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Design and Synthesis of Aminostilbene–Arylpropenones as Tubulin Polymerization Inhibitors

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A series of aminostilbene—arylpropenones were designed and synthesized by Michael addition and were investigated for their cytotoxic activity against various human cancer cell lines. Some of the investigated compounds exhibited significant antiproliferative activity against a panel of 60 human cancer cell lines of the US National Cancer Institute, with 50% growth inhibition (GI_{50}) values in the range from <0.01 to 19.9 μM. One of the compounds showed a broad spectrum of antiproliferative efficacy on most of the cell lines, with a GI_{50} value of <0.01 μM. All of the synthesized compounds displayed cytotoxicity against A549 (non-small-cell lung cancer), HeLa (cervical carcinoma), MCF-7 (breast cancer), and HCT116 (colon carcinoma) with 50% inhibitory concentration (IC_{50}) values ranging from 0.011 to 8.56 μM. A cell cycle assay revealed that these compounds arrested the G2/M phase of the cell cycle. Two compounds exhibited strong inhibitory effects on tubulin assembly with IC_{50} values of 0.71 and 0.79 μM. Moreover, dot-blot analysis of cyclin B1 demonstrated that some of the congeners strongly induced cyclin B1 protein levels. Molecular docking studies indicated that these compounds occupy the colchicine binding site of tubulin.

Introduction

Tubulin binding agents are known to play a major role in cancer chemotherapy, and well-known tubulin binders such as paclitaxel, vinca alkaloids, vincristine, and vinblastine are routinely employed as anticancer agents.[1] Microtubule, a polymer of tubulin with α and β subunits, is involved in signal transduction pathways of mammalian cells, particularly in cell division.[2] Hence, antitubulin agents block the transition from interphase to mitosis[3] and, thus, effectively block cell-cycle progression, which results in apoptosis.[4] Antimitotic agents derived from natural and synthetic products generally exert their effect as microtubule stabilizers or polymerizing agents, such as Taxol, paclitaxel, and docetaxel,[5] which block microtubule disassembly. They bind at the β-tubulin site in the microtubules and are used in the treatment of carcinomas, such as lung, breast, ovarian, and bladder. In contrast, microtubule destabilizers such as colchicinium,[6] vinca alkaloids,[7] and combretastatin A-4[8] bind at the β-tubulin site in microtubules and cause the depolymerization of the microtubules. Many of such agents manifest different limitations in their clinical utility owing to drug resistance and neurotoxicity;[9] therefore, the development of new microtubule targeting agents is of significance.

Combretastatin A-4 (CA-4, **1a**; Figure 1) is an excellent tubulin polymerization inhibitor that binds to the colchicine binding site of tubulin and demonstrates cytotoxicity against a broad spectrum of human cancer cell lines including multidrug-resistant cancer cells. However, the in vivo efficiency of CA-4 is limited owing to its poor pharmacokinetics resulting from its high lipophilicity and low water solubility. Structural modification of CA-4 has led to the development of a number of new CA-4 derivatives that are potent tubulin polymerization inhibitors,[10] including combretastatin A-4 phosphate[11,12] (CA-4P, **1b**; Figure 1) and CA-1 disodium phosphate (CA-1P, Oxi 4503) as prodrugs. The water-soluble prodrugs CA-4P and CA-1P have reached the most advanced stage of preclinical development. Currently, CA-4P is under investigation in phase II trails for ovarian, lung, and anaplastic thyroid cancers, and it displays selective toxicity towards the vasculature of the tumor.[13] However, as its potentiality appears to be uncertain,[14] many research groups are looking to improve this scaffold by different structural modifications. Some recent studies have shown that amino-substituted combretastatin derivatives have potential for further development. In addition, increased water solubility of a serine prodrug of combretastatin amine (**1d**, ombrabulin, AVE-8062) has a stronger effect on tumor blood flow; this leads to complete blockage of the nutrient supply to the solid tumor and thereby leads to necrosis.[15,16]

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Supporting information (^1H NMR, ^{13}C NMR, and HRMS spectra of aminostilbene–arylpropenones **3a–h**, **4a–h**, and **5a–f**) for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201402256.

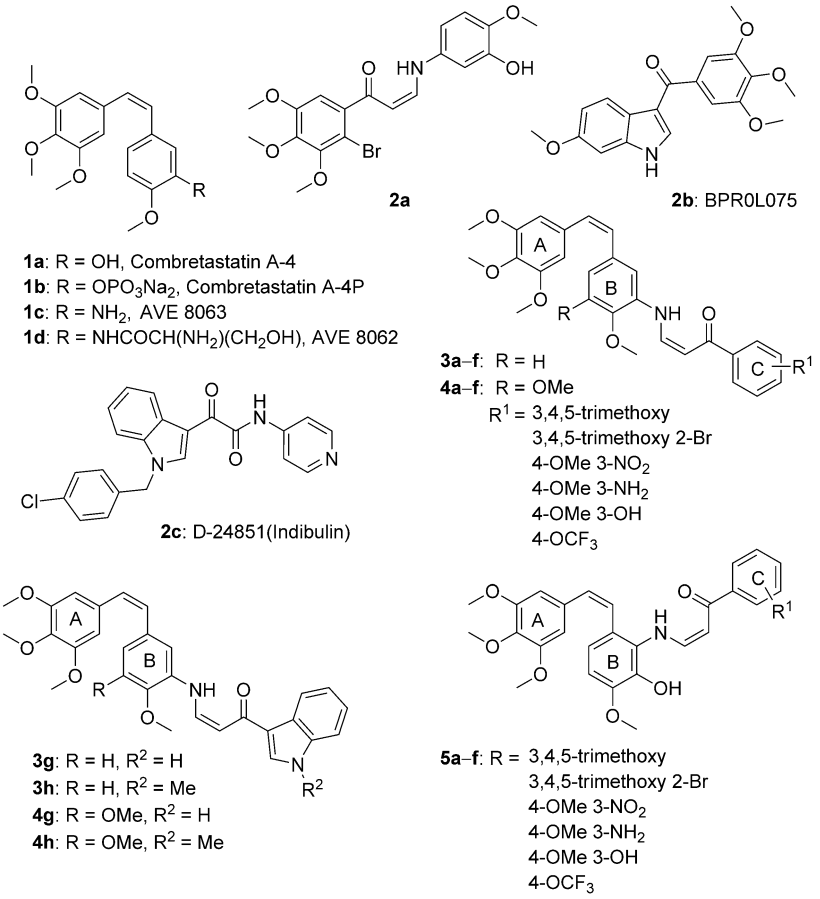


Figure 1. Chemical structure of microtubule targeting agents. CA-4 (**1a**), CA-4P (**1b**), AVE8063 (**1c**), AVE8062 (**1d**), (*Z*)-1-(2-bromo-3,4,5-trimethoxyphenyl)-3-[(3-hydroxy-4-methoxyphenyl)amino]prop-2-en-1-one (**2a**), BPR0L075 (**2b**), D-24851 (Indibulin, **2c**), aminostilbene–arylpropenones **3a**–**h**, **4a**–**h**, and **5a**–**f**.

Structure–activity relationship (SAR) studies of CA-4 analogues have shown that both hydroxy and amino substituents contribute to improve the antimitotic activity and provide the opportunity to structurally modify this scaffold.[17]

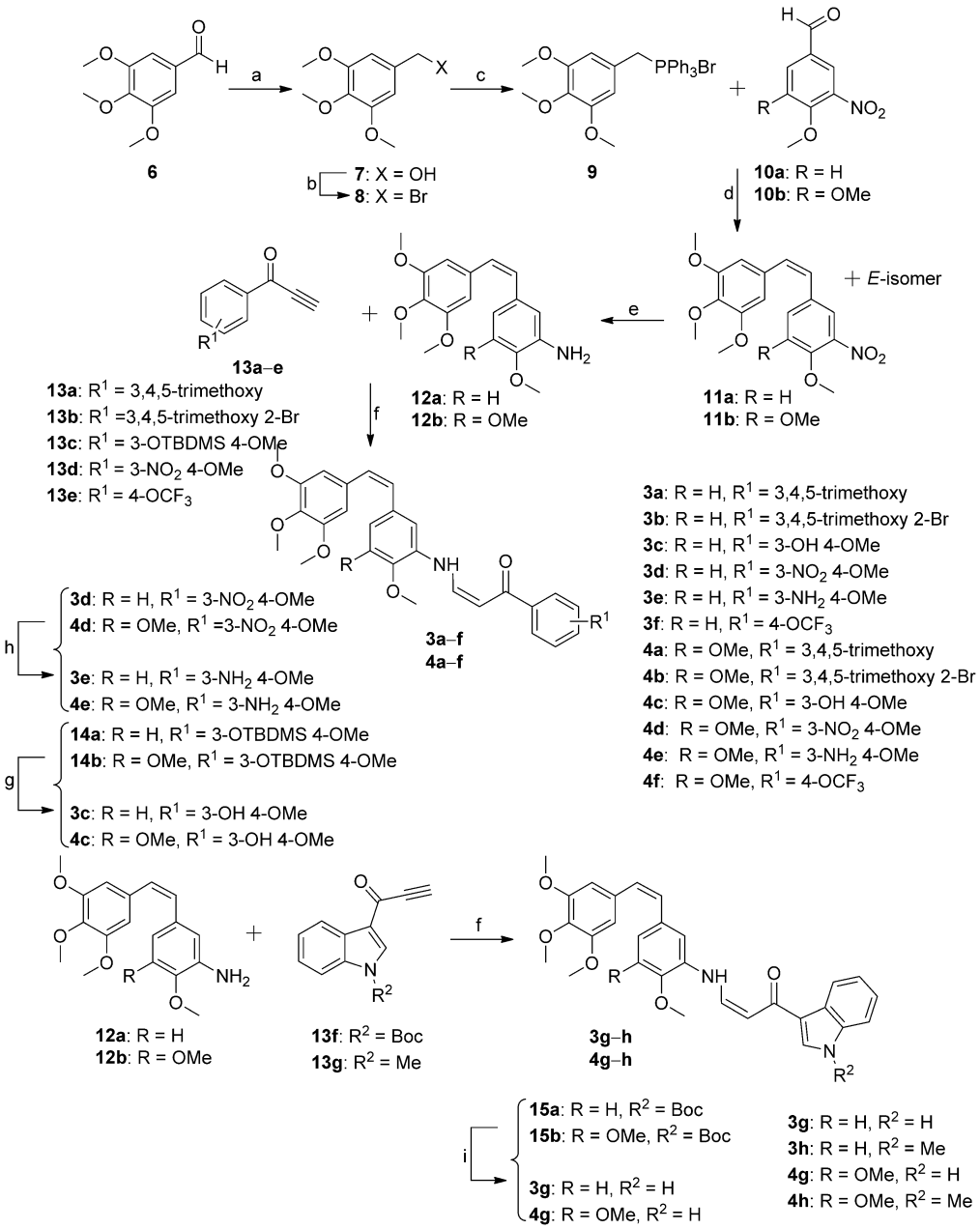
Recently, a series of antiproliferative compounds based on the (*Z*)-1-aryl-3-arylamino-2-propen-1-one scaffold of **2a** (Figure 1) were reported to significantly inhibit multidrug-resistant cancer cells and were also found to arrest the cells in the G2/M phase of the cell cycle.[18] A number of tubulin polymerization inhibitors containing an indole core nucleus have been obtained from natural sources and generated through design and synthesis. Some of the indole-containing compounds exhibit excellent antimitotic activity such as D-64131, D-24851, BPR0L075, BLF 61-3, and methyl 5-methoxy-3-((3,4,5-trimethoxyphenyl)thio)-1*H*-indole-2-carboxylate (ATI) derivatives.[19] 6-Methoxy-3-(3′,4′,5′-trimethoxybenzoyl)-1*H*-indole (BPR0L075, Figure 1), a microtubule depolymerizing agent, exhibits antiangiogenic activities.[20,21] Indibulin {*N*-(pyridin-4-yl)-[1-(4-chlorbenzyl)indol-3-yl]glyoxyl amide, D-24851; Figure 1a} is a synthetic small molecule with microtubule destabilizing activity.[22]

Thus, it is clear from the background presented above that the pharmacophores represented by aminostilbene **1c** and arylpropenones **2a** offer excellent possibilities for hybridization towards the design of improved tubulin polymerization inhibitors. In addition, the presence of an aryl ring in **2a** provides an opportunity to introduce a privileged indole scaffold in such a design. We have been previously involved in the development of new heterocyclic scaffolds such as combretastatin–amidobenzothiazole conjugates, and benzofurans were shown to be potential inhibitors of tubulin polymerization with significant cytotoxic activity.[23,24] In continuation of our earlier efforts and on the basis of literature findings, we linked amino-substituted combretastatin with arylamino-2-propenones to generate aminostilbene–arylpropenone hybrids. The 3,4,5-trimethoxyphenyl unit constitutes the A ring, which is utilized without any structural modification in view of its pharmacophoric importance. The aryl units represented by the B ring and C ring are varied to incorporate different substituents including the indole framework in some cases.

Results and Discussion

Chemistry

The syntheses of aminostilbene–arylpropenones **3a**–**h**, **4a**–**h**, and **5a**–**f** described in this study are outlined in Schemes 1 and 2. The final step was performed by Michael condensation between (*Z*)-3-substituted-2-methoxy-5-(3,4,5-trimethoxystyryl)aniline **12a**/**12b** or (*Z*)-2-amino-6-methoxy-3-(3,4,5-trimethoxystyryl)phenol (**21**) and substituted phenylprop-2-yn-1-ones **13a**–**f** or *N*-substituted-3-indolyl prop-2-yn-1-ones **13g**/**13h** in ethanol. The key intermediates, **12a**/**12b**, were prepared in five steps. 3,4,5-Trimethoxybenzaldehyde (**6**) was reduced by using sodium borohydride in methanol to give (3,4,5-trimethoxyphenyl)methanol (**7**). This was further treated with phosphorus tribromide in dichloromethane to produce 5-(bromomethyl)-1,2,3-trimethoxybenzene (**8**), which upon further reaction with triphenylphosphine in toluene gave 3,4,5-trimethoxybenzyltriphenylphosphonium bromide (**9**)[25] in good yield. The Witting salt thus formed was treated with substituted benzaldehydes **10a**/**10b** in the presence of sodium hydride in dichloromethane to produce (*Z*)-1,2,3-trimethoxy-5-(substituted-nitrostyryl)benzenes **11a**/**11b** and their *E* isomers in a 1:1 ratio.

