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Synthesis of Imidazothiazole–Chalcone Derivatives as Anticancer and Apoptosis Inducing Agents

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A new class of imidazo[2,1-*b*]thiazole chalcone derivatives were synthesized and evaluated for their anticancer activity. These chalcone derivatives show promising activity, with $\log GI_{50}$ values ranging from −7.51 to −4.00. The detailed biological aspects of these derivatives toward the MCF-7 cell line were studied. Interestingly, these chalcone derivatives induced G_0/G_1-phase cell-cycle arrest, down-regulation of G_1-phase cell-cycle regulatory proteins such as cyclin D1 and cyclin E1, and up-regulation of CDK4. Moreover, these compounds elicit the characteristic features of apoptosis such as enhancement in the levels of p53, p21, and p27, suppression of NF-κB, and up-regulation of caspase-9. One of these chalcone derivatives, **3d**, is potentially well suited for detailed biological studies, either alone or in combination with existing therapies.

Introduction

Chalcones are naturally occurring compounds that belong to the flavonoid family and have been associated with a wide variety of anticancer effects.[1] These compounds have also been reported to possess various other biological activities, such as antimalarial,[2,3] anti-HIV,[4] and tyrosinase inhibition.[5] The remarkable biological potential of these chalcones is due to their possible interactions with various proteins related to apoptosis and cell proliferation. Recent studies have shown that these chalcones induce apoptosis in a variety of cell types, including breast cancers.[6–9] Over the last few years, research into the antitumor properties of chalcones has received significant attention, and the majority of the naturally occurring chalcones contain either hydroxy or methoxy groups in both the A and B rings. The 3,4,5-trimethoxyphenyl ring is thought to be essential for the anticancer activity of chalcones. Some of the recent advances in the development of anticancer agents involve structural modification of chalcones to improve their bioavailability and to study the role of various substituents on aryl or heteroaryl rings.[10] Moreover, heterocyclic derivatives of chalcones in which the B ring is replaced by a heterocyclic ring have been systematically investigated. Imidazothiazoles are also well-known compounds, and many derivatives of this fused ring system have been evaluated for potential biological activity, particularly antitumor activity.[11,12] Some representative chalcones and imidazothiazole derivatives are illustrated in Figure 1.

Considering the potent bioactivities of compounds that possess an imidazothiazole core, we synthesized new chalcone derivatives that incorporate an imidazothiazole skeleton and evaluated their anticancer activity. The promising activity observed prompted us to investigate the role of these new compounds in the proliferation and apoptosis of a human breast cancer cell line (MCF-7). We also investigated the effect of these compounds on proteins that regulate cell-cycle progression.

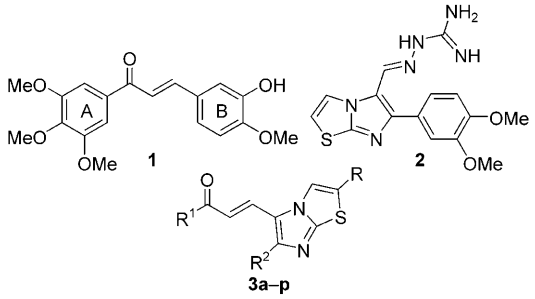

Figure 1. Structures of chalcone **1**, imidazothiazole guanylhydrazones **2**, and imidazothiazole–chalcone derivatives **3a–p**.

Results and Discussion

Chemistry

The imidazothiazole–chalcone derivatives **3a–p** were prepared by Claisen–Schmidt condensation[13] of the appropriately substituted acetophenones upon treatment with imidazo[2,1-*b*]thiazole aldehydes **8a–i** in the presence of 10% sodium hydroxide, as shown in Scheme 1. The imidazo[2,1-*b*]thiazole aldehydes **8**[14] were obtained by Vilsmeier reaction with the corresponding imidazo[2,1-*b*]thiazoles **7**, which were prepared from the appropriate 2-aminothiazoles **5** and bromoketones **4**. The intermediate compounds **6** were isolated and used in the subsequent step without further purification.

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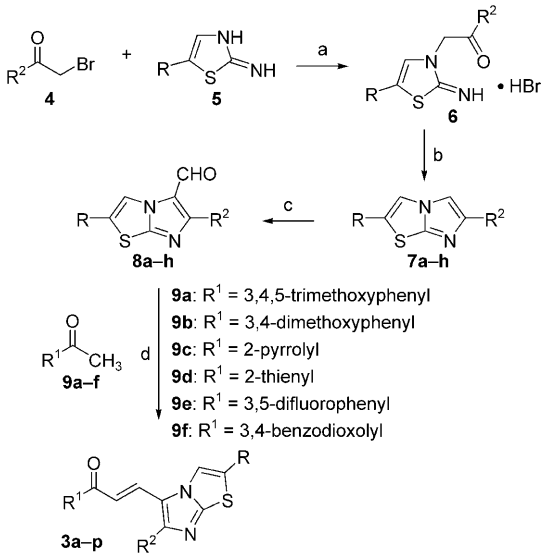


3a: R = H; R^1 = 3,4,5-trimethoxyphenyl; R^2 = 4-methoxyphenyl
3b: R = H; R^1 = 3,4,5-trimethoxyphenyl; R^2 = 4-fluorophenyl
3c: R = H; R^1 = 3,4,5-trimethoxyphenyl; R^2 = 2-thienyl
3d: R = H; R^1 = 3,4,5-trimethoxyphenyl; R^2 = trifluoromethyl
3e: R = H; R^1 = 3,4,5-trimethoxyphenyl; R^2 = 3,4-dimethoxyphenyl
3f: R = H; R^1 = 3,4-dimethoxyphenyl; R^2 = trifluoromethyl
3g: R = Me; R^1 = 3,4,5-trimethoxyphenyl; R^2 = 4-methoxyphenyl
3h: R = Me; R^1 = 3,4,5-trimethoxyphenyl; R^2 = 4-fluorophenyl
3i: R = Me; R^1 = 3,4,5-trimethoxyphenyl; R^2 = 2-thienyl
3j: R = H; R^1 = 2-pyrrolyl; R^2 = 4-methoxyphenyl
3k: R = H; R^1 = 2- thienyl; R^2 = 4-methoxyphenyl
3l: R = H; R^1 = 3,5-difluorophenyl; R^2 = 4-methoxyphenyl
3m: R = H; R^1 = 3,4-benzodioxolyl; R^2 = 4-methoxyphenyl
3n: R = H; R^1 = 3,4-benzodioxolyl; R^2 = 4-fluorophenyl
3o: R = H; R^1 = 3,4-dimethoxyphenyl; R^2 = 4-methoxyphenyl
3p: R = H; R^1 = 3,4-dimethoxyphenyl; R^2 = 4-fluorophenyl

Scheme 1. Reagents and conditions: a) acetone, reflux, 6–8 h; b) 2 N HCl, reflux, 1 h, 85–95%; c) $POCl_3$, DMF, reflux, 1 h, 70–80%; d) 10% $NaOH_{(aq)}$, 12 h, room temperature, 75–85%.

Biological results

Anticancer activity

The synthesized compounds[15] **3a**–**p** were evaluated for their anticancer activity by the National Cancer Institute (NCI) in a 60-cell-line screen. In a preliminary test at a single concentration (100 μM), a given compound was considered active if it decreased the growth of any of the cell lines down to 32% or less; from there it was included for evaluation against the full panel of 60 cell lines. This panel is organized into sub-panels representing leukemia, melanoma, and cancers of the lung, colon, kidney, ovary, breast, prostate, and central nervous system (CNS). Among the 19 derivatives tested by the NCI, 16 were active in the primary test, and nine were evaluated in a panel of 60 cell lines; the results are listed in Table 1. Specifically, compound **3c** exhibited excellent cytotoxicity, with GI_{50} values ranging from 0.26 to 4.74 μM in tests against leukemia (CCRF-CEM, K-562, RPMI-8226, SR), colon (HCC-2998, HCT-15, KM12), prostate (PC-3, DU-145), and breast (MCF-7, T-47D, MDA-MB-468) cancer cell lines, as well as NCI-H23, NCI-H460, NCI-H522 (non-small-cell lung cancer), SF-295, U251 (CNS cancer), IGROV1, NCI/ADR-RES, OVCAR-8 (ovarian cancer), UO-31 (renal cancer), and MDA-MB-435, SK-MEL-2, SK-MEL-5 (melanoma). Compound **3d** also showed promising activity against the SR (leukemia) and HCT-116 (colon cancer) cell lines. Moreover, the other compounds also showed significant activity in the sub-micromolar range against all the cell lines tested. Compounds with trifluoromethyl and 2-thienyl substituents at the 6-position are the most active derivatives. However, a methyl group at the 2-position of the imidazothiazole system (compounds **3g**–**i**) leads to a decrease in activity. Moreover, removal of one methoxy group (compounds **3d** and **3f**) in the A ring also decreases activity.

Cell viability assays were carried out to identify the cytotoxic effects of some of these chalcone derivatives (compounds **3b**–**e** and **3h**) toward MCF-7 cells at a concentration of 4 μM. The activities of parent chalcone **1** and doxorubicin as a positive control were also examined to substantiate the activities the chalcone derivatives tested (**3b**–**e** and **3h**). Interestingly, the test compounds show higher cytotoxicity than chalcone **1** toward MCF-7 cells, as shown in Figure 2, and the data for these chalcone derivatives are statistically significant relative to those of chalcone **1** (Table 2).

