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ARTICLE TYPE

Aryl-Imidazothiadiazole Analogues as Microtubule Disrupting Agents

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Two series of 2-ethyl -6-(3',4',5'-trimethoxyphenyl)-5-aryl and 2-cyclopropyl -6-(3',4',5'-trimethoxyphenyl)-5-aryl-imidazothiadiazoles were designed, synthesized and evaluated for anti-proliferative activity in various human cancer cell lines. A common starting material 2-bromo-1-(3-,4-,5-trimethoxyphenyl)-ethanone was employed to generate the two scaffolds. Among them, compounds functionalized with N,N-dimethyl aniline (**5i** and **6i**) and napthyl (**5k**) groups showed significant cytotoxic activity with an average IC_{50} values of 1.7-2.9 μM respectively. Moreover, structure-activity relationship was elucidated by incorporating the different substitutions on the aryl moiety at the 5th-position of the imidazothiadiazole ring. Treatments with **5i**, **6i** and **5k** arrested cells at G2/M phase, with cell death proceeding through an apoptotic pathway that was dependent on activation of caspase-3. Immunocytochemistry analysis revealed that loss of intact microtubule structures in treated cells. Treatments with **5i**, **6i** and **5k** manifested that an increased mRNA and protein levels of the G2/M marker, Cyclin B1. Molecular docking studies of the most potent compounds **5i**, **5k** and **6i** showed that these compounds interact and bind efficiently to the active site of tubulin. This was further confirmed by a colchicine competitive binding assay.

Introduction

The microtubule system of eukaryotic cells is a critical element in a variety of fundamental cellular processes such as cell division, formation and maintenance of cell shape, regulation of motility, cell signaling, secretion and intracellular transport.[1] Among the various strategies developed to block mitosis, microtubules represent an attractive target for numerous small natural and synthetic molecules that inhibit the formation of the mitotic spindle.[2] A variety of compounds, such as vinca alkaloids or colchicine (**1**) acts on microtubules or tubulin to disrupt the cellular microtubule structure and cause mitotic arrest (Figure **1**).[3] Combretastatin A-4 (**2a**) is one of the most potent antimitotic agent that strongly inhibits tubulin polymerization by binding to the colchicine site.[4,5] CA-4 shows potent cytotoxicity against a wide variety of human cancer cell lines, including those that are multidrug resistant.[6] Moreover, CA-4 is also structurally similar to colchicine and possesses a higher affinity for the colchicine binding site on tubulin.[7] However, the low water solubility of CA-4 limits its *in vivo* efficacy. A water-soluble sodium phosphate prodrug (CA-4P **2b**), is currently in phase III trials for the treatment of anaplastic thyroid cancer.[8]

Previous SAR studies have demonstrated that both the 3',4',5'-trimethoxy substitution pattern on the A-ring and the *cis*-olefin configuration at the bridge were fundamental requirements for maximize activity in a large series of inhibitors of tubulin polymerization,[9] while some B-ring structural modifications were tolerated by the target.[10] However, the *cis*-configuration of CA-4 is prone to isomerize to the thermodynamically more stable *trans*-form during storage and metabolism, resulting in the dramatic decrease in the antitumor activity.[11] Thus, to retain the appropriate geometry of the two adjacent aryl groups required for potent bioactivity; chemically stable *cis*-restricted derivatives of CA-4 were obtained by incorporating the olefinic double bond into vicinally diaryl-substituted five-member aromatic heterocyclic rings (**3**). The moieties such as pyrazole, oxazole, imidazole, thiazole, 1,2,3,4-tetrazole, furazan, 1,2,5-oxadiazole, isoxazole, 1,2,3-thiadiazole and triazole were employed to generate various congeners of stable trans-form of CA-4.[12-18] Nitrogen-bridge head fused heterocycles containing an imidazole ring are a common structural moiety in many pharmacologically important molecules that display a wide range of activities for diverse number of targets. The imidazo[2,1-*b*][1,3,4]thiadiazole (**4**) exhibits wide spectrum of biological activities, they act as antibacterial,[19] antifungal,[20] antitubercular[21] and anticancer inhibitors.[22-26] Our earlier efforts toward the discovery of new synthetic small molecules led to the development of a numerous hybrids/analogues as potent cytotoxic agents.[27-30] Therefore in the present study, design and synthesis of imidazo[2,1-*b*][1,3,4]thiadiazole as the core moiety was performed. Previous reports indicate that imidazothiadiazoles exhibit tubulin polymerization inhibition and show G2/M cell cycle arrest.[31]

Based on these observations we describe modifications on CA-4 scaffold which contain trimethoxyphenyl moiety identical to the A-ring of CA-4. Also incorporation of ethyl or cyclopropyl imidazothiadiazole moieties by replacement of *cis* restricted olefinic double bond and the B-ring of CA-4 was performed. Further congeners were generated with a naphth-2-yl moiety or electron-withdrawing (CF_3, OCF_3) or electron-releasing (Me, Et, OMe) substituents on the 5th-position of the imidazothiadiazole ring. Thus the resultant moiety possesses an olefin bridge of imidazothiadiazole that functions to analogue A-ring of CA-4, and B-ring of aryl moieties (**5a-p** and **6a-p**). Thus the rationale of these moieties was to conserve the privileged structures of trimethoxyphenyl and imidazothiadiazole groups. In addition, these groups can also serve as anchors that could be harnessed for further diversification. Based on results obtained from the SRB assay, the most cytotoxic analogues (**5i**, **5k** and **6i**) were used for further mechanistic studies, such as tubulin polymerization inhibition, immunohistochemistry and apoptosis induction. In addition, these analogues were also investigated for competitive binding assay, as well as molecular docking studies.

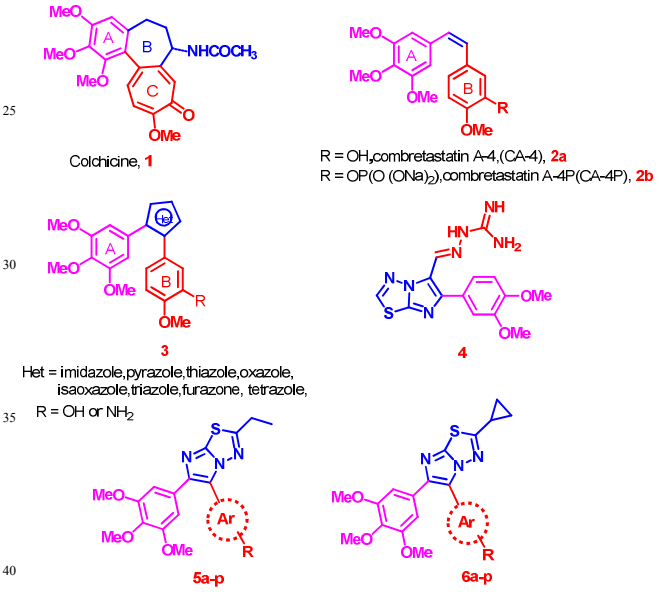
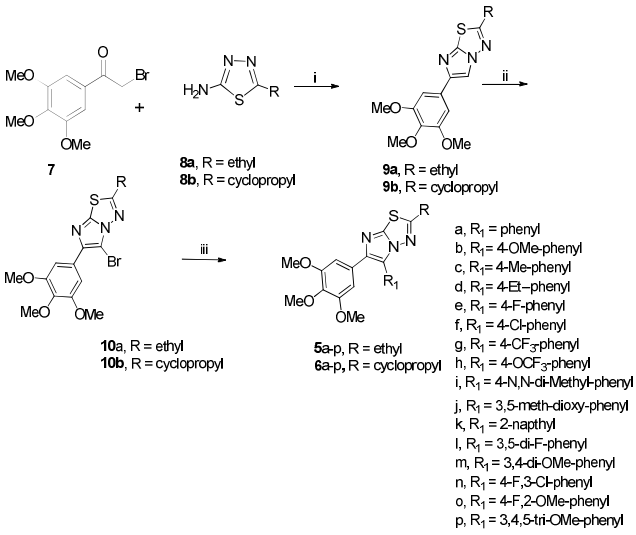


Fig 1: Structures of anticancer agents of tubulin polymerization inhibitors (1[3], 2a[4-6], 2b[8], 3[12-18]) and imidazothiadiazole guanylhydrazone derivative (4)[31] and target molecules (5a-p) & (6a-p).

Results and Discussion

Chemistry

The synthetic protocol employed for the preparation of 2-ethyl/cyclopropyl -6-(3',4',5'-trimethoxyphenyl)-5-aryl-imidazothiadiazoles analogues is shown in Scheme 1. 2-ethyl/cyclopropyl -6-(3',4',5'-trimethoxyphenyl)-5-aryl-imidazothiadiazole was easily obtained in good yield by condensation of 2-bromo-1-(3-,4-,5-trimethoxyphenyl)-ethanone (**7**) and substituted 2-aminothiadiazoles (**8a** and **8b**) in refluxing ethanol to afford 2-ethyl/cyclopropyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazoles **9a** and **9b**. These upon bromination with NBS in $CHCl_3$ yielded 5-bromo imidazothiadiazoles (**10a** and **10b**) respectively. The target compounds (**5a-p** and **6a-p**) were obtained by Suzuki cross-coupling conditions with **10a** and **10b** in the presence of the appropriate arylboronic acids under heterogeneous conditions [$PdCl_2(PPh_3)_2$], Cs_2CO_3 as the base in DME at 110 °C in good yields which were characterized by ^{1}H NMR, ^{13}C NMR, mass and HRMS spectral analysis.



Scheme 1 Reagents and conditions (i) EtOH, reflux, 12 h; (ii) NBS, $CHCl_3$, 0 °C to rt, 3 h; (iii) ArB(OH)$_2$, $Pd(PPh_3)_2Cl_2$, Cs_2CO_3, DME, 110 °C, 12 h.