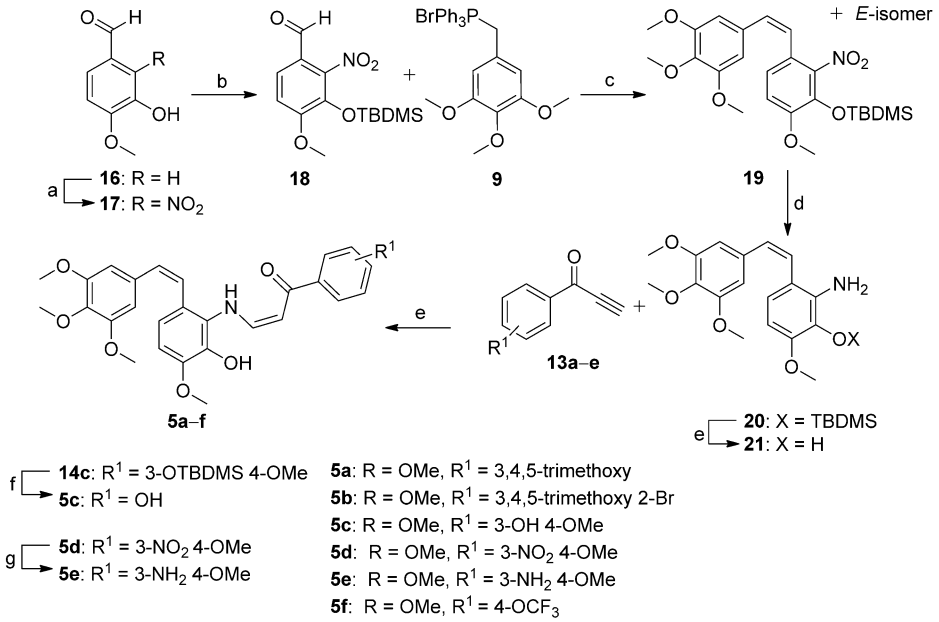


Scheme 1. *Reagents and conditions*: a) $NaBH_4$, MeOH, 3 h, 15–20 °C; b) PBr_3, CH_2Cl_2, 2 h, 15–20 °C; c) PPh_3, toluene, 12 h, 80 °C; d) NaH, CH_2Cl_2, 18 h, 15–20 °C; e) Zn AcOH, 4 h, RT; f) EtOH, RT, 4 h; g) TBAF (1 M in THF), THF 0 °C, 4 h; h) Zn, ammonium formate, EtOH, 4 h; i) TFA, CH_2Cl_2, 6 h, 0–5 °C.

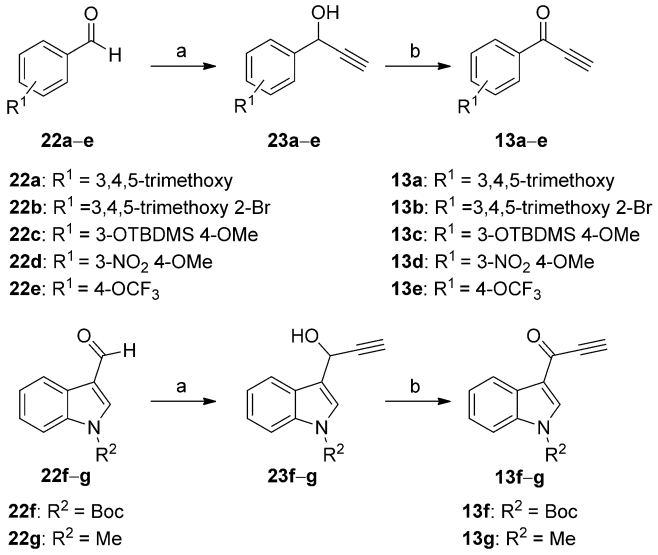
Compounds **11a**/**11b** were reduced with Zn in acetic acid to afford **12a**/**12b**.

Key intermediate **21** was prepared in five sequential steps. Isovaniline (**16**) was nitrated with nitronium tetrafluoroborate to give 2-nitro 3-hydroxybenzaldehyde (**17**). This was further treated with *tert*-butyldimethylsilyl chloride (TBDMSCl) and imidazole in dichloromethane to produce 3-(*tert*-butyldimethylsilyloxy)-4-methoxy-2-nitrobenzaldehyde (**18**). The obtained compound was treated with 3,4,5-trimethoxybenzyltriphenylphosphonium bromide (**9**) in the presence of sodium hydride in dichloromethane to produce (*Z*)-*tert*-butyl[6-methoxy-2-nitro-3-(3,4,5-trimethoxystyryl)phenoxy]dimethylsilane (**19**). Then, **19** was further reduced with Zn in acetic acid to give (*Z*)-2-(*tert*-butyldimethylsilyloxy)-3-methoxy-6-(3,4,5-trimethoxystyryl)aniline (**20**). Later, **20** was deprotected by using tetrabutylammonium fluoride (TBAF, 1 N in THF) to give derivative **21**.[25]

Aryl/heteroaryl prop-2-yn-1-ones **13a**–**g** were prepared in two steps, as shown in Scheme 3. Substituted benzaldehydes **22a**–**g** were treated with ethynylmagnesium bromide (0.5 N in THF) to give aryl/heteroaryl prop-2-yn-1-ols **23a**–**g**. These secondary alcohols were oxidized with 2-iodoxybenzoic acid (IBX) in dimethyl sulfoxide (DMSO) to produce aryl/heteroaryl prop-2-yn-1-ones **13a**–**g** in good yields.



Scheme 2. *Reagents and conditions*: a) Nitronium tetrafluoroborate, CH_3NO_2/CH_2Cl_2 (6:4), −40 °C, 8 h; b) TBDMSCl, imidazole, CH_2Cl_2, 3 h, 15–20 °C; c) NaH, CH_2Cl_2, 18 h, 15–20 °C; d) Zn AcOH, 4 h, RT; e) EtOH, RT, 4 h; f) TBAF (1 M in THF), THF 0 °C, 4 h; g) Zn, ammonium formate, EtOH, 4 h.



Scheme 3. *Reagents and conditions*: a) Ethynylmagnesium bromide (0.5 N in THF), THF, 8 h, 15–20 °C; b) IBX, DMSO, 15–20 °C, 2 h.

Final compounds **3e**, **4e**, and **5e** were prepared by the reduction of **3d**, **4d**, and **5d**, respectively, with Zn and ammonium formate in methanol. Compounds **14a**, **14b**, and **14c** upon deprotection with TBAF (1 N in THF) afforded **3c**, **4c**, and **5c**, respectively. Compounds **3g** and **4g** were prepared from **15a** and **15b** by deprotection of the *tert*-butoxycarbonyl (Boc) group by using trifluoroacetic acid (TFA) in dichloromethane.

Biological studies

Antiproliferative activity

Congeners **3a–h**, **4a–h**, and **5a–f** (Scheme 1) and **2** were evaluated for their cytotoxic activity by the US National Cancer Institute (NCI) in a 60-cell-line screen. Among the 22 compounds, compounds **3g**, **4a**, **4f**, **4g**, and **4h** were selected for a preliminary test at a single concentration (10 μM), and these compounds exhibited significant growth inhibition. Apart from **4h**, the other compounds were further evaluated in a five-dose screen. Compounds **3g**, **4a**, **4f**, and **4g** showed remarkable antiproliferative activity in a nine cancer panel of this NCI screen (leukemia, lung, colon, central nervous system, melanoma, ovary, kidney, prostate, and breast cancers). Compound **3g** with a methoxy group in the B ring and an indole moiety in the C ring emerged as the most promising in this series. Notably, **3g** demonstrated a 50% growth inhibition (GI_{50}) value of <0.01 μM in most of the cell lines of the 60-cell-line panel; thus, it possesses a broad spectrum of cytotoxicity. Compounds **4a**, **4f**, and **4g** have a common dimethoxy group in the B ring as a substituent, and presumably, the presence of trimethoxy (for **4a**), 4-trifluoromethoxy (for **4f**), and indole (for **4g**) groups in the C rings of these molecules resulted in cytotoxic activity in the NCI screen (see Table 1). Therefore, all of the compounds of this series were evaluated for their antiproliferative activities. Interestingly, similar to the NCI screening results, compounds **3a–h** (Figure 2) possessing a methoxy group in the B ring showed profound cytotoxic activity with a 50% inhibitory concentration (IC_{50}) value range of 0.011–4.68 μM. Among them, compound **3e**, which possess *meta*-amine and *para*-methoxy groups on the C ring, inhibited the growth of A549 cells with a IC_{50} value of 11 nM, as shown in Table 2. Moreover, compounds **4a–h** with *meta*,*para*-dimethoxy-substituted B rings also inhibited cell growth with an IC_{50} value in the range from 0.09 to 7.59 μM. Notably, **4e** with *meta*-amino and *para*-methoxy substitutions in the C ring inhibited growth of A549 cells with an IC_{50} value of 90 nM. In addition, hydroxy and methoxy substituents in the B rings of **5a–h** resulted in less-potent cytotoxic activities with IC_{50} values of 0.91–7.33 μM. Compound **5e** with *meta*-amino and *para*-methoxy groups in the C ring showed potent cytotoxic activity against MCF-7 cells with an IC_{50} value of 0.91 μM. Taken together, these results suggest that congeners with methoxy or dimethoxy moieties in the B ring and indole or *meta*-amino and *para*-methoxy substitutions in the C ring exhibit potent antiproliferative activities. Moreover, on the basis of the five-dose results of the NCI, we

Table 1. In vitro cytotoxic effect of conjugates **3g**, **4a**, **4f**, **4g**, and **1a** against a panel of 60 human cancer cells.

Cell line	GI_{50} [μM][a]					Cell line	GI_{50} [μM][a]				
	3g[b]	**4a**[c]	**4f**[d]	**4g**[e]	**1a**[f]		**3g**[b]	**4a**[c]	**4f**[d]	**4g**[e]	**1a**[f]
Leukemia						***Ovarian***					
CCRF-CEM	< 0.01	2.93	3.55	1.37	0.002	IGROV1	< 0.01	6.06	7.07	1.23	0.015
HL60(TB)	< 0.01	3.24	2.80	–[h]	–[h]	OVCAR-3	< 0.01	2.31	3.48	1.72	0.001
K-562	< 0.01	3.38	2.93	1.54	0.002	OVCAR-4	< 0.01	2.91	7.54	1.52	0.015
MOLT-4	< 0.01	4.62	3.99	2.53	0.003	OVCAR-5	< 0.01	3.64	8.20	2.45	0.1
RPMI-8226	< 0.01	3.04	3.66	0.63	0.003	OVCAR-8	< 0.01	4.70	4.59	3.33	0.003
SR	< 0.01	2.03	2.75	0.46	0.003	NCI/ADR-RES	< 0.01	3.12	3.04	3.49	0.001
						SK-OV-3	< 0.01	2.91	5.38	12.3	0.063
Non-small-cell lung						***Renal***					
A549/ATCC	< 0.01	5.14	4.54	14.5	0.015	786-0	< 0.01	3.76	4.34	2.04	0.1
HOP-62	< 0.01	2.46	7.85	11.6	0.002	A498	< 0.01	2.40	1.83	1.32	0.006
HOP-92	0.01	1.14	12.6	1.50	0.002	ACHN	< 0.01	3.99	5.56	1.75	0.006
NCI-H226	0.02	5.45	19.9	7.28	0.003	CAKI-1	< 0.01	3.37	4.96	1.58	0.025
NCI-H23	< 0.01	4.22	4.38	3.75	0.003	RXF 393	–[h]	–[h]	2.18	1.69	0.002
NCIH322M	–[g]	7.71	1.10	1.20	0.003	SN12C	0.02	4.03	4.85	2.44	0.006
NCI-H460	< 0.01	3.93	3.34	5.60	0.007	TK-10	–[g]	5.93	4.79	1.86	0.1
NCI-H522	–[h]	–[h]	1.96	1.76	0.001	UO-31	< 0.01	2.63	5.42	0.32	0.019
Colon						***Prostate***					
COLO-205	< 0.01	2.31	2.98	1.51	0.1	PC-3	< 0.01	2.89	–[h]	3.44	0.001
HCC-2998	< 0.01	8.92	3.71	3.34	0.063	DU-145	< 0.01	3.64	4.11	2.06	0.001
HCT-116	< 0.01	2.78	3.58	1.76	0.003						
HCT-15	< 0.01	3.08	3.42	2.32	0.003	***Breast***					
HT29	< 0.01	3.12	3.07	2.28	0.1	MCF7	< 0.01	2.34	4.08	0.72	0.005
KM12	< 0.01	3.21	3.69	3.69	0.005	MDA-MB-31/ATCC	< 0.01	4.01	3.00	1.73	0.001
SW-620	< 0.01	4.12	4.09	1.63	0.003	HS 578T	< 0.01	2.76	6.44	2.21	0.001
						BT-549	< 0.01	–[h]	5.90	1.64	10
Melanoma						T-47D	–[g]	1.68	4.16	1.55	10
LOX IMVI	< 0.01	3.54	5.95	0.44	0.003	MDA-MB-435	< 0.01	1.88	3.16	1.28	–[g]
MALME-3M	5.00	2.91	14.7	1.27	0.019	MDA-MB-435	< 0.01	1.88	3.16	1.28	–[g]
M14	< 0.01	2.53	3.46	2.87	0.002						
MDA-MB-435	< 0.01	1.22	1.84	2.43	0.001	***CNS***					
SK-MEL-2	< 0.01	4.54	–[h]	2.21	0.003	SF-268	< 0.01	6.04	8.90	3.57	0.006
SK-MEL-28	1.59	4.28	10.4	1.86	0.007	SF-295	< 0.01	2.40	4.12	7.94	0.003
SK-MEL-5	< 0.01	1.69	3.73	3.14	0.003	SF-539	< 0.01	1.92	2.26	1.70	0.002
UACC-257	–[g]	–[g]	4.33	3.16	0.003	SNB-19	< 0.01	4.49	4.97	3.92	0.003
UACC-62	4.38	3.11	6.86	1.39	0.005	SNB-75	< 0.01	1.39	2.65	1.47	0.007
						U251	< 0.01	3.84	3.46	3.84	0.007

[a] Data are reported as the GI_{50} value (concentration required to cause 50% inhibition of cell growth after an incubation time of 48 h). [b] NSC 777174. [c] NSC 777180. [d] NSC 773189. [e] NSC 777183. [f] NSC 613729. [g] Not active. [h] Not tested.