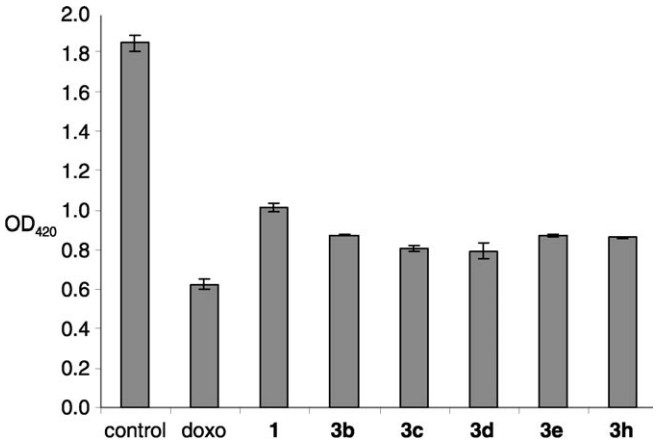


Figure 2. Effect of chalcone derivatives **3b**–**e** and **3h** on cell viability. MCF-7 cells were treated with chalcone compounds at a concentration of 4 μM as indicated for 24 h in 96-well plates seeded with ~10^4 cells per well. Optical density (OD) readings were taken at λ 420 nm. Doxorubicin was used as positive control; negative control: cells treated with DMSO vehicle alone.

Chalcone-based compounds have been shown to exhibit promising anticancer activity through various mechanisms. These mechanisms include interference with microtubule formation,[16–18] promotion of Bax protein expression and activation of caspases,[18,19] and inhibition of cell signaling pathways[9,20] such as blockage of nuclear factor κB (NF-κB). There are also reports on mitochondria-mediated apoptosis, and apoptosis involving the down-regulation of anti-apoptotic proteins such as Bcl-2 and Bcl-XL.[21] In the present investigation, as some of the chalcones exhibited promising anticancer activity, we were interested in getting a better understanding of their mode of action, particularly for the most potent compounds (Table 3).

Table 1. Anticancer activity of imidazothiazole–chalcone derivatives **3a–i** in human cancer cell lines.

Cancer panel / Cell line	Growth inhibition: log GI_{50} (M)								
	3a	**3b**	**3c**	**3d**	**3e**	**3f**	**3g**	**3h**	**3i**
Leukemia									
CCRF-CEM	−5.51	−5.49	−6.25	−5.91	−5.58	−5.63	−5.51	−5.14	−5.71
HL-60(TB)	−5.78	−5.84	−5.93	−5.74	−5.74	−6.00	−5.60	−5.54	−5.57
K-562	−5.76	−5.66	−6.35	−5.76	−5.62	−5.65	−5.50	−5.26	−5.68
MOLT-4	−5.60	−5.65	−5.99	−5.72	−5.68	−5.65	−5.63	−5.79	−5.89
RPMI-8226	–	−5.59	−6.35	−5.97	−5.93	−5.79	−5.65	−5.64	−5.78
SR	−5.63	−5.95	−6.58	−6.27	−5.89	−6.31	−5.68	−6.10	−5.95
Non-small-cell lung									
A549/ATCC	−5.65	−5.51	−5.80	−5.46	−5.58	−5.33	−5.47	−5.03	−5.57
EKVX	−5.51	−5.48	−5.62	−5.57	−5.41	−5.35	−5.39	−5.64	−5.70
HOP-62	−5.66	−5.77	−5.62	−5.71	−5.59	−5.22	−5.37	−4.07	−5.24
HOP-92	−6.61	−5.56	–	−5.15	–	−5.23	–	−6.81	−6.33
NCI-H226	−5.59	−5.63	−5.61	−5.66	−5.66	−5.16	−5.38	−5.02	−5.34
NCI-H23	−5.66	−5.62	−6.09	−5.76	−5.67	−5.56	−5.56	−5.65	−5.30
NCI-H322M	−5.43	−5.81	−5.48	−5.71	−5.37	−5.50	−5.25	−5.06	−5.17
NCI-H460	−5.57	−5.78	−6.22	−5.78	−5.70	−5.71	−5.50	−5.41	−5.73
NCI-H522	−5.71	−5.12	−6.59	−5.69	−5.90	−5.54	−6.06	−5.53	−5.87
Colon									
COLO-205	−5.60	−5.73	−5.78	−5.71	−5.62	−5.34	−5.33	−5.31	−5.40
HCC-2998	−5.75	−5.71	−6.09	−5.77	−5.57	−5.29	−5.43	−5.00	−5.41
HCT-116	−5.72	−5.64	−5.96	−6.00	−5.64	−5.85	−5.42	−5.53	−5.42
HCT-15	−5.46	−5.55	−6.08	−5.74	−5.49	−5.59	−5.47	−5.43	−5.47
HT29	−5.51	−5.65	−5.95	−5.74	−5.46	−5.70	−5.40	−5.16	−5.41
KM12	−5.58	−5.66	−6.47	−5.83	−5.69	−5.70	−5.57	−5.44	−5.55
SW-620	−5.50	−5.55	–	−5.78	–	−5.83	–	−5.27	–
CNS									
SF-268	−5.54	−5.65	−5.88	−5.76	−5.63	−5.46	−5.45	−4.82	−5.42
SF-295	−5.56	−5.51	−6.02	−5.59	−5.51	−5.50	−5.35	−5.63	−5.43
SF-539	−5.78	−5.84	−5.76	−5.81	−5.75	−5.63	−5.37	−5.21	−5.67
SNB-19	−5.45	−5.52	−5.67	−5.57	−5.61	−5.37	−5.34	−4.06	−5.54
SNB-75	−5.64	−5.87	−5.89	−5.73	−5.70	−5.16	−5.39	−4.30	−5.60
U251	−5.52	−5.58	−6.04	−5.86	−5.59	−5.73	−5.39	−5.39	−5.43
Ovarian									
IGROV1	−6.66	−4.93	−6.34	−5.57	5.92	−5.50	−5.78	−5.06	−5.60
OVCAR-3	−5.65	−5.62	−5.99	−5.73	−5.66	−5.67	−5.63	−5.44	−5.55
OVCAR-4	−5.39	−5.55	−5.71	−5.52	−5.54	−5.39	−5.50	−5.64	−5.54
OVCAR-5	−5.70	−5.75	−5.32	–	−5.61	−5.38	−4.00	−4.00	−4.09
OVCAR-8	−5.70	−5.54	−6.19	−5.51	−5.61	−5.62	−5.49	−5.31	−5.92
NCI/ADR-RES	−5.59	−5.70	−6.33	–	−5.64	−5.73	−5.55	−5.58	−5.68
SK-OV-3	−5.48	−5.62	−5.67	−5.54	−5.54	−5.47	−5.38	−5.21	−5.47
Renal									
786-0	−5.59	−5.59	5.82	5.75	−5.47	−5.25	−5.28	−5.05	−5.43
A498	−5.52	−5.69	–	−5.72	−5.59	−5.12	–	–	−5.82
ACHN	−5.39	−5.52	−5.67	−5.74	−5.49	−5.29	−5.38	−5.23	−5.29
CAKI-1	−5.92	−5.46	−5.75	−5.75	−5.52	−5.61	−5.29	−5.75	−5.53
RXF393	–	−6.02	–	5.69	–	−5.60	–	−5.24	–
SN12C	−5.54	−5.67	−5.72	−5.81	−5.51	−5.53	−5.39	−5.05	−5.28
TK-10	−5.30	−5.65	−5.68	−5.64	−5.32	−5.06	−5.26	−4.55	−5.21
UO-31	−5.95	−5.39	−6.11	−5.73	−6.19	−5.07	−5.64	−5.53	−5.74
Prostate									
PC-3	−5.51	−5.43	−6.14	−5.58	−5.66	−5.27	−5.64	−5.62	−5.55
DU-145	−5.58	−5.59	−6.29	−5.85	−5.63	−5.79	−5.43	−5.12	−5.52

Table 1. (Continued)

Cancer panel Cell line	Growth inhibition: log GI_{50} (M)								
	3a	**3b**	**3c**	**3d**	**3e**	**3f**	**3g**	**3h**	**3i**
Breast									
MCF7	−5.70	−5.79	−6.14	−5.90	−5.65	−6.12	−5.50	−5.74	−5.63
MDA-MB-231/ATCC	−5.59	−5.66	−5.81	−5.53	−5.54	−5.23	−5.42	−5.27	−5.53
HS-578T	−5.72	−5.75	–	–	–	−5.60	–	−5.08	–
BT-549	−5.46	−7.51	−5.75	−5.54	−5.61	−5.49	−5.38	−5.43	−5.42
T-47D	−5.65	−5.79	−6.10	−5.67	−5.76	−5.54	−5.62	−5.53	−5.75
MDA-MB-468	−5.53	−5.60	−6.03	–	−5.67	−5.21	−5.56	−5.62	−5.65
Melanoma									
LOX-IMVI	−5.62	−5.76	−5.97	−5.87	−5.59	−6.22	−5.45	−5.60	−5.42
MALME-3M	−5.27	−5.72	−5.48	−5.80	−5.48	−5.23	−5.59	−5.57	−5.50
M14	−5.63	−5.70	−5.60	–	−5.51	−5.57	−5.40	5.29	−5.25
MDA-MB-435	−5.55	−5.60	−6.23	−5.79	−5.55	−5.73	−5.58	−5.76	−5.57
SK-MEL-2	−5.62	−4.91	−6.23	−5.65	−5.73	−5.39	–	−5.24	−5.56
SK-MEL-28	−5.39	−5.25	–	−5.77	–	−5.07	–	−5.15	–
SK-MEL-5	−5.89	−5.95	−6.50	−5.80	−5.81	−5.38	−5.82	−6.29	−6.02
UACC-257	–	−4.85	−5.94	−5.72	−5.58	−5.11	−5.60	−5.32	−5.41
UACC-62	−5.68	−5.55	−5.86	−5.82	−5.67	−5.31	−5.55	−5.54	−5.60

Table 2. Inhibition of MCF-7 cell viability by chalcone derivatives **3b–e** and **3h** at 4 μM.

