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RESEARCH ARTICLE

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Design, synthesis and biological evaluation of benz-fused five-membered heterocyclic compounds as tubulin polymerization inhibitors with anticancer activities

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Abstract

A series of benz-fused five-membered heterocyclic compounds were designed and synthesized as novel tubulin inhibitors targeting the colchicine binding site. Among them, compound 4d displayed the highest antiproliferative activity against four cancer cell lines with an IC_{50} value of 4.9 μM in B16-F10 cells. Compound 4d effectively inhibited tubulin polymerization in vitro (IC_{50} of 13.1 μM). Further, 4d induced cell cycle arrest in G2/M phase. Finally, 4d inhibited the migration of cancer cells in a dose-dependent manner. In summary, these results suggest that compound 4d represents a new class of tubulin inhibitors deserving further investigation.

KEYWORDS

anticancer agents, CA-4 analogs, colchicine binding site, tubulin inhibitor

1 | INTRODUCTION

Out of the many clinically used antitumor drugs, microtubule targeting agents (MTAs) (e.g., taxanes and vinca alkaloids) have achieved considerable success in the treatment of various types of tumors such as melanoma and prostate cancer. However, those tubulin-targeting agents are naturally derived with high structural complexity which hampers further structural optimizations (Li et al., 2019; Lu et al., 2012; Vuuren et al., 2015). Of the four types of tubulin inhibitors which bind the taxane-, vinca alkaloid-, laulimalide- and colchicine-site, colchicine binding site inhibitors (CBSI) are generally more amenable to modifications due to the relative structural simplicity as compared to the other three types of MTAs

Buduma Komuraiah and Yichang Ren contributed equally.

with a natural origin (Bai et al., 1990; Bennett et al., 2012; Bollag et al., 1995; Kingston, 2009; Risinger et al., 2009; Wei et al., 2004). Therefore, the development of CBSIs has been intensified in the past two decades with numerous novel and potent CBSIs being discovered (Figure 1), for example, ATCAA (Lu et al., 2009), AICA (Lu et al., 2010), SMART (Lu et al., 2011), PAT (Lu et al., 2014), ABI-I (Chen et al., 2010), ABI-II (Chen et al., 2011), ABI-III (Chen et al., 2012), and RABI (Xiao et al., 2013).

We previously discovered a series of 2-aryl-4-benzoyl-imidazole (ABI-II) analogs as antiproliferative agents targeting the colchicine binding site in tubulin for treating melanoma and prostate cancer. The ABI-II analogs were designed based on the first generation ABI-I analogs utilizing a ring-fusion strategy, namely, fusing the A- and B-ring of ABI-I (Figure 1). However, we only synthesized a very limited number of compounds (<5) that showed moderate antiproliferative activities with IC_{50} in the micromolar range (e.g., ~10 μM). To extend the structure-activity relationship study of this scaffold, we designed two series of target compounds (Benz-fused five-membered heterocyclic compounds, Figure 2) based on the following rationales: 1) incorporating different five-membered heterocyclic B-ring (e.g., thiazole, oxazole, pyrrole, triazole) to understand the tolerability of the B-ring modifications (Series 1), as benzimidazole and benzothiazole moieties are found in many existing tubulin inhibitors (Ashraf et al., 2016; Fu et al., 2020). 2) shifting the position of the 3,4,5-trimethoxyphenyl (TMP) group from 2 to 1 to explore the effects of different geometric configurations (Series 2); With the TMP moiety at position-1, series 2 is essentially a structure mimic of CA-4, which is a highly potent CBSI in clinical trials. Herein, we describe the synthesis and biological evaluation of these compounds as detailed in the following section.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

As outlined in Scheme 1, the synthesis of series 1 compounds started with commercially available 4- or 5-substituted 2-nitro phenol (**1**, X = O). Initially, the nitro group was reduced by H_2 in the presence of Pd/C to obtain the amine derivatives (**2**, X = O) which were converted to benzoxazoles (**3**, X = O) using triethyl orthoformate in the presence of 3A° molecular sieves (MS) at 130°C (Scheme 1, step b). The same synthetic strategy was applied to the synthesis of substituted thiophenol derivatives (Scheme 1, step b). The synthesis of compound 4 (a-j) was achieved by oxidative C-H activation at the C-2 position of benzoxazole or benzothiazole with 3,4.5-trimethoxy benzaldehyde using ammonium persulphate and tetrabutyl ammonium bromide (Siddaraju et al., 2014) as a phase transfer catalyst (Scheme 1, step c). Compound 5f was prepared through Suzuki coupling by reacting 4f with 3-fluoro-4-meth oxy-phenylboronic acid (Scheme 1, step d).