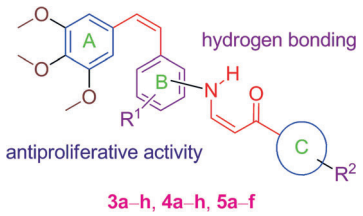


Figure 2. Structure–activity relationship of aminostilbene–arylpropenones.

hypothesize that these compounds inhibit a common target in all cell lines.

Dot-blot analysis for cyclin B1

Given that the congeners contain a combretastatin moiety, these molecules are expected to interfere with tubulin polymerization and arrest cells at the G2/M phase of the cell cycle.[26] As the majority of the molecules in this series exhibit significant cytotoxicity, we tested their ability to induce cyclin B1 protein, a marker for mitosis. To test this possibility, A549 cells were treated with various congeners at 1 μM concentrations for 24 h. Subsequently, dot-blot analysis for cyclin B1 indicated that most of the congeners strongly induced cyclin B1 protein levels. Notably, **3e**, **3g**, **4e**, and **5e** robustly activated cyclin B1. These results suggest that the congeners indeed arrest cells at the G2/M phase of the cell cycle; actin was employed as a loading control (Figure 3).

Effect on cell-cycle arrest

To investigate the effect of potential compounds **3e**, **3g**, and **4e** on the cell-cycle distribution of A549 cells, flow cytometry analysis was performed at a concentration of 1 μM for 24 h.

Table 2. In vitro cytotoxic effect of aminostilbene–arylpropenones **3a–h**, **4a–h**, and **5a–f**.

Compd	IC_{50} [μM][a]			
	A549[b]	HeLa[c]	MCF-7[d]	HCT116[e]
3a	1.68 ± 0.12	0.26 ± 0.03	0.27 ± 0.03	1.51 ± 0.02
3b	3.51 ± 0.3	0.25 ± 0.02	2.95 ± 0.78	3.98 ± 0.8
3c	0.77 ± 0.04	0.23 ± 0.012	1.59 ± 0.2	0.83 ± 0.05
3d	3.81 ± 0.51	2.21 ± 0.09	4.68 ± 0.47	1.29 ± 0.14
3e	0.011 ± 0.001	0.052 ± 0.013	0.025 ± 0.001	0.028 ± 0.004
3f	2.69 ± 0.23	0.26 ± 0.024	1.32 ± 0.09	2.95 ± 0.3
3g	0.015 ± 0.003	0.053 ± 0.001	0.031 ± 0.001	0.033 ± 0.001
3h	0.67 ± 0.04	0.46 ± 0.02	1.07 ± 0.049	0.37 ± 0.05
4a	6.25 ± 0.24	3.72 ± 0.35	3.08 ± 0.1	4.32 ± 0.021
4b	0.28 ± 0.02	1.58 ± 0.07	5.67 ± 0.27	0.3 ± 0.01
4c	0.92 ± 0.01	0.91 ± 0.016	2.09 ± 0.04	3.24 ± 0.13
4d	4.15 ± 0.09	1.2 ± 0.08	1.55 ± 0.31	4.79 ± 0.25
4e	0.09 ± 0.001	0.19 ± 0.045	0.11 ± 0.08	0.19 ± 0.08
4f	5.21 ± 0.019	2.86 ± 0.06	4.75 ± 0.07	7.59 ± 0.49
4g	8.56 ± 0.02	1.67 ± 0.05	3.02 ± 0.05	2.48 ± 0.21
4h	0.14 ± 0.015	0.89 ± 0.03	3.39 ± 0.2	1.95 ± 0.6
5a	2.25 ± 0.024	1.56 ± 0.014	2.23 ± 0.15	3.63 ± 0.41
5b	7.08 ± 0.56	2.78 ± 0.38	3.19 ± 0.4	2.69 ± 0.32
5c	1.58 ± 0.09	4.17 ± 0.14	2.2 ± 0.16	6.76 ± 0.54
5d	7.33 ± 0.21	2.66 ± 0.42	2.6 ± 0.02	3.98 ± 0.73
5e	1.31 ± 0.02	2.51 ± 0.08	0.91 ± 0.04	1.75 ± 0.026
5f	1.31 ± 0.4	7.08 ± 0.042	2.12 ± 0.063	1.94 ± 0.02
1a (CA-4)	0.031 ± 0.001	0.058 ± 0.007	0.058 ± 0.003	0.398 ± 0.46

[a] Concentration required to inhibit 50% cell growth following 48 h treatment with the tested drug. Values represent mean ± standard deviation from three different experiments performed in triplicate. [b] A549: non-small cell lung cancer. [c] HeLa: cervix cancer. [d] MCF-7: breast cancer. [e] HCT116: colon cancer cell line.

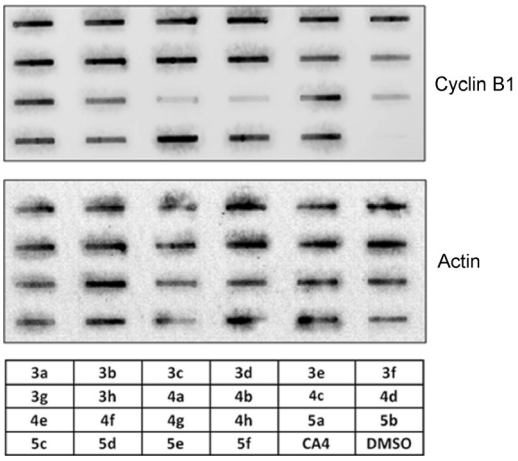


Figure 3. Dot-blot analysis of cyclin B1: A549 cells were treated with 1 μM concentrations of aminostilbene–arylpropenones for 24 h. Later, cells were harvested and whole-cell lysates were blotted on nitrocellulose membranes. The blotted proteins were probed with antibodies against cyclin B1. Actin was employed as loading control.

The results indicate that they caused G2/M cell-cycle arrest with a large accumulation of cells. Cells treated with **3e** at 1 μM showed 76.4% arrest of cells in the G2/M phase, whereas A549 cells treated with **3g** and **4e** at 1 μM showed arrest of cells in the G2/M phase by 73 and 71%, respectively (Figure 4). In contrast, vehicle- or DMSO-treated cells showed the majority of the population in the G1 phase (67.7%) of the cell cycle.[27]

Inhibition of tubulin polymerization

To further investigate whether the antiproliferative activities of compounds **3e**, **3g**, and **4e** were derived from interaction with tubulin, they were evaluated for their inhibition of tubulin polymerization (Table 3). The tubulin assembly assays revealed that these molecules showed potent inhibition of tubulin polymerization with IC_{50} values of 0.71 μM for **3e** and 0.79 μM for **3g**; these values are comparable to that for CA-4 (**1a**, Table 3).

Effect of 3e, 3g, and 4e on microtubule network

The presence of anomalous spindle fibers owing to altered microtubule dynamics is a hallmark of cells treated with microtubule inhibitors.[28] These aminostilbene–arylpropenones significantly decreased cell growth, inhibited tubulin assembly, and stalled cells in the G2/M phase of the cell cycle. Therefore, we were prompted to evaluate their ability to disrupt microtubule networks in the cells.[29] Consequently, A549 cells were treated with **3e**, **3g**, and **4e** at 1 μM concentrations for 24 h. Immunofluorescence analysis revealed that the cells exhibited rounded morphology typical of a mitotic-arrest population, and moreover, chromatin was condensed in the nuclei, which suggests metaphase arrest (Figure 5).

Effect of 3e, 3g, and 4e on cytosolic tubulin

Given that the aminostilbene–arylpropenones markedly inhibited in vitro tubulin polymerization, we investigated their ability to inhibit endogenous tubulin assembly. Microtubules exist in a dynamic equilibrium between free and polymerized tubulin,[30] and the amount of free tubulin increases in cells challenged with microtubule inhibitors. To test this possibility, A549 cells were treated with **3e**, **3g**, and **4e** at 1 μM concentrations for 24 h. Subsequently, the cells were permeabilized with detergents, and soluble (free tubulin) and polymerized fractions were collected. Immunoblot analysis suggested that the DMSO-treated cells contain fairly equal amounts of tubulin in both fractions (Figure 6.). In comparison, the compound-treated cells showed a significant amount of tubulin in the soluble fraction. Thus, these results suggest that the congeners strongly inhibit tubulin assembly in in vitro assays as well as in cell-based assays.

Molecular modeling studies

A molecular modeling study was performed to elucidate the binding characters of conjugates **3e**, **3g**, and **4e** with tubulin. Coordinates of the protein structure of tubulin–colchicine was obtained from the Protein Data Bank (PDB ID 3E22).[32] Docking studies of all compounds were accomplished into the colchicine binding site of tubulin by using AutoDock 4.2 software.[33] Congeners **3a–h**, **4a–h**, and **5a–f** possess two *cis*-olefinic units, and they were expected to occupy the colchicine bind-

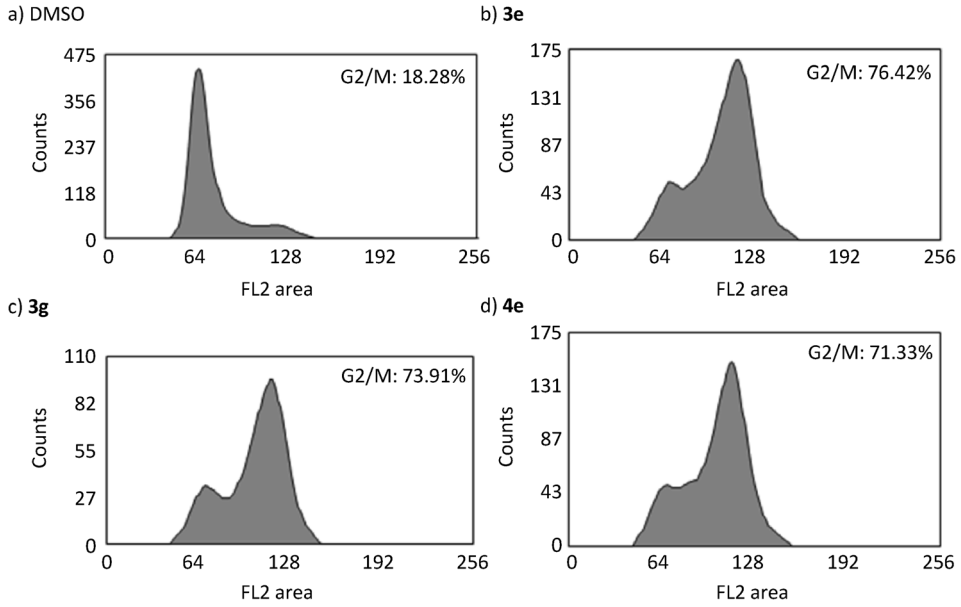


Figure 4. Antimitotic effects of **3e**, **3g**, and **4e** by fluorescence-activated cell sorting (FACS) analysis. Induction of cell-cycle G2/M arrest by compounds **3e**, **3g**, and **4e**. A549 cells were harvested after treatment at 1 μM for 24 h. Untreated cells and DMSO-treated cells served as controls. The percentage of cells in each phase of the cell cycle was quantified by flow cytometry.

Table 3. Antitubulin activity of aminostilbene–arylpropenones **3e**, **3g**, and **4e**.[a]

Compd	IC_{50} [μM]
3e	0.71 ± 0.01
3g	0.79 ± 0.04
4e	1.68 ± 0.43
1a (CA-4)	0.91 ± 0.12

[a] Inhibition of tubulin polymerization (IC_{50}) values for **3e**, **3g**, and **4e** were determined from tubulin polymerization assays.

ing site of tubulin. Conjugate **3e** exhibited a hydrogen-bonding interaction between the oxygen atom of the carbonyl group on the methoxyaniline ring with βLys254. Hydrophobic interactions were found between the methoxyaniline ring of **3e** with αAsn101 and βAsn249. The amine group of methoxyaniline was involved in a hydrogen-bonding interaction with αAla250 and in a hydrophobic interaction with βAsn250. The A ring (trimethoxyphenyl) of **3e** was buried in the hydrophobic pockets of βLys254, βAsn258, βVal238, βLeu242, βAla250, and βLeu255 in a manner similar to that of the trimethoxyphenyl group of colchicine. Some hydrophobic interactions were formed by the B ring in both the α- and β-tubulin interface with αSer178, αThr179, βLys352, and βAsn258. Conjugate **3g** showed a hydrogen-bonding interaction between indole and αGlu183, and this indole ring was buried in hydrophobic interactions with αTyr224, αGly142, and αAla180.

The trimethoxyphenyl group (A ring) showed hydrophobic interactions with βLeu255, βLeu248, βAla250, βLys254, and βLys255, and the methoxy group of the A ring established a hydrogen-bonding interaction with βLeu255. The methoxy group of the B ring showed hydrophobic interactions with βLeu254, βLeu255, and βAla354. The carbonyl group on the indole ring established a hydrophobic interaction with βLeu248. Conjugate **4e** established a hydrogen-bonding interaction between the methoxy group on aniline and βLys254. The carbonyl group on the methoxyaniline moiety showed a hydrogen-bonding interaction with αThr179 and a hydrophobic interaction with αSer178. The methoxyaniline group showed hydrophobic interactions with αTyr224, βGly247, and αGln11. The trimethoxyphenyl group (A ring) was buried in the hydrophobic pockets of βLeu255, βLys254, βLeu248, and βAla250.