Biology

Cytotoxic activity

To explore the cytotoxicity of these ethyl/cyclopropyl 6-(3',4,'5'-trimethoxyphenyl)-5-aryl imidazothiadiazoles (**5a-p** and **6a-p**), an SRB assay was performed against a panel of four human cancer cell lines (MIAPaCa-2, MCF-7, HeLa and DU-145) using CA-4[32,33] (**3a**) as the reference compound, IC_{50} values obtained are listed in Table **1**. Among the thirty two (32) analogues, some of them like **5a**, **5e**, **5i**, **5k**, **6b** and **6i** showed strong inhibitory effect. Structure-activity relationship studies showed that there was a considerable influence of substituents on 5-position of ring B and insignificant effect of the substituents on imidazothiadiazole ring on cytotoxicity.

Compound **5k** with 5-(naphth-2-yl) substitution exhibited most promising antiproliferative activity with an IC_{50} value 1.7 µM as an average against the cell lines, whereas, **5i** and **6i** possessing *N*,*N*-dimethylaniline substitution showed IC_{50} values ranging between 1.9-3.4 µM and 2.5-3.45 µM against MIAPaCa-2, MCF-7, HeLa and DU-145 cells respectively. EWGs at the C5 position of imidazothiadiazole ring bestowed better activity than ERGs with few exceptions wherein **5i** and **6i** containing *N*,*N*-dimethylaniline substitution showed better activity. Among the ERGs, analogues **5i** and **5k** showed better activity, but **5d**, **5j** and **5m** exhibit similar activity in all cell lines with exceptions. Presence of additional of methoxy groups as in **5j**, **5m** and **5p** moderately enhanced the potency. However presence of another halogen atom (**5l** and **5n**) leads to a loss of activity. Compounds with different disubstitutions on the phenyl ring such as in **5o** manifested low inhibitory activity suggesting that multi-substitution on the phenyl ring had a negative effect. Interestingly, replacement of phenyl group (**5a, 6a**) with napthylgroup (**5k, 6k**) substantially improved the activity probably due to the inherent steric factors of naphthyl ring.

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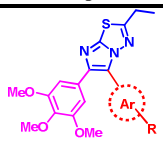
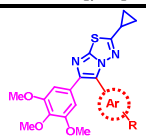
Table 1 Cytotoxicity of compounds **5a-p** and **6a-p** on MIA PaCa-2, MCF-7 and HeLa human cancer cell lines as IC_{50} [μM][a]



5a-p



6a-p

Entry	R	MIA PaCa-2	MCF-7	HeLa	DU-145	Entry	R	MIA PaCa-2	MCF-7	HeLa	DU-145
5a	Phenyl	13.4±0.2	11.5±0.1	10.8±0.5	14.56 ± 0.12	**6a**	Phenyl	31.5±0.4	42.3±0.5	35.4±0.1	25.57 ± 0.61
5b	4-OMe-Phenyl	30.5±0.7	25.4±0.21	21.6±0.4	25.4 ± 0.25	**6b**	4-OMe-Phenyl	16.9±0.5	11.6±0.15	14.6±0.3	10.45 ± 0.5
5c	4-Me-Phenyl	20.4±0.8	16.7±0.6	14.8±0.7	21.72 ± 0.53	**6c**	4-Me-Phenyl	29.8±0.1	26.1±0.6	20.5±0.6	18.49 ± 0.1
5d	4-Et-Phenyl	14.6±0.21	12.5±0.18	12.4±0.14	24.35 ± 0.22	**6d**	4-Et-Phenyl	39.8±0.5	29.3±0.23	36.2±0.9	13.47 ± 0.62
5e	4-F-Phenyl	10.5±0.16	9.8±0.7	11.3±0.6	16.6 ± 0.3	**6e**	4-F-Phenyl	27.6±0.1	19.6±0.4	15.6±0.3	15.06 ± 0.18
5f	4-Cl-Phenyl	15.4±0.6	12.2±0.4	13.7±0.08	19.11 ± 0.14	**6f**	4-Cl-Phenyl	17.4±0.09	22.9±0.8	19.2±0.21	11.61 ± 0.2
5g	4-CF_3-Phenyl	14.8±0.09	11.8±0.14	10.5±0.23	18.28 ± 0.46	**6g**	4-CF_3-Phenyl	23.4±0.1	18.4±0.27	43.5±0.56	15.21 ± 0.32
5h	4-OCF_3-Phenyl	16.4±0.5	14.8±0.09	13.4±0.16	23.53 ± 0.2	**6h**	4-OCF_3-Phenyl	18.2±0.6	16.8±0.15	11.3±0.72	12.97 ± 0.4
5i	4-$N(CH_3)_2$-Phenyl	2.5±0.09	1.9±0.04	2.2±0.01	2.75 ± 0.15	**6i**	4-$N(CH_3)_2$-Phenyl	2.5±0.1	3.4±0.03	2.9±0.06	3.45 ± 0.25
5j	3,5-(OCH_2O)-Phenyl	13.1±0.64	11.5±0.3	8.7±0.24	18.82 ± 0.13	**6j**	3,5-(OCH_2O)-Phenyl	19.4±0.3	25.6±0.42	16.8±0.24	12.78 ± 0.31
5k	2-Napthyl-Phenyl	1.2±0.03	2.4±0.01	1.6±0.02	4.49 ± 0.1	**6k**	2-Napthyl-Phenyl	21.6±0.34	15.3±0.7	19.4±0.54	45.18 ± 0.3
5l	3,5-DiF-Phenyl	30.6±0.42	36.4±0.25	26.4±0.5	21.63 ± 0.2	**6l**	3,5-DiF-Phenyl	29.6±0.5	23.4±0.16	17.3±0.46	16.88 ± 0.16
5m	3,4-$(OCH_3)_2$-Phenyl	14.6±0.1	11.4±0.15	14.6±0.1	13.26 ± 0.11	**6m**	3,4-$(OCH_3)_2$-Phenyl	19.6±0.7	12.9±0.42	15.6±0.7	16.56 ± 0.45
5n	3-F,4-Cl-Phenyl	17.3±0.4	14.5±0.07	13.9±0.6	11.39 ± 0.44	**6n**	3-F,4-Cl-Phenyl	20.7±0.4	18.7±0.2	15.5±0.6	13.65 ± 0.2
5o	2-OMe,4-F-Phenyl	29.1±0.35	30.2±0.5	28.1±0.4	35.18 ± 0.6	**6o**	2-OMe,4-F-Phenyl	40.9±0.8	34.8±0.6	25.9±0.35	22.37 ± 0.32
5p	3,4,5-$(OCH_3)_3$-Phenyl	19.6±0.8	20.4±0.3	17.5±0.4	13.4 ± 0.26	**6p**	3,4,5-$(OCH_3)_3$-Phenyl	39.6±0.2	36.5±0.4	13.7±0.13	52.1 ± 0.51
CA-4	-	0.087±0.001	0.134±0.004	0.150±0.001	0.12 ± 0.003	-	-	-	-	-	-

[a]Cell lines were treated with various concentrations of compounds for 48 h as described in the Experimental Section. Cell viability was measured using the SRB assay. IC_{50} values are given as the mean ± SD of three independent experiments.

On the other hand, no consistent effects of the substituents on imidazothiadazole ring on antiproliferative activity were observed. Taken together, the SAR studies of both the series conclude that the order of anti-proliferative activities elucidated from four-cell line screen were **5k** > **5i** > **6i**.

Effect on tubulin polymerization

The inhibition of tubulin polymerization is one of the goals for cancer therapeutics.[34,35] The 2-ethyl or cyclopropyl 6-(3', 4,'5'-trimethoxyphenyl)-5-aryl imidazothiadiazoles, such as **5i**, **5k** and **6i** potently inhibited cell growth. Since these analogues have a subunit similar to the CA-4 moiety in their structure, it was considered apt to examine their ability to inhibit the tubulin polymerization. These imidazothiadiazoles **5i**, **5k** and **6i** were employed for tubulin assembly assays, wherein CA-4

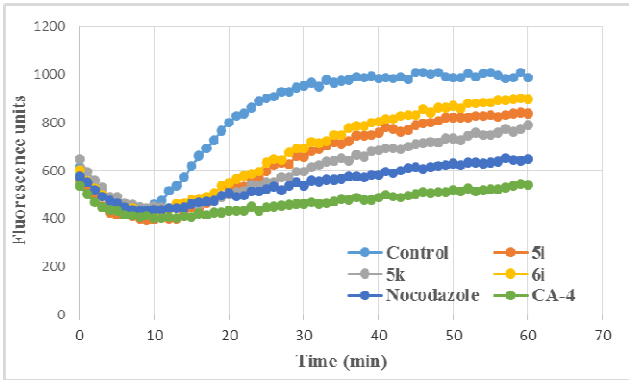


Fig. 2 Effect of compounds on tubulin polymerization. Inhibition of tubulin polymerization in presence of the compounds **5i**, **5k** and **6i** at a

concentration of 5 μM were determined employing an *in vitro* tubulin polymerization assay.

and nocodazole were used as positive controls. These analogues inhibited the tubulin polymerization at 5 μM concentration in a significant manner of **5i** (66.8%), **5k** (75.1%) and **6i** (60.9%) inhibited tubulin polymerization (Figure **2**). The positive controls exhibited remarkable inhibition tubulin assembly with CA-4 (87.2 %) and nocodazole (79.2 %), (Figure **2**). Furthermore, these three potential analogues were evaluated for their tubulin polymerization inhibition at different concentrations. They showed potent inhibition of tubulin polymerization with IC_{50} values 3.1, 1.7 and 3.8 μM respectively, as shown in Table **2**. Whereas, CA-4 showed IC_{50} values of 1.1 μM respectively.

Table **2**. Inhibition of tubulin polymerization (IC_{50}) of analogues **5i, 5k and 6i** and CA-4.

Compound	(IC_{50} in μM)
5i	3.1 ± 0.2
5k	1.7 ± 0.3
6i	3.8 ± 0.6
CA-4	1.1±0.01

[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.