The synthesis of series 2 compounds [1-(3,4,5-trimethox ybenzoyl)-benzimidazoles, 10a-10f)] is shown in Scheme 2. Briefly, the substituted 1,2-diaminobenzene 8 was reacted with ethyl formate to generate the benzimidazole 9. Compound 9 was acylated by 3,4.5-trimethoxy benzaldehyde at the N-1 position to give the desired 1-(3,4,5-trimethoxybe nzoyl)-benzimidazoles 10a-10f.

In addition to the 1-(3,4,5-trimethoxybenzoyl)-benzim idazole compounds 10a-10f, we also synthesized series 2 compounds with the 3,4,5-trimethoxybenzoyl moiety at the 1-position of the benz-, pyridine or pyrimidine-fused five-membered heterocycles, as shown in Scheme 3.

2.2 | Biological evaluation

2.2.1 | In vitro antiproliferative activity and structure-activity relationship

The antiproliferative activities of the newly synthesized compounds were evaluated against four cancer cell lines (MCF-7, H1299, HeLa and B16-F10) by an MTT assay with CA-4 and colchicine as the positive controls. As presented in Table 1, most of the compounds are not very active with IC_{50} > 10 μM. Compounds 4d, 4f, 4g exhibited moderate potency with IC_{50} ranging from 4.9 to 10 μM. In general, the benzothiazole analogs (e.g., 4d-4g, IC_{50} of

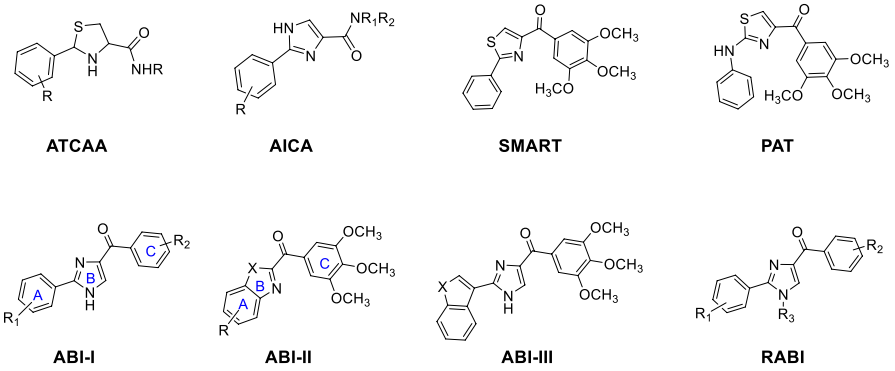


FIGURE 1 Structures of known CBSIs [Colour figure can be viewed at wileyonlinelibrary.com]

4.9~18.6 μM) exhibited slightly higher potency than that of the benzoxazole (e.g., 4a-c, IC_{50} of 16.5 ~ 46.2 μM) and benzimidazole (e.g., 10a-f, IC_{50} of 12.58~46.8 μM) compounds. For the R_1 and R_2 substituents on the benz-fused ring systems, when R_1 is a hydrogen, the compounds are generally less potent than that of the corresponding analogs with R_2 as a hydrogen (4b vs. 4a; 4e vs. 4d; 4i vs. 4h, 10b vs. 10a; 10d vs. 10c). Also, the geometrically "extended" Series 1 compounds are slightly better than their geometrically "bent" counterparts Series 2 compounds (4b vs. 10f; 4f vs. 10a; 4d vs. 10c; 4e vs. 10d). Specifically, for series 1 compounds, converting the oxazole (4a) to thiazole (4g) led to improved potency. Compounds with electron-withdrawing groups on the benzothiazole ring displayed higher potency than the ones with electron-donating groups (e.g., 4d, 4f, 4g and 4h; -Br>-Cl>-OCH_3>-NH_2). Among them, compound 4d (6-Br benzothiazole) exhibited the highest activity with an average IC_{50} value of 7.219 μM. The 6-substituted benzothiazole derivatives are less potent than that of 5-substituted benzothiazole derivatives [4d (IC_{50} = 7.219 μM) vs. 4e (IC_{50} > 10 μM)]. These results indicated that the 6-Br benzothiazole moiety is optimal for activity. Series 2 compounds (10a-f, 11–15) showed low activities (IC_{50} > 10 μM), which might be due to the shift of the position of C-Ring (3,4,5-trimethoxy benzoyl group) from 2-position to 1-position of the imidazole ring. Compound 4j with a N-methyl indolyl group as C-ring showed essentially a total loss of activity (IC_{50} > 10 μM), comparing to compound 4f, suggesting that the 3,4,5-trimethoxypheny