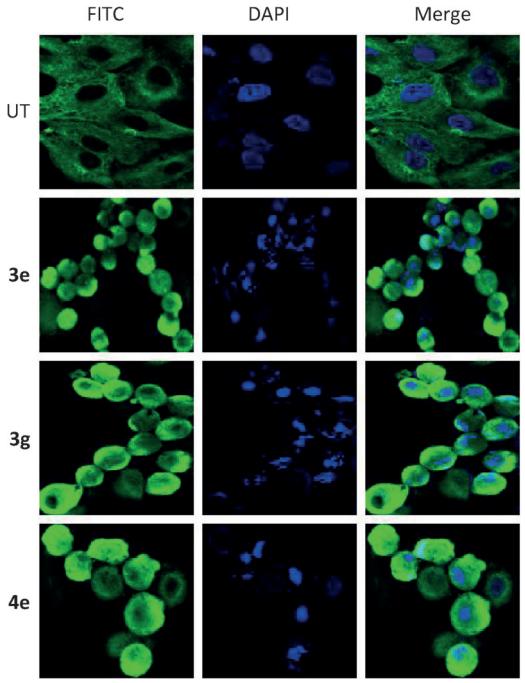


Figure 5. Effect of **3e**, **3g**, and **4e** on microtubules and nuclear condensation. A549 cells were independently treated with **3e**, **3g**, and **4e** at 1 μM for 24 h. Following treatment, cells were fixed and stained for tubulin by using 4′,6-diamidino-2-phenylindole (DAPI) as a counterstain. Photographs were taken by using an Olympus confocal fluorescence microscope equipped with fluorescein isothiocyanate (FITC) and DAPI filter settings. Cells stained for tubulin and DAPI from the same field of views are represented.

The dimethoxyphenyl group (B ring) showed hydrophobic interactions with βMet259, αAla180, βAla316, βLeu255, and βLys352. Conjugates **3e**, **3g**, and **4e** all interacted with the

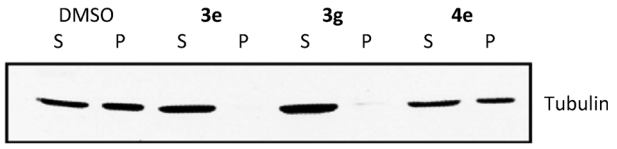


Figure 6. Distribution of tubulin in polymerized versus soluble fractions analyzed by immunoblotting in A549 cells treated with **3e**, **3g**, and **4e**. A549 cells were treated with 1 μM of **3e**, **3g**, and **4e** for 24 h. The fractions containing soluble and polymerized tubulin were collected and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Tubulin was detected by immunoblot analysis. S = soluble, P = polymerized.

α,β-tubulin interface in the colchicine binding pocket (Figure 7).

Conclusions

A library of novel aminostilbene–arylpropenones (i.e., **3a–h**, **4a–h**, and **5a–f**) comprising an A,B,C ring system was synthesized by using the Michael addition reaction, and the compounds were evaluated for their cytotoxic activity against human cancer cell lines. Representative congeners **3g**, **4a**, **4f**, and **4g** were evaluated in a 60-cell-line panel of the NCI five-dose screen. Among them, **3g**, which possesses a *para*-methoxy group in the B ring and an indole moiety as the C ring, exhibited excellent cytotoxicity in most human cancer cell lines with GI_{50} values <0.01 μM. Compounds **3a–h**, **4a–h**, and **5a–f** all displayed antiproliferative activity against four human cancer cell lines, including A549 (non-small cell lung cancer), HeLa (cervical carcinoma), MCF-7 (breast cancer), and HCT116 (colon carcinoma) with IC_{50} values ranging from 0.011 to 8.56 μM, and some of these values are comparable to that of CA-4 (**1a**) The cell-cycle assays revealed that congeners **3e**, **3g**, and **4e** caused cell-cycle arrest and accumulated cells in the G2/M phase. Furthermore, these compounds effectively inhibited microtubule assembly and induced cyclin B1 protein expression. As hypothesized, lead compounds **3e**, **3g**, and **4e** exert their cytotoxic activity by inhibition of tubulin polymeri-

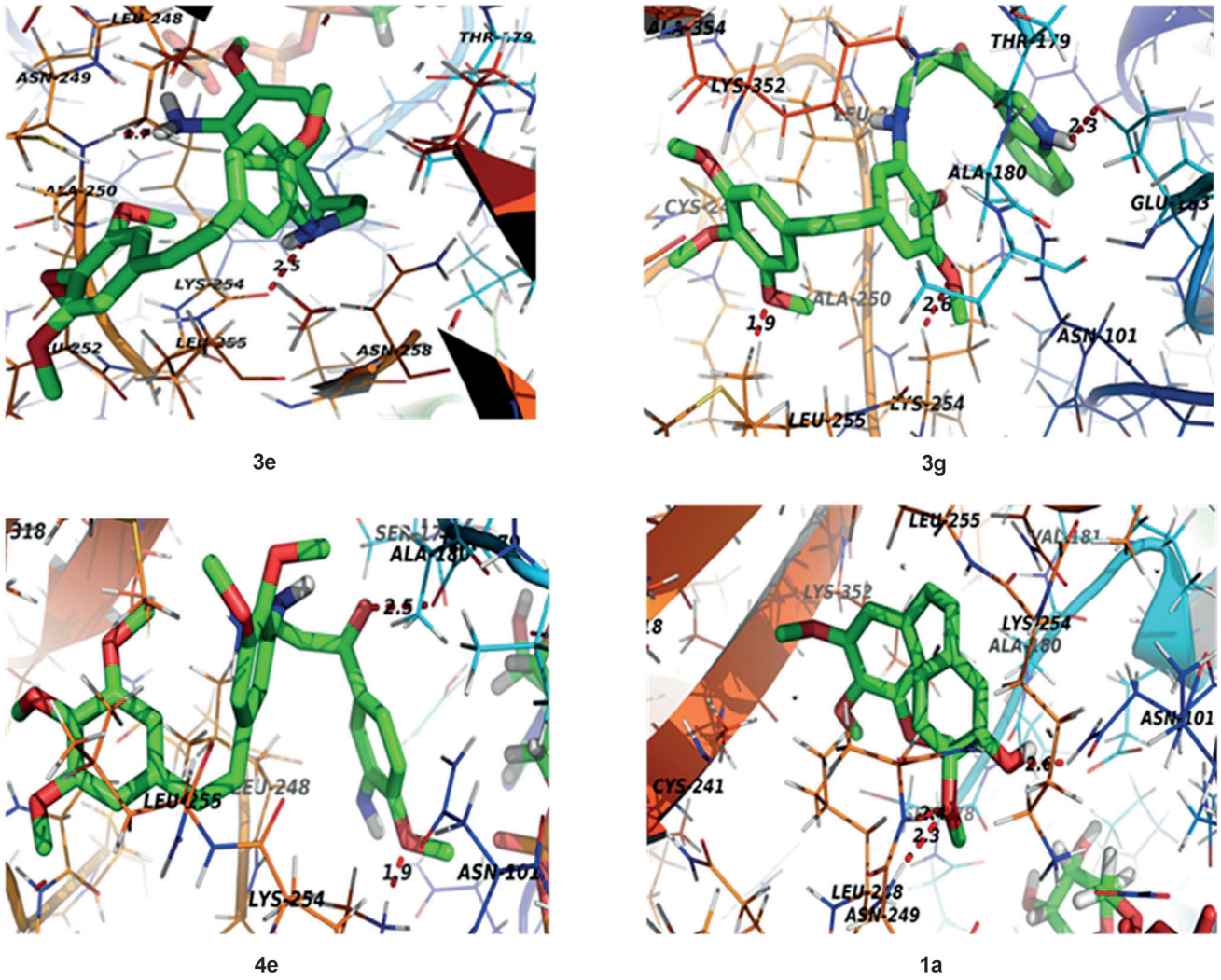


Figure 7. Interaction of **3e**, **3g**, **4e**, and **1a** (CA-4) with the colchicine binding site of tubulin. Probable hydrogen bonds are shown in red. This figure was generated by using the PyMOL software.

zation, with IC_{50} values of 0.71, 0.79, and 1.68 μM, respectively. The binding of these conjugates is at the colchicine binding site of tubulin, as indicated by molecular modeling studies. These results demonstrate that aminostilbene–arylpropenones are potent inhibitors of tubulin polymerization, and they are also amenable for further structural modifications in the discovery and development of effective chemotherapeutics for the treatment of cancer.

Experimental Section

Chemistry

General: 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 GF254, and visualization was performed by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H NMR spectra were recorded with a Bruker UXNMR/XWIN-NMR (300 MHz) or Inova Varian VXR-Unity (400 MHz) instrument. Chemical shifts (δ) are reported in ppm downfield from internal $(CH_3)_4Si$ standard. ESI-MS data were recorded with a Micromass Quattro LC instrument by using ESI+ software with a capillary voltage of 3.98 kV and an ESI mode positive ion trap detector. HRMS data were recorded with a QSTAR XL Hybrid MS–MS mass spectrometer. Melting points were determined with an Electrothermal melting point apparatus.

(*Z*)-1,2,3-Trimethoxy-5-(4-methoxy-3-nitrostyryl)benzene (11 a): A solution of 4-methoxy-3-nitrobenzaldehyde (**10 a**; 2.0 g, 0.011 mol) and triphenyl(3,4,5-trimethoxybenzyl)phosphonium bromide (**9**; 6.34 g, 0.012 mol) in dry CH_2Cl_2 (100 mL) was stirred under a nitrogen atmosphere. Sodium hydride (1.06 g, 0.044 mol) was added to the mixture at 0 °C. Then, the temperature of the mixture was slowly increased to RT, and the mixture was stirred for 18 h. The progress of the reaction was monitored by TLC (EtOAc/hexane = 3:7), and water was added after completion of reaction (until foaming stopped). The organic layer was separated, and the aqueous layer was extracted with $CHCl_3$. The organic layer was washed with brine, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure to afford the crude compound, which was purified by column chromatography (15% EtOAc/hexanes). Yield: 1.8 g, 42%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.79 (d, J = 2.26 Hz, 1 H), 7.42 (dd, J = 2.26 Hz, 8.3 Hz, 1 H), 6.94 (d, J = 8.3 Hz, 1 H), 6.58 (d, J = 12.09 Hz, 1 H), 6.47 (s, 2 H), 6.46 (d, J = 12.09 Hz, 1 H), 3.93 (s, 3 H), 3.85 (s, 3 H), 3.71 ppm (s, 6 H); MS (ESI): m/z: 346 $[M+H]^+$.

(*Z*)-5-(3,4-Dimethoxy-5-nitrostyryl)-1,2,3-trimethoxybenzene (11 b): Compound **11 b** was prepared according to the method described for compound **11 a** by employing 4,5-dimethoxy 3-nitrobenzaldehyde (**10 b**; 2.0 g, 9.5 mmol), triphenyl(3,4,5-trimethoxybenzyl)phosphonium bromide (**9**; 5.45 g, 0.0104 mol), and sodium hydride (0.910 g, 0.038 mol) to obtain pure **11 b** as a yellow solid. Yield: 1.5 g, 42%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.21 (s, 1 H), 6.93 (s, 1 H), 6.56 (d, J = 11.8 Hz, 1 H), 6.43 (s, 2 H), 6.40 (d, J = 11.8 Hz, 1 H), 3.91 (s, 3 H), 3.79 (s, 3 H), 3.71 (s, 6 H), 3.66 ppm (s, 3 H); MS (ESI): m/z: 376 $[M+H]^+$.

(*Z*)-2-Methoxy-5-(3,4,5-trimethoxystyryl)aniline (12 a): Zinc (1.46 g, 0.023 mol) was added to a stirred solution of **11 a** (2.0 g, 5.78 mmol) in acetic acid (20 mL) under a nitrogen atmosphere at 0 °C. Then, the temperature of the mixture was slowly increased to RT, and the mixture was stirred for 6 h. The progress of the reaction was monitored by TLC (EtOAc/hexane = 1:1). The mixture was basified with saturated $NaHCO_3$ solution and then extracted with EtOAc. The organic layer was separated and washed with brine, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure to afford the crude compound, which was purified by column chromatography (25% EtOAc/hexanes). Yield: 1.4 g, 77%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.91 (d, J = 2.26 Hz, 1 H), 6.80 (dd, J = 2.26 Hz, 8.31 Hz, 1 H), 6.73 (d, J = 8.31 Hz, 1 H), 6.42–6.36 (m, 2 H), 6.33 (dd, J = 1.51 Hz, 9.82 Hz, 2 H), 3.86 (s, 3 H), 3.81 (s, 6 H), 3.65 ppm (s, 3 H); MS (ESI): m/z: 316 $[M+H]^+$.

(*Z*)-2,3-Dimethoxy-5-(3,4,5-trimethoxystyryl)aniline (12 b): Compound **12 b** was prepared according to the method described for compound **12 a** by employing **11 b** (2.0 g, 5.32 mmol) and zinc (1.34 g, 0.0213 mol) to obtain pure **12 b** as a yellow solid. Yield: 1.5 g, 82%; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.70 (d, J = 1.51 Hz, 1 H), 6.68 (s, 2 H), 6.55 (s, 1 H), 6.45 (d, J = 12.1 Hz, 1 H), 6.36 (d, J = 12.1 Hz, 1 H), 3.85 (s, 3 H), 3.83 (s, 3 H), 3.70 ppm (s, 3 H); MS (ESI): m/z: 346 $[M+H]^+$.

(*Z*)-3-({2-Methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (3 a): 1-(3,4,5-Trimethoxyphenyl)prop-2-yn-1-one (**13 a**; 69.8 mg, 0.317 mmol) was added to a stirred solution of **12 a** (100 mg, 0.317 mmol) in ethanol (5 mL). The mixture was stirred at 25–35 °C for 4 h, and the progress of the reaction was monitored by TLC (hexane/EtOAc = 6:4). Then, water was added to the mixture. A yellow color solid appeared, which was filtered and washed with ethanol. Yield: 120 mg, 71%; m.p. 61–63 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 12.09 (d, J = 12.5 Hz, 1 H), 7.23–7.21 (m, 1 H), 7.20 (s, 2 H), 7.15 (d, J = 1.8 Hz, 1 H), 6.96 (dd, J = 6.56, 1.8 Hz, 1 H), 6.82 (d, J = 8.3 Hz, 1 H),6.54 (s, 2 H), 6.49 (q, J = 12.2 Hz, 2.44 Hz, 2 H), 5.92 (d, J = 7.9 Hz, 1 H), 3.96 (s, 3 H), 3.93 (s, 6 H), 3.90 (s, 3 H), 3.85 (s, 3 H), 3.71 ppm (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 189.7, 152.9, 152.8, 147.5, 142.9, 141.0, 137.0, 134.6, 132.7, 129.9, 129.2, 129.1, 124.6, 113.3, 110.6, 105.7, 104.5, 94.1, 60.8, 56.16, 55.98, 55.9 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3452, 2934, 2859, 1630, 1588, 1553, 1505, 1472, 1429, 1408, 1363, 1326, 1275, 1199, 1166, 1125, 1002, 914, 845, 769, 580 cm^{-1}; MS (ESI): m/z: 536 $[M+H]^+$; HRMS (ESI): m/z: calcd for $C_{30}H_{34}O_8N$: 536.22789 $[M+H]^+$; found: 536.22690.