Effect on cell cycle

The agents that inhibit the polymerization of tubulin stall cell cycle at the G2/M phase, thus preventing further cell division or subsequently resulting in mitotic slippage, which eventually leads to mitotic catastrophe or apoptosis.[36]

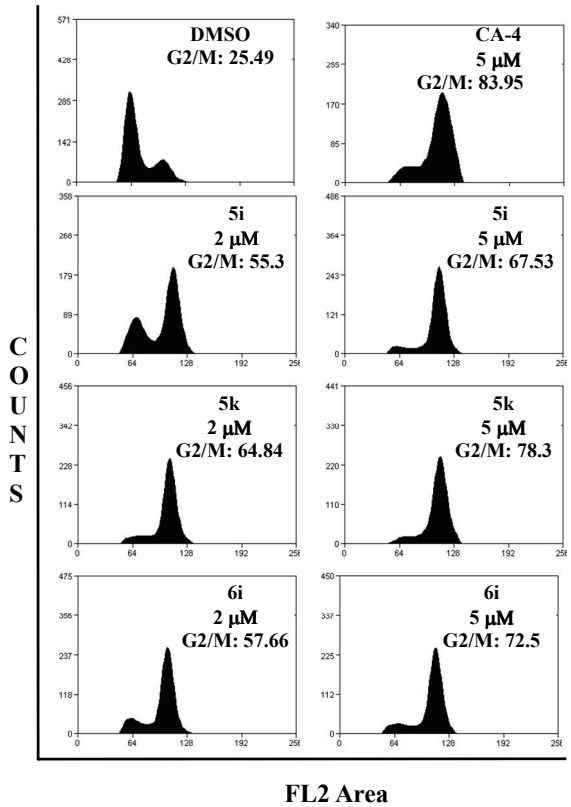


Fig. 3 Anti-mitotic effects of **5i**, **5k** and **6i** by FACS analysis: Induction of cell cycle G2/M arrest by compounds **5i**, **5k** and **6i**. HeLa cells were harvested after treatment at 5 μM and for 24 h. DMSO treated cells served as controls. The percentage of cells in each phase of cell cycle was quantified by flow cytometry.

To evaluate this possibility, HeLa cells were treated in a dose-dependent manner with **5i**, **5k** and **6i** for 24 h, and interestingly, they arrested cells at G2/M phase. Moreover, increased accumulation of cells at G2/M phase was observed with increasing concentration of these compounds. Therefore, these results suggest that **5i**, **5k** and **6i** arrest cells at G2/M phase through inhibition of tubulin assembly (Figure **3**).

Effect on microtubule network

Microtubule depolymerizing agents cause improper chromosome separation by inhibiting the organization of the mitotic spindle, and predominantly arrest chromosomes in metaphase of mitosis.[37] Since these imidazothiadiazoles exhibit significant inhibition of tubulin assembly and arrest cells at G2/M phase of the cell cycle, we investigated their ability to alter the microtubule network. Therefore, it was of interest to examine the intracellular effect of **5i**, **5k** and **6i** by monitoring cellular microtubules, as well as nuclear condensation. HeLa cells were treated with 5 μM of these compounds for 24 h cells upon staining and showed severe disruption in microtubule organization. CA-4 was employed as the positive control, however, vehicle-control/DMSO treated cells manifested normal microtubule network as shown in Figure **4**.

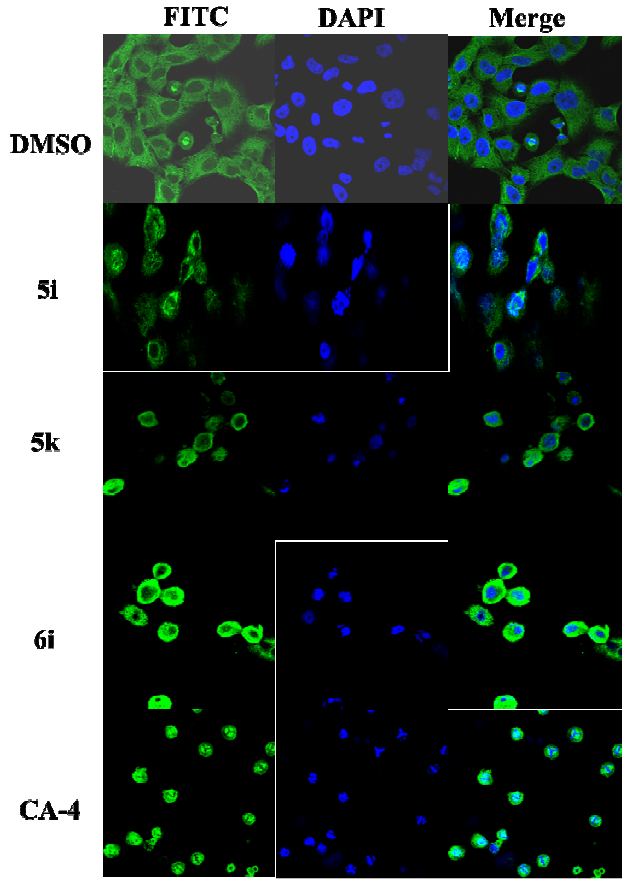


Fig. 4 Effect of **5i**, **5k** and **6i** on microtubules and nuclear condensation HeLa cells were independently treated with **5i**, **5k** and **6i** at 5 μM concentration for 24 h. Following the termination of experiment, cells were fixed and stained for tubulin. DAPI was used as counter stain. The merged images of cells stained for tubulin and DAPI are represented.

Colchicine competitive binding assay

Tubulin targeting agents are classified by the region of the protein to which they bind. The colchicine binding site, the taxol binding site, and the Vinca alkaloid domain are established binding regions. As CA-4 elicit potential cytotoxicity by binding at the colchicine binding site, it was considered of interest to determine the binding of the most active compound **5k** on this site. Therefore, a fluorescence based colchicine competitive binding assay was performed by comparing CA-4 as positive and taxol as negative controls.[38] Tubulin (3 μM) was incubated with the various concentrations of compound **5k**, CA-4 and taxol in the presence of colchicine (3 μM) at 37 ºC for 60 min and the fluorescence of tubulin–colchicine complex was monitored. As shown in Figure **5**, the fluorescence intensity of tubulin–colchicine complex was significantly decreased with increasing the concentration of **5k** indicating this compound competitively binds at the colchicine binding site of the tubulin. However, the intensity of fluorescence does not alter in the case of paclitaxel a negative control that binds at taxol binding site.

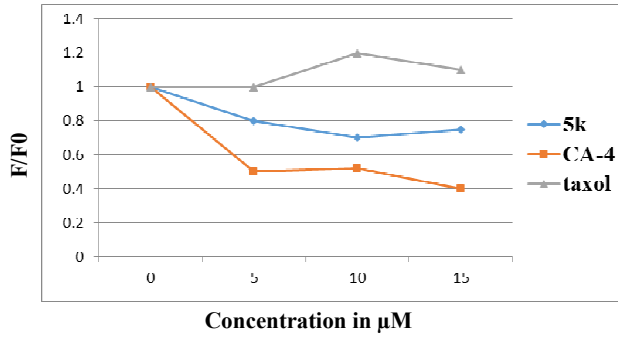


Fig. 5 **5k** binding to tubulin directly and inhibiting tubulin polymerization. Tubulin was co-incubated with indicated concentrations of Taxol and **5k** for 1 h, then 5 μmol/L colchicine was added. The fluorescence was measured by spectrofluorometer. All assays were repeated twice and representative data were shown.

Activation of caspase 3

Previously, it has been well established that cell-cycle arrest at G2/M phase leads to induction of apoptosis.[40] Caspase-3, an executioner of apoptosis is considered as an index of apoptotic type of cell death.[40] Therefore, we examined the activity of caspase-3 to assess the cytotoxic effect of these imidazothiadiazoles mediated through apoptotic signaling pathway. Hence, HeLa cells were treated with **5i**, **5k** and **6i** at two different concentrations (2 μM and 4 μM) for 24 h, and CA-4 was used as the positive control. It was observed that there was a 2.5 to 3.5 fold increase in caspase-3 activity relative to the control (Figure **7**). Hence, these results suggest that **5i**, **5k** and **6i** induced apoptosis in HeLa cells by activation of caspase-3.

Effect of 5i, 5k and 6i on cyclin B1 protein levels

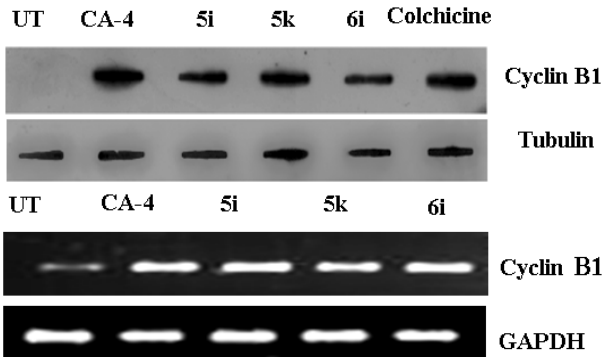


Fig. 6 Western blot and RT-PCR analysis of cyclin B1 in 5i, 5k and 6i treated HeLa cells. A) The cells were treated with 5 μM of CA-4, 5i, 5k and 6i and colchicine for 24 h. Tubulin was used as loading for equal loading of protein samples. B) Semi-quantitative RT-PCR analysis of Cyclin B1 gene expression in compounds 5i, 5k and 6i treated HeLa cells. GAPDH was used as an internal control.

Cyclin B1 is induced at the G2/M boundary to promote cell division. The anti-mitotic agents that arrest cells at G2/M phase are known to induce cyclin B1 protein levels.[39] CA-4 is known to cause mitotic arrest that leads to an induction of Cyclin B1 protein levels. Thus our compounds also exhibit the same mode of action. Since these imidazothiadiazoles inhibit tubulin polymerization and arrest cells at G2/M phase, we investigated their effect on cyclin B1 protein levels. Therefore, HeLa cells were treated with 5 μM concentration of these compounds for 24 h, wherein, CA-4 and colchicine were employed as positive controls. Immunoblot analysis revealed that these imidazothiadiazoles (**5i**, **5k** and **6i**) strongly induce cyclin B1 protein levels and tubulin was used as the loading control (Figure **6**). To further validate that the increase in protein levels of Cyclin B1 was not due to increased stability of cyclin B1 protein in the presence of compounds (**5i**, **5k** and **6i**) by any cross-reactivity of the compounds with cyclin B1 protein. Thus we examined the mRNA levels of cyclin B1 in control and compound treated cells. As expected, **5i**, **6i** and **6k** robustly activated Cyclin B1 mRNA levels. GAPDH was used as an internal control.