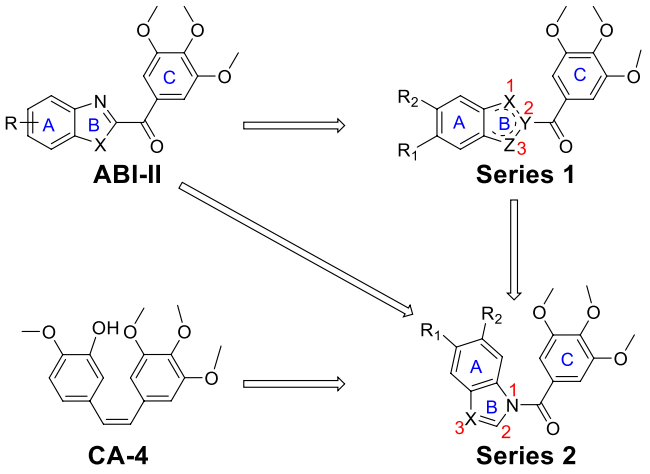
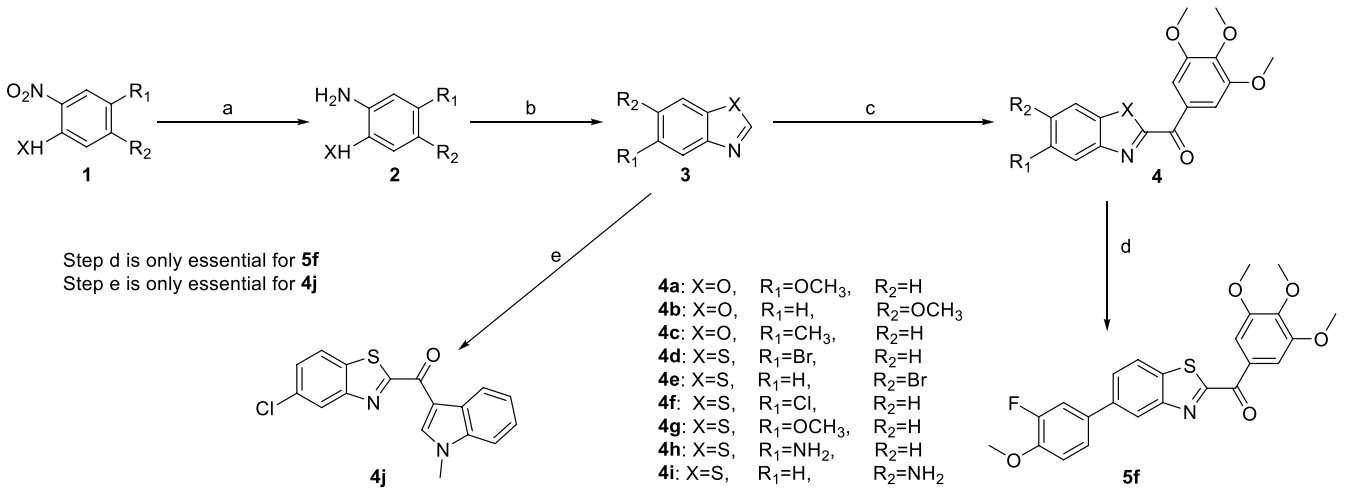
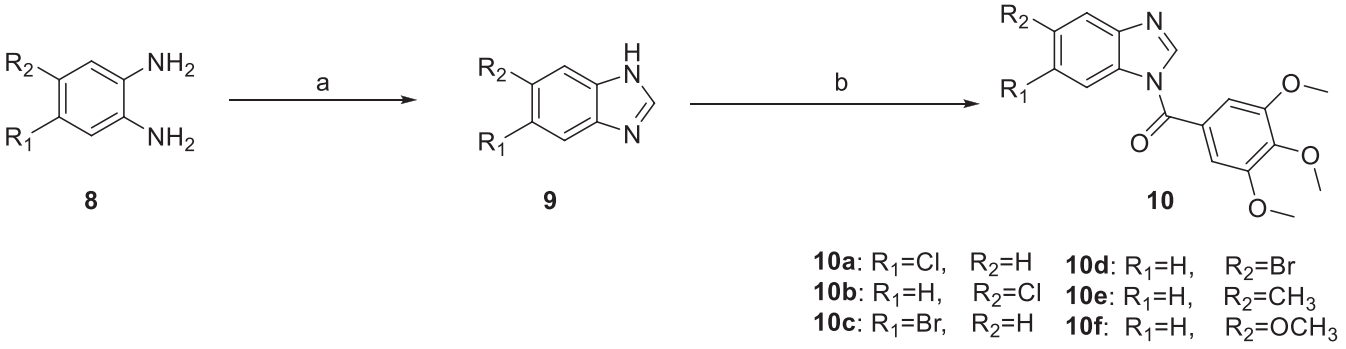