Compd	Viability $[OD_{420}]$[a]
control	1.843 ± 0.037
1	1.013 ± 0.021
3b	0.873 ± 0.005
3c	0.808 ± 0.016
3d	0.794 ± 0.038
3e	0.871 ± 0.007
3h	0.862 ± 0.006
doxorubicin	0.623 ± 0.025

[a] Values represent the mean ± SD of three independent experiments and are statistically significant compared with **1** by Student's *t*-test ($p < 0.001$).

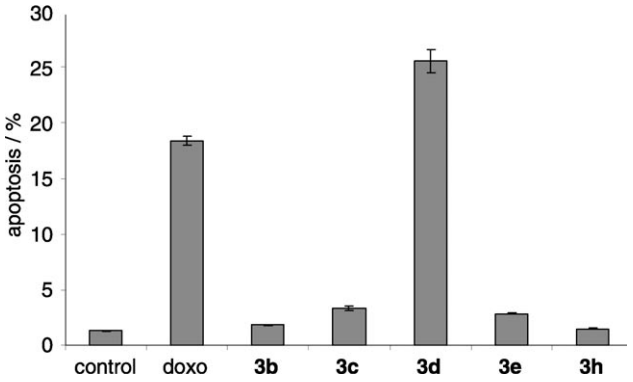


Figure 3. Histogram depicting the percentage of cells in G_0 phase, an indicator of percent apoptosis for the chalcone derivatives **3b–e** and **3h**.

Effect of imidazothiazole–chalcone derivatives on cell cycle

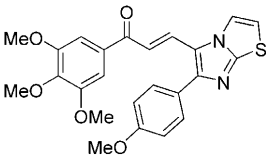
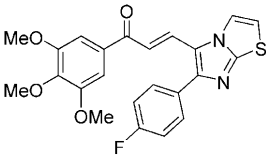
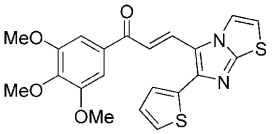
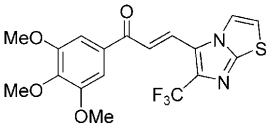
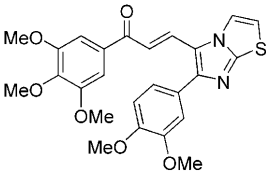
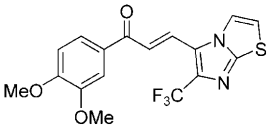
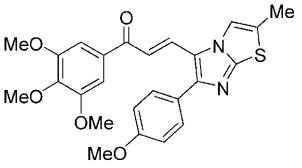
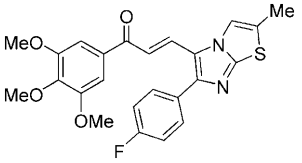
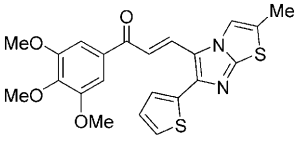
To investigate the effect of these chalcones on the cell cycle phase distribution of MCF-7 cells, flow cytometry analysis was performed at a test compound concentration of 4 μM for 24 h exposure. Results indicate that the chalcone derivatives induce G_0/G_1 cell-cycle arrest (Table 4), as the majority of cells in each case were found to be in the G_0/G_1 phase: 67% (**3b**), 73% (**3c**), 91% (**3d**), 58% (**3e**), 58% (**3h**), and 93% (doxorubicin) relative to control (DMSO vehicle-treated) cells (55%). Thus, modulation of G_1 phase cell-cycle regulators may be a key mechanism of action of these chalcones in MCF-7 cells. A decrease in the S-phase population was observed, indicating a block in the $G_1 \rightarrow S$ transition. The percentage of apoptosis mediated by compound **3d** is greater than that induced by doxorubicin (Figure 3).

In subsequent studies, compounds **3c** and **3d** were evaluated for G_1 arrest in a 5-bromo-2′-deoxyuridine (BrdU) incorporation assay. This assay determines a compound's ability to block the $G_1 \rightarrow S$ transition. Treatment of cells with compounds **3c** and **3d** at 4 μM resulted in a decreased incorporation of BrdU into cellular DNA. This level of inhibition of BrdU incorporation is indicative of a G_1-phase cell-cycle arrest by these chalcone derivatives, as shown in Figure 4.

Effect of chalcone derivatives on cell-cycle regulatory proteins

On the basis of the results of cell-cycle distribution, we next investigated the cell-cycle regulatory proteins related to G_1 phase,[22] such as cyclins D1, E1, and A, and their associated CDK2 and CDK4/CDK6, which are essential for cell-cycle progression from G_1 to S phase. MCF-7 cells were treated with compounds **3c** and **3d** at 4 μM for 24 h, and western blot analysis was then carried out. As shown in Figure 5, the protein levels of cyclin D1, cyclin A, and cyclin E1 were decreased significantly, and their associated CDK2 levels remain unchanged, whereas the CDK4 levels increased significantly relative to control. These results indicate that both chalcones **3c** and **3d** induced cell-cycle arrest at G_0/G_1 phase.

Table 3. Compounds selected by the National Cancer Institute (NCI) for the 60-cell-line panel.

Compd	NSC number[a]	Structure
3a	748733	
3b	748734	
3c	750743	
3d	749801	
3e	750741	
3f	749802	
3g	750742	
3h	749804	
3i	750744	

[a] NSC = National Service Center.

Phosphorylation of the retinoblastoma (Rb) protein, mediated by both cyclin D/CDK4 and cyclin E/CDK2, is required for cells to progress from G_1 to S phase in those cells possessing functional Rb; phospho-Rb releases (thereby activating) the previously Rb-bound transcription factor E2F-1, thus permitting cell-cycle progression.[23] To elucidate the arrest point of these chalcones (**3c** and **3d**), western blot analysis was carried out

Table 4. Cell-cycle distribution of MCF-7 cells in the presence of compounds **3b–e**, **3h**, and doxorubicin at 4 μM.[a]

Compd	G_0/G_1 [%]	S [%]	G_2/M [%]
control	55 ± 1.2	16 ± 0.7	29 ± 0.5
3b	67 ± 0.8	21 ± 0.5	12 ± 0.2
3c	73 ± 1.0	20 ± 0.5	7.0 ± 0.9
3d	91 ± 0.9	6 ± 0.7	3 ± 0.3
3e	58 ± 0.5	28 ± 0.8	14 ± 0.5
3h	58 ± 0.2	20 ± 0.5	22 ± 0.2
doxorubicin	93 ± 0.4	4.0 ± 0.2	3 ± 0.2

[a] Values represent the mean ± SEM of three determinations.

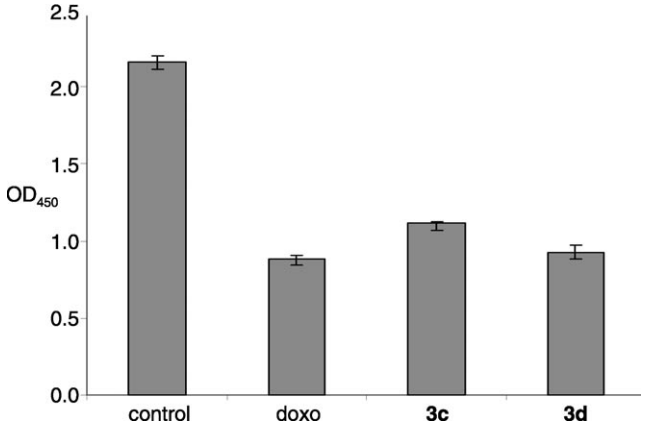


Figure 4. The BrdU cell proliferation assay. MCF-7 cells were seeded in 96-well plates at a density of ~10^4 cells per well and were grown for 24 h. After 24 h the compounds (doxorubicin, **3c**, and **3d**) were added to the respective wells at a final concentration of 4 μM. The colorimetric intensity at λ 450 nm was measured and is proportional to the amount of BrdU incorporated in proliferating cells. Lower OD values denote lower BrdU concentrations in the sample, thus an indirect reflection of a lower cell proliferation rate.

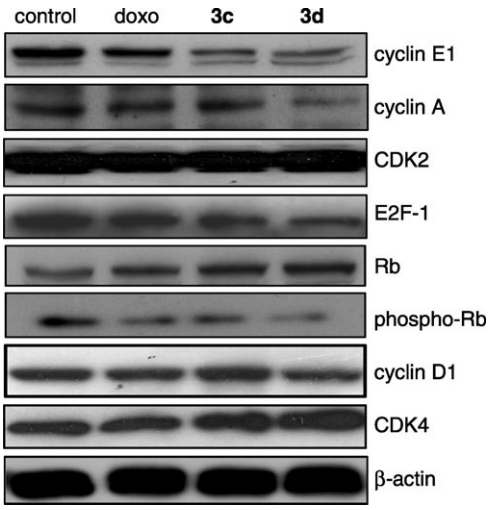


Figure 5. Effect of chalcone derivatives on the expression of cyclin and associated proteins. MCF-7 cells were treated with doxorubicin, **3c**, and **3d** at 4 μM. Western blot analysis was carried out with antibodies against cyclins (cyclin E1, cyclin A, cyclin D1), CDKs (CDK2 and CDK4), Rb, phospho-Rb (Ser780), and E2F-1; β-actin was used as a loading control.

at 4 μM for 24 h. From Figure 5, it is apparent that the expression level of Rb protein increased, and the amount of phospho-Rb (Ser780) decreased significantly, and thus the release of E2F-1 also decreased significantly relative to control. These results indicate that compound **3d** is more effective and thereby blocks the $G_1 \rightarrow$S-phase transition.

