5 min in the dark with 100 µL of hypotonic lysis buffer (1 mM$MgCl_2$, 2 mM EGTA, 0.5% NP-40, 20 µg/mL apportioning, 20µg/mL eupeptic, 1 mMorthovanadate, 2 mM PMSF, and 20 mMTris-HCl, and pH 6.8). After a brief but vigorous vortex, the samples were centrifuged at 14000 rpm (21000 g) for 10 min. The 100 µL supernatants containing soluble (cytosolic) tubulin were separated from the pellets containing polymerized (cytoskeleton) tubulin. The pellets were resuspended in 100 µL of lysis buffer. The total concentrations of proteins in the soluble fraction and pellet fraction were estimated separately by the Bradford method. Equal amounts (50 µg) of each sample were added with SDS polyacrylamide gel electrophoresis sample buffer and run in a 10% SDS polyacrylamide gel. The sample was then analyzed by Western blotting and probed with the antibody against α-tubulin.

Hoechst staining

Cells were seeded at a density of 10,000 cells over 18-mm cover slips and incubated for 24 h. After incubation, cells were treated with the compounds **6q**, **6r** and **6s** at 3 µM concentration for 24 h. Hoechst 33258 (Sigma Aldrich) was added to the cells at a concentration of 0.5 mg/mL and incubated for 30 min at 37°C. Later, cells were washed with phosphate buffered saline (PBS). Cells from each cover slip were captured from randomly selected fields under fluorescent microscope (Leica, Germany) to qualitatively determine the proportion of viable and apoptotic cells based on their relative fluorescence and nuclear fragmentation.

Caspase -3 Assay

There are some reports that the cell cycle arrest at G2/M phase takes place by the induction of cellular apoptosis. Hence, it was considered of interest to understand the correlation of cytotoxicity with that to apoptosis by compounds **6q**, **6r**, **6s** and E7010. 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 DU-145 cells were treated with compounds **6q**, **6r** and **6s** along with the positive control E7010 and examined the activation of caspase-3. The results indicate that there is nearly 4 to 9 fold induction in caspase-3 activity in cells treated with 3 µM concentration by these compounds. Interestingly under similar conditions, E7010 (3 µM) also induced the caspase activity .Therefore activation of caspase-3 by **6q**, **6r** and **6s** indicate that they have the capacity to induce apoptosis in DU-145 cells.

Flow cytometric evaluation of apoptosis

DU-145 (1×10^6) were seeded in six-well plates and allowed to grow overnight. The medium was then replaced with complete medium containing 3 µM concentration of compounds, **6q**, **6r** and **6s** for 48 h along with vehicle alone (0.001% DMSO) as control. After 48 h of drug 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. [50] Annexin V-FITC was analyzed using excitation and emission settings of 488 nm and 535 nm (FL-1 channel); PI, 488 nm and 610 nm (FL-2 channel). Debris and

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clumps were gated out using forward and orthogonal light scatter.

Mesurement of Mitochondrial Membrane Potential

The mitochondrial membrane potential was measured with the lipophilic cationic dye JC-1 (Molecular Probes), as described.[51]Cultures were treated with the test drugs for 48 h. After drug treatment the cells were incubated with JC-1 dye for 20 min at 37 °C. After incubation cultures were used for the measuring mitochondrial membrane potiential (ΔΨm), according to the manufacturer's instructions. The shift of the membrane potential1 was measured by flow cytometry using JC-1 (Molecular Probes), as previously described.

DNA fragmentation analysis

Cells were seeded (1×10^6) in six well plates and incubated for 24 h. After incubation, cells were treated with compounds **6q**, **6r** and **6s** at 3 µM concentration for 48 h. After 48 h of drug treatment cells were collected and centrifuged at 2500 rpm for 5 min at 4 °C. Pellet was collected and washed with Phosphate buffered saline (PBS), added 100 µL of Lysis buffer, centrifuged at 3000 rpm for 5 min at 4 °C and collected supernant. And add 10 µL of 10% SDS and 10 µL of (50 mg/mL) RNase-A and incubated for 2 h at 56 °C. After that 10 µL of Proteinase K (25 mg/mL) was added and incubated at 37 °C for 2 h. After incubation, 65 µL of 10 M Ammonium acetate and 500 µl of ice cold ethanol was added and mixed well. And this sample was incubated at 80 °C for 1 h. After that samples were centrifuged at 12000 rpm for 20 min at 4 °C and washed with 80% ethanol followed by air dried for 10 min at room temperature. Dissolved pellet in 50 µLof TE buffer. After that, DNA laddering was determined by 2% agarose gel electrophoresis.

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