(*Z*)-1-(2-Bromo-3,4,5-trimethoxyphenyl)-3-({2-methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (3 b): Compound **3 b** was prepared according to the method described for compound **3 a** by employing 1-(2-bromo-3,4,5-trimethoxyphenyl)-prop-2-yn-1-one (**13 b**; 94.9 mg, 0.317 mmol) to obtain pure **3 b** as a yellow solid. Yield: 150 mg, 77%; m.p. 54–57 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 11.92 (d, J = 12.8 Hz, 1 H), 7.28–7.19 (m, 1 H), 7.14 (s, 1 H), 6.97 (d, J = 8.3 Hz, 1 H), 6.87 (s, 1 H), 6.81 (d, J = 8.3 Hz, 1 H), 6.49 (d, J = 9.06 Hz,4 H), 5.64 (d, J = 8.30 Hz, 1 H), 3.94 (s, 3 H), 3.91 (s, 3 H), 3.90 (s, 3 H), 3.87 (s, 3 H), 3.84 (s, 3 H), 3.70 ppm (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 192.2, 152.9, 152.6, 147.5, 144.0, 142.8, 138.3, 137.13, 132.6, 130.01, 129.4, 129.1, 125.9, 124.8, 113.6, 110.6, 107.9, 106.1, 105.7, 98.4, 61.09, 60.91, 60.91, 56.08, 55.99, 55.91 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3418, 2933, 2839, 2359, 1629, 1581, 1501, 1465, 1424, 1328, 1379, 1202, 1163, 1125, 1102, 1007, 928, 850, 793, 743 cm^{-1}; MS (ESI): m/z: 614 $[M+H]^+$; HRMS (ESI): m/z: calcd for $C_{30}H_{33}O_8NBr$: 614.13841 $[M+H]^+$; found: 614.13838.

(*Z*)-1-[3-(*tert*-Butyldimethylsilyloxy)-4-methoxyphenyl]-3-({2-methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (14 a): Compound **14 a** was prepared according to the method described for compound **3 a** by employing 1-[3-(*tert*-butyldimethylsilyloxy)4-methoxyphenyl]prop-2-yn-1-one (**13 c**; 92.07 mg,

0.317 mmol) to obtain pure **14a** as a yellow solid. Yield: 150 mg, 78%.

(*Z*)-1-(3-Hydroxy-4-methoxyphenyl)-3-({2-methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (3c): 1 M TBAF in THF (0.3 mL, 0.272 mmol) was added to a stirred solution of **14a** (150 mg, 0.247 mmol) in THF (15 mL) at 10–15 °C. Then, the temperature of the mixture was slowly increased to RT, and the mixture was stirred for 6 h. The progress of the reaction was monitored by TLC (hexane/EtOAc = 1:1). Upon completion of the reaction, THF was evaporated, and the mixture was partitioned between water and EtOAc. The compound was purified by column chromatography (hexane/EtOAc = 6:4). Yield: 100 mg, 82%; m.p. 70–72 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.06 (d, J = 12.1 Hz, 1H), 7.50 (d, J = 6.7 Hz, 2H), 7.24–7.18 (m, 1H), 7.12 (s, 1H), 6,96–6.87 (m, 2H), 6.79 (d, J = 8.3 Hz, 1H), 6.49 (d, J = 13.6 Hz, 4H), 5.91 (d, J = 8.3 Hz, 1H), 5.68 (s, 1H), 3.94 (s, 6H), 3.85 (s, 3H), 3.71 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 187.5, 152.9, 147.3, 140.3, 137.4, 133.8, 132.2, 130.02, 129.8, 129.5, 128.9, 126.3, 123.4, 122.7, 122.4, 121.7, 112.7,110.4, 109.4, 105.79, 96.4, 60.9, 55.93 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3431, 2992, 2945, 2843, 1628, 1614, 1581, 1533, 1501, 1475, 1439, 1412, 1348, 1323, 1272, 1241, 1151, 1124, 1081, 1045, 1008, 871 cm^{-1}; MS (ESI): *m/z*: 492 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{28}H_{30}O_7N$: 492.20168 $[M+H]^+$; found: 492.20145.

(*Z*)-1-(4-Methoxy-3-nitrophenyl)-3-({2-methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (3d): Compound **3d** was prepared according to the method described for compound **3a** by employing 1-(4-methoxy-3-nitrophenyl)prop-2-yn-1-one (**13d**; 65.05 mg, 0.317 mmol) to obtain pure **3d** as a yellow solid. Yield: 130 mg, 79%; m.p. 164–166 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.15 (d, J = 12.8 Hz, 1H), 8.44 (s, 1H), 8.14 (d, J = 9.06 Hz, 1H), 7.32–7.26 (m, 1H), 7.12 (d, J = 8.13 Hz, 2H), 6.98 (d, J = 8.3 Hz, 1H), 6.81 (d, J = 9.06 Hz, 1H), 6.50 (d, J = 8.3 Hz, 4H), 5.91 (d, J = 7.55 Hz, 1H), 4.02 (s, 3H), 3.96 (s, 3H), 3.85 (s, 3H), 3.70 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 186.9, 155.0, 152.9, 147.6, 143.8, 139.2, 137.15, 133.0, 132.6, 131.6, 130.0, 129.4, 129.07, 128.9, 125.0, 124.9, 113.7, 113.0, 110.7, 105.7, 93.2, 60.9, 55.9,56.7, 56.03 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3427, 2934, 2837, 1630, 1612, 1579, 1531, 1503, 1473, 1437, 1414, 1350, 1325, 1270, 1237, 1149, 1183, 1125, 1085, 1047, 1012, 879, 682 cm^{-1}; MS (ESI): *m/z*: 521 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{28}H_{29}O_8N_2$: 521.19184 $[M+H]^+$; found: 521.19148.

(*Z*)-1-(4-Amino-3-methoxyphenyl)-3-({2-methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (3e): Zinc (77.12 mg, 1.22 mmol) and ammonium formate (78.34 mg, 1.22 mmol) were added to a stirred solution of **3d** (150 mg, 0.306 mmol) in ethanol (10 mL) at RT. The mixture was stirred for 4 h, and the progress of the reaction was monitored by TLC (EtOAc/hexane = 7:3). The mixture was then filtered through a bed of Celite, and the filtrate was evaporated under reduced pressure. Then, water (10 mL) and EtOAc (30 mL) were added to the concentrate. The organic layer was separated, dried with anhydrous Na_2SO_4, and concentrated to afford the crude product, which was further purified by column chromatography (EtOAc/hexane = 6:4) to obtain a pure yellow solid. Yield: 110 mg, 78%; m.p. 71–73 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.04 (d, J = 12.04 Hz, 1H), 7.36–7.32 (m, 2H), 7.21–7.13 (m, 1H), 7.12 (d, J = 1.51 Hz, 1H), 6.95–6.91(dd, J=1.51 Hz, 8.3 Hz, 1H), 6.82–6.78 (m, 2H), 6.54 (s, 2H), 6.48 (d, J = 2.26 Hz, 2H), 5.91 (d, J = 7.55 Hz, 1H), 3.94 (s, 3H), 3.90 (s, 3H), 3.85(s, 3H),3.70 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 190.2, 152.9, 150.1, 147.4, 142.1, 137.08, 135.9, 132.7, 132.3, 129.8, 129.5, 129.3, 129.1, 124.6, 118.8, 113.6, 113.1, 110.5, 109.3, 105.7, 94.2, 60.9, 55.9, 55.5 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3444, 3341, 2934, 2836, 1629, 1587, 1551, 1505, 1474, 1363, 1325, 1275, 1204, 1177, 1127, 1022, 878, 854, 776, 657, 591 cm^{-1}; MS (ESI): *m/z*: 491 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{28}H_{31}O_6N_2$: 491.21766 $[M+H]^+$; found: 491.21770.

(*Z*)-3-({2-Methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-[4-(trifluoromethoxy)phenyl]prop-2-en-1-one (3f): Compound **3f** was prepared according to the method described for compound **3a** by employing 1-[4-(trifluoromethoxy)phenyl]prop-2-yn-1-one (**13e**, 67.88 mg, 0.317 mmol) to obtain pure **3f** as a yellow solid. Yield: 120 mg, 72%; m.p. 103–105 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.15 (d, J = 13.4 Hz, 1H), 7.99 (d, J = 7.17 Hz, 2H), 7.26 (s, 3H), 7.14 (s, 1H), 6.97 (d, J = 8.49 Hz, 1H), 6.82 (d, J = 8.49 Hz, 1H), 6.50 (d, J = 10.09 Hz, 4H), 5.95 (d, J = 7.55 Hz, 1H), 3.96 (s, 3H), 3.85 (s, 3H), 3.71 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 189.03, 153.0, 147.6, 143.6, 137.6, 132.7, 130.0, 129.9, 129.4, 129.1, 129.0, 124.8, 120.3, 113.6, 110.7, 105.8, 93.9, 60.9, 55.9, 56.0 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3432, 2935, 2833, 1628, 1592, 1558, 1508, 1472, 1454, 1434, 1416, 1363, 1325, 1298, 1279, 1257, 1180, 1129, 1037, 1015, 1006, 963, 866, 844, 795, 779, 723 cm^{-1}; MS (ESI): *m/z*: 530 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{28}H_{27}O_6NF_3$: 530.17850 $[M+H]^+$; found: 530.17740.

***tert*-Butyl-3-[(*Z*)-3-({2-methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)acryloyl]-1*H*-indole-1-carboxylate (15a)**: Compound **15a** was prepared according to the method described for compound **3a** by employing *tert*-butyl 3-propioloyl-1*H*-indole-1-carboxylate (**13f**; 85.4 mg, 0.317 mmol) to obtain pure **15a** as a yellow solid.

(*Z*)-1-(1*H*-indol-3-yl)-3-({2-methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]-phenyl}amino)prop-2-en-1-one (3g): TFA (0.01 mL, 0.188 mmol) was added to a stirred solution of **15a** (100 mg, 0.171 mmol) in CH_2Cl_2 at 0 °C. Then, the temperature of the mixture was slowly increased to RT, and the mixture was stirred for 2 h. The progress of the reaction was monitored by TLC (EtOAc/hexane = 7:3). The mixture was neutralized by adding a saturated solution of $NaHCO_3$. The mixture was filtered through Celite, and the aqueous layer was extracted with $CHCl_3$. The organic layer was washed with brine, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure to afford the crude compound. Yield: 60 mg, 72%; m.p. 95–97 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 11.87 (d, J = 12.3 Hz, 1H), 8.62 (s, 1H), 8.45–8.42 (m, 1H), 7.80 (d, J = 2.8 Hz, 1H), 7.42–7.39 (m, 1H), 7.30–7.27 (m, 1H), 7.13–7.05 (m, 2H), 6.9 (dd, J = 8.3 Hz, 1.7 Hz, 1H), 6.82 (d, J = 8.3 Hz, 1H), 6.56 (s, 2H), 6.50 (d, J = 4.7 Hz, 2H), 5.85 (d, J = 7.9 Hz, 1H), 3.95(s, 3H), 3.86 (s, 3H), 3.71 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 188.1, 152.9, 147.3, 140.6, 136.5, 132.8, 129.86, 129.83, 129.5, 128.9, 125.5, 123.6, 122.9, 122.0, 121.7, 118.7, 112.8, 111.5, 110.5, 105.8, 96.4, 60.9, 55.9, 55.8 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3388, 2953, 2859, 1628, 1580, 1513, 1453, 1428, 1366, 1324, 1276, 1241, 1157, 1126, 1025, 1000, 966, 864, 790, 769, 758, 656, 579 cm^{-1}; MS (ESI): *m/z*: 485 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{29}H_{29}O_5N_2$: 485.20711 $[M+H]^+$; found: 485.20611.

(*Z*)-3-({2-Methoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(1-methyl-1*H*-indol-3-yl)prop-2-en-1-one (3h): Compound **3h** was prepared according to the method described for compound **3a** by employing 1-(1-methyl-1*H*-indol-3-yl)prop-2-yn-1-one (**13g**; 58.1 mg, 0.317 mmol) to obtain pure **3h** as a yellow solid. Yield: 115 mg, 73%; m.p. 122–124 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 11.87(d, J=12.1 Hz, 1H), 8.45–8.41 (m, 1H), 7.67(s, 1H), 7.35–7.23 (m, 3H), 7.11–7.03 (s, 2H), 6.92 (d, J = 8.3 Hz, 1H), 6.78 (d, J = 8.3 Hz, 1H), 6.58 (s, 2H), 6.49 (qt, J = 12.1 Hz, 5.3 Hz, 2H), 5.79 (d, J = 7.55 Hz, 1H), 3.96 (s, 3H), 3.83 (s, 6H), 3.70 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 187.5, 152.9, 147.3, 140.3, 137.4, 133.2, 132.8, 130.0, 129.8, 129.5, 128.9, 126.3, 123.4, 122.7, 122.4, 121.7, 112.7, 110.4, 109.4, 105.7, 96.4, 61.0, 60.9, 55.9 ppm; IR (KBr):

$\tilde{\nu}_{max}$ = 3434, 2929, 2835, 1631, 1604, 1578, 1524, 1488, 1469, 1433, 1415, 1368, 1327, 1277, 1217, 1187, 1146, 1126, 1087, 1026, 1005, 947, 877, 857, 783, 752 cm^{-1}; MS (ESI): *m/z*: 499 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{30}H_{31}O_5N_2$: 499.22275 $[M+H]^+$; found: 499.22339.