Effect on p53, p21, p27, and chk2 protein expression

The activity of tumor suppressors p53, p21, and p27 blocks the formation of tumors.[24] To understand the mechanism underlying the G_1 cell-cycle arrest in our chalcone derivatives, we examined the G_1/S checkpoint-associated tumor suppressor proteins p53, p21, p27, as well as chk2.

The activation of tumor suppressor genes such as *p53*, *p21*, *p27* and *chk2* was found to be important for the regulation of apoptotic pathways induced by various stimuli.[25–27] In order to understand the effect of chalcone derivatives on p53-, p21-, p27-, and chk2-dependent apoptotic pathways, western blot analysis was carried out with test compounds at a concentration of 4 μM for 24 h. As shown in Figure 6, the expression levels of p53, p21, p27, and chk2 were up-regulated in the case of compounds **3c** and **3d** relative to the untreated control; indeed, the effects of compound **3d** were even more prominent than those observed with the doxorubicin positive control. Therefore, up-regulation of these tumor suppressor genes is involved in G_1 cell-cycle arrest.

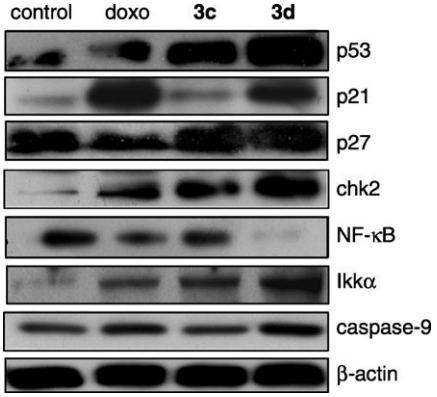


Figure 6. Effect of chalcone derivatives **3c** and **3d** on the expression of tumor suppressor proteins (p53, p21, p27, and chk2), apoptotic proteins, and their interactive partners (NF-κB, Ikkα). MCF-7 cells were treated with compounds **3c** and **3d** at 4 μM, and doxorubicin was used as a positive control. Cell lysates were collected, and western blot analysis was carried out with the aforementioned antibodies; β-actin was used as a loading control.

Activation of p53 induces apoptosis in many tumor cells, and this provides an effective anticancer therapy. One of the key proteins that modulate the apoptotic response is NF-κB, a transcription factor that can protect or contribute to apoptosis. Suppression of NF-κB activity is required for the induction of an apoptotic response, which is a prerequisite in the selection of drugs for the treatment of cancer.[28,29] NF-κB is bound to an inhibitory protein (Ikkα)[30] in the cytoplasm when it is in an inactive form. To investigate whether chalcone derivatives can suppress the activation of NF-κB and its inhibitory protein Ikkα, western blot analysis was carried out with test compounds at 4 μM for 24 h as shown in Figure 6. Ikkα protein levels were significantly increased, and NF-κB levels were significantly decreased after treatment with compounds **3c** and **3d**. Our data suggest that these chalcone derivatives affect cell growth by inhibiting the NF-κB signaling pathway and that compound **3d** has potential for further development.

Furthermore, induction of apoptotic cell death is mediated by caspases; hence the involvement of various caspases and their role in the process of apoptosis was also investigated. The aspartic acid specific protease caspase-9 has been linked to the mitochondrial death pathway, which is activated during programmed cell death (apoptosis). Caspase-9 is also the effector caspase in the case of the intrinsic pathway. MCF-7 cells were treated with compounds **3c** and **3d** at 4 μM for 24 h, and western blot analysis was carried out. Caspase-9 levels were up-regulated with the treatment, particularly with compound **3d**. However, the TNFR1 levels remained unchanged (data not shown). These data indicate the presence of an intrinsic pathway for these new chalcone derivatives (Figure 6).

Conclusions

In summary, we synthesized a new class of chalcones, wherein the B ring is replaced by an imidazo[2,1-*b*]thiazole scaffold. Compounds **3c** and **3d** showed significant anticancer activity in the NCI 60-cell-line panel and cell viability assays; these two compounds also showed cytotoxicity toward MCF-7 cells at a concentration of 4 μM. The flow cytometry analysis also showed a greater population of cells in the sub-G_1 phase, indicating that these chalcone derivatives have the ability to induce apoptosis. These effects were accompanied by changes in expression of key proteins in the G_1 phase of the cell cycle. Further modulation of the expression and function of the cell-cycle regulatory proteins provide the mechanism for the inhibition of growth. From the results, we also observed down-regulation of cyclins and up-regulation of CDK4, thereby resulting in down-regulation of phospho-Rb (Ser780), suggesting cell-cycle block in the G_1 phase. Furthermore, we observed that the levels of G_1/S checkpoint-associated tumor suppressor proteins such as p53, p21, p27, and chk2 were up-regulated, thus inducing cell-cycle arrest in the G_1 phase. Moreover, the balance between p53 and NF-κB were found to control the cell cycle and thus cell proliferation, as reported in earlier studies.[31] Our results also support that the up-regulation of p53 and concomitant down-regulation of NF-κB by these chalcones ultimately leads to apoptosis. This study involved an extensive examination into the cell-cycle regulatory role as well as apoptosis-inducing ability of these new chalcones. Compound **3d** is a particularly suitable candidate for detailed biological investigations, especially in the treatment of breast cancer.

Experimental Section

Chemistry

All chemicals and reagents were obtained from 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 glass plates coated with silica gel containing 60 GF_{254}, and visualization was carried out by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H NMR spectra were recorded on Bruker UXNMR/XWIN-NMR (300 MHz) or Inova Varian VXR-Unity (400 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from internal $(CH_3)_4Si$ standard. ESI-MS data were recorded on a Micromass Quattro LC instrument using ESI+ software with a capillary voltage of 3.98 kV and an ESI mode positive ion trap detector. HRMS data were recorded on a QSTAR XL Hybrid MS–MS mass spectrometer. Melting points were determined with an Electrothermal melting point apparatus and are uncorrected.

General procedure for the synthesis of imidazo[2,1-b]thiazoles 7 a–h

The appropriate 2-aminothiazole **4** (20 mmol) was dissolved in acetone (100 mL) and treated with the appropriate 2-bromoacetophenone **5** (20 mmol, 1 equiv). The mixture was held at reflux for 6–8 h, and the resulting salt was collected by filtration, washed with acetone, and held at reflux for 1 h with 200 mL 2 N HCl. Before complete cooling, the resulting solution was cautiously basified with 15% NH_4OH, in order to precipitate the crude compound **9**, which was crystallized from ethanol at yields of 85–95%.

6-(4-methoxyphenyl)imidazo[2,1-*b*][1,3]thiazole (7 a): White solid (218 mg, 95% yield): R_f = 0.4 (EtOAc/Hex 2:3); mp: 176–178 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.83 (s, 3H), 6.78 (d, J = 3.9 Hz, 1H), 6.93 (d, J = 8.5 Hz, 2H), 7.40 (d, J = 4.6 Hz, 2H), 7.63 (s, 1H), 7.74 ppm (d, J = 8.5 Hz, 1H); ESI-MS: m/z 231 $[M+1]^+$.

6-(4-fluorophenyl)imidazo[2,1-*b*][1,3]thiazole (7 b): White solid (200 mg, 92% yield): R_f = 0.35 (EtOAc/Hex 1:3); mp: 114–116 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 6.77 (s, 1H), 7.04 (t, J = 7.6 Hz, 2H), 7.37 (s, 1H), 7.60 (s, 1H), 7.73 ppm (d, J = 6.7 Hz, 2H); ESI-MS: m/z 218 $[M+1]^+$.

6-(thien-2-yl)imidazo[2,1-*b*]thiazole (7 c): White solid (185 mg, 90% yield): R_f = 0.38 (EtOAc/Hex 2:3); mp: 134–136 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.80 (d, J = 4.5 Hz, 1H), 7.02 (dd, J = 8.4 Hz, J = 1.3 Hz, 1H), 7.20 (dd, J = 6.0 Hz, J = 3.9 Hz, 1H), 7.30 (dd, J = 4.7 Hz, J = 2.4 Hz, 1H), 7.38 (d, J = 4.5 Hz, 1H), 7.61 ppm (s, 1H); ESI-MS: m/z 207 $[M+1]^+$.

6-(trifluoromethyl)imidazo[2,1-*b*][1,3]thiazole (7 d): White solid (173 mg, 90% yield): R_f = 0.4 (EtOAc/Hex 1:2); mp: 133–135 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.97 (d, J = 4.5 Hz, 1H), 7.47 (d, J = 4.5 Hz, 1H), 7.75 ppm (s, 1H); ESI-MS: m/z 193 $[M+1]^+$.

6-(3,4-dimethoxyphenyl)imidazo[2,1-*b*]thiazole (7 e): White solid (226 mg, 87% yield): R_f = 0.35 (EtOAc/Hex 2:3); mp: 101–103 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.88 (s, 3H), 3.95 (s, 3H), 6.76 (d, J = 4.5 Hz, 1H), 6.82 (d, J = 8.3 Hz, 1H), 7.22 (dd, J = 9.8 Hz, J = 6.7 Hz, 1H), 7.37 (d, J = 4.5 Hz, 1H), 7.40 (d, J = 1.5 Hz, 1H), 7.59 ppm (s, 1H); ESI-MS: m/z 261 $[M+1]^+$.