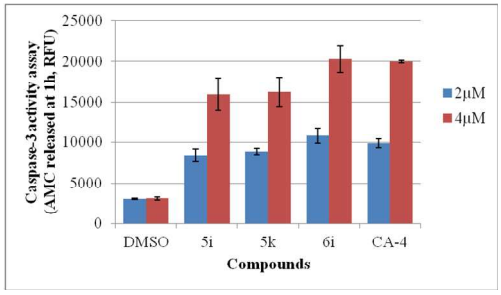


Fig. 7 Effect of compounds **5i**, **5k** and **6i** and CA-4 on caspase-3 activity. HeLa cells were treated for 24 with compounds **5i**, **5k** and **6i** (each at 2 or 4 μM), and CA-4. DMSO (0.5%) was used as vehicle control.

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Molecular docking studies

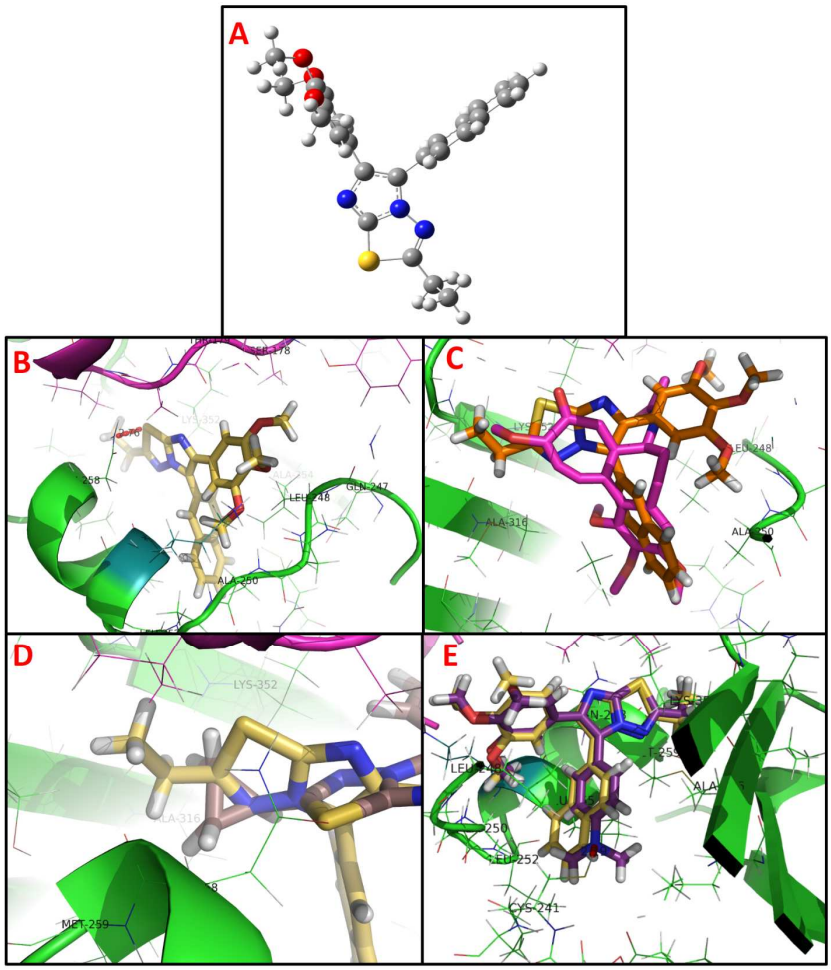

Figure **8**: Binding poses for **5k**, colchicine, **6k** and **5i** in tubulin protein in which α-chain shown in purple color and β-chain shown in green color. Hydrogen bonds were shown in red dotted line A) Optimized geometry of **5k**. B) Binding pose of **5k** C) Superimpose pose for **5k** and colchicine. D) Superimpose pose for **5k** and **6k** E) Superimpose pose for **5k** and **5i**.

To understand the binding mode and SAR for this series of molecules molecular modeling studies were performed. These analogues (**5i**, **5k** and **6i**) inhibit tubulin polymerization which prompted us to perform docking studies to understand their binding modes on colchicine binding site of tubulin. Tubulin crystal structure were obtained from Protein Data Bank (PDB ID 3E22).[41] Necessary corrections to the protein were done using Protein Preparation Wizard from Schrodinger package. Geometry of the molecule was optimized in Gaussian 09 using PM3 semi-empirical method.[42] Further docking studies were performed using AutoDock 4.2 docking software.[43] The geometry of the compounds is like 'Y' shaped in which imidazothiadiazole is like tail while naphthyl group and trimethoxypheyl ring are like arms (Figure **8A**). Binding pose of the most active compound **5k** shows that Y shaped ligand delimited by complementary cavity at the colchicine binding site of tubulin. The naphthyl ring was in the β chain buried deeply in hydrophobic cavity while imidazothiadiazole ring and trimethoxyphenyl rings were at the interface of α-β chains (Figure **8B**). The superimposed pose of co-crystalized ligand colchicine with **5k** shows that the trimethoxyphenyl group of colchicine superimposed with naphthyl group of **5k** and C ring of colchicine is superimposed with the imidazothiadiazole ring of **5k** in which carbonyl group of colchicine is coincides on the sulphur (Figure **8C**). The Y shaped geometry of the compounds demonstrate the trimethoxyphenyl group was not superimposed with the colchicine trimethoxyphenyl group. The naphthyl moiety interacts with Thr239, Cys241, Leu242, Ala250,

Leu252, Leu255, Val318, amino acids of β chain. The imidazothiadiazole moiety interacts with Asn258, Met259, Thr314, Val315, Ala316, Asn350, Val351, and Lys352 amino acids of β chain and Ala180 and Val181 amino acids of α chain. However trimethoxyphenyl ring interacts with the Gln247, Leu248, Asn249, Lys254 amino acids of β chain and Asn101, Ser178, Thr179, Tyr224 amino acids of the α chain. Also ring sulphur shows hydrogen bonding interaction with side chain of Asn258. Biological studies manifest that series with ethyl substitution having more activity than with cyclopropyl ring. By comparing the superimposed pose of **5k** and **6k** show due to the steric nature of the cyclopropyl group **6k** pushed it slightly towards the left site may be the reason for overall decrease in the activity of series (Figure **8D**). Also potency for various substituted compounds as follows naphthyl>Dimethylaniline>Fluoro>. To understand this superimposed poses of the different compounds are explored in detail. Superimposed pose of **5k** and **5i** (Figure **8E**) foremost active compounds reviles that **5i** shows additional hydrogen bonding interaction of nitrogen of aniline with thiol of Cys242. Interestingly hydrophobic interactions of naphthyl group in hydrophobic pocket including overcome the weak hydrogen bonding interactions.

Conclusion

2-Ethyl/cyclopropyl 6-(3',4,'5'-trimethoxyphenyl)-5-aryl imidazothiadiazoles were synthesized and investigated for their antiproliferative activity in various human cancer cell lines in comparision of CA-4. Among them **5i, 5k** and **6i** showed significant cytotoxic activity with IC_{50} values with an average of 2.2, 1.7 and 2.9 μM respectively. These three analogues (**5i, 5k** and **6i**) emerged as the promising molecules with broad spectrum of anticancer activity against all the tested tumor cell lines. These analogues accumulated cells in the G2/M phase, inhibited tubulin assembly and immunocytochemistry analysis revealed loss of intact microtubule structure in cells treated with them. Caspase-3 activity demonstrates that analogues **5i, 5k** and **6i** induce apoptotic cell death. Increased expression of cyclin B1 in cells treated with **5i, 5k** and **6i** corroborated cell cycle arrest at G2/M phase. Molecular docking studies of the most potent compounds **5i, 5k** and **6i** revealed that these compounds interact and bind efficiently in the active site of the tubulin and are involved in a series of interactions with the protein. This was further confirmed from competitive colchicine binding assay carried out on more potent analogue **5k**. Due to their relative ease of synthesis, the compounds of these series are further amenable for structural modifications and will be useful as templates for the design of new anticancer agents.

Experimental

Materials and Methods

All chemicals and reagents are commercially available and were used directly 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. ^1H and ^{13}C NMR spectra were recorded 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. ^1H NMR spectroscopic data are reported in the following order: multiplicity (s, singlet; brs, 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. The purity of tested compounds was P95% as determined by HPLC performed on a Shimadzu LCMS-2020 apparatus equipped with a SPD-M20A diode array detector and a Shimadzu SIL-20AC auto injector using C18 column (Phenomenex luna 5 μm C18, 4.6 mm × 250 mm column). Elution conditions: mobile phase A (75%)-acetonitrile; mobile phase B (25%)-water containing 0.1% formic acid + 10 mmol NH_4OAc. The flow rate was 1.0 mL/min and the injection volume was 5 μL at 25 °C and detection at 254 nm.

Chemistry

General Procedure for the Preparation of 9a and 9b

The mixture of 2-bromo-1-(3',4',5')-trimethoxyphenyl)ethanone (1.0 mmol) and 2-amino 5-ethyl/cyclopropyl thiadiazole (1 mmol) in anhydrous EtOH (30 mL) was heated at reflux for 12 h. After that, the solvent was removed in vacuo, and saturated aqueous $NaHCO_3$ (2× 50 mL) was added to make the mixture basic (pH = 8-9). Then the mixture was extracted with CH_2Cl_2 (3× 25 mL). The combined organic phases were washed with brine (1×25 mL) and dried with anhydrous Na_2SO_4. After removal of the solvent, the residue was stirred for 20 min with petroleum ether (40 mL) and filtered to afford as a pure product.

2-Ethyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (9a)

Compound **9a** was prepared according to the general procedure, using 2-amino 5-ethyl thiadiazole **8a** (3 g, 23.25 mmol) and 2-bromo-1-(3',4',5'-trimethoxyphenyl)ethanone **7** (6.7 g, 23.25 mmol) to obtain pure product **9a** as pale yellow solid (5.93 g, 80% yield); mp: 110-115 °C.