(*Z*)-3-({2,3-Dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (4a): Compound **4a** was prepared according to the method described for compound **3a** by employing **12b** (100 mg, 0.289 mmol) and **13a** (63.8 mg, 0.289 mmol) to obtain pure **4a** as a yellow solid. Yield: 120 mg, 73%; m.p. 61–63 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.12 (d, *J* = 12.1 Hz, 1H), 7.30–7.23 (m, 1H), 7.19 (s, 2H), 6.79 (s, 1H), 6.58–6.49 (m, 5H), 5.93 (d, *J* = 8.3 Hz, 1H), 3.96 (s, 3H), 3.94 (s, 6H), 3.90 (s, 3H), 3.84 (s, 3H), 3.82 (s, 3H), 3.72 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 189.7, 152.7, 152.5, 142.9, 140.8, 137.05, 136.6, 134.64, 134.6, 133.6, 133.2, 130.1, 129.2, 128.4, 127.4, 107.6, 106.2, 105.7, 104.4, 103.3, 94.06, 60.7, 60.6, 56.9, 55.75, 55.58 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3424, 2935, 2832, 1629, 1585, 1554, 1503, 1470, 1425, 1367, 1327, 1277, 1235, 1200, 1165, 1125, 1075, 1000, 913, 862, 838, 772, 734, 659, 585 cm^{-1}; MS (ESI): *m/z*: 566 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{31}H_{36}O_9N$: 566.23846 $[M+H]^+$; found: 566.23749.

(*Z*)-1-(2-Bromo-3,4,5-trimethoxyphenyl)-3-({2,3-dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (4b): Compound **4b** was prepared according to the method described for compound **3a** by employing **12b** (100 mg, 0.289 mmol) and **13b** (86.5 mg, 0.289 mmol) to obtain pure **4b** as a yellow solid. Yield: 130 mg, 69.7%; m.p. 55–57 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.04 (d, *J* = 12.8 Hz, 1H), 7.31–7.24 (m, 1H), 6.87 (s, 1H), 6.76 (d, *J* = 6.7 Hz, 1H), 6.57 (s, 1H), 6.52–6.47 (m, 4H), 5.67 (d, *J* = 7.55 Hz, 1H), 3.96 (s, 3H), 3.90 (d, *J* = 3.02 Hz, 9H), 3.87 (s, 3H), 3.71 ppm (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 192.4, 152.9, 152.7, 150.8, 144.1, 142.9, 138.3, 136.8, 133.6, 133.4, 132.3, 130.4, 129.3, 108.0, 107.9, 106.5, 106.1, 105.9, 98.6, 61.12, 60.8, 60.9, 55.7, 55.9, 56.1 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3434, 2935, 2833, 1630, 1582, 1505, 1466, 1423, 1381, 1329, 1276, 1239,1203, 1166, 1126, 1104, 1075, 1005, 932, 864, 850, 794, 657 cm^{-1}; MS (ESI): *m/z*: 644 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{31}H_{35}O_9NBr$: 644.14897 $[M+H]^+$; found: 644.14957.

(*Z*)-1-[3-(*tert*-Butyldimethylsilyloxy)-4-methoxyphenyl]-3-({2,3-dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (14b): Compound **14b** was prepared according to the method described for compound **3a** by employing **12b** (100 mg, 0.289 mmol) and **13c** (83.9 mg, 0.289 mmol) to obtain pure **14b** as a yellow solid. Yield: 150 mg, 81%.

(*Z*)-3-({2,3-Dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(3-hydroxy-4-methoxyphenyl)prop-2-en-1-one (4c): Compound **4c** was prepared according to the method described for compound **3c** by employing **14b** (150 mg, 0.236 mmol) and TBAF (1 N in THF) (0.3 mL, 0.26 mmol) to obtain pure **4c** as a yellow solid. Yield: 99 mg, 80%; m.p. 64–66 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.09 (d, *J* = 12.8 Hz, 1H), 7.53 (d, *J* = 6.7 Hz, 2H), 7.29–7.22 (m, 1H), 6.89 (d, *J* = 9.06 Hz, 1H), 6.75 (s, 1H), 6.53–6.51 (m, 5H), 5.94 (d, *J* = 8.3 Hz, 1H), 5.78 (s, 1H), 3.95 (s, 3H), 3.94 (s, 3H), 3.83 (s, 3H), 3.71 ppm (s, 9H), ^{13}C NMR (75 MHz, $CDCl_3$): δ = 189.9, 152.9, 152.7, 149.4, 145.2, 142.6, 137.2, 136.7, 134.1, 133.3, 132.9, 132.4, 130.2, 129.4, 120.4, 113.6, 109.8, 107.4, 106.4, 105.9, 94.2, 60.8, 60.7, 55.9, 55.7 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3331, 2990, 2965, 2935, 2837, 1632, 1580, 1546, 1504, 1464, 1435, 1370, 1328, 1282, 1203, 1178, 1162, 1127, 1074, 1054, 1026, 999, 974,945, 853, 775, 691 cm^{-1}; MS (ESI): *m/z*: 522 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{29}H_{32}O_8N$: 522.21224 $[M+H]^+$; found: 522.21105.

(*Z*)-3-({2,3-Dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(4-methoxy-3-nitro phenyl)prop-2-en-1-one (4d): Compound **4d** was prepared according to the method described for compound **3a** by employing **12b** (100 mg, 0.289 mmol) and **13d** (59.3 mg, 0.289 mmol) to obtain pure **4d** as a yellow solid. Yield: 120 mg, 75%; m.p. 144–147 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.21 (d, *J* = 12.8 Hz, 1H), 8.43 (s, 1H), 8.14 (d, *J* = 9.06 Hz, 1H), 7.37–7.30 (m, 1H), 7.12 (d, *J* = 9.06 Hz, 1H), 6.77 (s, 1H), 6.57–6.47 (m, 5H), 5.92 (d, *J* = 7.5 Hz, 1H), 4.03 (s, 3H), 3.97 (s, 3H), 3.84 (s, 3H), 3.74 ppm (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 187.1, 155.0, 152.9, 143.9, 139.1, 137.2, 136.9, 133.5, 133.4, 133.1, 131.6, 130.4, 129.2, 125.06, 113.06, 108.0, 106.5, 105.8, 93.3, 60.9, 60.8, 56.7, 55.9, 55.7 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3433, 2963, 2937, 2838, 1636, 1592, 1556, 1503, 1464, 1429, 1420, 1324, 1295, 1282, 1235, 1128, 1075, 1013, 999, 926 cm^{-1}; MS (ESI): *m/z*: 551 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{30}H_{31}O_9N_2$: 551.2029 $[M+H]^+$; found: 551.2031.

(*Z*)-1-(3-Amino-4-methoxyphenyl)-3-({2,3-dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (4e): Compound **4e** was prepared according to the method described for compound **3e** by employing **4d** (150 mg, 0.288 mmol), zinc (72.71 mg, 1.15 mmol), and ammonium formate (73.67 mg, 1.15 mmol) to obtain pure **4e** as a yellow solid. Yield: 111 mg, 78%; m.p. 68–71 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.1 (d, *J* = 12.1 Hz, 1H), 7.34 (d, *J* = 7.4 Hz, 2H), 7.23–7.19 (m, 1H), 6.81 (d, *J* = 8.49 Hz, 1H), 6.75 (s, 1H), 6.53–6.51 (m, 5H), 5.96 (d, *J* = 7.9 Hz, 1H), 3.95 (s, 3H), 3.90 (s, 3H), 3.83 (s, 3H), 3.72 ppm (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 190.4, 152.9, 152.7, 150.1, 142.2, 137.1, 136.6, 135.9, 134.1, 133.3, 132.4, 132.3, 130.1, 129.4, 118.8, 113.6, 109.2, 107.2, 106.3, 105.8, 94.4, 60.8, 60.7, 55.9, 55.7, 55.5 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3444, 3371, 2933, 2834, 1630, 1584, 1552, 1504, 1469, 1428, 1367, 1326, 1276, 1204, 1176, 1146, 1126, 1076, 999, 919, 854, 774 cm^{-1}; MS (ESI): *m/z*: 521 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{29}H_{33}O_7N_2$: 521.22823 $[M+H]^+$; found: 521.22722.

(*Z*)-3-({2,3-Dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-[4-(trifluoromethoxy)phenyl]prop-2-en-1-one (4f): Compound **4f** was prepared according to the method described for compound **3a** by employing **12b** (100 mg, 0.289 mmol) and **13e** (61.9 mg, 0.289 mmol) to obtain pure **4f** as a yellow solid. Yield: 120 mg, 74%; m.p. 107–110 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.21 (d, *J* = 12.8 Hz, 1H), 7.98 (d, *J* = 8.3 Hz, 2H), 7.35–7.31 (m, 1H), 7.27–7.25 (m, 3H), 6.77 (s, 1H), 6.57–6.46 (m, 4H), 5.93 (d, *J* = 8.3 Hz, 1H), 3.96 (s, 3H), 3.83 (s, 3H), 3.71 ppm (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 182.9, 152.9, 152.7, 151.4, 143.7, 137.6, 137.2, 136.9, 133.6, 133.4, 132.3, 132.0, 130.3, 129.3, 129.1, 128.0, 120.9, 107.9, 106.5, 105.9, 94.03, 60.89, 60.8, 55.9, 55.7 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3432, 2947, 2842, 1621, 1534, 1514, 1501, 1450, 1411, 1321, 1315, 1292, 1255, 1189, 1165, 1117, 1052, 1001, 925, 845, 818, 789 cm^{-1}; MS (ESI): *m/z*: 560 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{29}H_{29}O_7NF_3$: 560.18906 $[M+H]^+$; found: 560.18821.

***tert*-Butyl 3-[(*Z*)-3-({2,3-dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]-phenyl}amino)acryloyl]-1*H*-indole-1-carboxylate (15b)**: Compound **15b** was prepared according to the method described for compound **3a** by employing **13f** (77.8 mg, 0.289 mmol) to obtain pure **15b** as a yellow solid.

(*Z*)-3-({2,3-Dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(1*H*-indol-3-yl)prop-2-en-1-one (4g): Compound **4g** was prepared according to the method described for compound **3g** by employing **15b** (100 mg, 0.163 mmol) and TFA (0.01 mL, 0.178 mmol) to obtain pure **4g** as a yellow solid. Yield: 62 mg, 74%; m.p. 88–90 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 11.96 (d, *J* = 11.9 Hz, 1H), 8.47 (s, 1H), 8.37–8.36 (m, 1H), 7.82 (d, *J* = 2.89 Hz,

1H), 7.44–7.39 (m, 2H), 7.32–729 (m, 1H), 7.15 (dd, $J=4.1$ Hz, 8.1 Hz, 1H), 6.75 (d, $J=1.2$ Hz, 1H), 6.53 (d, $J=12.1$ Hz, 4H), 5.86 (d, $J=8.1$ Hz, 1H), 3.96 (s, 3H), 3.84 (s, 3H), 3.72 (s, 3H), 3.71 ppm (s, 6H); IR (KBr): $\tilde{\nu}_{max}=3415$, 2931, 2839, 1627, 1580, 1516, 1427, 1372, 1310, 1275, 1239, 1158, 1125, 1078, 1036, 1007, 997, 889, 771 cm^{-1}; MS (ESI): *m/z*: 515 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{30}H_{31}O_6N_2$: 515.21766 $[M+H]^+$; found: 515.21847.

(*Z*)-3-({2,3-Dimethoxy-5-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(1-methyl-1*H*-indol-3-yl)prop-2-en-1-one (4h): Compound **4h** was prepared according to the method described for compound **3a** by employing **13g** (52.9 mg, 0.289 mmol) to obtain pure **4h** as a yellow solid. Yield: 112 mg, 73%; m.p. 112–115 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=12.01$ (d, $J=12.1$ Hz, 1H), 8.50 (s, 1H), 7.73 (s, 1H), 7.37 (s, 3H), 7.24–7.17 (m, 1H), 6.8 (s, 1H), 6.59 (d, $J=12.1$ Hz, 5H), 5.90 (d, $J=7.55$ Hz, 1H), 4.05 (s, 3H), 3.92 (s, 3H), 3.87 (s, 3H), 3.72 ppm (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=187.5$, 153.02, 140.5, 137.5, 134.8, 133.3, 133.1, 132.5, 132.0, 129.6, 126.4, 122.7, 122.4, 121.8, 117.8, 109.4, 107.0, 106.3, 106.1, 96.7, 61.03, 60.9, 60.7, 56.02, 55.8 ppm; IR (KBr): $\tilde{\nu}_{max}=3432$, 2931, 2832, 1628, 1528, 1522, 1505, 1487, 1469, 1425, 1366, 1326, 1276, 1216, 1147, 1125, 1087, 999, 947, 859, 791, 770 cm^{-1}; MS (ESI): *m/z*: 529 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{31}H_{33}O_6N_2$: 529.23331 $[M+H]^+$; found: 529.23274.

3-Hydroxy-4-methoxy-2-nitrobenzaldehyde (17): Nitromethane (20 mL) and nitronium tetrafluoroborate (9.2 g, 0.069 mol) were added to a stirred solution of isovaniline (**16**; 10 g, 0.066 mol) in CH_2Cl_2 (30 mL) at −40 °C. Then, the mixture was stirred for 6 h. The progress of the reaction was monitored by TLC (EtOAc/hexane = 2:8). Water was added to the mixture at RT. The mixture was then concentrated, and the aqueous layer was extracted with diethyl ether (3×). The organic layer was washed with water and brine, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure to afford the crude compound, which was purified by column chromatography (EtOAc/hexane = 3:7). Yield: 6.0 g, 46%; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=9.79$ (s, 1H), 7.39 (d, $J=8.3$ Hz, 1H), 7.08 (d, $J=8.4$ Hz, 1H), 4.02 ppm (s, 3H); MS (ESI): *m/z*: 220 $[M+Na]^+$.