6-(4-methoxyphenyl)-2-methylimidazo[2,1-*b*][1,3]thiazole (7 f): White solid (207 mg, 85% yield): R_f = 0.32 (EtOAc/Hex 1:4); mp: 108–110 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.40 (s, 3H), 3.82 (s, 3H), 6.90 (d, J = 9.0 Hz, 2H), 7.07 (s, 1H), 7.47 (s, 1H), 7.69 ppm (d, J = 9.0 Hz, 2H); ESI-MS: m/z 245 $[M+1]^+$.

6-(4-fluorophenyl)-2-methylimidazo[2,1-*b*][1,3]thiazole (7 g): White solid (209 mg, 90% yield): R_f = 0.34 (EtOAc/Hex 2:3); mp: 177–179 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.41 (s, 3H), 7.05 (s, 1H), 7.08–7.12 (m, 2H), 7.53 (s, 1H), 7.74 ppm (dd, J = 13.5, J = 3.0 Hz, 2H); ESI-MS: m/z 233 $[M+1]^+$.

6-(thien-2-yl)-2-methyl-imidazo[2,1-*b*]thiazole(7 h): White solid (187 mg, 85% yield): R_f = 0.37 (EtOAc/Hex 3:7); mp: 177–179 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.40 (s, 3H), 7.01 (t, J = 3.9 Hz, 1H), 7.06 (s, 1H), 7.17 (d, J = 4.8 Hz, 1H), 7.26 (d, J = 3.9 Hz, 1H), 7.46 ppm (s, 1H); ESI-MS: m/z 221 $[M+1]^+$.

General procedure for the synthesis of aldehydes 8 a–h

The Vilsmeier reagent was prepared at 0–5 °C by dropping $POCl_3$ (20 mmol) into a stirred solution of DMF (25 mmol) in $CHCl_3$ (5 mL). Compound **9** (10 mmol) in $CHCl_3$ (60 mL) was added dropwise to the Vilsmeier reagent while maintaining stirring and cooling. The reaction mixture was kept at room temperature for 3 h and under reflux for 1–24 h (according to a TLC test). $CHCl_3$ was removed under reduced pressure, and the resulting oil was poured onto ice. The crude aldehyde **8** thus obtained was collected by filtration and crystallized from ethanol with a yield of 70–80%.

6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-carbaldehyde (8 a): Brown solid (206 mg, 80% yield): R_f = 0.28 (EtOAc/Hex 3:7); mp: 147–149 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.87 (s, 3H), 6.96–7.01 (m, 3H), 7.72 (d, J = 8.3 Hz, 2H), 8.38 (d, J = 4.5 Hz, 1H), 9.86 ppm (s, 1H, CHO); ESI-MS: m/z 259 $[M+1]^+$.

6-(4-fluorophenyl)imidazo[2,1-*b*]thiazol-5-carbaldeyde (8 b): Yellow solid (192 mg, 78% yield): R_f = 0.32 (EtOAc/Hex 2:3); mp: 169–171 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.05 (d, J = 4.5 Hz, 1H), 7.20 (t, J = 8.3 Hz, 2H), 7.78 (dd, J = 13.5, J = 3.0 Hz, 2H), 8.39 (d, J = 4.5 Hz, 1H), 9.87 ppm (s, 1H, CHO); ESI-MS: m/z 247 $[M+1]^+$.

6-(thien-2-yl)imidazo[2,1-*b*]thiazol-5-carbaldehyde (8 c): Brown solid (171 mg, 73% yield): R_f = 0.24 (EtOAc/Hex 1:3); mp: 129–131 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.02 (d, J = 4.3 Hz, 1H), 7.15 (dd, J = 8.7, J = 1.4 Hz, 1H), 7.48 (d, J = 5.1 Hz, 1H), 7.56 (d, J = 3.6 Hz, 1H), 8.37 (d, J = 4.3 Hz, 1H), 10.1 ppm (s, 1H, CHO); ESI-MS: m/z 235 $[M+1]^+$.

6-(trifluoromethyl)imidazo[2,1-*b*]thiazol-5-carbaldehyde (8 d): White solid (154 mg, 70% yield): R_f = 0.22 (EtOAc/Hex 3:7); mp: 110–112 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.24 (d, J = 4.5 Hz, 1H), 8.42 (d, J = 4.5 Hz, 1H), 10.01 ppm (s, 1H, CHO); ESI-MS: m/z 221 $[M+1]^+$.

6-(3,4-dimethoxyphenyl)imidazo[2,1-*b*]thiazol-5-carbaldehyde (8 e): White solid (216 mg, 75% yield): R_f = 0.28 (EtOAc/Hex 2:3); mp: 183–185 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.94 (s, 3H), 3.96 (s, 3H), 6.94 (d, J = 8.7 Hz, 1H), 7.01 (d, J = 4.3 Hz, 1H), 7.28 (dd, J = 9.5, J = 6.5 Hz,1H), 7.36 (d, J = 2.1 Hz, 1H), 8.39 (d, J = 4.3 Hz, 1H), 9.89 ppm (s, 1H, CHO); ESI-MS: m/z 289 $[M+1]^+$.

6-(4-methoxyphenyl)-2-methylimidazo[2,1-*b*]thiazol-5-carbaldehyde (8 f): White solid (190 mg, 70% yield): R_f = 0.3 (EtOAc/Hex 1:4); mp: 160–162 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.51 (s, 3H), 3.86 (s, 3H), 6.98 (d, J = 8.9 Hz, 2H), 7.70 (d, J = 8.9 Hz, 2H), 8.09 (s, 1H), 9.82 ppm (s, 1H, CHO); m/z ESI-MS: 273 $[M+1]^+$.

6-(4-fluorophenyl)-2-methylimidazo[2,1-*b*]thiazol-5-carbaldehyde (8g): White solid (190 mg, 73% yield): $R_f = 0.32$ (EtOAc/Hex 1:4); mp: 157–159 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.52$ (s, 3H), 7.17 (t, $J = 8.7$ Hz, 2H), 7.75 (dd, $J = 13.6$, $J = 3.9$ Hz, 2H), 8.09 (s, 1H), 9.82 ppm (s, 1H, CHO); ESI-MS: *m/z* 261 $[M+1]^+$.

6-(thien-2-yl)-2-methyl-imidazo[2,1-*b*]thiazol-5-carbaldehyde (8h): Yellow solid (186 mg, 75% yield): $R_f = 0.26$ (EtOAc/Hex 2:3); mp: 111–113 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.53$ (s, 3H), 7.13 (dd, $J = 8.8$, $J = 1.3$ Hz, 1H), 7.45 (dd, $J = 6.0$, $J = 4.1$ Hz, 1H), 7.52 (dd, $J = 4.7$, $J = 2.6$ Hz, 1H), 8.08 (d, $J = 1.3$ Hz, 1H), 10.04 ppm (s, 1H, CHO); ESI-MS: *m/z* 249 $[M+1]^+$.

Synthesis of (*E*)-3-(6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (3a): A 10% aqueous solution of NaOH (5 mL) was added to a stirred solution of 3,4,5-trimethoxyphenyl acetophenone (**9a**) (210 mg, 1 mmol) and 6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-carbaldehyde (**8a**) (258 mg, 1 mmol) in ethanol (20 mL). The reaction mixture was stirred at room temperature 27 °C for 4 h and the reaction was monitored by TLC using EtOAc/Hex (5:5) as a solvent system. The solvent was evaporated under vacuum, then the residue was dissolved in EtOAc/H_2O. The organic layer was washed with brine and evaporated. This was further purified by column chromatography using EtOAc/Hex (5:5) as a solvent system to obtain the pure product as yellow solid (361 mg, 80% yield): $R_f = 0.36$ (EtOAc/Hex 1:2); mp: 177–179 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.86$ (s, 3H), 3.92 (s, 9H), 6.96–7.06 (m, 4H), 7.18 (s, 2H), 7.19 (d, $J = 15.5$ Hz, 1H), 7.66 (d, $J = 8.5$ Hz, 1H), 7.83 (d, $J = 3.8$ Hz, 1H), 8.04 ppm (d, $J = 15.5$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 188.5$, 160.1, 153.4, 153.3 (2C), 142.2, 140.1, 133.6, 130.7, 130.2 (2C), 126.1, 119.1, 115.7 (2C), 114.1, 113.7, 105.8 (2C), 60.8, 56.3 (2C), 55.2 ppm; IR (KBr): $\tilde{\nu} = 3420$, 3120, 2924, 2834, 1647, 1557, 1501, 1455, 1382, 1330, 1298, 1243, 1162, 1121, 1005, 933, 841, 809, 767, 711 cm^{-1}; ESI-MS: *m/z* 451 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{24}H_{23}N_2O_5S$ calcd: 451.1327, found: 451.1338 $[M+1]^+$.

(*E*)-3-(6-(4-fluorophenyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(3,4,5-trimethoxyphenyl)pro-2-en-1-one (3b): This compound was prepared according to the method described for compound **3a**, employing compound **9a** (210 mg, 1 mmol) and 6-(4-fluorophenyl)imidazo-[2,1-*b*]thiazol-5-carbaldehyde (**8b**) (246 mg, 1 mmol) to obtain the pure product **3b** as a yellow solid (359 mg, 78% yield): $R_f = 0.38$ (EtOAc/Hex 1:2); mp: 222–225 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.89$ (s, 3H), 3.91 (s, 6H), 7.04 (d, $J = 4.3$ Hz, 1H), 7.12 (s, 2H), 7.15 (d, $J = 15.8$ Hz, 1H), 7.17 (d, $J = 8.0$ Hz, 1H), 7.24 (d, $J = 8.0$ Hz, 1H), 7.71 (dd, $J = 13.9$, $J = 3.6$ Hz, 2H), 7.83 (d, $J = 4.3$ Hz, 1H), 7.96 ppm (d, $J = 15.8$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 188.2$, 164.6, 161.3, 153.0 (2C), 152.0, 142.4, 133.4, 130.7, 129.7 (2C), 120.3, 119.1, 116.3 (2C), 115.8, 115.5, 114.1, 105.8 (2C), 60.8, 56.2 ppm (2C); IR (KBr): $\tilde{\nu} = 3434$, 3099, 2927, 1649, 1569, 1531, 1456, 1381, 1332, 1250, 1226, 1191, 1157, 1118, 1068, 990, 938, 846, 773, 709 cm^{-1}; ESI-MS: *m/z* 439 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{23}H_{19}N_2O_4FNaS$ calcd: 461.0947, found: 461.0930 $[M+Na]^+$.