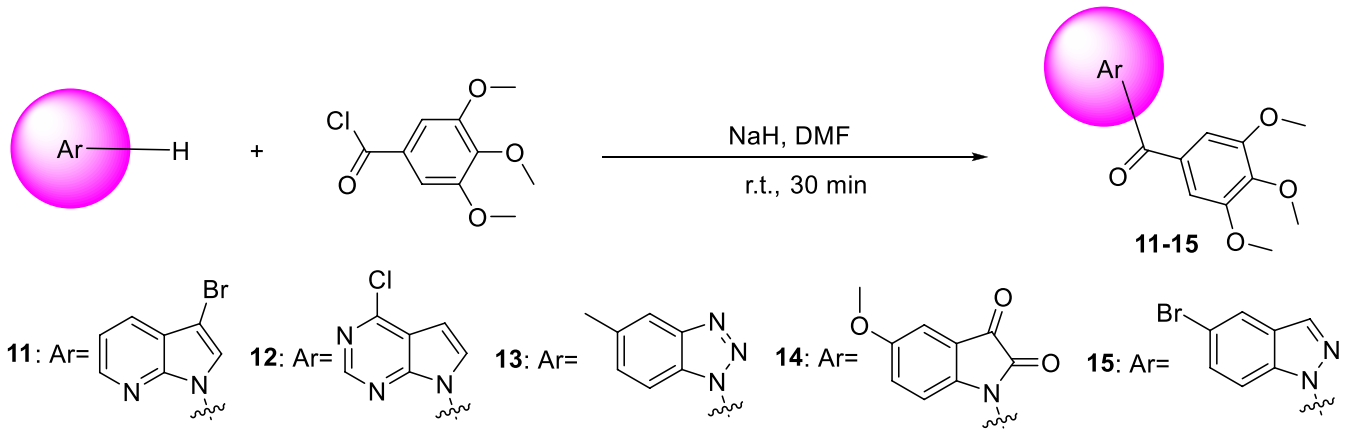
FIGURE 2 Rationale for the design of target compounds [Colour figure can be viewed at wileyonlinelibrary.com]



SCHEME 1 Synthesis of series 1 compounds



SCHEME 2 Synthesis of series 2 compounds (10a-10f)



SCHEME 3 Synthesis of series 2 compounds (11–15) [Colour figure can be viewed at wileyonlinelibrary.com]

play an important role in maintaining the cytotoxic activity. The positions of substituents on the benz-fused rings (10a, 10c vs. 10b, 10d) do not affect the potencies, but electron-withdrawing groups (10a-d, 11, 12) seem to be better than that of electron-donating substituents (10e-f, 13, 14). In addition, the cytotoxicity of these compounds was examined against a normal cell line HUVEC (human umbilical vein endothelial cell). As shown in Table 1, all the compounds demonstrated little toxicity to HUVEC with $IC_{50} > 50$ μM, suggesting that these compounds cause selective toxicity to cancer cells over normal cells. Interestingly, similar benzimidazole and benzothiazole analogs have been reported (Ashraf et al., 2016; Fu et al., 2020), but our compounds showed slightly lower potencies probably due to a higher fraction of drugs being transported out of cells by efflux pumps (Figure S55). Also the newly synthesized compounds are generally less potent than that of CA-4 and of the natural ligand colchicine, partially because of the lower binding affinities in the colchicine binding site of tubulin, as seen from the molecular docking studies of colchicine and compound 4d (Figure S54). However, the advantages of these compounds may be the higher stability (Figure S57) over CA-4 because there will be no cis-trans conversion due to the presence of a double bond as in the case of CA-4.

Cells were exposed to different concentrations of compounds for 48 hr to determine the cell viability through MTT assay. IC_{50} values are presented as the mean ± *SD* of at least three independent experiments.

2.2.2 | In vitro tubulin polymerization assay

To explore the mechanism of action, compound 4d was chosen to evaluate the effects on tubulin polymerization with CA-4 and colchicine as positive controls. The results indicated that compound 4d inhibited tubulin polymerization with an IC_{50} of 13.1 μM (Figure 3a), weaker than that of CA-4 (IC_{50} = 1.84 μM, Figure 3b) and colchicine (IC_{50} = 7.15 μM, Figure 3c). Furthermore, compound 4d inhibited tubulin polymerization in a dose-dependent manner (Figure 3d).