3-(*tert*-Butyldimethylsilyloxy)-4-methoxy-2-nitrobenzaldehyde (18): Imidazole (0.789 g, 0.0116 mol) and *tert*-butylchlorodimethylsilane (1.68 g, 0.0116 mol) were added to a stirred solution of **17** (2.0 g, 0.0101 mol) in CH_2Cl_2 (30 mL) at 0 °C. The temperature was slowly increased to RT, and the mixture was stirred for 3 h. The progress of the reaction was monitored by TLC (EtOAc/hexane = 2:8). Upon completion of the reaction, water was added to mixture, which was extracted with CH_2Cl_2. The organic layer was washed with water and brine and dried with anhydrous Na_2SO_4. Finally, the solvent was evaporated under reduced pressure to afford the pure compound. Yield: 2.9 g, 91%; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=9.56$ (s, 1H), 7.26 (d, $J=8.31$ Hz, 1H), 6.82 (d, $J=8.31$ Hz, 1H), 3.74 (s, 3H), 0.93 (s, 9H), 0.09 ppm (s, 6H); MS (ESI): *m/z*: 312 $[M+H]^+$.

(*Z*)-*tert*-Butyl[6-methoxy-2-nitro-3-(3,4,5-trimethoxystyryl)phenoxy]dimethylsilane (19): Compound **19** was prepared according to the method described for compound **11a** by employing **18** (3.69 g, 7.06 mmol) to obtain pure **19** as a yellow solid. Yield: 2.7 g, 48%; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.77$ (s, 2H), 6.61 (d, $J=12.0$ Hz, 1H), 6.41 (s, 2H), 6.38 (d, $J=12.0$ Hz, 1H), 3.82 (s, 3H), 3.81 (s, 3H), 3.67 (s, 6H), 0.94 (s, 9H), 0.21 ppm (s, 6H). MS (ESI, *m/z*): 476 $[M+1]^+$.

(*Z*)-2-(*tert*-Butyldimethylsilyloxy)-3-methoxy-6-(3,4,5-trimethoxystyryl)aniline (20): Compound **20** was prepared according to the method described for compound **12a** by employing **19** (900 mg, 1.89 mmol) and zinc (484.9 mg, 7.57 mmol) to obtain pure **20** as a light brown solid. Yield: 700 mg, 83%; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.70$ (d, $J=8.3$ Hz, 1H), 6.51 (s, 2H), 6.47 (d, $J=4.5$ Hz, 2H), 6.27 (d, $J=8.3$ Hz, 1H), 3.81 (s, 3H), 3.76 (s, 3H), 3.63 (s, 6H), 1.02 (s, 9H), 0.17 ppm (s, 6H); MS (ESI): *m/z*: 446 $[M+H]^+$.

(*Z*)-2-Amino-6-methoxy-3-(3,4,5-trimethoxystyryl)phenol (21): Compound **21** was prepared according to the method described for compound **3c** by employing **20** (100 mg, 0.224 mmol) to obtain pure **21** as a yellow liquid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=$ 6.67 (d, $J=8.3$ Hz, 1H), 6.53–6.43 (m, 4H), 6.32 (d, $J=8.3$ Hz, 1H), 3.85 (s, 3H), 3.81 (s, 3H), 3.63 ppm (s, 6H); MS (ESI): *m/z*: 332 $[M+H]^+$.

(*Z*)-3-({2-Hydroxy-3-methoxy-6-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (5a): Compound **5a** was prepared according to the method described for compound **3a** by employing **21** (100 mg, 0.301 mmol) and **13a** (66.2 mg, 0.301 mmol) to obtain pure **5a** as a yellow solid. Yield: 123 mg, 73%; m.p. 61–63 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=11.93$ (d, $J=12.2$ Hz, 1H), 7.84–7.75 (m, 1H), 7.18 (s, 2H), 6.80 (d, $J=$ 8.12 Hz, 1H), 6.67 (d, $J=11.8$ Hz, 1H), 6.61–6.55 (m, 2H), 6.44 (s, 2H), 5.85–5.81 (m, 2H), 3.92 (s, 6H), 3.91 (s, 3H), 3.90 (s, 3H), 3.81 (s, 3H), 3.58 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=189.6$, 152.8, 152.6, 149.8, 146.3, 137.2, 137.0, 135.0, 132.3, 132.0, 125.6, 125.2, 122.3, 121.1, 106.1, 105.9, 104.5, 92.7, 60.89, 60.8, 56.2, 56.1, 55.6 ppm; IR (KBr): $\tilde{\nu}_{max}=3411$, 2953, 2925, 2852, 1625, 1583, 1550, 1496, 1463, 1410, 1327, 1286, 1201, 1166, 1125, 1088, 1002, 919, 855, 833, 773 cm^{-1}; MS (ESI): *m/z*: 552 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{30}H_{34}O_9N$: 552.22281 $[M+H]^+$; found: 552.22399.

(*Z*)-1-(2-Bromo-3,4,5-trimethoxyphenyl)-3-({2-hydroxy-3-methoxy-6-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (5b): Compound **5b** was prepared according to the method described for compound **3a** by employing **13b** (89.6 mg, 0.301 mmol) to obtain pure **5b** as a yellow solid. Yield: 135 mg, 74%; m.p. 54–56 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=11.86$ (d, $J=$ 12.6 Hz, 1H), 7.93–7.84 (m, 1H), 6.85–6.80 (m, 2H), 6.68 (d, $J=$ 11.89 Hz, 1H), 6.61–6.55 (m, 2H), 6.46 (s, 2H), 5.89 (s, 1H), 5.54 (d, $J=7.55$ Hz, 1H), 3.90 (s, 6H), 3.89 (s, 3H), 3.87 (s, 3H), 3.81 (s, 3H), 3.63 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=199.1$, 152.5, 150.8, 149.6, 146.3, 143.9, 138.4, 136.9, 132.6, 131.9, 125.3, 124.9, 122.2, 121.1, 108.0, 106.1, 106.0, 97.04, 61.1, 60.9, 60.8, 56.3, 56.1, 55.7 ppm; IR (KBr): $\tilde{\nu}_{max}=3425$, 2957, 2935, 2836, 1625, 1580, 1504, 1461, 1423, 1381, 1330, 1285, 1204, 1164, 1126, 1104, 1006, 850, 790 cm^{-1}; MS (ESI): *m/z*: 630 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{30}H_{33}O_9NBr$: 630.13332 $[M+H]^+$; found: 630.13385.

(*Z*)-1-[3-(*tert*-Butyldimethylsilyloxy)-4-methoxyphenyl]-3-({2-hydroxy-3-methoxy-6-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-prop-2-en-1-one (14c): Compound **14c** was prepared according to the method described for compound **3a** by employing **13c** (82.4 mg, 0.301 mmol) to obtain pure **14c** as a yellow solid. Yield: 138 mg, 73%.

(*Z*)-3-({2-Hydroxy-3-methoxy-6-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(3-hydroxy-4-methoxyphenyl)prop-2-en-1-one (5c): Compound **5c** was prepared according to the method described for compound **3c** by employing **14c** (100 mg, 0.161 mmol) to obtain pure **5c** as a yellow solid. Yield: 63 mg, 76%; m.p. 65–67 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=11.95$ (d, $J=12.08$ Hz, 1H), 7.82–7.73 (m, 1H), 7.52–7.48 (m, 2H), 6.86 (d, $J=8.3$ Hz, 1H), 6.79 (d, $J=$ 8.3 Hz, 1H), 6.67 (d, $J=12.08$ Hz, 1H), 6.58–6.54 (m, 2H), 6.44 (s, 2H), 5.84–5.81 (m, 2H), 5.64 (s, 1H), 3.95 (s, 3H), 3.94 (s, 3H), 3.90 (s, 3H), 3.81 (s, 3H), 3.59 ppm (s, 6H), ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=$

189.6, 152.5, 149.4, 146.3, 145.2, 137.05, 136.9, 132.9, 132.1, 132.07, 125.8, 125.2, 122.1, 120.9, 120.2, 113.6, 109.8, 105.9, 105.8, 92.6 60.7, 56.2, 55.8, 55.6 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3417, 2959, 2935, 2837, 1626, 1608, 1587, 1548, 1496, 1462, 1421, 1327, 1265, 1125, 1008, 1020, 849, 775 cm^{-1}; MS (ESI): *m/z*: 508 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{28}H_{30}O_8N$: 508.19659 $[M+H]^+$; found: 508.19559.

(*Z*)-3-({2-Hydroxy-3-methoxy-6-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-(4-methoxy-3-nitrophenyl)prop-2-en-1-one (5 d): Compound **5 d** was prepared according to the method described for compound **3 a** by employing **13 d** (61.7 mg, 0.301 mmol) to obtain pure **5 d** as a yellow solid. Yield: 124 mg, 76%; m.p. 120–124 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 11.98 (d, *J* = 12.5 Hz, 1 H), 8.42 (d, *J* = 2.1 Hz, 1 H), 8.14–8.12 (m, 1 H), 7.88–7.84 (m, 1 H), 7.11 (d, *J* = 8.85 Hz, 1 H), 6.81 (d, *J* = 8.85 Hz, 1 H), 6.69 (d, *J* = 11.9 Hz, 1 H), 6.61 (d, *J* = 8.3 Hz, 1 H), 6.56 (d, *J* = 11.7 Hz, 1 H), 6.43 (s, 2 H), 5.88 (s, 1 H), 5.80 (d, *J* = 7.7 Hz, 1 H), 4.01 (s, 3 H), 3.91 (s, 3 H), 3.80 (s, 3 H), 3.59 ppm (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 186.6, 154.8, 152.6, 150.6, 146.3, 139.1, 137.2, 137.0, 133.0,132.6, 131.9, 125.3, 124.9, 122.4, 121.12, 112.9, 106.4, 105.9, 91.9, 60.8, 56.6, 56.3, 55.6 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3400, 2955, 2937, 2863, 1627, 1592, 1556, 1496, 1463, 1421, 1328, 1258, 1163, 1126, 1087, 1041, 1013, 963, 922, 855, 776, 736 cm^{-1}; MS (ESI): *m/z*: 537 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{28}H_{29}O_9N_2$: 537.18676 $[M+H]^+$; found: 537.18637.

(*Z*)-1-(3-Amino-4-methoxyphenyl)-3-({2-hydroxy-3-methoxy-6-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)prop-2-en-1-one (5 e): Compound **5 e** was prepared according to the method described for compound **3 e** by employing **5 d** (100 mg, 0.186 mmol) to obtain pure **5 e** as a yellow solid. Yield: 70 mg, 74%; m.p. 75–78 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.08 (d, *J* = 12.08 Hz, 1 H), 7.36–7.33 (m, 2 H), 7.23–7.19 (m, 1 H), 6.82–6.75 (m, 2 H), 6.51 (d, *J* = 5.28 Hz, 6 H), 5.95 (d, *J* = 7.93 Hz, 1 H), 3.95 (s, 3 H), 3.90 (s, 3 H), 3.83 (s, 3 H), 3.71 ppm (s, 6 H); IR (KBr): $\tilde{\nu}_{max}$ = 3426, 2995, 2938, 2837, 1610, 1579, 1532, 1505, 1462, 1421, 1353, 1327, 1283, 1237, 1185, 1126, 1089, 1009, 855, 827, 774 cm^{-1}; MS (ESI): *m/z*: 507 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for C_{28} $H_{31}O_7N_2$: 507.21258 $[M+H]^+$; found: 507.21359.

(*Z*)-3-({2-Hydroxy-3-methoxy-6-[(*Z*)-3,4,5-trimethoxystyryl]phenyl}amino)-1-[4-(trifluoromethoxy)phenyl]prop-2-en-1-one (5 f): Compound **5 f** was prepared according to the method described for compound **3 a** by employing **13 e** (64.4 mg, 0.301 mmol) to obtain pure **5 f** as a yellow solid. Yield: 130 mg, 78%; m.p. 97–99 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.01 (d, *J* = 12.8 Hz, 1 H), 7.96 (d, *J* = 8.3 Hz, 2 H), 7.87–7.80 (m, 1 H), 7.23 (d, *J* = 8.3 Hz, 3 H), 6.79 (d, *J* = 8.3 Hz, 1 H), 6.65 (d, *J* = 11.3 Hz, 1 H), 6.60–6.51 (m, 2 H), 6.43 (s, 2 H), 5.79 (d, *J* = 7.5 Hz, 1 H), 3.85 (s, 3 H), 3.80 (s, 3 H), 3.56 ppm (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 188.7, 152.5, 151.2, 150.5, 146.3, 137.8, 137.2, 137.1, 132.5, 132.01, 129.02, 125.4, 125.05, 122.4, 122.01, 121.07, 120.23, 106.3, 105.9, 105.7, 92.5, 60.7, 56.2, 55.6 ppm; IR (KBr): $\tilde{\nu}_{max}$ = 3429, 3311, 2935, 2898, 2837, 1625, 1606, 1550, 1503, 1462, 1421, 1327, 1284, 1257, 1176, 1126, 1088, 1007, 855, 775 cm^{-1}; MS (ESI): *m/z*: 546 $[M+H]^+$; HRMS (ESI): *m/z*: calcd for $C_{28}H_{27}O_7NF_3$: 546.1739 $[M+H]^+$; found: 546.1766.

General procedure for the preparation of 1-arylprop-2-yn-1-one (13): 0.5 N Ethynylmagnesium bromide in THF (1.5 mmol) was added to a stirred solution of substituted benzaldehyde **22 a–g** (1 mmol) in THF at 0 °C. Then, the temperature of the mixture was slowly increased to RT, and the mixture was stirred for 6–8 h. The progress of the reaction was monitored by TLC (EtOAc/hexane = 1:1). A solution of saturated ammonium chloride was added, and the mixture was concentrated and extracted with EtOAc. The organic layer was washed with brine, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure to afford pure **23 a–g**. Compound **23 a–g** (1 mmol) was dissolved in DMSO, and a solution of IBX (1.1 mmol) in DMSO (10 mL) was added at 10–15 °C. Then, the temperature of the mixture was slowly increased to RT, and the mixture was stirred for 4–6 h. The progress of the reaction was monitored by TLC (EtOAc/hexane = 3:7). Water was added, and the mixture was filtered through Celite. The aqueous layer was extracted with EtOAc. The organic layer was washed with water and brine, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure to afford the crude product, which was purified by column chromatography (EtOAc/hexane = 3:7).