(*E*)-3-[6-(2-thienyl)imidazo[2,1-*b*][1,3]thiazol-5-yl]-1-(3,4,5-trimethoxyphenyl)-2-propen-1-one (3c): This compound was prepared according to the method described for compound **3a**, employing compound **9a** (210 mg, 1 mmol) and 6-(thien-2-yl)imidazo[2,1-*b*]thiazol-5-carbaldehyde (**8c**) (234 mg, 1 mmol) to obtain the pure product **3c** as a yellow solid (349 mg, 82% yield): $R_f = 0.32$ (EtOAc/Hex 3:2); mp: 178–180 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.92$–3.95 (br, 9H), 7.05 (d, $J = 4.3$ Hz, 1H), 7.14 (d, $J = 4.3$ Hz, 1H), 7.16 (d, $J = 16.8$ Hz, 1H), 7.20 (s, 2H), 7.44 (t, $J = 3.6$ Hz, 2H), 7.82 (d, $J = 4.3$ Hz, 1H), 8.19 ppm (d, $J = 16.1$ Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): $\delta = 188.6$, 163.3, 153.0 (2C), 147.0, 142.3, 135.9, 133.5, 129.9 (2C), 128.3, 127.9, 127.2, 126.8, 119.8, 119.2, 116.5 (2C), 114.1, 105.8 (2C), 60.8, 56.3 ppm (2C); IR (KBr): $\tilde{\nu} = 3528$, 3113, 2969, 2934, 1650, 1568, 1502, 1456, 1417, 1376, 1331, 1255, 1189, 1161, 1123, 1069, 997, 967, 937, 831, 773, 712 cm^{-1}; ESI-MS: *m/z* 427 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{21}H_{18}N_2O_4NaS_2$ calcd: 449.0605, found: 449.0604 $[M+Na]^+$.

(*E*)-3-(6-(trifluoromethyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (3d): This compound was prepared according to the method described for compound **3a**, employing compound **9a** (210 mg, 1 mmol) and 6-(trifluoromethyl)imidazo-[2,1-*b*]thiazol-5-carbaldehyde (**8d**) (220 mg, 1 mmol) to obtain the pure product **3d** as a yellow solid (350 mg, 85% yield): $R_f = 4.00$ (EtOAc/Hex 7:3); mp: 177–179 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.89$ (s, 3H), 3.94 (s, 6H), 7.21 (d, $J = 4.5$ Hz, 1H), 7.22 (s, 2H), 7.34 (d, $J = 15.8$ Hz, 1H), 7.82 (d, $J = 4.5$ Hz, 1H), 7.90 ppm (d, $J = 16.6$ Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): $\delta = 186.7$, 151.8 (2C), 151.4, 141.3, 130.2, 125.2, 121.3, 119.9, 119.4, 118.8, 115.9, 105.1 (2C), 104.3, 59.4, 55.1 ppm; IR (KBr): $\tilde{\nu} = 3448$, 3105, 2939, 1667, 1583, 1503, 1463, 1413, 1333, 1283, 1234, 1161, 1123, 1072, 1006, 965, 941, 833, 733 cm^{-1}; ESI-MS: *m/z* 413 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{18}H_{15}N_2O_4F_3NaS$ calcd: 435.0602, found: 435.0605 $[M+Na]^+$.

(*E*)-3-(6-(3,4-dimethoxyphenyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (3e): This compound was prepared according to the method described for compound **3a**, employing compound **9a** (210 mg, 1 mmol) and 6-(3,4-dimethoxyphenyl)imidazo[2,1-*b*]thiazol-5-carbaldehyde (**8e**) (288 mg, 1 mmol) to obtain the pure product **3e** as a yellow solid (360 mg, 75% yield): $R_f = 0.38$ (EtOAc/Hex 1:2); mp: 179–181 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.91$ (s, 9H), 3.93 (s, 3H), 3.94 (s, 3H), 6.95 (d, $J = 8.2$ Hz, 1H), 7.04 (d, $J = 4.1$ Hz, 1H), 7.17 (s, 2H), 7.22 (d, $J = 8.2$ Hz, 2H), 7.29 (d, $J = 17.1$ Hz, 1H), 7.83 (d, $J = 4.8$ Hz, 1H), 8.07 ppm (d, $J = 15.8$ Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): $\delta = 188.5$, 153.0 (2C), 149.6, 133.6, 130.8, 126.2, 122.0, 121.8, 120.1, 119.2, 118.4, 115.8, 113.9, 111.5, 111.0, 110.7, 105.8 (2C), 60.9, 56.3 ppm (3C); IR (KBr): $\tilde{\nu} = 3437$, 3107, 2924, 2843, 1649, 1584, 1557, 1500, 1455, 1409, 1378, 1327, 1230, 1160, 1125, 1024, 995, 904, 862, 806, 766, 707 cm^{-1}; ESI-MS: *m/z* 481 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{25}H_{24}N_2O_6NaS$ calcd: 503.1252, found: 503.1262 $[M+Na]^+$.

(*E*)-3-(6-(trifluoromethyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(3,4-dimethoxyphenyl)prop-2-en-1-one (3f): This compound was prepared according to the method described for compound **3a**, employing compound **9b** (180 mg, 1 mmol) and 6-(trifluoromethyl) imidazo-[2,1-*b*]thiazol-5-carbaldehyde (**8d**) (220 mg, 1 mmol) to obtain the pure product **3f** as a yellow solid. (325 mg, 85% yield): $R_f = 0.42$ (EtOAc/Hex 2:3); mp: 166–169 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.97$ (s, 6H), 6.93 (d, $J = 9.0$ Hz, 1H), 7.19 (d, $J = 4.5$ Hz, 1H), 7.41 (d, $J = 15.8$ Hz, 1H), 7.59 (dd, $J = 6.7$, $J = 2.2$ Hz, 2H), 7.83 (d, $J = 4.5$ Hz, 1H), 7.91 ppm (d, $J = 15.8$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 187.3$, 153.5, 151.8, 149.3, 138.0, 130.6, 126.3, 122.9, 122.5, 121.5, 119.9, 118.6, 116.6, 110.6, 109.9, 56.0, 55.9 ppm; IR (KBr): $\tilde{\nu} = 3448$, 3124, 3006, 2954, 1658, 1600, 1575, 1511, 1467, 1419, 1379, 1328, 1298, 1257, 1167, 1116, 1019, 971, 922, 875, 804, 757, 705 cm^{-1}; ESI-MS: *m/z* 383 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{17}H_{13}N_2O_3F_3NaS$ calcd: 405.0496, found: 405.0488 $[M+Na]^+$.

(*E*)-3-(6-(4-methoxyphenyl)-2-methylimidazo[2,1-*b*]thiazol-5-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (3g): This compound was prepared according to the method described for compound **3a**, employing compound **9a** (210 mg, 1 mmol) and 6-(4-methoxyphenyl)-2-methylimidazo[2,1-*b*]thiazol-5-carbaldehyde (**8f**) (272 mg, 1 mmol) to obtain the pure product **3g** as a yellow solid

(362 mg, 78% yield): R_f = 0.42 (EtOAc/Hex 2:3); mp: 193–195 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.53 (s, 3H), 3.85 (s, 6H), 3.91 (s, 6H), 6.80 (d, J = 8.0 Hz, 1H), 6.98 (d, J = 8.0 Hz, 1H), 7.11 (d, J = 14.6 Hz, 1H), 7.15 (s, 2H), 7.51 (s, 1H), 7.63 (d, J = 7.3 Hz, 2H), 7.96 ppm (d, J = 16.8 Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): δ = 196.7, 188.7, 152.9 (2C), 133.7, 131.4, 130.9, 129.9 (2C), 127.8, 126.2, 122.7, 119.8, 115.7 (2C), 114.7, 114.0, 113.3, 105.8 (2C), 60.8, 56.2 (2C), 55.2 ppm; IR (KBr): $\tilde{\nu}$ = 3420, 3101, 2937, 2834, 1671, 1650, 1567, 1533, 1502, 1486, 1462, 1411, 1381, 1327, 1300, 1269, 1241, 1192, 1159, 1127, 1070, 1027, 996, 977, 932, 864, 842, 793, 737, 710 cm^{-1}; ESI-MS: *m/z* 465 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{25}H_{24}N_2O_5NaS$ calcd: 487.1303, found: 487.1317 $[M+Na]^+$.