^1H NMR (300 MHz, $CDCl_3$): δ 7.85 (s, 2H), 7.29 (s, 2H), 3.95 (s, 6H), 3.90 (s, 3H), 3.09 (d, J = 7.2 Hz, 2H), 1.46 (t, J = 6.9 Hz, 3H); MS (ESI, m/z): 320 $[M+H]^+$.

2-Cyclopropyl-6-(3',4',5-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (9b)

Compound **9b** was prepared according to the general procedure, using 2-amino 5-cyclopropyl thiadiazole **8b** (3 g, 21.27 mmol) and 2-bromo-1-(3',4',5'-trimethoxyphenyl)ethanone **7** (6.12 g, 21.27 mmol) to obtain pure product **9b** as pale yellow solid (5.98 g, 85% yield); mp: 156-159 °C.

^1H NMR (500 MHz, $CDCl_3$): δ 7.84 (s, 1H), 7.00 (s, 2H), 3.91 (s, 6H), 3.85 (s, 3H), 2.20 (tt, J = 8.3, 5.0 Hz, 1H), 1.24 – 1.10 (m, 4H); MS (ESI, m/z): 332 $[M+H]^+$.

General Procedure for the Preparation of 10a and 10b

A solution of compound **9a** and **9b** (1 mmol) in anhydrous chloroform (20 mL) was cooled to 0 °C and treated with NBS (1.1 mmol) under nitrogen. The reaction was allowed to warm to room temperature after 15 min and then stirred for 4 h. After quenching with saturated $Na_2S_2O_3$ (20 mL), the resulting mixture was diluted with water (20 mL) and extracted with CH_2Cl_2 (2×30 mL). The combined organics were dried (Na_2SO_4) and evaporated to give a yellow solid, which was purified by column chromatography, to afford as a pure product.

5-Bromo-2-ethyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (10a)

Compound **10a** was prepared according to the general procedure, using **9a** (5 g, 15.67 mmol) and NBS (3.03 g, 17.24 mmol) to obtain pure product **10a** as Pale yellow solid (6.22 g, 83% yield); mp: 124-129 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.28 (s, 2H), 3.93 (s, 6H), 3.88 (s, 3H), 3.06 (q, *J* = 7.5 Hz, 2H), 1.44 (t, *J* = 7.6 Hz, 3H); MS (ESI, m/z): 399 $[M+H]^+$.

5-Bromo-2-cyclopropyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (10b)

Compound **10b** was prepared according to the general procedure, using **9b** (5 g, 15.10 mmol) and NBS (2.9 g, 16.61 mmol) to obtain pure product **10b** as Pale yellow solid (5.26 g, 85% yield); mp: 176-179 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.25 (s, 2H), 3.93 (s, 6H), 3.88 (s, 3H), 2.32 – 2.26 (m, 1H), 1.32 – 1.11 (m, 4H); MS (ESI, m/z): 411 $[M+H]^+$.

General procedure for Suzuki coupling of 5a-p and 6a-p

To a solution of **10a** and **10b** (1 mmol) in DME (2 mL) and H_2O (0.4 ml), appropriate boronicacid (2.5 mmol), $[PdCl_2(PPh_3)_2]$ (0.06 mmol) and CS_2CO_3 (5 mmol) were added at room temperature. The mixture was refluxed for 12 h. After cooling, the reaction mixture was extracted with ethyl acetate. The combined organic extracts were dried with Na_2SO_4 and concentrated under vacuum to give crude products. Purification by column chromatography on silica gel afforded pure products.

2-Ethyl-5-phenyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5a)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5a** as a white solid (123 mg, 72%); mp 148-150 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.64 (d, *J* = 7.1 Hz, 2H), 7.45 (t, *J* = 7.3 Hz, 2H), 7.41 – 7.34 (m, 1H), 6.87 (s, 2H), 3.86 (s, 3H), 3.70 (s, 6H), 3.02 (t, *J* = 7.6 Hz, 2H), 1.42 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.75, 153.08, 144.44, 141.74, 137.32, 130.07, 129.57, 129.32, 128.66, 128.37, 123.47, 104.58, 60.93, 55.87, 25.81, 13.30; MS (ESI): *m/z* 396 $[M+H]^+$; HRMS calcd for $C_{21}H_{22}O_3N_3S[M+H]^+$396.13651, found 396.13660. HPLC: tR 6.142 min, purity 98.93%.

2-Cyclopropyl-5-phenyl-6-(3,4,5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6a)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6a** as a white solid (158 mg, 72%); mp 108-110 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.62 (d, *J* = 7.3 Hz, 2H), 7.44 (t, *J* = 7.3 Hz, 2H), 7.37 (t, *J* = 7.1 Hz, 1H), 6.85 (s, 2H), 3.85 (s, 3H), 3.70 (s, 6H), 2.27 (m, 1H), 1.27 – 1.07 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.00, 153.07, 147.77, 147.61, 141.30, 130.09, 123.73, 122.81, 109.95, 108.63, 104.51, 101.31, 60.95, 55.96, 13.38, 10.56; MS (ESI): *m/z* 408 $[M+H]^+$; HRMS calcd for $C_{22}H_{22}O_3N_3S[M+H]^+$408.13630, found 408.13636. HPLC: tR 6.329 min, purity 99.627%.

2-Ethyl-5-(4-methoxyphenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5b)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5b** as a white solid (137 mg, 69%); mp 152-155 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.56 (d, *J* = 7.6 Hz, 2H), 6.99 (d, *J* = 8.5 Hz, 2H), 6.88 (s, 2H), 3.84 (s, 6H), 3.72 (s, 6H), 3.07 (q, *J* = 7.6 Hz, 2H), 1.42 (t, *J* = 7.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.63, 159.64, 153.07, 143.83141.12, 130.96, 130.27, 130.20, 121.60, 114.16, 111.72, 104.38, 60.92, 55.91, 55.36, 25.80, 13.32; MS (ESI): *m/z* 426 $[M+H]^+$; HRMS calcd for $C_{22}H_{24}O_4N_3S[M+H]^+$426.14619, found 426.14642. HPLC: tR 6.892 min, purity 99.479%.

2-Cyclopropyl-5-(4-methoxyphenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6b)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6b** as a white solid (163 mg, 62%); mp 111-113 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.54 (d, *J* = 8.7 Hz, 2H), 6.98 (d, *J* = 8.7 Hz, 2H), 6.88 (s, 2H), 3.85 (s, 6H), 3.71 (s, 6H), 2.29 – 2.23 (m, 1H), 1.23 – 1.09 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.82, 159.67, 153.05, 143.17, 141.03, 137.12, 130.94, 130.28, 123.30, 121.66, 114.11, 104.35, 60.93, 55.90, 55.37, 13.36, 10.51; MS (ESI): *m/z* 438 $[M+H]^+$; HRMS calcd for $C_{23}H_{24}O_4N_3S[M+H]^+$438.14551, found 438.14569. HPLC: tR 6.011 min, purity 99.346%.

2-Ethyl-5-(p-tolyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5c)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5c** as a white solid (153 mg, 74%); mp 160-162 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.52 (d, *J* = 8.1 Hz, 2H), 7.27 (d, *J* = 8.1Hz, 2H), 3.86 (s, 3H), 3.71 (s, 6H), 3.03 (q, *J* = 7.6 Hz, 2H), 2.40 (s, 3H), 1.41 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.65, 153.04, 144.10, 141.36, 138.32, 137.22, 130.21, 129.45, 129.37, 126.32, 123.55, 104.50, 60.94, 56.42, 25.12, 21.41, 13.33; MS (ESI): *m/z* 410 $[M+H]^+$; HRMS calcd for $C_{22}H_{24}O_3N_3S[M+H]^+$410.15175, found 410.115191. HPLC: tR 5.856 min, purity 99.206%.

2-Cyclopropyl-5-(p-tolyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6c)

Following general procedure B, the crude residue, purified by

flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6c** as a white solid (167 mg, 82%); mp 156-158 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.52 (d, J = 8.1 Hz, 2H), 7.27 (d, J = 8.1Hz, 2H), 3.86 (s, 3H), 3.71 (s, 6H), 3.03 (q, J = 7.6 Hz, 2H), 2.29 – 2.23 (m, 1H), 1.23 – 1.09 (m, 4H);

^{13}C NMR (125 MHz, $CDCl_3$): δ 153.04, 134.02, 133.96, 133.90, 133.86, 133.80, 133.69, 129.44, 129.33, 128.21, 128.14, 128.04, 126.98, 123.53, 104.78, 104.55, 60.96, 55.90, 21.43, 13.37, 10.54; MS (ESI): *m/z* 422 $[M+H]^+$; HPLC: tR 6.856 min, purity 97.76%.

2-Ethyl-5-(4-ethylphenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5d)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5d** as a white solid (164mg, 76%); mp 134-136 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.56 (d, J = 8.1 Hz, 1H), 7.30 (d, J = 8.2 Hz, 1H), 3.87 (s, 3H), 3.71 (s, 6H), 3.05 (q, J = 7.6 Hz, 1H), 2.71 (q, J = 7.6 Hz, 1H), 1.43 (t, J = 7.6 Hz, 1H), 1.27 (t, J = 7.6 Hz, 1H); MS (ESI): *m/z* 424 $[M+H]^+$; HRMS calcd for $C_{23}H_{22}O_5N_3S[M+H]^+$424.16719, found 424.16720. HPLC: tR 6.695 min, purity 98.146%.

2-Cyclopropyl-5-(4-ethylphenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6d)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6d** as a white solid (156 mg, 77%); mp 94-97 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.56 (d, J = 8.1 Hz, 1H), 7.30 (d, J = 8.2 Hz, 1H), 3.87 (s, 3H), 3.71 (s, 6H), 3.05 (q, J = 7.6 Hz, 1H), 2.71 (t, J = 7.6 Hz, 1H), 2.29 – 2.23 (m, 1H), 1.23 – 1.09 (m, 4H); MS (ESI): *m/z* 436 $[M+H]^+$; HRMS calcd for $C_{24}H_{25}O_3N_3S[M+H]^+$436.16755, found 436.16751. HPLC: tR 6.687 min, purity 98.206%.