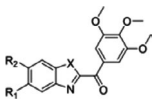
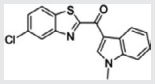
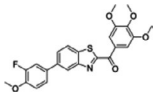
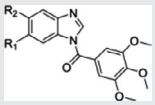
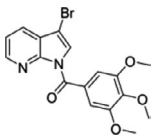
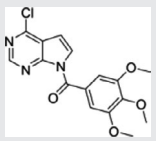
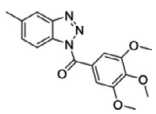
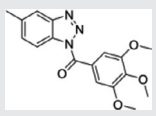
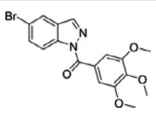
2.2.3 | Immunofluorescence studies

It is known that microtubule dynamics play an important role in cancer cell growth. To validate whether compound 4d might influence microtubule dynamics, an immunofluorescent assay in B16-F10 cells was performed. As showed in Figure 4, in the control group, the microtubule networks exhibited a normal arrangement with slim and fibrous microtubules (green) wrapped around the cell nucleus (blue). However, when cells were exposed to compound 4d (1, 5 and 10 μM) or CA-4 (10 nM) for 6 hr, the microtubule networks were disrupted in comparison with the control. These results suggest that compound 4d might induce the collapse of the microtubule networks in a dose-dependent manner.

2.2.4 | Cell cycle study by flow cytometry

Tubulin polymerization inhibitors have been previously known to impact cell division. Thus, we evaluated the effects of compound 4d on the cell cycle of B16-F10 cells with flow cytometry. As shown in Figure 5, compound 4d arrested cell cycle at G2/M phase (Figure 5a) in a dose-dependent manner. When B16-F10 cells were exposed to increasing concentrations of compound 4d (1, 5 and 10 μM), the percentage of cells in the G2/M phase was significantly increased from 8.27% to 82.90%.

TABLE 1 In vitro antiproliferative activity of series 1 and 2 compounds

Structure	Compound	X	R_1	R_2	IC_{50} (μM)				
					MCF−7	H1299	HeLa	B16-F10	HUVEC
	4a	O	OCH_3	H	21.58 ± 1.23	24.60 ± 0.98	19.58 ± 0.47	16.49 ± 1.05	>50
	4b	O	H	OCH_3	32.85 ± 2.51	44.12 ± 3.41	27.65 ± 1.87	30.17 ± 0.69	>50
	4c	O	CH_3	H	46.18 ± 1.96	38.66 ± 0.82	29.78 ± 2.54	30.06 ± 3.81	>50
	4d	S	Br	H	9.450 ± 0.292	7.652 ± 0.215	6.862 ± 0.144	4.912 ± 0.088	>50
	4e	S	H	Br	16.79 ± 0.35	18.62 ± 0.54	12.85 ± 0.40	21.39 ± 0.63	>50
	4f	S	Cl	H	10.00 ± 0.14	6.761 ± 0.171	7.605 ± 0.238	7.273 ± 0.411	>50
	4g	S	OCH_3	H	9.253 ± 0.065	6.538 ± 0.152	15.72 ± 0.28	13.14 ± 0.32	>50
	4h	S	NH_2	H	25.47 ± 1.36	32.18 ± 2.17	22.07 ± 0.66	19.03 ± 0.25	>50
	4i	S	H	NH_2	50.25 ± 3.87	61.24 ± 5.66	39.87 ± 3.25	41.08 ± 2.96	>50
	4j				16.20 ± 0.68	13.88 ± 0.60	19.67 ± 1.02	13.56 ± 0.24	>50
	5f				32.90 ± 1.94	38.62 ± 1.03	41.37 ± 1.26	22.53 ± 1.97	>50
	10a		Cl	H	13.77 ± 0.51	24.18 ± 0.85	31.21 ± 2.09	19.84 ± 2.45	>50
	10b		H	Cl	17.22 ± 0.62	27.17 ± 3.15	18.96 ± 0.67	22.68 ± 0.55	>50
	10c		Br	H	12.58 ± 1.21	20.73 ± 2.30	25.36 ± 1.71	17.52 ± 1.37	>50
	10d		H	Br	15.29 ± 0.68	32.08 ± 1.59	22.14 ± 0.96	18.81 ± 1.09	>50
	10e		H	CH_3	35.68 ± 3.76	52.12 ± 4.63	45.39 ± 2.64	38.47 ± 2.35	>50
	10f		H	OCH_3	29.60 ± 2.33	46.97 ± 1.26	39.85 ± 1.80	33.46 ± 2.11	>50
	11				18.30 ± 4.51	27.24 ± 0.81	34.12 ± 2.07	23.59 ± 0.58	>50
	12				20.88 ± 2.01	19.05 ± 1.70	24.86 ± 2.31	30.17 ± 1.06	>50
	13				32.90 ± 3.14	25.83 ± 0.77	36.10 ± 1.27	45.92 ± 3.32	>50
	14				69.17 ± 3.61	72.64 ± 2.08	48.19 ± 2.15	67.83 ± 6.18	>50
	15				55.28 ± 7.23	69.40 ± 4.28	74.32 ± 3.49	60.12 ± 2.37	>50
	CA-4				0.013 ± 0.002	0.014 ± 0.002	0.009 ± 0.005	0.007 ± 0.001	>50
	Colchicine				0.024 ± 0.006	0.120 ± 0.016	0.045 ± 0.004	0.068 ± 0.012	>50