(*E*)-3-(6-(4-fluorophenyl)-2-methylimidazo[2,1-*b*]thiazol-5-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (3h): This compound was prepared according to the method described for compound **3a**, employing compound **9a** (210 mg, 1 mmol) and 6-(4-fluorophenyl)-2-methylimidazo[2,1-*b*]thiazol-5-carbaldehyde (**8g**) (260 mg, 1 mmol) to obtain the pure product **3h** as a yellow solid (362 mg, 80% yield): R_f = 0.4 (EtOAc/Hex 2:3); mp: 159–162 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.54 (s, 3H), 3.79 (s, 3H), 3.90 (s, 6H), 7.31 (t, J = 8.6 Hz, 3H), 7.33 (s, 2H), 7.50 (d, J = 15.5 Hz, 1H), 7.68 (dd, J = 13.8, J = 3.4 Hz, 2H), 7.82 (d, J = 15.5 Hz, 1H), 8.22 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 186.8, 151.5 (2C), 149.4, 140.7, 132.1, 129.3 (2C), 128.7, 128.5, 126.6, 118.8, 115.8 (2C), 114.8, 114.3, 114.0, 104.6 (2C), 59.3, 54.9 ppm; IR (KBr): $\tilde{\nu}$ = 3432, 2937, 1644, 1591, 1569, 1531, 1506, 1485, 1452, 1412, 1382, 1353, 1325, 1275, 1230, 1170, 1136, 1038, 994, 972, 939, 864, 836, 810, 721, 709 cm^{-1}; ESI-MS: *m/z* 453 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{24}H_{21}N_2O_4FNaS$ calcd: 475.1103, found: 475.1120 $[M+Na]^+$.

(*E*)-3-(6-(thien-2-yl)-(2-methylimidazo[2,1-*b*]thiazol-5-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (3i): This compound was prepared according to the method described for compound **3a**, employing compound **9a** (210 mg, 1 mmol) and 6-(thien-2-yl)-2-methyl-imidazo[2,1-*b*]thiazol-5-carbaldehyde (**8h**) (248 mg, 1 mmol) to obtain the pure product **3i** as a yellow solid (361 mg, 82% yield): R_f = 0.44 (EtOAc/Hex 2:3); mp: 169–171 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.51 (s, 3H), 3.91 (s, 3H), 3.93 (s, 6H), 7.07 (s, 1H), 7.09 (d, J = 15.6 Hz, 1H), 7.11 (dd, J = 8.8, J = 1.3 Hz, 1H), 7.17 (s, 2H), 7.39 (dd, J = 7.3, J = 0.9 Hz, 1H), 7.50 (d, J = 1.3 Hz, 1H), 8.10 ppm (d, J = 15.6 Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): δ = 188.6, 176.8, 153.0 (2C), 147.0, 135.9, 133.5, 129.9, 127.9, 126.8, 119.8, 116.5, 114.1, 105.8 (2C), 60.8, 56.3 ppm (2C); IR (KBr): $\tilde{\nu}$ = 3426, 3100, 2937, 2833, 1660, 1570, 1504, 1461, 1411, 1331, 1259, 1222, 1190, 1159, 1124, 1000, 930, 851, 810, 707 cm^{-1}; ESI-MS: *m/z* 441 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{22}H_{20}N_2O_4NaS_2$ calcd: 463.0762, found: 463.0755 $[M+Na]^+$.

(*E*)-3-(6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(1*H*-pyrrol-2-yl)prop-2-en-1-one (3j): This compound was prepared according to the method described for compound **3a**, employing compound **9c** (109 mg, 1 mmol) and 6-(4-methoxyphenyl)imidazo [2,1-*b*]thiazol-5-carbalhedyde (**8a**) (258 mg, 1 mmol) to obtain the pure product **3p** as a yellow solid (286 mg, 82% yield): R_f = 0.34 (EtOAc/Hex 3:2); mp: 177–179 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.86 (s, 3H), 6.25 (dd, J = 5.8, J = 1.4 Hz, 2H), 7.04 (d, J = 8.7 Hz, 3H), 7.29 (d, J = 16.0 Hz, 1H), 7.36 (d, J = 5.1 Hz, 1H), 7.63 (d, J = 8.7 Hz, 2H), 7.89 (d, J = 16.0 Hz, 1H), 8.45 ppm (d, J = 4.3 Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): δ = 177.0, 160.1, 157.0, 154.9, 148.7, 136.3, 129.7, 126.9, 124.4, 122.9, 120.7, 119.5, 116.3, 114.6, 113.7, 109.6, 54.7 ppm; IR (KBr): $\tilde{\nu}$ = 3237, 3113, 2957, 1643, 1609, 1565, 1456, 1407, 1381, 1327, 1296, 1240, 1177, 1135, 1110, 1050, 1032, 943, 866, 835, 771, 742 cm^{-1}; ESI-MS: *m/z* 350 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{19}H_{15}N_3O_2NaS$ calcd: 372.0782, found: 372.0792 $[M+Na]^+$.

(*E*)-3-(6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(thien-2-yl)prop-2-en-1-one (3k): This compound was prepared according to the method described for compound **3a**, employing compound **9d** (126 mg, 1 mmol) and 6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-carbalhedyde (**8a**) (258 mg, 1 mmol) to obtain the pure product **3k** as a yellow solid (275 mg, 75% yield): R_f = 0.38 (EtOAc/Hex 1:2); mp: 169–172 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.86 (s, 3H), 7.00 (d, J = 8.3 Hz, 2H), 7.03 (s, 1H), 7.11 (d, J = 15.8 Hz, 1H), 7.15 (d, J = 4.5 Hz, 1H), 7.62–7.68 (m, 3H), 7.76 (d, J = 3.0 Hz, 1H), 7.84 (d, J = 4.5 Hz, 1H), 8.06 ppm (d, J = 15.8 Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): δ = 181.2, 160.0, 153.7, 145.5, 133.2, 131.0, 130.2, 128.1, 125.9, 119.8, 119.4, 115.2, 114.1, 113.7, 55.2 ppm; IR (KBr): $\tilde{\nu}$ = 3424, 3136, 3111, 2932, 1633, 1555, 1517, 1452, 1376, 1331, 1286, 1196, 1180, 1026, 977, 891, 852, 771, 726 cm^{-1}; ESI-MS: *m/z* 367 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{19}H_{15}N_2O_2S_2$ calcd: 367.0574, found: 367.0568 $[M+1]^+$.

(*E*)-3-(6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazo-5-yl)-1-(3,5-difluorophenyl)prop-2-en-1-one (3l): This compound was prepared according to the method described for compound **3a**, employing compound **9e** (156 mg, 1 mmol) and 6-(4-methoxyphenyl) imidazo[2,1-*b*]thiazol-5-carbalhedyde (**8a**) (258 mg, 1 mmol) to obtain the pure product **3L** as a yellow solid (325 mg, 82% yield): R_f = 0.32 (EtOAc/Hex 3:7); mp: 234–236 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.87 (s, 3H), 7.02 (d, J = 9.0 Hz, 2H), 7.06 (d, J = 15.8 Hz, 1H), 7.46 (dd, J = 10.5, J = 6.0 Hz, 3H) 7.65 (d, J = 8.3 Hz, 2H), 7.86 (d, J = 4.5 Hz, 2H), 8.08 ppm (d, J = 15.1 Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): δ = 188.7, 165.7 (2c), 160.6, 141.1, 136.5, 135.9, 128.5, 127.3, 125.3, 122.5, 117.2, 114.8 (2c), 112.1, 110.5, 55.8 ppm; IR (KBr): $\tilde{\nu}$ = 3421, 3105, 2924, 2852, 1652, 1566, 1453, 1389, 1310, 1254, 1194, 1096, 1032, 974, 858, 805 cm^{-1}; ESI-MS: *m/z* 397 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{21}H_{15}N_2O_2F_2S$ calcd: 397.0822, found: 397.0827 $[M+1]^+$.

(*E*)-3-(6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(Benzo[*d*]1,3]dioxol-5-yl)prop-2-en-1-one (3m): This compound was prepared according to the method described for compound **3a**, employing compound **9f** (164 mg, 1 mmol) and 6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-carbalhedyde (**8a**) (258 mg, 1 mmol) to obtain the pure product **3m** as a yellow solid (323 mg, 80% yield): R_f = 0.30 (EtOAc/Hex 3:2); mp: 189–191 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.86 (s, 3H), 6.05 (s, 2H), 6.86 (d, J = 8.0 Hz, 1H), 7.01 (dd, J = 8.8, J = 4.4 Hz, 3H), 7.17 (d, J = 16.1 Hz, 1H), 7.46 (d, J = 2.2 Hz, 1H) 7.55 (d, J = 8.0 Hz, 2H), 7.65 (d, J = 8.8 Hz, 1H), 7.84 (d, J = 4.4 Hz, 1H), 8.03 ppm (d, J = 15.4 Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): δ = 189.7, 160.6, 154.6, 149.3, 145.7, 135.7, 131.5, 128.3, 125.3, 123.2, 121.6, 116.5, 113.9 (2c), 112.6, 101.5, 54.8 ppm; IR (KBr): $\tilde{\nu}$ = 3419, 3122, 2912, 1654, 1563, 1533, 1500, 1440, 1388, 1352, 1290, 1257, 1172, 1143, 1092, 1034, 956, 923, 832, 798, 756 cm^{-1}; ESI-MS: *m/z* 405 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{22}H_{17}N_2O_4S$ calcd: 405.0909, found: 405.0915 $[M+1]^+$.