2-Ethyl-5-(4-fluorophenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5e)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5e** as a white solid (143 mg, 62%); mp 152-154 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.67 – 7.60 (m, 2H), 7.16 (t, J = 8.7 Hz, 2H), 6.85 (s, 2H), 3.88 (s, 3H), 3.74 (s, 6H), 3.05 (q, J = 7.6 Hz, 2H), 1.44 (t, J = 7.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.18, 160.85, 153.14, 143.51, 141.71, 137.36, 131.42, 129.94, 125.35, 115.88, 115.59, 104.52, 60.95, 55.91, 25.92, 13.40; MS (ESI): *m/z* 414 $[M+H]^+$; HRMS calcd for $C_{21}H_{21}O_3N_3FS[M+H]^+$414.12688, found 414.12688. HPLC: tR 6.168 min, purity 96.212%.

2-Cyclopropyl-5-(4-fluorophenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6e)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6e** as a white solid (123 mg, 72%); mp 135-137 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.60 (dd, J = 8.6, 5.4 Hz, 2H), 7.14 (t, J = 8.6 Hz, 2H), 6.83 (s, 2H), 3.86 (s, 3H), 3.72 (s, 6H), 2.29 – 2.25 (m, 1H), 1.26 – 1.10 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.18, 164.15, 160.85, 153.14, 141.71, 137.36, 131.42, 129.94, 125.39, 115.88, 115.59, 104.52, 60.95, 55.91, 13.37, 10.62; MS (ESI): *m/z* 426 $[M+H]^+$; HRMS calcd for $C_{22}H_{21}O_3N_3FS$ $[M+H]^+$426.12827, found 426.12811; HPLC: tR 6.358 min, purity 98.861%.

5-(4-chlorophenyl)-2-ethyl-6-(3,4,5-trimethoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazole (5f)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5f** as a white solid (136 mg, 67%); mp 93-95 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 7.56 (d, J = 8.4 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 6.71 (s, 2H), 3.89 (s, 2H), 3.64 (s, 3H), 3.13 – 3.00 (m, 2H), 1.43 (t, J = 7.5 Hz, 3H). MS (ESI): *m/z* 430 $[M+H]^+$; HPLC: tR 5.614 min, purity 97.979%.

5-(4-Chlorophenyl)-2-cyclopropyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6f)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6f** as a white solid (136 mg, 67%); mp 93-95 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.58 (dd, J = 8.8, 2.0 Hz, 2H), 7.43 – 7.39 (m, 2H), 6.83 (s, 2H), 3.87 (s, 3H), 3.73 (s, 6H), 2.31 – 2.22 (m, 1H), 1.28 – 1.09 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 153.19, 133.90, 133.80, 130.48, 128.85, 128.24, 127.74, 123.85, 122.36, 105.02, 60.98, 55.97, 29.74, 13.34, 10.75; MS (ESI): *m/z* 442 $[M+H]^+$; HRMS calcd for $C_{23}H_{21}O_3N_3ClS$ $[M+H]^+$442.09891, found 442.09884. HPLC: tR 7.730 min, purity 96.997%.

2-Ethyl-5-(4-(trifluoromethyl)phenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5g)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5g** as a white solid (164 mg, 82%); mp 171-173 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81 (d, J = 8.3 Hz, 2H), 7.69 (d, J = 8.3 Hz, 2H), 6.81 (s, 2H), 3.88 (s, 3H), 3.72 (s, 6H), 3.06 (q, J = 7.6 Hz, 2H), 1.44 (t, J = 7.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.18, 153.20, 148.86, 144.71, 142.36, 137.58, 130.91, 129.59, 128.10, 121.98, 121.20, 104.71, 60.94, 55.83, 25.82, 13.27.; MS (ESI): *m/z* 464 $[M+H]^+$; HRMS calcd for $C_{22}H_{21}O_3N_3FS$ $[M+H]^+$464.12263, found 464.12315. HPLC: tR 6.486 min, purity 97.906%.

2-Cyclopropyl-5-(4-(trifluoromethyl)phenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazole (6g)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6g** as a white solid (189 mg, 82%); mp 117-119 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.78 (d, J = 8.1 Hz, 2H), 7.68 (d, J = 8.3 Hz, 2H), 6.80 (s, 2H), 3.87 (s, 3H), 3.72 (s, 6H), 2.33 – 2.24 (m, 1H), 1.29 – 1.11 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.48, 153.27, 144.36, 143.09, 137.87, 132.89, 129.66, 129.25, 125.46, 125.42, 121.90, 105.08, 60.95, 55.96, 13.36, 10.70; MS (ESI): *m/z* 476 $[M+H]^+$; HRMS calcd for $C_{23}H_{21}O_3N_3F_3S[M+H]^+$476.12464, found 476.12436. HPLC: tR 7.832 min, purity 98.404%.

2-Ethyl-5-(4-(trifluoromethoxy)phenyl)-6-(3',4',5'-

trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5h)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5h** as a white solid (145 mg, 62%); mp 172-175 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.73 (d, *J* = 8.1 2H), 7.32 (d, *J* = 8.1 Hz, 2H), 6.83 (s, 2H), 3.88 (s, 3H), 3.73 (s, 6H), 3.07 (q, *J* = 7.6 Hz, 2H), 1.45 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.18, 153.20, 148.86, 144.71, 142.36, 137.58, 130.91, 129.59, 128.10, 121.98, 121.20, 104.71, 60.94, 55.83, 25.82, 13.27.; MS (ESI): *m/z* 480 $[M+H]^+$; HRMS calcd for $C_{23}H_{22}O_5N_3S[M+H]^+$480.12700, found 480.12712. HPLC: tR 6.284 min, purity 99.256%.

2-Cyclopropyl-5-(4-(trifluoromethoxy)phenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6h)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6h** as a white solid (157 mg, 62%); mp 146-148 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.69 – 7.65 (m, 2H), 7.29 (d, *J* = 8.0 Hz, 2H), 6.80 (s, 2H), 3.86 (s, 3H), 3.71 (s, 6H), 2.28 (tt, *J* = 8.3, 4.9 Hz, 1H), 1.28 – 1.11 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.30, 153.21, 148.84, 143.85, 142.34, 137.65, 130.89, 129.73, 128.13, 121.92, 104.77, 60.93, 55.85, 13.35, 10.60; MS (ESI): *m/z* 492 $[M+H]^+$; HRMS calcd for $C_{23}H_{22}O_4N_3F_3S[M+H]^+$492.11980, found 492.11960. HPLC: tR 8.088 min, purity 99.422%.

4-(2-Ethyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazol-5-yl)-N,N-dimethylaniline (5i)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5i** as a white solid (146 mg, 72%); mp 182-185 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.50 – 7.46 (m, 2H), 6.95 (s, 2H), 6.80 – 6.76 (m, 2H), 3.86 (s, 3H), 3.73 (s, 6H), 3.04 – 2.98 (m, 8H), 1.40 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.27, 152.99, 150.39, 143.52, 140.40, 136.92, 130.63, 130.47, 124.13, 116.63, 112.25, 104.22, 60.92, 55.91, 40.43, 25.79, 13.37; MS (ESI): *m/z* 439 $[M+H]^+$; HRMS calcd for $C_{23}H_{27}O_3N_4S[M+H]^+$439.17984, found 439.17818. HPLC: tR 7.813 min, purity 98.931%.

4-(2-Cyclopropyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazol-5-yl)-N,N-dimethylaniline (6i)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6i** as a white solid (167 mg, 72%); mp 170-172 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.48 – 7.45 (m, 2H), 6.93 (s, 2H), 6.80 – 6.75 (m, 2H), 3.85 (s, 3H), 3.72 (s, 6H), 3.00 (s, 6H), 2.26 (ddd, *J* = 10.0, 6.6, 4.2 Hz, 1H), 1.22 – 1.08 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.38, 152.98, 150.34, 142.65, 140.38, 137.01, 130.66, 130.46, 124.06, 116.19, 112.22, 104.39, 60.92, 55.92, 40.43, 13.15, 10.19. MS (ESI): *m/z* 451 $[M+H]^+$; HRMS calcd for $C_{24}H_{27}O_3N_4S[M+H]^+$451.17951, found 451.17939. HPLC: tR 6.546 min, purity 99.000%.

5-(Benzo[*d*][1,3]dioxol-5-yl)-2-ethyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5j)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5j** as a white solid (187 mg, 72%); mp 135-140 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.12 (d, *J* = 1.7 Hz, 1H), 7.11 (d, *J* = 1.7 Hz, 1H), 6.90 – 6.88 (m, 3H), 6.02 (s, 2H), 3.86 (s, 3H), 3.75 (s, 6H), 3.03 (q, *J* = 7.6 Hz, 2H), 1.42 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.82, 153.09, 147.81, 147.67, 141.38, 137.31, 134.82, 130.05, 128.15, 122.82, 109.97, 108.67, 104.55, 101.33, 60.95, 55.96, 25.81, 13.36. MS (ESI): *m/z* 440 $[M+H]^+$; HRMS calcd for $C_{22}H_{21}O_5N_3S[M+H]^+$440.12537, found 440.12549. HPLC: tR 6.356 min, purity 99.143%.

5-(Benzo[*d*][1,3]dioxol-5-yl)-2-cyclopropyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6j)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6j** as a white solid (159mg, 68%); mp 128-130 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.10 (d, *J* = 6.2 Hz, 2H), 6.89 (d, *J* = 6.2 Hz, 3H), 6.01 (s, 2H), 3.86 (s, 3H), 3.74 (s, 6H), 2.32 – 2.23 (m, 1H), 1.27 – 1.06 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.97, 153.10, 147.79, 147.64, 143.15, 141.35, 137.36, 130.11, 123.76, 123.05, 122.86, 109.98, 108.64, 104.61, 101.32, 60.95, 55.99, 13.38, 10.54. MS (ESI): *m/z* 452 $[M+H]^+$; HRMS calcd for $C_{23}H_{22}O_5N_3S[M+H]^+$452.12700, found 452.12712. HPLC: tR 5.833 min, purity 98.320%.