2.2.5 | Inhibition of cancer cell migration

Knowing that cancer cells are able to migrate to distant organs and microtubule play an important role in cell migration. A wound healing assay was performed in order to confirm the effects of compound 4d in cell migration. When

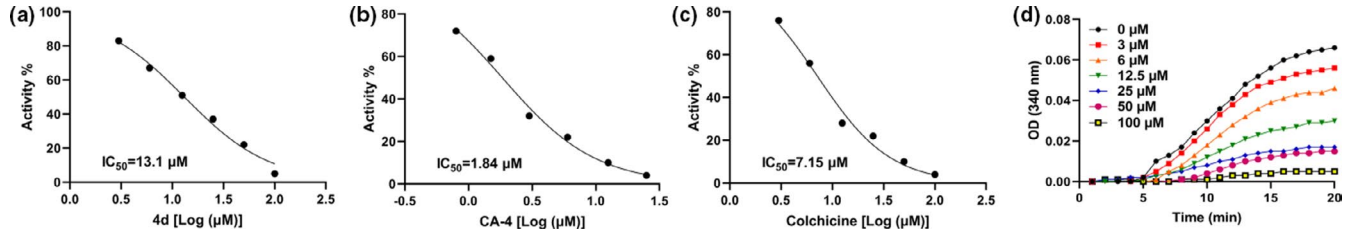


FIGURE 3 Inhibition of tubulin polymerization in vitro. (a) The inhibition of tubulin polymerization by compound 4d. (b) The inhibition of tubulin polymerization by CA-4. (c) The inhibition of tubulin polymerization by colchicine. (d) Compound 4d exhibited a dose-dependent inhibition of tubulin polymerization [Colour figure can be viewed at wileyonlinelibrary.com]