(*E*)-3-(6-(4-fluorophenyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(Benzo[*d*]-[1,3]dioxol-5-yl)prop-2-en-1-one (3n): This compound was prepared according to the method described for compound **3a**, employing compound **9f** (164 mg, 1 mmol) and 6-(4-fluorophenyl)imidazo[2,1-*b*]thiazol-5-carbalhedyde (**8b**) (246 mg, 1 mmol) to obtain the pure product **3n** as a yellow solid (323 mg, 80% yield): R_f = 0.38 (EtOAc/Hex 3:2); mp: 205–207 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.07 (s, 2H), 6.88 (d, J = 8.1 Hz, 1H), 7.07 (d, J = 4.5 Hz, 1H), 7.18 (d, J = 15.6 Hz, 1H), 7.20 (d, J = 8.6 Hz, 1H), 7.47 (d, J = 2.2 Hz, 1H), 7.55 (dd, J = 8.1 Hz, 2H), 7.70 (d, J = 8.8 Hz, 2H), 7.86 (d, J = 4.5 Hz, 1H), 8.01 ppm (d, J = 15.6 Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): δ = 185.6, 158.5, 152.3, 151.7, 150.0, 146.7, 131.4, 128.8 (2c), 128.4, 124.7, 123.3, 119.6, 118.7, 118.3, 113.8 (2c), 112.7,

106.8, 106.5, 100.5, 53.9 ppm; IR (KBr): $\tilde{\nu}$ = 3368, 3136, 1653, 1574, 1528, 1496, 1440, 1392, 1354, 1284, 1258, 1159, 1092, 1038, 923, 837, 807, 782, 757 cm^{-1}; ESI-MS: *m/z* 393 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{21}H_{14}N_2O_3FS$ calcd: 393.0709, found: 393.0713 $[M+1]^+$.

(*E*)-3-(6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(3,4-dimethoxyphenyl)prop-2-en-1-one (**3o**): This compound was prepared according to the method described for compound **3a**, employing compound **9b** (180 mg, 1 mmol) and 6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazol-5-carbalhedyde (**8a**) (258 mg, 1 mmol) to obtain the pure product **3p** as a yellow solid (328 mg, 78% yield): R_f = 0.30 (EtOAc/Hex 3:7); mp: 195–197 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.86 (s, 3H), 3.95 (s, 6H), 6.87 (d, *J* = 8.3 Hz, 1H), 6.97 (s, 1H), 7.01 (d, *J* = 4.5 Hz, 2H), 7.23 (d, *J* = 15.1 Hz, 1H), 7.51 (d, *J* = 1.5 Hz, 1H), 7.54 (s, 1H), 7.65 (d, *J* = 9.0 Hz, 2H), 7.85 (d, *J* = 4.5 Hz, 1H), 8.02 ppm (d, *J* = 15.8 Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): δ = 187.6, 160.0, 153.0, 152.4, 149.1, 131.2, 130.0, 125.5, 122.4, 120.0, 119.4, 115.8, 114.1, 113.9, 110.6, 109.8, 55.9, 55.2 ppm; IR (KBr): $\tilde{\nu}$ = 3422, 3035, 2923, 1656, 1575, 1518, 1451, 1381, 1335, 1274, 1248, 1160, 1028, 966, 822, 792, 767 cm^{-1}; ESI-MS: *m/z* 421 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{23}H_{20}N_2O_4NaS$ calcd: 443.1041, found: 443.1046 $[M+Na]^+$.

(*E*)-3-(6-(4-fluorophenyl)imidazo[2,1-*b*]thiazol-5-yl)-1-(3,4-dimethoxyphenyl)prop-2-en-1-one (**3p**)**:** This compound was prepared according to the method described for compound **3a**, employing compound **9b** (180 mg, 1 mmol) and 6-(4-fluorophenyl)-imidazo-[2,1-*b*]thiazol-5-carbalhedyde (**8b**) (246 mg, 1 mmol) to obtain the pure product **3o** as a yellow solid (306 mg, 75% yield): R_f = 0.38 (EtOAc/Hex 2:3); mp: 221–223 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.96 (s, 6H), 6.91 (d, *J* = 8.3 Hz, 1H), 7.07 (d, *J* = 4.5 Hz, 1H), 7.17 (d, *J* = 8.3 Hz, 2H), 7.29 (d, *J* = 15.8 Hz, 1H), 7.54 (d, *J* = 2.2 Hz, 1H), 7.57 (s, 1H), 7.72 (dd, *J* = 9.0, *J* = 8.3 Hz, 2H), 7.86 (d, *J* = 4.5 Hz, 1H), 8.02 ppm (d, *J* = 15.8 Hz, 1H); ^{13}CNMR (75 MHz, $CDCl_3$): δ = 187.0, 163.8, 152.7, 148.5, 130.6, 129.8, 128.7, 127.9, 122.7, 120.3, 115.8, 115.3, 114.2, 111.5, 110.3, 109.9, 55.5 ppm; IR (KBr): $\tilde{\nu}$ = 3368, 3124, 2994, 2920, 1639, 1595, 1555, 1464, 1420, 1373, 1329, 1299, 1244, 1166, 1148, 1013, 981, 870, 837, 802, 768, 742 cm^{-1}; ESI-MS: *m/z* 409 $[M+1]^+$; HRMS (ESI *m/z*) for $C_{22}H_{17}N_2O_3FNaS$ calcd: 431.0841, found: 431.0857 $[M+Na]^+$.

Biology

Cell lines: The MCF-7 (human breast cancer) cell line was obtained from the American Type Culture Collection (ATCC, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% fetal calf serum and 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin sulfate (Sigma). The cells were passaged and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability (**MTT-based assays**)**:** Cell viability was assessed by the MTT-based assay using WST-1 (premixed WST-1 cell proliferation assay system, Takara), which is more sensitive than MTT. Briefly, MCF-7 cells were seeded in a 96-well plate (TPP) at a cell density of ~10^4 cells per well. After incubation overnight, the cells were treated with compounds **3b**–**e**, **3h**, or doxorubicin and incubated for a further 24 h. The medium was then discarded and replaced with fresh medium (100 μL) followed by the addition of WST-1 dye (10 μL). Plates were incubated at 37 °C for 30 min. Optical density (OD) was read at λ 420 nm using a Multimode Varioskan FLASH instrument (Thermo-Fischer Scientific).

Cell-cycle analysis: MCF-7 cells (~5×10^5) were seeded in a 60 mm dish and were allowed to grow for 24 h. Compounds **3b**–**e**, **3h**, or doxorubicin at a concentration of 4 μM were added to the culture media, and the cells were incubated for an additional 24 h. Cell harvest was carried out with trypsin–EDTA, fixed with ice-cold 70% ethanol at 4 °C for 30 min, washed with PBS, and incubated with a 1 mg mL^{-1} solution of RNase A (Sigma) at 37 °C for 30 min. Cells were collected by centrifugation at 2000 rpm (fixed angle rotor, 24×4g) for 5 min and further stained with 250 μL DNA staining solution [10 mg propidium iodide (PI), 0.1 mg trisodium citrate, and 0.03 mL Triton X-100 dissolved in 100 mL sterile Milli-Q water at room temperature for 30 min in the dark]. The DNA content of 20000 events were measured by flow cytometer (Dako Cytomation, Beckman Coulter, Brea, CA, USA). Histograms were analyzed using Summit Software.

Cell proliferation assays: These assays were carried out by using the 5-bromo-2′-deoxyuridine (BrdU) cell proliferation assay kit (Millipore) to asses the effect of doxorubicin and compounds **3c** and **3d** on the proliferation of MCF-7 cells. Cells (~10^4) were seeded and allowed to grow for 24 h. BrdU was added and allowed to incorporate for 5 h followed by the addition of test compound at a concentration of 4 μM for 24 h. Fixation was done for 30 min at room temperature. The cells were then washed, anti-BrdU antibody was added which binds to BrdU that was incorporated in the cells. After 1 h incubation, 100 μL anti-BrdU goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution) was added and incubated for 30 min. Washing procedures were followed according to the manufacturer's instructions. TMB substrate (100 μL) was added, incubated for another 30 min at room temperature, and OD values were read at λ 450 nm. Lower OD values reflect lower BrdU concentrations in the sample, and thus an indirect depiction of a low cell proliferation rate.

Protein extraction and western blot analysis: Total cell lysates were isolated from cultured MCF-7 cells after treatments with test compounds as mentioned above by lysing the cells in ice-cold RIPA buffer (1×PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS containing protease inhibitors). After centrifugation at 12000 rpm (fixed angle rotor, 24×4g) for 10 min, the protein in the supernatant was quantified by the Bradford method (Bio-Rad) using a Multimode Varioskan instrument (Thermo-Fischer Scientific). Protein (30 μg per lane) was subjected to 12% SDS-PAGE. After electrophoresis, the protein was transferred to a polyvinylidine difluoride (PVDF) membrane (Amersham Biosciences). The membrane was blocked at room temperature for 2 h in TBS + 0.1% Tween-20 (TBST) containing 5% blocking powder (Santa Cruz Biotechnology). The membrane was washed with TBST for 5 min, and primary antibody was added and incubated at 4 °C overnight. Rabbit polyclonal antibodies p53 (53 kDa), CDK2 (32 kDa), E2F-1 (50 kDa), and cyclin D1 (35 kDa) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies cyclin E1, (40 kDa), β-actin (38–53 kDa), Rb (110 kDa); mouse monoclonal antibodies chk2 (60 kDa), cyclin A (50 kDa), NF-κB (65 kDa); and goat polyclonal antibodies p27 (27 kDa) were purchased from Imgenex Pvt. Ltd. (Orissa, India). Mouse monoclonal Ikkα antibody was purchased from Abcam Co. Mouse monoclonal p21 antibody (21 kDa) was purchased from Ana Spec Co. Phospho-Rb (Ser780; 110 kDa) rabbit polyclonal antibody was purchased from Cell Signalling Technology. Rabbit polyclonal antibody CDK4 was purchased from Pro Scientifics. After three TBST washes, the membrane was incubated with corresponding HRP-labeled secondary antibody (1:2000; Santa Cruz Biotechnology) at room temperature for 1 h. Membranes were washed with TBST three times for 15 min, and the protein blots were visualized with chemiluminescence reagent

(Thermo-Fischer Scientific). The X-ray films were developed with developer and fixed with fixer solution (Konica Minolta).

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