2-Ethyl-5-(naphthalen-2-yl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5k)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5k** as a white solid (178 mg, 72%); mp 148-150 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.22 (s, 1H), 7.91 – 7.82 (m, 3H), 7.67 (dd, *J* = 8.6, 1.5 Hz, 1H), 7.51 (dd, *J* = 6.2, 3.2 Hz,2H), 6.92 (s, 2H), 3.86 (s, 3H), 3.63 (s, 6H), 3.06 (q, *J* = 7.5 Hz, 2H), 1.43 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.83, 153.13, 144.60, 142.07, 137.43, 133.35, 132.93, 130.14, 128.47, 128.23, 128.11, 127.76, 127.07, 126.57, 126.44, 123.46, 104.69, 60.95, 55.91, 25.85, 13.31. MS (ESI): *m/z* 446 $[M+H]^+$; HRMS calcd for $C_{25}H_{24}O_3N_3S[M+H]^+$446.15115, found 446.15122. HPLC: tR 6.284 min, purity 99.256%.

2-Cyclopropyl-5-(naphthalen-2-yl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6k)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6k** as a white solid (167mg, 72%); mp 118-122 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.20 (s, 1H), 7.89 – 7.82 (m, 3H), 7.66 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.54 – 7.47 (m, 2H), 6.91 (s, 2H), 3.86 (s, 3H), 3.63 (s, 6H), 2.29 (tt, *J* = 8.3, 4.9 Hz, 1H), 1.28 – 1.08 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.00, 153.12, 143.73, 142.03, 137.43, 133.35, 132.90, 130.15, 128.47, 128.22, 128.05, 127.75, 127.09, 126.54, 126.42, 123.40, 104.70, 60.94, 55.91, 13.69, 10.60; MS (ESI): *m/z* 458 $[M+H]^+$; HRMS calcd for $C_{26}H_{24}O_3N_3S[M+H]^+$458.15307, found 458.15326. HPLC: tR

7.709 min, purity 96.317%.

5-(3,5-Difluorophenyl)-2-ethyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5l)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5l** as a white solid (158 mg, 77%); mp 154-156 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.24 (d, J = 7.4 Hz, 3H), 6.85 (s, 3H), 6.81 – 6.76 (m, 2H), 3.88 (s, 3H), 3.76 (s, 6H), 3.06 (dd, J = 14.9, 7.3 Hz, 2H), 1.44 (t, J = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.40, 153.30, 145.26, 143.24, 137.98, 132.07, 130.02, 129.41, 111.87, 111.52, 105.16, 103.69, 103.36, 103.02, 60.99, 56.05, 25.84, 13.26; MS (ESI): *m/z* 432 $[M+H]^+$; HRMS calcd for $C_{21}H_{20}O_3N_3F_2S$ $[M+H]^+$432.11663, found 432.11685. HPLC: tR 5.681 min, purity 94.513%.

2-Cyclopropyl-5-(3,5-difluorophenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6l)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6l** as a white solid (154 mg, 67%); mp 175-177 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.24 (d, J = 6.5 Hz, 2H), 6.86 (s, 2H), 6.81 (t, J = 8.8 Hz, 1H), 3.90 (s, 3H), 3.79 (s, 6H), 2.35 – 2.28 (m, 1H), 1.32 – 1.15 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.60, 164.68, 164.50, 161.39, 161.22, 153.30, 144.40, 143.20, 138.02, 129.45, 111.85, 111.50, 105.20, 103.62, 103.29, 102.95, 60.98, 56.06, 13.38, 10.77. MS (ESI): *m/z* 444 $[M+H]^+$; HRMS calcd for $C_{22}H_{20}O_3N_3$ $F_2S[M+H]^+$444.11853, found 444.11856. HPLC: tR 7.251 min, purity 99.148%.

5-(3,4-Dimethoxyphenyl)-2-ethyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5m)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5m** as a white solid (165 mg, 75%); mp 172-174 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.23 (d, J = 8.3Hz, 1H), 7.13 (s, 1H), 6.96 (d, J = 8.3 Hz, 1H), 3.92 (s, 3H), 3.85 (s, 3H), 3.80 (s, 3H), 3.73 (s, 6H), 3.03 (q, J = 7.5 Hz, 7.4 Hz, 2H), 1.42 (t, J = 7.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.68, 153.07, 149.10, 148.89, 143.87, 141.21, 137.22, 130.21, 123.30, 122.26, 121.74, 112.57, 111.24, 104.54, 60.93, 55.96, 25.82, 13.24. MS (ESI): *m/z* 456 $[M+H]^+$; HRMS calcd for $C_{23}H_{26}O_5N_3S[M+H]^+$456.15625, found 456.15650. HPLC: tR 6.925 min, purity 96.706%.

2-Cyclopropyl-5-(3,4-dimethoxyphenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6m)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6m** as a white solid (158 mg, 75%); mp 152-154 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.23 (dd, J = 8.3, 1.2 Hz, 1H), 7.12 (s, 1H), 6.95 (d, J = 8.3 Hz, 1H), 6.90 (s, 2H), 3.93 (s, 3H), 3.85 (s, 6H), 3.81 (s, 3H), 3.73 (s, 6H), 2.32 – 2.23 (m, 1H), 1.27 – 1.09 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.94, 153.17, 149.15, 148.96, 143.23, 141.27, 137.38, 130.35, 123.35, 122.33, 121.86, 112.68, 111.33, 104.67, 61.03, 56.06, 55.97, 55.93, 34.64, 13.47, 10.65; MS (ESI): *m/z* 468 $[M+H]^+$; HRMS calcd for $C_{24}H_{26}O_5N_3S[M+H]^+$468.15828, found 468.15810. HPLC: tR 5.079 min, purity 98.965%.

5-(3-Chloro-4-fluorophenyl)-2-ethyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5n)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5n** as a white solid (125 mg, 72%); mp 136-138 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (dd, J = 7.0, 2.0 Hz, 1H), 7.51 (ddd, J = 8.4, 4.5, 2.1 Hz, 1H), 7.20 (t, J = 8.7 Hz, 1H), 6.85 (s, 2H), 3.88 (s, 3H), 3.75 (s, 6H), 3.06 (q, J = 7.6 Hz, 2H), 1.44 (t, J = 7.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.32, 159.34, 156.02, 153.26, 144.81, 142.37, 137.71, 131.28, 131.22, 129.58, 129.27, 126.50, 126.45, 121.41, 121.05, 116.95, 116.66, 104.72, 60.94, 55.96, 25.83, 13.29. MS (ESI): *m/z* 448 $[M+H]^+$; HRMS calcd for $C_{21}H_{20}O_3FClN_3S[M+H]^+$448.08718, found 448.08730. HPLC: tR 4.960 min, purity 98.446%.

5-(3-Chloro-4-fluorophenyl)-2-cyclopropyl-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6n)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6n** as a white solid (167 mg, 72%); mp 150-152 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.75 (dd, J = 7.0, 1.9 Hz, 1H), 7.54 – 7.45 (m, 1H), 7.20 (t, J = 8.7 Hz, 1H), 6.83 (s, 1H), 3.87 (s, 1H), 3.75 (s, 1H), 2.38 – 2.18 (m, 1H), 1.31 – 1.07 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 156.68, 153.28, 143.95, 142.33, 137.73, 131.27, 129.61, 129.30, 129.24, 126.52, 126.49, 121.01, 116.87, 116.70, 104.74, 60.99, 56.04, 13.41, 10.75; MS (ESI): *m/z* 460 $[M+H]^+$; HRMS calcd for $C_{22}H_{20}O_3N_3ClFS[M+H]^+$460.08869, found 460.08869. HPLC: tR 7.869 min, purity 97.248%.

2-Ethyl-5-(4-fluoro-2-methoxyphenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5o)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5o** as a white solid (128 mg, 77%); mp 132-137 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 (dd, J = 16.4, 8.9 Hz, 1H), 7.29 (d, J = 6.5 Hz, 1H), 7.17 – 7.04 (m, 1H), 6.85 (s, 2H), 3.96 (s, 1H), 3.88 (s, 3H), 3.76 (s, 6H), 3.05 (q, J = 7.6 Hz, 2H), 1.43 (t, J = 7.5 Hz, 3H); MS (ESI): *m/z* 444 $[M+H]^+$; HPLC: tR 7.571 min, purity 98.629%.

2-Cyclopropyl-5-(4-fluoro-2-methoxyphenyl)-6-(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6o)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6o** as a white solid (156 mg, 72%); mp 165-167 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 (t, J = 7.6 Hz, 1H), 6.82 (s, 2H), 6.77 (d, J = 7.6 Hz, 2H), 3.83 (s, 3H), 3.69 (s, 9H), 2.23 (td, J = 8.3, 4.2 Hz, 1H), 1.25 – 1.04 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.61, 166.04, 162.75, 159.28, 152.94, 143.29, 142.05, 136.94, 133.59, 133.46, 130.37, 107.69, 107.40, 103.30, 100.06, 99.71, 60.90, 55.83, 55.77,

13.30, 10.48. MS (ESI): *m/z* 456 $[M+H]^+$; HRMS calcd for $C_{23}H_{23}O_5N_3S[M+H]^+$456.13817, found 456.13799.

HPLC: tR 6.356 min, purity 99.143%. HPLC: tR 5.431 min, purity 98.306%.

2-Ethyl-5,6-bis(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (5p)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **5p** as a white solid (156 mg, 72%); mp 199-200 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.59 – 7.53 (m, 1H), 7.02 – 6.96 (m, 1H), 6.89 (s, 1H), 3.86 (s, 2H), 3.72 (s, 9H), 3.03 (q, *J* = 7.6 Hz, 1H), 1.41 (t, *J* = 7.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.85, 153.33, 153.06, 141.63, 130.03, 124.52, 123.27, 106.62, 104.70, 60.93, 56.17, 55.96, 25.86, 13.14.; MS (ESI): *m/z* 486 $[M+H]^+$; HRMS calcd for $C_{24}H_{28}O_6N_3S[M+H]^+$486.16708, found 486.16732. HPLC: tR 6.021 min, purity 89.530%.