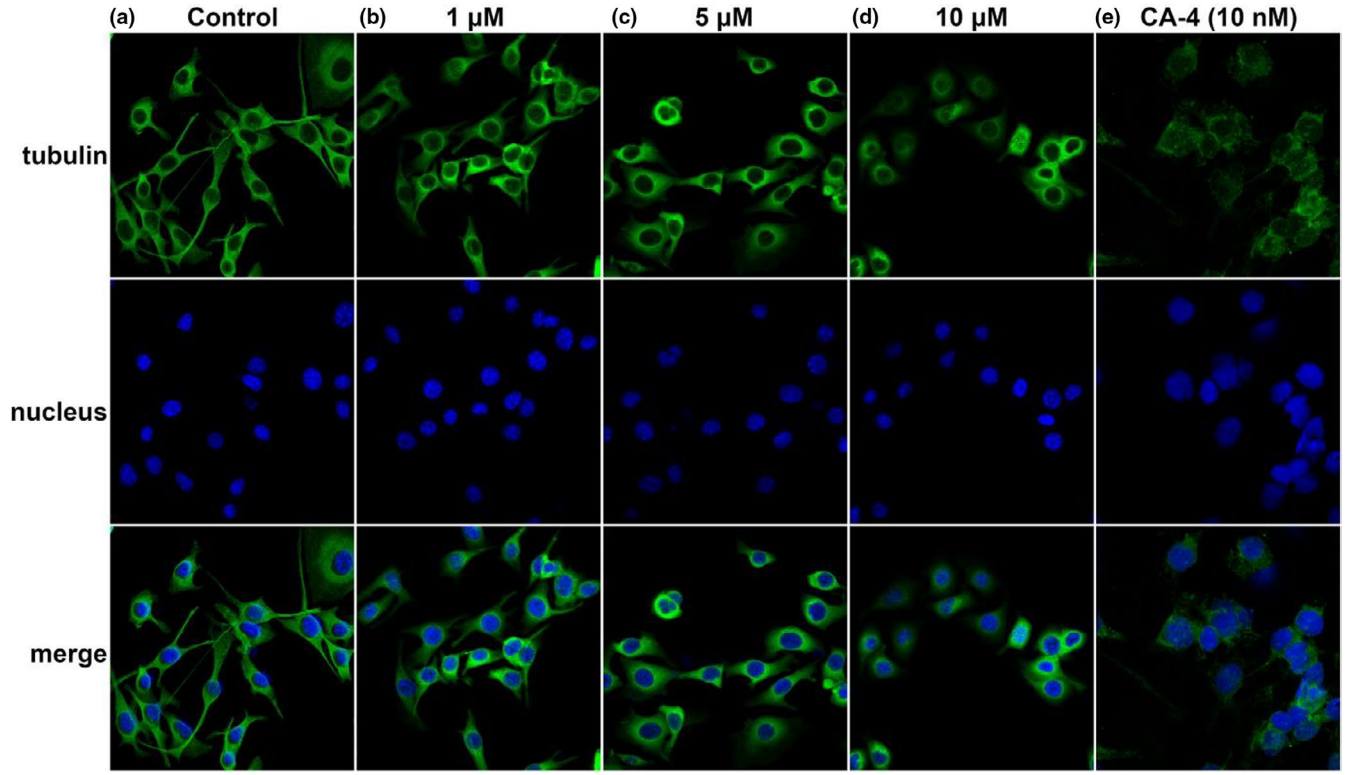


FIGURE 4 Effects of compound 4d on microtubules. B16-F10 cells were treated with vehicle control 0.1% DMSO (a), compound 4d (1 μM) (b), compound 4d (5 μM) (c), compound 4d (10 μM) (d), CA-4 (10 nM) (e) for 6 hr. Microtubules were visualized with an anti-β-tubulin antibody (green), and the cell nucleus was visualized with DAPI (blue). Fluorescence images were collected by LSM 880 laser confocal microscope (Carl Zeiss, Germany) [Colour figure can be viewed at wileyonlinelibrary.com]

treating the B16-F10 cells with 1, 5 and 10 μM of compound 4d, the wound closure was potently suppressed (Figure 6), showing that the migration of cancer cells was inhibited by compound 4d in a dose-dependent manner.

3 | CONCLUSION

In summary, a series of benz-fused five-membered heterocyclic compounds were designed and synthesized as tubulin polymerization inhibitors and a focused SAR study was performed. Most of these compounds exhibited low antiproliferative potency with IC_{50} values larger than 10 μM. Several of them (4d, 4f, 4g) demonstrated moderate potency against a panel of cancer cell lines with IC_{50} values in the micromolar range (e.g., 4.7–10 μM). Structure-activity relationships revealed that the relative position of the 3,4,5-trimethoxybenzoyl group on the five-membered heterocyclic ring has significant influence on the biological activities of the compounds, with 2-position being optimal. Among the newly synthesized compounds, 4d displayed the highest antiproliferative activity against four cancer cell lines in vitro. In addition, compound 4d was able to inhibit tubulin polymerization in vitro in a dose-dependent manner.