1-(3,4,5-Trimethoxyphenyl)prop-2-yn-1-one (13 a): Compound **13 a** was prepared according to the method described for compound **13** by employing 1-(3,4,5-trimethoxyphenyl)prop-2-yn-1-ol (**23 a**; 750 mg, 3.38 mmol) and IBX (1.04 g, 3.7 mmol) to obtain pure **13 a** as a light brown solid. Yield: 650 mg, 88%; m.p. 123–125 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.43 (s, 2 H), 3.95 (s, 3 H), 3.93 (s, 6 H), 3.43 ppm (s, 1 H); MS (ESI): *m/z*: 221 $[M+H]^+$.

1-(2-Bromo-3,4,5-trimethoxyphenyl)prop-2-yn-1-one (13 b): Compound **13 b** was prepared according to the method described for compound **13** by employing 1-(2-bromo-3,4,5-trimethoxyphenyl)-prop-2-yn-1-ol (**23 b**; 750 mg, 2.49 mmol) and IBX (767 mg, 2.74 mmol) to obtain pure **13 b** as a brown solid. Yield: 640 mg, 86%; m.p. 79–80 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.46 (s, 1 H), 3.98 (s, 3 H), 3.93 (s, 3 H), 3.89 (s, 3 H), 3.50 ppm (s, 1 H); MS (ESI): *m/z*: 298 $[M+H]^+$.

1-[4-(*tert*-Butyldimethylsilyloxy)-3-methoxyphenyl]prop-2-yn-1-one (13 c): Compound **13 c** was prepared according to the method described for compound **13** by employing 1-[3-(*tert*-butyldimethylsilyloxy)-4-methoxyphenyl]prop-2-yn-1-ol (**23 c**; 750 mg, 2.57 mmol) and IBX (790 mg, 2.82 mmol) to obtain pure **13 c** as a brown solid. Yield: 620 mg, 83%; m.p. 80–82 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.67 (dd, *J* = 6.41 Hz, 0.92 Hz, 1 H), 7.43 (dd, *J* = 0.92 Hz, 1.52 Hz, 1 H), 6.73 (d, *J* = 8.54 Hz, 1 H), 3.71 (s, 3 H), 3.19 (s, 1 H), 0.83 (s, 9 H), 0.12 ppm (s, 6 H); MS (EI): *m/z*: 291 $[M+H]^+$.

1-(3-Methoxy-4-nitrophenyl)prop-2-yn-1-one (13 d): Compound **13 d** was prepared according to the method described for compound **13** by employing 1-(4-methoxy-3-nitrophenyl)prop-2-yn-1-ol (**23 d**; 750 mg, 3.62 mmol) and IBX (1.12 g, 3.98 mmol) to obtain pure **13 d** as a brown solid. Yield: 500 mg, 74%; m.p. 120–123 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.61 (d, *J* = 2.0 Hz, 1 H), 8.31 (dd, *J* = 2.0 Hz, 8.85 Hz, 1 H), 7.19 (d, *J* = 8.85 Hz, 1 H), 4.06 (s, 3 H), 3.51 ppm (s, 1 H). MS (EI): *m/z*: 206 $[M+H]^+$.

1-[3-(Trifluoromethoxy)phenyl]prop-2-yn-1-one (13 e): Compound **13 e** was prepared according to the method described for compound **13** by employing 1-[4-(trifluoromethoxy)phenyl]prop-2-yn-1-ol (**23 e**; 750 mg, 3.47 mmol) and IBX (1.07 g, 3.82 mmol) to obtain pure **13 e** as a brown solid. Yield: 600 mg, 80%; m.p. 97–99 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.83 (d, *J* = 8.3 Hz, 2 H), 7.21 (d, *J* = 8.3 Hz, 2 H), 3.15 ppm (s, 1 H); MS (EI): *m/z*: 215 $[M+H]^+$.

***tert*-Butyl 3-propioloyl-1*H*-indole-1-carboxylate (13 f)**: Compound **13 f** was prepared according to the method described for compound **13** by employing *tert*-butyl 3-(1-hydroxyprop-2-yn-1-yl)-1*H*-indole-1-carboxylate (**23 f**; 750 mg, 2.76 mmol) and IBX (851 mg, 3.04 mmol) to obtain pure **13 f** as a brown solid. Yield: 620 mg, 83%; m.p. 150–152 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.43 (s, 1 H), 8.32 (d, *J* = 8.1 Hz, 1 H), 8.12 (s, *J* = 8.1 Hz, 1 H), 7.41–7.35 (m, 2 H), 3.27 (s, 1 H), 1.72 ppm (s, 9 H); MS (EI): *m/z*: 291 $[M+H]^+$.

1-(1-Methyl-1*H*-indol-3-yl)prop-2-yn-1-one (13 g): Compound **13 g** was prepared according to the method described for compound

13 by employing 1-(1-methyl-1*H*-indol-3-yl)prop-2-yn-1-ol (**23 g**; 800 mg, 4.32 mmol) and IBX (1.33 g, 4.75 mmol) to obtain pure **13 g** as a brown solid. Yield: 800 mg, 73%; m.p. 180–183 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.38$–8.35 (m, 1H), 7.95 (s, 1H), 7.36 (t, $J = 2.83$ Hz, 5.09 Hz, 2H), 7.33–7.32 (m, 1H), 3.88 (s, 3H), 3.16 ppm (s,1H); MS (EI): *m/z*: 184 $[M+H]^+$.

Biology

Cell cultures, maintenance, and antiproliferative evaluation: All the cell lines used in this study were purchased from the American Type Culture Collection (ATCC, United States). A549, MCF-7, HeLa, and HCT116 were grown in Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C). Cells were trypsinized when subconfluent from T25 flasks/60 mm dishes and seeded in 96-well plates. The aminostilbene–arylpropenones were evaluated for their in vitro antiproliferative activity in four different human cancer cell lines. A protocol of 48 h continuous drug exposure was used, and a sulforhodamine B (SRB) cell proliferation assay was employed to estimate cell viability or growth. The cell lines were grown in their respective media containing 10% fetal bovine serum and were seeded into 96-well microtiter plates in 200 μL aliquots at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to the addition of the experimental drugs. Aliquots of 2 μL of the test compounds were added to the wells already containing 198 μL of cells, which resulted in the required final drug concentrations. For each compound, four concentrations (0.1, 1, 10, and 100 μM) were evaluated, and each was done in triplicate wells. Plates were incubated for another 48 h, and the assay was terminated by the addition of 100 μL of 10% *wt/vol* cold trichloroacetic acid and incubated at 4 °C for 1 h. The supernatant was discarded. The plate was washed with tap water (4×) and was allowed to air dry. The cells were then stained with 0.057% SRB dissolved in 1% acetic acid for 30 min at RT. Unbound SRB was washed away with four washes of 1% acetic acid. The plate was again allowed to air dry, and the bound SRB stain, representing surviving cells, was dissolved in Tris base (10 mM, 50 μL). The optical density was determined at $\lambda = 510$ nm by using a microplate reader (Enspire, PerkinElmer, USA). Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. The above determinations were repeated thrice. The growth inhibitory effects of the compounds were analyzed by generating dose response curves as a plot of the percentage surviving cells versus compound concentration. The sensitivity of the cancer cells to the test compound was expressed in terms of IC_{50}, a value defined as the concentration of compound that produced 50% reduction relative to the control absorbance. IC_{50} values are indicated as means ± standard deviations of three independent experiments.[27]

Dot-blot assay: A549 cells were treated with 1 μM **3 a**–**h**, **4 a**–**h**, and **5 a**–**f** for 24 h. Subsequently, cells were harvested and proteins were quantified by using Amido Black followed by densitometry analysis. Equal amounts of protein were blotted on a nitrocellulose membrane by using a Bio-Dot SF microfiltration apparatus (Bio-Rad). Briefly, the nitrocellulose membrane and three filters papers (Whatmann 3) were soaked in 1× Tris-buffered saline (TBS) solution for 10 min. Later, the filter papers and membrane were arranged in the apparatus and connected to a vacuum pump (Millipore). The membranes were rehydrated by using 1× TBS (100 μL) by vacuum filtration. Subsequently, the samples (50 μL) were blotted on the membranes and washed with 1× TBS (200 μL) through application of vacuum. The blot was blocked with 5% blotto for 1 h at RT. Immunoblot analysis was performed as described previously by using UVP, biospectrum 810 imaging system.[27]

Tubulin polymerization assay: An in vitro assay for monitoring the time-dependent polymerization of tubulin to microtubules was performed by employing a fluorescence-based tubulin polymerization assay kit (BK011, Cytoskeleton, Inc.) according to the manufacturer's protocol. The reaction mixture in a final volume of 10 μL in PEM buffer [80 μM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 0.5 μM ethylene glycol tetraacetic acid (EGTA), 2 μM $MgCl_2$, pH 6.9] in 384-well plates contained bovine brain tubulin (2 mg mL^{-1}), 10 μM fluorescent reporter, 1 mM guanosine-5′-triphosphate (GTP) in the presence or absence of test compounds at 37 °C. Tubulin polymerization was followed by monitoring the fluorescence enhancement resulting from the incorporation of a fluorescence reporter into the microtubules as the polymerization proceeded. Fluorescence emission at $\lambda = 420$ nm ($\lambda_{exc} = 360$ nm) was measured for 1 h at 1 min intervals in a multimode plate reader (Tecan M200). To determine the IC_{50} values of the compounds against tubulin polymerization, the compounds were preincubated with tubulin at varying concentrations (0.01, 0.1, 1, 10, and 100 μM). Assays were performed under conditions similar to those employed for polymerization assays as described above.[29]

Analysis of cell cycle: A549 cells in 60 mm dishes were incubated for 24 h in the presence or absence of test compounds **3 e**, **3 g**, and **4 e** at 1 μM concentration. Cells were harvested with trypsin–EDTA and fixed with ice-cold 70% ethanol at 4 °C for 30 min; then, ethanol was removed by centrifugation, and the cells were stained with DNA staining solution [1 mL; 0.2 mg of propidium iodide (PI), and 2 mg RNase A] for 30 min as described earlier. The DNA contents of 20000 events were measured by flow cytometer (BD FACSCanto II). Histograms were analyzed by using FCS express 4 plus.

Immunohistochemistry of tubulin and analysis of nuclear morphology: A549 cells were seeded on glass cover slip and incubated for 24 h in the presence or absence of test compounds at a concentration of 1 μM. Cells grown on coverslips were fixed in 3.5% formaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 10 min at RT. Cells were permeabilized for 6 min in PBS containing 0.5% Triton X-100 (Sigma) and 0.05% Tween-20 (Sigma). The permeabilized cells were blocked with 2% bovine serum albumin (Sigma) in PBS for 1 h. Later, the cells were incubated with the primary antibody for tubulin (Sigma) at 1:200 diluted in blocking solution for 4 h at RT. Subsequently, the antibodies were removed, and the cells were washed with PBS (3×). Cells were then incubated with fluorescein isothiocyanate labeled anti-mouse secondary antibody (1:500) for 1 h at RT. Cells were washed with PBS (3×) and mounted in medium containing 4′,6-diamidino-2-phenylindole. Images were captured by using the Olympus confocal microscope FLOW VIEW FV 1000 series and analyzed with FV10ASW 1.7 series software.

Western blot analysis of soluble versus polymerized tubulin: Cells were seeded in 12-well plates at 1×10^5 cells per well in complete growth medium. Following individual treatment of cells with compounds **3 e**, **3 g**, and **4 e** for 24 h, the cells were washed with PBS and subsequently soluble and insoluble tubulin fractions were collected. To collect the soluble tubulin fractions, cells were permeabilized with prewarmed lysis buffer [200 μL; 80 μM PIPES-KOH (pH 6.8), 1 μM $MgCl_2$, 1 μM EGTA, 0.2% Triton X-100, 10% glycerol, 0.1% protease inhibitor cocktail (Sigma–Aldrich)] and incubated for 3 min at 30 °C. The lysis buffer was gently removed and mixed with 3×Laemmli's sample buffer [100 μL; 180 μM Tris-Cl pH 6.8, 6% sodium dodecyl sulfate (SDS), 15% glycerol, 7.5% β-mercap-

toethanol, 0.01% bromophenol blue]. Samples were immediately heated to 95 °C for 3 min. To collect the insoluble tubulin fraction, 1× Laemmli's sample buffer (300 μL) was added to the remaining cells in each well, and the samples were heated to 95 °C for 3 min. Equal volumes of samples were run on an SDS–10% polyacrylamide gel and were transferred to a nitrocellulose membrane employing semidry transfer at 50 mA for 1 h. Blots were probed with mouse anti-human α-tubulin diluted 1:2000 mL (Sigma) and stained with rabbit anti-mouse secondary antibody coupled with horseradish peroxidase, diluted 1:5000 mL (Sigma). Bands were visualized by using an enhanced chemiluminescence protocol (Pierce) and radiographic film (Kodak).[31]

Molecular modeling

Optimizations of all compounds were performed in Gaussian 09 by using PM3 semiempirical methods. The protein cocrystal structure was downloaded from RSCB-Protein Data Bank with ligand colchicine (PDB ID 3E22). [32] Autodock 4.2 software[33] was used to perform the docking studies. The visualization and analysis of the interactions were performed by using PyMOL (ver. 0.99). [34]

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Keywords: antiproliferation · cell-cycle arrest · dot-blot analyses · molecular docking · polymerization

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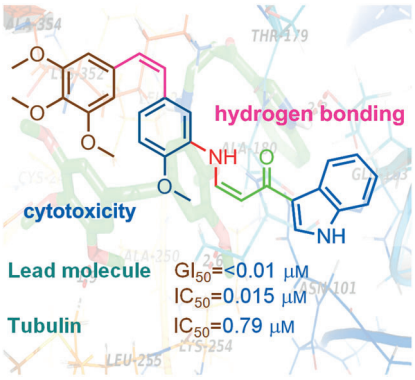
Design and Synthesis of Aminostilbene–Arylpropenones as Tubulin Polymerization Inhibitors



Sittin' on the docking site of tubulin: A series of aminostilbene–arylpropenones are designed, synthesized, and evaluated for their cytotoxic activity. These compounds inhibit microtubule assembly and induce cyclin B1 protein expression. Docking experiments reveal that these compounds bind with the colchicine binding site of tubulin.