2-Cyclopropyl-5,6-bis(3',4',5'-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (6p)

Following general procedure B, the crude residue, purified by flash chromatography using ethyl acetate: petroleum ether 6:4 (v:v) as eluent, furnished **6p** as a white solid (167 mg, 69%); mp 140-145 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.90 (s, 2H), 6.87 (s, 2H), 3.89 (s, 3H), 3.85 (s, 3H), 3.79 (s, 6H), 3.74 (s, 6H), 2.29 (tt, *J* = 8.3, 4.9 Hz, 1H), 1.28 – 1.09 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.08, 153.27, 153.05, 141.55, 137.92, 137.32, 130.07, 128.05, 124.51, 123.18, 106.51, 104.64, 61.11, 56.15, 55.95, 13.40, 10.68. MS (ESI): *m/z* 498 $[M+H]^+$; HRMS calcd for $C_{23}H_{22}O_5N_3S[M+H]^+$498.16897, found 498.16875. HPLC: tR 5.099 min, purity 98.9454%.

Biology

Cell Cultures, Maintenance and Anti proliferative Evaluation

The cell lines, MIA PaCa-2, MCF-7, HeLa and DU-145 (pancreatic, breast, cervical and prostate cancer) used in this study were procured from American Type Culture Collection (ATCC), USA. The synthesized test compounds were evaluated for their *in vitro* anti-proliferative activity in these four different human cancer cell lines. A protocol of 48 h continuous drug exposure was used and an SRB cell proliferation assay was used to estimate cell viability or growth. All the cell lines were grown in Dulbecco's modified Eagle's medium (containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C). Cells were trypsinized when sub-confluent from T25 flasks/60 mm dishes and seeded in 96-well plates in 100 µL aliquots at plating densities depending on the doubling time of individual cell lines. The microtitre plates were incubated at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to the addition of experimental drugs and were incubated for 48 h with different doses (0.01, 0.1, 1, 10, 100 µM) of the prepared derivatives. After incubation at 37 °C for 48 h, the cell monolayers were fixed by the addition of 10% (wt/vol) cold trichloroacetic acid and incubated at 4 °C for 1 h and were then stained with 0.057% SRB dissolved in 1% acetic acid for 30 min at room temperature. Unbound SRB was washed with 1% acetic acid. The protein–bound dye was dissolved in 10 mM Tris base solution for OD determination at 510 nm using a microplate reader (Enspire, Perkin Elmer, USA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as:

[(Ti-Tz)/(C-Tz)] x 100 for concentrations for which Ti>/=Tz

[(Ti-Tz)/Tz] x 100 for concentrations for which Ti<Tz.

The dose response parameter, growth inhibition of 50% (GI_{50}) was calculated from [(Ti-Tz)/(C-Tz)] × 100 = 50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. Values were calculated for this parameter if the level of activity is reached; however, if the effect is not reached or is exceeded, the value for that parameter was expressed as greater or less than the maximum or minimum concentration tested.

Tubulin polymerization assay

A fluorescence based in vitro tubulin polymerization assay was performed according to the manufacturer's protocol (BK011, Cytoskeleton, Inc.). Briefly, the reaction mixture in a total volume of 10 lL contained PEM buffer, GTP (1 µM) in the presence or absence of test compounds (final concentration of 5 µM). Tubulin polymerization was followed by a time dependent increase in fluorescence due to the incorporation of a fluorescence reporter into microtubules as polymerization proceeds. Fluorescence emission at 420 nm (excitation wavelength is 360 nm) was measured by using a Varioscan multimode plate reader (Thermo scientific Inc.). Combretastatin A-4 was used as positive control in each assay. The 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 5 µM concentrations. Polymerization was monitored by increase in the Fluorescence as mentioned above at 37 °C.[44,45] 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, 3 and 5 µM). Assays performed under similar conditions as employed for polymerization assays as described above.

Cell Cycle analysis

Human cervical cancer cell line (HeLa) in 60 mm dishes were incubated for 24 h in the presence or absence of test compounds **5i**, **5k** and **6i** (5 µM). Cells were harvested with Trypsin-EDTA, fixed with ice-cold 70% ethanol at 4 °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. The DNA contents of 20,000 events were measured by flow cytometer (BD FACSC anto II). Histograms were analyzed using FCS express 4 plus.

Immunohistochemistry of tubulin and analysis of nuclear morphology

HeLa cells were seeded on glass cover slip, incubated for 24 h in the presence or absence of test compounds **5i, 5k** and **6i** at a concentration of 5 µM. Cells grown on coverslips 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 4 h 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.

Colchicine Competitive binding assay

For colchicine competitive binding assay, tubulin was co incubated with indicated concentrations of **5k** and CA-4 at 37 °C for 1 h. Then colchicine was added to a final concentration of 3 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 ofthe 5 µmol/L colchicine-tubulin complex, and F is the fluorescence of a given concentration of **5k** or CA-4 at 3 µM competition with the 5 µmol/L colchicine-tubulin complex. Taxol, not binding in the colchicine-site of tubulin, was added as a negative control.

Dot-blot assay:

Cells were trypsinized when sub-confluent from T25 flasks/60 mm dishes and seeded in 6-well plates. These imidazothiadiazole derivatives (**5i**, **5k** and **6i**) were evaluated for their activity against Cyclin B1. HeLa cells were treated with 5 µM concentrations of **5i**, **5k** and **6i** 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 1X TBS 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 vaccum. The blot was blocked with 5% blotto for 1 h at room temperature. Immunoblot analysis was performed using UVP, biospectrum 810 imaging system. Semi-quantitative reverse transcription PCR (RT-PCR)-Total RNA was extracted using RNase mini kit (Qiagen) and reverse transcribed into cDNA using superscript II reverse transcriptase (Invitrogen life technologies).The PCR primers for Cyclin B1 were 5'-CTACCTTTGCACTTCCTTCGG-3'as forward and 5'-CCTGCTGCAATTTGAGAAGG-3'as reverse primers.For GAPDH, the forward primer was 5'-CAAGGTCATCCATGACAACTTTG-3'and the reverse was 5'- CTTCACCACCTTCTTGATGTCATC-3'. The PCR conditions for Cyclin B1 and GAPDH were, 95°C 2 minutes, followed by 21 cycles of 95°C-15sec, 60°C-30 sec, and 72°C-30sec. GAPDH was used as an internal control. The products were electrophoresed on agarose gel (2.5%) followed by staining with ethidium bromide and visualized under U.V. light. The signal intensity of respective bands was measured by means of the quantity one version 4.1.1 software using Bio-Rad image analysis system.[46]

Caspase-3 Assay:

HeLa cells were seeded in 12 well plates as mentioned above and were treated with imidazothiadiazole compounds (**5i**, **5k** and **6i**) at 2 and 4 µM concentration. After 24 h of incubation with the compound, cells were lysed using lysis buffer and incubated at 4 ^{o}C for 10mins which then collected and spun at 4 ^{o}C, 10000 rpm for 10 mins. The supernatant was collected which contains the protein. The protein was quantified employing Bradford Assay. Equal protiens were added with Assay buffer and caspase substrate which gives fluorescence units thereby calculated the activity against caspase.[47]

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Notes and references

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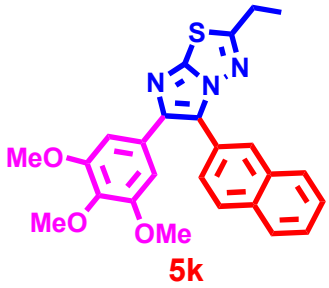

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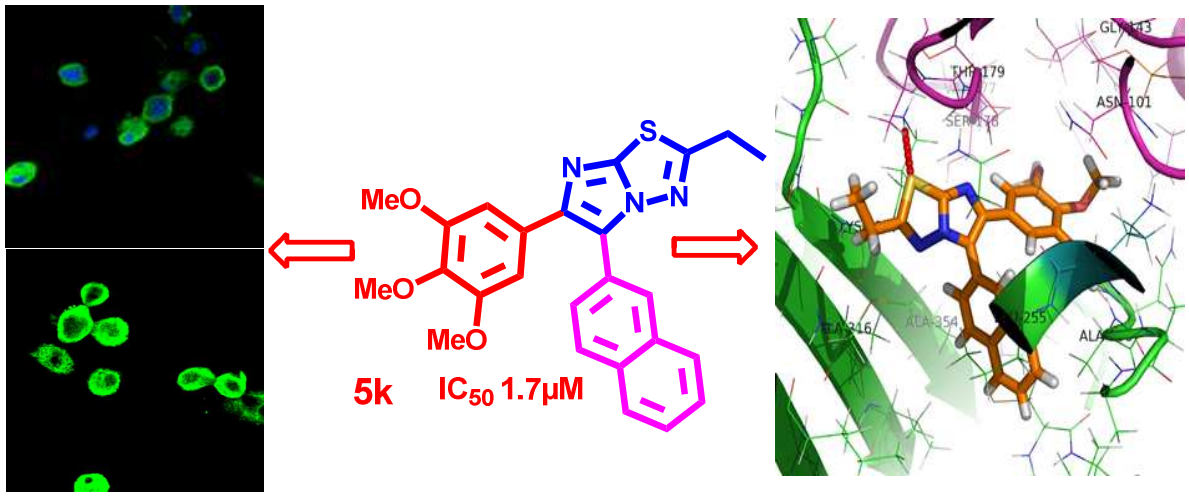
Ahmed Kamal, A.V. Subba Rao, T. Srinivasa Reddy, Sowjanya Polepalli Chandrakant Bagul, Siddiq Pasha Shaik, M.V.P.S. Vishnuvardhan and Nishant Jain

Aryl-Imidazothiadiazole Analogues as Microtubule Disrupting Agents

Graphical abstract

Aryl-Imidazothiadiazole Analogues as Microtubule Disrupting Agents



Disruption of tubulin polymerization Docked pose of 5k in colchicine binding site of tubulin