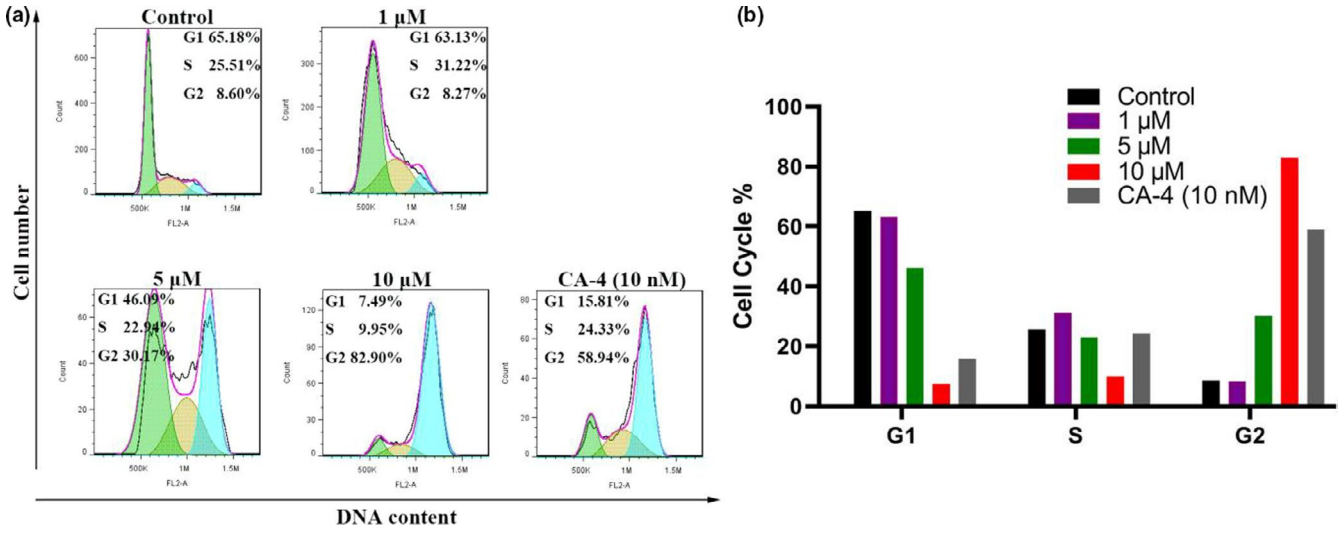


FIGURE 5 Cell cycle arrested by compound 4d. (a) Compound 4d induced G2/M arrest in B16-F10 cells. B16-F10 cells were incubated with varying concentrations of compound 4d (1, 5 and 10 μM) and CA-4 (10 nM) for 48 hr. The percentages of cells in different phases of the cell cycle were analyzed by FlowJo 7.6.1. (b) Histograms displayed the percentage of cell cycle distribution after treatment with compound 4d and CA-4 [Colour figure can be viewed at wileyonlinelibrary.com]

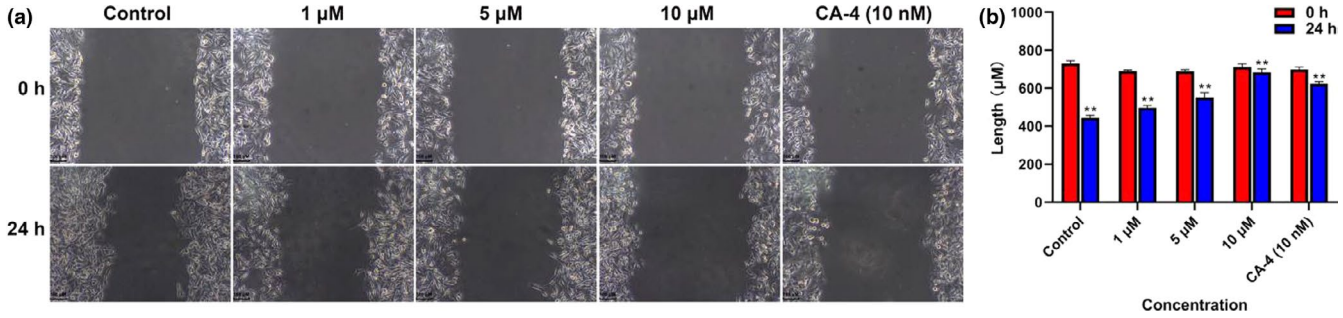


FIGURE 6 Effects on the B16-F10 cells migration. (a) Representative images of wound area in a wound healing assay. Images were obtained at 0 hr and 24 hr after treatment with 0, 1, 5 and 10 μM compound 4d and 10 nM CA-4. (b) Histograms displayed the length of the scratches at 0 hr and 24 hr. ($n = 3$, $^{**}p < .05$ vs. DMSO control) [Colour figure can be viewed at wileyonlinelibrary.com]

Further mechanism studies indicated that compound 4d induced cell cycle arrest in G2/M phase. Finally, compound 4d inhibited the migration of cancer cell in a dose-dependent manner. Taken together, these results suggest that compound 4d represents a promising tubulin inhibitor deserving further investigation.

3.1 | Supporting information availability

The supporting information contains ^{1}H NMR, ^{13}C NMR and mass spectra (Figure S1 to Figure S50). The purity of compounds 4d, 4f and 4g was determined by HPLC (Figure S51 to Figure S53). Molecular modeling of colchicine and compound 4d at colchicine binding site of tubulin was shown in Figure S54. The concentrations of compounds 10c and 11 at various time points in cell media (with cells co-cultured) were determined by HPLC (Figure S55, Figure S56). The concentrations of compounds 10c and 11 at various time points in cell media (without cells) were determined by HPLC (Figure S57, Figure S58).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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